Bioénergétique systémique moléculaire dans les cellules nerveuses et musculaires: Compartimentation et hétérogénéité de la diffusion de l'ATP, Interactosome Mitochondrial

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Claire Monge, Nathalie Béraud et Valdur Saks.

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LISTE DES ABREVIATIONS

ADP	Adénosine DiPhosphate
AMP	Adénosine MonoPhosphate
ANT	Adénine Nucléotide Translocase
AK	Adénylate Kinase
ATP	Adénosine TriPhosphate
BBCK	Isoforme cytosolique de la Créatine Kinase
CK	Créatine Kinase
CoQ	Coenzyme Q
Cr	Créatine
GAPDH	Glycéraldéhyde phosphate déshydrogénase
HK	Hexokinase
ICEU	IntraCellular Energetic Unit
kDa	KiloDalton
LDH	Lactate DesHydrogénase
MAP	Microtubule Associated Protein
MMCK	Isoforme cytoplasmique de la Créatine Kinase
MT	Microtubule
MEM	Membrane Externe Mitochondriale
NADH	Nicotinamine Adénine Dinucléotide
NESS	Non-Equilibrium Steady State
OxPhos	Oxydation Phosphorylante
PCr	PhosphoCréatine
Pi	Phosphate Inorganique
PK	Pyruvate Kinase
RES	Rapid Equilibrium State
RMN	Résonance Magnétique Nucléaire
SERCA	Sarcoplasmic Endoplasmic Reticulum Calcium ATPase
sMtCK	Isoforme sarcomérique mitochondrial de la créatine kinase
STOP	Stabilisizing Tubule Only Polypeptide
uMtCK	Isoforme ubiquitaire mitochondrial de la Créatine Kinase
VDAC	Voltage Dépendant Anion Channel

Préambule

La Biologie systémique, ou Biologie des Systèmes, est une philosophie émergente et une façon intégrative d'appréhender la Biologie. Les fonctions cellulaires sont parfois étonnamment insensibles aux variations et perturbations de leur environnement, et cette robustesse est largement due à la complexité et la plasticité des processus biologiques.

Mais cette philosophie n'est pas à proprement parler actuelle. Au IVème siècle avant Jésus Christ, Aristote écrivait déjà dans 'La Metaphysique' (1045a10) : « L'ensemble est plus que la somme de ses parties ». Et c'est exactement dans cette lignée que s'inscrit ce travail : considérer les phénomènes biologiques comme un tout, un ensemble d'interactions et de réseaux interconnectés.

Cette étude balayera le champ de la Bioénergétique à partir de la diffusion de molécules d'ATP jusqu'aux variations métaboliques associées au cancer.

L´imagination est plus importante que le connaissance. La connaissance est limitée alors que l´imagination englobe le monde entier, stimule le progrès, suscite l´évolution.
Albert Einstein, 1929

I. INTRODUCTION

La vie parait être un comportement ordonné et réglementé de la matière, comportement qui n´est pas basé exclusivement sur sa tendance à passer de l´ordre au désordre, mais basé en partie sur un ordre qui se maintient.

Erwin Schrödinger, 1944 « What is life ? »

I. 1. Cadre philosophique

I. 1.1 Biologie systémique

I. 1.1.1 Réductionnisme

Dans les années 90, un nouveau paradigme émerge dans les sciences biologique : la Biologie Systémique (ou Biologie des Systèmes, ou encore Biologie Intégrative). Comme son nom l'indique, elle étudie les phénomènes biologiques en les traitant comme des systèmes, en étudiant leur complexité, en considérant les aspects moléculaires, cellulaires, physiologiques et médicaux du gène à l'organisme, voire même à la population. Le but de la biologie systémique est de décrire quantitativement les systèmes intégrés à différentes échelles pour comprendre et caractériser ces systèmes complexes et multi-composants.

Cette méthodologie est opposée à celle plus ancienne appelée 'réductionnisme' qui étudie les objets dans leur état isolé, c'est-à-dire sorti de leur contexte cellulaire, physico-chimique, biochimique etc…Le réductionnisme est une étape absolument nécessaire à la détermination de propriétés et de caractéristiques intrinsèques des objets qui ne seraient pas révélées dans leur environnement physiologique, ou impossible à déterminer s'ils ne sont pas étudiés dans leur état fondamental. C'est ainsi que grâce à une grande représentante du réductionnisme, la biologie structurale, il a été possible de déterminer la structure de protéines ou de complexes protéiques. De même que la génétique a, pendant plusieurs décennies, permis des avancements phénoménaux sur la connaissance des processus cellulaires. Dès la fin des années 40 et depuis la description de la structure en double hélice de l'ADN par Watson et Crick en 1953 [Watson Crick, Nature], l'ADN fut communément considéré comme vecteur de l'hérédité. Certes. Cependant, la génétique a longtemps été prépondérante et omnisciente dans les sciences biologiques si bien qu'a très vite émergé le génocentrisme plaçant l'ADN comme la « cause » de la vie. Cette conception du gène « égoïste », actuellement un peu dérangeante, stipule que Tout découle des gènes, de leurs mutations et de leur traduction, et qu'un gène pourra égoïstement assurer sa survie, sa transmission en conférant un avantage sélectif à un organisme [Richard Dawkins, le gène égoïste]. Denis Noble dans son livre « The Music of Life » a parfaitement schématisé la situation : le **Schéma 1** est la représentation réductionniste d'une causalité initiée par l'ADN et linéairement dirigée vers l'organisme. Il est vrai que la

base de notre constitution est encodée dans notre patrimoine génétique transmis pas nos parents, mais ce déterminisme génétique est une négation du Reste.

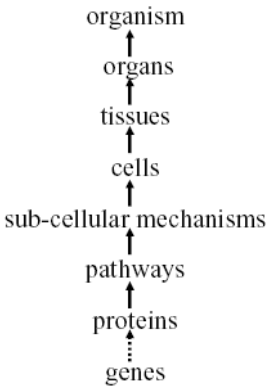


Schéma 1 : Causalité réductionniste ascendante ou « bottom-up ». Cette chaîne débute par les gènes puis le flux progresse linéairement jusqu'à l'organisme. [D'après Noble, 2006].

Denis Noble décrit parfaitement ce qu'est le Reste à travers le **Schéma 2** représentant l'évolution des pensées concernant la place du gène en Biologie. Le **Schéma 2** représente le **Schéma 1** complété par des boucles de régulation, telles que des boucles de signalisation cellulaire et des boucles de régulation génique, assurant une régulation fine et un haut degré d'organisation cellulaire.

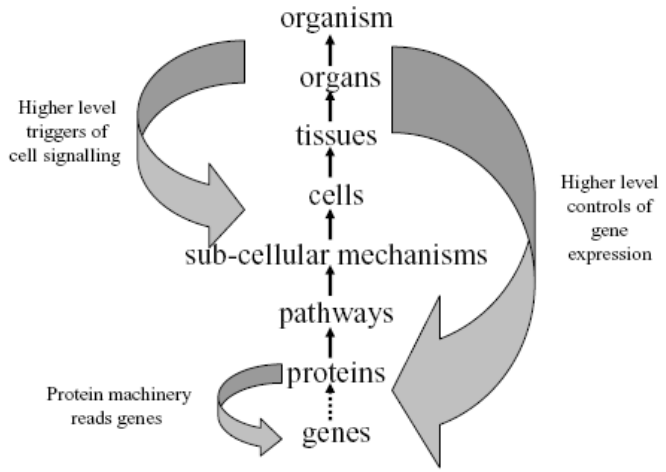


Schéma 2 : Causalité descendante ou « downward ». Le schéma 1 a été complété par une représentation de la signalisation cellulaire et la régulation rétrograde de l'expression des gènes. [D'après Noble, 2006].

Nous voyons finalement que loin d'être égoïste, le gène est plutôt au service de l'organisme, en interaction avec son environnement et doit chercher à coopérer avec d'autres gènes ou protéines pour assurer une fonction physiologique.

Dès 1944, Erwin Schrödinger dans son très célèbre livre « What is Life? » décrit la vie comme un 'cristal apériodique' qui ne répond à aucune séquence ordonnée ou régulière. Il prédisait déjà que la vie n'était pas juste une séquence chimique régulière mais que le code se trouve plutôt dans les protéines que dans l'ADN [Schrödinger, 1944].

I. 1.1.2 Biologie systémique et médecine

L'étude des systèmes n'est évidemment pas purement épistémologique. Georg Wilhelm Friedrich Hegel, un philosophe allemand de la fin XVIIIème-début XIXème siècle, dans sa recherche de l'Idée Absolue [Hegel, The encyclopedia logic], a déterminé une méthodologie basée sur la logique pour appréhender l'Ensemble plutôt que les parties isolées de la pensée. La célèbre dialectique hégélienne à trois moments : thèse, antithèse, synthèse, peut très facilement être adaptée à la volonté de la Biologie Systémique de regrouper ce qui pourrait sembler être des oppositions au niveau méthodologique pour les réconcilier sur le plan idéologique. La médecine (thèse), par ses observations phénoménologiques, est opposée au réductionnisme (antithèse) et l'étude des objets dans leur état fondamental, et la biologie systémique (synthèse) s'appuie sur les résultats des domaines réductionnistes pour étudier les propriétés d'un système (**Schéma 3**). Cette dernière est elle-même mise au service de la médecine grâce notamment aux mathématiques appliquées et aux outils informatiques qui permettent la modélisation de processus physiologiques ou physiopathologiques.

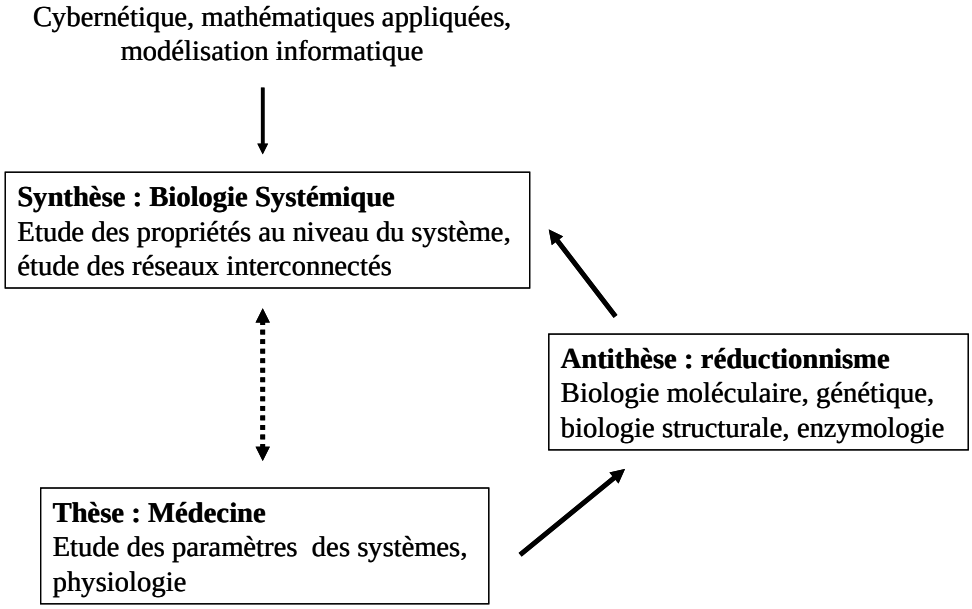


Schéma 3 : Logique hégélienne de réflexion et d'approche des sciences biologiques. [Modifié d'après Saks 2009].

Effectivement, au-delà de la stricte étude de processus biologiques, la richesse de la biologie systémique réside dans sa capacité à être complémentaire d'outils puissants tels que la modélisation mathématique ou la biophysique pour accéder à une dimension plus quantitative. Au début de siècle dernier un mathématicien américain, Norbert Wiener, imagina un mot pour donner une identité au transfert des idées traitant de communication et de contrôle, de la technologie vers la biologie : la cybernétique [Wiener, N. 1948]. Quand les concepts de dynamique, de régulation et de contrôle des fonctions biologiques furent combinés à des modèles mathématiques bien établis (obtenus au départ à partir du réductionnisme) alors l'approche systémique de la biologie prit forme. Le but idéaliste de la biologie des systèmes combiné avec l'informatique et les mathématiques a été très bien exposé par le physiologiste français Claude Bernard à la fin du XIXème siècle. Claude Bernard est considéré comme l'un des premiers fondateurs de la biologie systémique actuelle [Noble, 2006] (bien qu'à l'époque le terme employé était 'matérialisme holistique'). Il a, en effet, introduit le terme d' « équation générale » pour décrire les mécanismes de la vie à travers la modélisation mathématique [Claude Bernard, Introduction à l'étude..]. Cependant à l'époque de Claude Bernard la science ne disposait que d'assez peu de données expérimentales : « C'est par elle seule [l'application mathématique] que, dans la suite, la science se constituera ; seulement j'ai la conviction que l'équation générale est impossible pour le moment, l'étude qualitative des phénomènes devant nécessairement précéder leur étude quantitative ». Désormais, la biologie systémique dispose des données expérimentales nécessaires pour relever le défi de « l'équation générale » [Saks, 2009, article 1].

I. 1.2 Bioénergétique systémique moléculaire

La bioénergétique systémique moléculaire est une nouvelle direction scientifique, faisant partie intégrante de la biologie systémique et qui décrit la bioénergétique cellulaire à partir des processus moléculaires jusqu'au niveau systémique. Elle étudie et décrit non seulement le métabolisme énergétique comme un réseau intégré d'interactions mais aussi ses aspects spatiaux (organisation) et temporaux (dynamique). En effet, la bioénergétique systémique moléculaire considère l'organisation intracellulaire comme étant dynamique et dont la topologie elle-même est porteuse d'information. C'est pourquoi l'étude des propriétés au niveau du système est essentielle à la compréhension des phénomènes cellulaires.

I. 2 Importance de l'organisation intracellulaire pour la régulation métabolique

Les précédents travaux réductionnistes ont élargis le champ de connaissance des processus moléculaires laissant la place à présent pour une bioénergétique systémique moléculaire basée sur l'étude des interactions. En effet, la régulation métabolique passe par des interactions directes ou indirectes avec l'environnement. Le chemin parcouru jusqu'à la mitochondrie ou d'autres organites est déterminant pour cette régulation car il existe plusieurs obstacles à sa diffusion : les interactions protéine-protéine, les barrières physiques (membranes, réseau de protéines du cytosquelette …), les processus métaboliques AT/DP dépendants, les phénomènes d'absorption-désorption sur les surfaces [Wheatley, 1995], l'encombrement moléculaire (**Figure 1**). Le volume intracellulaire est en effet occupé par 20 à 30 % de protéines qui peuvent atteindre la concentration de 300 mg/ml [Fulton, 1982 ; Srere, 2000]. Dans la mitochondrie, la densité en enzymes et autres protéine est encore plus élevée et représente plus de 60 % du volume matriciel [Scalettar, 1991].

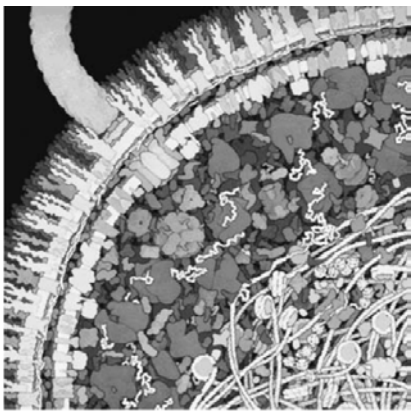


Figure 1 : Illustration de l'encombrement moléculaire intracellulaire. [D'après Goodsell, 1999].

En plus de l'encombrement moléculaire, les membranes et les surfaces dues au cytosquelette sont autant d'obstacles à la diffusion et au transport des métabolites. Gershon et Peters [Gershon, 1983 ; Peters, 1986] ont déterminé que pour une cellule de 1000 μm^3 (volume cytoplasmique) le cytosquelette occuperait plus de 20 % du volume total pour une surface de l'ordre de 10^6 μm^3.

Cet encombrement intracellulaire aboutit à la proximité des différents acteurs métaboliques et donc à une interaction facilitée. Par conséquent, il existe dans les cellules des propriétés au niveau du système qui n'existe pas dans un contexte hors cellule, c'est-à-dire *in vitro*.

I. 2.1 Compartimentation et micro-compartimentation

Le terme de compartimentation est généralement rattaché à l'existence de macro-compartiments, qui sont d'une dimension supérieure à la dimension moléculaire, et de micro-compartiments dont la taille est de l'ordre des métabolites. Le terme compartiment évoque des régions où les réactions biochimiques sont cinétiquement isolées du reste des processus cellulaires [Saks, 1994 ; Friedrich, 1985]. Les micro-compartiments impliquant les notions de complexes multienzymatiques et de canalisation métabolique sont la base de l'organisation et de la régulation du métabolisme énergétique [Saks, 2006, article ; Saks, 2007, article].

La canalisation métabolique d'un intermédiaire entre deux enzymes (ou entre un transporteur et une enzyme) peut se produire via des micro-compartiments ou un transfert direct [Srivastava, 1986 ; Qian, 2002 ; Huang, 2001]. Dans les deux cas il en résulte un couplage fonctionnel (couplage fonctionnel = canalisation métabolique + microcompartimentation [Saks, 2004]). Ces couplages fonctionnels permettent ainsi l'accumulation de produits et d'intermédiaires de réaction dans un espace restreint. La micro-compartimentation doit être un processus dynamique qui résulte dans la coexistence d'un jeu de réseaux métaboliques organisés [Friedrich, 1985]. L'importance de l'existence de tels phénomènes réside en partie dans le fait que des systèmes multienzymatiques associés physiquement ont le potentiel d'exprimer des propriétés catalytiques uniques en contraste à des systèmes ou des enzymes isolées [Gaertner, 1978]. La compartimentation peut concerner aussi bien les métabolites que les enzymes. Dans le cas des enzymes, le terme 'metabolon', proposé par Paul Srere [Srere, 1985], s'applique à des complexes multi-enzymatiques impliqués dans des cascades de signalisation ou des processus métaboliques tels que la glycolyse [Kurganov, 1985 ; Maughan, 2005] ou le cycle de Krebs [Ve´lot, 1997]. Dans les muscles squelettiques, les enzymes de la glycolyse sont liées à l'actine et des protéines associées [Knull, 1990 ; Masters, 1996]. Dans le cerveau 5 enzymes (glycéraldéhyde phosphate isomérase, glycéraldéhyde phosphate déshydrogénase (GAPDH), aldolase, pyruvate kinase (PK) et la lactate déshydrogénase (LDH)) sont associées à la membrane plasmique via l'actine (**Figure 9**) [Knull, 1980] ce qui permet un cycle production- consommation d'ATP efficace entre la glycolyse et les pompes membranaires Na+/K+ ATPases [Knull, 1978] et Ca2+ ATPase [Paul, 1989 ; Hardin, 1992]. D'ailleurs, le découplage des enzymes glycolytiques productrices d'ATP avec la membrane plasmique devient pathologique pour la cellule et dégrade les fonctions cognitives [Ueda, 2007].

Les premières expériences menant à penser qu'il existe une compartimentation des métabolites, et plus particulièrement de l'ATP, ont été menées par Gudbjamason [Gudbjamason, 1970] et Neely [Neely, 1973] sur le métabolisme cardiaque en condition ischémique (**Figure 2**). Ils ont parallèlement observé une très nette diminution de la quantité de PCr pendant toute la durée d'une contraction (diminution d'environ 80 %) alors que le contenu en ATP n'a diminué que d'environ 10 % pendant le même temps.

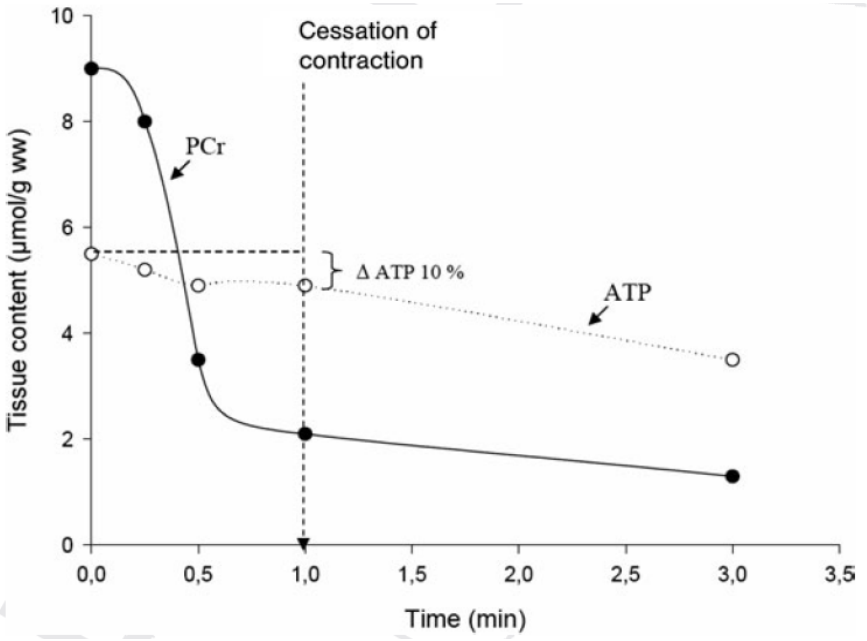


Figure 2 : Contenu cellulaire en phosphates de 'haute énergie' durant la contraction en conditions ischémiques. Alors que la concentration en phosphocréatine (PCr) est très fortement diminuée pendant la contraction, la quantité d'ATP de subit une variation que minime de 10 %. [D'après Saks, 2007, article 3].

Plusieurs hypothèses furent émises pour expliquer le fait que la contraction est stoppée alors qu'il subsiste encore 90 % d'ATP dans les tissus : changement de pH, variation de la sensibilité de la troponine au calcium…Mais l'explication la plus probable en tenant compte de l'échelle de temps, fut celle de la compartimentation de l'ATP. En effet, cette théorie explique très bien le fait que sur l'ATP total contenu dans une cellule, uniquement 10 % est situé au niveau des sites les plus énergivore pendant la contraction, le reste n'étant pas sollicité. D'autres travaux ont montré que le contenu cellulaire en ATP pouvait être diminué de 70 % sans influencer la contraction [Neely, 1984 ; Kupriyanov, 1987 ; Kupriyanov, 1991]. Les processus impliqués dans la contraction musculaire semblent donc dépendre d'une petite fraction d'ATP (dizaine de %). Les processus métaboliques étant extrêmement sensibles aux concentrations et rapports de concentration des nucléotides adényliques (ATP, ADP, AMP),

la question de leur compartimentation est un thème central dans la régulation métabolique mais aussi dans les processus contractiles ou de mobilité cellulaire. Le maintien d'un ratio ATP/ADP a été montré essentiel dans la régulation de la contraction musculaire [De Groof, 22002].

Jusqu'à présent les concentrations d'ATP obtenues dans la littérature sont essentiellement déduites de techniques mesurant les concentrations cellulaires totales d'ATP (^{31}P-RMN…). Des études basées sur une technique de perméabilisations contrôlées a permis de déterminer que, dans plusieurs types cellulaires, 74 % des nucléotides totaux sont localisés dans le cytoplasme, 20 % dans la mitochondrie et 6 % dans des structures cellulaires [Geisbuhler, 1984]. Mais il n'y avait encore aucun moyen de mesurer des concentrations locales d'ATP, jusqu'au développement de techniques d'imagerie assez sophistiqués pour avoir accès aux informations de concentrations micro-compartimentées et de micro-concentrations. Par exemple, les techniques de comptage de photons émis par bioluminescence ont permis une distinction dans les concentrations intracellulaires d'ATP selon les compartiments sondés (cytosol, mitochondrie, sites submembranaires) [Kennedy, 1999].

Très récemment, Inamura et al [Inamura, 2009] ont développé une technique basée sur la fluorescence de transfert d'énergie de résonance (FRET). Deux sondes ont été construites sur la sous-unité ε de l'ATP -synthase qui, en présence d'ATP qui provoque leur proximité spatiale, permettent une mesure locale des concentrations d'ATP, de 2 µM à 8 mM. Un adressage mitochondrial, nucléaire et cytoplasmique des sondes a permis de conclure que la concentration d'ATP mitochondriale est nettement inférieure à celles obtenues dans le noyau ou le cytoplasme des cellules cancéreuses Hela utilisées [Inamura, 2009]. Il apparaît maintenant comme une évidence que ce n'est pas la concentration globale d'ATP qui est importante mais plutôt l'ATP disponible dans les micro-compartiments cellulaires.

I. 2.2 La diffusion des métabolites

L'organisation et l'architecture intracellulaire déterminent la distribution spatiale et temporelle des métabolites. Il semble donc très difficile d'imaginer qu'il puisse exister une diffusion libre des métabolites dans le cytosol, et à plus forte raison des nucléotides adényliques qui sont impliqués dans de très nombreuses réactions. Cette question de la diffusion de l'AD/TP est actuellement un grand sujet de débat [Saks, 2008].

Deux écoles de pensée s'affrontent sur cette question sur le terrain glissant du métabolisme énergétique. Le premier courant de pensée considère que le milieu intracellulaire est

homogène et que par conséquent la diffusion des métabolites (et donc de l'ATP) est homogène [Barros, 2007 ; Kongas, 2000]. Le deuxième considère la richesse des structures intracellulaires (**Figure 1)** et processus métaboliques aboutissant à une hétérogénéité de la diffusion [Selivanov, 2007 ; Vendelin, 2008]. La différence majeure entre ces 2 directions est que la première est basée uniquement sur de la modélisation mathématique théorique alors que la deuxième intègre des données expérimentales à sa modélisation. Ces études sont toutes deux basées sur la modélisation de la diffusion des nucléotides adényliques dans le muscle et une analyse comparative de ces 2 différents modèles sera proposée à la section I. 5.3.

I 2.2.1 Equation de Fick

La diffusion en solution a été décrite de manière macroscopique (phénoménologique) par Adolph Fick au milieu du XIX^{ème} siècle [Fick, 1855]. La première loi de Fick,

$$J = -D\frac{\delta c}{\delta x} \qquad (1)$$

relie linéairement le flux instantané J en x_0 à l'instant t, au coefficient de diffusion (ou de diffusivité) D et au gradient de concentration ($\delta c / \delta x$) de la molécule. D'après cette loi, la diffusion est le mouvement des molécules d'une région de forte concentration vers une région de faible concentration dans un milieu aqueux 'continu'. A l'époque de Fick, beaucoup de scientifiques pensaient pouvoir expliquer tout les phénomènes biologiques par la physique (mécanique, thermodynamique, optique, électricité et magnétisme) [Agutter, 2000]. Ce courant émergent, appelé le matérialisme mécanistique, rendit la théorie de la diffusion de Fick largement populaire en dépit de son absence de relevance en condition intracellulaire.
En effet, étant donné l'encombrement, la compartimentation et les barrières physiques intracellulaires, les principes de diffusion classique (en solution) ne sont pas appropriés pour l'étude de la diffusion des métabolites dans leur environnement cellulaire. En effet, la diffusion d'une molécule est fonction de l'agitation thermique (mouvement des molécules en fonction de la température) et peut être considérée comme la résultante de collisions, de chocs avec les molécules ou structures environnantes créant un déplacement stochastique.

I 2.2.2 Equation d'Einstein-Smoluchowski

Cette notion de mouvement aléatoire est modélisée par la théorie du mouvement brownien (également appelé random walk) décrite par le botaniste Robert Brown en 1827 [Brown,

1828] après observation au microscope de particules de pollen flottant dans de l'eau et décrivant des trajectoires aléatoires. Dans les années 1870, ce mouvement commence à être expliqué comme la résultante de collisions entre les particules en suspension et les molécules du fluide. Au tout début du XX^{ème} siècle, Albert Einstein et Gabriel Stockes ont établit une relation entre la viscosité du milieu et le coefficient de trainée, qui correspond à la résultante des pressions subies par la particule mobile dans un fluide :

$$\gamma = 6\pi\eta R \qquad (2)$$

où γ est le coefficient de trainée (drag force), η le coefficient de viscosité du milieu et R le rayon de la particule.

La mobilité de la particule (molécule unique, protéine…) est reliée au coefficient de trainée et au coefficient de diffusion par l'équation d'Einstein-Stokes :

$$D = \mu p k_B T \qquad (3)$$

où D est le coefficient de diffusion, μ_P la mobilité de la particule, k_B la constante de Boltzmann (k_B = 1.38065× 10^{-23} J.K^{-1}) et T la température absolue. Comme $\mu_{P=1/}\ \gamma$, d'après les équations (2) et (3) on obtient :

$$D = \frac{k_B T}{6\pi\eta R} \qquad (4)$$

Cette équation représente le mouvement d'une particule sphérique évoluant dans un fluide.

Albert Einstein [Einstein, 1905 ; Einstein, 1956] et Marian Smoluchowski [Smoluchowski, 1906] décrivent indépendamment la diffusion à un niveau plus microscopique, en tenant compte du caractère stochastique de la diffusion (mouvement brownien).

L'équation d'Einstein-Smoluchowski définit le coefficient de diffusion en fonction du déplacement λ et du temps t de ce déplacement :

$$D = \frac{\lambda^2}{2t} \qquad (5)$$

Ce coefficient de diffusion D a été établit pour un mouvement unidimensionnel, mais peut être étendu à des mouvements bi-dimensionnels $D = \frac{\lambda^2}{4t}$, ou tri-dimensionnels $D = \frac{\lambda^2}{6t}$.

Cependant, les phénomènes diffusifs intracellulaires ne répondent pas simplement à une équation mais sont une somme de différents comportements fluctuant selon les influences environnementales.

I 2.2.3 Théorie de la diffusion appliquée au milieu intracellulaire

Le transport métabolique intracellulaire est une combinaison de différents facteurs faisant intervenir la diffusion des particules mais ne peut être entièrement représenté par la diffusion brownienne. La canalisation des métabolites permet en effet le transfert direct d'un intermédiaire d'une protéine ou d'une enzyme à une autre sans passer pas une diffusion libre en phase aqueuse. Ainsi, la compartimentation métabolique ou enzymatique peut aboutir à la perte du caractère aléatoire de la diffusion pour dériver vers une diffusion plus coordonnée et vectorielle [Agutter, 1995 ; Srivastava, 1986]. Même si l'approche brownienne contribue significativement aux mouvements particulaires, le nombre de molécules ayant un mouvement aléatoire dans un compartiment subcellulaire serait trop petit pour que les prédictions statistiques de la théorie de la diffusion soient applicables [Donnan, 1927 ; Schrödinger, 1944 ; Halling, 1989]. De même, la loi de Fick n'est adaptée que pour des milieux considérés homogènes et même si dans le cytoplasme certains petits volumes peuvent être considérés comme homogènes, le niveau de complexité et d'intensité des interactions et des liaisons physico-chimiques qui s'y déroulent rendent la notion de diffusion plus difficilement interprétable [Agutter, 1995].

De la même façon, considérant les travaux de Smoluchowski [Smoluchowski, 1906], la généralisation des coefficients de diffusion n'est appropriée que si le milieu homogène contient un nombre assez important de particules identiques (i.e. de même masse), ce qui n'est souvent pas le cas dans le cytoplasme. C'est pourquoi Tyrrell [Tyrrell, 1961] s'interrogea si les paramètres mesurés concernent effectivement le mouvement des molécules dans le système (diffusion pure) ou s'ils concernent les vitesses d'échange entre particule et solution (par solution comprendre : cytoplasme et tout ce qui s'y trouve). Il attira alors l'attention sur difficulté d'interprétation des coefficients de diffusion et Agutter [Agutter, 1995] conseilla d'utiliser le terme 'coefficient empirique de transport'. Plusieurs auteurs utiliseront par la suite le terme de coefficient apparent de diffusion [De Graaf, 2000 ; Saks, 2003].

I. 3. Bioénergétique fondamentale

Qu'est-ce que la bioénergétique ?

La bioénergétique est l'étude des flux, des échanges, des conversions énergétiques cellulaires via la rupture de liaisons hautement énergétiques. Elle s'intéresse à divers processus cellulaires et en particulier la respiration mitochondriale qui fournit l'une des molécules les plus riches en énergie (au travers de l'hydrolyse de la liaison γ-phosphate), l'ATP.

I.3.1 Historique

I. 3.1.1 La mitochondrie et l'oxydation phosphorylante

C'est au milieu du XIXème siècle qu'un médecin allemand, Rudolph Albert von Kölliker, décrit la structure de la mitochondrie par microscopie photonique. Il révèle sa structure compartimentée : membrane externe, espace intermembranaire, membrane interne et matrice. Les modèles les plus récents de structure mitochondriale ont été proposé par Manella en 2006 [Manella 2006] par tomographie cryo-éléctronique (**Figure 3**) et mettent en évidence la présence de cristae et la dynamique de la membrane interne selon l'état fonctionnel de la mitochondrie.

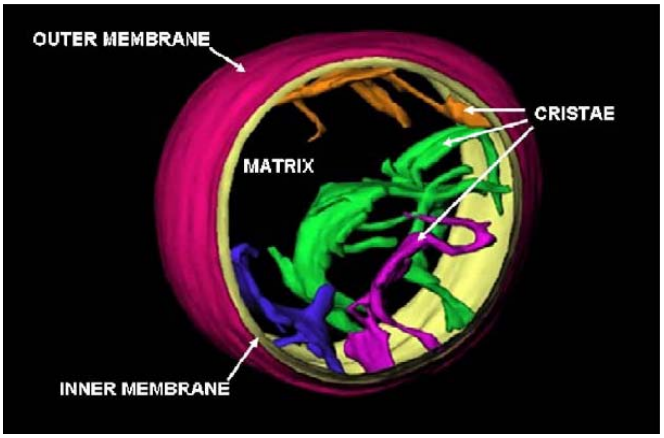


Figure 3 : Modèle de structure mitochondriale dérivé de tomographie cryo-éléctronique de mitochondries isolées de foie de rat. Le diamètre approximatif de cette mitochondrie est de 700 nm. [D'après Manella , 2006].

Wladimir A. Engelhardt en 1930 [Engelhardt, 1930, Engelhardt 1932] étudia une corrélation négative entre la respiration cellulaire et l'accumulation de phosphates inorganiques dans les erythrocytes. C'est à cette époque qu'apparu le terme phosphorylation oxydative (OxPhos). L'implication de la mitochondrie dans la production de l'ATP (découvert par Karl Lohmann en 1929 à partir d'extraits de muscles et de foie [K. Lohmann, 1929]) ne survient qu'en 1949, quand Kennedy et Lehninger montrent, à partir de mitochondries isolées de foie, que le cycle

de Krebs, la bêta-oxydation des acides gras et l'OxPhos ont lieu dans la mitochondrie [Lehninger, 1949, Kennedy, 1949].

I. 3.1.2 La théorie chimio-osmotique

Ce n'est qu'en 1961 que Peter Mitchell formule la théorie chimio-osmotique [Mitchell, 1961] et émet l'hypothèse que le processus de l'OxPhos ne met pas en jeu un intermédiaire chimique de 'haute énergie'comme il était couramment admis [Slater, 1953] mais un potentiel électrochimique. Le **Figure 4** décrit la composition de la chaîne respiratoire dans la membrane interne mitochondriale. Le chaîne de transport d'électrons est constitué de 4 complexes : le complexe I (NADH CoQ oxydoréductase), le complexe II (succinate CoQ oxydoréductase), le complexe III (CoQ-cytochrome c oxydoréductase) et le complexe IV (cytochrome c oxydase).

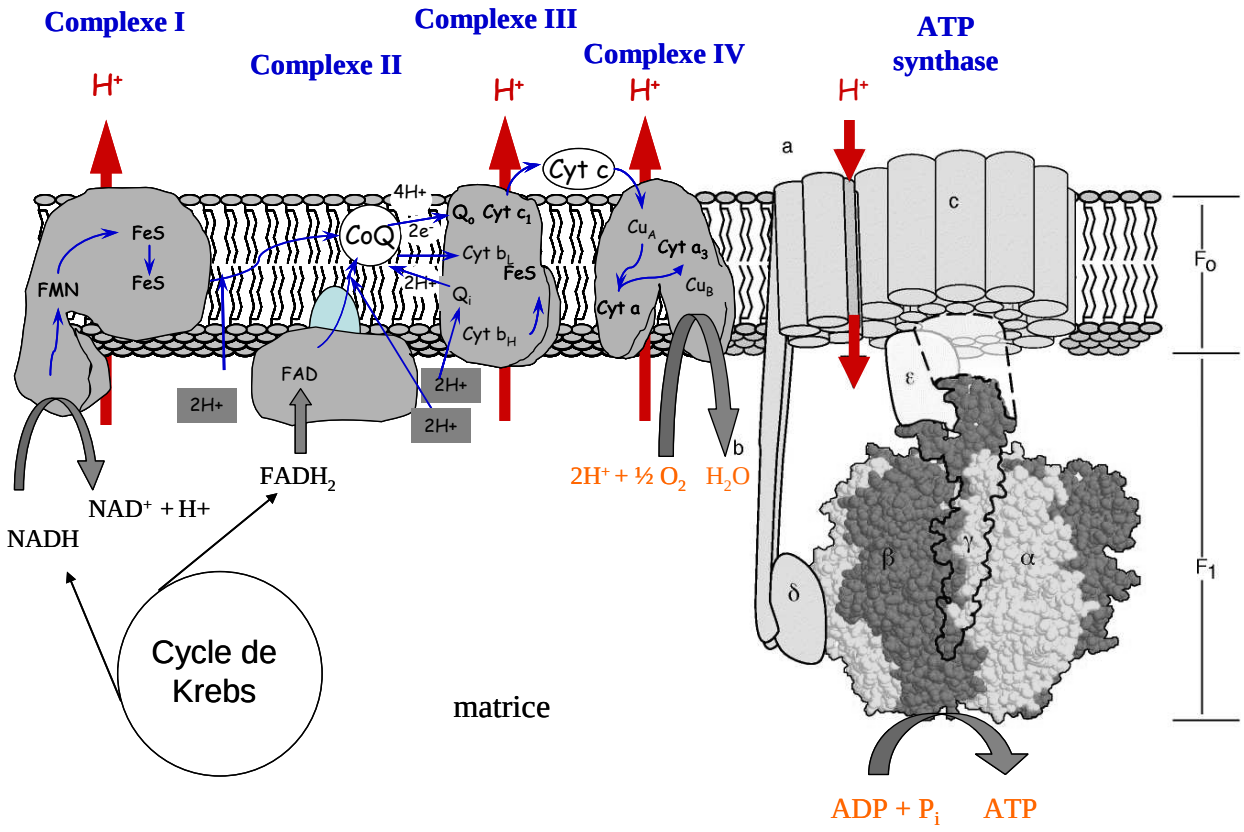


Figure 4 : Composition de la chaîne respiratoire mitochondriale. Cyt : cytochrome, CoQ : coenzyme Q.

Le passage des protons par les complexes I, III et IV conduit à la création d'un gradient de concentration de protons et donc une différence de pH entre l'espace inter-membranaire et la matrice (ΔpH). Dans le même temps, le transfert des électrons dans la chaîne crée une différence de potentiel électrique (ΔΨ). La théorie chimio-osmotique de Mitchell repose sur la force proton-motrice (Δp) engendrée par le pompage des protons et s'exprime de la façon suivante : $\Delta p = \Delta\Psi - Z\,\Delta pH$, où $Z = 2{,}3RT/F$.

C'est en se servant de cette force proton-motrice que l'ATP synthase peut phosphoryler un ATP à partir d'un ADP et d'un phosphate inorganique (Pi), selon la réaction de base de l'énergétique cellulaire :

$ADP + Pi + H^+ \rightarrow ATP + H_2O$

La mitochondrie convertit donc l'énergie libre des réactions d'oxydo-réduction en énergie libre d'hydrolyse [Westerhoff, 1987].

I. 3.1.3 Le potentiel de phosphorylation

L'ATP, de par la richesse énergétique libérée par la rupture de la liaison γ -phosphate, est la principale source d'énergie des processus cellulaires (c'est pourquoi par la suite il sera utilisé le terme général 'énergie' pour désigner l'énergie libérée par l'hydrolyse des liaisons γ -phosphate). Le métabolisme cellulaire est un couplage entre catabolisme (utilisant protéines, glucides et lipides) et anabolisme (**Figure 3**).

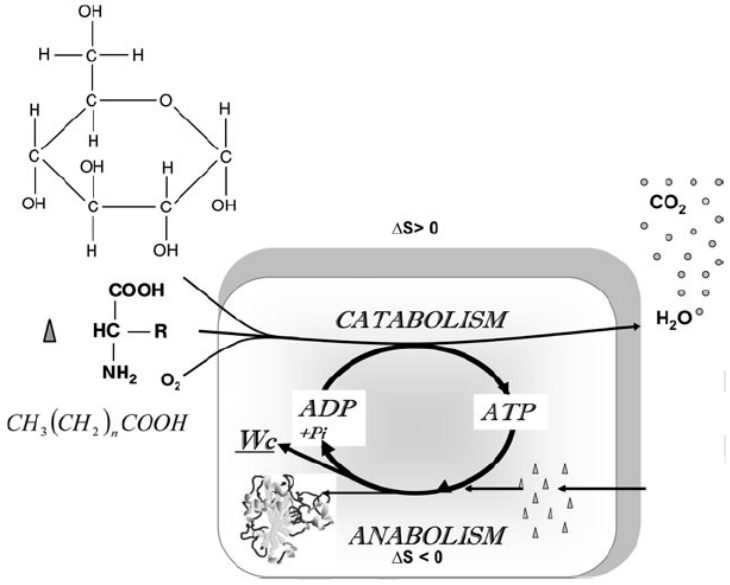


Figure 5 : Métabolisme cellulaire basé sur un couplage entre processus cataboliques (augmentation de l'entropie (ΔS>0) et production d'ATP) et processus anaboliques (diminution de l'entropie (ΔS<0) et utilisation d'ATP). Wc : travail cellulaire. [D'après Saks 2007, article 2]

L'ATP (ou plus largement les processus de phosphorylation/déphosphorylation) ont été choisi par l'évolution comme mécanisme ubiquitaire de la transduction des signaux cellulaires [Qian, 2007]. En effet, les concentrations intracellulaires d'ATP, d'ADP, d'AMP et de Pi sont des régulateurs allostériques de plusieurs processus métaboliques fondamentaux. Cependant, leur seule présence ne suffit pas à engendrer du travail cellulaire (Wc) comme le précisent Nicholls et Ferguson [Nicholls, 2002] « l'Océan Pacifique pourrait être rempli d'une mixture d'ATP, d'ADP et de Pi à l'équilibre, mais l'ATP n'aurait aucune capacité à engendrer du travail ». Ce ne sont donc pas *stricto sensu* les molécules de nucléotides adényliques qui permettent les conversions énergétiques mais bien les rapports des concentrations en ATP, ADP et Pi. Par exemple, le rapport ATP/ADP, qu'on appelle aussi potentiel phosphate, est un facteur régulateur de la respiration mitochondrial et contrôle le flux d'OxPhos. Ces rapports de concentration sont directement reliés à l'énergie du système. En effet, les processus de phosphorylation sont caractérisés par le potentiel de phosphorylation qui représente l'énergie libre disponible pour les processus cellulaires :

$$\Delta G_{ATP} = \Delta G^{0}_{ATP} + RT \ln \Gamma$$

où $\Gamma = \frac{[ATP]}{[ADP][P_i]}$ et correspond au **rapport d'action de masse** de la synthèse d'ATP,

et $\Delta G^{0}_{ATP} = - RT \ln K_{eq} = 32$ kJ/mol

Le rôle de la mitochondrie ne se limite pas à une « usine d'ATP » mais s'étend au maintien du ratio Γ à une valeur élevée (10^{10} fois plus élevé que la constante d'équilibre : $K_{eq} \sim 10^{-5}$ M et $\Gamma \sim 10^{5}$ M) et par conséquent permettent le maintien d'un haut potentiel de phosphorylation ~ 60 kJ/mole et l'apport d'énergie libre pour le travail cellulaire [Nicholls, 2002].

Mais l'ATP n'est pas la seule molécule de haute énergie et dans beaucoup de cellules une autre molécule fonctionne en synergie avec l'ATP pour le contrôle, la signalisation et la régulation du métabolisme énergétique : la phosphocréatine (PCr), synthétisée par la créatine kinase.

I. 3.2 Les créatine kinases

La réaction enzymatique de la créatine kinase (CK) a été mise en évidence par Karl Lohmann en 1934 [Lohmann, 1934]. Elle suit un mécanisme BiBi aléatoire en quasi-équilibre, d'après

la classification de Cleland **(Schéma 4**) et catalyse le transfert réversible d'un groupement phosphoryl de l'ATP vers la créatine (Cr): $MgATP^{-2} + Cr \rightarrow MgADP + PCr^{-2} + H^{+}$

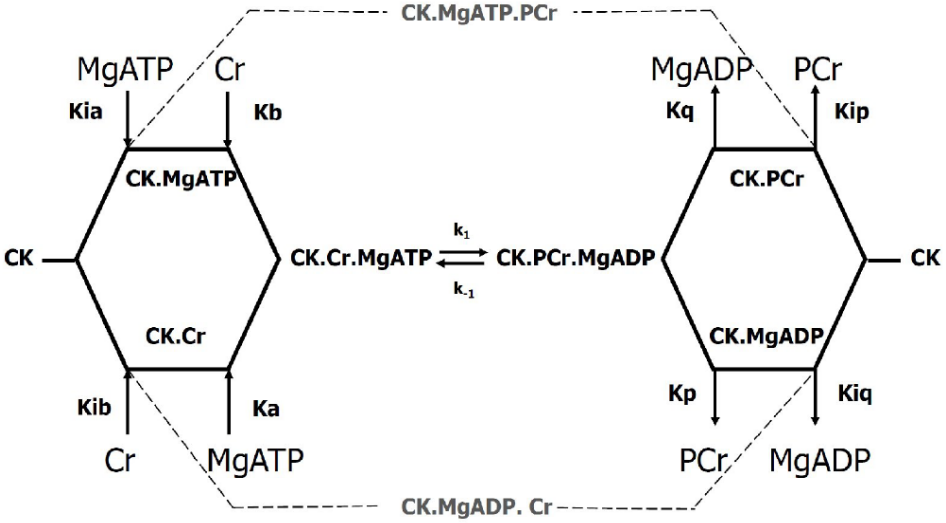


Schéma 4 : Réaction enzymatique de la créatine kinase.

Comme l'indique le **Schéma 4**, il existe deux états intermédiaires dans la réaction enzymatique : formation d'un complexe binaire CK.Cr ou CK.MgATP caractérisé par une constante de dissociation Kib ou Kia, respectivement, puis formation d'un complexe tertiaire CK.Cr.MgATP caractérisé par les constantes de dissociation Ka ou Kb selon le chemin suivi.

La CK est une enzyme clé du métabolisme énergétique car elle fournit la PCr dont le ΔG^{o} est proche de celui de l'ATP (ΔG^{0} PCr = - 42,8 kJ/mol). La PCr est une molécule plus petite et moins chargée que l'ATP et qui peut par conséquent diffuser plus librement. Elle intervient donc en couplage avec des processus demandeurs d'énergie plus ou moins éloignés des sites de production d'ATP. Dans la plupart des cellules, les capacités de la CK à régénérer l'ATP dépassent d'une part la consommation cellulaire en ATP et d'autre part la production propre d'ATP par les 2 principales sources, l'OxPhos et la glycolyse. Cependant, les pools intracellulaires d'ATP sont assez bas. Dans les cellules excitables (muscle, neurone...), la concentration d'ATP totale est d'environ 2-5 mM, quantité qui serait utilisée en quelques secondes d'activité [Infante, 1965] si la CK ne régénérait pas continuellement et efficacement l'ATP à partir de large pools de PCr. En effet, les concentrations de PCr peuvent atteindre 20-35 mM ou plus selon le type de muscle [Fitch, 1977 ; Carlson, 1974] et 5-10 mM dans le cerveau, les muscles lisses ou les reins [Iyengar, 1984 ; Erecinska, 1989].

L'activité des CKs est largement tissu-dépendante : les cellules cardiaques ont une activité CK très basse ainsi que les muscles lisses, tandis que les muscles rapides (dits muscles

glycolytiques) ont une activité CK plusieurs fois supérieure à celle des cardiomyocytes. Les créatines kinases ne sont pas exprimées dans le foie, qui sécrète cependant la créatine cellulaire.

Les CKs sont divisées en deux groupes : cytosoliques et mitochondriales (**Figure 6**).

I. 3.2.1 Les CK cytosoliques

Les isofomes cytosoliques de la CK sont dimériques et ont un poids moléculaire d'environ 86 kiloDalton (kDa). Les différents monomères sont soit dits musculaires (M, muscle) soit dits cérébraux (B, brain) (**Figure 4).** L'isoforme MMCK est assez spécifique des muscles striés et du muscle cardiaque et l'isoforme BBCK est prédominante dans le cerveau [Eppenberger 1964, Eppenberger 1967] mais son activité est élevée également dans les reins, les muscles lisses, les cellules épithéliales et l'intestin. Il existe aussi une isoforme hybride MBCK moins exprimée dans la plupart des tissus mais présentes dans le cœur et dans les muscles squelettiques en développement où s'opère une transition de l'isoforme BBCK vers l'isoforme MMCK.

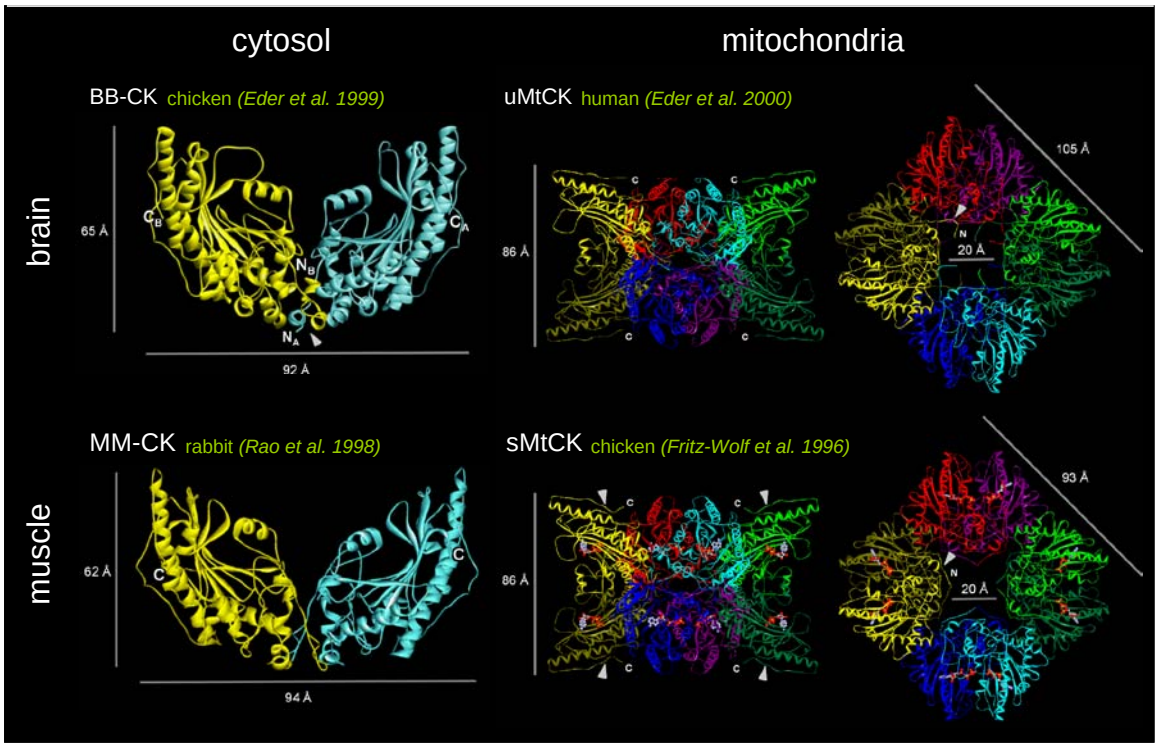


Figure 6 : Les différentes isoformes de créatine kinases. [Présentation originale de Uwe Schlattner]

I. 3.2.2 Les CK mitochondriales

Se sont dans les laboratoires de Martin Klingenberg que furent découvertes les isoformes mitochondriales de la CK [Jacobs, 1964]. Les CKs mitochondriales sont exprimées de façon tissu-sélective. Dans les muscles striés se trouvent l'isoforme sMtCK (sarcomeric mitochondrial CK) et dans le cerveau et les muscles lisses notamment se trouvent préférentiellement l'isoforme uMtCK (ubiquitous mitochondrial CK) (**Figure 6**). La sMtCK représente dans le cœur environ 40 % de l'activité CK cellulaire totale [Saks, 1974]. Ces isoformes sont octamériques et se situent sur la face externe de la membrane interne mitochondriale, dans l'espace inter-membranaire. Le récepteur membranaire des CKs mitochondriales est un composé phospholipidique, la cardiolipine [Müller, 1985 ; Schlattner 2009], qui est seul phospholipide de la membrane interne à être chargé négativement [Hovius, 1990].

I. 3.2.3 Le système CK/PCr et les couplages fonctionnels

Dans les cellules, les CKs sont généralement situées à proximité de sites de production d'ATP (la glycolyse, et en particulier la phosphofructokinase, la glycéraldéhyde phosphate déshydrogénase [Bloch, 1971] et l'aldolase [Kraft, 2000], et l'OxPhos) et des sites de consommation d'ATP tels que les ATPases myofibrillaires, les Ca^{2+}-ATPases (dont SERCA, la pompe calcique de reticulum sarcoplasmique ATP-dépendante) [Wallimann, 1984, Rossi, 1990] et les Na^{+}/K^{+}-ATPases [Blum, 1991]. En effet, l'isoforme MMCK par sa proximité avec les ATPases myofibrillaires et les ATPases calciques du reticulum sarcoplasmique (SERCA) permettent an apport rapide et efficace d'ATP aux processus contractiles [De Groof, 2002]. De même, l'isoforme BBCK a un rôle prépondérant dans l'apport énergétique de processus dynamiques ATP-dépendants liés au cytosquelette, et en particulier à l'actine [Kuiper, 2008], dans des cellules non-musculaires. Le groupe de Bé Wieringa, aux Pays-Bas, a montré que BCK contrôle la mobilité et l'extension de fibroblastes en compartimentant et donc en régulant les concentrations locales d'ATP à la membrane plasmique [Kuiper 2009, van Horssen, 2008]. Dans le cerveau, la localisation subcellulaire de la CK est très peu étudiée (ceci étant dû à l'hétérogénéité cellulaire du système nerveux central), néanmoins certaines études ont montré son association avec les vésicules synaptiques [Friedhoff, 1997] et les membranes plasmiques en conjonction avec les pompes ATPasiques Na^{+}/K^{+} [Lim, 1983 ; Blum, 1991].

I. 3.2.3.1 Le système CK/PCr

Le système CK/PCr à travers ses différents couplages fonctionnels a plusieurs rôles physiologiques.

1/ La phosphocréatine est usuellement appelée 'stock' d'énergie. Les hautes concentrations de PCr intracellulaires sont en effet un moyen pour la cellule de disposer d'une réserve d'énergie immédiate (fonction temporelle de tampon) [Wallimann, 1992].
2/ Les couplages fonctionnels de la CK permettent également de transporter l' « énergie » des sites de production d'ATP (OxPhos, glycolyse, synthèse d'ATP *de novo*) vers les sites de consommation (ATPases). Cette fonction est apellée fonction spatiale de tampon ou navette Cr/PCr, comme le proposa Bessman au début des années 80 [Bessman 1981]. Cette proximité physique CK/ATPase et les pools importants de PCr intracellulaires permettent donc un renouvellement efficace, local et rapide de l'ATP comme représenté sur la **Figure 7.**
3/ Le système CK/PCr maintient un niveau très bas d'ADP qui s'avère essentiel à la régulation métabolique cellulaire. En effet, les concentrations en ADP cellulaire sont de l'ordre de 50 à 100 µM dans les cellules cardiaques [Meyer, 1984] et de l'ordre de la centaine de µM dans le système nerveux central [Erecinska, 1989]. L'ADP étant un inhibiteur compétitif efficace des ATPases, un niveau d'ADP bas permet également d'éviter leur l'inhibition, le Ki (constante d'inhibition) étant proche de 200 µM [Yamashita, 1994].
4/ L'homéostasie énergétique dépend d'une composante essentielle qui est le rapport des concentrations en ATP sur celles en ADP (ATP/ADP). Le système CK/PCr a pour fonction de fournir un ratio local ATP/ADP approprié aux sites de couplages fonctionnels, et augmenter ainsi l'efficacité thermodynamique des processus d'hydrolyse de l'ATP [Kammermeier, 1987].
5/ La réaction de la CK utilise un proton dans le sens de la synthèse d'ATP, c'est à dire quand il y a une demande d'ATP à proximité des ATPases. Cette utilisation de proton permet d'éviter une acidification du milieu notamment en cas d'effort musculaire intense.

La théorie de navette Cr/PCr est basé sur l'idée de compartimentation subcellulaire (**Figure 7**) et une notion sous-jacente de ce concept est que le transport d' « énergie » est assuré par la PCr, et non pas par l'ATP directement [Srere, 1978, Sers, 1982].

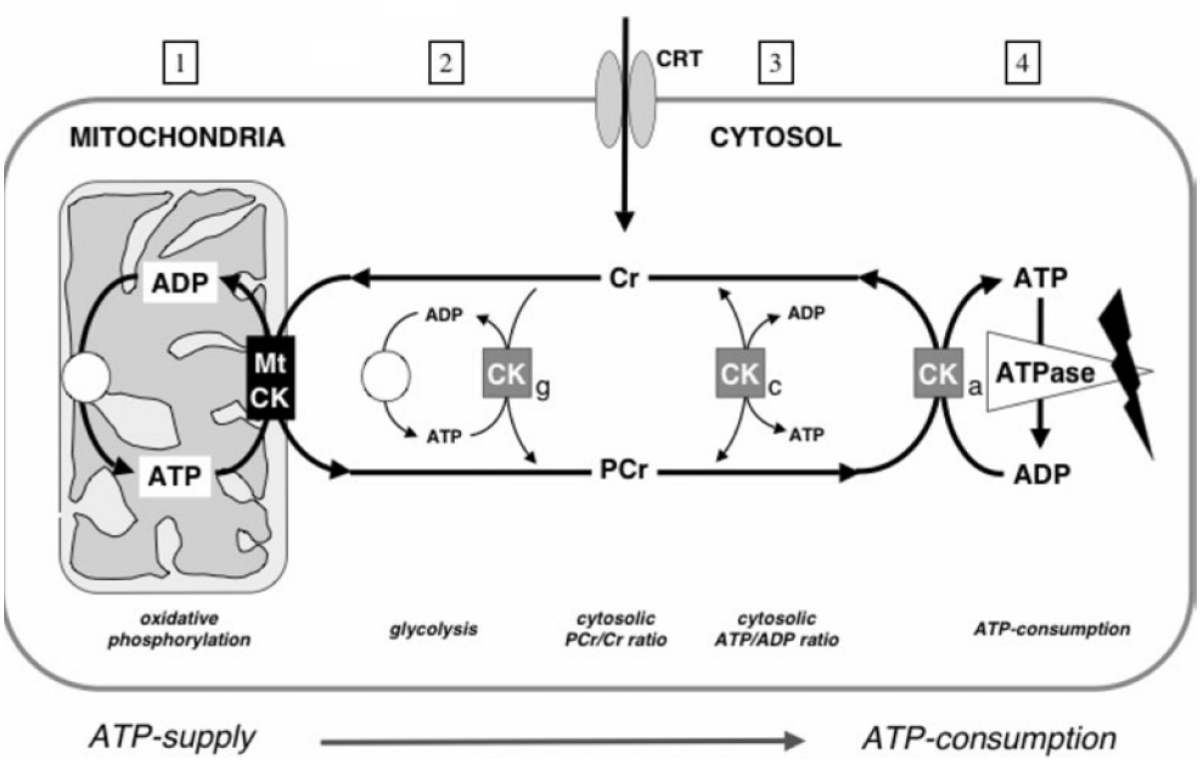


Figure 7 : Circuit créatine / phosphocréatine (Cr/PCr). Les créatines kinases (CKs) sont compartimentées pour permettre le maintien de l'homéostasie énergétique au niveau cellulaire. 1/ La CK mitochondriale permet une régénération local d'ADP et une production efficace de PCr. 2/ 3/ La CK cytosolique est couplée aux processus glycolytiques de production d'ATP et permet la constance des ratios ATP/ADP. 4/ Les ATPases membranaires étant de fortes consommatrices d'ATP, la CK cytosolique peut assurer l'apport en ATP nécessaire au fonctionnement des pompes ATPasiques à partir des pools intracellulaires de PCr. [D'après Wallimann, 2007].

Ce système est très efficace en terme de flux énergétiques et permet une certaine robustesse à la régulation métabolique tant pour les cellules musculaires striées que pour les cellules cardiaques ou les cellules nerveuses.

En résumé, le système CK/PCr diminue les [ATP] et régénère l'ADP aux sites de production d'ATP et diminue les [ADP] et régénère l'ATP aux sites de consommation d'ATP. Un exemple bien décrit de couplage fonctionnel entre CK et site de production d'ATP se situe dans la mitochondrie.

I. 3.2.3.2 La créatine kinase mitochondriale et le transporteur d'AT/DP

Les premières expériences qui ont mis en évidence un couplage fonctionnel des CKs mitochondriales avec l'OxPhos montraient que la consommation d'oxygène était stimulée par la Cr ce qui produisait de la PCr avec un rapport phosphate/oxygène (P/O) d'environ 3 [Belitzer, 1939]. Les mêmes auteurs ont observé qu'il se produit le même phénomène de stimulation de la respiration par la Cr sans addition d'aucuns nucléotides adényliques dans des homogénats de muscles squelettiques. De même, il a été observé qu'en présence d'ATP, la Cr augmentait significativement le stade 4* de respiration [Bessman, 1966 ; Vial, 1972] et qu'à une concentration physiologique de 10-15 mM l'ajout de Cr stimulait maximalement la respiration jusqu'au stade 3* [Jacobus, 1973] (**Figure 8**). Ces résultats préliminaires révélaient déjà l'utilisation efficace de l'ADP par les CKs et le contrôle de la production de PCr par l'OxPhos.

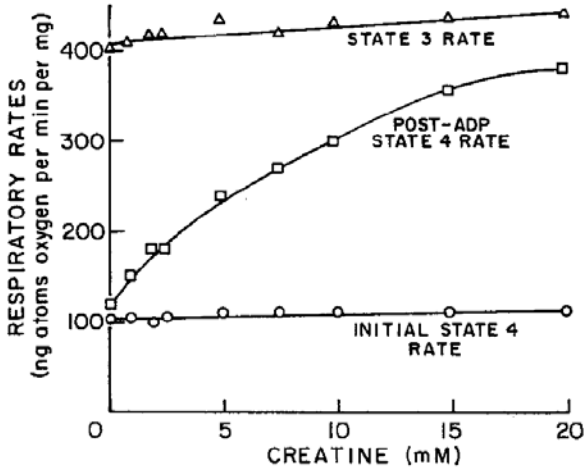


Figure 8 : Dépendance de la respiration mitochondriale à des concentrations croissantes de créatine dans une suspension de mitochondries isolées de cœur. La vitesse de respiration au stade 2 (initial state 4 rate,○) correspond à la vitesse de respiration a vant addition d'ADP, la vitesse de respiration au stade 3 (state 3 rate, Δ) correspond à celle mesurée immédiatement après addition de 300 µM d'ADP et la vitesse de respiration au stade 4 (post-ADP state 4 rate, □) correspond à celle mesurée après phosphorylation de tout l'ADP ajouté (300µM).

Les CKs mitochondriales (MtCK) sont en effet couplées fonctionnellement à la production d'ATP mitochondriale. Ce couplage a été mis en évidence dans les muscles par des techniques biochimiques, cinétiques, thermodynamiques et d'analyses radioisotopiques [Bessman, 1966 ; Jacobus, 1982 ; Gellerich, 1982; Kottke, 1991].

* Stade 1, stade de respiration sans substrats respiratoires ni ADP exogènes. Stade 2, respiration après addition de substrats de la chaîne respiratoire. Stade 3, respiration après addition de substrats de la chaîne respiratoire et d'ADP exogène (stade de respiration maximale). Stade 4, niveau de respiration quand tout l'ADP a été consommé (c'est-à-dire, phosphorylé en ATP). Stade 5, anoxie.

Il se manifeste par un transfert direct de l'ATP nouvellement synthétisé par l'ATP synthase dans la matrice mitochondriale vers la MtCK dans l'espace inter-membranaire via l'adenine nucleotide translocase (ANT, ou échangeur ATP/ADP) (**Figure 9**). Des études de stoechiométrie ont montré des quantités equimolaires de MtCK et d'ANT dans des mitochondries de muscles squelettiques et de cœur [Kuznetsov, 1986]. La position stratégique de la MtCK permet donc de phosphoryler la Cr en PCr, régénérant ainsi l'ADP. Cet ADP est disponible pour retourner dans la matrice via l'ANT et activer l'OxPhos en servant de substrat à l'ATP synthase.

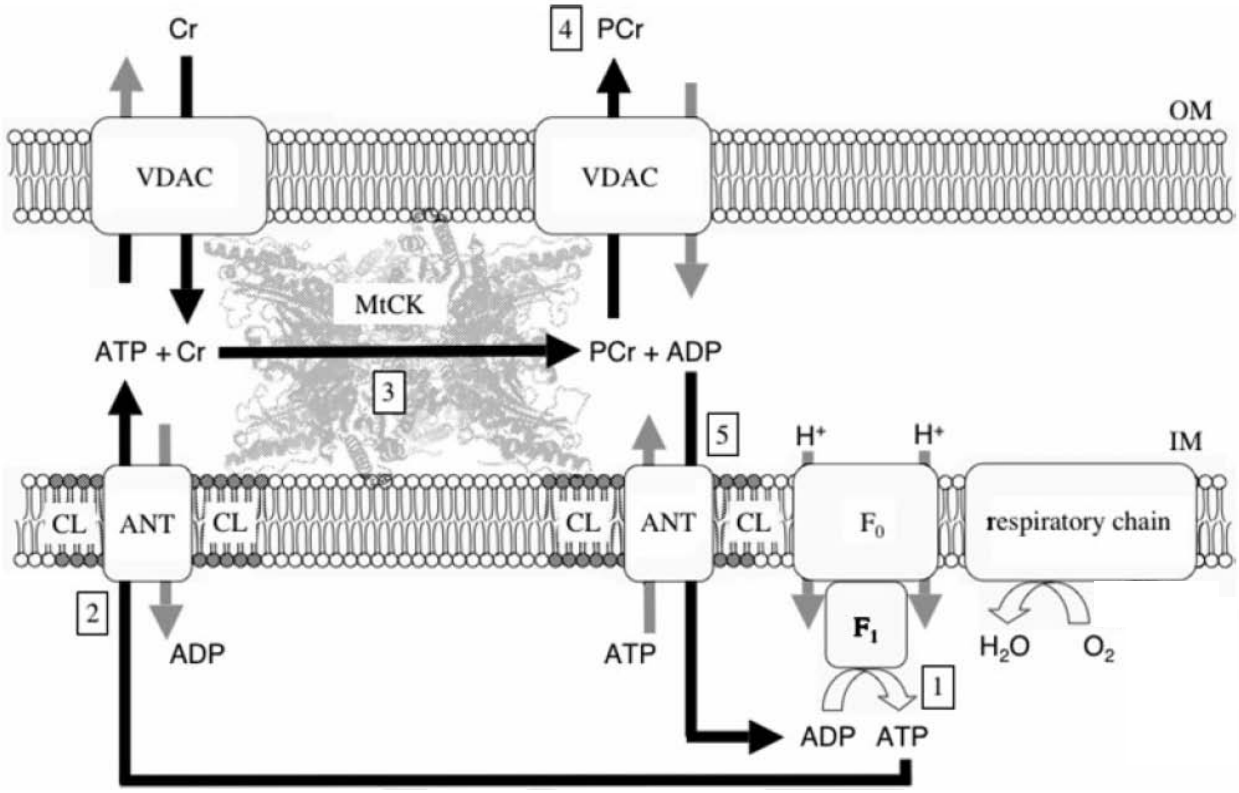


Figure 9 : Couplage fonctionnel entre la créatine kinase mitochondriale (MtCK) et l'adenine nucleotide translocase (ANT). (1) L'ATP est synthétisé par l'ATP synthase. (2) L'ATP nouvellement synthétisé est transporté de la matrice vers l'espace inter-membranaire via l'ANT. (3) L'ATP est hydrolysé par la MtCK et le groupement phosphate est transféré à la Cr. Les produits de cette réaction sont la PCr (qui sort de la mitochodnrie par le VDAC) et l'ADP. (5) L'ADP retourne dans la matrice via l'ANT et peut de nouveau activer l'OxPhos pour synthétiser une nouvelle molécule d'ATP. PCr : phosphocréatine, Cr : créatine, CL : cardiolipine, OM : membrane externe, IM : membrane interne, VDAC : voltage dependant anion channel. [D'après Wallimann, 2007].

Ce renouvellement rapide et local d'ADP (ou turnover) rend très efficace l'OxPhos et permet le maintien d'une haute concentration de PCr dans le cytoplasme. Ainsi la MtCK, l'ANT et le VDAC forment un canal efficace pour le transport et la régulation des flux énergétiques cellulaires en maintenant les concentrations en PCr et en ATP au niveau optimal et en minimisant l'énergie libre requise à la synthèse d'ATP. Ce couplage a d'importants rôles

physiologiques et physiopathologiques car il a été montré qu'il intervient dans la prévention de l'ouverture du pore de transition de perméabilité (PTP) mitochondriale et, donc, s'avère central pour la vie cellulaire [Dodler, 2003]. Des études de modélisation mathématique permettront de mettre en évidence les mécanismes cinétiques impliqués dans ce couplage (cf section I. 5.2) [Vendelin, 2004].

I. 3.2.3.3. Etat stationnaire hors équilibre des créatine kinases

Dans les réactions enzymatiques, il existe l'état d'équilibre rapide (RES, rapid equilibrium state) et l'état stationnaire hors équilibre (NESS, non-equilibrium steady state), en reprenant la terminologie du mathématicien Hong Qian [Qian, 2007]. L'équilibre d'une réaction enzymatique est en fait un cas particulier d'état stationnaire. Pour comparer ces deux états, considérons la réaction enzymatique :

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{P}$$

où les k_1 et k_{-1} sont les constantes cinétiques.

La vitesse nette V_n de réaction correspond à la différence des vitesses de réaction directe et inverse. Pour cette réaction : $V_n = 0$ pour un RES et $V_n \neq 0$ pour un NESS. En d'autres termes, le NESS est un système chimique où toutes les concentrations et les fluctuations sont stationnaires, où il existe des flux et qui dissipe de la chaleur. La vitesse nette ne peut exister qu'en cas de différence de potentiel chimique, c'est-à-dire en présence d'un gradient de concentration. Ce gradient n'est possible qu'en cas de système ouvert qui échange masse et énergie avec le milieu environnant. C'est pourquoi les systèmes ouverts tendent à être en NESS alors que les systèmes fermés vont tendre vers l'équilibre. Les énergies (découlant des différences de potentiels chimiques) impliquées dans le transport d'information doivent être également considérées en termes de production d'entropie, qui est en fait le concept central du NESS [Ruelle, 2003].

La deuxième loi de la thermodynamique stipule que dans un système isolé la tendance est à l'augmentation de l'entropie (correspondant à une augmentation du désordre, $\Delta S>0$). Une question fondamentale fût posée par Erwin Schrödinger : comment font les organismes vivants pour contredire cette deuxième loi de la thermodynamique ? Comment font-ils pour survivre et éviter leur propre désintégration ou décomposition rapide vers un état inerte d'équilibre ? La réponse à ses questions se trouve dans des phénomènes tels que la compartimentation et le métabolisme vectoriel qui compensent l'augmentation naturelle

d'entropie. Selon Schrödinger : « L'organisme vivant évite le déclin rapide vers l'état inerte d'équilibre en soutirant continuellement l'entropie négative (négentropie) de son milieu environnant » [Schrödinger, 1944]. En effet, les systèmes ouvert hors équilibre sont aussi appelés structures dissipatives [Nicolis, 1977] car en absence de travail ses systèmes dissipent leur énergie sous forme de chaleur et la transforme en entropie (qui deviendra de l'entropie négative pour l'environnement), impliquant donc que toutes les réactions biochimiques cellulaires hors équilibre sont des sources potentielles d'énergie libre.

Historiquement, la réaction de la CK a toujours été considérée comme étant à l'équilibre alors qu'en considérant les données précédentes de compartimentation, il ne semble pas raisonnable de n'imaginer aucun gradient de concentration d'ATP, d'ADP ou de Pi entre différents (micro-)compartiments, ce qui reviendrait à une concentration d'ATP ou d'ADP homogène dans le cytoplasme, et donc une vitesse nette nulle.

Dans le cas de la CK, l'orientation et le transport unidirectionnels des échanges de groupements phosphoryls permettent fortement d'augmenter l'efficacité thermodynamique cellulaire des processus anaboliques et compenser la tendance catabolique (augmentation de l'entropie) naturelle.

I. 3.2.3.4 Le démon de Maxwell

La seconde loi de la thermodynamique a toujours suscitée beaucoup de passion et d'interrogations car elle allait à l'encontre de la Vie. Effectivement, une augmentation constante de l'entropie ne serait pas compatible avec l'ordre et l'organisation que nécessitent les organismes vivants. Ces principes sont parfaitement illustrés par la théorie thermodynamique du démon de Maxwell.

En 1871, le physicien James Maxwell propose une expérience de pensée appelée par la suite 'le démon de Maxwell' [Maxwell, 1871]. Le cadre de l'expérience est un système fermé, en équilibre thermodynamique : pression et température constantes, pas d'échanges avec l'extérieur, et donc pas de travail possible (**Figure 10A**). Ce système est alors divisé en deux parties indépendantes (**Figure 10B**). Jusqu'ici les deux nouveaux systèmes sont identiques. Imaginons maintenant un minuscule trou dans lequel se trouve le démon (de taille moléculaire) qui réussi à faire passer une particule à la fois et qui sélectionne celles qui ont une vitesse supérieure à la moyenne (à température constante les vitesses des particules n'étant pas toutes égales mais distribuées selon la fonction de Boltzmann) (**Figure 10C**).

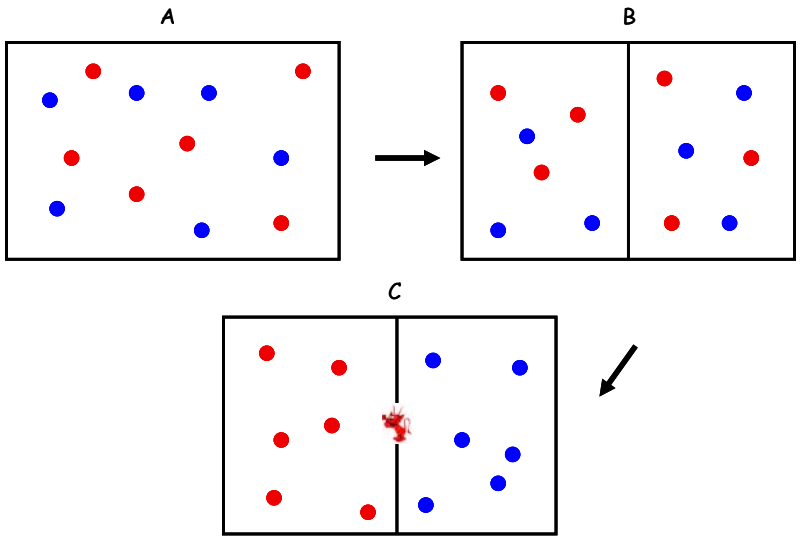


Figure 10 : Expérience de pensée de James Maxwell qui contredit la deuxième loi de la thermodynamique. A/ Système isolé à température et pression constantes. B/ Le système est divisé en deux systèmes égaux. C/ Le démon de Maxwell (de taille moléculaire) sélectionne le passage des particules dont la vitesse est supérieure à la moyenne, ce qui conduit à deux systèmes aux températures différentes.

Cette action du petit démon tend à perturber l'équilibre et à créer deux systèmes différents ayant chacun leur température propre (**Figure 10C**), ce qui est une violation totale de la deuxième loi de la thermodynamique. En effet, la conséquence de la perturbation de l'équilibre initial est que la différence de température (ou la chaleur ΔQ, correspondant à l'agitation thermique qui est alors interprétée à l'échelle microscopique comme une manifestation de l'agitation moléculaire) permet maintenant au système d'effectuer un travail W sans aucun apport d'énergie extérieur ce qui était a priori impossible.

La première loi de la thermodynamique (loi de la conservation d'énergie) :

$$\Delta E = \Delta W + \Delta Q$$

où ΔE est l'énergie totale du système, ΔW la partie de cette énergie qui correspond au travail échangé avec le milieu extérieur, et ΔQ la quantité d'énergie mise en jeu sous forme de chaleur,

stipule qu'il est impossible que la chaleur passe spontanément d'un corps froid à un corps chaud, et qu'elle se transforme complètement en travail si la température est la même partout dans un système. Donc, le démon de Maxwell autorise ce travail dans un système qui initialement ne le permettait pas. Dans les systèmes biologiques, on trouve également quantité de processus physico-chimique contredisant le second principe.

C'est par exemple le cas des CK mitochondriales dans l'espace confiné intermembranaire. Le petit démon prendrait une molécule d'ATP directement à la sortie de l'ANT pour le diriger

dans le site actif de la CK puis prendrait en échange un ADP qu'il conduirait vers l'ANT (**Figure 11**). Cette opération permettrait alors à la réaction d'éviter une augmentation inutile d'entropie, en tirant de l'entropie négative de son environnement. Car, en fait, en biologie, le démon de Maxwell porte le nom de couplage fonctionnel.

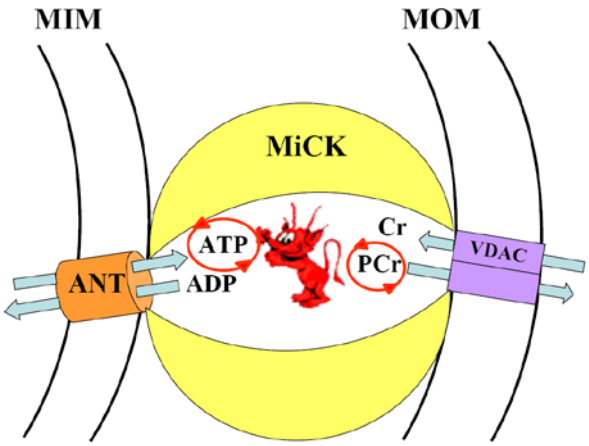


Figure 11 : Le démon de Maxwell et le couplage fonctionnel entre la créatine kinase mitochondriale (MtCK) et le transporteur ADP/ATP (ANT).

Ce couplage fonctionnel implique que la réaction est unidirectionnelle du fait de l'évacuation systématique d'un produit de réaction (PCr) et va, par conséquent, maintenir la réaction hors de l'équilibre en maintenant une vitesse nette non nulle.

De plus, le ratio ATP/ADP matriciel est 25 fois inférieur à de celui de l'espace intermembranaire, ceci étant dû à l'éléctrogénicité de l'ANT : l'échange d'un ATP chargé 4 fois négativement contre un ADP chargé 3 fois négativement crée un potentiel négatif dans la matrice qui favorise l'export de l'ATP et l'import d'ADP [Klingenberg, 1968] favorisant les flux en faveur d'une régénération d'ADP par la MtCK et donc permettant une régulation de la respiration mitochondriale.

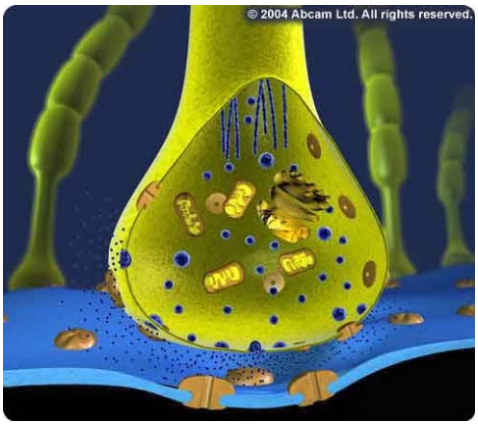
I. 4 Mécanismes de la régulation de la respiration mitochondriale

I. 4.1 Métabolisme et respiration neuronale

Le cerveau, bien qu'il ne représente chez l'adulte que 2 % de notre masse corporelle, utilise à lui seul 25 % de notre énergie. C'est un organe qui recèle beaucoup de mystères et reste très difficile à étudier car il est l'organe le plus hétérogène du corps. Il compte, en effet, trois types cellulaires bien différenciés : les neurones, la microglie et les astrocytes, lesquels peuvent encore être divisés en plusieurs catégories. Pour plus de simplicité, seul le métabolisme des neurones et le processus de transmission synaptique seront abordés.

Les neurones sont des cellules compartimentées de par leur structure particulière : le soma (ou pericaryon) qui contient le noyau et toute la composition cellulaire ordinaire, les axones qui sont les prolongations nerveuses et les synapses qui sont les terminaisons nerveuses et qui ne contiennent essentiellement que du cytosquelette, des mitochondries, des récepteurs et canaux membranaires et des vésicules diverses de neurotransmetteurs (**Figure 12A**).

A



B

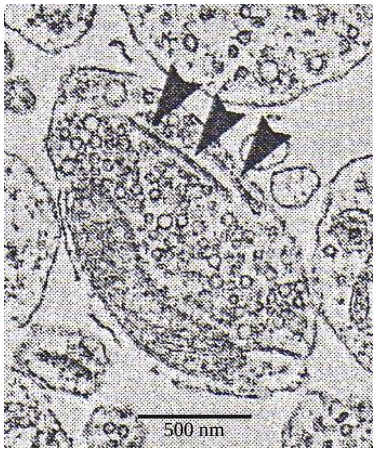


Figure 12 : A/ Représentation d'une synapse ou bouton synaptique. Cette espace restreint contient des mitochondries (en jaune), des vésicules synaptiques (sphères bleues) enchâssées dans des réseaux de protéines du cytosquelette (filaments bleus) et des canaux et récepteurs membranaires (en marron). [D'après Abcam (www.abcam.com)]. B/ Microscopie électronique d'un synaptosome. Les flèches indiquent le réseau microtubulaire. [D'après Dunkley, 1988].

La principale fonction des cellules nerveuses est de maintenir un déséquilibre ionique nécessaire à la formation et la transmission des messages nerveux. La synapse permet en effet la communication d'un neurone à un autre par l'intermédiaire de courant électrique (potentiel d'action) parcourant les axones jusqu'à la synapse. Ce potentiel d'action agit sur des canaux ioniques dont les perturbations ioniques occasionnées vont provoquer l'exocytose de neurotransmetteurs (acétylcholine, dopamine, glutamate, adrénaline…) jusqu'à la cellule post-synaptique (dendrite). Les principaux canaux ioniques en jeu dans la transmission nerveuse

sont : les Na^+/K^+ ATPases et Ca^{2+} ATPases membranaires et les H^+ ATPases des vésicules pré-synaptiques.

La pompe Na^+/K^+ ATP-dépendante est la principale consommatrice d'énergie car elle est responsable de la maintenance et de la restauration des gradients ioniques et donc la génération des potentiels électriques. Elle consomme en effet entre 50 et 80 % de l'énergie cellulaire [Erecinska, 1989 ; Ames, 2000 ; Atwell, 2001]. Les autres processus essentiels sont le recyclage des neurotrasmetteurs, le transport cellulaire, le turnover lipidique dû aux cycles endo-exocytose, et les dynamiques actomyosine-dépendantes [Attwell, 2001]. Les cellules nerveuses sont caractérisées par un import important de glucose, une navette lactate très active entre neurone et astrocyte, un niveau élevé d'OxPhos, un haut niveau d'enzymes de combustion du glucose et une très haute consommation d'ATP [Mehrabian, 2005].

En conditions physiologiques, le glucose est le substrat préférentiel du cerveau [Siesjo, 1978]. Dans certaines conditions, des corps cétoniques ou des acides aminés (en particulier glutamine, alanine et leucine) peuvent être utilisés [Erecinska, 1996]. Mais là encore, en considérant l'hétérogénéité de cet organe, il est possible que les substrats utilisés varient selon les compartiments étudiés [Erecinska, 1996].

Etant donné l'accessibilité difficile aux données *in vivo* des neurones, un modèle simplifié a été introduit par Gray et Whittaker [Gray et Whittaker, 1962] et De Robetis et al [De Robetis, 1962] : les synaptosomes, qui sont les parties pré-synaptiques vesicularisées (**Figure 12B**). Les synaptosomes possèdent les mêmes caractéristiques anaboliques et cataboliques que les cellules intactes, répondent aux stimulations électriques et présentent les mécanismes de largage et recapture des neurotransmetteurs [De Belleroche, 1972 ; Blaustein, 1975]. Selon David Nicholls [Nicholls, 2002], un synaptosome peut être considéré comme une « super-mitochondrie » entourée de cytoplasme, de membrane et de processus associés. Il a en effet été montré que les synaptosomes ne renferment que quelques mitochondries [Booth, 1978]. Les synaptosomes incubés avec du glucose puisent 95 % de leur énergie de l'OxPhos [Gleitz, 1993] (sachant que cette valeur peut fortement varier selon le type cellulaire étudié) en utilisant le pyruvate dérivé du glucose (**Figure 13**). De ce fait, l'addition de roténone (inhibiteur du complexe I de la chaîne respiratoire) augmente la synthèse de lactate d'un facteur 10 [Kauppinen, 1986]. Dans les cellules 'au repos' et en présence d'oxygène, la glycolyse contribue donc seulement à 5 % ou moins de la production totale d'ATP (ce qui n'est plus le cas des neurones en cultures qui dévient très vite vers un métabolisme glycolytique alors que le métabolisme global du cerveau serait plutôt oxydatif) [Erecinska, 1996].

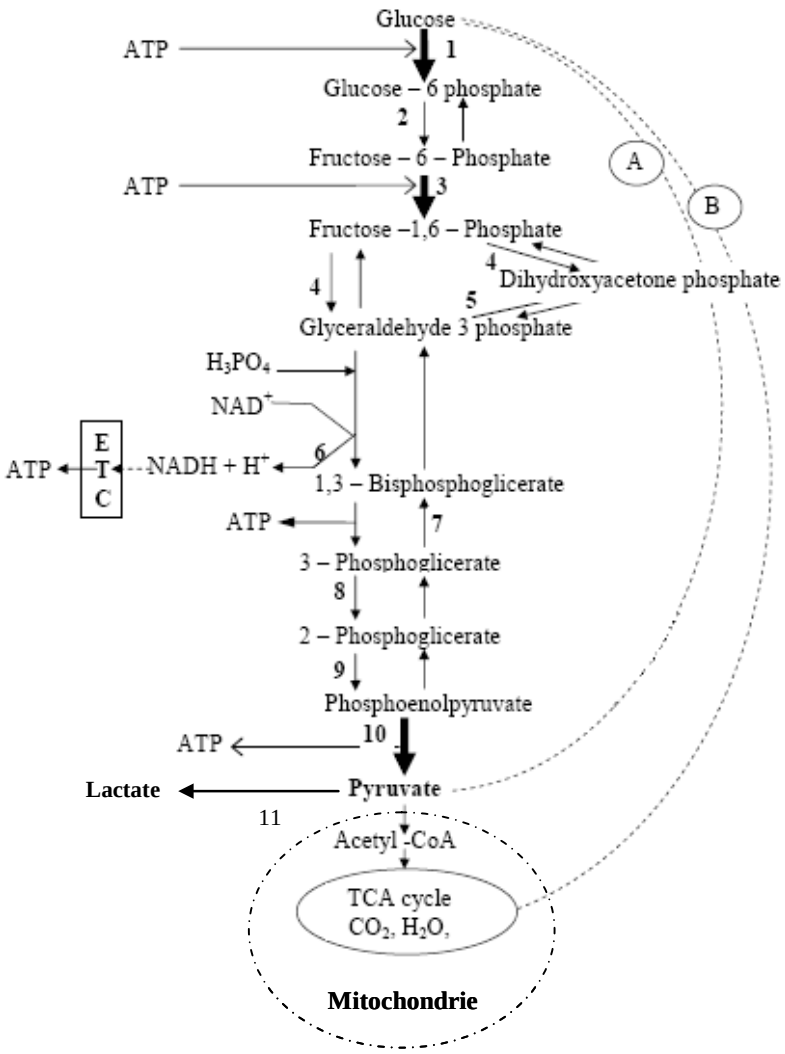


Figure 13 : Voie A/ Glycolyse aérobie, voie B/ oxydation complète du glucose. Enzymes : 1 : hexokinase, 2 : phosphohexose isomérase, 3 : phosphofructokinase, 4 : aldolase, 5 : triose phosphate isomérase, 6 : glycéraldéhyde 3 phosphate déshydrogénase, 7 : phosphoglycérate kinase, 8 : phosphoglycéromutase, 9 : enolase, 10 : pyruvate kinase, 11 : lactate déshydrogénase. ETC : chaîne de transport des électrons, TCA cycle : cycle des acides tricarboxyliques ou cycle de Krebs.

L'augmentation de la respiration et de la glycolyse peut être supprimée par l'addition de ouabaïne (inhibiteur des pompes Na^+/K^+), ce qui suggère bien que la production d'énergie est subséquente à la stimulation des pompes Na^+/K^+. Une réduction substantielle d'apport en glucose extracellulaire diminue l'exocytose de glutamate sans changement apparent du niveau d'ATP, montrant l'importance de la localisation subcellulaire de l'ATP [Fleck, 1993]. Il existe donc une stabilité métabolique dans les cellules nerveuses.

La complexité du milieu intérieur cellulaire et l'incompatibilité d'une diffusion libre dans le cytoplasme ont été établit par Clegg [Clegg, 1984] qui conclue de ses travaux que la taille des cellules ne permet pas un 'solution-based metabolism'. Des études de spectroscopie H^1-RMN ont indiqué que les coefficients de diffusion des métabolites dans le fluide intracellulaire est

plus bas que ceux trouvés en solution d'au moins au ordre de magnitude [Pfeuffer, 2000]. En effet, les cellules nerveuses (entre autres) ont développé des moyens d'échapper 'au chaos de la chimie des solutions' en développant des connections intimes entre les différentes structures cellulaires et la machinerie métabolique [Clegg, 1984 ; Ames, 2000]. En effet, si l'apport en substrats est complètement interrompu, les réserves énergétiques totales du cerveau (en incluant le glycogène) ne seraient suffisantes que pour maintenir l'activité énergétique standard pendant 80 s [Ames, 2000]. Des études de comparaison entre la production et la demande énergétique ont aboutit à la conclusion d'une balance énergétique qui permettrait de préserver les niveaux d'ATP en cas de limitation d'énergie [Ames, 2000]. Ces moyens sont connus sous le nom de phosphotransferts, représentés par le système CK/PCr et l'adénylate kinase (AK, réaction réversible : AMP + ATP → 2 ADP) (**Figure 14**).

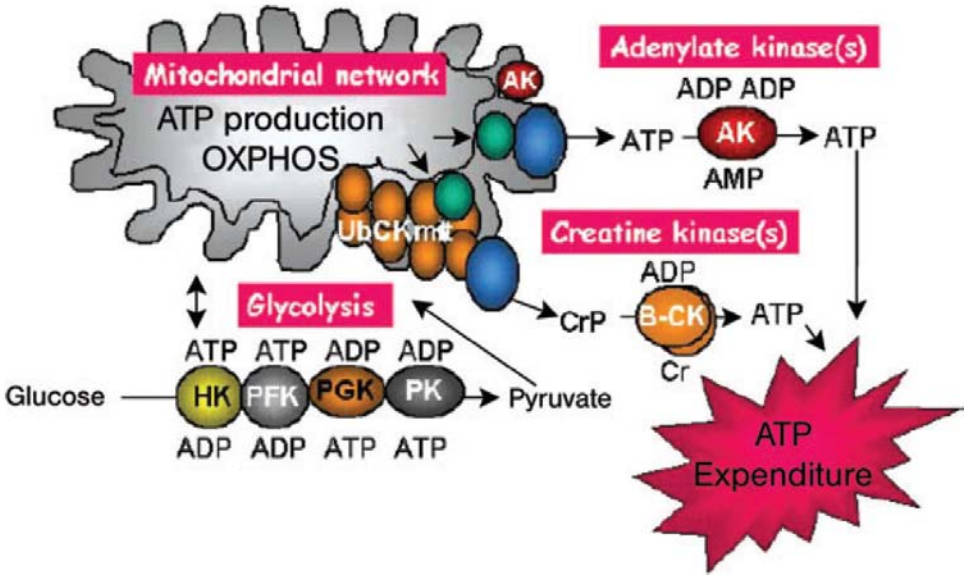


Figure 14 : Schéma des enzymes et réseaux métaboliques impliqués dans les phosphotransferts et la production d'ATP. AK : adénylate kinase, B-CK : créatine kinase cytosolique, UbCKmit : créatine kinase mitochondriale, HK : hexokinase, PFK, phosphofructokinase, PGK : phosphoglycérate kinase, PK : pyruvate kinase. [D'après Streijger, 2007].

Des études de manipulation génétique de cellules souches embryonaires, ont permis d'obtenir des souris portant soit une délétion du gène B-CK (souris B-CK^{-}/$^{-}$) soit du gène uMtCK (appelé UbCKmit $^{-}$/$^{-}$) soit des deux (souris CK $^{-}$ $^{-}$/$^{-}$ $^{-}$) [Streijger, 2005]. Le phénotypage des fonctions cérébrales de ces souris a été effectué par analyses histo-chimiques et par des approches biophysiques telles que la spectroscopie à résonance magnétique ou l'imagerie à résonance magnétique (IRM). Etant donné l'importance du système CK/PCr pour les cellules à haute plasticité cellulaire et forte activité membranaire tels que les neurones, de fort changements phénotypiques pourraient être attendus. Pourtant, aucune des mutations ne se sont avérées directement létales pour les animaux. Il semblerait cependant que les doubles

mutants CK $^{-}$ $^{-}$/$^{-}$ $^{-}$ souffrent de troubles du comportement et de l'apprentissage. Cependant, certains résultats tendent à penser que le système CK/PCr est impliqué dans la perte cellulaire (neurodégénérescence) et l'organisation métabolique. La survie des animaux mutants est essentiellement due à la remarquable plasticité neuronale qui permet une extraordinaire adaptabilité [Streijger, 2005 ; Streijger, 2007].

Dans certaines pathologies, une supplémentation à la Cr s'est avérée efficace pour retarder certains symptômes et a montré des effets neuroprotecteurs, comme dans la sclérose latérale amyotrophique (ALS), et a réduit la formation d'agrégats protéiques dans des maladies neurodégénératives telles que les maladies d'Alzheimer, d'Huntington ou de Parkinson [Bürklen, 2006 ; Andres, 2008].

Cependant, la bioénergétique et la régulation métabolique des cellules nerveuses sont encore assez méconnus, contrairement aux tissus musculaires dont le métabolisme a été plus largement traité.

I. 4.2 Métabolisme, contraction et respiration cardiaque

Le cœur est un tissu au métabolisme oxydatif, c'est-à-dire que la plus grande partie de l'ATP produit provient de l'OxPhos (95 %) et très peu de la glycolyse (5 %). Cependant, tout comme pour les cellules nerveuses, l'ATP provenant de la glycolyse a été souvent relié à des processus de transport ionique [Weiss, 1985]. C'est donc la β-oxydation des acides gras qui pourvoit la plus grande partie de l'énergie en fournissant les substrats nécessaires à alimenter le cycle de Krebs, et par conséquent la chaîne respiratoire et la production d'ATP. Dans le muscle cardiaque, plus de la moitié de la production d'énergie est dédiée à la contraction, ce qui signifie que les mécanismes contractiles sont intimement liés à la respiration mitochondriale.

I. 4.2.1 Loi de Frank-Starling et homéostasie énergétique cardiaque

Les aspects phénoménologiques de la régulation de la respiration ont été mis en évidence pour la première fois dans entre 1914 et 1926, avant la découverte de l'ATP et bien avant la découverte de l'OxPhos. Les travaux d'Otto Frank [Frank, 1885] et d'Ernest Starling [Starling, 1926] ont permis l'établissement de la loi de Frank-Starling et de son interprétation métabolique reliant linéairement le travail et la respiration à l'augmentation du volume ventriculaire diastolique (**Figure 15**).

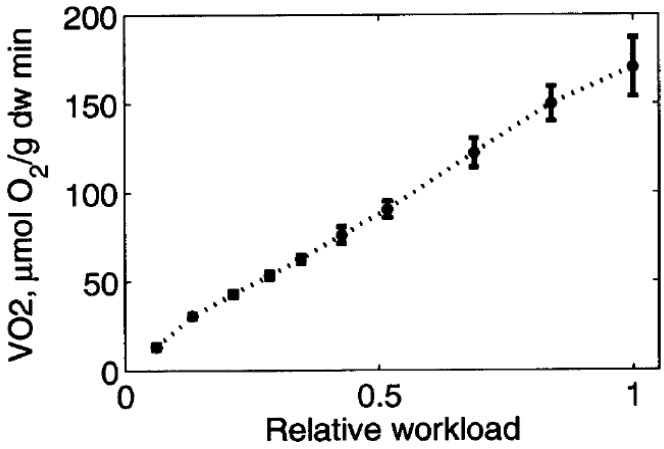


Figure 15 : Interprétation métabolique de la loi de Frank-Starling. Illustration de la dépendance linéaire de la vitesse de consommation d'oxygène (VO_2) et du travail cardiaque pour des cœurs travaillant perfusés de rat. Le travail maximal correspond à 0,6 kg/m pour une vitesse de remplissage ventriculaire de 56 ml/min. [D'après Williamson, 1976] ***"Any increase in the work demanded of the heart is met by corresponding increase in the oxygen consumption and in the amount of chemical changes taking place" [Starling, 1926].***

L'une des principales caractéristiques de l'énergétique cardiaque est de permettre une augmentation linéaire du taux de consommation d'oxygène (VO_2) en fonction du travail cardiaque sans changements mesurables de concentrations cellulaires d'ATP, de PCr ou de concentration cytoplasmique apparente d'ADP [Neely, 1972 ; Balaban, 1986, par la technique de ^{31}P-RMN ; Wan, 1993] (**Figure 16**). Williamson et al [Williamson, 1976] ont montré que la vitesse de respiration peut être augmentée de 15 à 20 fois toujours sans variations mesurables. Si l'on considère l'ensemble des données concernant la régulation métabolique (réseaux de phospho-transferts, compartimentation des métabolites et des enzymes, diffusion hétérogène des métabolites, restriction de diffusion), nous pouvons rester perplexes en abordant la question de la stabilité métabolique. Peut-il y avoir une compatibilité entre ces différentes notions : une activité cellulaire variable (demande énergétique fluctuante) et un maintien constant de l'homéostasie énergétique ? Compatibilité ou paradoxe ?

Cette remarquable stabilité métabolique des concentrations en métabolites 'riches en énergie', sous-jacente à la loi de Frank-Starling, est aussi appelée 'homéostasie métabolique' [Balaban, 2002] ou 'paradoxe de stabilité' [Hochachka, 2003] si l'on considère les importantes variations de demande énergétique des cellules excitables.

Cette stabilité n'est pas retrouvée dans les muscles rapides (au métabolisme glycolytique) qui sont plus rapidement fatigables (le contenu cellulaire en PCr diminue après 15 min d'exercice puis revient au niveau initial après 30 min) [Kushmerick, 1992].

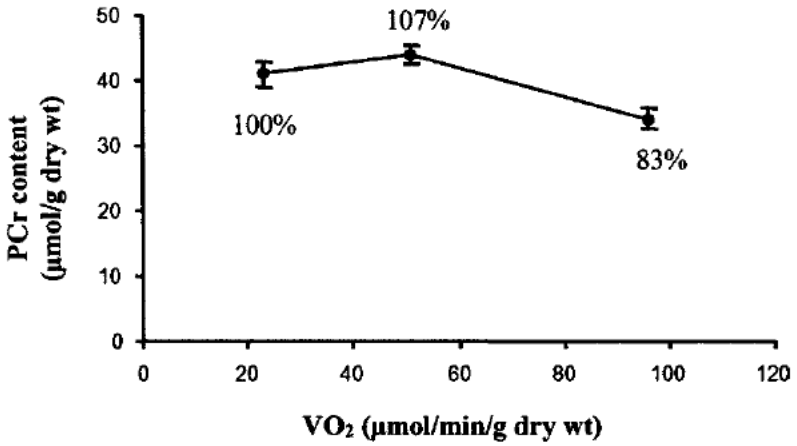


Figure 16 : Stabilité métabolique cardiaque. La concentration en phosphocréatine (PCr) ne quasiment varie pas en fonction de la vitesse de respiration (VO_2). [D'après Wan, 1993].

Pour être déchiffrée, l'étude la régulation de la respiration mitochondriale doit porter d'une part sur les mécanismes permettant la balance énergétique entre les processus production et consommation d'ATP *in vivo* et d'autre part sur les acteurs de la signalisation métabolique.

I. 4.2.2 Théorie du 'calcium only'

Une explication répandue des mécanismes de régulation de la respiration mitochondriale est basée sur l'activation parallèle de la contraction et de la respiration par le calcium [McCormack, 1990 ; Jouaville, 1999 ; Territo, 2001 ; Balaban, 2002 ; Bianchi, 2004 ; Brookes, 2004 ; Gunter, 2004]. En effet, le calcium étant un activateur important de déshydrogénases du cycle de Krebs, une augmentation intramitochondriale de calcium participe à l'augmentation des capacités de l'OxPhos [Denton, 1978 ; McCormack, 1990 ; Hansford, 1998 ; Balaban, 2002]. Le cycle mitochondrial du calcium [Carafoli, 2003] est un système d'import-export du calcium qui permet de réguler les fluctuations calciques cytoplasmiques locales [Berridge, 2003 ; Mackenzie, 2004] et qui protège la mitochondrie des surcharges de calcium [Bernardi, 1999 ; Rizzuto, 2000 ; Gunter, 2004 ; Jacobson, 2004]. Il a donc été déduit que le calcium relie les demandes callulaires en ATP à sa production mitochondriale, et par conséquent régule la respiration mitochondriale [Jouaville, 1999 ; Territo, 2001 ; Balaban, 2002 ; Bianchi, 2004]. Des expériences *in vivo* d'activation de la respiration mitochondriale par le calcium [Territo, 2001] et une modélisation mathématique du métabolisme mitochondrial [Cortassa, 2003] ont aboutit au même résultat : des variations dans les concentrations calciques cellulaires peuvent au maximum activer la respiration cellulaire d'un facteur 2 (par rapport à la valeur de la respiration en absence de calcium) alors

que par les mécanismes de la loi de Frank-Starling (augmentation du volume de remplissage du ventricule droit de cœur perfusés de rat) la vitesse de respiration est multipliée par un facteur au moins 10 (par rapport à la valeur de respiration obtenue à partir de cœurs au repos) (**Figure 17**).

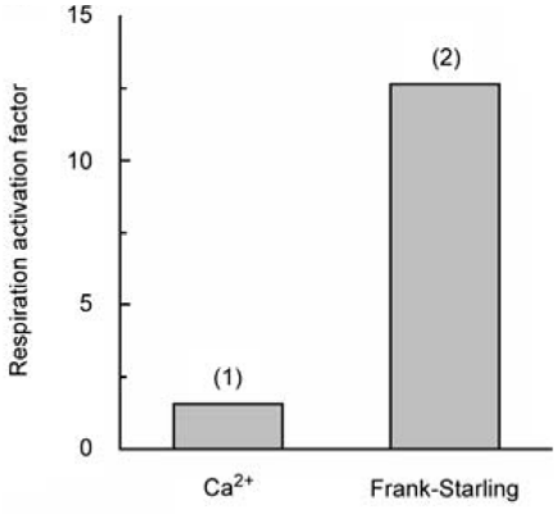


Figure 17 : Différence d'activation de la respiration mitochondriale *in vivo* par le calcium (1) et par les mécanismes de la loi Frank-Starling (variation du volume de remplissage du ventricule droit de cœur perfusés de rat) (2). [D'après Saks, 2006b]

Territo et al. ont montré que, à concentration de calcium nulle, la vitesse de respiration des mitochondries isolées correspond déjà à 60 % de la vitesse maximale (Vmax) obtenue à des concentrations en calcium supérieures à 600 nM [Territo, 2001]. Donc, au regard des concentrations intracellulaires et de la quantité de calcium nécessaire à l'activation très rapide de la chaîne respiratoire, la vitesse de respiration serait toujours très proche de sa vitesse maximale Vm. La théorie du calcium ne permet donc pas de subvenir aux fortes et rapides variations des besoins énergétiques des cellules excitables et procurer une régulation efficace. De plus, de fortes augmentations de concentrations calciques intracellulaires seraient dangereuses pour les cellules car elles provoqueraient une inhibition de la synthèse d'ATP [Holmuhamedov, 2001] et une ouverture du pore de transition de perméabilité (PTP) [Bernardi, 1999 ; Rizzuto, 2000].

Hibberd et al [Hibberd, 1982], entre autres, ont confirmé que le mécanisme cellulaire découlant de la loi Frank-Starling est une relation force/longueur, et que l'affinité la plus forte des filaments fins pour le calcium est mesurée pendant la phase d'élongation et non de raccourcissement du sarcomère [Allen, 1982 ; Landesberg, 1996 ; Gordon, 2001]. Seule une petite augmentation dans les concentrations transitoires de calcium fut observée après un changement de longueur sarcomérique [Allen, 1982]. Par ces mécanismes d'activation

dépendant de la longueur du sarcomère, les vitesses de consommation d'ATP (et de respiration mitochondriale) peuvent varier largement sans changement dans le cycle du calcium. L'hypothèse du 'calcium only' ne permet donc pas d'expliquer la régulation de la respiration mitochondriale et des mécanismes de contraction.

Nous avons montré jusqu'ici que la vitesse de respiration pouvait être augmentée sans variations de concentration de calcium ni de variation dans les niveaux de phosphate de 'haute énergie' (ATP et PCr). En 2004, Marco Colombini [Colombini, 2004] se pose la question suivante : Quel signal intracellulaire peut primer sur la mitochondrie pour augmenter la vitesse de respiration dans un environnement intracellulaire contenant déjà un haut niveau d'« energie » ?

Cette interrogation et le paradoxe apparent de l'homéostasie énergétique vont être résolus grâce à la mise en évidence des compartimentations cellulaires et de la régulation métabolique rétrograde (metabolic feedback regulation).

I. 4.3 Les Unités Energétiques Intracellulaires

La nature modulaire de la régulation des fonctions biologiques dans les muscles [Saks, 2001] aussi bien que dans les neurones [Wheatley, 1997] est une notion qui englobe différents aspects de la régulation métabolique et implique des notions biochimiques, physico-chimiques et biophysiques. Pour schématiser les différentes échelles de régulation et souligner l'étroite relation qu'il existe entre structure et fonction, Saks et al [Saks, 2001] ont proposé d'examiner les cellules cardiaques comme des complexes fonctionnels : les Unités Energétiques Intracellulaires (IntraCellular Energetic Units, ICEUs) (**Figure 16**).

L'organisation de ces modules énergétiques en fait des structures finement régulées. Dans la **Figure 18** sont schématisées les principales sources d'ATP (mitochondrie, glycolyse, et synthèse d'ATP *de novo* par l'adénylate kinase AK) et les principaux sites de consommation d'ATP (les ATPases membranaires, sarcoplasmiques et myofibrillaires). Le cycle du calcium via le réticulum sarcoplasmique est représenté car il représente un acteur essentiel à la contraction cardiaque. Dans cette représentation, les processus métaboliques ont lieu dans un espace réduit de quelques micromètres résultant en un fonctionnement synchronisé de ses unités métaboliques répétées [Saks, 2006, article 2].

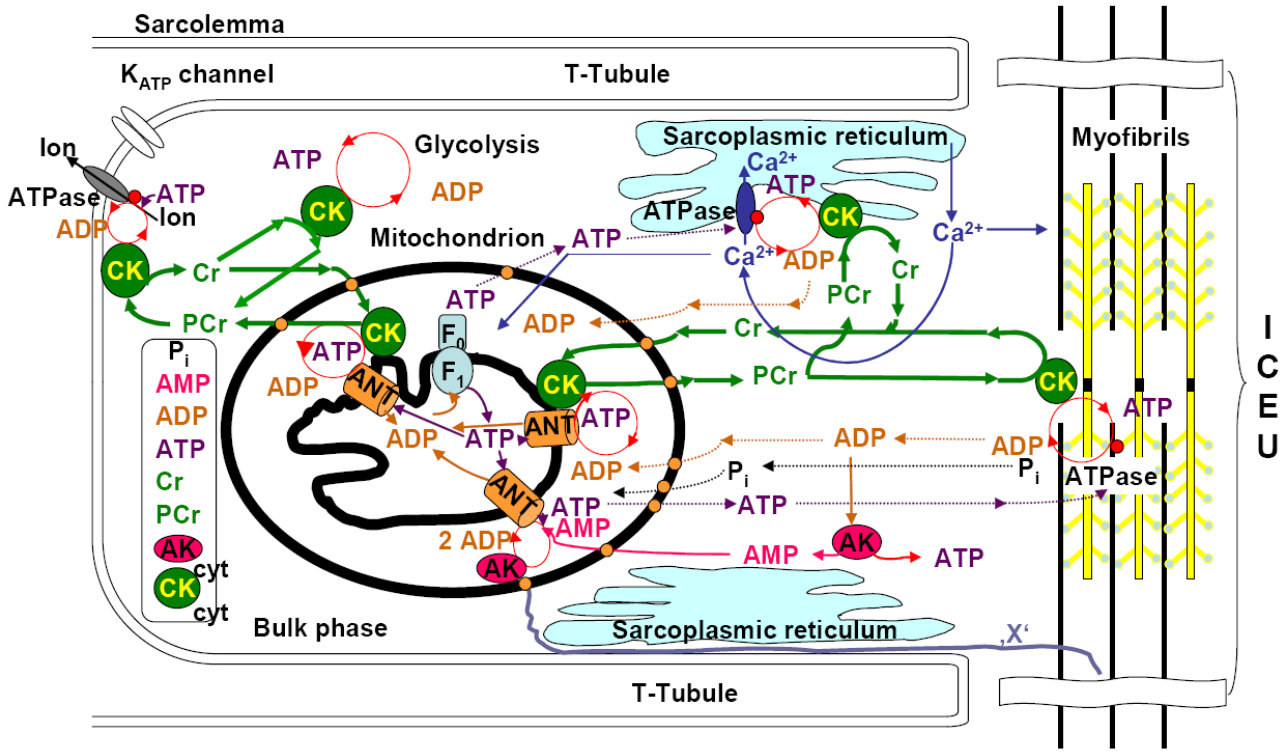


Figure 18 : Représentation des Unités Energétiques Intracellulaires (IntraCellular Energetic Unit (ICEU)). Imbriquée entre tubules-T et myofibrilles, la mitochondrie est au cœur des ICEUs. Des navettes de phosphotransferts (créatine kinase, CK et adénylate kinase, AK) assurent un rôle prépondérant dans le maintien de l'homéostasie énergétique. Les CK sont représentées couplées aux sites de production d'ATP (mitochondrie, glycolyse) et de consommation d'ATP (ATPases). Le fonctionnment synchronisé des ICEUs se produit par l'intermédiaire des métabolites énergétiques et/ou par des relargages synchronisés de calcium pendant le processus couplé d'excitation-contraction [D'après Saks, 2006 (article 2), schéma original de Christian Linke].

Ce concept d'ICEU fournit une explication topologique aux observations de Gudbjarnason [Gudbjarnason, 1970] concernant l'existence de pools d'ATP connectés à des pools de PCr. En effet, la PCr est rapidement utilisable, ce qui aboutit à l'accumulation d'ATP à l'intérieur des ICEU et donc au déclenchement de la relaxation-ATP dépendante et par conséquent à l'inhibition de la contraction en dépit de la présence de forte concentration d'ATP dans le cytoplasme.

Dans les neurones, il semblerait qu'il existe une telle organisation modulaire [Wheatley, 1997] mais aucune représentation schématique complète n'a été établie à ce jour.

I. 4.4 La régulation métabolique rétrograde de la respiration

Considérant le haut degré d'organisation et la complexité de l'architecture intracellulaires, les acteurs de la régulation métabolique énergétique sont soumis à différentes interactions. La régulation métabolique rétrograde est basée sur la communication métabolique, c'est-à-dire les échanges métaboliques entre les sites de production d'ATP et de consommation d'ATP dont les principaux phénomènes régulateurs sont : la (micro-)compartimentation, les couplages fonctionnels conduisant à un métabolisme vectoriel (canalisation métabolique, conduction orientée des intermédiaires enzymatiques).

Pendant la contraction cardiaque, et l'ADP et la Cr doivent être rapidement renouvelés pour maintenir la stabilité métabolique et pour ce faire ils doivent être fournis à la mitochondrie. C'est pourquoi les flux métaboliques rétrogrades via les réseaux de phosphotransferts sont les mécanismes les plus probables pour réguler la respiration mitochondriale. La signalisation métabolique rétrograde est donc représentée par les changements cycliques d'ADP dont les amplitudes dépendent du travail cardiaque et peuvent être fortement amplifiées par les phénomènes de couplages fonctionnels ou de variations d'autres métabolites tel que le Pi [Vendelin, 2000].

Les réactions catalysées par des groupements enzymatiques ont donc dû évoluer en groupements orientés (métabolons) pour faciliter la linéarité des échanges. Par conséquent, il en résulte une plus grande rapidité de réaction et une plus grande efficacité de conduction, qui ne serait par réalisable dans un milieu à distribution métabolique homogène. Cette canalisation des flux métaboliques permet ainsi le maintien des concentrations locales de métabolites intermédiaires.

Etant donné l'importance capitale de l'ADP dans le métabolisme énergétique, les moindres fluctuations critiques de concentrations cellulaires en ADP font intervenir tout un réseau de senseurs métaboliques autres que les CK. Cette signalisation métabolique fait intervenir différents processus de réseaux parallèles de phosphotransferts pour la conduction vectorielle des ligands à travers des systèmes multienzymatiques cytoplasmiques organisés tels que la glycolyse et l'adénylate kinase (AK) [Mitchell, 1979].

Ces phénomènes font partie de la régulation métabolique rétrograde qui ne peut être observée que dans des systèmes hautement organisés. La complexité et la richesse intracellulaires font des cellules des objets très difficile à étudier *in vivo*. C'est pourquoi la modélisation mathématique est un outil puissant pour comprendre les phénomènes de la régulation mitochondriale *in vivo* à partir de données expérimentales.

I. 5 Modélisation mathématique

La modélisation mathématique est un appui essentiel à la bioénergétique systémique moléculaire. Elle permet d'accéder à des informations que les techniques actuelles ne peuvent pas fournir. Mais il y a certains risques à utiliser la modélisation si, par exemple, la quantité de données expérimentales est insuffisante ou si aucunes données expérimentales ne sont utilisées, le modèle final peut s'avérer erroné. Certains auteurs considèrent même que leur modèle est a priori correct et que ce sont les données expérimentales qui doivent être changées (!) [Kongas, 2002].
La richesse des données expérimentales est la garantie d'un modèle le plus fidèle et le plus fiable possible. Mais il faut toujours garder à l'esprit qu'un modèle ne sera jamais le système étudié mais une représentation de ce système ou d'une partie de ce système. La modélisation n'est qu'une image de la complexité d'un système et il est finalement assez aisé d'aboutir à la création de phénomènes virtuels loin de la réalité, comme il sera discuté dans la section I. 5.3.

I. 5.1 Modèle mathématique du couplage fonctionnel MtCK/ANT

Les mécanismes du couplage fonctionnel entre MtCK et ANT ont été mis en évidence par modélisation mathématique [Vendelin, 2004] par détermination des différentes constantes cinétiques et sur la base de faits expérimentaux portant sur deux mécanismes distincts : i/ la compartimentation dynamique de l'ADP et de l'ATP [Gellerich, 1987] ou ii/ le transfert direct d'ADP et d'ATP entre protéines basés notamment sur des études de cinétique complète d'activation des CKs mitochondriales [Jacobus, 1982]. Dans le modèle établit, le premier mécanisme signifie que l'ATP et l'ADP sont libérés dans l'espace inter-membranaire puis liés à l'ANT ou à la MtCK, et par conséquent passent par une phase de diffusion en solution (sans restriction de la diffusion) (**Figure 19A**). Contrairement au deuxième mécanisme où les nucléotides adényliques sont acceptés directement par les enzymes sans aucun passage en phase aqueuse, et donc sans équilibre de concentration avec le milieu environnant (**Figure 19B**).
Les résultats obtenus par modélisation montrent que le mécanisme de compartimentation dynamique dans l'espace inter-membranaire existe mais qu'il est insuffisant pour reproduire les changements expérimentaux des constantes cinétiques de la réaction de la MtCK couplée à l'OxPhox dans les mitochondries isolées de rat [Jacobus, 1982]. Par contre, un transfert direct des nucléotides de l'ANT à la MtCK et inversement permet de reproduire les données

expérimentales. Cependant, par simplification, le premier mécanisme admet une diffusion des nucléotides sans tenir compte de restrictions de diffusion et le second mécanisme est basé uniquement sur la canalisation directe, c'est-à-dire sans aucun passage du tout dans le milieu environnant, ce qui n'est possible que dans certains cas : la MtCK est libre (d'où formation d'un complexe binaire) ou n'est lié qu'à une molécule de Cr ou de PCr (formation d'un complexe ternaire). Dans les autres cas, un passage en solution, même très bref serait probable.

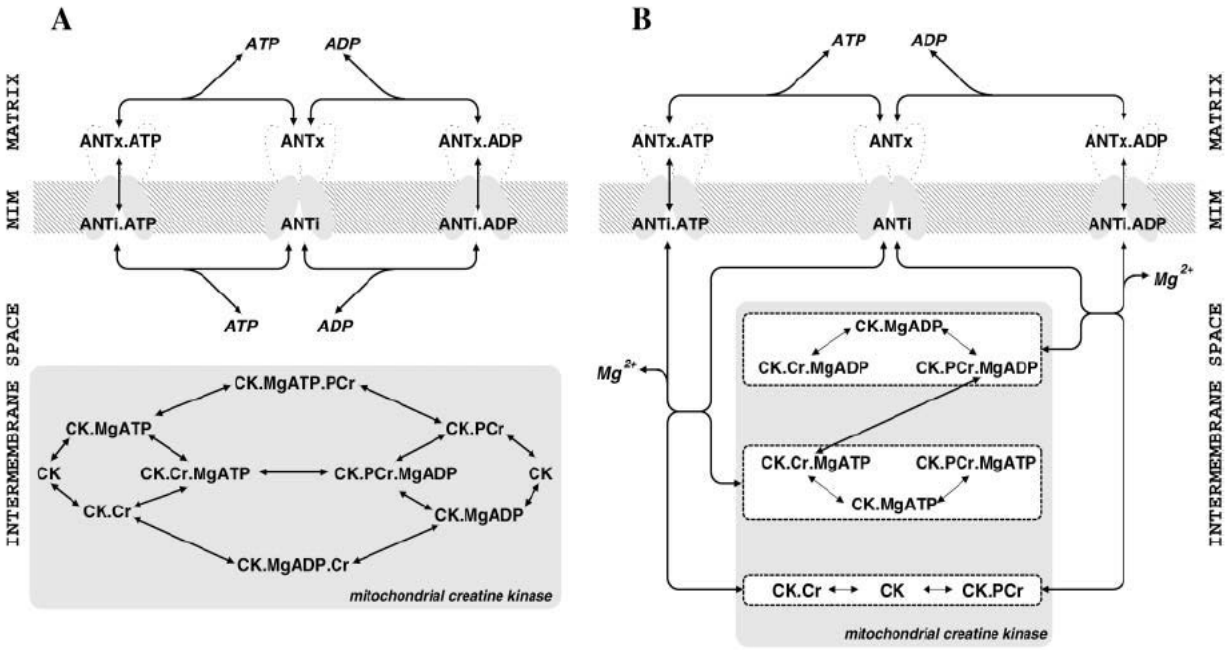


Figure 19 : Schéma de couplage fonctionnel entre la créatine kinase mitochondriale (MtCK) et le transporteur ATP/ADP (ANT) dans l'espace inter-membranaire. Deux mécanismes de couplages sont proposés : A / mécanisme de compartimentation dynamique où l'ATP et l'ADP diffusent librement en solution avant de se lier aux enzymes (le modèle ne comprend pas de restriction de diffusion), et B/ mécanisme de transfert direct des nucléotides de la MtCK à l'ANT et inversement sans aucun passage en solution. [D'après Vendelin, 2004].

Il semblerait donc tout de même que le modèle soit un peu restrictif et que les 2 mécanismes puissent être complémentaires *in vivo* [Vendelin, 2004]. En effet, considérant une restriction de la diffusion à la membrane externe mitochondriale (cf section I. 6), il serait envisageable de combiner transfert direct, compartimentation dynamique et restriction de la diffusion de l'ADP et de l'ATP dans un nouveau modèle.

Des preuves directes des restrictions de la diffusion des métabolites dans les cellules in vivo restent encore à apporter pour établir définitivement l'absence de diffusion homogène dans le cytoplasme, comme le pense encore certains groupes [Kongas, 2002 ; Barros, 2007].

I. 5.2 Modèles mathématiques de la régulation métabolique cellulaire

L'une des premières modélisations mathématiques de la régulation de la respiration mitochondriale fût proposée par Bohnensack [Bohnensack, 1985] puis développée par Korzeniewski [Korzeniewski, 1998] pour les cellules musculaires. Cependant leurs modèles n'impliquaient pas les couplages fonctionnels de la CK et considéraient le calcium comme un activateur parallèle.

En 1997, Mayis Aliev et Valdur Saks [Aliev, 1997] établissent un modèle des systèmes de transferts compartimentés d'énergie dans les cellules cardiaques qui s'avéra être le plus proche de la réalité expérimentale. La **Figure 20** représente le schéma général des transferts compartimentés d'énergie où sont considérées les diffusions d'ATP, d'ADP, de PCr, de Cr et de Pi entre les myofibrilles et la mitochondrie. Le modèle permet de tracer les flux métaboliques dans un espace de 13 unités de 0,1 µm chacune.

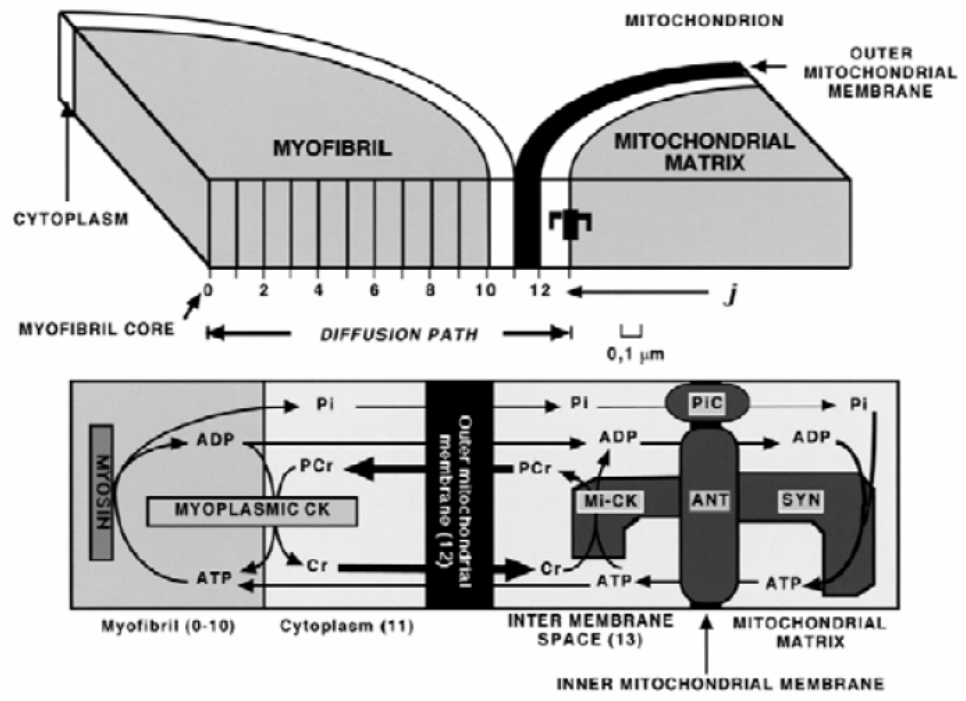


Figure 20 : Schéma général de la compartimentation des transferts énergétiques dans les cellules cardiaques. Les diffusions d'ATP, ADP, phosphocréatine (PCr), créatine (Cr) et phosphate inorganique (Pi) sont considérées le long d'une voie de diffusion de 13 unités de 0,1 µm chacune comprenant un espace myofibrillaire (10 unités), un espace cytoplasmique (1 unité), un espace représentant la membrane externe mitochondriale (1 unité) et un espace correspondant à l'espace inter-membranaire (1 unité). Le panneau inférieur représente les couplages fonctionnels des créatines kinases (CKs) : la créatine kinase mitochondriale (MiCK) et le transporteur ATP/ADP (ANT) (à droite) et la créatine kinase myofibrillaire (myoplasmicCK) et les ATPases liées à la myosine (à gauche). PIC : Pi Carrier, SYN : ATP synthase [D'après Aliev, 1997].

Ce modèle intègre les principes fondamentaux de la cinétique chimique et des lois d'action de masse. Il considère pleinement la dynamique des évènements du métabolisme énergétique : l'hydrolyse de l'ATP par les ATPases myofibrillaires durant la contraction, les phénomènes de diffusion durant les échanges métaboliques entre mitochondrie et myofibrilles, les restrictions de diffusion de l'ATP et de l'ADP à la membrane externe mitochondriale, la synthèse d'ATP par l'ATP synthase et dans les réactions couplées des CKs ainsi que la production de PCr.

Un profil des fluctuations du contenu des 5 métabolites étudiés a pu être tracé (**Figure 21**). Il démontre la rapidité des variations des concentrations en nucléotides adényliques qui retrouvent leurs valeurs initiales après 90 ms. Ce profil illustre bien la stabilité métabolique observée car pour les concentrations en ATP, PCr et Cr qui sont relativement élevées, la variation observée pendant la contraction (quelques centaines de µM) ne représente que quelques %.

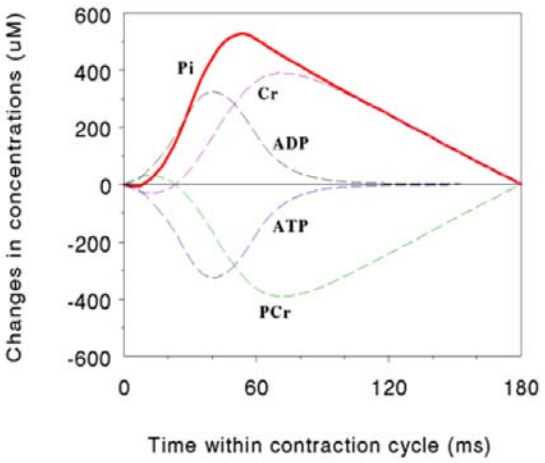


Figure 21 : Variations phasiques des différents métabolites énergétiques pendant un cycle de contraction cardiaque. Pi : phosphate inorganique, Cr : créatine, PCr : phosphocréatine [D'après Aliev, 1997].

Les résultats obtenus sur les flux métaboliques ont montré que pratiquement aucun équilibre cinétique n'est possible concernant les réactions des CKs pendant la contraction cardiaque. En effet, les données de simulation indiquent que l'équilibre cinétique de l'isoforme myofibrillaire MMCK est fortement déplacé dû à des augmentations cycliques des niveaux cytoplasmiques d'ADP pendant la diastole (contraction). Seul pendant la phase systolique (relaxation), alors que les concentrations d'ADP diminuent, la réaction des MMCK peut être redéplacée vers une position plus proche de l'équilibre. De plus, ces variations d'ADP n'ont pu être compensées même par une simulation d'augmentation d´un facteur 10 de l'activité MMCK. L'état stationnaire métastable est donc l'état cinétique qui correspond le mieux aux

variations métaboliques observées d'après les données expérimentales. En effet, le concept de 'système métastable' introduit par Daut [Daut, 1987] correspond bien à la cinétique enzymatique de la CK in vivo, car ce concept représente un système qui n'est pas en équilibre thermodynamique mais qui présente une configuration stable en état stationnaire [Aliev, 1997].

Ce modèle, après simulation d'inhibition des différentes CK, souligne la séparation des fonctions des différents isoformes de CK pendant les cycles contractiles : MMCK devient responsable de la régénération de l'ATP dans le myoplasme pendant la systole, créant ainsi une diminution de PCr qui est compensée dans la mitochondrie par la réaction de la MiCK à partir de Cr myoplasmique et par le couplage fonctionnel avec l'ANT tout au long du cycle contraction-relaxation. La question des restrictions de la diffusion des métabolites a été abordée en simulant les flux d'export de l'ATP et de la PCr, en faisant varier la perméabilité de la membrane externe mitochondriale (MOM) pour les nucléotides adényliques (**Figure 22**).

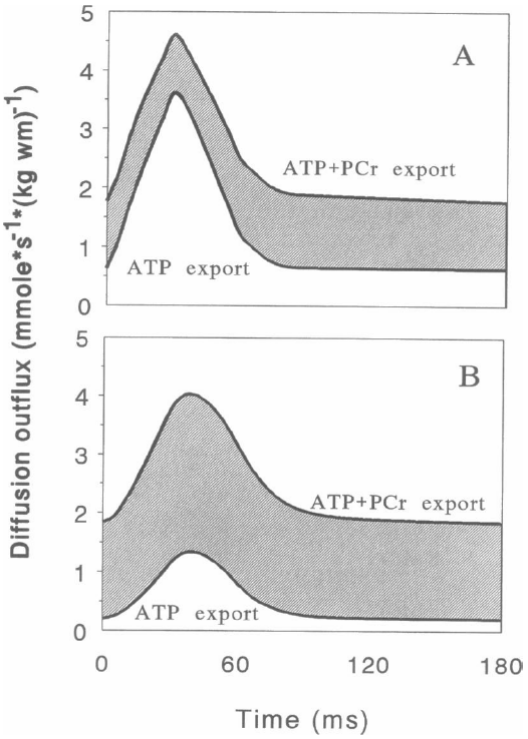


Figure 22 : Flux d'export de l'ATP et de la PCr à travers la membrane externe mitochondriale sans (A) ou avec (B) restriction de la diffusion des nucléotides adényliques (aboutissant à un Km apparent pour l'ADP de 400 µM). [D'après Aliev, 1997].

En simulant une restriction de la diffusion aboutissant à un Km apparent pour l'ADP de 400 µM (Km apparent obtenu *in situ* sur cardiomyocytes perméabilisés), les auteurs ont montré que cette restriction n'influence pas la vitesse de synthèse de l'ATP par l'ATP synthase car la

principale source d'ADP est la réaction couplée de la MiCK et donc un facteur de contrôle majeur de la respiration. De plus, les résultats de simulation de flux d'export de la PCr et de l'ATP en condition de restriction de la perméabilité de la MOM on démontré que 80 % de l' « énergie » sortant de la mitochondrie est portée par la PCr (**Figure 22B**), ce qui confirme les précédentes études sur la compartimentation des CKs et le système navette CK/PCr [Wallimann, 1992]. Ces résultats laissent donc à penser que la perméabilité de la MOM est sélective et diminue pour les nucléotides adényliques.

En 2000, Vendelin et al [Vendelin, 2000] perfectionnent le modèle de Aliev et Saks en construisant un modèle de réaction-diffusion intégrant i / les réactions de l'adénylate kinase (AK), ii/ la modération des couplages fonctionnels par fuite diffusionnelle des intermédiaires et iii/ d'autres métabolites tels que le NADH, le cytochrome c ou le coenzyme Q. D'après leur modèle, les auteurs retrouvent par simulation la dépendance linéaire de la consommation d'oxygène VO_2 et du travail cardiaque selon la loi de Frank-Starling (**Figure 23**). Cette dépendance linéaire peut être décrite par leur modèle de réaction-diffusion des transferts compartimentés d'énergie, tenant compte des activités enzymatiques expérimentales et de la localisation subcellulaire des CKs.

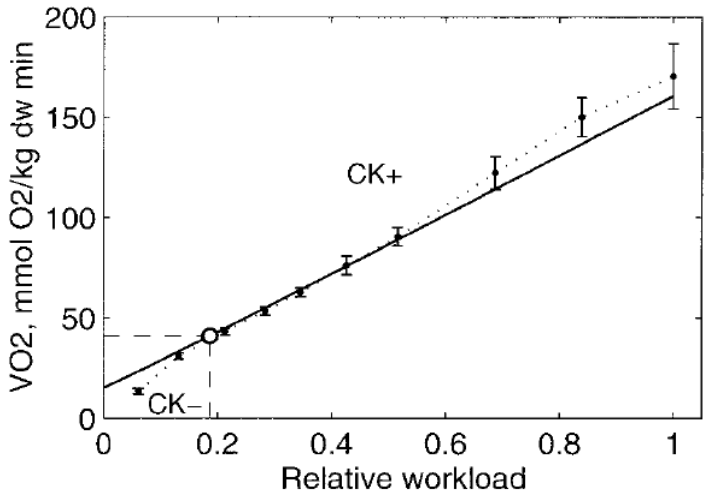


Figure 23 : Dépendance linéaire de la vitesse de consommation d'oxygène (VO_2) et du travail cardiaque selon la loi de Frank-Starling d'après des données expérimentales (ligne pointillées) ou par simulation (ligne continue) [D'après Vendelin, 2000].

Les auteurs aboutissent à plusieurs conclusions concordantes avec de précédentes observations : d'une part, dans leur modèle il ne peut pas y avoir d'équilibre dans les réactions du système compartimenté des CKs car les activités des différentes isoformes s'ajustent très bien aux oscillations des concentrations d'ADP, faisant donc partie intégrante du système de

signalisation métabolique rétrograde entre les phénomènes de contraction et la production d'énergie tout en maintenant constants les niveaux des autres métabolites ; et d'autre part la stabilité métabolique (rapport PCr/ATP constant) n'est observée que dans les cas intégrant le couplage fonctionnel MiCK/ANT et une restriction de diffusion à la MOM.

Un autre modèle de la régulation de la respiration mitochondriale été établit par Cortassa et al [Cortassa, 2003] comprend la description thermodynamique des transferts d'électrons de la chaîne respiratoire et les cinétiques précises des réactions du cycle de Krebs, incluant les effets du calcium sur les déshydrogénases. Ils se basent sur l'analyse du contrôle métabolique (Metabolic Control Analysis, MCA) de l'OxPhos basé sur les flux à travers la chaîne respiratoire, le système de phosphorylation et la fuite protonique (proton leak) pour décrire les processus de régulation de la respiration en perturbant le système par différentes concentrations d'ADP et/ou de calcium. D'après leur modèle, les auteurs concluent que le calcium (après variation de concentration de 20 à 660 nM) active la vitesse de respiration VO_2 de 23 % et la vitesse de synthèse d'ATP de 18 %. Cet effet modeste du calcium est expliqué par le fait que le calcium a deux effets opposés sur la mitochondrie : un effet stimulant sur l'activité de l'α-céto glutarate déshydrogénase et de l'isocitrate déshydrogénase du cycle de Krebs (d'où une production de NADH stimulée) et un effet dissipateur du potentiel de membrane dû à l'influx de cations divalents [Cortassa, 2003]. Ces résultats de modélisation corroborent parfaitement les résultats obtenus précédemment par la méthode expérimentale [Territo, 2001] et confirme que la régulation de la respiration cardiaque se fait principalement par le phénomène de signalisation rétrograde par canalisation des métabolites (et en particulier ADP, Cr et AMP) via les réseaux organisées de phosphotransferts [Wallimann, 1992 ; Saks, 1998 ; Dzeja, 1998]. La régulation métabolique étant basée sur des flux énergétiques, l'étude des diffusions métaboliques est un point d'étude central pour comprendre comment est régulée la respiration mitochondriale.

I. 5.3 Modèles mathématiques de la diffusion métabolique

L'organisation structurale des mitochondries en complexes fonctionnels avec les myofibrilles et le réticulum sarcoplasmique dans les ICEUs est la base de l'organisation du métabolisme énergétique dans les muscles oxydatifs et peuvent conduire à une hétérogénéité de la diffusion des métabolites.

Un phénomène important a été découvert par Kinsey et al [Kinsey, 1999] et De Graaf et al [de Graaf, 2000] sur la diffusion des métabolites phosphorés dans les muscles squelettiques

rouges et blancs (oxydatifs et glycolytiques, respectivement). C'est par spectroscopie ^{31}P-RMN à champ pulsé *in vivo* que fut mise en avant l'anisotropie de la diffusion (qui dépend de la direction de mesure) de l'ATP et de la PCr par la mesure des coefficients apparents de diffusion (correspondant aux dans différentes directions en fonction du temps de diffusion. Cette méthode permet de mesurer le déplacement d'une molécule pour un temps de diffusion t_{dif} et le coefficient apparent de diffusion D^{app} donné par l'équation d'Einstein-Smoluchowski (equation (5), section I. 2.2.2) : $\lambda^2 = 2\, D^{app}\, t_{dif}$ [de Graaf, 2000]. Les résultats obtenus ont montré un coefficient de diffusion radial plus petit que l'axial (dans la direction d'orientation de la fibre cylindrique) montrant ainsi l'anisotropie de la diffusion. Les auteurs déduisent que les restrictions de la diffusion de l'ATP et de la PCr ne sont par imposées par le sarcolemme mais pas d'autres structures intracellulaires [de Graaf, 2000].

En 2008, Vendelin et al [Vendelin, 2008] ont également confirmé le caractère anisotropique de la diffusion de l'ATP dans les cardiomyocytes par spectroscopie à corrélation d'image (RICS, Raster Image Correlation Spectroscopy) à l'aide d'un analogue d'ATP fluorescent, ATP-Alexa Fluor 647. La méthode développée a permis de déterminer et de comparer les coefficients de diffusion de la sonde en solution et dans les cardiomyocytes. Les auteurs ont déterminé une diminution des coefficients de diffusion de plus de 2 fois dans le sens longitudinal et de plus de 3 fois dans le sens transversal en comparaison des coefficients de diffusion en solution.

L'hétérogénéité de la diffusion des métabolites est un sujet encore très controversé qui ne fait pas encore l'unanimité. Certains auteurs considèrent que les restrictions de diffusion résultant à une hétérogénéité de diffusion ne peuvent être retrouvées que dans les fibres musculaires qui peuvent être considérées comme un système homogène avec une distribution uniforme d'enzymes mitochondriales et d'ATPases cellulaires, sans limitation de diffusion de l'ADP, comme en milieu aqueux [Kongas, 2002]. Cette théorie prédit que les fortes restrictions ne peuvent être obtenus que dans des faisceaux de diamètre > 70-100 µm et résultant d'un simple gradient de concentration d'ADP dépendant de l'activité des ATPases. Ces assertions sont en totales contradictions avec les données expérimentales (discutées plus loin, section I. 6.1).

Considérant un milieu intracellulaire homogène ou les concentrations métaboliques sont uniformes et les coefficients de diffusion d'ATP très haut [Hubley, 1995], les processus consommateurs d'ATP seraient toujours activés maximalement et aucune régulation de serait possible. Pourtant, la plupart des modèles mathématiques de diffusion moyennent les concentrations de métabolites, ne tenant ainsi pas compte de leur distribution spatiale.

C’est par exemple le cas dans les études de Barros et Martinez [Barros, 2007] où la seule donnée expérimentale utilisée est un coefficient de diffusion égal à 500 µm²/s mesuré par ^{31}P-RMN dans des muscles squelettiques [de Graaf, 2000] (ce résultat correspond également à celui obtenu dans des muscles blancs ou glycolytiques [Hubley, 1995]). Seulement, de Graaf et al [de Graaf, 2000] ont effectivement déterminé ce coefficient de diffusion de l’ATP mais en condition « unbounded » c'est-à-dire en l’absence de liaison, ce qui revient quasiment à mesurer la diffusion de l’ATP en solution. Sachant que l’ATP peut être transporté par 2 mécanismes différents : 1/ par transfert direct [Jacobus, 1985] ou 2/ par diffusion facilitée via la navette Cr/PCr [Meyer, 1984 ; Wallimann, 1992], et que la diffusion libre joue un rôle mineure dans l’activité métabolique [Agutter, 1995 ; Wheatley, 1998], la spectroscopie RMN permet de mesurer les déplacements moléculaires mais n’est pas capable de discriminer une diffusion brownienne d’une diffusion « assistée » [de Graaf, 2000]. De ce fait, les travaux de modélisation de Barros et Martinez ne sont pas valides pour mesurer un coefficient de diffusion de l’ATP dans les cardiomyocytes n’ayant tenu compte dans leur modèle ni des phosphotransferts, ni de la distribution spatiale des métabolites et enzymes, ni des activités des différentes kinases et ATPases impliquées dans le métabolisme énergétique cardiaque.

La même année, dans le même journal, une méthode de détermination du coefficient de diffusion de l’ATP a été proposée par Selivanov et al [Selivanov, 2007]. Ils proposent en effet un modèle mathématique de réaction-diffusion cohérent avec la composition cellulaire et intégrant les données connues et obtenues expérimentalement sur la distribution spatiale des métabolites et les propriétés des kinases pour déterminer la distribution spatio-temporelle de l’ATP dans des myofibrilles *in situ*. Leur modèle inclus les flux enzymatiques provoqués par la CK mais également par l’AK ; l’activité des ATPases ainsi que les distances de diffusion ont été prises en compte dans le modèle.

L’estimation du coefficient de diffusion, via une analyse bidimensionnelle (radiale et axiale) de myofibrilles, a été traitée en considérant les gradients de concentrations d’ATP crées par les ATPases myofibrillaires. Sur la **Figure 24**, sont représentées les diffusions d’ATP (de l’extérieur vers le centre) et d’ADP (de centre vers l’extérieur) à l’intérieur d’une myofibrille cylindrique de 1 µm de rayon.

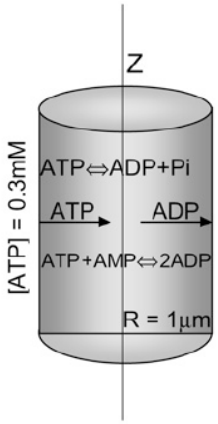


Figure 24 : Modèle schématisé d'une myofibrille et des gradients d'ATP et d'ADP crées par l'activité des ATPases. La réaction de l'adénylate kinase est également représentée. [D'après Selivanov, 2007].

Dans un premier temps, les simulations d'activités ATPasiques radiales en fonction de différentes valeurs de coefficients de diffusion ont révélé que celle qui permettait la reproduction la plus fidèle des données expérimentales était D = 0,1 µm/s. Dans un deuxième temps, les simulations ont intégré la dimension axiale et ont pour déterminer les distributions spatiales des CK et AKs. La **Figure 25** représente la distribution des activités enzymatiques le long d'une myofibrille. L'activité des ATPases est la plus élevée dans les zones A où actine et myosine se chevauchent. Par contre, les activités CK et AK sont maximales aux stries Z et bandes M. Ce résultat confirme la distribution inhomogène des enzymes et par conséquent l'existence de gradients de concentration localisés (micro-compartimentation).

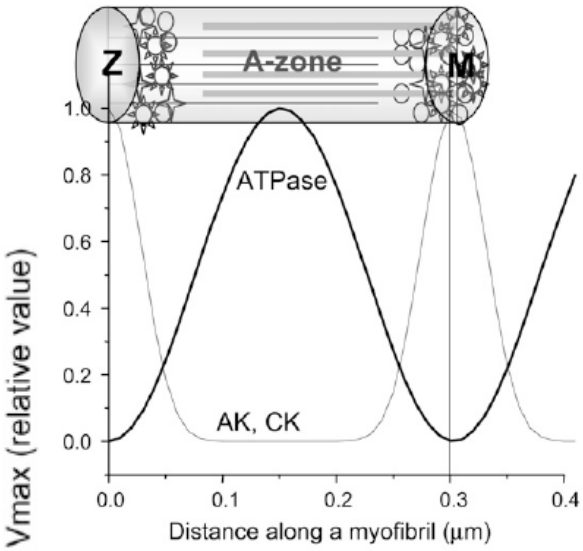


Figure 25 : Distribution enzymatique déterminée par un modèle bidimensionnel de réaction-diffusion de la consommation d'ATP dans les myofibrilles. [Selivanov, 2007].

Les modèles intégrant les phosphotransferts et la distribution spatiale intracellulaire aboutissent à une diffusion lente de l'ATP, au minimum 3 fois inférieure à celle obtenue par ^{31}P-RMN. Ces résultats sont cohérents sachant que les cellules cardiaques sont constituées principalement de myofibrilles et de mitochondries, qu'il n'existe pratiquement pas de cytosol « libre » et que la diffusion est restreinte.

I. 5.4 Modèles mathématiques des restrictions mitochondriales de diffusion

La régulation de la respiration mitochondriale est fonction de la quantité d'ADP disponible pour l'activer. C'est pourquoi la question des restrictions de la diffusion de l'ADP est au cœur des études de régulation métabolique. La **Figure 26** représente des modèles de diffusion avec et sans restriction autour de la mitochondrie [Vendelin, 2004].

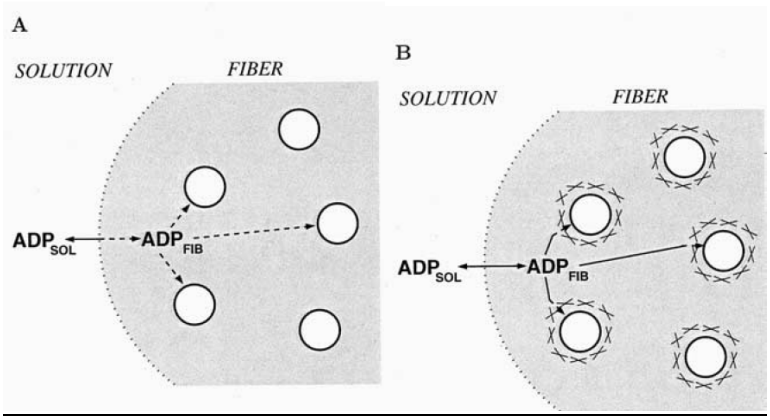


Figure 26 : Modèles de diffusion de l'ADP avec (A) et sans (B) restriction de la diffusion de l'ADP à la membrane externe mitochondriale. [D'après Vendelin, 2004].

Les simulations d'activation de la respiration en conditions de restriction à la membrane externe ont démontré que les restrictions de la diffusion de l'ADP sont localisées dans certaines zones et ne sont pas distribuées de façon homogène et que ces restrictions reproduisaient parfaitement les mesures expérimentales. De plus, par un modèle plus récent met en évidence que les restrictions de diffusion dues à la membrane externe elle-même sont très modérées tandis que celles provoquées par le reticulum sarcoplasmique ou des éléments du cytosquelette sont beaucoup plus accentuées [Ramay, 2009].
Il est maintenant clair que des éléments restreignent la diffusion de l'ADP mais la nature précise de ces éléments reste à déterminer.

I. 6 Restriction de diffusion mitochondriale et facteur X

I. 6.1 Différences de régulation mitochondriale *in vitro* et *in vivo*

L'un des paramètres cinétiques fondamentaux pour l'étude de la respiration mitochondriale est le Km apparent de l'OxPhos pour l'ADP. Il représente la disponibilité en ADP pour activer la respiration mitochondriale et sa valeur est fortement tissu-dépendante et peut varier en fonction de l'état physio-pathologiques des cellules. Le terme « apparent » a été introduit pour ne pas assimiler ce paramètre à une constante enzymatique pure (d'association, de dissociation, d'inhibition). En effet, le Km apparent ne correspond pas à une affinité enzyme/substrat mais reflète plutôt une accessibilité de l'ADP pour l'OxPhos. En condition *in vitro*, c'est-à-dire à partir de mitochondries isolées, le Km apparent mesuré par oxygraphie est environ égal au Km de l'ANT pour l'ADP [Klingenberg, 1970], soit environ 10-20 µM [Vignais, 1976 ; Jacobus, 1982]. Cependant, *in vivo* ou *in situ* (mesures en cellules ou fibres perméabilisées à la saponine), cette valeur peut atteindre 20 fois cette valeur soit environ 300 µM pour les cardiomyocytes ou les fibres cardiaques [Kay, 1998 ; Saks, 1998 ; Saks, 2001 ; Seppet, 2001 ; Dos Santos, 2002] (**Tableau 1**). Des valeurs élevées de Km apparent ont également été montrées dans d'autres muscles oxydatifs [Kay, 1997], dans des hépatocytes [Fontaine, 1995] mais pas dans les muscles squelettiques rapides [Kuznetsov, 1996 ; Veksler, 1995]. Ce phénomène est donc bien tissu-spécifique et ne dépend pas ni des distances de diffusion ni du diamètre cellulaire [Kay, 1997, **Tableau 1**]. De plus, l'extraction de myosine des cardiomyocytes (cardiomyocytes fantômes ou « ghost ») n'a pas d'influence sur le Km apparent dont la valeur reste élevée (**Tableau 1**), ce qui implique que la régulation mitochondriale de la respiration ne dépend pas de la présence des myosine-ATPases et qu'il existe toujours des restrictions de diffusion qui empêche l'ADP de diffuser librement jusqu'à la matrice mitochondriale et qui, de ce fait, restreignent (et contrôlent) la respiration cellulaire.

Cependant, la présence de Cr permet de shunter ces restrictions de diffusion, comme le montre le **Tableau 1**. En effet, l'addition de (10-20 mM) de Cr sur les préparations cellulaires diminue le Km apparent de 60-70 % en moyenne, témoignant du rôle central de la CK dans la régulation de la respiration in situ dans les cellules musculaires et de l'efficacité du couplage fonctionnel entre CK mitochondriale et ANT.

Comme l'on montré plusieurs études de modélisation [Aliev, 1997 ; Saks, 2003 ; Vendelin, 2009], des restrictions de diffusion existent autour de la mitochondrie et corroborent une ancienne théorie : la théorie du facteur X.

Tableau 1 : Km apparents de la respiration mitochondriale pour l'ADP pour différents matériels biologiques musculaires dans différentes conditions : conditions normales, après un traitement à la trypsine (0,125 mg/ml, 15 min) ou après addition de créatine (Cr, 20 mM). Dans certains cas, le diamètre cellulaire est indiqué.

Préparation	Diamètre, µm	Km Apparent (ADP), µM	Km Apparent (ADP) avec créatine, µM	Km Apparent (ADP) après traitement à la trypsine , µM
Homogénat de tissu cardiaque		228 ± 16		36 ± 16
Cardiomyocytes	10–25	–	35,6 ± 5,6	
		329 ± 50		
	16	250 ± 38		
	20	200 – 250		
Cardiomyocytes fantômes	20	200 – 250		
Fibres cardiaques perméabilisées	20	297 ± 35	85 ± 5	83 ± 22
		260 ± 50	79 ± 8	
		300 ± 23	102 ± 35	
		300 – 400		
		227 ± 40		
		370 ± 70		
		234 ± 24		
	22	–		
	18*	324 ± 25		
		320 ± 36		
Fibres perméabilisées de muscle rapide	80–100	7.5 ± 0.5		
		8 – 22		
Mitochondries isolées de cœur	~1	17.6 ± 1	13,6 ± 4,4	17.6 ± 1
		13,9 ± 2,6		

I .6.1 La théorie du facteur X

L'histoire du facteur X remonte à 1995 quand Saks et al [Saks, 1995] montrent que des traitements à la trypsine appliquée sur des cardiomyocytes, des fibres perméabilisées et un

homogénat de tissu cardiaque diminuaient très fortement le Km apparent alors que la valeur du Km apparent pour les mitochondries isolées ne montrait pas de variation avant et après traitement protéolytique (**Tableau 1**).

Les auteurs déduisent donc de ses observations qu'un facteur protéique influence la régulation mitochondriale. Ils émettent l'hypothèse que ce facteur, appelé le facteur X, serait associé au cytosquelette (**Figure 27**).

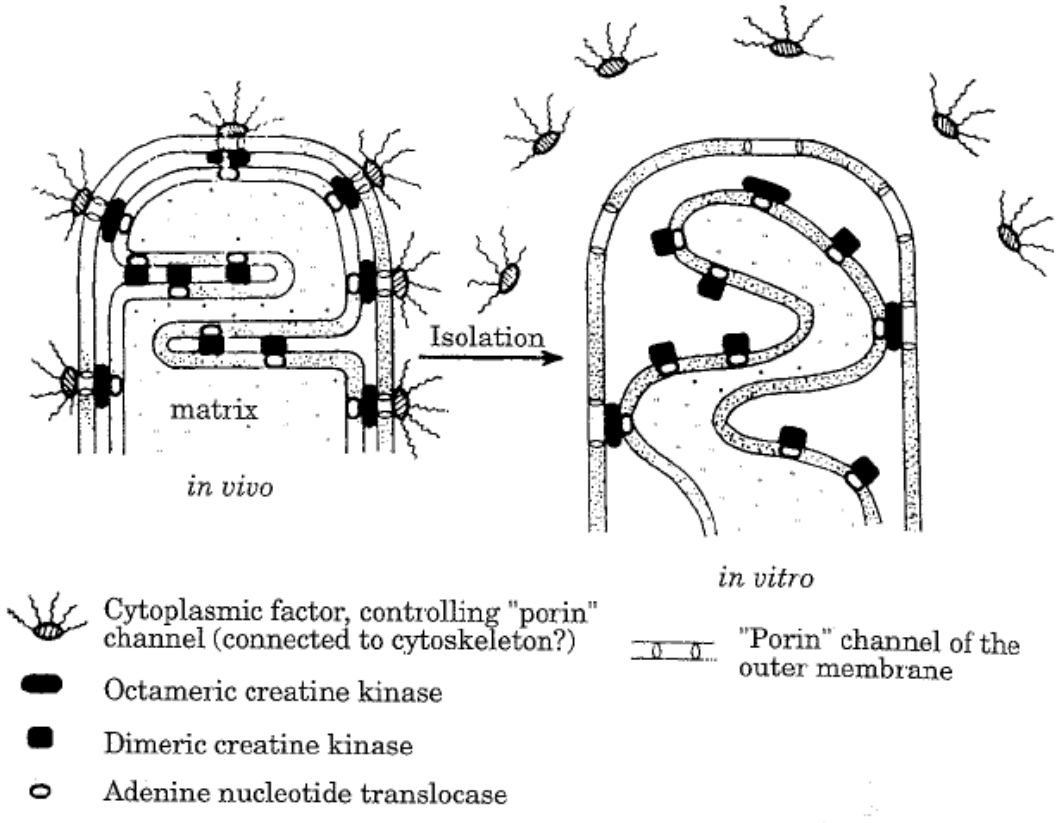


Figure 27 : Schéma de l'association du facteur X à la mitochondrie via le Voltage Dependant Anion Channel (VDAC). L'isolement des mitochondries conduirait au détachement de ce facteur. [D'après Saks, 1995].

La différence de régulation entre les mitochondries isolées et les mitochondries *in situ* (dans les cellules perméabilisées) serait donc due au facteur X qui serait lié à la membrane externe *in vivo* et *in situ* mais qui se détacherait de la mitochondrie lors de la procédure d'isolement aboutissant à une diminution du Km apparent. En effet, sans la présence du facteur X, la membrane serait plus perméable à l'ADP et par conséquent il serait plus accessible pour activer la respiration.

Ce facteur influerait donc sur la perméabilité de la membrane externe mitochondriale, c'est-à-dire sur la perméabilité de la porine mitochondriale, le Voltage Dependant Anion Channel (VDAC).

I. 6.2 Le Voltage Dependant Anion Channel

Le VDAC a été identifié par Marco Colombini [Colombini, 1979] et porte ce nom car il est sensible aux variations de potentiel électrique (études pratiquées sur des protéines isolées et purifiées), bien qu'aucun courant ne traverse la membrane externe mitochondriale. Le VDAC a été observe pour la première fois après reconstitution de protéine purifiée dans une bicouche phospholipidique plane [Colombini, 1980]. L'application de l'équation d'Einstein-Stokes (équation (2), section I. 2.2.2) dans un electrolyte connu a permis de déterminer le diamètre du pore à environ 3 nm [Colombini, 1980 ; Manella, 1982]. Ce pore est donc assez large et permet la translocation d'une grande quantité d'ions moléculaires [Schein, 1976 ; Colombini, 1989] et de composés de poids moléculaires elevé (jusqu'à 5-10 kDa) [Benz, 1990]. Il possède plusieurs positions fonctionnelles : un état ouvert qui est anion-sélectif, un état fermé qui est cation sélectif, et des états intermédiaires réversibles [Peng, 1992 ; Colombini, 1996 ; Pavlov, 2005] (**Figure 28**).

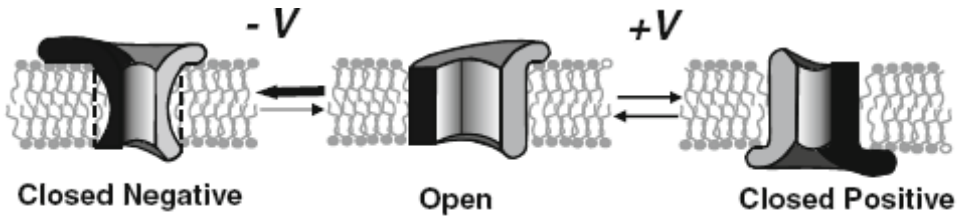


Figure 28 : Schéma représentant l'asymétrie du VDAC. Il existe un état ouvert et deux états fermés dépendants du signe du voltage appliqué. [D'après Song, 1998].

La famille des gènes du VDAC comporte trois isoformes chez les mammifères : VDAC 1, VDAC 2 et VDAC 3, qui ont deux à deux 70 % d'homologie de séquence. Chez la souris, la délétion de VDAC 1 ou VDAC 2 entraîne une réduction des capacités respiratoires de 30 % [Wu, 1999].

De récentes études sur VDAC ont mis en évidence sa régulation par différents facteurs (protéines, lipides, co-facteur), tant au niveau cytoplasmique que membranaire [Rostovtseva, 2008], répertoriés dans le **Tableau 2**. Les protéines Bcl-$_{XL}$ (protéine anti-apoptotique) et tBid (proteine pro-apoptotique) sont des régulateurs du VDAC qui se retrouve au centre des processus de mort cellulaire [Lemasters, 2006 ; Tan, 2007]. Un des modèles proposé pour la participation du VDAC dans les processus anti-apoptotiques est l'attachement de l'hexokinase

à la mitochondrie régulé par le facteur de croissance Akt et dont l'association pourrait prévenir le relargage du cytochrome c [Robey, 2006]. La liaison hexokinase / VDAC est retrouvée dans la plupart des cellules cancéreuses [Mathupala, 2009 ; Pedersen, 2008 ; Pedersen, 2007].

Tableau 2 : Régulateurs cytoplasmiques et membranaires du Voltage Dependant Anion Channel.

Agent	Mechanism of action
Bcl-x_L	Favors open state
NADH	Favors closed states
tBid	Induces irreversible closure
Non-lamellar lipids, PE and cardiolipin	Favors closed states of smaller conductance
Tubulin	Induces permeation block
Actin	Favors closed states of smaller conductance
Hexokinase-I	Induces closure[a]

Le VDAC n'est donc pas seulement un canal mais également, de par sa position en interface, un régulateur global de la fonction mitochondriale [Lemasters, 2006].

In vivo, la perméabilité du VDAC, et donc de toute la membrane externe, crée une dynamique de compartimentation, voire de micro-compartimentation, dans l'espace inter-membranaire [Gellerich, 1987] qui contribue à la formation d'un gradient de concentration d'ADP et d'ATP en séparant les pools cytoplasmiques et mitochondriaux [Adrienko, 2003]. La perméabilité du VDAC aux nucléotides adényliques a été étudiée par des techniques de bioluminescence via le couple luciférase/luciférine [Rostovtseva, 1997]. Les flux d'ATP à travers le VDAC (protéines purifiées insérés dans une membrane phopholipidique) ont été mesurés et les auteurs ont confirmé que l'ouverture du VDAC était suffisante pour supporter tous les efflux d'ATP provenant de la mitochondrie [Rostovtseva, 1997]. Par contre, quand un voltage élevé est appliqué, tous les flux d'ATP sont stoppés, comme indiqué sur la **Figure 29**.

Ces résultats *in vitro* peuvent être transférés *in vivo* en remplaçant le voltage positif (car il n'y a pas de différence de potentiel à la membrane externe) par un facteur régulateur [Rostovtseva, 2008].

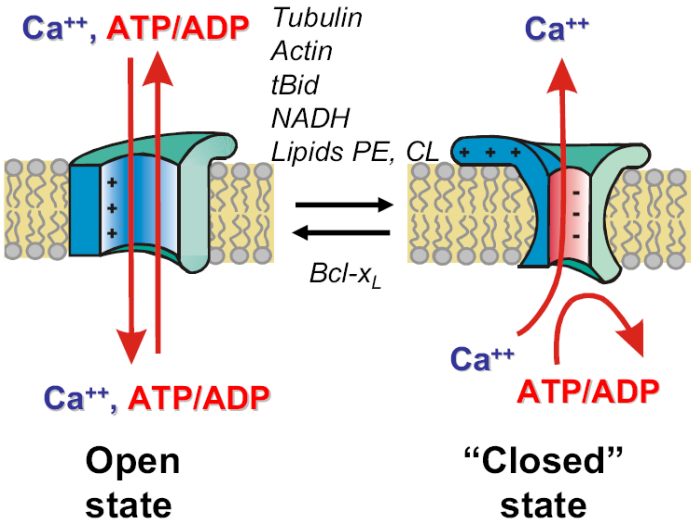


Figure 29 : Perméabilité du VDAC aux nucléotides adényliques et au calcium. L'application d'un voltage positif transfert le VDAC vers un état fermé où le volume du pore est réduit et ne permet plus le passage de l'ADP et de l'ATP, alors qu'il reste perméable aux ions calcium. *In vivo*, la membrane externe mitochondriale n'étant pas chargée, ce sont les régulateurs cytoplasmiques ou membranaires qui modifient l'état fonctionnel du VDAC. [D'après Rostovtseva, 2008].

Cette imperméabilité du VDAC aux nucléotides adényliques en position fermée est donc dépendante des régulateurs de perméabilité. Parmi tous les régulateurs possibles, une seule protéine a présenté, *a priori*, des effets compatibles avec une régulation de la respiration mitochondrial et serait un potentiel facteur X : la tubuline.

I. 6.4 La tubuline

I. 6.4.1 Tubuline et microtubules

La tubuline est une protéine abondante retrouvée dans la plupart des cellules eucaryotes et représente à elle seule 20 % des protéines neuronales. Elle a été identifiée comme étant une protéine soluble capable de se lier à une drogue anti-mitotique, la colchicine [Wilson, 1966 ; Borisy, 1967]. C'est une protéine hétérodimèrique (**Figure 30**) composée d'un monomère α et d'un monomèreβ. Des isoformes γ, δ, et ε existent mais sont moins abondantes [Wolff, 2009].

Chaque monomère a une masse moléculaire d'environ 50 kDa et une extrémité C-terminal acide (appelée queue) qui définissent les différents isoformes de tubuline et sont sujets à de nombreuses modifications post-traductionnelles.

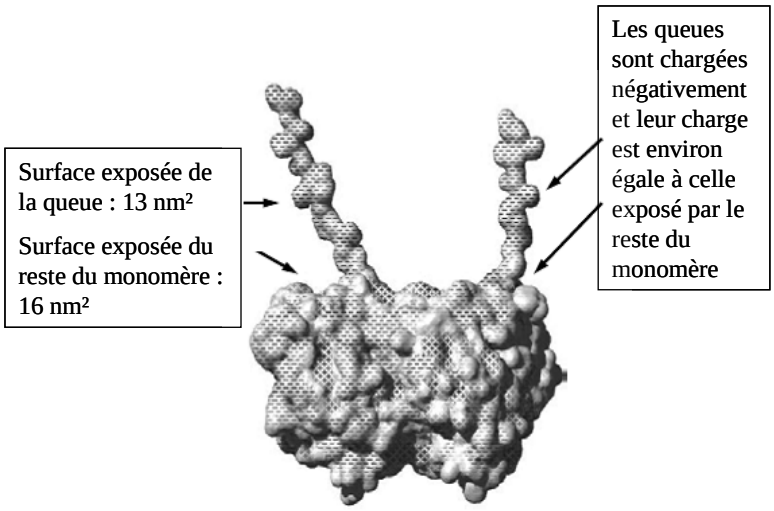


Figure 30 : Hétérodimère de tubuline. Leurs extremités (queues) C-terminales sont chargées négativement. [D'après Sataric, 2003].

La tubuline est une protéine très dynamique. Elle peut se présenter dans les cellules soit sous forme dimérique libre (70 %) soit sous forme polymérisée en réseau microtubulaire (30 %) [Tagawa, 1998]. Les dimères, une fois chargés en GTP, s'associent pour former des protofilaments de 25 nm de diamètre. L'association de 10 à 15 protofilaments rend la structure plus rigide puis l'association de nouveaux dimères à l'extrémité positive du microtubule (MT) permet son élongation (**Figure 31**). Dans le cytoplasme les dimères sont en équilibre dynamique avec les microtubules. Chaque mm de longueur de MT est composé de 1650 hétérodimères.

Le réseau microtubulaire participe à la croissance, au guidage et au branchage axonal [Dent, 2003] de même qu'il est impliqué dans la ségrégation des chromosomes pendant la division cellulaire, la maintenance de la forme cellulaire et le trafic intracellulaire de macromolécules et d'organelles [Dutcher, 2001]. C'est pourquoi les MTs sont une des principales cibles d'une famille d'agents anticancéreux, appelés les agents anti-tubuline. Ces drogues ciblées suppriment la dynamique microtubulaire essentielle à la formation du réseau mitotique pendant la division cellulaire et par conséquent inhibent la prolifération tumorale [Gonçalves, 2000].

Certains agents chimiques peuvent provoquer la prolifération et/ou la stabilisation du réseau microtubulaire (taxol, thiorédoxine) [Kahn, 1991] ou au contraire le détruirent (colchicine). En effet, l'exposition de cardiomyocytes adultes à la colchicine a révélé une augmentation de la quantité de tubuline libre [Kerfant, 2001].

La tubuline peut être phosphorylée par différentes kinases [Verde, 1990] et subit à son extrémité C-terminale de nombreuses modifications posttraductionnelles, telles que glutamylation, glycosylation, tyrosynation, acetylation, acylation [MacRae, 1997 ;

Westermann, 2003], nitrotyrosynation [Phung, 2006] ou nitration [Rayala, 2007] capables de moduler la fonction microtubulaire. Des pools de α-tubuline tyrosynée et détyrosynée coexistent dans les cellules et interviennent dans la stabilité et le cycle assemblage-désassemblage des MTs [Schulze, 1987 ; Webster, 1987 ; Peris, 2009].

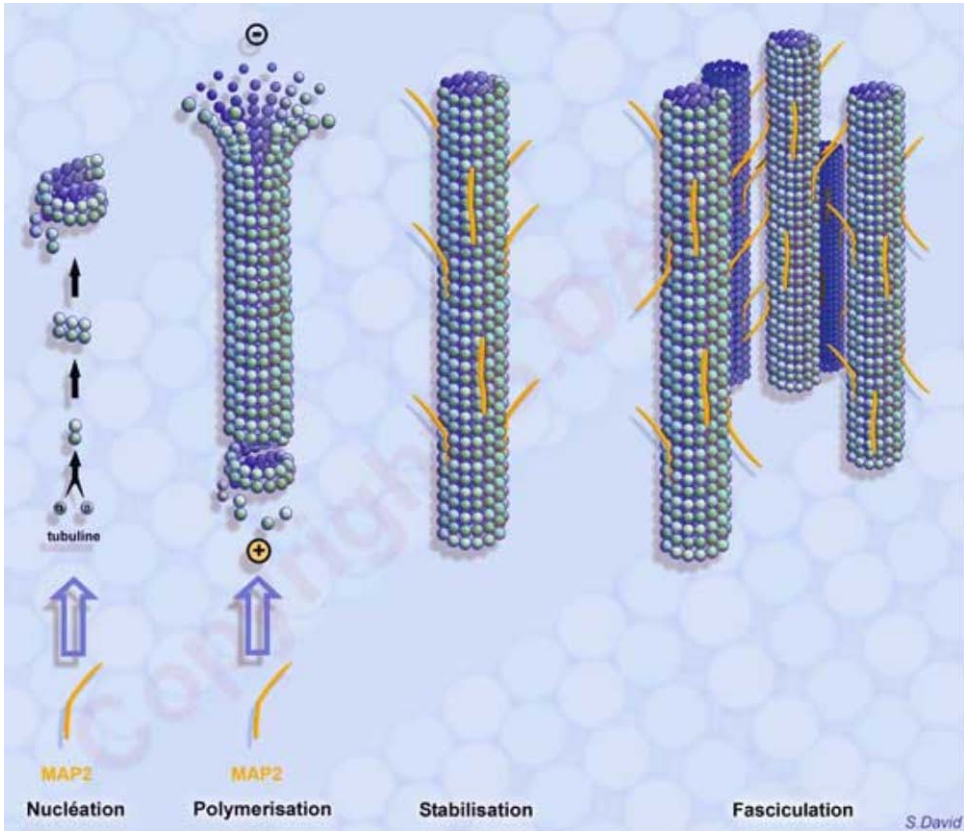


Figure 31 : Polymérisation de la tubuline en microtubules. Cette polymérisation dynamique est catalysée par des Microtubule Associated Protein (MAP), dont la MAP-2. [D'après un schéma original de Sébastien David].

I. 6.4.2 Les protéines associées aux microtubules

Deux catégories majeures de protéines interagissent avec les MTs : les protéines motrices (dynéine et kinésine) intervenant dans le trafic cellulaire [Vale, 1985] et les protéines associées aux MTs, les MAPs (Microtubule Associated Protein). Contrairement aux liaisons des protéines motrices qui sont GTP-dépendantes, l'association MT-MAP ne dépend d'aucun nucléotide. Les MAPs copolymérisent avec les MTs à travers les cycles d'assemblage-désassemblage stimulant ainsi leur nucléation et stabilisant les polymères [Olmsted, 1991 ; Schoenfeld, 1994 ; Mandelkow, 1995]. La stabilité des MTs peut être différente *in vitro* et *in vivo*. En effet, *in vitro*, les MTs se désorganisent à 4°C alors que dans les cellules ils sont résistants au froid [Lieuvin, 1994]. Cette stabilité *in vivo* est due principalement à une

protéine controlée par la calmoduline appelée STOP (Stabilizing Tubule Only Polypeptide) [Job, 1983 ; Guillaud, 1998]. Il existe plusieurs variantes de STOP protéines toutes associées aux MTs et à la calmoduline : N-STOP (neuronal STOP), E-STOP (Early STOP) et la moins abondante, F-STOP (fibroblastic STOP) [Bosc, 1999].

Une délétion du gène codant pour la protéine STOP chez des souris (STOP -/-) a permis d'établir un phénotypage de ces animaux dépourvus de microtubules neuronaux [Andrieux, 2002]. Cette délétion s'est avérée viable pour les animaux qui ne présentaient pas d'anomalies majeures de l'anatomie de cerveau ou du système nerveux en général, si ce n'est une diminution de la densité de vésicules synaptiques, une hyperdopaminergie et des altérations de la plasticité synaptique à court et moyen terme. Cependant les souris porteuses de la délétion ont révélé de multiples troubles sévères du comportement : activité désorganisée, troubles de la mémoire, anxiété, troubles nutritionnels, retrait social et négligence vis à vis de leur progéniture entrainant le décès de ceux-ci dans les 24 à 48 h suivant leur naissance. Ces animaux ont été traité par différents dosages soit d'anxiolytique (diazepam) soit de neuroleptiques (chlorpromazine, haloperidol ou clozapine) [Andrieux, 2002]. Ces troubles ont pu être atténués par un traitement aux neuroleptiques. Sachant que ces antipsychotiques sont utilisés en cas de troubles schizophréniques chez l'Homme, cette observation corroborerait certaines études reliant la schizophrénie à des désordres synaptiques [Harrison 1997 ; Mirnics, 2001].

Les souris déficientes en protéine STOP semblent donc s'approcher d'un modèle pour l'étude des troubles de type schizophréniques sensibles aux neuroleptiques car les troubles cognitifs et sociaux retrouvés chez les animaux STOP -/- sont apparentés à ceux retrouvés chez les patients atteints de cette pathologie [Andrieux, 2004 ; Hanaya, 2008 ; Begou, 2008]. Une nouvelle cible thérapeutique (l'épothelione D, un stabilisateur de MTs) a déjà été testée avec succès à partir de ce modèle [Andrieux, 2006].

I. 6.4.3 Différentes isoformes de tubuline

Chez les vertébrés, les tubuline α et β ont plusieurs isoformes qui peuvent différer drastiquement selon l'organisme. Chez la souris, on retrouve majoritairement 2 isoformes α (Mα1 et Mα2) et 5 isoformes β (Mβ1, Mβ2, Mβ3, Mβ4 et Mβ5).

Les travaux de clonage de Lewis et al [Lewis, 1985] ont donné les résultats suivants :

L'isoforme Mα1 est exprimé majoritairement dans le cerveau et les poumons et l'isoforme Mα2 montre des niveaux plus élevés dans la rate, le thymus, le cœur, le cerveau en developpement, les poumons et le foie.

Le pattern Mβ2 montre une forte présence dans le cerveau et les poumons. Mβ4 est exprimé exclusivement dans le cerveau et Mβ5 majoritairement dans le cerveau en developpement, les poumons, la rate et le thymus. Il semblerait donc que les isoformes Mα1 et Mβ2 soit co-exprimés ainsi que les isoformes Mα2 et Mβ5.

D'autres études ont montré que l'isoforme Mβ3 est moins abondante que les formes 1 et 2 mais s'est avéré être un marqueur de la résistance aux drogues anticancéreuses [Cicchillitti, 2008]. Il est exprimé notamment dans le cerveau et les testicules [Cleveland, 1985] et une abondance de Mβ3 déstabiliserait les MTs ce qui serait corrélé avec la résistance aux drogues [Verdier-Pinard, 2003 ; Kamath, 2005].

Il a été observé par co-immunoprécipitation que, dans plusieurs tissus (dont des tissus humains), l'isoforme Mβ3 est retrouvé assez abondamment dans les membranes mitochondriales où il exerce une fonction inconnue sur le VDAC [Bernier-Valentin, 1982 (travaux sur foie de rat) ; Carre, 2002b], contrairement à l'isoforme Mβ4 qui est retrouvé moins largement au niveau mitochondrial mais largement dans le cytoplasme. La tubuline est donc bien une protéine inhérente des mitochondries et représente 0,5 % des protéines mitochondriales totales et 2,2 % de la tubuline cellulaire [Bernier-Valentin, 1982 ; Carre, 2002a ; Linden, 1989].

Il a été reporté que des agents anti-tubuline permettent de provoquer le relargage mitochondrial de cytochrome c [Carre, 2002a]. Il y a donc bien une corrélation entre tubuline, VDAC et régulation mitochondriale mais les mécanismes de ses interactions sont encore mal connus.

Des tests préliminaires électrophysiologiques ont montré que la tubuline libre provoque la fermeture du VDAC isolé inséré dans une membrane reconstitué de phospholipidique [Rostovtseva, 2008]. Ces résultats *in vitro* sont très encourageants mais seuls des tests en condition plus physiologique permettrait de déterminer si la tubuline est le facteur X.

II. HYPOTHESE DE TRAVAIL ET OBJECTIFS SCIENTIFIQUES

Hypothèse de travail et objectifs scientifiques

Notre hypothèse de travail est que le milieu intracellulaire n'est pas un milieu homogène, mais est un milieu extrêmement organisé tant dans les cardiomyocytes que les synaptosomes. Sur la base de cette théorie, le but de ce travail est de mettre en évidence l'importance des interactions entre mitochondrie et structures ou processus extra-mitochondriaux par l'étude des propriétés au niveau du système, pour comprendre les mécanismes de régulation de la respiration entre mitochondrie *in vivo*. Les questions posées dans ce travail sont :

1- Quelle est la nature des structures responsables de la restriction de la diffusion des nucléotides adényliques à travers la membrane externe mitochondriale ? Quelle est la nature du facteur X ?

2- Existe-t-il un couplage fonctionnel entre la CK mitochondriale et l'ANT dans les mitochondries de cerveau ? Quel est sa fonction dans le métabolisme énergétique neuronal ?

3- Comment sont organisés les flux métaboliques entre la mitochondrie et le cytoplasme en tenant compte des restrictions de diffusion à la membrane externe mitochondriale et des couplages fonctionnels ?

4- Quels sont les changements dans la relation structure-fonction de l'énergétique cellulaire dans la pathologie, et en particulier dans les cellules cancéreuses ?

5- Peut-on étudier de manière quantitative l'hétérogénéité de la diffusion de l'ATP ?

Working hypothesis and scientific aims

Our working hypothesis is that the intracellular medium of cardiomyocytes and synaptosomes is not homogeneous but is a highly organized medium. Based on this theory, the aim of this work is to highlight the importance of interactions between the mitochondrion and cellular structures or processes by studying the system level properties to understand the mechanisms of regulation of mitochondrial respiration *in vivo*. The questions studied in this work are:

1- What is the nature of the structures responsible of restriction of diffusion of the adenine nucleotides across the mitochondrial outer membrane? What is the nature of the factor X?

2- Is there a functional coupling between the mitochondrial CK and ANT in brain mitochondria? What is its function in neuronal energy metabolism?

3- How metabolic fluxes between mitochondria and cytoplasm are organized taking into account diffusion restrictions and functional coupling?

4- How the structure-function relationships of cellular energetics are changed in pathology, in particular in cancer cells?

5- Is it possible to study quantitatively the heterogeneity of ATP diffusion?

III. RESULTATS ET DISCUSSION

III. 1 Découverte du facteur X (articles 4 et 5)

Article 4

Rostovtseva TK, Sheldon KL, Hassanzadeh E, Monge C, Saks V, Bezrukov SM, Sackett DL (2008) Tubulin binding blocks mitochondrial voltage-dependent anion channel and regulates respiration. Proc Natl Acad Sci U S A. 105(48):18746-18751.

Les dissimilitudes existant entre les valeurs de Km dans les mitochondries *in vitro* et *in situ* ont été expliquées par l'hypothèse du facteur X une protéine induisant une restriction de la diffusion des nucléotides adényliques à la membrane externe mitochondriale. Après sélection de plusieurs candidates, la protéine qui montra une action sur la perméabilité membranaire via le VDAC fût la tubuline.

En effet, les résultats montrent que la tubuline hétérodimérique à des concentrations nanomolaires provoque une augmentation de la sensibilité de VDAC au voltage quand il est purifié puis introduit dans une bicouche phospholipidique reconstituée et induit sa fermeture réversible à de faibles voltages (**Figure 32**).

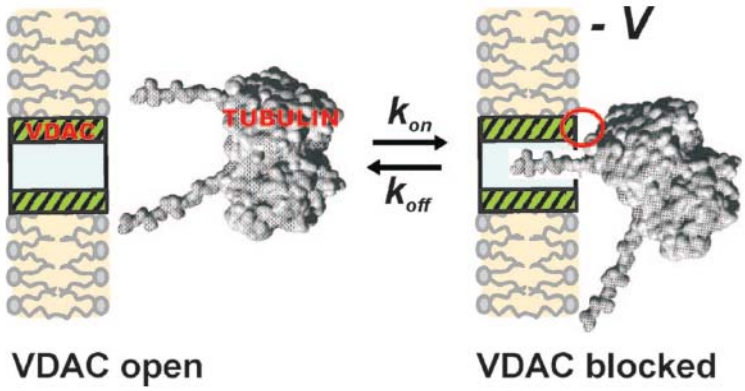


Figure 32 : Modèle de mécanisme de l'interaction tubuline hétérodimérique-Voltage Dependant Anion Channel (VDAC). La tubuline provoque la fermeture réversible du VDAC à de très faibles concentrations (insuffisantes pour la polymérisation et en absence de GTP et Mg^{2+}).

L'utilisation d'un isoforme mutant de tubuline auquel l'extrémité C-terminale anionique (CTT) a été tronquée a montré que l'interaction tubuline-VDAC nécessite la présence de la CTT. Dans le modèle proposé, la CTT négativement chargée pénètre dans le canal de la porine et interagit avec une boucle positivement chargée du VDAC (**Figure 32**).

Ces mesures *in vitro* mettent en évidence l'interaction directe entre la tubuline et le VDAC mais son implication plus physiologique dans la régulation de la respiration mitochondriale a été montrée par des mesures d'oxygraphie.

Pour cela, un protocole de reconstitution partielle de l'environnement extra-mitochondrial par incubation de mitochondries isolées de cerveau et de cœur avec la tubuline dimérique (1 µM) a été utilisé. Les résultats d'oxygraphie prouvent que la tubuline régule la respiration en créant des barrières de restriction pour l'ADP car en présence de tubuline le Km apparent pour l'ADP exogène augmente drastiquement. Le Km apparent des mitochondries contrôle (mitochondries isolées sans tubuline) est d'environ 10 µM alors que celui des mitochondries incubées avec la tubuline atteint des valeurs proches de celles mesurées *in situ* : pour les mitochondries de cœur incubées avec la tubuline : Km apparent = 330 ± 47 µM correspondant au Km apparent de cardiomyocytes (environ 300 µM) et Km apparent mitochondries de cerveau incubées avec la tubuline = 169 ± 52 µM correspondant au Km apparent des synaptosomes (environ 100 µM, cf article 6). Ces hautes valeurs de Km ont été déterminées à partir d'une deuxième composante obtenue après linéarisation des vitesses de respiration, la première composante ayant une valeur de Km similaire à celle retrouvée avec les mitochondries contrôle (sans tubuline). La présence de 2 composantes peut être expliquée par les différences d'affinités existant entre le tubuline et les différents isoformes de VDAC. Une autre possibilité serait l'association d'autres protéines au VDAC qui agirait en compétition avec la tubuline. Certaines protéines pro- ou anti-apoptotiques sont connues pour s'associer au VDAC ainsi que l'hexokinase (HK). Ces hypothèses restent à vérifier.

Des alignements de séquences ont permis de remarquer que les propriétés de la tubuline (longueur et charge) ne sont conservées dans l'évolution que dans les organismes possédant des mitochondries fonctionnelles.

Ces résultats ont permis de confirmer l'importance de l'interaction de la tubuline avec la mitochondrie dans la régulation *in vivo* de la respiration et d'identifier un rôle fonctionnel pour la tubuline dimérique.

Article 5

Guerrero K, Monge C, Brückner A, Puurand U , Kadaja L, Käämbre T, Seppet E, Saks V (2009) Study of possible interactions of tubulin, microtubular network and STOP protein with mitochondria in muscle cells. Mol. Cell Biochem., in press.

Les rôles fonctionnels de la tubuline et de la protéine STOP ont été étudiés dans les muscles. En effet les microtubules ne montrent par les mêmes propriétés de stabilité *in vitro* et *in vivo*. Alors qu'ils se désorganisent à 4°C *in vitro*, dans les cellules, ils sont résistants au froid

[Lieuvin, 1994]. Cette stabilité *in vivo* est due principalement à la protéine STOP (Stabilizing Tubule Only Polypeptide) [Job, 1983 ; Guillaud, 1998]. Cette protéine a été bien étudiée dans les cellules nerveuses via des souris déficientes en protéine STOP [Andrieux, 2002] cependant sont rôle dans le muscle est mal étudié. Ce travail propose donc d'étudier si la protéine STOP, par son rôle stabilisateur de microtubules, est impliquée dans la régulation de la respiration, comme la tubuline libre (hétérodimérique).

Les études oxygraphiques et génétiques pratiquées ont permis de conclure que l'expression de la tubuline, de la protéine STOP mais également que l'organisation microtubulaire sont tissu-spécifiques. Ces protéines sont beaucoup plus exprimées dans les muscles oxydatifs que dans les muscles glycolytiques et l'isoforme Mβ4 n'est pas exprimée exclusivement dans le cerveau (comme précédemment établit par Lewis [Lewis, 1985]) mais également dans le muscle cardiaque et le muscle oxydatif soléaire.

Il a été observé que le réseau microtubulaire n'apparaît que dans les fibres cardiaques. Cependant, l'utilisation de souris déficientes en protéine STOP n'a révélé aucune implication de cette protéine et de son rôle stabilisateur de microtubules dans la régulation de la respiration des fibres musculaires étudiées (pas de variations significatives de Km apparent pour l'ADP exogène entre mesures sur muscles de souris sauvages ou déficientes en protéine STOP). De même, la présence de colchicine, qui désorganise ce réseau, n'a que très peu d'influence sur la régulation de la respiration par la tubuline, suggérant que la présence de microtubules n'est pas requise pour l'interaction avec le VDAC. L'apparition d'une deuxième composante au Km apparent assez bas et représentant 40 % de la population mitochondriale peut être expliquée par le détachement de certains dimères de tubuline du VDAC dû à l'action de la colchicine qui est un agent chimique puissant.

Il n'y a donc pas de connexion directe entre la présence d'un réseau microtubulaire organisé dans les cardiomyocytes et la régulation de la respiration. Il semblerait que seule la tubuline libre soit requise pour créer les barrières de restrictions de diffusion.

La reproduction du protocole de reconstruction partielle du cytosquelette en présence de Cr a montré que l'activation de la MtCK permet de shunter les restrictions de diffusion imposées par la tubuline car le Km apparent en présence de Cr diminue fortement (23 ± 6µM) confirmant le rôle central de la CK dans l'énergétique cellulaire.

III. 2 Mise en évidence du couplage fonctionnel entre uMtCK et ANT dans les cellules nerveuses et caractérisation de la régulation de la respiration dans les synaptosomes (article 6)

Monge C, Beraud N, Kuznetsov AV, Rostovtseva T, Sackett D, Schlattner U, Vendelin M, Saks V (2008) Regulation of respiration in brain mitochondria and synaptosomes: restrictions of ADP diffusion in situ, roles of tubulin, and mitochondrial creatine kinase. Mol. Cell. Biochem. 318(1-2):147-165.

Dans les mitochondries de tissu cardiaque, le couplage fonctionnel de la sMtCK et de l'ANT a été découvert par des mesures cinétiques de la réaction de la sMtCK et la détermination des constantes de dissociation de l'ATP et de la Cr [Jacobus, 1982 ; Aliev, 1993]. Cependant, ce couplage n'a jamais été étudié dans les cellules nerveuses. C'est pourquoi ce travail, sur la base de celui de Jacobus et Saks [Jacobus, 1982], propose d'analyser la cinétique complète de l'activation de la uMtCK et d'identifier les 4 constantes de dissociation de l'ATP (K_{ia} et K_a) et de la Cr (K_{ib} et K_b) avec ou sans inhibition de l'OxPhos, selon la réaction indiquée **Figure 33**.

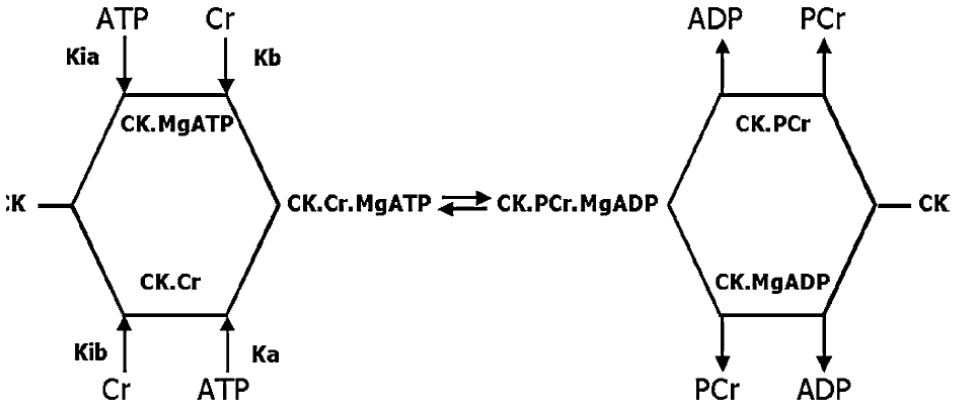


Figure 33 : Schéma de la réaction de la créatine kinase. Les constantes de dissociation de l'ATP (K_{ia} et K_a) et de la Cr (K_{ib} et K_b) sont indiquées.

Les constantes de dissociation ont ainsi pu être comparée selon les conditions : OxPhos activée par l'ATP et la Cr et du succinate, substrat du complexe II (mesures oxygraphiques) ou OxPhos inhibée par de la roténone et de l'oligomycine qui sont des inhibiteurs du complexe I et de l'ATP synthase, respectivement (mesures spectrophotométriques).

L'analyse cinétique complète est basée sur une analyse primaire puis secondaire de l'activation de réaction de la uMtCK.

L'analyse primaire s'effectue en deux temps : dans un premier temps la concentration d'ATP est fixée et après linéarisation en double inverse, la vitesse de réaction s'exprime ainsi :

$$\frac{1}{V} = \frac{1}{[\mathrm{MgATP}]}\left(\frac{K_a}{\mathrm{Vm}}\left(\frac{K_{ib}}{[\mathrm{Cr}]}+1\right)\right)+\frac{1}{\mathrm{Vm}}\left(\frac{K_b}{[\mathrm{Cr}]}+1\right)$$

Et dans un deuxième temps, la concentration de Cr est fixée et après linéarisation en double inverse, la vitesse de réaction s'exprime ainsi :

$$\frac{1}{V} = \frac{1}{[\mathrm{Cr}]}\left(\frac{K_b}{\mathrm{Vm}}\left(\frac{K_{ia}}{[\mathrm{MgATP}]}+1\right)\right)+\frac{1}{\mathrm{Vm}}\left(\frac{K_a}{[\mathrm{MgATP}]}+1\right)$$

L'analyse secondaire consiste à utiliser les différentes pentes et ordonnées à l'origine obtenues par linéarisation en double inverse des vitesses, puis à les exprimer en fonction de la concentration en Cr ou en ATP selon la constante à déterminer.

Cette analyse a permis de confirmer l'existence du couplage fonctionnel entre la uMtCK et l'ANT. En effet, la diminution d'un facteur 7 des constantes de dissociation K_{ia} et K_a de l'ATP en condition d'OxPhos activée atteste de l'augmentation de l'efficacité du recyclage de l'ADP (turnover) dans l'espace inter-membranaire. Ce recyclage rapide et local n'est possible que par le couplage fonctionnel de l'uMtCK et de l'ANT induit par leur proximité spatiale et créant de ce fait une micro-compartimentation métabolique. Ces résultats ont été confirmés par simulation informatique.

Il en résulte un contrôle de l'OxPhos par ce système couplé et une production efficace de PCr utilisable dans le cytoplasme.

Un schéma de l'énergétique synaptosomale a été construit sur la base d'un schéma de Nicholls [Nicholls, 2002] (**Figure 34**). Les résultats obtenus dans cette étude ont permis d'ajouter au schéma initial de Nicholls le couplage fonctionnel uMtCK-ANT, la présence de tubuline régulant la perméabilité du VDAC, le système Cr/PCr qui agit comme une 'navette énergétique' entre les sites de production d'ATP (principalement la mitochondrie) et de consommation d'ATP (principalement les pompes Na^+/K^+, mais aussi les pompes Ca^{2+} et les processus d'exocytose des neurotransmetteurs). Il n'existe que très peu de preuves directes du couplage entre l'isoforme cytoplasmique BBCK et les pompes membranaires ou les vésicules synaptiques (d'où le point d'interrogation sur le schéma de la **Figure 34**) pourtant ces couplages sont des éléments clés dans la régulation énergétique cellulaire.

La PCr utilisée pour le recyclage de l'ATP à proximité des ATPases provient donc de l'ATP mitochondrial et de la réaction hors équilibre de la CK mitochondriale dont la direction de réaction (synthèse de PCr) est dirigée par le couplage fonctionnel.

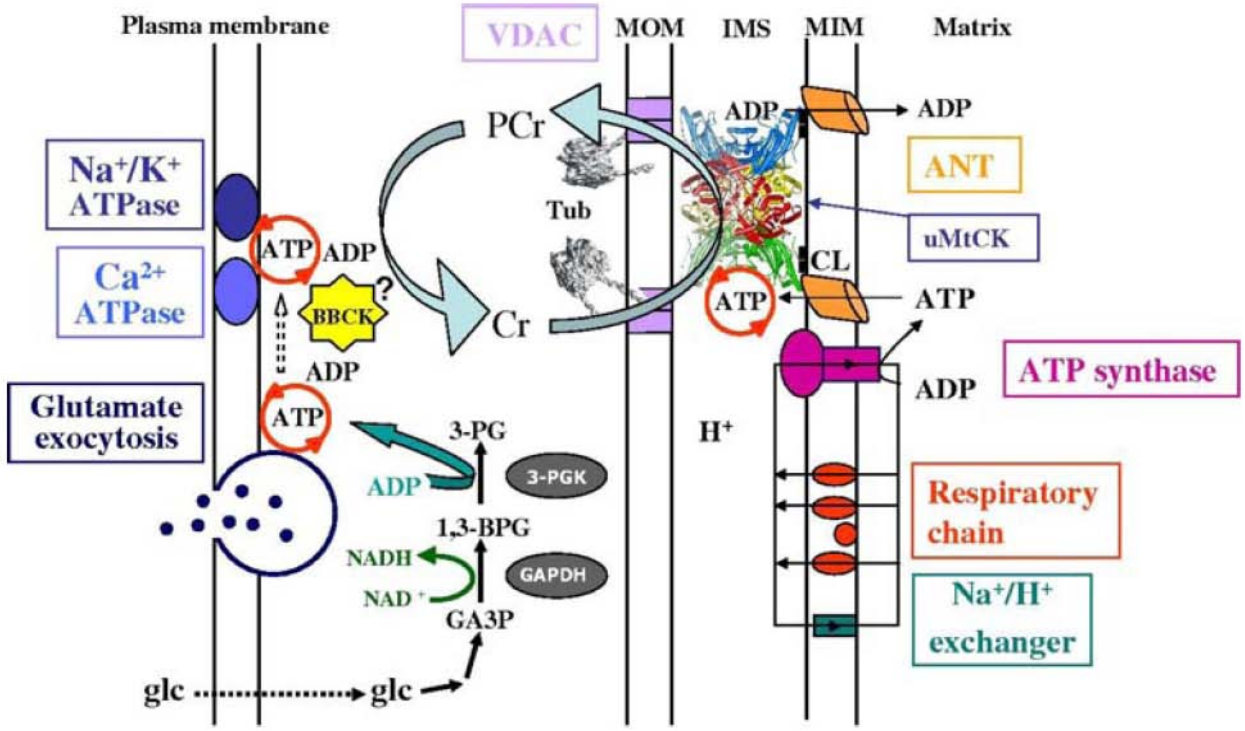


Figure 34 : Schéma du métabolisme énergétique synaptosomal. L'existence du couplage fonctionnel entre uMtCk et ANT et la présence de latubuline entraine une micro-compartimentation métabolique. Glc : glucose, GA3P : glycéraldéhyde 3 phosphate, GAPDH : glycéraldéhyde 3 phosphate déshydrogénase, 1,3-BPG : 1,3 biphosphoglycérate, 3-PGK : 3 phosphoglycérate kinase, 3-PG : 3 phosphoglycérate, Cr : créatine, PCr : phosphocréatine, VDAC : voltage dependant anion channel, ANT : adenine nucleotide translocase, CL : cardiolipine, Tub : tubuline, BBCK : créatine kinase cytosolique, MOM : membrane externe mitochondriale, IMS : espace inter-membranaire, MIM : membrane interne mitochondriale.

Des tests oxygraphiques de maintien de l'état 3 de respiration en présence d'ADP et de Cr, de même que des tests d'activation de la respiration par des concentrations croissantes de Cr, ainsi que la forte diminution du Km apparent dans les synaptosomes après addition de Cr (de 110 ± 11 à 25 ± 1 µM) témoignent de la fonction cruciale de la CK dans l'énergétique du tissu nerveux. Les résultats obtenus expliquent parfaitement les effets bénéfiques rencontrés après supplémentation en Cr chez certains patients atteints de maladies neurodégénératives telle que la maladie de Parkinson [Andres, 2008]. En effet, un apport en Cr par le sang (les cellules musculaires et nerveuses ne synthétisant par de Cr) permet de maintenir de hauts niveaux de PCr et donc de renouveler efficacement l'AD/TP via les couplages fonctionnels des CKs et par conséquent d'entretenir un métabolisme énergétique robuste.

III.3 L'Interactosome Mitochondrial : mécanismes et bases structurales du couplage fonctionnel *in situ* (articles 7et 8)

Article 7

Guzun R, Timohhina N, Tepp K, Monge C, Kaambre T, Sikk P, Kuznetsov AV, Pison C, Saks V. (2009) Regulation of respiration controlled by mitochondrial creatine kinase in permeabilized cardiac cells in situ Importance of system level properties. Biochim. Biophys. Acta. 1787(9):1089-105.

Les couplages fonctionnels impliquant les CKs mitochondriales ont toujours été étudiés *in vitro* dans les mitochondries isolées. Cette approche réductionniste a permis d'élaborer les bases moléculaires de ces couplages fonctionnels. Cependant des études *in vivo* permettraient d'inclure ses couplages dans la régulation métabolique cellulaire.
Cette étude est donc une analyse systémique expérimentale de la régulation de la respiration *in vivo*, basée sur l'étude cinétique de la respiration activée par la CK mitochondriale, dont le protocole expérimental est représenté **Figure 35**.

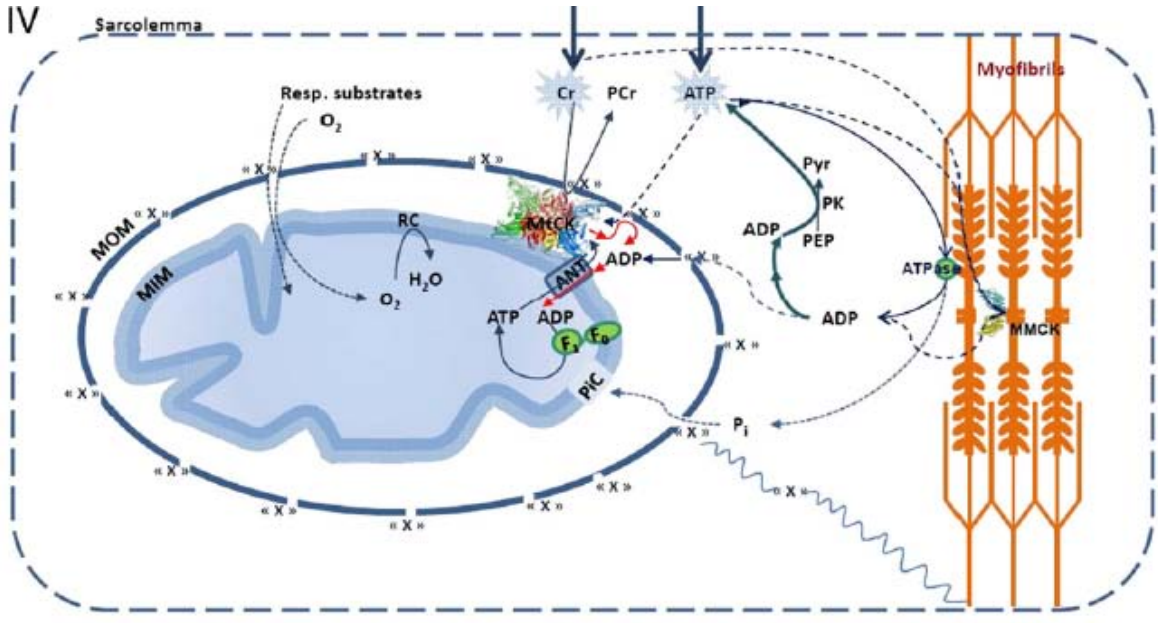


Figure 35 : Schéma du protocole expérimental d'étude du couplage fonctionnel entre la créatine kinase mitochondriale (MtCK) et l'adenine nucleotide translocase (ANT) dans des cardiomyocytes perméabilisés. La créatine (Cr) et l'ATP sont ajoutés à des concentrations connues et fixées. Le phosphoenolpyruvate (PEP) et la pyruvate kinase (PK) constituent un système piège d'ADP cytoplasmique (produit par les ATPases membranaires et la créatine kinase myofibrillaire MMCK) et régénèrent ainsi l'ATP dans le cytoplasme. MOM : membrane

externe mitochondriale, IMS : espace inter-membranaire, MIM : membrane interne mitochondriale, PIC : canal à phosphate inorganique, PCr : phosphocréatine.

La comparaison des cinétiques d'activations et la détermination des constantes de dissociation de l'ATP, de la Cr et de la Pcr dans la réaction de la CK dans les mitochondries isolées de cœur et les cardiomyocytes perméabilisés en présence ou en absence d'un système piège d'ADP cytoplasmique (basé sur la réaction de la pyruvate kinase (PK) en présence de phosphoénolpyruvate (PEP) mimant l'effet de la glycolyse) ont permis d'étudier les cinétiques de la réaction de la CK dans les cardiomyocytes en excluant l'influence des réactions cytoplasmiques consommatrices d'ATP et les flux cytoplasmiques d'ADP.

Les résultats de cette étude montrent clairement que la régulation de la respiration et des flux métaboliques dépend de l'interaction entre mitochondrie et d'autres structures ou processus métaboliques intracellulaires, telle que la glycolyse. Ces régulations ont donc des propriétés propres élevées au niveau du système, qui ne pourraient pas être mesurables *in vitro*, en mitochondries isolées.

Tout d'abord, dans une suspension de mitochondries isolées dont la respiration est activée par l'ATP et la Cr, la présence du système PEP/PK diminue de 50 % la vitesse de respiration indiquant que ce système est capable de piéger l'ADP produit par la réaction de la MtCK dans l'espace inter-membranaire. Cette fuite d'ADP à travers la membrane externe n'est plus observée dans les mitochondries *in situ*.

Des mesures des concentrations cytoplasmiques d'ATP et de PCr ainsi que la comparaison des constantes de dissociation de la PCr (K_{ip}) et de la Cr (Kb et Kib) ont clairement montré que la perméabilité du VDAC est hautement sélective. La diffusion des nucléotides adényliques (ADP et ATP) est fortement restreinte par la présence de tubuline contrairement aux composés guanidiniques (Cr et PCr) qui diffusent plus librement. La présence de tubuline et la sélectivité de la perméabilité ont pour effet une augmentation du taux de recyclage de l'ADP par le couplage fonctionnel MtCK-ANT. Ces résultats expliquent donc l'absence d'inhibition de la respiration par la système PEP/PK dans les cardiomyocytes permeabilisés où la présence de la tubuline en interaction avec le VDAC entraîne une séquestration de l'ADP dans la mitochondrie et un transfert direct de l'ADP dans la matrice mitochondriale via le couplage fonctionnel MtCK-ANT.

La micro-compartimentation de tous les ADP produits dans la mitochondrie par la réaction de la MtCK sans fuite dans le milieu extra-mitochondrial est une des plus importantes propriétés de notre modèle expérimental de cardiomyocyte perméabilisé.

La détermination des constantes des Kb et Kib *in vitro* et *in situ* a mis en évidence l'augmentation très nette de l'affinité de la MtCK pour la Cr dans les cardiomyocytes par rapport aux mesures en mitochondries isolées. Cette différence avec les valeurs *in vitro* peut être expliquée par les changements conformationnels subit par les mitochondries isolées. En effet, dans les cardiomyocytes, la conformation interne des cristae des mitochondries est maintenue et permet le positionnement idéal de tous les acteurs mitochondriaux de la régulation de la respiration. Cette forte affinité de la MtCK pour la Cr *in vivo* dénote l'importance de la Cr dans la régulation de l'Oxphos et du recyclage de l'AD/TP via le couplage fonctionnel MtCK-ANT dans les mitochondries de cardiomyocytes.
Le couplage fonctionnel MtCK-ANT, la présence de la tubuline et la conformation idéale de la membrane interne mitochondriale sont autant d'éléments permettant une production efficace de PCr *in vivo*. Les résultats de cette étude confirment les prédictions des modèles mathématiques basés sur les transferts énergétiques compartimentés. Les données récoltées pourraient être utilisées pour un modèle mathématique complet de l'énergétique cardiaque incluant les processus mitochondriaux de conversion d'énergie et les flux métaboliques à travers la membrane externe.

Article 8

Timohhina N, Guzun R, Tepp K, Monge C, Varikmaa M, Vija H, Sikk P, Kaambre T, Sackett D, Saks V (2009) Direct measurement of energy fluxes from mitochondria into cytoplasm in permeabilized cardiac cells in situ: some evidence for mitochondrial interactosome. J. Bioenerg. Biomemb. 41:259-275.

Dans cette étude, les flux énergétiques ont été mesurés directement dans des cardiomyocytes perméabilisés dans des conditions proches de celles rencontrées *in vivo*, c'est-à-dire en présence du système PEP/PK représentant le système glycolytique et piégeant l'ADP cytoplasmique produit par les ATPases.
Les résultats mettent en évidence le rôle central du système MtCK-ANT-VDAC-tubulin dans la régulation de ces flux. Chen et Pedersen [Ko, 2003 ; Chen, 2004 ; Pedersen, 2007a] ont démontré l'existence de l'ATPsynthasome composé de l'ANT, du canal à phosphate inorganique (PIC) et de l'ATP synthase. Il a été proposé dans cette étude de réunir toutes ces structures autour de la chaîne respiratoire dans un super-complexe appelé l'Interactosome Mitochondrial (MI), représenté **Figure 36**. Son rôle est d'assurer le recyclage continuel des

nucléotides adényliques dans l'espace inter-membranaire, leur transphosphorylation, leur transfert direct (canalisation métabolique ou métabolisme vectoriel de la MtCK vers l'ANT) et l'export d'énergie libre dans le cytoplasme via la PCr.

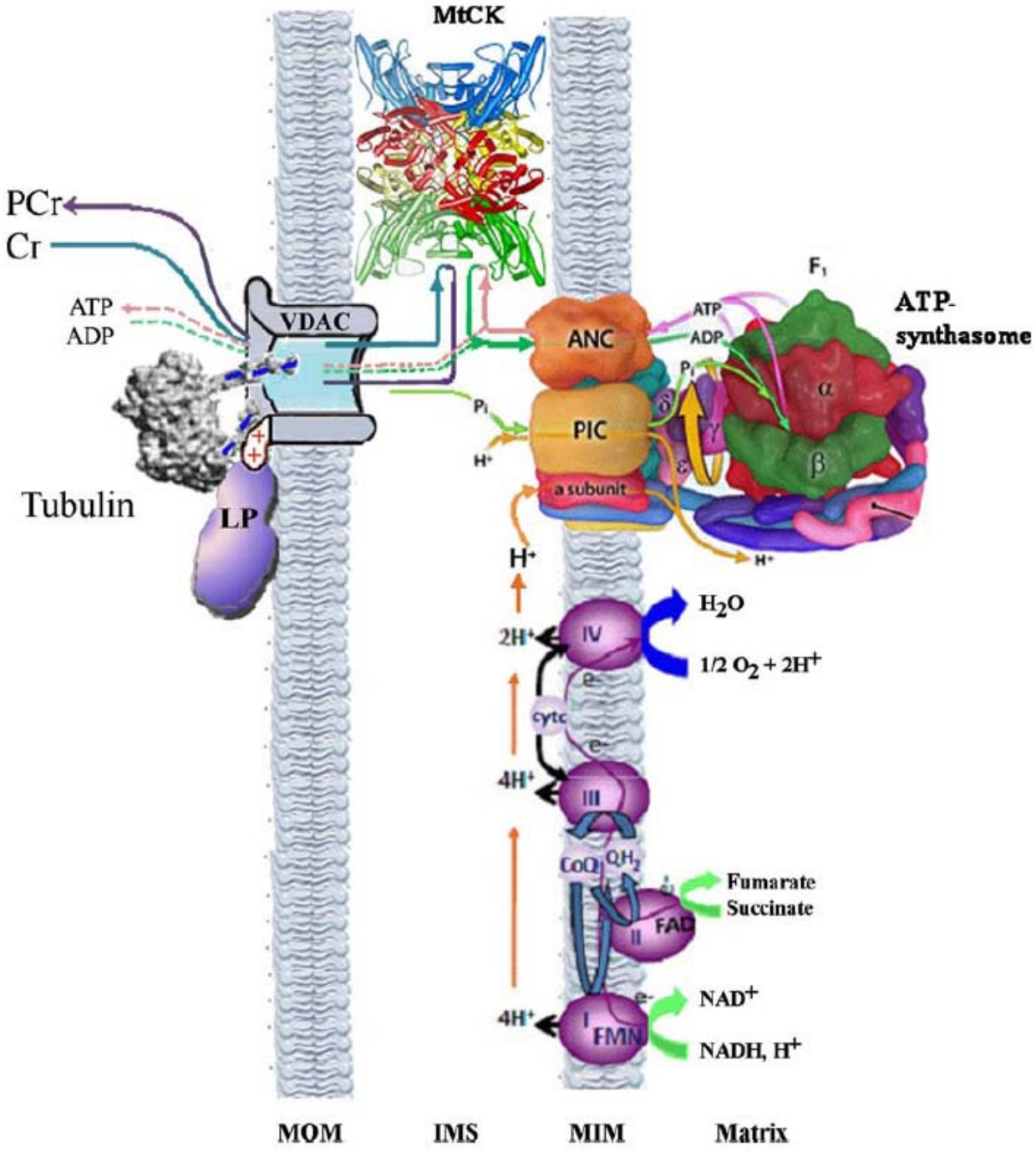


Figure 36 : Schéma de l'Interactosome Mitochondrial (MI). PCr : phosphocréatine, Cr : créatine, VDAC : voltage dependant anion channel, MtCK : créatine kinase mitochondriale, ANC : adenine nucleotide carrier = ANT, PIC : inorganic phosphate carrier, LP: linker proteína, MOM : membrane externe mitochondriale, IMS : espace inter-membranaire, MIM : membrane interne mitochondriale.

La mesure des concentrations de PCr par chromatographie en phase liquide à haute et ultra performance (HPLC/UPLC) combinée à des mesures oxygraphiques de vitesses de respiration ont permis de déterminer le rapport PCr/O_2 *in situ* (5,68 ± 0,14) qui s'est avéré très proche de la valeur théorique *in vivo* (6) déterminée par Nicholls et Ferguson [Nicholls, 2002]. De plus, ce rapport reste constant quelque soit la concentration de MgATP ajoutée (de 1 à 5 mM qui est la concentration physiologique). Ce résultat montre bien l'efficacité de la transmission énergétique (transmission duγ -phosphate de l'ATP à la PCr) dans le MI et confirme que le transport énergétique cellulaire est assuré par la PCr et non par le transfert direct du MgATP aux ATPases [Kaasik, 2001 ; Kuum, 2009], même quand les niveaux d'ATP cytoplasmique sont diminués.

Du fait de l'interaction tubuline-VDAC et du couplage fonctionnel MtCK-ANT, l'ATP produit par l'OxPhos est quasiment entièrement utilisé pour la production de PCr qui diffuse presque librement à travers la membrane externe. La structure du MI engendre donc une micro-compartimentation dynamique où les flux métaboliques mitochondriaux entrant et sortant sont contrôlés.

La présence d'autres protéines en interaction avec le VDAC est envisagée et représentée sur le schéma du MI par une protéine de liaison (LP, linker protein) qui pourrait renforcer ou affaiblir la liaison de la tubuline au VDAC (**Figure 36**).

III.4 Changements de structure de l'Interactosome Mitochondrial dans le cancer (articles 9 et 10)

Article 9

Monge C, Beraud N, Pelloux S, Tepp K, Chahboun S, Kaambre T, Tourneur Y, Ronot X, Kuznetsov AV, Seppet E, Saks V (2009) Comparative analysis of the bioenergetics of the adult cardiomyocytes and non-beating HL-1 cells. Respiratory chain activities, glycolytic enzyme profiles and metabolic fluxes. Can J Physiol Pharmacol. 87(4):318-26.

Cette étude est basée sur les différences de structure et de fonction entre les cardiomyocytes adultes (CMs) et des cellules cancéreuses issues d'une tumeur cardiaque (cellules HL-1) mais ayant conservé un phénotype cardiaque différencié.

Des observations de l'organisation mitochondriale dans les CMs et les cellules HL-1 par microscopie confocale ont montré des différences structurales majeures (**Figure 37**). Dans les CMs, les mitochondries ont une position fixée et régulière, comme dans un cristal, entre les myofibrilles [Vendelin, 2005] alors que dans les cellules HL-1, on observe un réseau mobile et filamenteux de mitochondries.

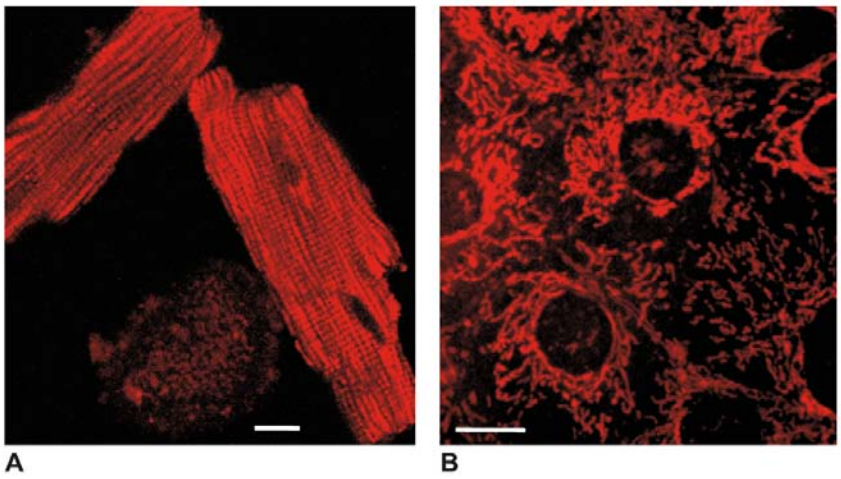


Figure 37 : Microscopie confocale de cardiomyocytes adultes (CMs, A) et de cellules HL-1 (B) marqués par une sonde mitochondriale sensible au potentiel de membrane, la tetramethylrhodamine methyl ester (TMRM). Les barres d'échelle correspondent à 10 µm.

Des mesures oxygraphiques des vitesses de respiration des deux types cellulaires ont montré que l'activité respiratoire des HL-1 est environ 4 à 8 fois inférieure dans les cellules HL-1. Ce résultat est expliqué par la diminution importante du contenu en cytochromes aa_3, b et cc_1 dans les cellules HL-1 reflétant un contenu diminué en complexes de la chaîne respiratoire. Le métabolisme oxydatif est donc fortement diminué dans ces cellules et ne représente pas la principale source d'ATP.

Le Km apparent mesuré dans les cellules HL-1 (30-50 µM) est nettement inférieur à celui mesuré dans les CMs (environ 300 µM). Ce résultat révèle un changement drastique de régulation de la respiration par des changements d'organisation métabolique.

La mesure par spectrophotométrie des activités de certaines enzymes de la glycolyse a révélé une augmentation de l'activité HK dans les cellules HL-1 (environ 5 fois par rapport aux CMs) caractéristique des cellules cancéreuses [Mathupala, 2006]. L'activité de la PK est elle aussi plus elevée dans les cellules HL-1 témoignant d'une forte production de lactate dans ces cellules et d'une activité glycolytique bien supérieure à celle des CMs.

Dans le même temps, l'activité de la CK est très fortement diminuée (plus de 15 fois) dans les cellules HL-1 (résultat également caractéristique des cellules cancéreuses [Eimre, 2008]), impliquant que, contrairement aux CMs, la régulation métabolique de ces cellules n'est pas basée sur les phosphotransferts via la CK.

La transformation des cardiomyocytes en cellules cancéreuses passe donc par une phase de déviation métabolique (d'un métabolisme oxydatif à un métabolisme glycolytique) où l'HK a un rôle central.

Article 10

Saks V, Guzun R, Timohhina N, Kersti T, Varikmaa M, ***Monge C****, Beraud N, Anmann T, Kaabre T, Kuznetsov A, Seppet E (2009) Molecular system bioenergetics : structure-function relationship in feedback regulation of cellular respiration in vivo in health and disease. BBA Bioenergetics. Accepted.*

Cette revue a pour but de décrire brièvement certains résultats expérimentaux obtenus sur le métabolisme énergétique intégré dans les muscles et les cellules nerveuses. Des applications cliniques de ces résultats sont décrites et proposées.

Concernant les cellules cancéreuses HL-1, des tests oxygraphiques ont montré que la respiration mitochondriale pouvait être activée par de l'ATP et du glucose. Cette observation signifie que l'HK est associée au VDAC et constitue un système de régénération de l'ADP.
Ces résultats sont cohérents avec les observations faites par Otto Warburg dans les années 20 puis précisé en 1956 [Warburg, 1924 ; Warburg, 1956]. En effet, Warburg décrit une augmentation de la production de lactate suite à une augmentation des flux glycolytiques en dépit de la présence d'oxygène dans les cellules cancéreuses. Ce phénomène de glycolyse aérobie est également appelé l'effet Warburg. L'HK-2 est l'enzyme la plus critique impliquée dans cet effet quand elle s'associe à la mitochondrie via le VDAC dans les cellules musculaires [Bustamante, 1977] (**Figure 38**). L'isoforme prépondérant dans les muscles est HK-2 alors que l'isoforme HK-1 est prépondérant dans le cerveau [Wilson, 1995]. La liaison de l'HK au VDAC protège de la mort cellulaire, favorise la prolifération et conduit à la tumorogénèse.

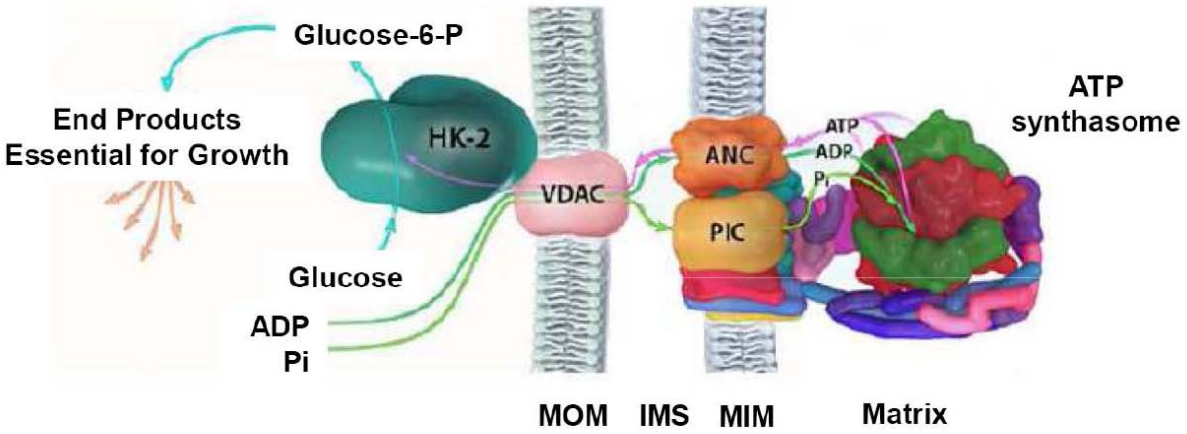


Figure 38 : Schéma des bases moléculaires de l'effet Warburg. Dans les cancers du muscle à métabolisme fortement glycolytique, l'HK, associée au VDAC, utilise l'ATP produit par l'OxPhos via l'ATP synthasome pour phosphoryler le glucose en glucose-6-phosphate qui est essentiel à la croissance cellulaire. HK-2 : hexokinase-2, VDAC : voltage dependant anion channel, Pi : phosphate inorganique, ANC : adenine nucleotide carrier, PIC : inorganic phosphate carrier, MOM : membrane externe mitochondriale, IMS : espace inter-membranaire, MIM : membrane interne mitochondriale. [D'après Pedersen, 2007].

C'est pourquoi cet effet du glucose sur la respiration n'est pas retrouvé dans les CMs ou les fibres musculaires où l'HK n'est pas associée au VDAC. Cette observation pourrait être le point de départ de l'explication de la déviation métabolique dans les cellules cancéreuses. En effet, la surexpression d'HK lors de la transformation cellulaire doit déplacer l'interaction de la tubuline au VDAC. Comme il a également été montré récemment que l'association de l'HK

à la mitochondrie est dépendante de la phosphorylation du VDAC, il est possible que des phosphorylations modifient les mécanismes reliant la tubuline au VDAC pour laisser place à l'HK. La glycogène synthase kinase 3 [Pastorino, 2005] et la protéine kinase C [Sun, 2008] peuvent phosphoryler le VDAC et donc modifier son interaction avec l'HK.

De plus, des modifications post-traductionnelles différentes ont été trouvées entre les cellules HL-1 et les CMs aboutissant à un réseau microtubulaire beaucoup plus stable dans les CMs [Belmadani, 2004]. Ces observations mériteraient des approfondissements.

Dans les cellules HL-1, le métabolisme énergétique est drastiquement différent de celui des CMs. Les données suivantes : l'expression de la CK diminuée, l'activité de l'HK fortement augmentée et le Km apparent très bas dans ces cellules montrant qu'il n'existe pas de restriction de la diffusion de l'ADP par la tubuline associée au VDAC, sont autant de preuves de la modification de structure du MI. Le MI est donc un complexe central dans le maintien d'un métabolisme énergétique sain, sachant qu'un déséquilibre dans la balance énergétique peut aboutir à de sérieuses pathologies. Ainsi l'altération du système CK/PCr dans le cerveau entraine également de graves pathologies, telle que la maladie d'Alzheimer due principalement à une inhibition de la CK par oxydation [Bürklen, 2006]. De même, pendant l'infarctus du myocarde, on observe une diminution rapide du contenu cellulaire en PCr due au manque d'apport en oxygène et aux changements pathologiques dans le système CK/PCr. Les variations d'ATP cellulaire étant très faibles en condition normale et étant totalement dissociées de la diminution rapide de la force contractile cardiaque, son utilisation totale et rapide entraîne la contracture prolongée du muscle cardiaque. Des études cliniques par imagerie et spectroscopy à résonance magnétique non-invasive sur des patients atteints de myopathies sévères ont permis de déterminer les paramètres de fonctionnement du système CK/PCr et les valeurs des rapports PCr/ATP chez ces patients et de fournir un pronostic vital [Neubauer, 2007].

Le rapport PCr/ATP est donc un paramètre important de diagnostic concernant les maladies cardiaques. De faibles concentrations de PCr et un rapport PCr/ATP faible reflètent la diminution de la concentration locale d'ATP qui n'est pas régénéré par les CKs dans les micro-compartiments qui sont, nous l'avons vu, essentiels au fonctionnement cardiaque, musculaire et neuronal. Il devient maintenant important et urgent de relever le défi de développer des biosondes pour visualiser ces micro-compartiments métaboliques d'ATP et coupler ces techniques avec la protéomique pour identifier directement les protéines impliquées dans leur formation.

III.5 Mesure directe de l'hétérogénéité de la diffusion par Spectroscopie à Corrélation de Fluorescence (article 11)

Monge C, Grichine A, Guzun R, Robert-Nicoud M, Saks V (2009) ATP diffusion is heterogeneous in cardiomyocytes: direct evidence from Fluorescence Correlation Spectroscopy. Submitted to BBRC.

La technique de Spectroscopie à Corrélation de Fluorescence (FCS) combinée à l'utilisation d'un analogue fluorescent de l'ATP, ATP-Alexa 647 (Fluo-ATP), pour étudier la diffusion de l'ATP, a révélé une diffusion non homogène de l'ATP aussi bien dans une suspension de mitochondries isolées de cœur que dans des cardiomyocytes.

Les mesures des coefficients de diffusion en solution ont montré : 2,0 ± 0,1.10^{-6} cm²/s (et temps de diffusion τ_{diff} = 66 ± 4 µs) à 20°C dans l'eau, et 1,4 ± 0,1.10^{-6} cm²/s (τ_{diff} = 92 ± 6 µs) dans la solution de respiration Mitomed. Ces résultats sont cohérents avec ceux obtenus précédemment par Vendelin [Vendelin, 2008] en Raster Image Correlation Spectroscopy (RICS) : 1,83 ± 0,27.$10^{-6}$$cm^2$/s à 22°C et par Hubley par des mesures de coefficient de diffusion de l'ATP non marqué [Hubley, 1996] : 3,68 ± 0,14.10^{-6} cm^2/s à 25°C. Ces résultats sont radicalement différents de ceux obtenus en cardiomyocytes (**Figure 39**) ou suspension de mitochondries isolées.

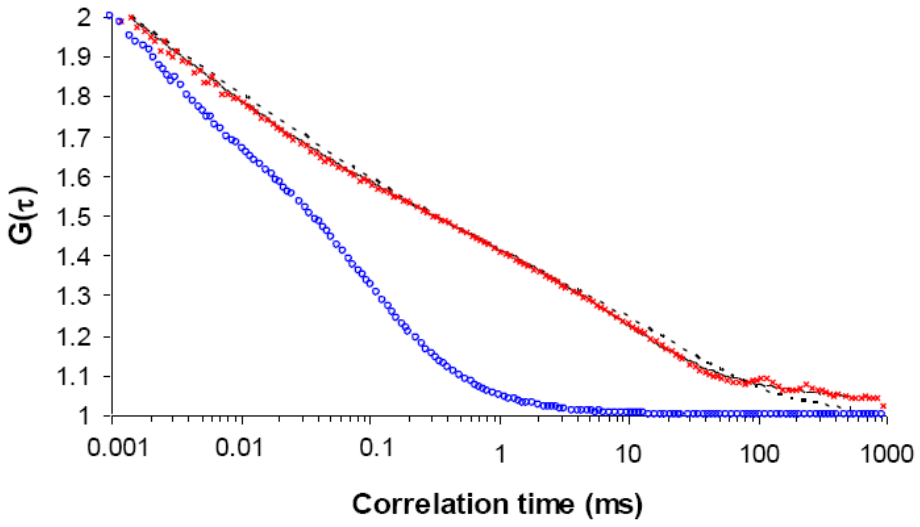


Figure 39 : Courbes d'autocorrélation normalisées pour le Fluo-ATP (ATP-Alexa 647) dans la solution Mitomed (courbe bleue) et dans un cardiomyocyte perméabilisé en présence d'ADP et de substrats respiratoires (courbe rouge).

Il a en effet été déterminé qu'il existe au moins 3 différents temps de corrélation, aux ordres de grandeur bien différents (de 10^{-2} à 10^{-4} s) dans les cardiomyocytes et les mitochondries isolées, par comparaison aux résultats obtenus en solution pour lesquels un seul temps de corrélation (ou temps de diffusion) n'est mesuré.

La répartition en % des temps de corrélation obtenus en fonction de la quantité totale de Fluo-ATP dans le volume focal a permis de caractériser quantitativement les changements dans ces temps de corrélation par la variation de leur distribution. Ainsi, il a pu être déterminé, dans les cardiomyocytes, que la présence d'ADP exogène et de substrats de la chaîne respiratoire (glutamate et malate, substrats du complexe I) provoquent l'apparition d'un temps de corrélation très court (de l'ordre de la centaine de µs) correspondant à 80 % des ATP-Fluo et qui n'était pas détectable en absence d'ADP et de substrats. Ce temps de corrélation très court indique une diffusion très rapide de l'ATP subséquente à l'activation de l'OxPhos. L'addition d'atractyloside (inhibiteur de l'ANT) fait au contraire apparaître un temps de corrélation très long correspondant à 6 % des Fluo-ATP et témoignant de la formation de 'pools' d'ATP également détectés dans les cardiomyocytes sans ADP ni substrats, c'est-à-dire en condition non-phosphorylante (OxPhos non activée ou inhibée). Des résultats similaires ont été obtenus dans les mitochondries isolées. L'addition de Cr sur cardiomyocytes ayant été incubés uniquement avec ADP et substrats a également provoqué l'apparition d'un temps de corrélation très long correspondant à 41 % des Fluo-ATP. Contrairement à l'atractyloside qui inhibe la respiration mitochondriale, la Cr va stimuler les CKs et engendrer les phénomènes de couplages fonctionnels bien caractérisés dans les cellules cardiaques. De ce fait, le temps de corrélation très long détecté après addition de Cr atteste de la micro- voire de la nano-compartimentation crée par le recyclage (turnover) de l'ATP aux sites de consommation d'ATP (ATPases membranaires et myofibrilaires) et de l'ADP aux sites de couplage fonctionnel entre les CKs mitochondriales et l'ANT. Ces résultats montrent que : i / la diffusion de l'ATP est hétérogène dans les cardiomyocytes et dépendante de l'état fonctionnel de la mitochondrie ; cette hétérogénéité est due aux compartimentations et restrictions de la diffusion, et que ii/ l'activité de la CK est capable d'influer sur la diffusion cellulaire de l'ATP en créant des micro-compartiments dynamiques.

IV. CONCLUSION ET PERSPECTIVES

Conclusion

Les résultats de ces travaux de thèse ont montré que :

1- Le facteur X est la tubuline et régule la perméabilité du VDAC.

2- Il existe un couplage fonctionnel entre uMtCK et ANT dans les mitochondries de cerveau.

3- Il existe un super-complexe tubuline-VDAC-MtCK-ATP synthasome, l' Interactosome Mitochondrial (MI), qui est la base d'une régulation efficace de la respiration mitochondriale.

4- La structure du MI peut varier en fonction de l'état pathologique, comme par exemple dans le cas du cancer où l'interaction tubuline-VDAC est remplacée par l'interaction HK-VDAC résultant donc à une modification de la régulation métabolique.

5- La diffusion de l'ATP est hétérogène dans les cardiomyocytes et peut être caractérisé en fonction de l'état fonctionnel de la mitochondrie**.**

Conclusion

The results of this thesis work showed that:

1- Tubulin is the factor X and it regulates VDAC permeability.

2- There is a functional coupling between uMtCK and ANT in brain mitochondria.

3- The tubulin-VDAC-MtCK-ATP synthasome super-complex, the Mitochondrial Interactosome (MI), is the basis of an effective regulation of the mitochondrial respiration.

4- MI structure changes in pathological state. For example, in cancer, tubulin-VDAC interaction is replaced by HK-VDAC interaction modifying the metabolic regulation.

5- ATP diffusion is heterogeneous in cardiomyocytes and can be characterized according the functional state of mitochondrion.

Perspectives

Les résultats de ces travaux ont permis de répondre à plusieurs interrogations mais certains points nécessiteraient des approfondissements.

- La tubuline est incontestablement un régulateur de la respiration mitochondriale de par son interaction avec le VDAC. Mais les mécanismes de cette interaction ne sont pas encore éclaircis. C'est pourquoi de futurs travaux sur la caractérisation de cette interaction, et plus particulièrement sur l'isoforme (α, β ou les deux) participant à cette interaction, permettrait d'élucider ce processus qui s'avère essentiel dans la régulation métabolique.

De plus, plusieurs autres protéines sont connues pour interagir avec le VDAC ou pourraient avoir une interaction indirecte sur l'association d'autres protéines. Il serait donc intéressant d'étudier le rôle d'autres protéines du cytosquelette, telles que la desmine ou la plectine, pour vérifier si elles font partie d'un complexe X modulaire de régulation.

- Le schéma de structure du MI a été construit à partir de données expérimentales concernant les cardiomyocytes et les mitochondries isolées de cœur. Cette théorie reste à confirmer pour le tissu nerveux (et en particulier les synaptosomes) par des études oxygraphiques incluant le système PEP/PK pour simuler la glycolyse, c'est-à-dire un système piège pour l'ADP extramitochondrial. De même, l'analyse complète de la cinétique de la uMtCK *in situ* pourrait établir le couplage fonctionnel entre uMtCK et ANT comme il a été établit dans les mitochondries isolées de cerveau.

- La déviation métabolique due au cancer est très probablement liée à des modifications d'interactions entre tubuline, VDAC et HK. Les mécanismes précis de l'effet Warburg ne sont que des hypothèses qui nécessitent des études quantitatives. Des études d'oxygraphies sur mitochondries isolées en présence de tubuline avec ou sans HK permettraient probablement de mieux comprendre ces mécanismes. De même qu'une caractérisation et une quantification des isoformes de tubuline présents dans les cellules cancéreuses HL-1 pourrait permettre de mieux comprendre les mécanismes de transformation cellulaire via les processus d'interaction au VDAC qui est un point stratégique de l'énergétique cellulaire.

- L'hétérogénéité de la diffusion de l'ATP pourrait être mesurée dans différents types cellulaires, dont les synaptosomes, pour élargir les connaissances du métabolisme neuronal et confirmer que les cellules nerveuses ont-elles aussi un milieu intracellulaire complexe et fortement organisé.

Further studies

The results of this work allowed answering several questions but further investigation is needed to go deeper into metabolism regulation understanding.

- Tubulin is unmistakably a mitochondrial respiration regulator by interacting with VDAC. Nevertheless precise mechanisms of this interaction are not well understood. Further studies of which isoform participate in this intercation (α, β or both) will allow to elucidate this essential process for metabolic regulation.

Furthermore, several other proteins are known to interact directly or indirectly with VDAC. It would be interesting to study the role of other cytoskeletal proteins, as desmin or plectin, to verify if there take part of a modular complex X of regulation.

- The scheme of MI structure was built from experimental data from cardiomyocytes and heart isolated mitochondria. This theory could be verified for brain cells, and in particular synaptosomes, by oxygraphic measurments with the PEP/PK ADP trapping system. Similary, complete kinetic analysis of the uMtCK activation *in situ* could give more information about the metabolic regulation of neuronal cells.

- Metabolic shift in cancer is probably due to modifications of interaction between tubulin, VDAC and HK. Precise mechanisms of Warburg effect are hypothesis which need quantitative studies. Oxygraphic measurements on isolated mitochondria in presence of tubulin and with or without HK will allow understanding of these mechanisms. Furthermore, characterization and quantification of tubulin isoforms present in cancerous HL-1 cells could lead to highlight mechanism of cell transformation via VDAC interaction processes which is a strategic element of cellular energetics.

- Heterogeneity of ATP diffusion could be measured in different cell types, including synaptosomes, to confirm that neuronal cells have a complex and highly organized intracellular medium, as cardiac cells for example.

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VI. ANNEXES

Article 1

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Review

Philosophical Basis and Some Historical Aspects of Systems Biology: From Hegel to Noble - Applications for Bioenergetic Research

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Abstract: We live in times of paradigmatic changes for the biological sciences. Reductionism, that for the last six decades has been the philosophical basis of biochemistry and molecular biology, is being displaced by Systems Biology, which favors the study of integrated systems. Historically, Systems Biology - defined as the higher level analysis of complex biological systems - was pioneered by Claude Bernard in physiology, Norbert Wiener with the development of cybernetics, and Erwin Schrödinger in his thermodynamic approach to the living. Systems Biology applies methods inspired by cybernetics, network analysis, and non-equilibrium dynamics of open systems. These developments follow very precisely the dialectical principles of development from thesis to antithesis to synthesis discovered by Hegel. Systems Biology opens new perspectives for studies of the integrated processes of energy metabolism in different cells. These integrated systems acquire new, system-level properties due to interaction of cellular components, such as metabolic compartmentation, channeling and functional coupling mechanisms, which are central for regulation of the energy fluxes. State of the art of these studies in the new area of Molecular System Bioenergetics is analyzed.

Keywords: Systems Biology; Molecular Systems Bioenergetics; cellular energy metabolism; regulation; compartmentation; energy transfer systems.

Contents

Wherever there is movement, wherever there is life,
Wherever anything is carried into effect in the actual word,
there Dialectic is at work. It is also the soul of all knowledge
which is truly scientific.

Hegel's Logic
Translated by William Wallace
Oxford University Press, Oxford UK, 2005,
p. 116

1. Systems Biology – New Paradigm and New Perspectives of Biological Research

Within last decade, biological sciences have witnessed a radical change of paradigms [1-19]. Reductionism, whch used to be a philosophical basis of biochemistry and molecular biology when everything – from genes to proteins and organelles – were studied in their isolated state is leaving its place to Systems Biology, which favours the study of integrated systems at all levels: molecular, cellular, organ, organism, and population [1-19]. The importance and rapid expansion of Systems Biology become clear when one opens PubMed with this keyword – tens of thousands of entries from different fields of the biological sciences appear. Hundreds, if not thousands of books have been published in recent years on this topic; references [1- 9] are just very few examples of them, mostly related to the topics of this article. Indeed, suddenly Systems Biology is everywhere. Given the very rapidly increasing number of publications, it may even be that the term Systems Biology is not always understood in the same way, but nevertheless, there are general and rather precise commonly accepted definitions of this scientific direction. In 2005 Alberghina and Westerhoff edited a whole book analyzing the definitions and perspectives of Systems Biology [4]. The shortest and most clear

definition of Systems Biology is given by Westerhoff's group: "Systems Biology is the science that aims to understand how biological function absent from macromolecules in isolation, arises when they are components of their systems" [15]. Very similar definitions have been given by many other authors [1-19].

These very intensive developments make it interesting and necessary to discuss its origins – the philosophical basis and historical aspects of System Biology. This is especially interesting for scientists who have spent almost all of their time and efforts in studies of mechanisms of integrated cellular metabolism (not even knowing before that what they study is Systems Biology). For many of them the topics are very familiar, but what is most helpful for them is the development of new concepts within Systems Biology that help to make general conclusions and give new tools for further research. One of these areas of research concerns the study of integrated energy metabolism in cells which we call now Molecular System Bioenergetics, as one can see from the titles of this Special Issue and a recent volume published by Wiley VCH [7]. Metabolic studies are not new, for example studies of cardiac metabolism started more than 50 years ago, after pioneering works by Richard Bing [20]. The great value of Systems Biology for metabolic research is mostly conceptual because of the clear definition of system-level properties [1-10,15]. System-level properties are the results of interactions between components of the system [1,2,4,7,10-15]. Systems Biology gives also the tools for these studies, most important among them are the quantitative methods of modeling and network analysis [4-6,8-19] .

Already several books and articles have been written on the philosophy and origin of the systemic approach in biology [1-4,13,21,22]. Among them are recent publications by Noble [2,13,14], who has traced Systems Biology back to the works by Claude Bernard [23], concluding that Systems Biology is in fact physiology at new higher level [2,13,14] and that a genuine, quantitative theory of biology is to be developed in future research [14]. This is a very optimistic conclusion for new generation of scientists - they still have immense task ahead to work on, and this task attracts both attention and funding. Landmark precedents of Systems Biology are the work of N. Wiener associated with the development of cybernetics, along with the impact of E. Schrödinger's contributions [24,25]. Another aim of this review is to show that the philosophical foundations of Systems Biology may be found in Hegel's dialectical philosophy, as applied to biology [26-30]. Finally, a critical analysis of the current state of the art in Molecular System Bioenergetics will be given, in addition to those discussed in our recent book [7]. We have found this general presentation of Systems Biology and Molecular System Bioenergetics very useful for explaining and teaching these new disciplines to doctoral students at Grenoble University.

2. Hegel's Dialectic and Systems Biology. Some Important Steps in History.

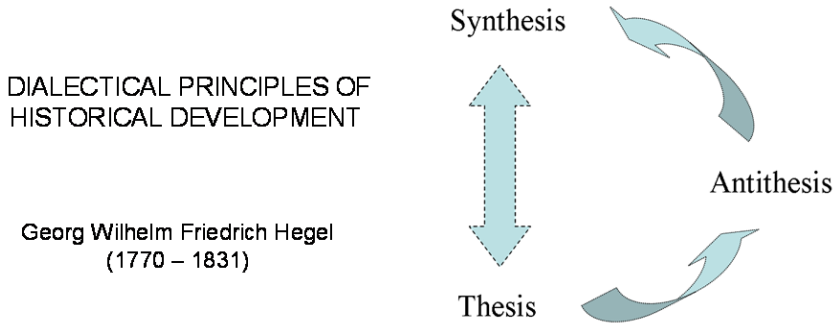
According to Thomas Kuhn's definition, a paradigm represents a specific, widely accepted way of viewing reality in science [26]. In this sense, Systems Biology is a new paradigm of biological sciences; it has been became widely popular within the last 10 - 15 years [1-19]. However, its history can be traced back into the two last centuries of biological and medical research, starting with Claude Bernard's theory of permanence of the internal milieu of organisms, later called homeostasis [13,23].

From that time and up to the modern times of Systems Biology, life sciences appear to perfectly fit and strictly follow the dialectic principles of general historical developments discovered by Hegel [27-30].

2.1. Hegel's dialectic laws

Georg Wilhelm Friedrich Hegel (1770 – 1831), one of most famous German philosophers, gave in his philosophy the most profound description of the logic and rules of historical developments [27-30]. An excellent description of this rather complicated philosophy is given by Bertrand Russell in his famous book "History of Western Philosophy" [30], explaining in easily way the dialectic laws. Hegel, as a most serious philosopher, was thinking about the relations of our thinking and the real world. For him, the real knowledge was to understand not only parts but the Whole, the Absolute Idea [27-30]. From our practical point of view (not to be involved in deep discussions between materialism and idealism in philosophy), Absolute Idea may be taken to represent the perfect, detailed knowledge of the integrated, whole systems. That in fact is what Systems Biology wants to find out, to know all about the life in its complexity, to comprehend the Whole, an Absolute Idea of the living systems. The process of development, the way to achieve this, the logic of finding out the Absolute Idea, according to Hegel, is the triadic movement called dialectic [30]. Dialectic consists of thesis, antithesis and synthesis [30]. Thus, knowledge as a whole has its triadic movement, and the process is essential to understand the results. To move from one stage to another, thinking as a dialectic process must "fall into the negative of itself" ([27], p. 35). Each later stage contains all the early stages, and all stages are given their proper place as a movement [30]. Thus, a thesis is first giving rise to its reaction, an antithesis which contradicts or negates the thesis, and the tension between the two being resolved by means of a synthesis [30].

Figure 1. Hegelian dialectic of historical movement from thesis to antithesis to synthesis.



These dialectic movements were explained by Hegel and his followers by three basic concepts: 1) everything is made out of opposing forces/opposing sides (contradictions); 2) gradual changes lead to turning points, where one force overcomes the other (quantitative change leads to qualitative change); 3) change moves in spirals not circles (sometimes referred to as "negation of the negation") [30]. These Hegelian dialectic rules are illustrated by the Scheme in Figure 1. Thomas Kuhn's description of the structure of scientific revolutions [26] gives an excellent illustration of the second basic concept of Hegelian dialectic when applied to science. When we look into the history of Systems Biology, we find an excellent illustration of the validity of these dialectic laws. If Hegel had been a biologist, he

could have predicted the appearance of Systems Biology as a necessary and inevitable step in our way to find out the final truth, the Absolute Idea of the life. What we know now is only the beginning of this long way.

2.2. Claude Bernard and the theory of permanence of internal milieu – homeostasis

Claude Bernard (1813-1878), a famous French physiologist, was a founder of experimental medicine, and according to Denis Noble [13] the first system biologist, one of the first instigators of Systems Biology (or integrative biology). The scope of his works was very wide: discovery of the pancreas function, discovery of the gluconeogenesis in liver, neurophysiology, toxicology, anesthesia and asphyxia. One of the main theories developed by Claude Bernard is the theory of the permanence of the *milieu intérieur* (later called homeostasis) due to integrated regulatory mechanisms. Analysis of the *milieu intérieur* is the study of the physiological mechanisms with which the organism can adapt itself to the *milieu extérieur* and maintain its functional balance in spite of the external constraints [23]. According to Claude Bernard "the fixity of the interior medium is the condition of a free and independent life" [23]. At his time, he had to separate himself and fight against popular theory of vitalism [21,22]. This theory postulated, in some way analogously with Systems Biology, that the whole living cell or organism is more than simple sum of its parts, but explained life by action of a vital force which neutralizes the "negative effects of physico-chemical forces" in living organism [21,22]. Bernard had to fight against this mystification of life [23]. He emphasized that an organism is able to adjust itself to external physical and chemical variations by maintaining permanence of its *milieu intérieur* and this adaptation is possible because the cells, the organs or the organisms are integrative systems. He was always attentive not to explain all his observations only by anatomy, claiming that anatomy has to serve physiology because of its complexity. He indicated that the function of an organ is not a strict one and that a function can be due to the interactions of two or several organs (for example the digestion process). According to him, physiologists must start from studies of physiological phenomena to explain them in the whole organism and not try to explain a function from an organ [23].

The strength of Claude Bernard's theories comes also from his ability to extrapolate his works to chemistry, physics and mathematics. He understood the importance of the mathematical modeling to understand the natural phenomena because *"Cette application des mathématiques aux phénomènes naturels est le but de toute science, parce que l'expression de la loi des phénomènes doit toujours être mathématique"* - "*This mathematical application at natural phenomena is the aim of all sciences, because the expression of the laws of phenomena should always be mathematical"* [23]. And time proved that he was right. Applied mathematics, cybernetics and computer sciences are now very powerful tools in biological research. But Bernard understood also that the application of mathematical modelling should be based on very firm experimental data, which were not available at his time: *« C'est par elle seule [l'application mathématique] que, dans la suite, la science se constituera; seulement j'ai la conviction que l'équation générale est impossible pour le moment, l'étude qualitative des phénomènes devant nécessairement précéder leur étude quantitative. » « It is by it alone [the mathematical application] that, in the continuation, science will be created but I have the conviction*

that the general equation is impossible at the moment, the qualitative study of phenomena must necessary precede their quantitative study" [23].

Thus, he understood the possible danger of misuse of the powerful method of mathematical modelling in biology: construction of mathematical models of a metabolic pathway or network can indeed lead to an erroneous model if the amount of physiological data is not sufficient. The richness of experimental data ensures the fidelity of the model. And we always have to keep in mind that a model is only a reflection of a complex system, a model will never be a system but only a representation of a system or a part of a system. The computer sciences are a very useful tool in Systems Biology but this tool must be used with caution inseparably from collection of experimental data, to avoid creating a virtual world far from reality. This very clever advice of Claude Bernard is still often forgotten or simply ignored in our times by a new generation of applied mathematicians coming into biological research with easy access to computing technologies but with rather weak knowledge of experimental data (see below). What we need to do first is to collect the maximally possible amount of experimental data describing the system level properties, with the aim to finally reach the "general equation" evoked by Claude Bernard. What did lack in Claude Bernard's time was a sufficient amount of quantitative experimental data. Systems Biology is now in much more favourable position and following the advices given by Claude Bernard, we can go ahead taking up the challenge of finding the general equation of life, its Absolute Idea.

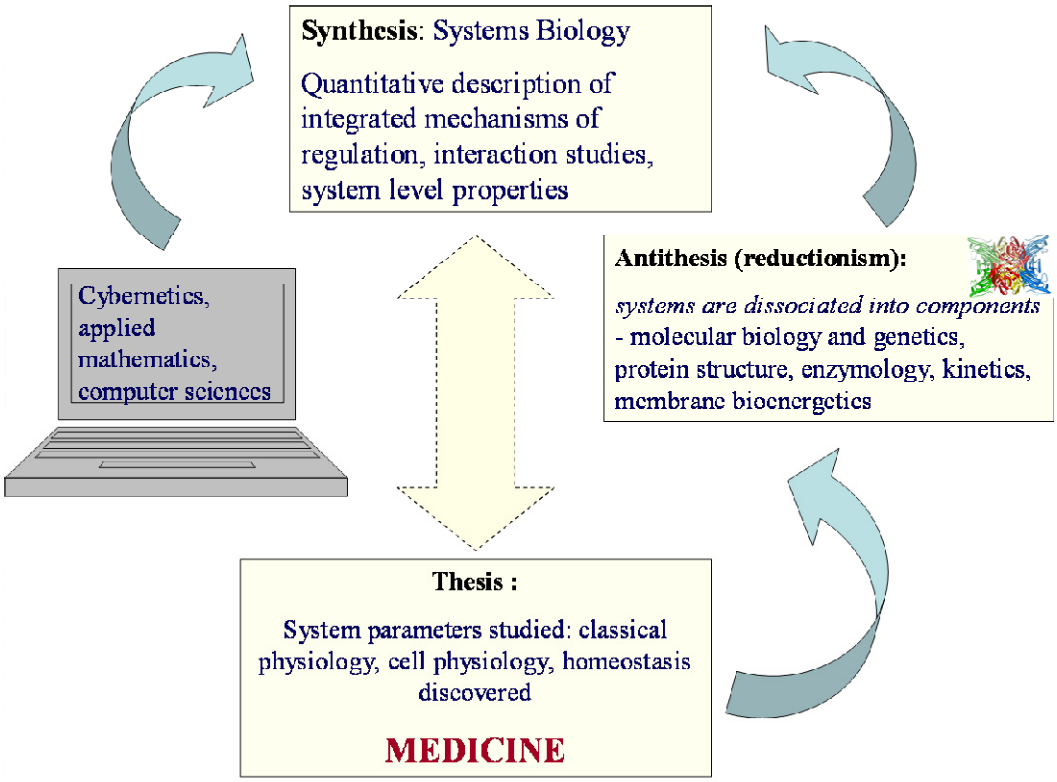
2.3. Cybernetics of Norbert Wiener and Systems Biology.

Norbert Wiener (1894 – 1964) was an American mathematician who studied the communication and control processes both in technical electronic systems and in biology, notably in physiology, by analysis of information transmission and treatment processes [24]. He was a founder of cybernetics, a science of control and governing, which studies the structure and function of regulatory systems [24] and has very wide application in computer sciences, engineering, logic modeling as in electronic and information network (including Internet) theories, in physiology, evolutionary biology, neuroscience, anthropology, psychology, sociology. Application of cybernetics in biology is now known in general as biocybernetics, which is a part of theoretical biology, and plays a major role in systems biology, seeking to integrate different levels of information to understand how biological systems function. One of the most important achievements of cybernetics developed by Wiener was the theory of feedback regulation and its application for explanation of the mechanisms of homeostasis [24] discovered by Claude Bernard and described above. Discovery of the feedback mechanisms by Wiener is still probably the most important contribution of cybernetics into Systems Biology. Another direct application of biocybernetics is network biology [18,19]. As it was emphasized by Barabasi in his reviews on network biology, "quantifiable tools of network theory offer unforeseen possibilities to understand the cell's internal organization and evolution, fundamentally altering our view of cell biology. The emerging results are forcing the realization that, notwithstanding the importance of individual molecules, cellular function is a contextual attribute of strict and quantifiable patterns of interactions between the myriad of cellular constituents" [19]. That tells us that understanding cell biology means understanding of system level properties.

2.4. Systems Biology: from Hegel to Noble.

Systems Biology uses the methods of both experimental studies and computing, focusing on the studies of interactions within the system with the aim of understanding the biological function. In this vast area, there are many new particular directions of research, such as the Physiome Project [17]. And there are very numerous scientists and groups who have made significant contributions into this area [1-19]. The philosophy, general principles of these important studies on cellular and organ level have been analyzed and described by Denis Noble, who has summarized them in 10 basic principles, "10 commandments" [14].

Figure 2. Presentation of development of biological sciences as Hegelian dialectic movement. In times of Claude Bernard, the problems of experimental physiology and medicine were formulated from the point of view of the theory of homeostasis at the organ level. To solve these problems, the components of the cell (proteins, genes, mitochondria etc.) were studied in the isolated state. In Systems Biology, these components are again studied in their interaction within the intact systems of interest.



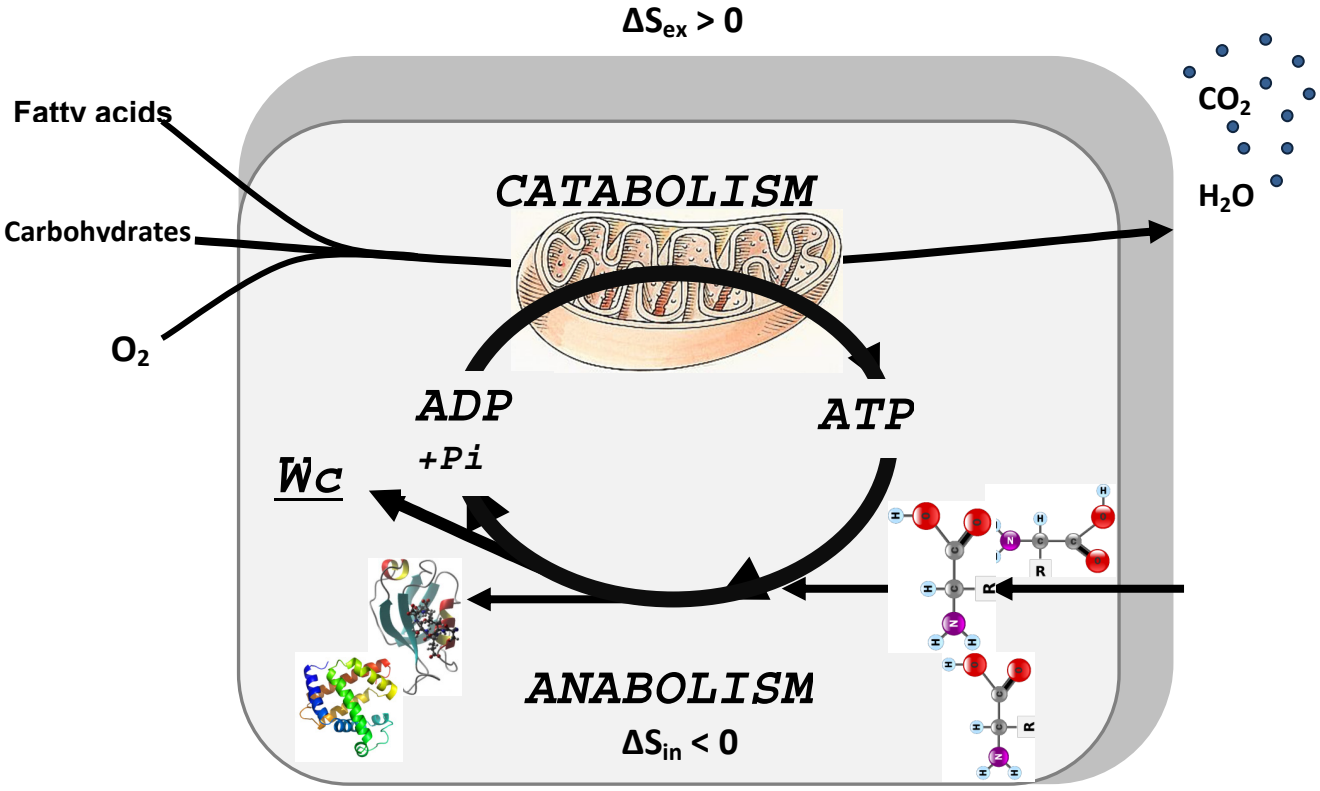
Among these principles, transmission of information by feedback mechanisms is most important, but these mechanisms are to be discovered yet [2,14]. Thus, the whole process of development of biological sciences during last 150 years, from the times of Claude Bernard (experimental physiology, medicine) to that of molecular and structural biology, enzymology, membrane bioenergetics and then to Systems Biology perfectly follows the Hegelian dialectic principles, with triadic movement from thesis (formulation of problems of experimental biology and medicine) to antithesis (the systems are

divided into components which are studied separately) to synthesis (coming back at new level to studies of biological function of whole system), as it is shown in Figure 2.

2.5. Erwin Schrödinger: negentropy production as a basis of metabolism, central role of bioenergetics.

In the history of biological sciences, one of the most influential events has been publication of Erwin Schrödinger's book "What is life?" in 1944 [25]. And it is still one of the most influential books in biology. Probably, it is not too much to say that scientists working in biology have successfully accomplished, with brilliant results, realizing the ideas described in the first chapters of this book related to foundation of molecular genetics, and are now busy in collectively reading the chapter 6 in this short book, related to the principles of organization of cellular metabolic processes, and all that together is now called Systems Biology.

Figure 3. General scheme of cellular metabolism. A cell is a thermodynamically open system, in accordance with the Schrödinger's principle of negentropy extraction. Increasing the entropy in extracellular medium, and decreasing it in the cell via metabolism is necessary for maintenance of the structural organization of both biopolymers as proteins, DNA and RNA, and also maintenance of the fine structural organization of the cell for effectively running compartmentalised metabolic processes. In this way, the cell can live in agreement with the thermodynamic laws (see the text). The Scheme shows the central role of bioenergetic processes in the cellular life by coupling catabolism with anabolism. Adapted from ref. [7].



The main conclusion made by Schrödinger was that the living cells need to be open systems with energy and mass exchange with surrounding medium, with the aim of maintaining their high structural and functional organization and thus internal entropy low, achieving this by means of increasing the entropy of the medium by catabolic reactions. Thus, Schrödinger wrote: " The essential thing in metabolism is that the organism succeeds in freeing itself from all entropy it cannot help producing while alive" [25]. In cellular metabolic systems, catabolic reactions which increase entropy in surrounding medium are coupled to anabolic reactions (biosynthesis) which maintain cell structure and organization with necessary decrease in entropy. Catabolic reactions are mostly oxidative degradation of fatty acids and carbohydrates such as glucose. They are also the source of metabolic energy for the performance of any kind of cellular work. This is shown by the general scheme in Figure 3 describing the integrated metabolism of the cell as an open system exchanging both energy and masses with surrounding medium. This Scheme shows that the processes of free energy conversion are central for coupling catabolism to anabolism, emphasizing the central role of bioenergetics in studies of integrated metabolism of the cells. To live, the metabolic systems need to be in the steady state far from equilibrium, and how they maintain intracellular organization and low entropy state is explained by non-equilibrium thermodynamics: the organized states are maintained by energy and matter dissipation, therefore they are also known as *dissipative structures* [31-34].

3. Application of the Systems Biology Approach to Metabolic Studies. Metabolic Compartmentation as System Level Property

In studies of integrated metabolic processes, one of the most important problems is that of diffusion in the organized intracellular medium. In fact, all mathematical models of metabolism, and practical values of these models depend upon the authors' views on cell structure and diffusion of metabolites, and still popular oversimplified theories of cell interior as a homogenous diluted solution of metabolites are sources of grave errors and may lead to meaningless models (see below). In our recent article in *International Journal of Molecular Sciences* we have analyzed in details the problems of diffusion of metabolites in organized intracellular medium [35]. Here, we emphasize some of the important conclusions made. The problem starts with the intracellular mobility of water, which is significantly reduced, leading to partitioning of metabolites between different water phases and to changes in binding constants [36-38]; then there is low-affinity adsorbtion of metabolites, especially if charged as ATP, to intracellular surfaces increasing the viscosity, and due to this the diffusion coefficient of metabolites is decreased by a factor of $(1+C/K_d)^{-1}$ where C is concentration of binding sites and Kd is dissociation constant of solute from these complexes [36]; macromolecular crowding and cytoskeletal structures create the barriers which increase the effective path-length of diffusion, and again diffusion coefficient is decreased by λ^{-2} where λ is relative increase in path attributable to the barriers [36-38]; finally, the movements of individual molecules become co-ordinated and vectorially directed due to organization of enzymes into the complexes, and the randomness of molecular events may be lost [36]. The results of these local diffusion restrictions are microcompartmentation of metabolites and their channeling within organized multienzyme complexes which need to be accounted for to explain biological phenomena [39,35]. Compartmentation and microcompartmentation of metabolites are system-level properties resulting from interactions between

cellular components. Indeed, none of important observations in cellular bioenergetics could be explained by a paradigm describing a viable cell as a "mixed bag of enzymes" with homogenous metabolite distribution still sometimes in use: this simplistic theory excludes any possibility of metabolic regulation of cellular functions [35]. Due to macromolecular crowding and hindered diffusion cells need to compartmentalize metabolic pathways in order to overcome diffusive barriers. Biochemical reactions can successfully proceed and even be facilitated by metabolic channeling of intermediates due to structural organization of enzyme systems into organized multienzyme complexes. Metabolite channeling directly transfers the intermediate from one enzyme to an adjacent enzyme without the need of free aqueous-phase diffusion [40,41,7,42-46]. Enzymes are able to associate physically in non-dissociable, static multienzyme complexes, which are not random associations but an assembly of sequentially related enzymes, very often due to their association with cytoskeleton [42,43, 47]. Thus, principal mechanisms of functioning and regulation of cell metabolism are system-level properties: macro- and microcompartmentation, metabolic channeling and functional coupling, resulting from specific structural interactions between cellular components. For this reason Systems Biology approaches are most important for further advancement of metabolic studies. At the cellular level, it is becoming clear that most of biological characteristics arise from complex interactions between the cell's numerous constituents, and based on protein-protein interactions, cellular metabolism is likely to be carried out in a highly modular manner within hierarchically organized networks [7,48]. The real problems and challenges for further studies are both to measure local concentrations of metabolites, including those of ATP in different cellular microcompartments and its metabolic channelling within microdomains (local fluxes), and to fully understand the nature of these restrictions of diffusion upon which intracellular compartmentation is based. This difficult work is necessary for reasonable computer modeling of the hierarchical modules of metabolic networks as a part of Molecular System Bioenergetics [7] and Systems Biology in general [11,17].

4. Molecular System Bioenergetics: Structural and Dynamic Organization of Cellular Energy Metabolism, Mitochondrial-Cytoskeletal Interactions, Mitochondrial Dynamics, Energetic Modules and Regulatory Mechanisms

Molecular Systems Bioenergetics is a science describing a new area of cellular bioenergetics in transition from molecular to the system level [7]. Miguel Aon has proposed the following definitions for this new direction of research [7]: *Molecular System Bioenergetics* is a broad research field accounting not only for metabolism as reaction networks but also for its spatial (organization) and temporal (dynamics) aspects. The main focus of Molecular System Bioenergetics are the processes of energy conversion both at molecular and cellular levels, with special emphasis on the structure and function of energy transfer and regulatory networks, mechanisms of interaction between their components, and a quantitative description of these networks by computational models. An important consequence of the organization of the enzymes into multienzyme complexes is vectorial metabolism and ligand conduction, a general principle proposed by Peter Mitchell after extensive enzymological studies and detailed characterization of mitochondrial proteins (reviewed in refs. [7] and [39]). The molecular system approach to the study of energy conversion in cells allows to fully explaining many classical observations in the cellular physiology of respiration, such as the metabolic aspects of the

Frank- Starling law of the heart and the regulation of substrate supply to the cell [7, 49]. This approach helps us to understand how a cell senses its energy status in adjusting its functional activity under stressful conditions, or others aspects of its life.

4.1. Unitary organization of energy metabolism and compartmentalized energy transfer in cardiac cells

As we have seen above, coupling of catabolism with anabolism (the metabolism) is the way through which "negentropy" or free energy is extracted from the medium. Evolution has selected the adenine nucleotides to fulfil this important task of coupling catabolism and anabolism (Figure 3). A possible explanation is the rather high standard free energy change (ΔG^o = 31.5 kJ/mol) and high affinity of ATP and ADP for many enzymes and carriers [50]. Cellular energetics is thus based on the reactions of ATP synthesis and utilization, $ADP + P_i \Leftrightarrow ATP + H_2O$. Taking water content as a constant and in excess (not changing in the reaction), the mass action ratio of the reaction of ATP synthesis is usually written as:

$$\Gamma = \frac{[ATP]}{[ADP][P_i]} \quad (1)$$

By maintaining high mass action ratio for the reaction of ATP synthesis, catabolic reactions supply also free energy for cellular work. Free energy available in the cellular system is a function of the ratio of Γ to the equilibrium constant of the ATP synthesis, or:

$$\Delta G_{ATP} = \Delta G^0_{ATP} + RT \ln \frac{[ATP]}{[ADP][P_i]} \quad (2)$$

This function is usually called phosphorylation potential [51,52]. The principal purpose of free energy transformation associated to catabolic reactions is to keep a high value of the phosphorylation potential which is mostly achieved through mitochondrial oxidative phosphorylation or photosynthesis in autotrophic organisms. The theory of phosphorylation potential for the analysis of the cellular life was first used by Veech *et al*. [51] and Kammermeier *et al*. [52].

However, now it has become clear that applying Eq. 2 as well as any quantitative theory of physical chemistry to the real intracellular medium is not a simple task, namely due to the complex organisation of cell structure and metabolism. It has become clear that it is not the global ATP content which is important, but the ATP and the free energy available in micro- and macrocompartments which have to be accounted for, as it will be described below.

Studies by using pulsed-gradient ^{31}P-NMR showed that the diffusion of ATP and phosphocreatine is anisotropic in muscle cells [53,54]. Recent mathematical modelling of the decreased affinity of mitochondria for exogenous ADP *in situ* in permeabilized cardiac cells also showed that the ADP or ATP diffusion in cells is heterogeneous and the apparent diffusion coefficient for ADP (and ATP) may be locally decreased (diffusion locally restricted) by an order, or even several orders of magnitude [55]. A similar limited diffusion of ATP in the subsarcolemmal area in cardiac cells was proposed by the Terzic and Dzeja group [56,57]

Application to cells of Eq. 2 in its general form is complicated by the compartmentation of ATP and adenine nucleotides, making the use of the easily measurable total ATP content very questionable and

practically useless. ATP compartmentation has been studied in normal and ischemic heart for a long time (reviewed in ref. [7]), and recently demonstrated in several cell types by imaging techniques [58]. Most important recent data showing the significance of compartmentation phenomenon for cardiac energy metabolism have been collected by Neubauer's group [59]. By using ^{31}P-NMR spectroscopy in combination with imaging for investigation of cardiac muscle energy metabolism in patients, the authors showed that in the patients with cardiac disease – dilated cardiomyopathy (DCM) the decreased PCr/ATP ratio (lower than 1.6) is very clear and strong diagnostic index of increased mortality. In the heart of patients with DCM the ATP content remained the same as in healthy control patients, but PCr decreased by 70 % as compared to control. This shows the vital importance of the phosphocreatine – creatine kinase energy transfer network described below for the cardiac muscle normal function and life.

4.2. Cardiac cells as highly organized metabolic systems

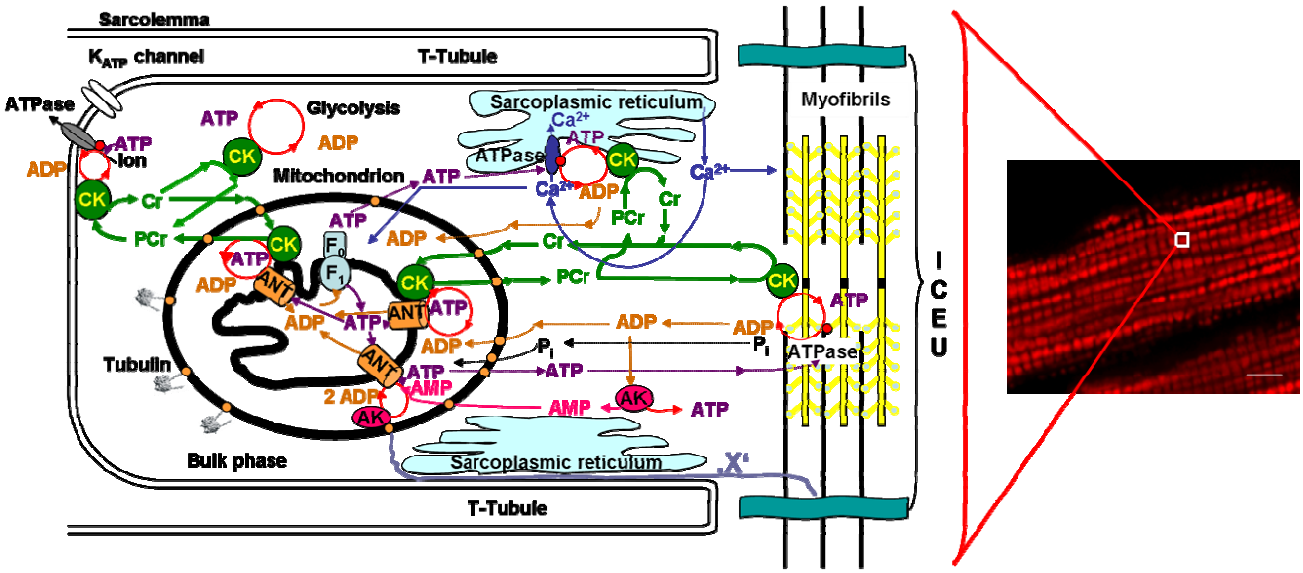
Cardiac cells present a highly organized structure where mitochondria are localized at the A-band level within the sarcomere [60-62]. These cells represent best examples the complexity of organization of intracellular energy metabolism. Intermyofibrillar mitochondria are arranged in highly ordered crystal-like patterns in a muscle-specific manner, with relatively small deviations in the distances separating neighboring mitochondria [62,61]. Contrary to many other cells with less developed intracellular structures, dynamic changes in mitochondrial position due to their fission and fusion [63-65] are not found in adult and healthy cardiac and skeletal muscle cells because of their rigid intracellular structural organization, and mitochondria in these cells are morphologically heterogeneous (see the paper by Kuznetsov in this Special Issue). In this structurally organized medium, energy transfer between different subcellular micro- and macrocompartments (shortly called compartmentalized energy transfer) are of central importance. Existence of these rather complicated networks of energy transfer and signaling is a direct consequence of the compartmentalization of adenine nucleotides in the cells [55-58,66-69]. This is due to significant heterogeneity and local restrictions in the diffusion of adenine nucleotides in cells, and the necessity of rapid removal of ADP from the vicinity of MgATPases to avoid their inhibition by accumulating product – MgADP [7,70-77]. ATP is not only delivered by diffusion, but intracellular energy transfer is facilitated *via* networks consisting of phosphoryl-group transferring enzymes such as creatine kinase (CK), adenylate kinase (AK) and glycolytic phosphoryl-transferring enzymes [39,70-82]. Most important among them is the creatine kinase system. CK catalyzes the reversible reaction of adenine nucleotides transphosphorylation, the forward reaction of phosphocreatine (PCr) and MgADP synthesis and the reverse reaction of creatine (Cr) and MgATP production:

$$MgATP^{2-} + Cr \leftrightarrow MgADP^{-} + PCr^{2-} + H^{+} \quad (3)$$

Four CK isoforms, each with compartmentalized cellular location, exist in mammals. Specific mitochondrial CK isoenzymes (MtCK), called ubiquitous (uMtCK) and sarcomeric (sMtCK), are functionally coupled to oxidative phosphorylation and produce PCr from mitochondrial ATP. PCr in turn is used for local regeneration of ATP by the muscle cytoplasmic isoform of CK (M-CK), driving myosin-ATPases or ion pump-ATPases [39,70-82].

In the heart as well as in oxidative skeletal muscle the intracellular energy transfer networks are structurally organized in the intracellular medium where macromolecules and organelles, surrounding a regular mitochondrial lattice, are involved in multiple structural and functional interactions [81-83]. Figure 4 summarizes available information about such an organized and compartmentalized energy metabolism in cardiac cells. This scheme also illustrates the view that mitochondria in muscle cells are structurally organized into functional complexes with myofibrils and sarcoplasmic reticulum [81-83]. These complexes were called 'intracellular energetic units", ICEUs, and taken to represent the basic pattern of organization of muscle energy metabolism [81].

Figure 4. Organization of compartmentalized energy transfer and metabolism in cardiac cells by intracellular energetic units (ICEU). Adapted from ref. [7].



The scheme shows the structural organization of the energy transfer networks of coupled CK and AK reactions within an ICEU. By interaction with cytoskeletal elements, the mitochondria and sarcoplasmic reticulum (SR) are precisely fixed with respect to the structure of sarcomere of myofibrils between two Z-lines and correspondingly between two T-tubules. Calcium is released from SR into the space in ICEU in the vicinity of mitochondria and sarcomeres to activate contraction and mitochondrial dehydrogenases. Adenine nucleotides within ICEU do not equilibrate rapidly with adenine nucleotides in the bulk water phase. The mitochondria, SR and MgATPase of myofibrils and ATP sensitive systems in sarcolemma are interconnected by metabolic channeling of reaction intermediates and energy transfer within ICEU by the creatine kinase – phosphocreatine and myokinase systems. The protein factors (still unknown and marked as "X"), most probably connected to cytoskeleton, fix the position of mitochondria. One of these proteins – tubulin-also controls the permeabilty of the VDAC channels for ADP and ATP. Adenine nucleotides within ICEU and bulk water phase may be connected by some more rapidly diffusing metabolites as Cr – PCr. Mitochondria were labelled by pre-incubation of cells with mitochondrial inner membrane potential sensitive probe MitoTracker Red (50 nM). Very regular arrangement of mitochondria and thus ICEUS is seen. Scale bar 10 μm.

There are no physical barriers between ICEUs, each mitochondria (or several adjacent mitochondria) can be taken to be in the centre of its own ICEU. This concept is consistent with very regular, crystal like arrangement of mitochondria in cardiac cells [60-62] and describes the organized functional connections of mitochondria with their neighbours. ICEUs are analogous to calcium release units, (CRUs), structurally organized sites of Ca^{2+} microdomains (Ca^{2+} sparks) which form a discrete, stochastic system of intracellular calcium signaling in cardiac cells [84,85].

This concept is supported by Weiss *et al*. [48], who presented a holistic view of cardiovascular metabolism, considering it from the perspective of a physical network, in which various metabolic modules are spatially distributed throughout the interior of the cell to optimize ATP delivery to specific ATPases [48]. In addition to a mitochondrial module (which is represented by ICEUs) the authors considered also a module consisting of glycolytic enzyme complexes serving for energy channeling to molecular complexes in sarcolemma and sarcoplasmic reticulum, and modules of calcium cycling (which Wang *et al*. called calcium release units, CRU [84]). These modules were further analyzed from the abstract perspective of fundamental concepts in network theory and dynamic perspective of interactions between modules [48]. Understanding the nature of these interactions within hiercharchical modular structures is a main challenge of research of cardiac metabolism to gain deeper understanding of possible mechanisms of cardioprotection [48].

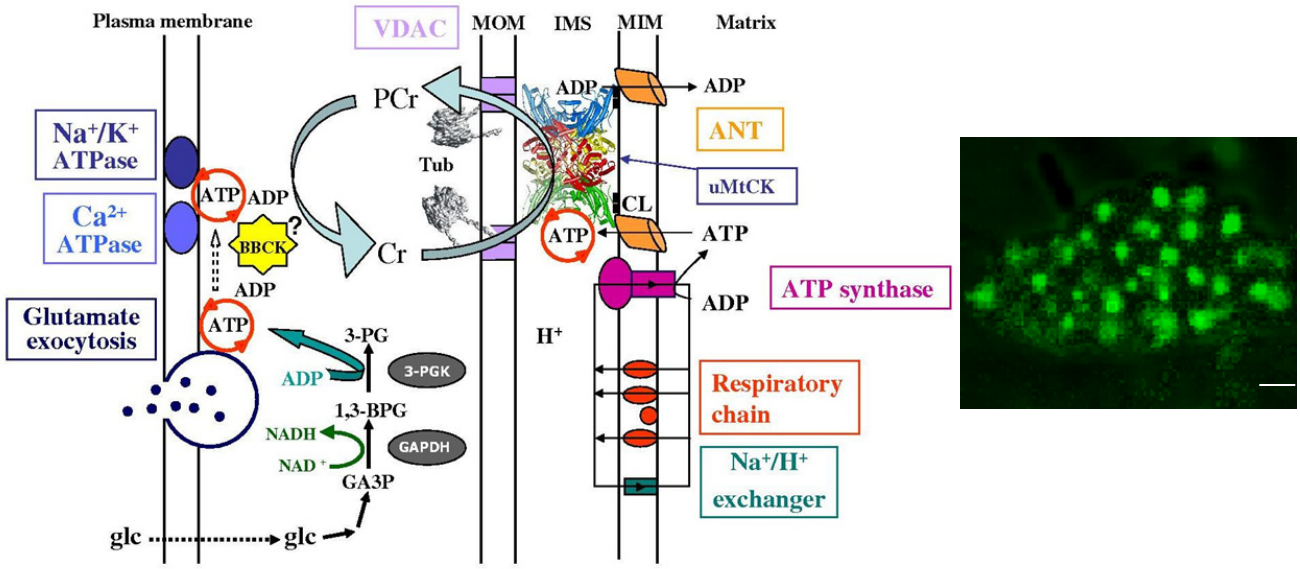
Metabolic compartmentation described above and unitary organization of energy metabolism is clear and important examples of system-level properties. Identification of cell components responsible for specific organization of energy transfer systems and intracellular diffusion merits further investigation. One of these components may be tubulin which is able to bind to the VDAC channels in mitochondrial outer membrane and in this way to decrease the apparent affinity of mitochondria for ADP [86, 87]. For further elucidation of the nature of cytoskeletal components responsible for specific organization of both mitochondrial arrangement and complex metabolic signalling and intracellular energy transfer pathways, further investigations on the proteome are needed.

The energy transfer network similar to that described above for cardiac cells is also functioning in brain cells, particularly in synaptosomes (Figure 5) [88].

4.3. Unitary organization of energy metabolism versus mitochondrial reticulum

Another important question is related to the role and cell specificity of mitochondrial fusion and fission. These processes are reviewed by Kuznetsov in his chapter in this Special Issue. Recent works in this area showed that the mitochondrial fusion and fission are potentially important for cell differentiation and pathophysiology [63, 89-92] . Many proteins responsible for fission and fusion such as Dynamin-related protein-1 (DRP1), Mitofusin-1 and Mitofusin-2 (Mnf1, Mnf2), OPA1 ─ have been identified [90-93], as being involved in cancer or apoptosis, during mitochondrial mobility changes by disrupting cytoskeletal architecture and in some other human pathologies.

Figure 5. Energetics of brain synaptosomes. Sites of ATP production (mitochondrial matrix) and sites of ATP consumption (ion transport across the plasma membrane and vesicle trafficking for neurotransmitter uptake and release, e.g. glutamate) are linked by an energy transfer pathway represented by the phosphocreatine/creatine kinase system. uMtCK bound to mitochondrial inner membrane (MIM) via cardiolipin (black squares). ATP consumed by the energy consuming reactions is reproduced locally by BBCK from PCr. GA3P, glyceraldehyde-3-phosphate; 1,3-BPG, 1,3 biphosphoglycerate; 3-PG, 3-phosphoglycerate; GAPDH, glycerate-3-phosphate-deshydrogenase; 3-PGK, 3-phosphoglycerate kinase; VDAC, voltage dependant anion channel; MOM, mitochondrial outer membrane; uMtCK, ubiquitous mitochondrial creatine kinase; Tub – αβ heterodimer of tubulin interacting with the VDAC channels and limiting its permeability for adenine nucleotides. Right panel shows the confocal image of isolated rat synaptosomes Mitochondria were labelled by pre-incubation with mitochondrial inner membrane probe MitoTracker Green (50 nM). Scale bar 1 μm. Adapted from reference [88].



All these numerous studies on mitochondrial dynamics, fusion and fission have been carried out mostly in yeast or various cultured cells (easy to grow and use in confocal microscopic studies), when the cells are usually at the stage of continuous division. Probably for this reason, the general conclusion has been made that fusion and fission phenomena are characteristic and necessary for normal functioning of mitochondria [90-92]. This general and rather enthusiastic conclusion is, however, not totally justified. Many works [61,94-99] have shown that fusion and fission of mitochondria are not observed in non-dividing adult cardiomyocytes. Thus, the fusion and fission are not necessary for normal functioning of mitochondria and for cardiac cell energetics in particular. Instead, mitochondrial localization and regular arrangement in muscle cells are controlled by cytoskeleton [86,87,100-102].By its nature, the contraction process needs very precise structural organization of sarcomeres and muscle cells [7]. Changes in the lattice spacing between actin and myosin filaments in sarcomeres due to alteration of titin orientation are the basis of length-dependent activation of sarcomere contraction and Frank-Straling law [49]. Mitochondria in muscle cells are in

fixed positions determined by their interactions with cytoskeleton and also with sarcoplasmic reticulum. Cytoskeleton plays an important role for mitochondrial and cell morphology and motility, intracellular traffic, mitosis [103,104]. Complex cytoskeletal network (microfilaments, microtubules, intermediate filaments) with specific cytoskeleton associated proteins interacts with mitochondria [86,87,97,100,105,106]. Very recently, Rostovtseva *et al*. have shown the ability of tubulin to bind directly to the VDAC channel on mitochondrial outer membrane and to control the permeability of this channel [86-88]. These interactions are thought to be responsible for mitochondrial regular arrangement into unitary structures (energetic modules, ICEUs) [48,81]. It is not yet completely clear; however, which components of the cytoskeleton and to which extent are responsible for the arrangement of mitochondria in cardiomyocytes into regular networks with modular organization. Organized and regularly arranged energetic units (modules) in adult cardiomyocytes represent a good example of regular networks, while NB HL-1 and many other cells [107] are examples of irregular networks.

It has to be mentioned that the rather popular hypothesis according to which the mitochondrial fusion is a necessary requirement for their normal function [90-92] evidently contradicts all the 50 years of experimental evidence in bioenergetics. During these five decades all laboratories all over the world have isolated mitochondria from heart, liver, skeletal muscle, brain etc. in perfectly granular shape and intact smooth outer and dynamic inner membranes [108-111] in functionally intact state, with all soluble Krebs cycle substrates including NADH in the matrix. If the mitochondria were in the cells in fused state as proposed by Twig *et al*. [92], rapid homogenisation of tissue should disrupt all mitochondrial membranes and release Krebs cycle substrates from matrix, and isolated mitochondria should represent only membrane fragments not capable to use Krebs-cycle linked substrates as pyruvte or glutamate malate – but this is never the case if isolation is performed carefully. Thus, systemic approach requires taking into account all available data before any general conclusion can be made; taking out only some fragmented data is nothing more than a relic of classical reductionism.

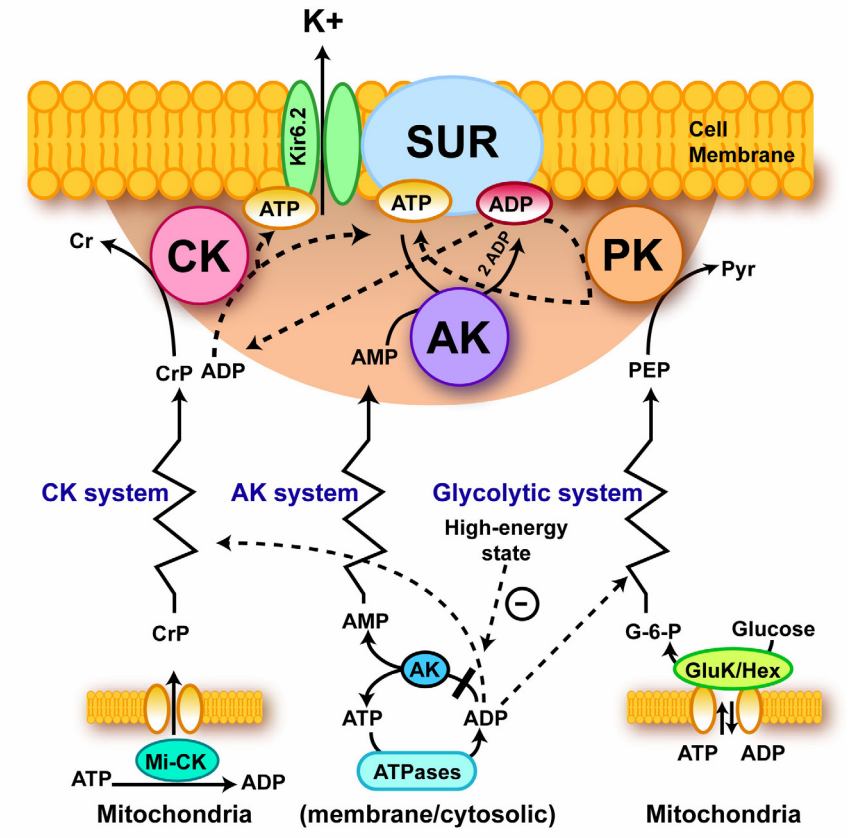
The very regular arrangement of distinct mitochondria into modular structures (ICEUs) evidently serves an important purpose of survival of heart cells under stress conditions. Under these conditions depolarization and functional damage of separate mitochondria does not result in complete breakdown of cell energetics, since contractile function of the heart is still maintained by other energetic units which continue to function in a well synchronized manner. The mechanism of this synchronization is still not precisely known and is under active studies in several laboratories [33,94,95,112]. On the contrary, fusion of mitochondria in heart cells is a clear sign of pathogenesis and cell death. Using electron microscopy Sun *et al*. showed already in 1969 that in perfused heart hypoxia resulted in formation of gigantic mitochondria due to the fusion process [113]. Thus, in cardiomyocytes mitochondrial fusion is most probably the beginning of their degradation and energetic breakdown of the cells.

4.4. Excitation-contraction coupling and cardiac energetics: membrane energy sensing

For modeling of the heart function within the projects of Systems Biology (as Physiome project), it is important to quantitatively describe relationships between energy metabolism and electrical activity of the cells. There is a clear need to account for the system level properties, such as metabolic

compartmentation and channeling phenomena, in modeling the energy sensing of ion currents across sarcolemma [49, 80]. In the control of the excitation – contraction coupling in the heart a principal step is the sarcolemmal membrane metabolic sensor complex [56,57,114-116]. Its main component is the sarcolemmal ATP sensitive K^+ (K_{ATP}) channel acting as an alarm system to adjust cell electrical activity to the metabolic state of the cell [56,57,114-116]. The sarcolemmal MM CK creatine kinase rephosphorylates the local ADP maintaining a high ATP/ADP level in these microcompartments for coordination of membrane electrical activity with cellular metabolic status, notably with PCr levels (see Figure 6).

Figure 6. A paradigm of phosphotransfer-mediated energetic signaling: coupling cellular metabolic and electrical activities. Dynamic interaction between creatine kinase (CK), adenylate kinase (AK) and glycolytic (represented by pyruvate kinase, PK) phosphotransfer relays determines a prototypic metabolic sensor - K_{ATP} channel behavior and subsequent cellular responses, such as excitability, hormone secretion, intracellular calcium homeostasis and vascular tone. The shadowed area represents a metabolic sensor "sensing zone", where intimate local changes in nucleotide ratios are sensed and transduced into an appropriate cellular response. Phosphotransfer circuits connect the "sensing zone" with cellular processes. Dashed lines indicate pathways signaling the high-energy state, while solid lines represent low-energy state signal transmission. Kir6.2 – potassium channel subunit; SUR – sulfonylurea receptor; GluK/Hex –glucokinase and hexokinase. Reproduced from ref. [7] with permission.



The K_{ATP} channel was discovered by Noma, and it was found that this channel has high affinity for ATP, about 100 μM [117,118]. Nevertheless, the channel is opened in the presence of millimolar ATP, as seen from rapid membrane repolarisation and shortening of action potential in ischemic and hypoxic hearts or as shown directly in experiments with internally perfused cardiomyocytes [56,57]. This is explained by strong diffusional restriction and thus ATP compartmentation in the subsarcolemmal area [119] and linked to the cellular pool of PCr via CK reactions. Pool exhaustion in the first minutes of ischemia certainly contributes to the cessation of contraction due to opening of K_{ATP} channel and decreased calcium entry. This energy transfer and control functions are shared by the whole system, including the creatine kinase, the adenylate kinase and glycolytic systems, as it was seen in experiments involving genetic manipulation [79,120,121]. Similar cell-membrane metabolic sensors may be also important in brain cells. The phosphotransfer relays communicate metabolic signals originating in mitochondria or at cellular ATPases to metabolic sensors conveying information about "high" or "low" cellular energy, oxygen supply or hormonal states [56,122].

Metabolic feedback signaling by phosphotransfer networks described above explains quantitatively the metabolic aspect of the classical Frank-Starling law regarding regulation of cardiac function and respiration under conditions of metabolic stability and unchanged calcium transients and changes of cardiac function in ischemia [49]. These systems are based on the compartmentalized energy transfer, whose deficit explains the rapid fall of contractile force in the first minutes of total ischemia. By regulating the sarcolemmal metabolic sensor - K_{ATP} channels, the CK-AK-glycolytic network affects the excitation-contraction process and the calcium cycle of the cell [80,39]. The latter system explains adrenergic modulation of cardiac cell function and energetics under stress [123-130]. Both systems may be activated simultaneously, as it is observed in the case of positive inotropy induced by β-adrenergic agents, when Frank-Starling curves are shifted upward [49].The physiological mechanism of respiration regulation described above has the important advantage of ensuring effective control of free energy conversion across the whole physiological range of workloads, without requiring a severe increase in cytoplasmic calcium and ADP concentrations. It thus avoids any danger of mitochondrial calcium overload that would open the mitochondrial permeability transition pore and thus lead to cell death [49]. Functioning of the coupled MtCK – ANT system in mitochondria prevents from the reactive oxygen species (ROS, oxygen free radicals) formation in mitochondrial respiratory chain and helps to avoid many problems related to ROS production, such as PTP opening, necrosis, apoptosis and rapid ageing [131]. In this way, the CK – PCr network may significantly contribute in the positive effects of physical exercise on the human health: exercise – induced increased fluxes via this pathway increase the ADP-ATP turnover in the coupled MtCK-ANT reactions in mitochondria and keeps the ROS production low.

Thus, effective cardiac work and fine metabolic regulation of respiration and energy fluxes need the organized and interconnected energy transfer and metabolic signaling systems. Direct transfer of ATP and ADP between mitochondria and different cellular compartments is not able to fulfill this important task efficiently.

4.5. Molecular system analysis of integrated mechanisms of regulation of fatty acid and glucose oxidation.

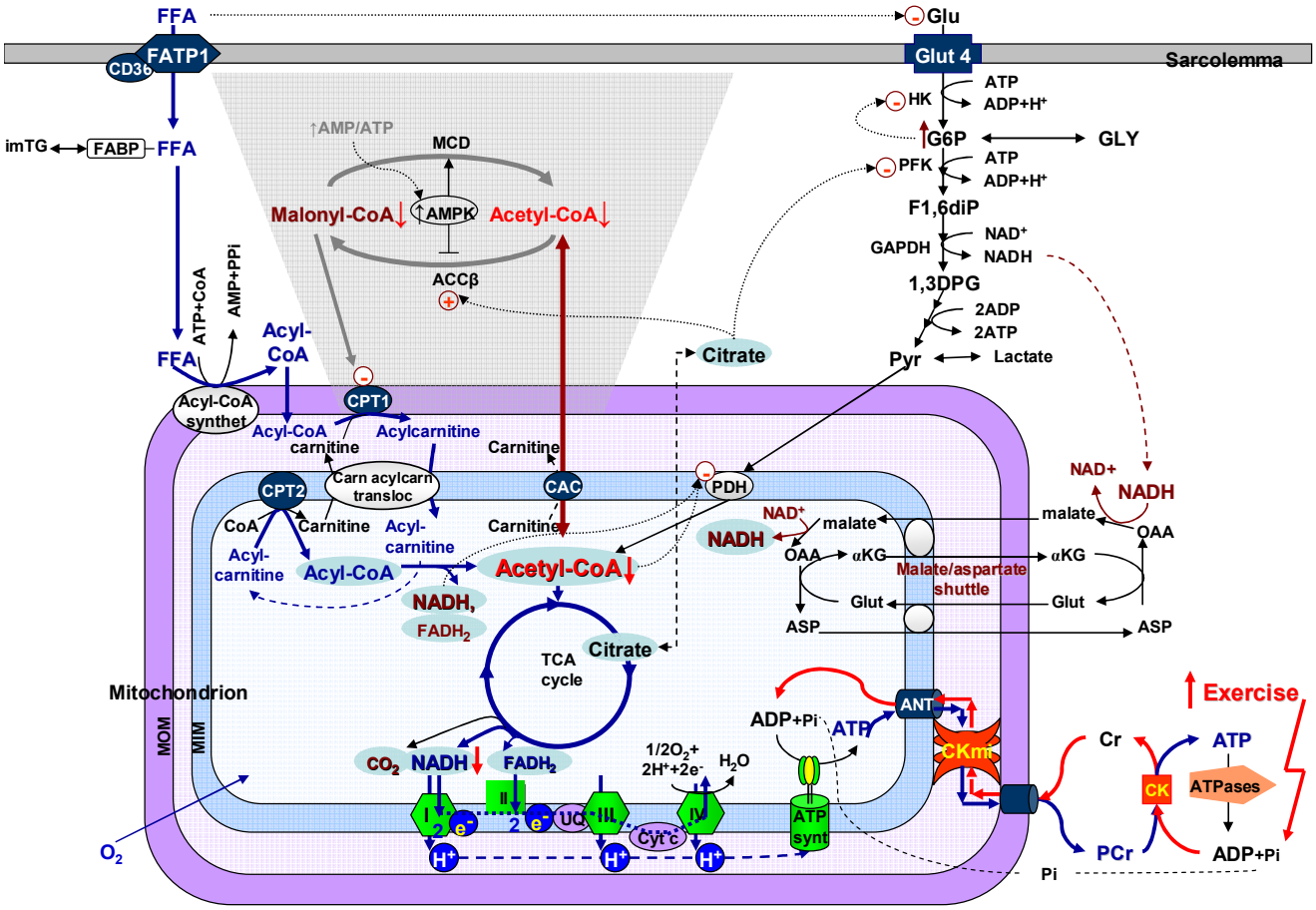
Molecular system analysis as a method is also useful for elucidation of the mechanisms of regulation of substrate supply for the heart [132]. In muscle cells, contractile function and cellular energetics are fuelled by oxidation of carbohydrate substrates and fatty acids [133-135]. The choice of substrates depends upon their availability, and the rates of their utilisation are very precisely regulated by multiple interactions between the intracellular compartmentalized and integrated bioenergetic systems of glycolysis, fatty acid oxidation and the Krebs cycle in the mitochondrial matrix, linked directly to the activity of the respiratory chain and the phosphorylation process catalysed by the ATP synthase complex [132,134]. The rates of all these processes are geared to the workload, mostly by the mechanism of the feedback metabolic regulation described above [132,134].

The network of reactions of main substrate supply for mitochondrial respiration in muscle cells and their multiple interactions and feedback mechanisms of regulation are illustrated in Figure 7. The choice of the substrates for oxidation depends on their availability, and if glucose and FFA are both present, FFA strongly inhibits the transport of glucose across the plasma membrane both in heart and in skeletal muscle [132,134,135]. At relatively low workloads, mitochondrial acetyl-CoA and NADH produced by beta-oxidation tend to inhibit the pyruvate dehydrogenase complex in the mitochondrial inner membrane, and citrate, the production of which is increased in the Krebs cycle, after transport across the inner mitochondrial membrane into the cytoplasm, inhibits PFK [134]. A glucose-fatty acid cycle (Randle hypothesis): if glucose and FFA are both present, FFA inhibit the transport of glucose across the plasma membrane, acyl-CoA oxidation increases the mitochondrial ratios of acetyl-CoA/CoA and of $NADH/NAD^+$, which inhibit the pyruvate dehydrogenase (PDH) complex, and increased citrate (produced in the TCA cycle) can inhibit phosphofructokinase (PFK). These changes would slow down oxidation of glucose and pyruvate (PYR) and increase glucose-6-phosphate (G6P), which would inhibit hexokinase (HK), and decrease glucose transport. The mitochondrial creatine kinase (miCK) catalyzes the direct transphosphorylation of intramitochondrially produced ATP and cytosolic creatine (Cr) into ADP and phosphocreatine, (PCr). ADP enters the matrix space to stimulate oxidative phosphorylation, while PCr is transferred via cytosolic Cr/PCr shuttle to functional coupling of CK to ATPases (acto-myosin ATPase and ion pumps), resulting in release of high free energy of ATP hydrolysis. If the workload increases, ATP production and respiration are increased due to feedback signalling via creatine kinase (CK) system, leading to decrease of mitochondrial acetyl-CoA content, which is transferred into cytoplasm with participation of carnitine acetyl carrier (CAC). Acetyl-CoA carboxylase (ACC) is responsible for converting acetyl-CoA to Malonyl-CoA, a potent inhibitor of CPT-I, with the aim to avoid overloading the mitochondria with fatty acid oxidation intermediates, when the workload is decreased. Inactivation of ACC occurs via phosphorylation catalyzed by AMP-activated protein kinase (AMPK). Phosphorylation and inactivation of ACC leads to a decrease in the concentration of malonyl-CoA. A fall in malonyl-CoA levels disinhibits CPT1, resulting in increased fatty acid oxidation. Malonyl-CoA is also converted back into acetyl-CoA in the malonyl–CoA decarboxylase (MCD) reaction. Increase in the workload increases the rate of acetyl-CoA consumption and that automatically decreases the malonyl-CoA content. The ACC and MCD regulation occur under stress conditions when the AMP/ATP ratios are increased, but are unlikely to

occur under normal work-load conditons of the heart. Thus, AMPK may be envisaged as a modulator, under situations of cellular stress, rather than as a master on-off switch of fatty acid oxidation.

It is the Krebs cycle, which is on the crossroads between the metabolic pathways of glucose and fatty acid oxidation, and its intermediates that play a very important role of feedback metabolic regulation of upstream pathways of substrate oxidation [132,134]

Figure 7. The scheme of substrate supply for mitochondrial respiration and the mechanisms of feedback regulation of the fatty acid and glucose oxidation during workload elevation in oxidative muscle cells: central role of TCA cycle intermediates. Reproduced from reference [132] with permission.



FFAs are taken up by a family of plasma membrane proteins (fatty acid transporter protein (FATP1), fatty acid translocase (CD36) and in cytoplasm associated with fatty acid binding protein (FABP). FFAs are esterified to acyl-CoA via fatty acyl-CoA synthetase. The resulting acyl-CoA is the transported through the inner membrane of the mitochondrion, via the exchange of CoA for carnitine by carnitine-palmitolyltransferase I (CPT I). Acylcarnitine is then transported by carnitine-acylcarnitine translocase into the mitochondrial matrix where a reversal exchange takes place through the action of carnitine-palmitoyltransferae II (CPT II). Once inside, the mitochondrion acyl-CoA is a substrate for the beta-oxidative pathway, resulting in acetyl-CoA production. Each round of beta-oxidation produces one mole of NADH, one mole of $FADH_2$ and one mole of acetyl-CoA. Acetyl-CoA enters the TCA cycle, where it is further oxidized to CO_2 with the concomitant generation of three

moles of NADH, one mole of $FADH_2$ and one mole of ATP. Acetyl-CoA which is formed in the mitochondrial matrix, can be transferred into the cytoplasm with participation of carnitine, carnitine acetyltransferases and carnitine acetyltranslocase (carnitine acetylcarnitine carrier complex, CAC).

Glucose (GLU) is taken up by glucose transporter-4 (GLUT-4) and enters the Embden-Meyerhof pathway, which converts glucose via a series of reactions into two molecules of pyruvate (PYR). As a result of these reactions, a small amount of ATP and NADH are produced. G6P – glucose 6-phosphate, HK – hexokinase, PFK – phosphofructokinase; GLY – glycogen; F1,6diP – fructose-1,6-bisphosphate, GAPDH – glyceralhehydephosphate dehydrogenase, 1,3DPG – 1,3-diphosphoglycerate. The redox potential of NADH is transferred into the mitochondrial matrix via the malate/aspartate shuttle; OAA – oxaloacetate, Glut – glutamate, αKG – alpha – ketoglutarate, ASP - aspartate. Malate generated in the cytosol enters the matrix in exchange for alpha-ketoglutarate (αKG) and can be used to produce matrix NADH. Matrix oxaloacetate (OAA) is returned to the cytosol by conversion to ASP and exchange with glutamate (Glut). Most of the metabolic energy derived from glucose can come from the entry of pyruvate into the citric acid cycle and oxidative phosphorylation via the acetyl-CoA production. NADH and $FADH_2$ are oxidized in the respiratory chain (complexes I, II, III and IV). These pathways occur under aerobic conditions. Under anaerobic conditions, pyruvate can be converted to lactate.

5. Mathematical Models of Energy Metabolism, Useful and Not Very Useful

Because of the high complexity of the processes involved, mathematical modeling is an increasingly important part of Systems Biology, including Molecular System Bioenergetics. Different models of cardiac energy metabolism have already been used to analyze the mechanisms of regulation of respiration and cellular energy fluxes [136-145], with rather contradictory results. There is no complete model available as yet, no "general equation" (as Claude Bernard could call it) of energy metabolism, and this will need further intensive work. However, the number of models developed is already significant, whch allows us to try to classify them by their impact and constructive contribution into our understanding of regulation of integrated metabolism, and to critically analyze some errors which have become evident. In general, the models can be easily classified as good, bad and very bad. Bad and very bad models are those which authors have ignored the wise advice of Claude Bernard to take into account the maximally possible amount of reliable experimental data (see above), and thus have only some virtual value. An extreme case of these models is that by Barros and Martinez [146] who consider the cell as a sphere where a metabolite is produced by a single source and diffuses freely in a homogenous, isotropic medium, the cytosol. The authors take the diffusion coefficients for metabolites determined for bulk water phase as the only realistic values, which for ATP would be equal to about 500 $\mu m^2/s$ [146]. The fact that these values for diffusion coefficients may be significantly different inside cells with a protein concentration of approximately 100 - 200 mg/mL were not considered, thus the possibility that physical barriers resulting from a dense cytoskeletal or mitochondrial network or from macromolecular crowding [35] were not taken into account at all. Taking this for granted, the authors conclude that "even under most favorable conditions that are compatible with the known physical constraints, it would be impossible that ATP pools could appear in the cytosol of a compact cell", and then conclude that "unrealistic conditions were needed to form ATP domains" [146]. No experimental data were analyzed to verify the correctness of this model and

the conclusions derived. Critical analysis of this model can be found in our previous article in IJMS [35]. The problem is that Barros and Martinez are not alone – there is whole group of authors who have produced models of cardiac energy metabolism based on the theory of homogenous cytoplasm as diluted solution of metabolites. Some of them (critically analysed in [147]) arrive at non-realistic conclusion that the cells must be much bigger than they are (these are very bad, non-realistic models). Some other authors [148,149] by applying otherwise correct theories of chemical thermodynamics of dilute solutions have recently discovered that the cardiac function is governed by a general, average value of phosphorylation potential (see equation 2). These authors ignore the pioneering experimental works of Kammermeyer's and Radda's groups [52,150]. In the beginning of metabolic research, including studies by use of ^{31}P-NMR method, the first problem to study was to investigate the question how changes of cardiac function might be dependent on average phosphorylation potential, and the result was very clear: there is no dependence, cardiac function can be changed manifold at almost constant value of phosphorylation potential [52,150]. Thus, the modeling result [149] contradicts experimental data – there is no way to call this model a good one. The reason is evidently that the model does not account for the compartmentation phenomenon described above.

The models which describe the mitochondrial metabolism, including respiratory chain and Krebs cycle, are in much better situation and have given some of the important results, consistent with experimental data – evaluation of the role of calcium in regulation of mitochondrial activities, role of Pi in respiration regulation [138-140]. Most important is to apply these models correctly for in vivo conditions taking into account the compartmentation phenomenon.

Finally, there are the compartmentalized energy transfer models aimed at describing quantitatively the results of *in vivo* studies of respiration regulation in the ICEUs under physiological conditions of the Frank-Starling law. These models are based on the concepts of ICEUs and include: kinetics of ATP hydrolysis by actomyosin ATPase during contraction cycle, diffusional exchange of metabolites between myofibril and mitochondrial compartments, VDAC-restricted diffusion of ATP and ADP across mitochondrial outer membrane, the mitochondrial synthesis of ATP by ATP synthase, ΔpH and $\Delta\Psi$ controlled Pi and ADP transport into mitochondrial matrix, PCr production in the coupled mitochondrial CK reaction and its utilization in cytoplasmic CK reaction [136,137]. These events are considered in a system consisted of a myofibril with a radius of 1 μm, a mitochondria, and a thin layer of cytoplasm interposed between them [136,137]. The modeling results show cyclic changes in the concentration of ADP in the core of myofibrils in ICEUs in a microcompartment containing myofibrillar-bound MM-CK, where ADP is first produced by actomyosin MgATPase during the contraction cycle of crossbridges, and then rephosphorylated by the CK due to a non-equilibrium state of the CK reaction [136,137]. Interestingly, these calculated cyclic changes in PCr, ATP and Cr which are in the range of 5–10 % of their cellular contents, are in good agreement with the multiple observations of the cyclic changes of these compounds in the contraction cycle published in the literature [151,152]. These changes in Cr, PCr and total ATP are however close to the experimental errors of their detection, thus giving an overall impression of metabolic stability. Changes in ADP and Pi concentrations are relatively much more significant because of very low initial values. Without CK, the changes of local ADP concentrations in these microcompartments will be much more dramatic [136,137]. Within the whole contraction cycle the rates of ADP and ATP cycling and thus the respiration in mitochondria coupled to the PCr production are increased with elevation of the

workload. Increasing cyclic changes in the local ADP production in myofibrils are immediately displacing the myofibrillar MM-CK reaction in the direction of local ATP regeneration. The amplitude of displacement of CK from equilibrium is proportionally increased with workload [136,137]. In this regard, CK, adenylate kinase and other phosphotransfer isoenzymes in different intracellular compartments are "pushed" or "pulled" from the equilibrium in opposite directions, depending on the activity of an associated process which drives steady-state high-energy phosphoryl flux [49]. The model quantitatively describes the experimental observations on the dependence of the respiration rate upon the workload [7,49]. Evidently, to find the "general equation", the complete model of integrated energy metabolism of muscle and brain cells we need to develop these constructive models which have already given us significant explanation of experimental data. The more complete model(s) should include all data and phenomena shown in Figures 4 – 7. Only these models can be included into more general project of Systems Biology and Molecular System Bioenergetics.

5. Conclusions

Systems Biology as a new paradigm of biological sciences which favours the study of integrated systems at all levels: cellular, organ, organism, and population with the aim of explaining biological function by interaction of system components provides new conceptual tools for studies of integrated metabolic processes. The aim of Systems Biology is the higher-level analysis of complex biological systems by using the wealth of information obtained in studies of isolated components, applying the methodological approaches of cybernetics, applied mathematics, network analysis, nonequilibrium thermodynamics of open systems. From a historical perspective the first systems biology approach was already applied by Claude Bernard about 150 years ago, and further important contributions were made by Norbert Wiener and Erwin Schrödinger. The developments of biological research during last 150 years follow very precisely the dialectical principles of development from thesis to antithesis to synthesis discovered by Hegel. The Systems Biology opens new perspectives for studies of the integrated processes of energy metabolism in different cells. These integrated systems acquire new, system-level properties due to interaction of cellular components, such as metabolic compartmentation, channeling and functional coupling mechanisms, which are central for regulation of the energy fluxes. All these mechanisms are functioning within phosphotransfer networks of the compartmentalized energy transport. These mechanisms explain such important physiological phenomena as metabolic aspects of Frank-Starling law of the heart and membrane sensing of cellular energy levels. Mathematical modeling of these important systems is a promising approach in Molecular System Bioenergetics.

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Article 1

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Biophysics of the organized metabolic networks in muscle and brain cells

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Summary

The biophysics of the energy transport by phosphotransfer networks which is mostly presented by the creatine kinase system in muscle and in brain cells, and metabolic feedback regulation of mitochondrial respiration and energy fluxes are analyzed in their historical perspective from the point of view of molecular system bioenergetics, including the description of development of mathematical

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modeling of these processes. The central cellular mechanism of functioning of these organized metabolic pathways is the functional coupling between the isoenzymes of the creatine kinase and mitochondrial adenine nucleotide translocase, on one hand, and MgATPases in the myofibrillar compartment in muscle cells and in subcellular membranes, at another hand, both in muscle and brain cells. The functional coupling phenomenon is well illustrated by its analogy with the philosophical principle of Maxwell demon. Mathematical modeling has been a very useful and important tool of the quantitative analysis of these structurally organized and integrated metabolic systems. Applications of these methods show the central importance of the metabolic feedback mechanisms in regulation of mitochondrial respiration and energy fluxes in the cells.

Introduction

The creatine kinase systems represent the major part of the energy transfer networks in many types of the adult mammalian cells, playing a role superior to that of the adenylate kinase and glycolytic systems (*1-5*). The mitochondrial respiration is intimately regulated by the availability of the ADP to the adenine nucleotide translocase, ANT (*6-10*). This regulatory mechanism known as respiratory control phenomenon was discovered in 1952 – 1955 by Lardy, Wellman, Chance and Williams in studies of isolated mitochondria *in vitro* (*6,7*). However, situation regarding the regulation of mitochondrial respiration in the intact cells *in vivo* seems to be much more complicated, many new factors coming into existence due to complex intracellular organization. In the cells with high energy requirements and fluxes as heart, skeletal muscles, brain and many others, the ADP necessary for regulation of respiration is supplied via energy transfer networks, mainly via the creatine kinase system (1–*5, 11-16*). In this review we describe the general principles and mechanisms of functioning of these coupled creatine kinase systems. The nature and significance of this phenomenon is well illustrated by its analogy with the philosophical concept of Maxwell demon. We analyze the experimental data revealing the molecular, supramolecular and cellular mechanisms involved in the regulatory actions of the creatine kinase systems. By using the methods of mathematical modeling we show that the main reason for their functioning is to overcome the heterogeneity and localized restrictions of the intracellular diffusion of adenine nucleotides (ATP and ADP) within the organized metabolic systems of the cells. Taken together, these results show the perspectivity of the integrated approach in the studies of cellular bioenergetics – molecular system bioenergetics (*2*).

I. Experimental description of the creatine kinase system

The connection between ATP and phosphocreatine, PCr, was understood in 1934, when Lohmann discovered the creatine kinase reaction (*17*). In 1939

Belitzer and Tsybakova showed that in muscle homogenates the oxygen consumption was stimulated by creatine and always resulted in phosphocreatine (PCr) production with the ratio of PCr/O about 3 (*18*). The latter result was the earliest indication of the functioning of the mitochondrial creatine kinase coupled to oxidative phosphorylation (see below). Mitochondrial isoforms of the creatine kinase were discovered in the Klingenbergs laboratory in 1964 (*19*) after the discovery of its MM and BB isoforms (*20*).

All creatine kinase isoenzymes are compartmentalized in the cells, significant fractions of the MM isozyme being connected structurally to the myofibrils, to the membrane of sarcoplasmic reticulum and to the sarcolemma in muscle cells (*1-5, 11, 21-26*). The molecular biology, expression, structure at the atomic resolution of the different creatine kinase isoenzymes – sarcomeric and ubiquitous mitochondrial isoforms sMtCK and uMtCK, respectively, muscle form MM and brain form BB - and the chemical mechanism of catalysis in their active center are described in many excellent earlier reviews (*1 ,4, 12 ,13, 27-31*). Given below is the description how the creatine kinase isoenzymes are integrated into the cellular energy metabolism and how they interact with other metabolic systems. It will be shown that these interactions always include a functional coupling mechanism.

a) Mitochondrial creatine kinases

Most of information of functional role of the mitochondrial creatine kinase has been obtained in studies of heart mitochondria, *s*MtCK and in some lesser extent in skeletal muscle *s*MtCK and also for brain and smooth muscle mitochondria, in both cases *u*MtCK (*27-28,30-31*). The work of Belitser and Tsybakova on muscle homogenates showing constant PCr/O_2 ratio was first to describe the activation of respiration by creatine, due to creatine kinase reaction, as it was already mentioned above (*18*). Bessman and Fonyo showed in isolated heart muscle mitochondria that addition of creatine increased the respiration rate in the State 4 (presence of ATP) (*32*). Similar data were reported by Vial et al. (*33*). In 1973, Jacobus and Lehninger studied the kinetics of the stimulatory effect of creatine on the State 4 respiration rate and found that at its physiological concentration, 10-15 mM, creatine stimulated the respiration maximally, to the State 3 level (*34*). From this important work, the ideas of coupling of the mitochondrial creatine kinase reaction with the oxidative phosphorylation as a mechanism of regulation of respiration started to take a shape. In 1974 Saks et al. published a paper (*22*) confirming the results reported by Jacobus and Lehninger, and in 1975 the same authors applied the kinetic analysis and simple methods of mathematical modeling to investigate the phosphocreatine production coupled to the oxidative phosphorylation (*35*). The results showed that the oxidative phosphorylation itself controls the phosphocreatine production in heart mitochondria. When

uncoupled from oxidative phosphorylation (if the latter is not activated, for example), the mitochondrial creatine kinase reaction does not differ kinetically and thermodynamically from other creatine kinase isoenzymes: the reaction always favours the ATP production and according to the Haldane relationship, ADP and phosphocreatine binding is more effective due to higher affinities than that of ATP or creatine, respectively (*22, 35*). When the calculated predicted rates of the reaction were compared with the experimental ones, good fitting for any experimental conditions was found in the absence of oxidative phosphorylation but not when the latter was activated: under conditions of oxidative phosphorylation the mitochondrial creatine kinase reaction was strongly shifted in direction of phosphocreatine synthesis (*35*). This was taken to show that ATP produced in mitochondrial oxidative phosphorylation was much more effective substrate for MtCK than the MgATP in medium, and it was proposed that this is due to direct transfer of ATP by adenine nucleotide translocase from matrix space to the creatine kinase, which should be located somewhere in the close proximity to ANT to make this direct channeling possible (*35*). To understand better the mechanism of this phenomenon, Jacobus and Saks undertook a joint study and performed a complete kinetic analysis of the creatine kinase reaction in isolated rat heart mitochondria under both conditions: with and without oxidative phosphorylation (*36*). While the kinetic constants for guanidino substrates - creatine and phosphocreatine - were not changed and were the same in both conditions, the oxidative phosphorylation had a specific effect on the kinetic parameters for adenine nucleotides. Under conditions of oxidative phosphorylation the dissociation constants can be measured only for a substrate - MgATP - in the medium, and the apparent affinity for this substrate (if creatine was already bound to MtCK) was seen to be increased by order of magnitude (*36*). The Haldane relationship for the creatine kinase reaction was no more valid, showing the involvement of some other processes – oxidative phosphorylation and ANT (*36*). The explanation proposed was the direct transfer of ATP from ANT to MtCK due to their spatial proximity which results also in increased uptake of ADP from MtCK (reversed direct transfer), and as a result, the turnover of adenine nucleotides is increased manifold at low external concentration of MgATP, this maintaining high rates of oxidative phosphorylation and coupled phosphocreatine production in the presence of enough creatine. This was the intuitive hypothesis of the direct transfer of ATP and ADP as a coupling mechanism for qualitative explanation of the decrease of the apparent (under these conditions) kinetic constants for MgATP in the MtCK reaction in the presence of oxidative phosphorylation (*22,35,36*). Further experiments confirmed these conclusions and in recent structural studies and in mathematical modeling of functional coupling, interesting quantitative features of this mechanism were revealed (see below).

The conclusions of the privileged access of mitochondrial ATP to MtCK and increased mitochondrial turnover of adenine nucleotides in the presence of creatine were directly confirmed by Barbour et al. with the use of isotopic method (*37*) and by the thermodynamic approach by De Furia (*38*), Saks et al. (*39*), and Soboll et al. (*40*). Finally, an effective competitive enzyme method for studying the functional coupling phenomenon, namely the pathway of ADP movement from MtCK back to mitochondria, was developed by Gellerich et al. (*41-45*). These authors used the phosphoenol pyruvate (PEP) – pyruvate kinase (PK) to trap ADP and thus to compete with ANT for this substrate. This competitive enzyme system was never able to suppress more than 50 % of the creatine - stimulated respiration in isolated heart mitochondria, this showing the rather effective channeling of ADP from MtCK to the ANT (*41*). The Gellerich group has preferred to explain these latter data by the hypothesis of dynamic compartmentation of adenine nucleotides in the intermembrane space, that meaning that there is some control of the permeability of the outer mitochondrial membrane and because of this, the formation of some ADP and ATP concentration gradients (*42-45*). This was an alternative hypothetical mechanism of coupling between MtCK and ANT without direct transfer of the substrates. Interestingly, this hypothesis focused attention on the role of mitochondrial outer membrane in the control of mitochondrial function, and foresaw many important aspects of the control of mitochondrial function *in vivo*, but appeared to be insufficient to explain quantitatively the functional coupling between MtCK and ANT.

Fig. 1 summarizes schematically the two important mechanisms of the effective functional coupling of MtCK to the ANT described above.

Fig. 2 gives kinetic evidences for the functional coupling between MtCK and ANT described above for the isolated mitochondria of the rat heart (A) and rat synaptosomes (B, C). In these experiments the isolated mitochondria were incubated in the presence of 10 mM creatine and the rate of the phosphocreatine production in the mitochondrial creatine kinase reaction measured either spectrophotometrically by a coupled enzyme assay (PEP – PK- lactate dehydrogenase), when the oxidative phosphorylation was inhibited by rotenone (10μM) and oligomycin (1 μM), or by measuring the rate of oxygen consumption coupled to the phosphocreatine production (*36*). This Figure shows that in both cases, the oxidative phosphorylation increases the rate of the mitochondrial creatine kinase reaction in direction of the phosphocreatine and ADP synthesis (35,36).

Interesting model experiments by Fossel showed that for the direct transfer to be efficient in the functional coupling of two enzymes, the distance between them should be shorter than 10 nm that is comparable with the size of a protein molecule (*46*).

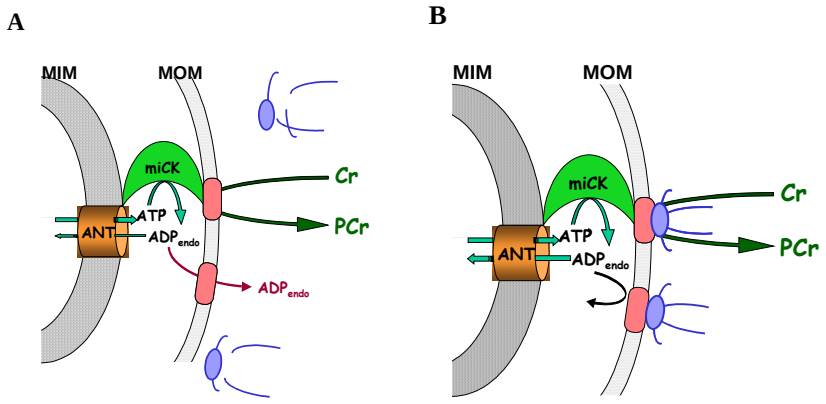
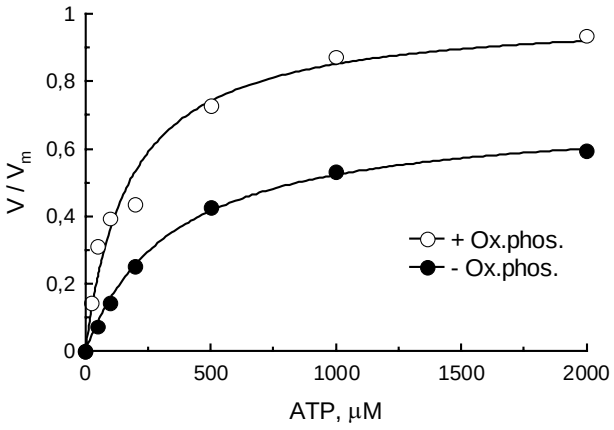


Figure 1. The mechanism of functional coupling between ANT and MtCK in vitro and in the cells in vivo. A. In isolated mitochondria or in trypsin-treated fibers the contacts of mitochondria with cytoskeleton are lost and the VDAC in the outer mitochondrial membrane are in open configuration. ATP is directly channeled from ANT to MtCK and used to produce phosphocreatine PCr and ADP which is partially channeled back (about 50 %) to ANT and another part may leave the intermembrane space via VDAC and is accessible for utilization by the PEP-PK system (*41, 125*). B. In intact cells the VDAC is in closed configuration due to contacts with some cytoskeletal proteins and all ADP is preferably taken up by ANT and thus inaccessible for pyruvate kinase. This increases the role of the creatine kinase in the mitochondrial cytoplasmic communications.

Figure 2

A.



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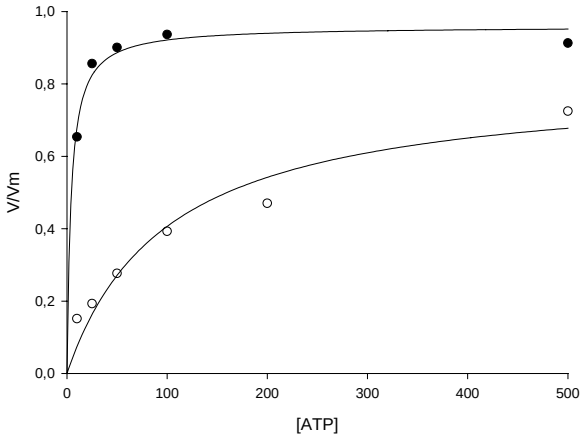


C.

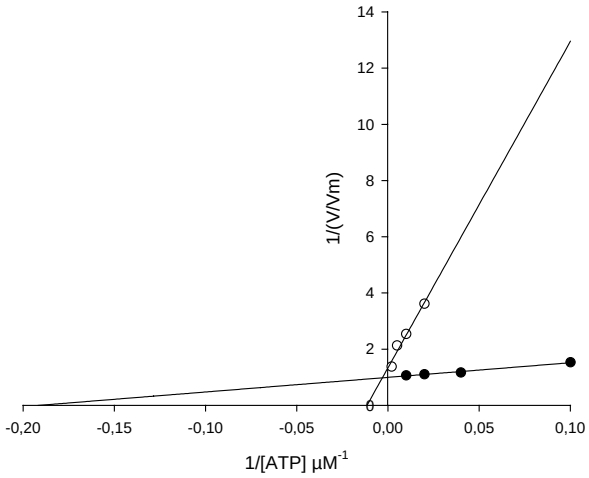


Figure 2. A. Normalized rates of CK kinetics in isolated cardiac mitochondria in the presence of 10 mM Cr with and without oxidative phosphorylation (OP). The results for respiration in presence of OP were obtained by oxygraphy measurements in the Mitomed medium of the following composition: 110 mM sucrose, 60 mM K-lactobionate, 0.5 mM dithiothreitol, 0.5 mM EGTA, 3 mM $MgCl_2$, 20 mM taurine, 3 mM KH_2PO_4, 20 mM K-HEPES, pH 7.1, with 5 mM glutamate, 2 mM malate, 2 mg/ml essentially fatty acid free bovine serum albumin, and those obtained in absence of OP

Figure 2. legend continued

were measured by spectophotometry in the same medium after inhibition of respiratory chain and ATP synthase by rotenone (10μM) and oligomycin (1μM), respectively. B, C. Comparison of exogenous ATP kinetics of regulation of the CK reaction in isolated mitochondria of rat brain with 10 mM of Cr in presence or absence of oxidative phosphorylation (OP). B) Michaelis-Menten representation of regulation of CK activity by exogenous ATP, C) Double-reciprocal representation of regulation of CK activity by exogenous ATP. The ordinate axis represents the normalized rate of respiration (Vm is the maximal rate of respiration). (●) presence of OP, (o) absence of OP.

Structural aspects of the MtCK and its functioning have been extensively studied in excellent experimental investigations by Theo Wallimann group (*29,47-49*). Among their achievements is description of a spatial structure of this enzyme at 2.8 Å resolution (*29*). The peculiarity of the MtCK, in contrast with other dimeric CK isoenzymes (MM and BB), is that it forms octameres (*1,48*). It is still not yet completely clear whether in intact mitochondria there are both forms (dimeric and octameric) of the MtCK present (*50*), but the existence of MtCK in mitochondria in octameric form can give even a strong further support and explanation for the functional coupling between ANT and MtCK. ANT in the inner mitochondrial membrane forms tight complexes with negatively charged cardiolipin in the ratio 1:6 (*51*). It has been shown that positively charged MtCK is fixed to this cluster by electrostatic forces due to three C-terminal lysines which strongly interact with the negatively charged cardiolipin in complex with ANT (*52-54*). The structure of the ANT was recently resolved at 2.2 Å resolution by Brandolin group in Grenoble (*55*). The translocation of both ATP and ADP in the Mg-free forms is related to the conformation changes of pore-forming monomeres (*55*). Klingenberg's group thinks that both monomeres within a dimere are taken to be alternatively involved in the translocation, one accepting for example ATP from matrix and translocating it, the second only releasing ADP translocated in previous cycle ("half-site reactivity") (*56-59*). This conformation change ("pore") mechanism leads in its simplest version to the Ping-Pong reaction mechanism of transport (*58*). On the other hand, the kinetics of ATP-ADP exchange conforms to sequential mechanism of the simultaneous binding of nucleotides on both sides (*60*). The structural data of Brandolin group and the kinetics of ATP-ADP exchange by ANT are well fitting with each other by the hypothesis that the dimeres with alternatively activated monomers function in coordinated manner in the tetrameric clusters, where the export of ATP from mitochondria by one monomer in a dimer occurs simultaneously with import of ADP by another monomer in another dimer (*61, 62*).

Thus, both structural and functional data available now show convincingly that the oxidative phosphorylation controls, via ANT, the MtCK reaction and forces it to produce the phosphocreatine in spite of unfavorable kinetic and thermodynamic characteristics for this reaction. At the same time, the MtCK plays back the same role for ANT and oxidative phosphorylation, by channeling ADP and thus directly controlling the rate of respiration. It is interesting to note that in their first classical experiments on the well washed skeletal muscle homogenates Belitser and Tsybakova observed strong stimulation of respiration by creatine without addition of adenine nucleotides (*18*). Much later Kim and Lee showed the same effect for isolated pig mitochondria (*63*). Both these experiments are explained by very effective use of the endogenous adenine nucleotides in coupled sMtCK reaction.

Experimentally, the role of functional coupling between MtCK and ANT was verified recently in the studies of the energy metabolism the heart of mice with knock-out of MtCK: as predicted by the theory described above, these heart had lower levels of the phosphocreatine and reduced post-ischemic recovery (*64,65*). A new important role of the control by MtCK over ANT is the prevention of opening of the mitochondrial permeability transition pore recently discovered by Dolder et al. in Wallimann's laboratory (*66*), this preventing from the cell death by inhibiting apoptosis and necrosis. This again illustrates the vital importance of the functional coupling phenomenon.

b) Myofibrillar creatine kinases

The myofibrillar end of the creatine kinase-phosphocreatine shuttle is a more general system in muscle cells than that of mitochondrial one, it exists and is fully active also in fast – twitch glycolytic muscles with very low content of mitochondria, in which contractile function is maintained mostly by the ATP production in the glycolysis, coupled to cytoplasmic phosphocreatine production (*67-84*). In spite of the very high activity of the glycolytic enzymes and of total creatine kinase activity in these muscles, the coupling between these two systems is weaker (except the specific compartments or microcompartments close to membranes) and the rate of phosphocreatine production lower that in heart cells, and no metabolic stability is observed (*67-70*). Instead, the muscle fatigue is a common phenomenon if the PCr pool is exhausted (71-74). The differences in the organization of the creatine kinase shuttles in different muscles have recently been extensively analyzed (*75*). In the cytoplasmic compartment, the MM creatine kinase seems to be in the classical quasi - equilibrium state, and the glycolysis seems to drive the phosphocreatine synthesis by the permanent removal of the ADP and thus shifting the equilibrium in direction of production of phosphocreatine (*69,76*), which is the substrate for the coupled creatine kinase in the myofibrillar compartment.

At the same time, all what concerns the mitochondrial respiration and its regulation, different muscle types seem to have a similar regulatory mechanism for respiration because of the presence of the sMtCK and its functional coupling to the ANT (*77,78*). As it has been described in the early works by Michael Mahler (*77*) and later by Kent Sahlin group (*78*) and by many others (*75,79*), one of the most important factors of the regulation of the respiration is the ratio of phosphocreatine to creatine, and also the total creatine content (*80*).

The myofibrillar end of creatine – phosphocreatine cycle is represented by MM isozyme of creatine kinase localized in different parts of sarcomere and functionally coupled to the actomyosine MgATPase (*81-88*). Indeed, within the contraction cycle the ADP release is a necessary step for new binding of the MgATP, dissociation of actomyosin crossbridges and for muscle relaxation, to start the new cycle of contraction (*71,73,74,84-90*). This step is often found to be the slowest one in contraction cycle and therefore the rate limiting one, since MgADP may compete with MgATP for the substrate site on myosin and inhibit crossbridge detachment by MgATP (*71,89,90*). Indeed, because of the structural similarity with MgATP, MgADP binds easily to the actomyosin with inhibition constant Ki being in the range of 200 μM both in MgATPase reaction and in sliding of fluorescent actin on myosin (*71,89,90*). Thus, accumulation of MgADP fixes the crossbridges in their rigor states and by inhibiting the contraction contributes in muscle fatigue (*72-74*). From kinetic point of view, the MgADP should be rapidly removed from actomyosin and the high local value of the MgATP/MgADP ratio and thus the local phosphorylation potential maintained. This task corresponds exactly to that initially proposed for the function of the creatine kinase made in Davies group's works (*91*), and this function – rapid removal of ADP and local production of MgATP perfectly fits both with the thermodynamic and kinetic characteristics of the creatine kinase.

The function and roles of myofibrillar creatine kinase have been studied and described very extensively (*75,82,92-94*). In this chapter, the interesting question is the state of the reaction: is it the classical equilibrium one or not, but rather in steady state, out of equilibrium in dependence of the rate of contraction. Wallimann group has shown that the MM CK is bound specifically to the M-line (*84,93,94*), and significant part of this isozyme is found in the space of I - band of sarcomeres (*81*). *In vitro*, the interactions of myosin and CK have been known for a long time (*83*). There is increasing amount of evidence that this MM creatine kinase is intimately involved in the contraction cycle at the level of the ADP release and ATP rebinding steps. First, multiple studies by Ventura-Clapier and Vassort have shown that phosphocreatine accelerates the release of muscle from rigor tension in the presence of exogenous ATP, decreasing the necessary ATP concentration by

order of magnitude (*75,92*). Second, Krause and Jacobus have shown close functional coupling between the actomyosin ATPase and the creatine kinase reaction in isolated rat heart myofibrils, seen as the decrease of the apparent Km value (*82*). In accordance with this, Sata et al. found that sliding velocity of fluorecently labeled actine on a cardiac myosin layer coimmobilized with creatine kinase showed significantly smaller apparent Km for MgATP than in the absence of CK (*95*). Ogut and Brozovich studied the kinetics of force development in skinned trabeculae from mice hearts and found that in spite of the presence of 5 mM MgATP, the rate of force development depended on the concentration of the phosphocreatine, and concluded that there is a direct functional link between the creatine kinase reaction and the actomyosin contraction cycle at the step of the ADP release in myofibrils (*96*). Most probably, this effective interaction occurs via small microcompartments of adenine nucleotides in myofibrils and is facilitated by anisotropy of their diffusion. Mathematical modeling of the myofibrillar CK reaction showed that it is clearly out of equilibrium during the contraction cycle (*97-99*). The results of ^{31}P-NMR inversion transfer studies by Joubert et al. directly confirmed this conclusion (*100*).

c) Membrane-bound creatine kinases

The next of important ATP consuming systems, besides the contractile one in muscle cells, are the membrane ATPases, both in the membranes of sarcoplasmic reticulum and in the plasmalemma (sarcolemma). Their function is to maintain the ionic homeostasis and particularly, the regulation of the calcium cycle. Here, the role of coupled creatine kinase (also adenylate kinase and compartmentalized glycolytic system) is of utmost importance. Indeed, these coupled systems represent the membrane sensor mechanisms connecting ion fluxes to the intracellular energy state. In their turn, the ion fluxes across the sarcolemma control the cell function. The two best examples of this kind of coupled systems is the MM creatine kinase connected to the sarcolemmal membrane of the the cardiomyocytes and to the membranes of sarcoplasmic reticulum (SR) of cardiac and skeletal muscle cells.

The role of the MM CK connected to the membrane of the SR and functionally coupled to the Ca, MgATP- dependent ATPase (SERCA) has been described in great details in many studies (*101-104*). This coupling has been shown both for isolated SR vesicles and for intact SR in the permeabilized cardiac fibers, and the introduction of the phosphocreatine increased the rate of the calcium uptake and the maximum SR Ca^{2+} content, while the exogenous ATP regenerating system (phosphoenol pyruvate and pyruvate kinase) was less effective (*104*). It was also shown in experiments with permeabilized cardiomyocytes that withdrawal of phosphocreatine from the medium reduced the frequency and amplitude but increased the duration of

spontaneous Ca^{2+} sparks (*105*). Thus, despite the presence of millimolar levels of cytosolic ATP, depletion of phosphocreatine impairs the Ca^{2+} uptake (*102-105*). All these data clearly show the importance of the MM CK, bound to the membrane of SR, in rapid rephosphorylation of local MgADP produced in the Ca,MgATPase reaction, independently from cytoplasmic situation and thus clearly in non-equilibrium manner. This is consistent with the results of studies by Be Wieringa's laboratory showing that the knock-out of MM CK gene resulted in remarkable adaptive changes in muscle cells morphology, and the most remarkable of these changes was the multifold increase of the volume SR system, to compensate for the loss of the efficiency of calcium uptake due to the absence of MM CK (*106*).

An important step in the control of the excitation – contraction coupling in the heart is the sarcolemmal membrane metabolic sensor complex. Its main part is the sarcolemmal ATP sensitive K^+ (K_{ATP}) channel acting as an alarm system to adjust cell electrical activity to the metabolic state of the cell (*107-109*). ATP closes the channel by interacting with its Kir6.2 subunit, but active membrane ATPases constantly reduce the local ATP concentration which is distinct from that in cytosol (*109,110*). It is the function of the sarcolemmal MM CK creatine kinase to rephosphorylate the local ADP and maintain the high ATP/ADP level in these microcompartments for coordination of membrane electrical activity with cellular metabolic status, notably with the phosphocreatine level. In this way, the phosphocreatine – creatine kinase network becomes the main intracellular regulatory pathway for cardiac cells, controlling electrical activity and cell excitability, calcium cycling, contraction and mitochondrial respiration. This energy transfer and control functions are shared by the whole hierarchical systems, including, besides the creatine kinase also the adenylate kinase and glycolytic systems, as it was seen in experiments with gene manipulation (*111,112*). The MM creatine kinase was first described in the purified rat heart sarcolemmal preparations already in 1977 (*23*). Later the CK was found to be physically associated with cardiac K_{ATP} channel in experiments with immunoprecipitation of guinea-pig cardiac membrane fraction with the antibodies against the K_{ATP} channel's subunit SUR2 (*110*). Abraham et al. (*109*) and Selivanov et al. (*111*) showed in experiments with permeabilization of isolated cardiomyocytes for open cell – attached patch formation that because of this sarcolemmal localization of the creatine kinase, the K_{ATP} channel's closed-open transitions are dependent upon the phosphocreatine concentration at ATP concentrations higher than threshold level for channel closure. In these experiments it was concluded also that there exist local strong restriction of ATP diffusion in the subsarcolemmal area bypassed by the creatine kinase flux in cardiac cells. This is in good concord with the results of studies by Sasaki et al. (*113*) showing that the activation of mitochondrial hydrolysis of the ATP by uncouplers activated also

the sarcolemmal K_{ATP} channels in dependence of the activity of the creatine kinase system, which was regulated by its inhibitor, 2,4 – dinitrofluorobenzene. Similar functional coupling of the creatine kinase with the K_{ATP} channel was described for pancreatic β-cells (*114*).

Functional coupling: A Maxwell demon principle

The phenomenon of functional coupling may be well illustrated by one of general philosophical concept in the history of physical sciences – by the concept of Maxwell's demon, which is very useful for understanding the general nature and principal importance of this type of mechanism of metabolic regulation.

The Maxwell's demon

In 1871 James Clerk Maxwell analyzed, in his book "Theory of Heat", the nature of the second law of thermodynamics and described the following imaginary situation. In the state of thermodynamic equilibrium all parameters of the system, such as temperature and pressure have constant values and no work is possible. This is due to the constant average value of the rate of movement of molecules, the constant average value of their kinetic energy. However, the average value is of the statistical nature due to large number of molecules, which have different rates distributed according to Boltzmann function. Maxwell proposed to consider the following situation: the homogenous system is divided into two parts separated by a small hole which can be closed or opened by an hypothetical being of intelligence but of molecular order. This hypothetical being, which was later nicknamed by William Thomson "a demon", permits the molecules with the rate higher than average one to pass the hole, but closes it for the molecules with the rate lower than the average one. In this way the "Maxwell demon" disturbs the equilibrium, creating the difference of the temperature between two parts of the system and making the work possible without any use of external energy supply. This imaginary experiment has immediately initiated vivid philosophical discussions up to our days and has been particularly useful in information theory, and it is often used for analysis of biological systems. And it is also very useful and refreshing to apply this concept for analysis of the mechanisms of the functioning of the creatine kinase systems (as well as of other kinases), and their role in regulation of respiration.

The equilibrium of the enzymatic reaction, in this case it is the creatine kinase reaction, means that all over the space of the cell cytoplasm the average concentrations of the substrates and products of the reaction and their ratios are constant, determined by the equilibrium constant value and thus the value of the standard free energy change, ΔG_0 of the reaction. The reaction equilibrium is always dynamic, that meaning that the direct and reverse reactions occur

with the same rate, but the net reaction rate is zero. This is the definition of the equilibrium. For simplicity, that may be taken to mean that any given enzyme molecule catalyses in average an equal number of the direct and reverse reactions in time unit in a random manner, in dependence on the frequencies of collisions with the substrate or product, and rate constants.

The multiple components of an enzymatic reaction system leave the Maxwell's demon much more choice of parameters to play with than it had in the classical situation of Maxwell's time. The most interesting and important game could be to look at each enzyme molecule and decide for the latter in which direction it will catalyse the reaction, simply by giving it a necessary substrate and removing at the same time the product from it. If the demon wishes, it can keep any given enzyme molecule always working in one direction and thus out of equilibrium. The other enzyme molecules can be kept working in the reverse direction, especially if there are other demons working at the same time, to keep the metabolic system in the overall permanent steady state. It is clear that this kind of action of the "demon" on the enzyme will be most effective if it stays always nearby the enzyme he wants to control, not to waste the time for looking for it. This principle of intelligence, the concept of Maxwell's demon is well realized in compartmentalized energy transfer pathways, such as creatine kinase systems with structurally fixed (bound) creatine kinase isoenzymes interacting with the adjacent ATP producing, transporting or consuming systems. The neighbouring systems which supply the substrate and remove the product in local microcompartment now fulfil the intelligent role of demons. It is important to note that the reverse is also true; the fixed kinases also play the demon's role for their neighbours. For regulation of mitochondrial respiration in many cells with high energy fluxes, this is the key mechanism.

The scheme in Figure 3 illustrates the analogy of the functional coupling between mitochondrial creatine kinase and ANT with the philosophical principle of Maxwell demon.

Thus, the intelligence of Maxwell's demon is realized in proper structural organization of the cellular systems. This helps the cell to obey the general principle of the life, and of bioenergetics in particular, formulated by Schroedinger in one of the most famous books in science, in his "What is life" (*115*). This is the law of permanent decrease of entropy (or production of negative entropy, "negentropy") in the living cells at the expense of the surrounding medium. By controlling the direct supply of substrate to the enzyme and removing the product, the intelligence of Maxwell's demon helps to avoid unnecessary increase of entropy. The molecular mechanisms of these "demonic" actions have been given the name of functional coupling, that meaning the metabolic channelling in some kind of microcompartment, or microdomain of molecular dimensions (*116*). Maxwell's demon principle is

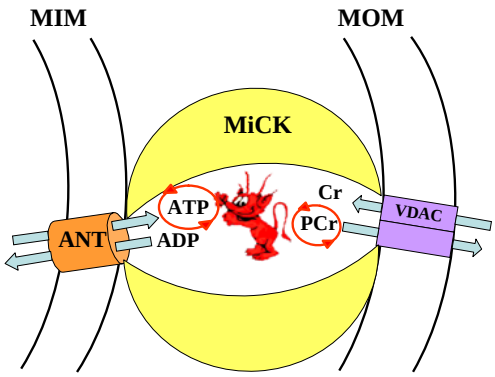


Figure 3. Illustration of the functional coupling between mitochondrial creatine kinase and ANT by the principle of Maxwell demon. The hypothetical being of molecular sizes (Maxwell demon) takes ATP molecules from ANT and puts them to the active site of the creatine kinase, from where it takes in exchange ADP and puts back to the ANT. This intelligence of Maxwell demon is realized by precise structural arrangement of MtCK and ANT (54). For further explanations see in the text.

also well represented by the sarcolemmal creatine kinase functionally closely coupled to the ATP sensitive and consuming systems of this membrane, the membrane metabolic sensors. Direct interactions within these coupled protein complexes and high local diffusion restrictions for ATP exclude the equilibrium mechanism of cellular creatine kinase this area. It even seems that the Maxwell's demon principle is a central one in governing the cardiac cell's energy metabolism and both electrical and contractile functional activities.

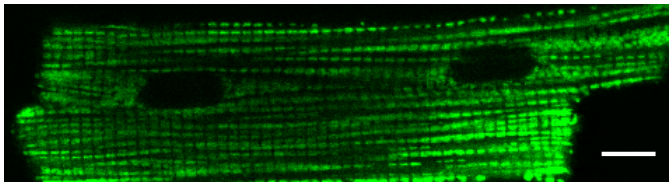
II. Cellular regulation of respiration

Heterogeneity of intracellular diffusion of ADP and feedback metabolic regulation in organized systems

The question of the equilibrium or non-equilibrium state of the creatine kinase systems in muscle cells is related to the much more general problem of cell biophysics: is the muscle cell an homogenous metabolic system, which can be described by simple kinetic and thermodynamic theories of homogenous solutions (*117,118*), or not? The equilibrium creatine kinase theory does not hesitate to state that it is the homogenous system, since it is itself based on the assumption of homogenous systems (*119*), for which the Maxwell demon is a stranger. However, the serious failure of this simple theory and its inconsistency with the experimental observations in the field of cardiac physiology and metabolism has been recently analysed in details (*2*). The importance and the mode of functioning of the creatine kinases are clearly

related to the specificity of the cell structure. It is enough to have just a short look on the electron or confocal micrographs of the myocytes to understand that the theory of the cell as homogenous solution is at least a naïve and a very rough approximation to the reality. Fig a. 4A shows the confocal imaging of mitochondria in isolated intact cardiomyocytes by using a fluorescent dye Mitotracker sensitive to the mitochondrial membrane potential. This imaging reveals a very regular mitochondrial arrangement of a crystal – like pattern in cardiomyocytes with permanent distances between neighbouring mitochondria

A.



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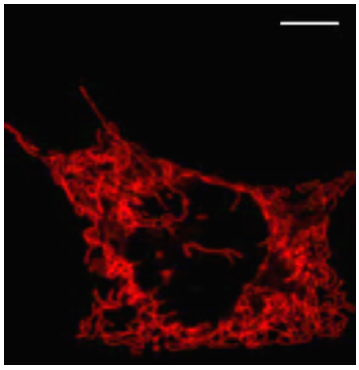


C. **D.** **E.**

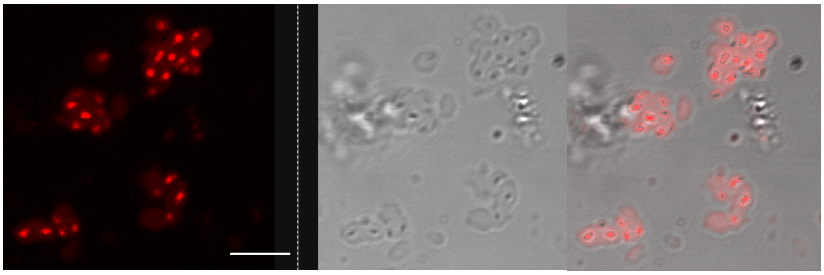
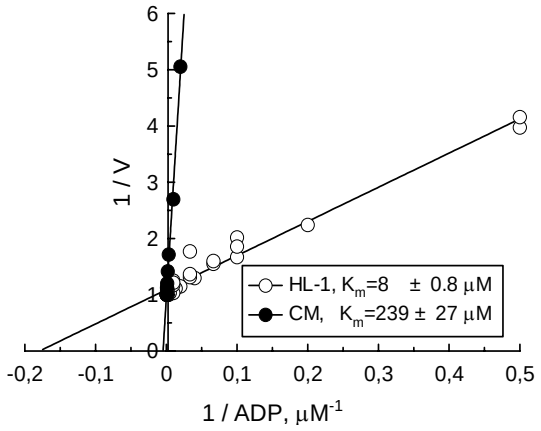


Figure 4. legend

A. Confocal image of mitochondria in isolated cardiomyocytes. Imaging of mitochondria using MitoTracker(R) Green CMXRos. Cardiomyocytes were incubated with MitoTracker(R) Green CMXRos 100 nM for 1 hour at 4°C in Flexiperm(R) chambers (from Vivascience, Hanau, Germany) in a solution with 5 mM glutamate, 2 mM malate and 2 mg/ml of bovin serum albumin. The fluorescence of this dye was measured (excitation and emission maxima at 516 nm and 490 nm, respectively). Note that mitochondria are regularly arranged in the cardiac cells. This conforms to the unitary nature of energy metabolism of cardiac cells according to the ICEUs concept. B. Mitochondrial arrangement and dynamics in nonbeating HL-1 cells were visualized by mitochondrial specific probe Mitotracker Deep Red (62.5 nM incubation 15 min at 37°C) in Tyrode medium. Cells were observed with a 63x (NA 0.9) water immersion objective. Excitation, 633 nm, emission 653-703 nm. A, B : Bars, 10 µm. C, D, E: Confocal fluorescent imaging of mitochondria in rat brain synaptosomes. The experimental protocol was as for cardiomyocytes (Fig.4 A). MitoTracker-Green, C; Transmission, D; Merge, E. Scale bar, 6 µm.

(*120*). Clearly, this highly organized and tightly packed system is very far from the homogenous solution. On the contrary, this is an excellent example of structural organization and the phenomenon of macromolecular crowding, the understanding of which needs new conceptual and experimental approaches (*116,121-123*). Fig. 4B shows the confocal image of another type of cardiac cells – nonbeating HL-1 cells (*124*). It is clear from the comparison of these two Figures that the mitochondrial arrangement in these cells is very different: it is very regular, crystal-like in adult cardiomyocytes, and chaotic in the HL-1 cells with filamentous dynamic mitochondria. Figure 4C-E show the abundant mitochondria in rat brain synaptosomes, with granular appearance in rather fixed position. Important information was obtained when regulation of mitochondrial respiration by added, exogenous ADP in permeabilized cardiac cells was studied. These studies clearly revealed the heterogeneity of the intracellular diffusion of adenine nucleotides (*125*). Fig. 5A shows in double reciprocal plots the kinetics of regulation of the respiration rate in permeabilized cardiomyocytes and in nonbeating HL-1 cells, and Figure 5B shows that for permeabilized rat brain synaptosomes. In the latter case, the kinetics was studied in the absence and in the presence of creatine (20 mM). Unusually high values of the apparent Km for exogenous ADP in permeabilized cardiac cells have been found in many laboratories since 1988 (*126-138*). Similar high values of this parameter were found in several other oxidative muscles (*132,143*), in hepatocytes (*139*), but not in fast skeletal muscle (*130,131,138*). Thus, this phenomenon is tissue specific; it certainly does not depend on the size of the cell and cannot be explained trivially by long diffusion distances due to the geometry of the fiber preparation (*132*). Indeed, Figure 5A shows that in the nonbeating HL-1 cells with no cellular

A.



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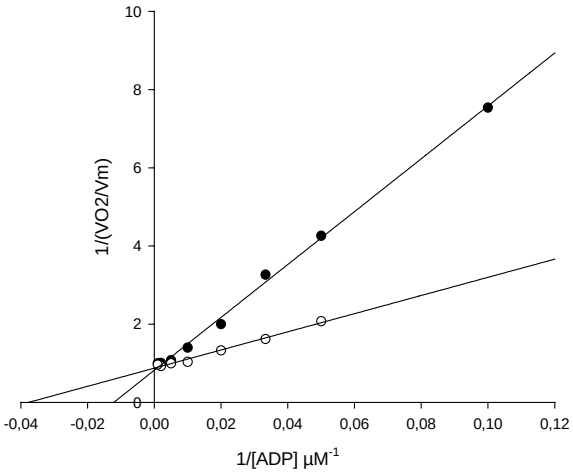


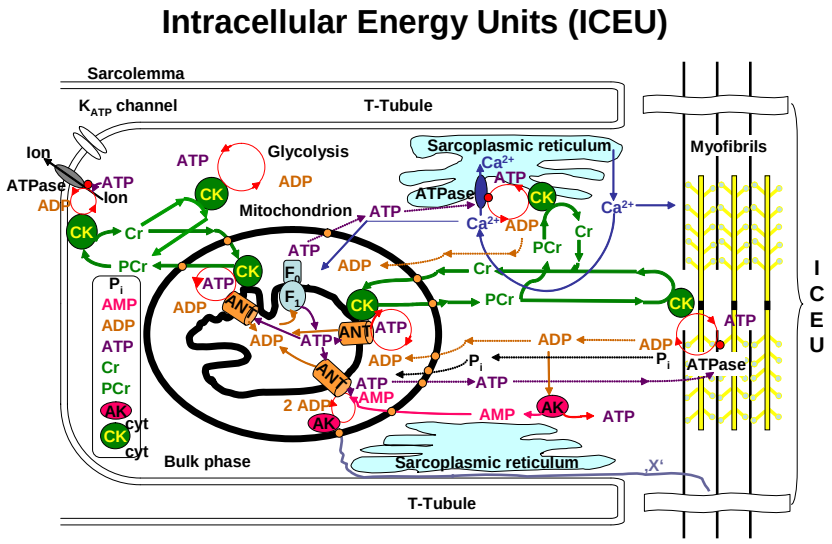
Figure 5. Kinetics of regulation of respiration by exogenous ADP in permeabilized cardiomyocytes and nonbeating HL-1 cells (A) and in permeabilized rat brain synaptosomes. ADP respiration rates were recorded using a two-channel high-resolution respirometer (Oroboros oxygraph-2k, Oroboros, Innsbruck, Austria), in respirometry medium Mitomed (see the legend to Fig. 2) with 5 mM Glutamate, 2 mM Malate, 2mg/mL bovine serum albumin. Cells were permeabilized by addition of 25 μg/ml of saponin, followed by incubation during 10 min at 25°C. A: Comparison of respiration kinetics as normalized respiration rates *versus* [ADP] in permeabilized adult

Figure 5. legend continued

cardiomyocytes (CM) and in permabilized HL-1 NB cells (HL-1) in double-reciprocal plots. The values of apparent Km for exogenous ADP are shown in box, they differ by factor of 30. For normalisation, the respiration rates were expressed as fractions of the maximal rates, Vmax, found by analysis of initial experimental data in double – reciprocal plots for both preparations. B: Double-reciprocal representation of the kinetics of regulation of respiration by exogenous ADP in permeabilized rat brain synaptosomes in absence or presence of 20 mM of creatine (Cr). The ordinate axis represents the normalized rate of respiration (Vm is the maximal rate of respiration). Permeabilisation was performed by 25 μg/ml of saponin. (●) absence of Cr, (o) presence of Cr 20 mM. The apparent value for exogenous ADP is decreased from 104 μM in absence of creatine to 28 μM in its presence due to activation of coupled uMtCK (see Fig. 2B and C).

organization of mitochondria, the value of the apparent Km for exogenous ADP is very low, as in isolated mitochondria. Thus, the mechanism of regulation of cellular respiration is clearly dependent on the cell structure. Interestingly, in permeabilized synaptosomes the value of this parameter, apparent Km for exogenous ADP, is intermediate between adult cardiomyocytes and HL-1 cells, and significantly decreased in the presence of creatine (Fig. 5B). The high values of apparent Km for exogenous ADP in permeabilized cardiac cells and direct channelling of ADP from endogenous ATPases to mitochondria is explained by the heterogeneity of ADP diffusion inside the cells, caused by contacts of mitochondria with cytoskeleton and other cellular systems, and thus, by intracellular organization (*125,140*). It has been concluded that mitochondria in oxidative muscle cells are included into the functional complexes with sarcomeres and sarcoplasmic reticulum, and all of them form together the intracellular energetic units, ICEUs (*141-143*). That means that all processes of energy metabolism may take place in a small space of the dimensions of several μm, a macrocompartment, the space occupied by ICEUs, and the energy metabolism of the cell is the result of synchronized functioning of these repeating metabolic units (Fig. 6A). And this is the space within the ICEUs where the mitochondrial respiration regulation mostly by the creatine kinase system takes place. In synaptosomes, there is also some organization of mitochondrial positions probably by the cytoskeletal proteins, and clear diffusion restrictions for ADP (ATP) which are overcome by the creatine kinase system (Fig. 6B). Studies of the effects of creatine on the mitochondrial endogenous ADP - dependent respiration in the presence of ADP – trapping system of PK + PEP supported both the conclusion of the central role of the mitochondrial creatine kinase in regulation of respiration, and importance of changes in outer mitochondrial membrane permeability for adenine nucleotides after treatment of fibers with trypsin. Fig.7B shows that

A.



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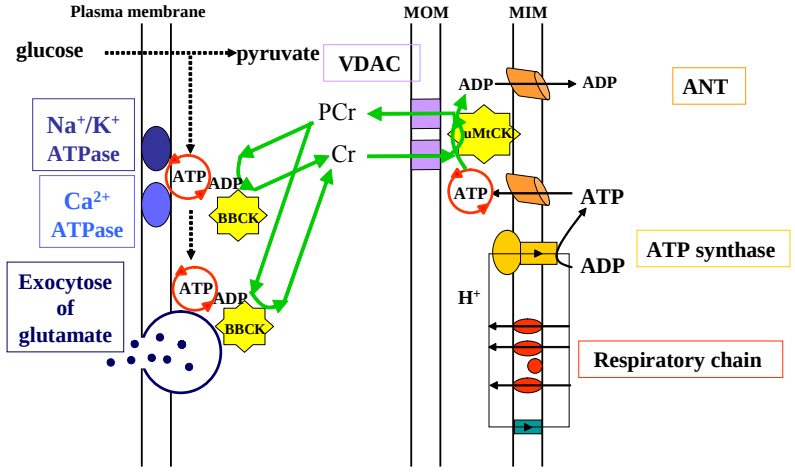


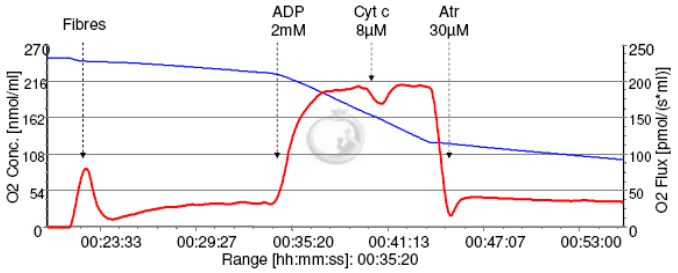
Figure 6. The Maxwell's demons in action: the coupled CK and AK reactions within the intracellular energetic units – ICEUs in muscle cells (A) and in the brain synaptosomes (B). (A) By interaction with cytoskeletal elements, the mitochondria and sarcoplasmic reticulum (SR) are precisely fixed with respect to the structure of

Figure 6. legend continued

sarcomere of myofibrils between two Z-lines and correspondingly between two T-tubules. Calcium is released from SR into the space in ICEU in the vicinity of mitochondria and sarcomeres to activate contraction and mitochondrial dehydrogenases. Adenine nucleotides within ICEU do not equilibrate rapidly with adenine nucleotides in the bulk water phase. The mitochondria, SR and MgATPase of myofibrils and ATP sensitive systems in sarcolemma are interconnected by metabolic channeling of reaction intermediates and energy transfer within ICEU by the creatine kinase – phosphocreatine and myokinase systems. The protein factors (still unknown and marked as "X"), most probably connected to cytoskeleton, fix the position of mitochondria and probably also controls the permeability of the VDAC channels for ADP and ATP. Adenine nucleotides within ICEU and bulk water phase may be connected by some more rapidly diffusing metabolites as Cr – PCr. Synchronization of functioning of ICEUs within the cell may occur by the same metabolites (for example, Pi or PCr) or/and synchronized release of calcium during excitation – contraction coupling process. This scheme is an artwork of Christian Linke, a student on Erasmus programme at Joseph Fourier University of Grenoble from Julius Maximilian University of Wurzburg, Germany. (B) In brain synaptosomes, mitochondrial coupled reactions are very close to those in heart mitochondria (A). The energy consuming reactions are related to the ion transport across the outer membrane and exocytose of glutamate (10) at the expense of ATP, produced locally by BB CK from PCr.

the addition of ATP in 2 mM concentration results in activation of respiration up to the level of 75 % of that seen with exogenous ADP (maximal State 3 activation, Fig. 7A). This activation of respiration is due to the endogenous ADP production which was not maximally activated in the absence of calcium. This respiration was decreased only to 25 % after addition of a very powerful ADP consuming system of phosphoenol pyruvate – pyruvate kinase. The activation of the MtCK reaction by stepwise addition of creatine up to 20 mM resulted in maximal activation of the respiration equal to that in the real State 3 (observed in experiments only in the presence of exogenous ADP in high concentration, Fig. 7A), in spite of the presence of the powerful ADP consuming PK-PEP system. That means that the local pools of ADP generated by the MtCK reaction near the ANT were completely protected from the PK-PEP system, in spite of some leaks of ADP into the intermembrane space (*144*), and the mtCK reaction exerted its central role in the control of respiration. Fig. 7C shows that this stimulating action of creatine on respiration is only slightly modified by the presence of phosphocreatine (20 mM), this meaning that this mechanism of respiration regulation is very effective under in vivo conditions in the cells. The effect of creatine was seen also after the treatment by trypsin, but in this case the maximal degree of activation was much lower than before trypsin treatment (125). Since the outer membrane was not broken by trypsin treatment (*125*), the explanation is the increase of the

A.



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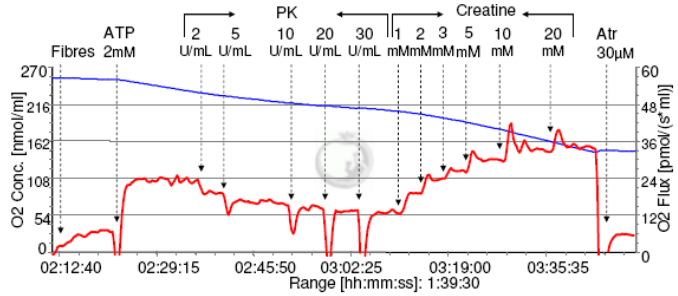

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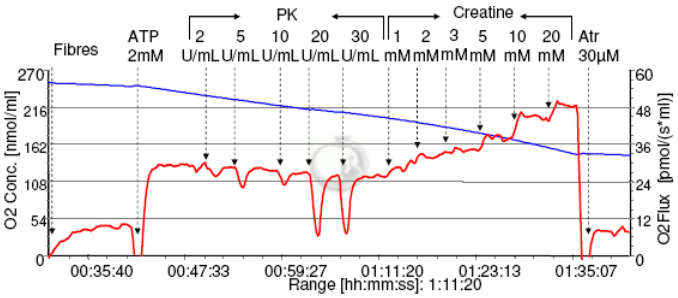


Figure 7. Effective regulation of the respiration in permeabilized cardiac fibers by creatine. ADP kinetic protocols and representative respiration traces recorded using a two-channel high-resolution respirometer (Oroboros oxygraph-2k, Oroboros, Innsbruck, Austria), in respirometry medium Mitomed (see the legend to Fig. 2) with 5 mM Glutamate, 2 mM Malate, 2mg/mL bovine serum albumin. Fibers were permeabilized by addition of 25 µg/ml of saponin, followed by incubation during

Figure 7. legend continued

10 min. A. Stability test and recording of the respiratory control ratio by ADP, 2 mM. At the end of the measure, cytochrome c (Cyt c, 8 μM) addition does not change the respiration, indicating that the outer membrane is intact. Atractyloside (Atr, 30 μM) results in a decrease in respiration back to Vo due to inhibition of adenine-dinucleotide translocase. The respiratory control index in this experiment was 6,2. B. The respiration was activated by addition of exogenous MgATP in the presence of 3 mM PEP, and then pyruvate kinase was added in increasing amounts up to 20 IU/ml. In the presence of this powerful PEP –PK system, stepwise addition of creatine maximally activated the respiration. **C.** The same as in B, in the presence of 20 mM phosphocreatine for simulation of the in vivo conditions in cardiac cells.

permeability of the outer mitochondrial membrane (VDAC channels) and the leak of some ADP from intermembrane space of mitochondria. This is confirmed by the results of the mathematical modeling (*125*).

The diffusion of ADP (and ATP) may be locally restricted inside the ICEUs at the level of mitochondrial outer membrane due to the interaction of some cytoskeletal proteins with the VDAC (*125*). Hypothetically, this interaction may involve the interaction of cytosolic (cytoskeletal) proteins with the unusually long loop of VDAC molecule facing the cytosol (*145*). Thus, these experiments show the importance of the mitochondrial outer membrane in strengthening the functional coupling between MtCK and ANT, originally proposed by Gellerich et al. (*41-45*) and shown in Fig. 1. As described above, functional coupling between ANT and MtCK involves direct transfer of ATP from ANT to MtCK, and the ADP produced may be channeled back to ANT or some part of it diffuse into the intermembrane space and leave mitochondria if the outer mitochondrial membrane permeability is high enough.

The impressive amount of fundamental work carried out by the laboratory of Be Wieringa in Niemingen, The Netherlands, on the genetic modification of the creatine and adenylate kinases has given us firm evidence of the importance of this system: the ‘knock-out’ of the creatine kinase and adenylate kinase genes results in very significant adaptive changes in the cells to compensate for the loss of this important system, causing structural remodeling of the cells (*146,147*). Thus, the specific demons can be replaced, but the Maxwell’s principle - not.

To explain how the feedback metabolic regulation of respiration by the CK system is achieved within this complex, precisely organized metabolic system as ICEUs under conditions of metabolic stability, and the description in details of this mechanism needs new quantitative methods of research – the mathematical modeling.

III. Mathematical models of metabolic regulation

The models of regulation of mitochondrial respiration

One of the first models of regulation of mitochondrial respiration was developed by Bohnensack (*148*). This model was developed further by Korzeniewski (*149*) and applied for analysis of the mitochondrial respiration regulation in the muscle cells. The author proposed that energy-producing and - consuming processes are activated in parallel leaving only the fine-tuning to the feedback mechanism. Using such parallel activation, Korzeniewski was able to reproduce asymmetry in transitions between low- and high-workload states, i.e. time-constants for transitions from low to high and from high to low workloads (*150*). In one of the versions of this model, the CK reaction in its equilibrium state was included (*151*). Neither realistic CK kinetics nor functional coupling between CK and ANT were considered (*150-152*). While the Korzeniewski's model of the respiratory chain is a reasonable one under limited conditions, no evidence has been found for his parallel activation theory. As it has been shown recently, the Ca^{2+} cannot pretend for the role of the parallel activator (*2*).

Much more complete and probably the best of the existing mathematical models of mitochondrial respiration was recently described by Cortassa et al. (*153*). This model includes both the thermokinetic description of the electron transfer in respiratory chain of mitochondria and the precise kinetics of the reactions of the Krebs cycle in the mitochondrial matrix, including the effects of calcium on the dehydrogenase of this cycle. The remarkable result of this modeling was that the authors reproduced the effects of the calcium on the respiration rate (*153*) in good accordance with the experimental data by Balaban group (*154,155*), and the effect of the calcium on respiration was again found to be not more than twofold, far from that needed to explain the classical observations by Starling, Visscher, Neely and Williamson (*156-159*), which show that the respiration rate of the heart may be increased by factor of 20 under physiological conditions of the Frank-Starling law (*2*). Cortassa et al. showed also that to model the red-ox changes in mitochondria in response to the work-jump (increased frequency of contraction) described by Brandes and Bers (*160-162*), the supply of ADP from cytoplasm was needed, in accordance with the theory of metabolic feedback regulation of respiration.

Modeling the feedback metabolic regulation of mitochondrial respiration

The mitochondrial respiration rate *in vivo* may vary 20 fold, from 8-10 μmol min^{-1} g^{-1} dry weight in resting (KCl-arrested) aerobic hearts to at least 170 μmol min^{-1} g^{-1} dry weight in rat hearts (*159*). As it has been shown by Neely et al. and Williamson et al. (*157-159*), the oxygen consumption of the heart muscle is linearly dependent on the heart workload under conditions when the heart is metabolically stable (*158,163*). The parallel activation of

energy-producing and -consuming processes is not needed to explain these observations in the heart muscle if CK compartmentation and the functional coupling mechanisms described above are taken into account. The model of compartmentalized energy transfer was initially developed by Aliev and Saks (*99*) and then adapted by Vendelin et al. (*125,164*).

Mathematical modeling of energy exchange in working cardiac cells was performed to gain some insight on fundamental questions of cell energetics still discussed up to time: 1) is the cellular CK reaction always in equilibrium state during the *in vivo* steady state contractions of heart muscle, and, therefore, can cellular ADP levels in the cytoplasm be predicted from measured cellular metabolite levels; 2) do *in vivo* mitochondria export energy by ATP, according to classical concept of Chance, or in the form of PCr, according to concept of CK shuttle by Jacobus and Saks; 3) do metabolite levels in the myoplasm oscillate during cardiac contractions *in vivo*; 4) how cell realizes its metabolic stability, when the manifold linearly interrelated increases in cardiac work and oxygen consumption take place at the practically constant metabolite levels and PCr/ATP ratio in the cells. Without an experimental method for direct monitoring of cellular ADP levels, the mathematical modeling of dynamic events in cell cytoplasm remains the only choice.

Description of a model

For these purposes we constructed a new class of dynamic mathematical models of intracellular compartmentalized energy transport in cardiac cells, which are based mainly on the principles of chemical kinetics and mass action law (*99,165*). These models consider the time dynamics of basic events of cell energetics (Fig. 8): ATP hydrolysis by actomyosin ATPase during contraction cycle, diffusional exchange of metabolites between myofibril and mitochondrial compartments, porine-restricted diffusion of ATP and ADP across mitochondrial outer membrane, the mitochondrial synthesis of ATP by ATP-synthase, ΔpH and $\Delta\Psi$ controlled Pi and ADP transport into mitochondrial matrix, PCr production in the coupled mitochondrial CK reaction and its utilization in cytoplasmic CK reaction. These events are considered in the system consisted by a myofibril with a radius of 1 μm, a mitochondria, and a thin layer of cytoplasm interposed between them (*99, 165*). The computations of diffusion and chemical events were performed for every 0.1-μm segment of chosen diffusion path at each 0.01 ms time step (*99*). This allows the simulation of the space-dependent changes throughout the entire cardiac cycle. This system with adjacent ADP producing systems in myofibrils and in sarcoplasmic reticulum is supposed to represent the basic pattern of organization of muscle cell energy metabolism, the "Intracellular Energetic Unit" (ICEU) of cardiac cell energetics (see above).

The mitochondrial block of the model is based on a simple chemical kinetic scheme of mitochondrial ATP synthase with parameters allowing the description of experimental ADP and Pi dependences of OP in isolated mitochondria (*99*). The basic experimental data were obtained in the laboratories of Wilson and Chance (*8,166*) (Fig. 9). Mitochondrial OP is activated by ADP and Pi produced from ATP hydrolysis by myosin in the myofibril compartment. The kinetics of ATP hydrolysis by myosin in contracting muscle was predicted from d*P*/d*t* change in isovolumic rat heart: a linear increase in ATP hydrolysis rate up to 30 ms, followed by its linear decrease to zero at the 60-th ms of contraction-relaxation cycle. The total length of this cycle was taken to be 180 ms (*99*).

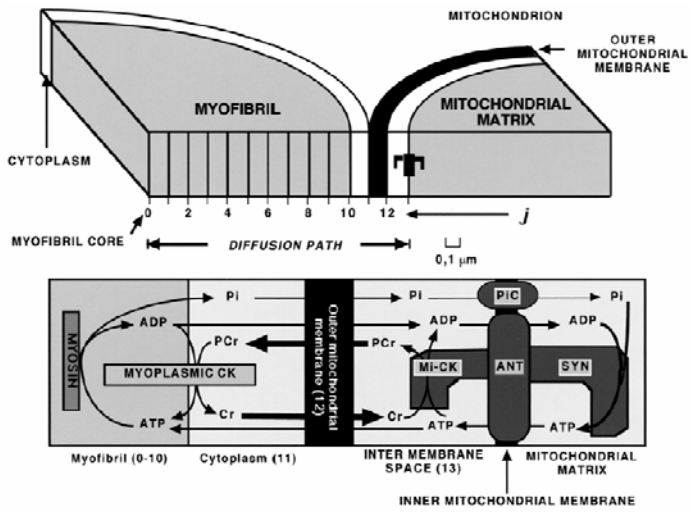


Figure 8. The general scheme of compartmentalized energy transfer in cardiac cell (Reproduced from (*165*) with permission). In model the diffusional exchange of ATP, ADP, PCr, Cr and Pi between myofibrils and mitochondria is considered along their radii and an interposed among them layer of cytoplasm. Diffusion path of 1.3 μm length includes ten 0.1 μm space units (j) in myofibril and 3 units in cytoplasm, mitochondrial outer membrane and intermembrane compartments. Lower part of Figure shows compartmentation of myoplasmic CK in myofibril and cytoplasm spaces, of mitochondrial CK (Mi–CK), adenine nucleotide translocase (ANT) and Pi Carrier (PiC) in mitochondrial intermembrane space and of mitochondrial ATP synthase (Syn) in mitochondrial matrix space. Myofibrillar myosin provides ATP hydrolysis during myofibrillar contraction. Mi–CK and ANT are proposed to be coupled by high local ATP concentration arising from restricted ATP diffusion in the narrow gap (microcompartment) between coupled molecules. Arrows indicate diffusion fluxes of metabolites in compartments and between them through mitochondrial outer membrane.

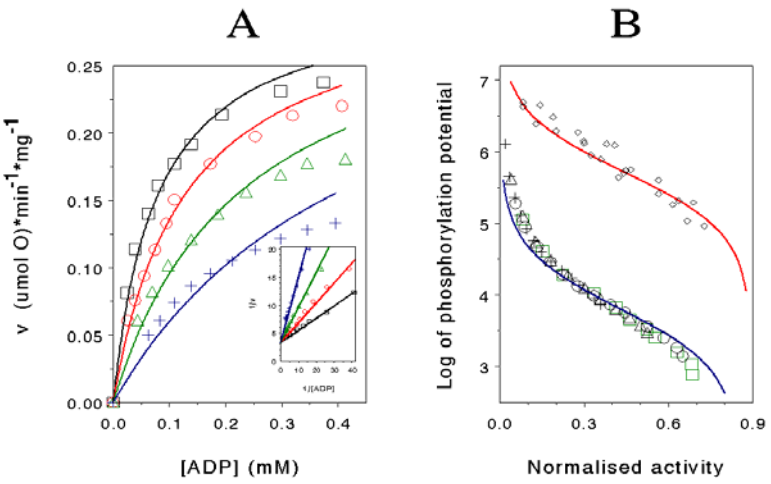


Figure 9. Experimental (symbols) and simulation (lines) data on kinetic and thermodynamic behavior of reconstituted in vitro systems of mitochondrial oxidative phosphorylation (Reproduced from (*99*) with permission). A. Double reciprocal plots of [ADP] dependence of oxygen consumption rates by heart mitochondria at constant initial [Pi], 4 mM, and varying fixed initial [ATP]: 1 mM (+), 2 mM (Δ), 4 mM (o) and 8 mM (□). *Experimental data* indicated by symbols were taken from Fig. 2B of Holian et al. (8). *Simulated data* are indicated by lines. B. Steady state values of extramitochondrial log phosphorylation ratio as a function of respiration (lower part, Holian et al. (*8*) or total ATPase (upper part, (*166*)) activities. Phosphorylation potential is defined as log ([ATP]/([ADP]*[Pi])). *Experimental* (symbols) and *simulated* (lines) *data* for lower part of drawing are those for Fig. 9A (data of Holian et al. (*8*)). *Experimental data* for upper part (◊) were taken from Fig. 3 of Gyulai et al. (*166*), *simulated data* are indicated by lines.

In mitochondria, the ATP/ADP translocase (ANT) and the Pi carrier regulate the matrix concentrations of ATP, ADP and Pi available for the ATP synthase activation. These carriers establish constant positive ADP and Pi gradients between the matrix and mitochondrial intermembrane space. In the latest version of the model (*165*) the ATP/ADP ratios in the matrix and activity of ATP synthase are dependent on ΔΨ, the electric component of mitochondrial membrane potential. The latest version of the model employs the complete mathematical model of the Pi carrier based on the probability approach, allowing to predict the dynamics of Pi accumulation in the matrix in exchange for matrix OH^- ions at the expense of mitochondrial proton-motive force, ΔpH (*165*).

The model of cellular events considers CK compartmentation (Fig. 8). The molecules of cytoplasmic isoenzyme of CK (MM-CK), 69% of total activity,

are freely distributed in the myofibrillar and cytoplasmic spaces. The intrinsic thermodynamic parameters of MM-CK favor its functioning in the reverse direction of CK reaction to transphosphorylate ADP to ATP at the expense of PCr utilization. A remaining part of cellular CK, mitochondrial isoenzyme of CK (Mi-CK), is localized in the mitochondrial compartment. In mitochondria, Mi-CK is tightly anchored to ANT and outer surface of inner mitochondrial membrane by cardiolipin molecules. The resulting close proximity of Mi-CK and ANT allows direct tunneling of adenine nucleotides between their adjacent active centers; this tunneling is the actual base for shifting the thermodynamic equilibrium of Mi-CK toward the synthesis of PCr from translocase-supplied ATP even at high levels of ATP in the myoplasm of in vivo heart cells.

Mathematical modeling of cellular CK shuttle was developed further by a special modeling of the kinetics of mitochondrial ANT by the probability approach and of functional coupling of translocase with Mi-CK (see below). In both versions of the model (*165,99*) functional coupling of Mi-CK to ANT was simulated by means of dynamically changing high local ATP concentrations in a 10-nm narrow space (microcompartment) between coupled molecules (Fig. 8). This simplified approach - coupling by local dynamic compartmentation - was used because of a large number of calculations in original probability model of coupling. The probability model was used to check the validity of calculations in a simplified approach (*99*).

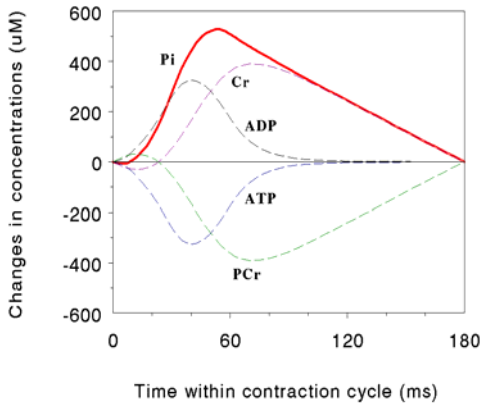
A distinctive feature of the given modeling is that we avoided, as much as possible, the formal description of chemical phenomena by adjustable mathematical terms. The living cell is a self-regulating chemical machine; therefore, the basing on the principles of chemical and enzyme kinetics decreases the probability of errors during mathematical modeling. The proper choice of maximum rates of enzyme activities, taken from *in vivo* and biochemical data (*99*), also serves for this goal. All details of modeling can be found in our publications (*165,99*).

Main results

Modeling revealed the oscillations of all metabolite levels in the cytoplasm (Fig. 10). Activation of contraction results in a small spike-like transient decrease in ATP and a symmetrical increase in ADP levels in the systole, 0-80^{th} ms, followed by deeper and longer changes in PCr (negative transient decrease) and Cr (positive transient decrease) concentrations. Pi concentrations change in a way similar to free Cr (*165,99*).

These changes are basic for respective changes in chemical reactions. At very high workload corresponding to the rate of oxygen consumption of 46 μ atoms of O * (g wet mass)$^{-1}$ * min^{-1}, the increase in myoplasmic ADP from diastolic basic level of 58 μM to a peak value of 383 μM is responsible for the

A.



B.

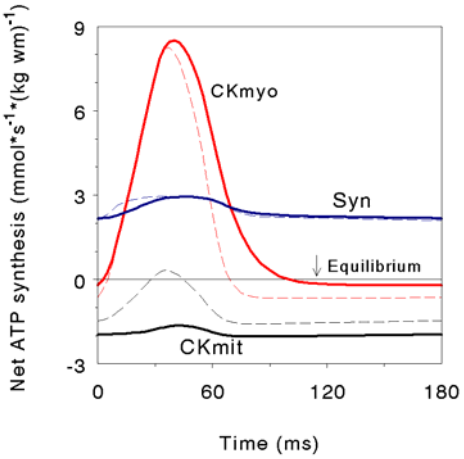


Figure 10. A.Phasic changes in metabolite concentrations in myofibril core during cardiac contraction cycle (Reproduced from Aliev and Saks (*99*) with permission). Modeling for the high workload corresponding to the rate of oxygen consumption of 46μ atoms of O*(g wm)$^{-1}$ *min^{-1}. B. Non-equilibrium behaviour of ATP-synthase (Syn), myofibrillar and mitochondrial CK (CKmyo and CKmit, respectively) in the systems with (solid lines) or without (dotted lines) restrictions for adenine nucleotide diffusion through mitochondrial outer membrane (Reproduced from Aliev a. Saks (*99*) with permission). An arrow indicates the position of equilibrium, when net ATP production is equal to zero.

activation of net ATP synthesis by MM-CK from -0.2 to 8.5 mmol ATP $*s^{-1}*(kg\ wm)^{-1}$ (*99*) with a mean value of 2.75 mmol ATP $*s^{-1}*(kg\ wm)^{-1}$ (Fig. 10 A).

Considered simulation data clearly indicate that myoplasmic MM-CK is sharply out of equilibrium in cyclically contracting cells. Non-equilibrium behavior of MM-CK is induced by cyclic increases in myoplasmic ADP levels during the systole of the cell. These oscillations cannot be completely damped even on artificial 10-fold increase in the activity of MM-CK (*97*).

In such a system, based on published experimental data, the regeneration of wasted myoplasmic PCr takes place mostly in mitochondria, as evidenced from permanent shift of mitochondrial Mi-CK towards the net PCr and ADP synthesis (*99*) (Fig. 10 B). The mean value of a steady state net PCr synthesis in mitochondrial compartment is 1.91 mmol PCr $*s^{-1}*(kg\ wm)^{-1}$. The sustained shift of Mi-CK towards the PCr synthesis results both from local coupling of Mi-CK to ANT and dynamic compartmentation, imposed by the restrictions of ADP diffusion through the mitochondrial outer membrane. The relative contribution of local coupling reaction into this phenomenon is about 54%: in the same system without restrictions for ADP diffusion (*99*) the mean value of net PCr synthesis by mitochondria falls to 1.03 mmol PCr $*s^{-1}*(kg\ wm)^{-1}$ (Fig.10 B). A shift of Mi-CK toward the PCr synthesis leads to prevailing, about 80%, export of total energy from mitochondria by PCr molecules (*99*) (Fig.11), the remaining minor part being due to direct activation of OP by ADP and Pi, according to classic mechanism of Chance. It must be noted, that the relatively small domination of local coupling effect in the considered phenomenon results from the decrease in efficiency of local coupling at high levels of MgATP, specific for heart cell myoplasm.

As a whole, in the system with compartmented CK the functional local coupling of CK to ANT leads to complete separation of functional roles of cellular CK: MM-CK becomes responsible for ATP regeneration in the myoplasm during the systole at the expense of PCr breakdown, while the regeneration of myoplasmic Cr to PCr takes place in mitochondria in the coupled Mi-CK reaction throughout the contraction-relaxation cycle (Fig. 11). Such a separation of functions is important for realization of metabolic stability of working heart (*165,167*).

Metabolic stability, i.e. practical constancy of PCr/ATP levels on the increase in workload, supposes respective constancy of myoplasmic mean Cr levels during manifold increase in workload. In the considered system with coupled Mi-CK, where OP activation is achieved by Pi and mostly by Cr, complete regeneration of increased bursts of Pi and Cr in steady state contractions can be realized only by an increase of the power of OP machine. Theoretically this increase can be realized in two ways: by an increase in the power of OP machine parallel to increase in the workload or by the general sustained increase in the power of OP machine.

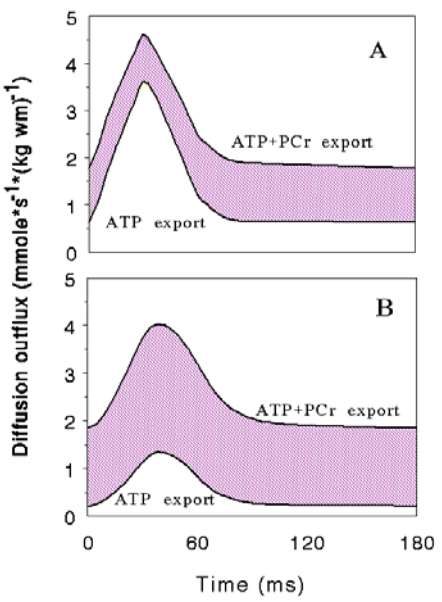


Figure 11. Diffusional ATP and PCr export through the mitochondrial outer membrane in the systems without (A) or with (B) restrictions for adenine nucleotide diffusion through mitochondrial outer membrane. Reproduced from Aliev a. Saks (*99*) with permission. The shaded area indicates the PCr outflux.

Recently we have analyzed two main sets of experimental data: the workload dependence of oxygen consumption and of metabolite profiles in rat hearts perfused by the media with glucose or with pyruvate (*165*). According to obtained experimental data, cellular PCr/ATP ratio decreases about 2-fold at high workloads in glucose-perfused hearts, but not in the pyruvate-perfused ones where this ratio was practically constant and high, about 2.3. Model well reproduced these data (*165*), showing that in pyruvate or lactate perfused hearts the increase in the workload takes place at practically constant diastolic levels of ADP and Cr in the myoplasm, in contrast to glucose perfused hearts (Fig. 12 A). In metabolically stabile hearts the increase in the workload is not followed by dramatic rise in the diastolic and systolic levels of Pi (Fig. 12B), as in glucose perfused hearts. Complete fitting of experimental data by the mathematical model allowed to predict the values of mitochondrial $\Delta\Psi$ in both sets of data, from -106 to -109 mV in glucose- perfused hearts, and from –150 to –166 mV in pyruvate-perfused ones (*165*). That is an exceptional power of mitochondrial OP machine, which allows complete damping of ADP and Pi birsts during the cardiac cycle at increased workloads.

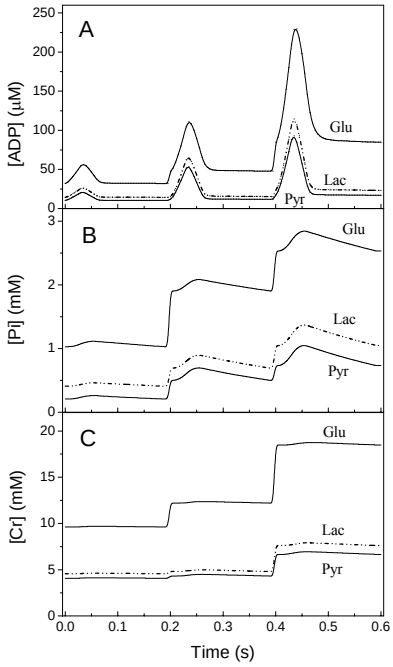


Figure 12. Simulated myoplasmic ADP (A), Pi (B) and Cr (C) dynamics at 3 workload levels: low (0-0.2 s), medium (0.2-0.4 s) and high (0.4-0.6 s). These workloads were induced by perfusate Ca2+ concentrations of 0.5, 1.25 and 3.5 mM, respectively, in media with Glucose (Glu), Lactate (Lac) or Pyruvate (Pyr) (*173*). Slightly modified from Aliev et al. (*62*) with permission.

The data of our modeling indicate that metabolic stability supposes the high power of mitochondrial OP machine. Activation of OP in these conditions is realized mainly by the cyclic transient increases in the Cr and Pi concentrations in the myoplasm and by additional activation of OP by coupled Mi-CK (*167*). The metabolic stability of perfused hearts at $\Delta\Psi$ = -160 mV was demonstrated also by Vendelin et al. (*164*) on the considerably modified clone of our model.

Modeling the functional coupling: Probability approach

The direct substrate channeling in the coupled enzymatic complexes, which certainly realises the function of "Maxwell's demon" in biological systems, may be quantitatively analysed on the basis of probability approach (*168-170*). This approach is most suitable in general for description of the substrate channeling between enzymes, and at the same time free of limitations

of the mass action law which requires the knowledge of the substrate concentrations, but which is not known for small volumes (microcompartments) within enzyme complexes (*171*). To illustrate this approach, it seems useful to consider the simplest system of two enzymes, Enz_1 and Enz_2, where product of first enzyme is the substrate for second one. The enzymes are structurally organized in pairs, Enz_1-Enz_2, in a manner ensuring 100% transfer of metabolite from Enz_1, the metabolite donor (D), to Enz_2, the metabolite recipient (R). If R receives substrate (S) only from D, it is obvious that in such unidirectional couple the extent of R activation will be equal to the extent of D activation from medium by the substrates of Enz_1. It is noteworthy that in such system significant activation of Enz_2 can take place without any substrate S in the medium, "Maxwell's demon" works with maximal efficiency!

The necessity of probability approach becomes evident if we allow in this simple system an activation of R also by the substrate S from medium. In this system the total activation of R is a sum of its mutually independent activations by the substrate from medium (free activation) and from D (local activation).

Let us assume the half-maximal, 50%, activation of Enz_1 (D); the concentration of substrate S in medium can be set equal to 1/3 of Km of Enz_2 for S. With these data, the fraction of free activation of R can be easily calculated according to modified Michaelis-Menten relation as 25% of the amount of R (S/(S+Km) = 0.33Km / (0.33Km + Km) = 100%*0.33/1.33). After the free activation of R, only 75% of the population of Enz_2 molecules remains accessible for local activation by D. The question is which part of this population of R molecules will be activated by the half-amount of population of activated molecules of D. Operationally we should find the probability of coincidence of activated molecules of D with free molecules of R.

According to Probability Theory, the probability of the event, arising from two independent partial probabilities, is the product of these partial probabilities. Dimensionless partial probabilities in our case can be expressed as an amount of the given enzyme form normalised to total number of enzyme molecules. Therefore, if the probability of activated forms of D ($\boldsymbol{P}^{D}$) is 0.5 and probability of free forms of R ($\boldsymbol{P}^{R}$) is 0.75, then the probability of local activation of R ($\boldsymbol{P}_{LocAct}$) is 0.375 (0.5*0.75):

$$\boldsymbol{P}_{LocAct} = \boldsymbol{P}^{D} \times \boldsymbol{P}^{R} \quad (1)$$

The probability of total activation of R ($\boldsymbol{P}^{R}{}_{TotAct}$) is the sum of its free ($\boldsymbol{P}_{FreeAct}$) and local ($\boldsymbol{P}_{LocAct}$) activations:

$$\boldsymbol{P}^{R}{}_{TotAct} = \boldsymbol{P}_{FreeAct} + \boldsymbol{P}_{LocAct} \quad (2)$$

In this sample $\boldsymbol{P}^{R}_{TotAct}$ amounts to 0.625 (0.25+0.375). Equation 1 clearly indicates, that extent of local coupling depends on the probability of metabolite accepting by recipient R: it is maximal when $\boldsymbol{P}^{R} = 1.0$ and zero when $\boldsymbol{P}^{R} = 0$.

Probability approach is the basic tool in analysing local coupling because the donor-recipient relations are common in molecular coupling phenomena. We have used this approach to model local coupling between mitochondrial CK and ANT (*168-170*). Model takes into account the following features of coupling based on its extensive experimental exploitation (*36*):

1. Bidirectional mode of coupling, i.e. both MtCK and ANT may be donors and recipients (Fig. 13). MtCK to ANT coupling in this respect differs from established couplings in rigid enzyme-enzyme complexes, which are mostly unidirectional (for review see (*171*)).
2. Local activations do not change the catalytic rate constants of participants.
3. Both participants of coupling can be activated by the substrates from medium, so the total activation of recipient is a sum of local and free activations, according to Equation 2.

Donors in considered system are two ternary kinetic forms of MtCK (CK.ATP.Cr and CK.ADP.PCr) and four ternary forms of ANT ($ANT.ATP_i.ADP_o$; $ANT.ADP_i.ATP_o$; $ANT.ATP_i.ATP_o$; $ANT.ADP_i.ADP_o$), finishing catalysis or transport cycle by the release of ATP or ADP into nana-gap between coupled molecular complexes. Main recipients are binary kinetic forms of MtCK (CK.Cr; CK.PCr) and ANT ($ANT.ATP_i$ and $ANT.ADP_i$), able to immediately produce catalysis or transport kinetic forms from tunneled adeninenucleotides. Locally supplied adeninenucleotides can not be accepted by the complexes with bound ATP or ADP on the binding site; unaccepted adeninenucleotides leave the coupling gap.

Basic in these calculations was assumption about closed conformation of catalytically effective complexes of MtCK reactions (CK.ATP.Cr and CK.ADP.PCr) formed from the ANT-supplied local adeninenucleotides. During catalysis these complexes, as well as transporting complexes of ANT, are not accessible for the exchanges with metabolites in the medium, so the local transfer phenomena are assumed to take place at tiny time intervals at the end of catalysis or transport cycles.

Model uses the partial probabilities to calculate the relative concentrations of locally activated kinetic forms of CK and ANT; the rate constants of mediated reactions are used to take into account the time persistence of the respective catalysis or translocation cycles. These calculations proceed until attaining steady state levels of all kinetic forms of MtCK and ANT created by local and free activations. Details of these calculations, employed basic assumptions, parameters of modeling and complete set of equations can be found in (*168-170*).

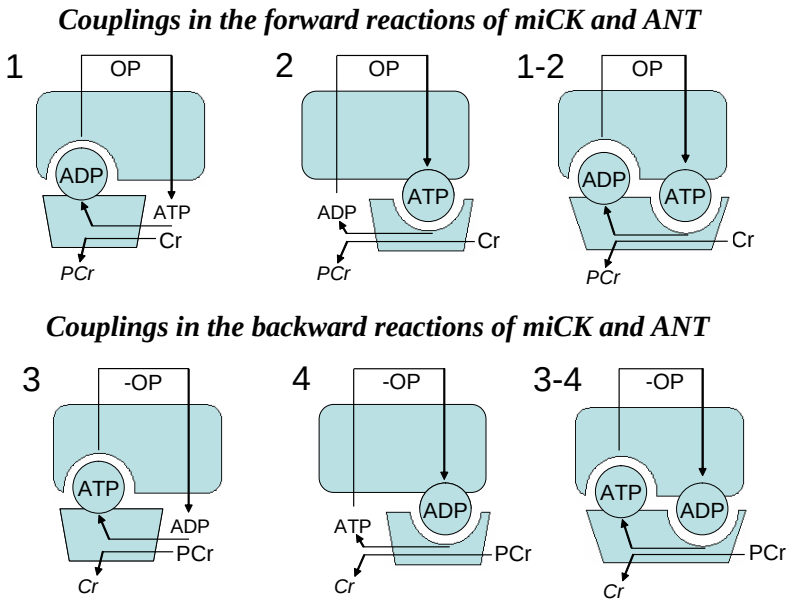


Figure 13. Schematic presentation of donor-acceptor relations in local coupling between mitochondrial CK (shaded rectangle) and ANT (shaded trapezium). Transferred adenine nucleotide is shown by a circle associated with the donor complex, nana-gap is shown by a notch in the acceptor complex, and arrows indicate the reactions of CK and mitochondrial OP. Couplings in the forward reactions of CK and ANT: 1 – local transfer of ADP from CK to ANT; in the sole unidirectional mode CK activates ANT being activated only by the substrates from medium; 2 – local transfer of ATP from ANT to CK; in the sole unidirectional mode ANT activates CK being activated by ADP from medium; 1-2– bidirectional local transfer of ADP and ATP in the forward reactions of CK and ANT; mutual activations of CK and ANT without interaction with the substrates in medium in the pure mode of local coupling. Couplings in the reverse reactions of CK and ANT (Schemes 3, 4, 3-4) are the mirror reflections of Schemes 1 to 1-2, ADP being substituted by ATP and vice versa, system accumulates Cr instead of ADP.

The probability model reproduces all experimental data in paper of Jacobus and Saks (*36*) and allows calculations at any ratios of local to free activations. Model well simulates (*169*) the experimental data by Saks et al. (*39*) on the respiration-induced shift of mitochondrial CK out of equilibrium. According to this phenomenon, in the presence of OP, when the mass action ratio of CK metabolites in the medium (Γ = ([MgADP]*[PCr])* ([MgATP]*[Cr])) favors the Cr production, rat heart mitochondria (*172*) and mitoplasts (*39*) produce the PCr at rather high rates. Persisting PCr production

is due to fact that CK to ANT coupling shifts the apparent equilibrium constant of CK reaction to the values higher than 'K_{eq} of free CK.

Simulations shown in Fig. 14 present this topic in a more general form, as a dependence of the equilibrium Γ-shift on the MgATP concentration in the medium with 30 mM [Cr+PCr]. The predicted values of the equilibrium Γ-shift are in the ranges of published experimental evaluations. For example, at 1 mM MgATP the predicted shift is 3.6-fold if the [Cr+PCr] level is 10 mM - at this experimental conditions Soboll et al. (*40*) determined the 3-5 change in this parameter. Data of Fig. 14 clearly show that value of the equilibrium Γ-shift from local coupling – the final measure of the thermodynamic profit of the work of "Maxwell's demon" - is not a fixed digit, it depends on experimental conditions. Careful analysis of basic data in simulations of Fig. 14 reveals that increase in the value of equilibrium Γ shift at low MgATP concentrations is due to increased proportion of locally activated CK forms in total CK activation. From the viewpoint of traditional kinetics it is evident that the decrease in the level of MgATP at constant high level of [Cr] leads to the

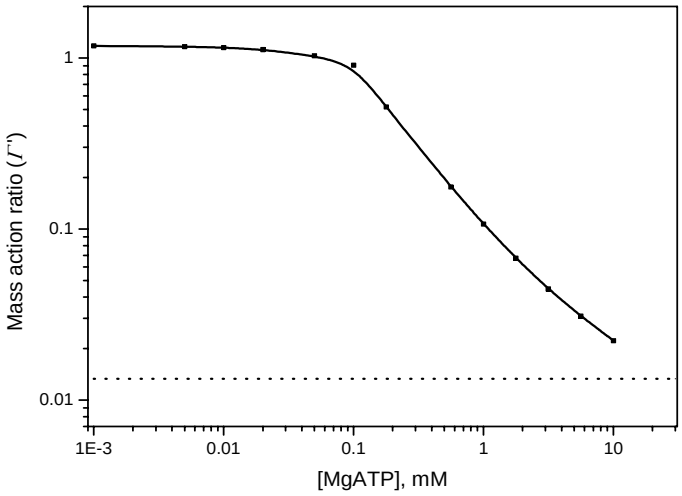


Figure 14. The MgATP-dependence of the shift in the equilibrium mass action ratio of CK metabolites in the medium, Γ', predicted by probability model. Activities of coupled CK were simulated for the medium, containing 30 mM of [Cr+PCr] and different amounts of total adenine nucleotide pool, AN_t. $[AN_t] = [MgATP] + [MgADP]$. Concentrations of MgADP were taken as $[AN_t]\times 0.01$ at $[MgATP]_{tot} < 0.11$ mM, and 1 μM at higher levels of $[AN_t]$. At each point of this graph we have changed the PCr/Cr ratio to attain zero net rate of Cr or PCr production by CK. ANT activation in these conditions do not exceeded 6.3% of its maximal rate.

increase in the proportion of receiving form of enzyme, CK.Cr, at the expense of a decrease in the content of non-receiving, CK-MgATP, form. An increase in the probability of receiving form of enzyme, $\boldsymbol{P}^{R}$, leads to higher probability of local activation, according to Eqn. 1.

The probability model well corresponds to experimental data. At the same time, the absence of metabolite dissociation from the locally formed E.Cr.ATP or E.PCr.ADP complexes of CK, postulated by the probability model, precludes the reconstruction of thermodynamic profiles of coupled CK reactions by the traditional approaches. The thermodynamic profiles of coupled CK reactions can be considered as a very important detailed thermodynamic report on the work of "Maxwell's demon".

Recently Vendelin et al. (*144*) attempted to fill this gap using the following set of main assumptions: the coupled CK-ANT complex with locally supplied ATP or ADP can exchange these adenine nucleotides with mitochondrial matrix through intrinsic exchanges of ANT; exchanges of locally supplied adenine nucleotides with mitochondrial intermembrane space through CK are completely prohibited; the coupled CK-ANT complex freely equilibrates with Cr and PCr in medium; model allows reversible catalytic CK.Cr.ATP to CK.PCr.ADP transformations in such coupled complexes. Model considers the local activations separately from free ones.

Formally model of Vendelin et al. (*144*) allows exchanges of tunneled metabolites with medium. But supposed ANT-mediated exchanges of locally supplied adeninenucleotides are too slow to be considered as the equivalents of free diffusional exchange and equilibration with medium. Problem was solved by the 10-fold increase in translocation rate constants of ANT. With these basic assumptions Vendelin et al. (*144*) managed to predict the thermodynamic profiles of coupled CK/ANT reactions and to simulate the different sets of experimental data. While the reliability of basic assumptions in models of Aliev and Saks (*168-170*) and Vendelin et al. (*144*) remains to be proven in direct experiments, it seems important to stress that both models use the probability approach in basic calculations of local coupling.

Probability approach can be used also in the modeling the kinetics of mitochondrial carriers. It is known that the majority of mitochondrial carriers function according to simultaneous (sequential) mechanism of transport (*173*). This kinetic mechanism supposes that the carrier performs the rate-limiting translocation step only when having the simultaneously bound metabolites on both its binding sites, with outer site (subscript "$_o$") directed to mitochondrial intermembrane space and inner site (subscript "$_i$") directed to matrix space of mitochondria. If we assume the independent probabilities of binding of metabolite "x" to outer site of carrier "C", $\boldsymbol{P}(C.x_o)$ and of metabolite "y" to its inner site $\boldsymbol{P}(C.y_i)$, then the probability of transmembrane counter transport of

metabolite x_o for metabolite y_i, $\boldsymbol{P}(C.x_o.y_i)$, will be, according to probability approach:

$$\boldsymbol{P}(C.x_o.y_i) = \boldsymbol{P}(C.x_o) * \boldsymbol{P}(C.y_i) \quad (3)$$

Partial probabilities of binding of any metabolite (metabolites) to inner and outer binding sites of carrier can be easily calculated using ordinary equations of steady-state kinetics; we used probability approach to model the kinetics of mitochondrial adenine nucleotide translocase (*168-170*) and mitochondrial Pi carrier (*165*).

The probability approach gives the reliable results even in this simplest form because it considers the main feature of mitochondrial carriers - simultaneous occupation of both binding sites. Really the kinetic schemes may be more complicated as the binding of metabolite on one site of carrier can change the affinity of the opposing one. Examples of the solution of such complex probability based kinetic schemes and discussion on encountered problems can be found in Aliev and Saks (*62*) and Metelkin et al. (*174*).

Commentaries for conclusion

As we have seen above, the Maxwell's demon has a real role to play in cellular energetics. The intelligence of this principle is realized in the functional coupling mechanisms, and the coupled creatine kinase isoenzymes are probably the best examples (at least best studied) among the many others to illustrate how this works. The other phenomena explained by this principle are the compartmentation of cyclic AMP in the cells, the metabolism of calcium organized into microdomains (calcium sparks) according to this principle etc. The mechanism of the functional coupling of the MtCK with ANT explains the most important phenomenon in the cardiac physiology and energy metabolism: the linear dependence of the rate of oxygen consumption on the workload regulated by the Frank-Starling mechanism under conditions of the metabolic stability (2). Also, the coupled creatine kinases at the cellular and subcellular membranes represent the membrane sensor mechanisms connecting the ion fluxes to the cellular energy state.

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Article 1

3
Integrated and Organized Cellular Energetic Systems: Theories of Cell Energetics, Compartmentation, and Metabolic Channeling

Valdur Saks, Claire Monge, Tiia Anmann, and Petras Dzeja

The essential thing in metabolism is that the organism succeeds in freeing itself from all entropy it cannot help producing while alive.

Erwin Schrödinger, *What Is Life?* (1944)

Abstract

Bioenergetics as a part of biophysical chemistry and biophysics is a quantitative science that is based on several fundamental theories. The definition of energy itself is given by the first law of thermodynamics, and application of the second law of thermodynamics shows that living cells can function as open systems only where the internal order (low entropy state) is maintained due to increasing the entropy in the surrounding medium (Schrödinger's principle of negentropy). For this, the exchange of mass is needed, which gives rise to metabolism as the sum of catabolism and anabolism, and the free energy changes during catabolic reactions supply energy for all cellular work – osmotic, mechanical, and biochemical. The free energy changes during metabolic reactions obey the rules of chemical thermodynamics, which deals with Gibbs free energy of chemical reactions and with electrochemical potentials. For application of these theories to the integrated systems *in vivo*, complex cellular organization should be accounted for: macromolecular crowding; metabolic channeling and functional coupling mechanisms due to close and tight protein–protein interactions; compartmentation of the enzymes due to their attachment to the subcellular membranes or connection to the cytoskeleton; and both macro- and microcompartmentation of substrates and metabolites in the cells. Because of this, almost all processes important for cell life are localized within small areas of the cell, and for their integration effective systems of communication, including compartmentalized energy transfer systems, are required. These are represented in muscle cells by the phosphotransfer networks, mostly by the creatine kinase and adenylate kinase systems, whose alterations under ischemic conditions contribute significantly to acute ischemic contractile failure of the heart.

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3.1 Introduction

Living cells belong to the group of open thermodynamic systems because they exchange both energy and mass with their environment [1–6]. This is necessary to maintain cell structure and function and to avoid decay into equilibrium, which would lead to death. This thermodynamic principle as a physical basis of life was first discovered and described some 60 years ago by Erwin Schrödinger in his famous book *What Is Life?* [7]. Mass and energy exchange are the functions of metabolism [7], including both catabolism and anabolism (see below), and these constitute a lawful way for cells to live in a thermodynamic sense. At the same time, metabolism is the basis of biological energy transformations in the cells, as any chemical or biochemical reaction is inevitably linked to free energy changes according to the laws of chemical thermodynamics. In the first part of this chapter, we describe briefly and in a general manner these theoretical bases of free energy conversion and metabolism. In the second part, we analyze the energy transfer networks in muscle and brain cells. Because of structural organization and macromolecular crowding, almost all processes important for cell life are localized within small areas of the cell, and for their integration effective systems of communication, including compartmentalized energy transfer systems, are required. These are represented in muscle cells by the phosphotransfer networks, mostly by the creatine kinase and adenylate kinase systems, whose alterations under ischemic conditions contribute significantly to acute ischemic contractile failure of the heart.

3.2 Theoretical Basis of Cellular Metabolism and Bioenergetics

3.2.1 Thermodynamic Laws, Energy Metabolism, and Cellular Organization

The first law of thermodynamics, formulated by Mayer in 1842, is the law of energy conservation for isolated systems. It is also useful for defining of energy itself as the capacity to perform any kind of work and/or to produce heat. This law says that a change in the internal energy of a system (dE)

$$dE = E_f - E_0 = \delta q + \delta w \tag{1}$$

is the result of heat exchanged and taken up by system δq and work done by the surrounding medium on the system δw (E_f and E_0 are the final and initial energy levels, respectively). By convention, if the system performs work, the sign of δw is negative.

The second law of thermodynamics describes a direction in which all processes can occur spontaneously. It tells us that irreversible processes move in the direc-

tion of the increase of entropy. The entropy function S was introduced by Clausius as $dS = \delta q/T$ (for reversible processes) to describe the Carnot cycle, and Boltzmann gave its statistical explanation in terms of molecular kinetic theory:

$$S = k_B \log W \tag{2}$$

Here, k_B is Boltzmann's constant and W is the thermodynamic probability function associated with the number of possible arrangements of molecules (each arrangement is called a ''microstate'') in the given ''macrostate'' of a system [1, 4]. Thus, the less organized a system is, the larger the number of possible arrangements of molecules in it (or number of ''microstates'') and the higher the entropy of the system will be. The second law of thermodynamics tells us that under irreversible conditions all processes in all systems move toward a state of a greater probability or greater disorder.

The two thermodynamic laws taken together describe the relationship between changes in the functions of the thermodynamic state of a system – Gibbs free energy G (which is the capacity to do useful work), enthalpy H (which is the maximal amount of exchangeable heat a system can produce), and entropy S (related to the amount of heat that cannot be used to perform useful work but related to the order exhibited by the system):

$$dG = dH - TdS = dE + pdV - TdS \leq 0 \tag{3}$$

According to these laws, all irreversible processes can proceed spontaneously only when the free energy decreases in the direction of equilibrium, at which it reaches a minimum and does not change anymore ($dG = 0$). The decrease in free energy in any spontaneous process is due mainly to an increase in entropy. If the system is under non-equilibrium steady-state conditions, the free energy is continuously dissipated (see below) [1, 2, 4–6].

Thus, the universal laws of thermodynamics described by Eq. (3) allow all processes and reactions to proceed only in the direction in which the free energy decreases and entropy increases and thus in which disorder increases. This seems to be in contradiction with what is known about cell life. In cells, biochemical catalysis needs protein catalysts with precise conformational structure; transmission of genetic information needs the permanence of the structure of DNA and RNA; and metabolic pathways are organized into supramolecular complexes, metabolons, etc. Schrödinger showed that the apparent contradiction is overcome if we consider that living cells are open systems that, by means of metabolism, decrease or maintain their low entropy state by increasing the entropy of the medium [7]. This is shown by the general scheme in Fig. 3.1 describing the energetics of the cell as an open system exchanging both energy and masses with surrounding medium. Catabolic reactions through coupling to anabolic reactions (biosynthesis) maintain cell structure and organization as an expression of the decrease in entropy. They are also the source of metabolic energy for the performance of any kind of cellular work.

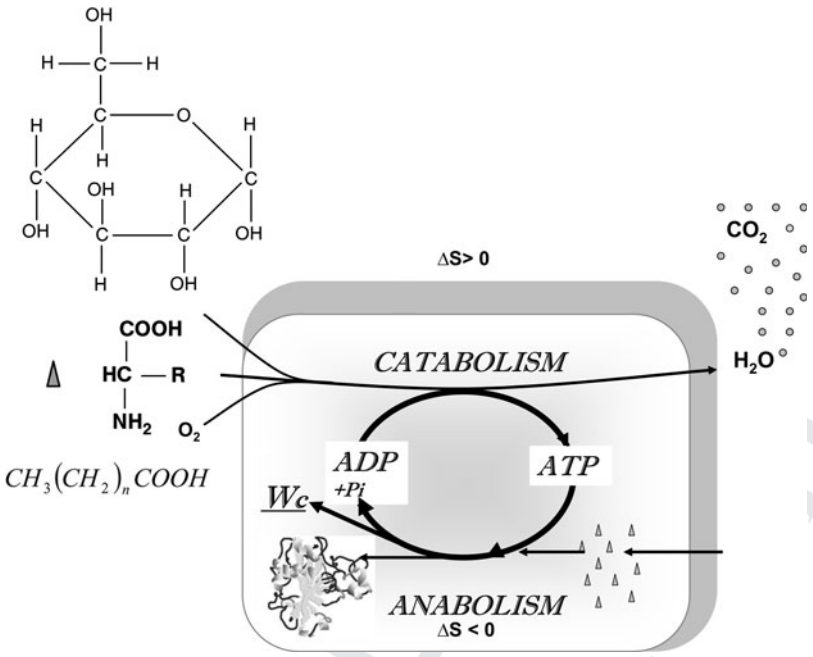


Fig. 3.1 General scheme of cellular metabolism. A cell is a thermodynamically open system. In accordance with Schrödinger's principle of negentropy, extraction (increasing the entropy in the extracellular medium and decreasing it in the cell) via metabolism is necessary for maintenance of the structural organization of both biopolymers as proteins, DNA and RNA, and for maintenance of the fine structural organization of the cell for effectively running compartmentalized metabolic processes. In this way, the cell can live in agreement with thermodynamic laws (see the text).

3.2.2
Chemical and Electrochemical Potentials: Energy of Transmembrane Transport and Metabolic Reactions

In general terms, the capacity to do useful work, quantified by the Gibbs free energy, is a function of the pressure p, temperature T, and chemical composition [1], and its full differential can be written as:

$$dG = \left(\frac{\partial G}{\partial p}\right)_{T,n_i} \cdot dp + \left(\frac{\partial G}{\partial T}\right)_{p,n_i} \cdot dT + \sum_i \left(\frac{\partial G}{\partial n_i}\right)_{p,T} \cdot dn_i \qquad (4)$$

where $\dfrac{\partial G}{\partial n_i} = \mu_i$ is a chemical potential of a component i.

Usually biochemical reactions occur at constant temperature and pressure, and thus $dP = 0$ and $dT = 0$. Under these conditions, the free energy changes are

caused by changes in the chemical composition, i.e., the free energy changes in the system are directly related to the chemical or biochemical reactions [1, 2]:

$$dG = \sum_i \left(\frac{\partial G}{\partial n_i}\right)_{T,p} \cdot dn_i = \sum_i \mu_i \cdot dn_i \tag{5}$$

or in the integral form:

$$G = \sum_i \mu_i \cdot n_i \tag{6}$$

The first derivative of the Gibbs free energy with respect to the molar concentration of component n_i, its chemical potential $\mu_i = (\partial G/\partial n_i)_{T,p}$ (see Eq. 4), is a function of the molar concentration C_i of component n_i. For species bearing electric charges z (cations or anions) in the presence of electrical potential Ψ, it becomes an electrochemical potential [1–3]:

$$\tilde{\mu}_i = \mu_i^0 + RT \ln C_i + zF\Psi \tag{7}$$

$F = 0.0965$ kJ mol^{-1} mV^{-1} is the Faraday constant [3], and $\mu^0{}_i$ is a standard chemical potential, corresponding to the standard reference state of the system, in which the concentration is $C^0 = 1$ M. To remind one that all concentrations should be given in reference to this state, the equation can be rewritten as:

$$\tilde{\mu}_i = \mu_i^0 + RT \ln \frac{C_i}{C^0} + zF\Psi \tag{8}$$

Equations (8) and (9) are the basic equations of bioenergetics and describe the energy conversion in biophysical or biochemical processes [1–6]. Thus, according to Mitchell's chemiosmotic theory (see ▮Chapter 2▮), the protons translocated by the respiratory chain (see ▮Chapter 2▮) result in a transmembrane gradient of H^+ or ΔpH and other ions (e.g., K^+, Na^+, Ca^{2+}) indirectly coupled to the H^+ gradient or $\Delta\Psi$, which together represent an electrochemical potential of ions or protonmotive force:

$$\Delta p = \frac{\Delta\mu_{H^+}}{F} = \Delta\Psi - \frac{2.3RT}{F} \cdot \Delta pH \tag{9}$$

The free energy changes in the metabolic reactions are in a similar way explained by changes of chemical potentials of reaction components, substrates and products. Thus, free energy change in the reaction of the conversion of substrate A into product P,

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} P$$

is given by the difference of chemical potentials of P and A:

$$\Delta G = \mu_P - \mu_A = \left(\mu_P^0 + RT \ln \frac{[P]}{C^0}\right) - \left(\mu_A^0 + RT \ln \frac{[A]}{C^0}\right)$$

$$= (\mu_P^0 - \mu_A^0) + RT \ln \frac{[P]}{[A]} \tag{10}$$

where $\Delta G^0 = (\mu_P^0 - \mu_A^0)$ is the standard free energy change and $\Gamma = \frac{[P]}{[A]}$ is the mass action ratio of the reaction (for multi-reactant reactions, this ratio is defined as $\Gamma = \frac{\prod_i [P]_i}{\prod_i [A]_i}$), and therefore:

$$\Delta G = \Delta G^0 + RT \ln \Gamma \tag{11}$$

At equilibrium,

$$\Delta G = \Delta G^0 + RT \ln \Gamma = 0 \tag{12}$$

and Γ corresponds to the equilibrium constant K_{eq}:

$$\Gamma_{eq} = K_{eq} = \frac{[P]_{eq}}{[A]_{eq}} = \frac{k_1}{k_{-1}} = e^{-(\mu_P^0 - \mu_A^0)/RT} \tag{13}$$

That is typically written as

$$\Delta G^0 = -RT \ln \mathrm{K}_{eq} \tag{14}$$

This is the famous equation relating the equilibrium constant to standard free energy change, first described by van't Hoff. This gives the final equation for the free energy change in any reaction:

$$\Delta G = -RT \ln K_{eq} + RT \ln \Gamma \tag{15}$$

This equation can be rearranged in the following way:

$$\Delta G = RT \ln \frac{\Gamma}{\mathrm{K}_{eq}} \tag{16}$$

Figure 3.2A shows the plot of the free energy G of a system calculated on the basis of Eq. (6) as functions of log Γ/K_{eq} (the ratio Γ/K_{eq} is shown in logarithmic scale), and Fig. 3.2B shows the corresponding ΔG. In Fig. 3.2, one can see a minimum of free energy G in the equilibrium position, where $\Delta G = 0$ [8]. If the reaction is shifted to the right in the direction of an increase in Γ/K_{eq}, free energy

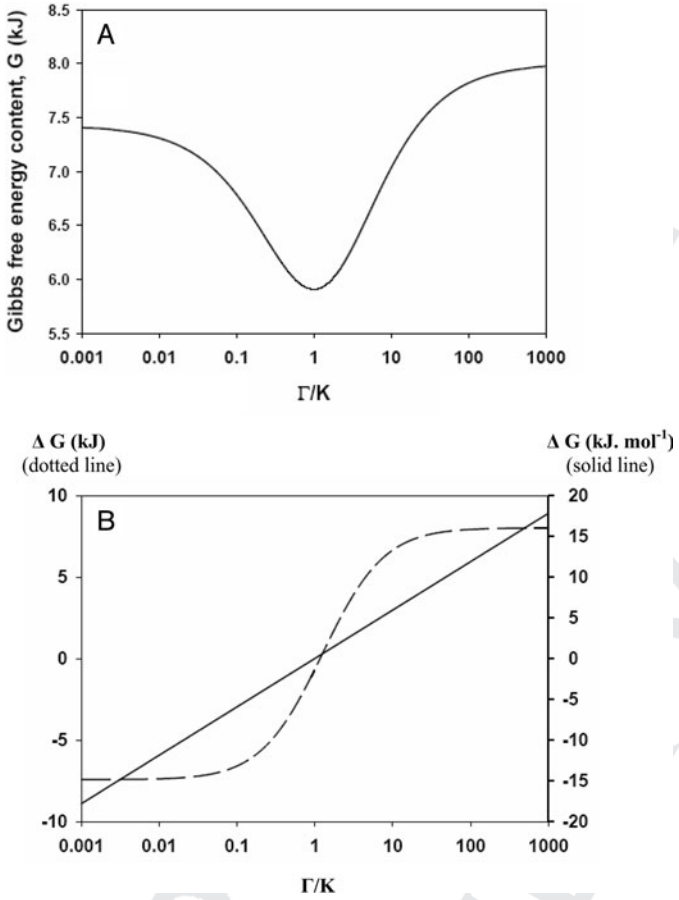


Fig. 3.2 (A) The free energy of a system as a function of the log Γ/K_{eq} (Γ/K_{eq} on the abscissa axis is given in the logarithmic scale). *A* and *P* indicate the free energies of pure compounds *A* and *P*, respectively. Γ is shown for the direction of conversion of *A* into *P*. Free energy of the system was calculated according to Eq. (6): $G = n_A\mu_A + n_P\mu_P$. (B) ΔG as a function of log Γ/K for two cases. The solid line shows ΔG calculated by using Eq. (16) and expressed in kilojoules per mole. This line shows the amount of the free energy, which could be obtained (in exergonic reactions) or used (in endergonic reactions) for conversion of one mole of substrate under given conditions. The dotted line shows the ΔG available in the "real" system according to our calculations, taking into account an amount of substrate present, $\Delta G = n_P\mu_P - n_A\mu_A$, in kilojoules. The calculations were made by Marc Jamin (Joseph Fourier University, France) using the following arbitrary conditions: $\mu_A^\circ = 7.4$ kJ mol^{-1}, $\mu_P^\circ = 8$ kJ mol^{-1}, $K = 0.8$, $[A]_0 = 1$ M, $[P]_0 = 0$ M. (The figure is adapted from [8] and is courtesy of Marc Jamin).

and ΔG increase (Fig. 3.2). Free energy of a system is also higher left of the equilibrium position of the reaction (Fig. 3.2A), but in this case ΔG is negative (Fig. 3.2B). This means that left of equilibrium, the reaction in the direction $A \rightarrow P$ is exergonic (spontaneous) and right of equilibrium it is endergonic (non-spontaneous). The opposite is true for the reaction $P \rightarrow A$, which is endergonic left of and exergonic right of equilibrium. Fig. 3.2B shows two ways of expression of ΔG: the solid line shows this parameter in kilojoules per mole of a substrate, which is a linear function of $\log \Gamma/K$ in accordance with Eq. (16). This is the free energy change during conversion of one mole of substrate into product under given conditions (given value of Γ). The dotted line shows ΔG available in the actual system by taking into account the number of moles present in the system, $\Delta G = n_P\mu_P - n_A\mu_A$, where n_A and n_P are the number of moles of A and P, respectively. This latter value of ΔG is directly related to ''real'' changes in G shown in Fig. 3.2A.

These relationships mean that any biochemical reaction in the cell that is away from equilibrium is a potential source of free energy.

3.2.3 Non-equilibrium, Steady-state Conditions

As has recently been shown by Qian and Beard, in the case of biochemical networks in living systems, when the metabolic processes are in the steady state far from equilibrium, Eq. (16) can be modified to include fluxes or reaction rates [5, 6]. For processes that are in the non-equilibrium steady state, for example, the reaction

$$\rightarrow A \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} P \rightarrow$$

the forward and backward fluxes are $J_+ = k_1[A]$, $J_- = k_{-1}[P]$, respectively, and by definition $J = J_+ - J_- \neq 0$ (only in equilibrium does $J = 0$). The equilibrium constant is $K_{eq} = k_1/k_{-1}$, and by replacing K_{eq} in Eq. (16) with this expression, for chemical potential differences in steady state of this reaction in the system, we obtain

$$\Delta\mu = RT \ln \frac{J_-}{J_+} \tag{17}$$

This represents the chemical driving force under steady-state conditions. In an open system, Eq. (17) also shows the amount of energy to be applied to the system in order to maintain the reactions in the steady state (constant concentrations of A and P), and in the absence of work done this is the energy dissipated as heat and transformed into entropy of the surroundings [5, 6]. This is explained by non-equilibrium thermodynamics [2, 9–11], according to which for open systems the entropy may be split into two contributing parts [9, 10]:

$$dS = d_iS + d_eS \tag{18}$$

where d_iS is the entropy production from irreversible catabolic processes inside the system, which always increases, and d_eS is the entropy flow from the surroundings, whose contribution is null in an isolated system since entropy always increases irreversibly in such a system. However, in systems open to the exchange of matter and energy, such as biological systems, d_eS may be negative, so that the system may decrease its entropy because statistical fluctuations may favor the organized state. The stabilization of an organized state after statistical fluctuations or instabilities (order through fluctuations) may occur because of the existence of nonlinear kinetic laws. These states are maintained by energy and matter dissipation; therefore, they are also known as *dissipative structures* [9]. This clarifies the answer given by Schrödinger to the question: How do living organisms avoid decay? Or, otherwise stated, how do they avoid contradicting the second law of thermodynamics? The basic answer given by Schrödinger is that organisms do not disobey the second law because as first formulated it applies to closed rather than open systems; more explicitly, although a living organism continually increases entropy (positive entropy or d_iS) and tends to approach the dangerous state of maximum entropy (or death), it keeps away from it by continually drawing negative entropy (d_eS) from its environment [7], or, more rigorously, free energy to keep its organization (see [11] for an excellent discussion).

3.2.4 Free Energy Changes and the Problem of Intracellular Organization of Metabolism

Coupling of catabolism with anabolism (the metabolism) is the way through which ''negentropy'' or free energy is extracted from the medium. Evolution has selected the adenine nucleotides to fulfill this important task of coupling catabolism and anabolism (Fig. 3.1). A possible explanation is the rather high standard free energy change ($\Delta G^o = 31.5$ kJ mol^{-1}) and high affinity of ATP and ADP for many enzymes and carriers [12]. Cellular energetics is thus based on the reactions of ATP synthesis and utilization: $ADP + P_i \Leftrightarrow ATP + H_2O$. Taking water content as a constant and in excess (not changing in the reaction), the mass action ratio of the reaction of ATP synthesis is usually written as:

$$\Gamma = \frac{[ATP]}{[ADP][P_i]} \tag{19}$$

By maintaining a high mass action ratio for the reaction of ATP synthesis, catabolic reactions also supply free energy for cellular work. The free energy available in the cellular system is, according to Eqs. (14)–(16), a function of the ratio of Γ to the equilibrium constant of the ATP synthesis, or:

$$\Delta G_{ATP} = \Delta G^0_{ATP} + RT \ln \frac{[ATP]}{[ADP][P_i]} \tag{20}$$

This function is usually called the phosphorylation potential [13, 14]. The principal purpose of free energy transformation associated with catabolic reactions is to

maintain the phosphorylation potential at a high value, which is achieved mostly through mitochondrial oxidative phosphorylation or photosynthesis in autotrophic organisms. The theory of phosphorylation potential for the analysis of cellular life was first used by Veech et al. [13] and Kammermeier et al. [14].

However, now it has become clear that applying Eq. (20) as well as any quantitative theory of physical chemistry to the real intracellular medium is not a simple task, because of the complex organization of cell structure and metabolism. It has become clear that it is not the global ATP content that is important; rather, the ATP and the free energy available in micro- and macrocompartments have to be accounted for, as will be described below.

3.2.5 Macromolecular Crowding, Heterogeneity of Diffusion, Compartmentation, and Vectorial Metabolism

The first phenomenon to be taken into account in all cells is macromolecular crowding: the high concentrations of macromolecules in the cells [15–22] decrease the volume available for free diffusion of substrates and accordingly make it difficult to use correctly the enzyme kinetics and equations usually worked out in enzymatic studies carried out in diluted solutions [20, 22, 23]. Cytoplasmic protein concentration may be as high as 200–300 mg mL^{-1}, which corresponds to a volume fraction of about 20–30% of intracellular medium occupied by these proteins [15, 16]. In the mitochondria the high density of enzymes and other proteins constitutes more than 60% of the matrix volume [17].

At first sight, this macromolecular crowding should cause real chaos by making intracellular communication by diffusion of reaction intermediates difficult. This is similar to the situation that occurs in any megalopolis at rush hour if the traffic lights are turned off. This chaos and the related problems are well described by Denis Noble in his recent book [24]. In reality, however, macromolecular crowding gives rise to new mechanisms based on specific protein–protein interactions – microcompartmentation, metabolic channeling, and functional coupling – and in this way leads to a fine organization and regulation of the metabolism. The traffic lights are on, and movements are perfectly organized. The origin of this order as well as the program and source of these specific interactions and cellular organizations are still a mystery hidden in undiscovered genetic laws, including feedback control of gene expression, and in rules governing system-level properties of cellular systems [24], and their elucidation is the main challenge for cell sciences in the post-genomic era [24–27].

3.2.5.1 Heterogeneity of Intracellular Diffusion and Metabolic Channeling

Many new experimental techniques have been developed to study the molecular networks formed by protein–protein interactions [28]. In the cells, the high protein density predominantly determines the major characteristics of the cellular environment, such as diffusion in heterogeneous compartments [23, 29–31]. There are distinct barriers to diffusion of solutes within the cells, i.e., binding

and crowding. Whereas molecular crowding and sieving restrict the mobility of very large solutes, binding can severely restrict the mobility of smaller solutes (see Ref. [30] and references therein). This also explains the heterogeneity of the diffusion behavior of ATP in cells. Studies using pulsed-gradient ^{31}P-NMR showed that the diffusion of ATP and phosphocreatine is anisotropic in muscle cells [31, 32]. Recent mathematical modeling of the decreased affinity of mitochondria for exogenous ADP *in situ* in permeabilized cardiac cells also showed that ADP or ATP diffusion in cells is heterogeneous and that the apparent diffusion coefficient for ADP (and ATP) may be locally decreased (diffusion locally restricted) by an order, or even several orders, of magnitude [33]. A similar limited diffusion of ATP in the subsarcolemmal area in cardiac cells was proposed by Terzic and Dzeja's group [34, 35]. There is also firm experimental evidence for compartmentalization of ATP in cardiomyocytes (see next section).

Because of molecular crowding and hindered diffusion, cells need to compartmentalize metabolic pathways in order to overcome diffusive barriers. Biochemical reactions can successfully proceed and even be facilitated by metabolic channeling of intermediates due to structural organization of enzyme systems into organized multi-enzyme complexes. Metabolite channeling directly transfers the intermediate from one enzyme to an adjacent enzyme without the need of free aqueous-phase diffusion [15, 18, 36–38]. This property was suggested to be a unique catalytic behavior of enzyme complexes due to their specific structural organization [37]. Gaertner [37] reported that "physically associated multienzyme systems (enzyme clusters) have the potential of expressing unique catalytic properties in contrast to their non-associated counterparts." It is quite clear that the enhanced probability for intermediates to be transferred from one active site to the other by sequential enzymes requires stable or transient interactions between the relevant enzymes. Enzymes are able to associate physically in non-dissociable, static multi-enzyme complexes, which are not random associations but an assembly of sequentially related enzymes.

3.2.5.2 Compartmentation Phenomenon and Vectorial Metabolism

The principal mechanisms of organization of cell metabolism are macro- and microcompartmentation, metabolic channeling, and functional coupling. By definition, the term compartmentation is usually related to the existence of intracellular *macrocompartments* – subcellular regions that are large relative to the molecular dimension – and *microcompartments* that are on the order of the size of metabolites. A compartment is a "subcellular region of biochemical reactions kinetically isolated from the rest of cellular processes" [39]. Macrocompartments are easy to understand, and they can be visualized by electron or confocal microscopy. They are compartments inside the organelles, such as mitochondria or lysosomes, that are insulated from the cytoplasm by membranes. The concept of microcompartmentation is more mysterious, because these microcompartments are not usually visible by electron microscopy. However, now it is becoming clear that microcompartments related to multi-enzyme complexes and metabolic channeling, as described above, are the principal basis of organization and compartmentation of

cellular metabolism. They are formed by specific protein–protein interactions within multi-enzyme complexes that are due to macromolecular crowding, anchoring of glycolytic [40–43] and other enzymes to the cytoskeleton, or membrane channels and transporters [43–48]. Investigations carried out in Clegg and Deutscher's laboratories have shown that mammalian cells behave as highly organized macromolecular assemblies dependent on the cytoskeleton [47, 48]. Multi-enzyme complexes may be of different size and may even include whole metabolic pathways; these are then called metabolons, according to the terminology introduced by Paul Srere in 1985 [49]. Thus, there are glycolytic metabolons [41, 45], Krebs cycle metabolons [50], and others [51, 52] in the cytoplasm. New techniques such as FRET have been developed to study and visualize microcompartmentation, e.g., the study of microcompartmentation of cyclic AMP [53, 54]. Microcompartmentation is sometimes taken to be synonymous with metabolic channeling [16, 39]. However, metabolic channeling of the reaction intermediate between two enzymes (or a transporter and an enzyme) may occur via microcompartment or by direct transfer [55, 56]. In both cases, it results in functional coupling (see below). Importantly, microcompartmentation may be of a dynamic nature, and this may result in the coexistence of a whole set of organized metabolic networks [55].

Interestingly enough, there is an exciting hypothesis that these phenomena, in particular metabolic channeling, are even older than life itself and are related to its origin. Edwards and others (see [57] for review) have put forward the hypothesis that on prebiotic Earth, when no enzyme or metabolic complexes were initially present, archetypal catalytic complexes were formed at the mineral surface (e.g., iron sulfide minerals) and biomolecules and catalysts were formed at specific sites relative to these complexes [57]. The evolution of metabolic pathways in this case would have been dictated by the relative positions of substrates and catalysts, where only closely juxtaposed species would have been allowed to interact [57]. Thus, the cell as a ''bag of enzymes'' probably never existed. It is now clear that living cells are much more complicated and better organized than previously thought [58, 59].

In the case of metabolic channeling of species via microcompartments with only a small number of molecules, the validity of introducing chemical potentials is questionable [6], whereas for macrocompartments and larger microdomains, this classical theory remains useful. In the first case, application of thermodynamic activities instead of concentrations [18–20] and stochastic kinetics based on probabilities of different states of enzymes are essential [60, 61].

An important consequence of the organization of enzymes into multi-enzyme complexes is vectorial metabolism and ligand conduction, a general principle proposed by Peter Mitchell after extensive enzymological studies and detailed characterization of mitochondrial proteins. One important example is the chemiosmotic coupling of energetic processes through the ''protonic current'' [62, 63]. It brought together ''transport and metabolism into one and the same chemiosmotic molecular level – biochemical process catalyzed by group-conducting or conformationally mobile group-translocating enzyme system'' [63]. In his re-

views, Mitchell encouraged the wider use of the chemiosmotic principle and the biochemical concept of specific ligand conduction to explain the organization and operation of metabolic and transport processes within the cell [63]. Today, this idea is receiving increased attention and is certainly another important insight by Mitchell into the understanding of cellular energy conversion processes.

3.3
Compartmentalized Energy Transfer and Metabolic Sensing

3.3.1
Compartmentation of Adenine Nucleotides in Cardiac Cells

The application to cells of Eq. (20) in its general form is complicated by the compartmentation of ATP and adenine nucleotides making the use of the easily measurable total ATP content very questionable and practically useless. For cardiac cells, macrocompartmentation of adenine nucleotides was demonstrated in Ruth Altschuld's laboratory by using a controlled permeabilization technique [64].

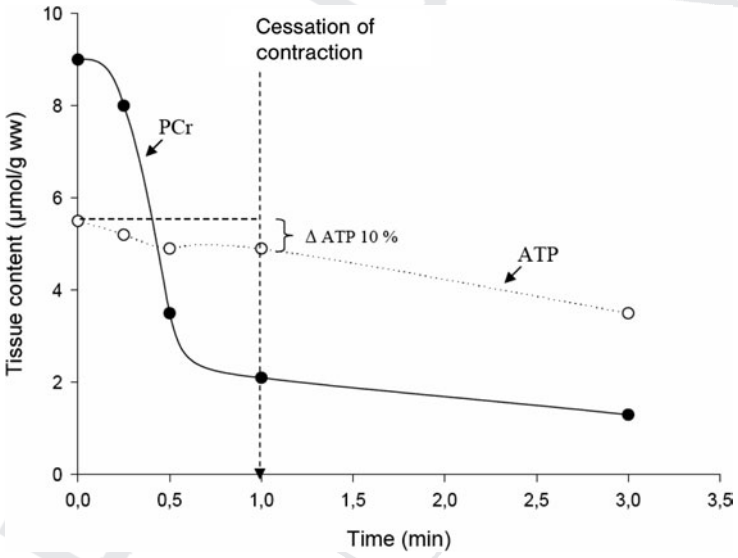


Fig. 3.3 Origin of the problem of the compartmentalization of adenine nucleotides and metabolic energy sensing in cardiac cells. The data show metabolic changes in totally ischemic dog hearts [65]. Within 1 min, PCr content falls by 80% and contraction stops, but 90% of cellular ATP is still intact. The general problems to explain are why the contraction stops when most of the ATP is not used up, and which mechanism allows sensing of the decrease in PCr level as the main source of cellular energy. For explanation, see the text and Fig. 3.5. (Data are redrawn from [65]; only the mean values of metabolites are shown).

They found that 74% of total nucleotides are localized in the cytoplasm, about 20% in mitochondria, and the remaining 6% in cellular structures. A similar distribution was found in other cells [64]. Before this, however, important data on the possible compartmentation of ATP were obtained in studies of the metabolism of ischemic heart by Gudbjarnason [65] and Neely [66]: they always observed very rapid decreases in contractile force in parallel with a decrease in PCr concentration (exactly as Lundsgaard detected in skeletal muscle; see the Introduction to this volume) and a complete interruption of contraction in the presence of about 80–90% intact ATP (see Fig. 3.3). These basic, classical observations show that there is a very precise energy-sensing mechanism present in the cells, responding

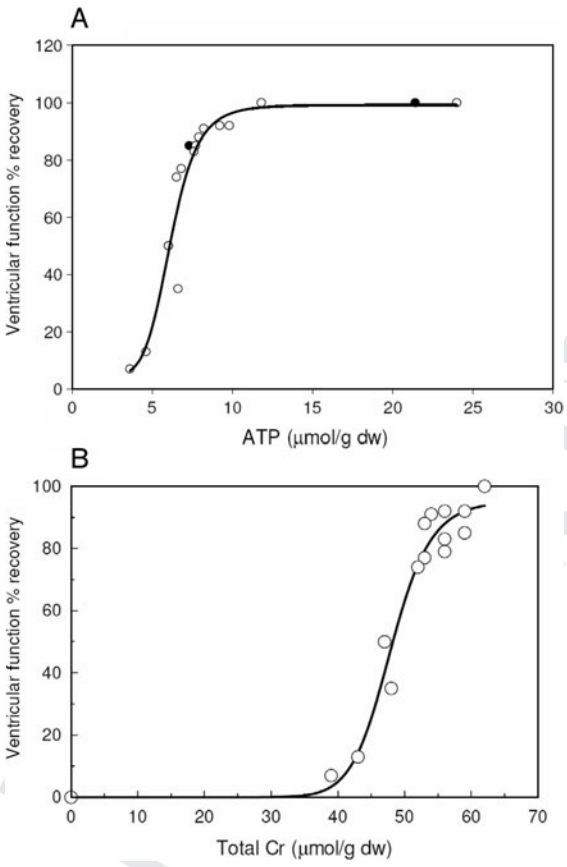


Fig. 3.4 (A) Absence of any correlation between total ATP content in the cells and contractile function of heart muscle. (Data are redrawn from [69–71]). ATP was changed by periods of hypoxic perfusion followed by reperfusion with normal solutions [69] (open circles) or by treatment of the hearts with 2-deoxyglucose [70, 71]. (B) Permanence of the total creatine in the experiments shown in Fig. 3.4A [69–70].

mostly to changes in PCr content (this mechanism will be discussed below). The interpretation of these experiments posed the following problem: Why does the heart stop contracting in the absence of oxygen supply so quickly if there is still plenty of ATP in the cells? Many of the explanations proposed – such as pH changes, changes in the sensitivity of troponin to calcium, etc. – are not sufficient because these changes occur at longer time intervals [67, 68]. The explanation proposed by Gudbjarnason highlights the importance of ATP compartmentation, only about 10% of which is in the functionally important pool(s) [65]. While the experiments described above showed almost constant ATP during a decrease in contraction force, further experiments with perfusion of heart during different hypoxic periods or perfusion with 2-deoxyglucose (a phosphate trap that decreases ATP content via adenylate kinase and 5′-nucleotidase reactions) [69–71] showed that the total content of ATP can be decreased by 70% (corresponding to the amount present in cytoplasm) without a significant effect on contraction (Fig. 3.4A), provided that total creatine content is not changed (Fig. 3.4B). Thus, the total content of ATP is dissociated from contractile force, which seems to depend only upon a fraction (not more than 20–30%) of ATP. At the same time, inactivation of the creatine kinase system by inhibition of creatine kinase or replacing creatine with less active analogues significantly decreases the maximal work capacity of the muscle [72, 73]. ATP compartmentation was confirmed in multiple indirect experiments performed on ischemic hearts, showing the importance of so-called glycolytic ATP, and in studies of energy supply to membrane ion pumps [74–77]. Compartmentation of ATP has been demonstrated recently by imaging techniques in several cell types [78].

Thirty-five years ago, the experiments described above initiated very intensive investigations that led to the discovery and description of phosphotransfer networks and metabolic signaling connecting different functional pools of ATP. Now, we have an answer to the question raised by Gudbjarnason and Neely concerning the complex phenomenon of ischemic heart failure. The answer is described below in this and other chapters of this book.

3.3.2
Unitary Organization of Energy Metabolism and Compartmentalized Energy Transfer in Cardiac Cells

Cardiac cells present a highly organized structure in which mitochondria are localized at the A-band level within the sarcomere limits [79–82]. Intermyofibrillar mitochondria are arranged in a highly ordered crystal-like pattern in a muscle-specific manner with relatively small deviations in the distances separating neighboring mitochondria [81, 82]. Contrary to many other cells with less developed intracellular structure, dynamic changes in mitochondrial position due to their fission and fusion [83–85] are not found in adult and healthy cardiac and skeletal muscle cells because of their rigid intracellular structural organization, and the mitochondria in these cells are morphologically heterogeneous (see ▌Chapter 5▐). In this structurally organized medium, energy transfer between different

subcellular micro- and macrocompartments (called compartmentalized energy transfer) is of central importance. The existence of these rather complicated networks of energy transfer and signaling is a direct consequence of the compartmentalization of adenine nucleotides in the cells [33–35, 62, 65, 71, 78, 86]. This is due to the significant heterogeneity and local restrictions in the diffusion of adenine nucleotides in cells and to the necessity of rapid removal of ADP from the vicinity of Mg-ATPases to avoid their inhibition by the accumulating product MgADP. As is described in many chapters of this book, not only is ATP delivered by diffusion but also intracellular energy transfer is facilitated via networks consisting of phosphoryl group–transferring enzymes such as creatine kinase (CK), adenylate kinase (AK), and glycolytic phosphoryl-transferring enzymes [39, 87–105]. Most important among them is the creatine kinase system. CK catalyzes the reversible reaction of adenine nucleotide transphosphorylation, the forward reaction of phosphocreatine (PCr) and MgADP synthesis, and the reverse reaction of creatine (Cr) and MgATP production:

$$MgATP^{2-} + Cr \leftrightarrow MgADP^{-} + PCr^{2-} + H^{+} \quad (21)$$

Four CK isoforms, each with compartmentalized cellular location, exist in mammals. Specific mitochondrial CK isoenzymes (MtCK), called ubiquitous (uMtCK) and sarcomeric (sMtCK), are functionally coupled to oxidative phosphorylation and produce PCr from mitochondrial ATP. PCr in turn is used for local regeneration of ATP by the muscle cytoplasmic isoform of CK (M-CK), driving myosin ATPases or ion pump ATPases [87–105]. Recent studies in CK-deficient transgenic animals indicate that energy transfer and communication between ATP-generating and ATP-utilizing sites within a muscle cell do not rely exclusively upon the activity of CK but rather may include a number of additional intracellular phosphotransfer systems such as AK and glycolysis [106–111]. AK-catalyzed reversible phosphotransfer between ADP, ATP, and AMP molecules may process cellular signals associated with ATP production and utilization [104, 105]. Cluster organization and the high rate of unidirectional phosphoryl exchange in phosphotransfer systems promote ligand conduction and signal communication at cellular distances, providing enhanced thermodynamic efficiency.

Remarkably, in the heart the intracellular energy transfer networks are structurally organized in the intracellular medium, where macromolecules and organelles surrounding a regular mitochondrial lattice are involved in multiple structural and functional interactions [112–114]. Figure 3.5 summarizes the available information about such an organized and compartmentalized energy metabolism in cardiac cells. This scheme also illustrates the view that mitochondria in muscle cells are structurally organized into functional complexes with myofibrils and sarcoplasmic reticulum [112–114]. These complexes are called ''intracellular energetic units'' (ICEUs) and are taken to represent the basic pattern of organization of muscle energy metabolism [112]. There are no physical barriers between ICEUs; each mitochondrion (or several adjacent mitochondria) can be taken to be in the center of its own ICEU. This concept is consistent with the very regular,

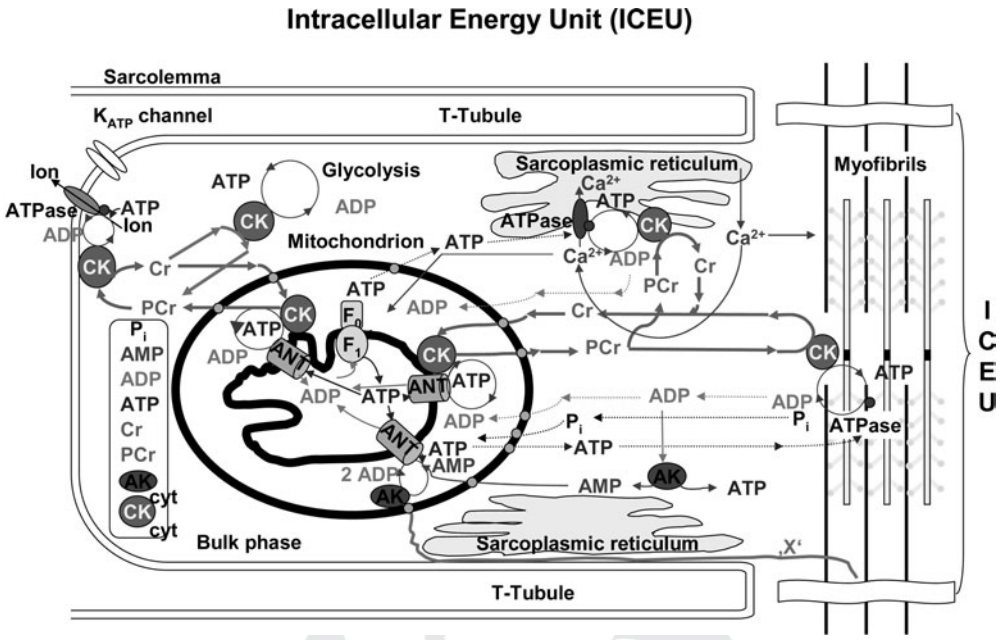


Fig. 3.5 Organization of compartmentalized energy transfer and metabolism in cardiac cells by intracellular energetic units (ICEUs). The scheme shows the structural organization of the energy transfer networks of coupled CK and AK reactions within an ICEU. By interaction with cytoskeletal elements, the mitochondria and sarcoplasmic reticulum (SR) are precisely fixed with respect to the structure of the sarcomere of myofibrils between two Z-lines and correspondingly between two t-tubules. Calcium is released from the SR into the space in the ICEU in the vicinity of mitochondria and sarcomeres to activate contraction and mitochondrial dehydrogenases. Adenine nucleotides within ICEUs do not equilibrate rapidly with adenine nucleotides in the bulk water phase. The mitochondria, SR, and Mg-ATPase of myofibrils and the ATP-sensitive systems in the sarcolemma are interconnected by metabolic channeling of reaction intermediates and energy transfer within ICEUs by the creatine kinase–phosphocreatine and myokinase systems. The protein factors (still unknown and marked as "X"), most likely connected to the cytoskeleton, fix the position of mitochondria and possibly also control the permeability of the VDAC channels for ADP and ATP. Adenine nucleotides within the ICEU and bulk water phase may be connected by more rapidly diffusing metabolites such as Cr–PCr. (Reproduced from [112] with permission).

crystal-like arrangement of mitochondria in cardiac cells [79–82] and describes the organized functional connections of mitochondria with their neighbors. ICEUs are analogous to calcium release units (CRUs), structurally organized sites of Ca^{2+} microdomains (Ca^{2+} sparks) that form a discrete, stochastic system of intracellular calcium signaling in cardiac cells [115, 116]. The structural organization of ICEUs results in local confinement of adenine nucleotides and Cr–PCr couples in discrete dynamic energetic circuits between actomyosin ATPases and mitochondrial ATPsynthases [90–106]. Similar discrete microdomains in cardio-

myocytes have been shown for cAMP in the range of approximately 1 μm exhibiting high local concentrations [53, 116–118].

The concept of ICEUs is a useful basis for the mathematical modeling of compartmentalized energy metabolism (see ▌Chapter 11▌). It allows dividing the complex problem of regulation of mitochondrial functions into two aspects: metabolic regulation of respiration inside these units and synchronization of the mitochondrial activities in the cell in all ICEUs. The first problem is analyzed in ▌Chapter 11▌, the second in ▌Chapter 4▌. Inside the ICEUs, the phosphotransfer pathways of energy channeling and metabolic signaling are based on (1) the mechanisms of functional coupling of compartmentalized CK isoenzymes with ANT in mitochondria and (2) Mg-ATPases in myofibrils and subcellular membranes. These will be analyzed below in more detail.

3.3.3 Functional Coupling of Mitochondrial Creatine Kinase and Adenine Nucleotide Translocase

The central mechanism in compartmentalized energy transfer is given by the functional coupling between the mitochondrial ATP/ADP translocase (ANT) and mitochondrial creatine kinase (MtCK). The structural basis of this coupling is described in ▌Chapter 7▌; here, we will analyze some functional peculiarities.

MtCK was discovered by Klingenberg's group in 1964 [119]. This CK isoenzyme is localized on the outer surface of the mitochondrial inner membrane, in close vicinity to the ANT [119–123]. The structure of the ANT was recently resolved at 2.2 Å [124]. The translocation of both ATP and ADP in the Mg^{2+}-free forms is related to the conformational changes in pore-forming monomers [125]. This conformational change ("gated pore") mechanism leads in its simplest version to the ping-pong transport mechanism [126–139], but the kinetics of ATP–ADP exchange conforms to the sequential mechanism with simultaneous binding of nucleotides on both sides [130]. The structural data and the kinetics of ATP–ADP exchange by ANT fit well together by assuming that the dimers with alternatively activated monomers function in a coordinated manner in the tetramers, where the export of ATP from mitochondria by one monomer in a dimer occurs simultaneously with import of ADP by another monomer in another dimer [131, 132], or both monomers in the dimmer may be active and transport nucleotides simultaneously in opposite directions [125, 133].

In the mitochondrial matrix, ANT forms a supercomplex, a synthesome with ATP synthase F_0F_1 and P_i carrier (PIC) [134]. ANT in the inner mitochondrial membrane forms tight complexes with negatively charged cardiolipin (1:6 ratio) [135–137]. It has been shown that positively charged MtCK is fixed to this cluster by electrostatic forces through three C-terminal lysines that strongly interact with the negatively charged cardiolipin in complex with ANT at the outer surface of the inner mitochondrial membrane [122, 123]. The peculiarity of the MtCK, in contrast with other dimeric CK isoenzymes (MM and BB), is that it forms octamers [90, 98, 100]. Thus, in the heart, brain, skeletal and smooth muscle, and some

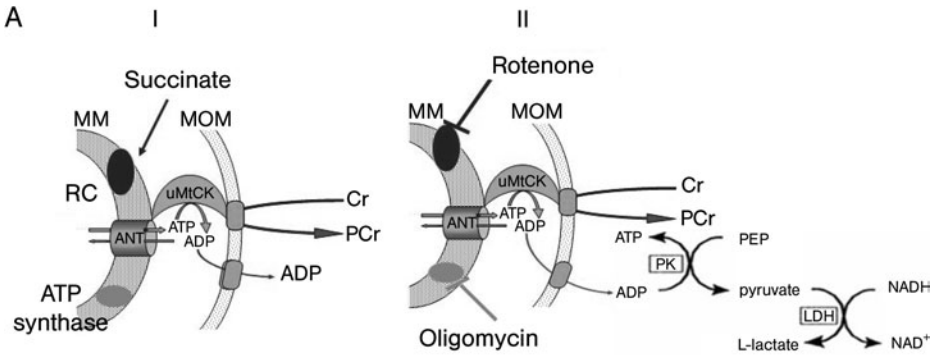
other cells, both ANT and MtCK function within a real supercomplex that connects mitochondrial ATP production with the cytoplasmic reactions of energy utilization via MtCK and VDAC (see also ▌Chapter 7▌) [138, 139].

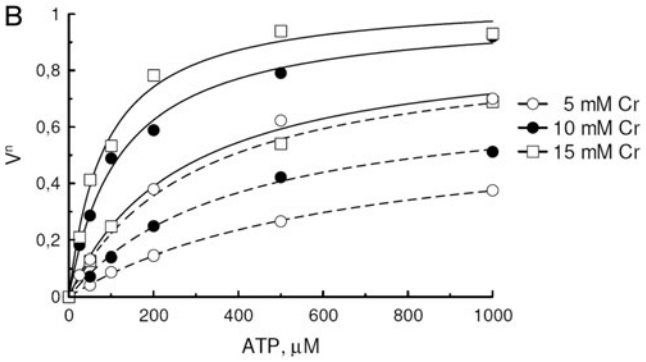
3.3.3.1 Kinetic Evidence of Functional Coupling

There is a good kinetic method for identifying and quantifying functional coupling between MtCK and ANT [140–145], the principles of which are shown in Fig. 3.6.

The creatine kinase reaction mechanism follows a Bi-Bi quasi-equilibrium random-type mechanism, according to the Cleland's classification, and is characterized by two dissociation constants for each substrate – from the binary and ternary (central) complexes of CK [146, 147] (see the scheme in Table 3.1). The values of these constants are easily determined by measuring reaction rates in series at different concentrations of both substrates. First, the concentration of one substrate is fixed and that of the other is changed. This procedure is repeated for several other fixed concentrations of the first substrate. In Fig. 3.6B, creatine is

Fig. 3.6 Kinetic evidence for the functional coupling mechanism in mitochondria: CK kinetics in isolated cardiac mitochondria without and with oxidative phosphorylation. Experiments and analyses were carried out according to the protocols and equations described in [143].
(A) Scheme of the protocols to study the kinetics of the creatine kinase reaction. MIM: mitochondrial inner membrane; MOM: mitochondrial outer membrane; RC: respiratory chain; ANT: adenine nucleotide translocase; MtCK: mitochondrial creatine kinase: Cr: creatine; PCr: phosphocreatine; I: with oxidative phosphorylation; II: without oxidative phosphorylation; PEP: phosphoenol pyruvate; PK: pyruvate kinase; LDH: lactate dehydrogenase.
(B) Initial rates of the MtCK reaction in isolated heart mitochondria as functions of MgATP concentration for different fixed Cr concentrations without oxidative phosphorylation (dotted lines) and with oxidative phosphorylation (solid lines).
(C) Double reciprocal plot of data shown in Fig. 3.6B.
(D) Secondary analysis of MtCK kinetic data from (C) for different fixed creatine and changing ATP concentrations. Values of intercepts of ordinate, i_o, in (C) are plotted as a function of 1/[Cr]; the abscissa intercepts correspond to reciprocals of a dissociation constant K_b of creatine from ternary complex [143]. No effect of oxidative phosphorylation is seen.
(E) Secondary analysis of the kinetic data when the reaction rates were measured for different fixed MgATP concentrations as functions of creatine concentration; from double reciprocal plots, the intercepts of ordinate were found as functions of 1/[ATP] (in these experiments, ATP means MgATP). Abscissa intercepts show the reciprocals of K_a, a dissociation constant for MgATP from ternary complex into medium [143]. This constant is decreased by oxidative phosphorylation by an order of magnitude (see Table 3.1), showing direct transfer of ATP from ANT to MtCK and recycling of ADP and ATP in the coupled reactions.
(F) Complete kinetic analysis of the forward MtCK reaction in heart mitochondria. The temperature dependencies of the kinetic constants (see scheme in Table 3.1) are shown at a semi-logarithmic scale in the presence (+) and absence (−) of oxidative phosphorylation. The dissociation constants were expressed in mM. Only K_a (see E) is changed by oxidative phosphorylation by an order of magnitude; smaller changes are seen for K_{ia}, but practically no changes are seen for the dissociation constant of creatine. (Reproduced from [96] with permission).





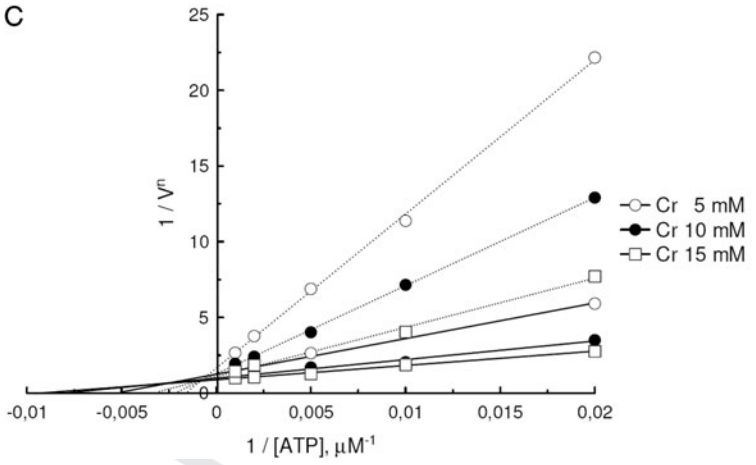


Fig. 3.6 (A–C) (legend see p. 77)

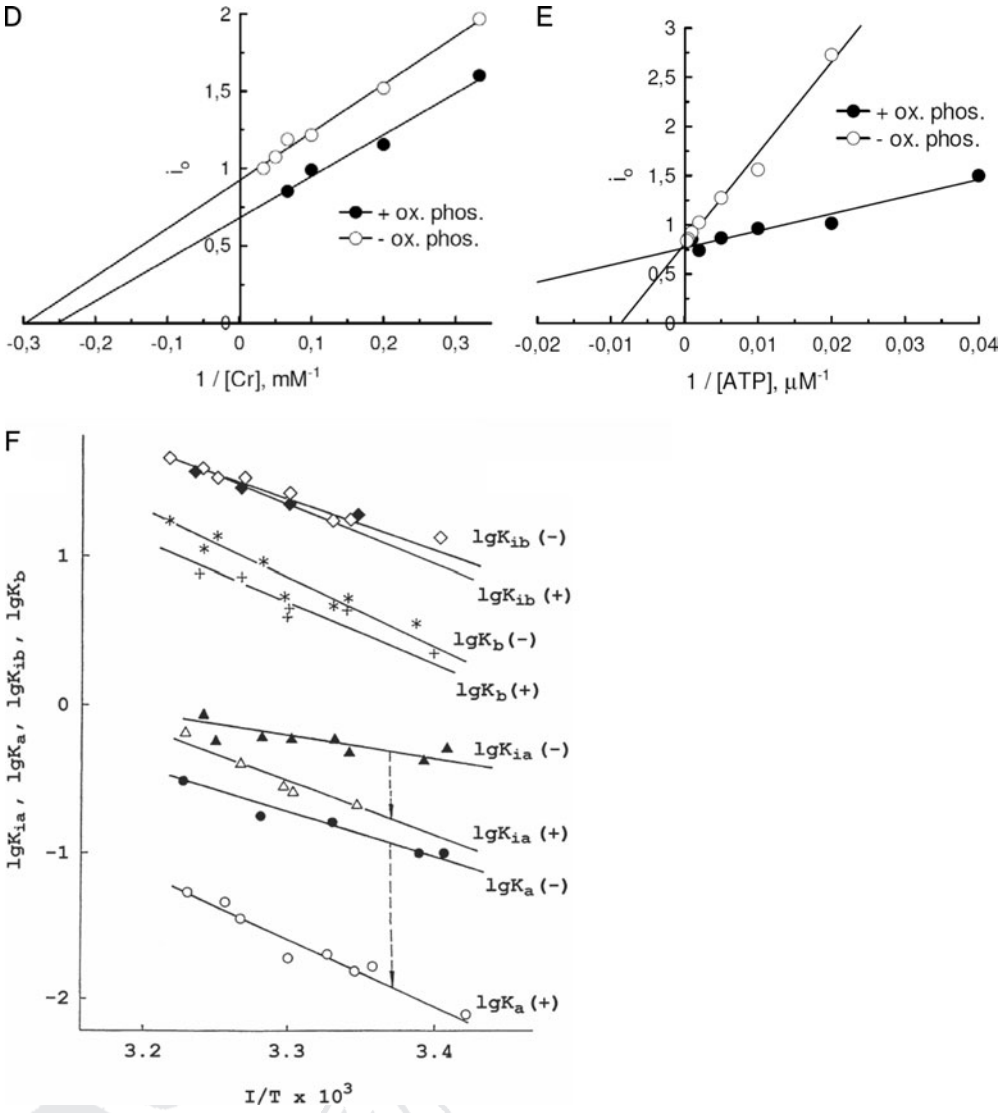
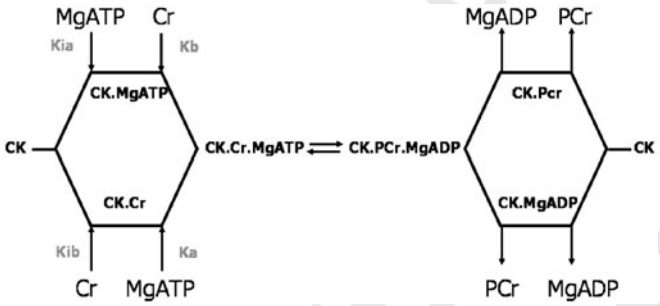


Fig. 3.6 (D–F) (legend see p. 77)

Table 3.1 The kinetic scheme of the Bi-Bi random type quasi-equilibrium creatine kinase reaction and dissociation constants for the forward MtCK reaction in the absence and presence of oxidative phosphorylation.



Dissociation constant	Without oxidative phosphorylation	With oxidative phosphorylation	Reference
K_{ia} (mM)	0.75 ± 0.06	0.29 ± 0.04	[143]
	0.64 ± 0.04	0.20 ± 0.03	[144]
	1.1 ± 0.1	0.3 ± 0.06	[145]
	0.92 ± 0.09	0.44 ± 0.08	1)
K_a (mM)	0.15 ± 0.01	0.014 ± 0.005	[143]
	0.14 ± 0.02	0.016 ± 0.001	[144]
	0.22 ± 0.03	0.02 ± 0.004	[144]
	0.12 ± 0.23	0.023 ± 0.003	1)
K_{ib} (mM)	28.8 ± 8.45	29.4 ± 12	[143]
	30 ± 4.5	30 ± 4	[144]
	51.1 ± 11	30 ± 1	[145]
	28.67 ± 7.15	24.54 ± 5.32	1)
K_b (mM)	5.2 ± 0.3	5.2 ± 2.3	[143]
	9.5 ± 4.5	3.5 ± 1	[144]
	9 ± 1	5 ± 1.2	[145]
	3.37 ± 0.41	3.96 ± 0.64	1)

1) Determined by Tiia Anmann for this publication (see Fig. 3.6).

the substrate used at different fixed concentrations, and MgATP is varied. When oxidative phosphorylation is not activated, the mitochondrial creatine kinase reaction does not differ kinetically and thermodynamically from that catalyzed by other creatine kinase isoenzymes: the reaction favors ATP production, ADP and phosphocreatine binding is more effective due to higher affinities than that of ATP or creatine, respectively, and the kinetic constants obey the Haldane relationship [142–146]. However, under conditions of oxidative phosphorylation, the mitochondrial creatine kinase reaction is strongly shifted in the direction of

PCr synthesis and may use all ATP produced in mitochondria for PCr production [96, 143–145]. This is clearly seen in Fig. 3.6B,C: for any creatine concentration (5, 10, and 15 mM), the rates of phosphocreatine production in the presence of active oxidative phosphorylation (solid lines) were much higher than in its absence (dotted lines). Secondary analysis of the data in double reciprocal plots (Fig. 3.6C–E) directly yields the dissociation constants. While the kinetic constants for creatine were not changed and were the same in both conditions, the oxidative phosphorylation had a specific effect on the kinetic parameters for adenine nucleotides [96, 143–145]. These changes in the MtCK kinetics induced by oxidative phosphorylation are very clearly illustrated in Fig. 3.6E,F. Complete kinetic analysis of the MtCK reaction under conditions of oxidative phosphorylation (Fig. 3.6F) revealed that both constants for MgATP dissociation from the MtCK–MgATP and MtCK–Cr–MgATP complexes, Kia and Ka, respectively, were decreased, most significantly Ka, which was dramatically decreased by one order of magnitude [96, 143–145] (see Fig. 3.6 and Table 3.1). The explanation proposed was the direct transfer of ATP from ANT to MtCK due to their spatial proximity, which also results in an increased uptake of ADP by ANT from MtCK (reversed direct transfer). As a result, the turnover of adenine nucleotides is increased manifold at low external concentrations of MgATP, thus maintaining high rates of oxidative phosphorylation and coupled phosphocreatine production in the presence of creatine. This was confirmed in direct experiments in which MtCK was detached from the inner mitochondrial membrane into the intermembrane space by isotonic KCl solutions: in this case the effect of oxidative phosphorylation on the MtCK kinetics was lost [145]. It is important to emphasize that the structural and functional coupling of the MtCK–ANT system does not prevent its participants from working independently under some conditions. For example, it is well known that in a medium with only ADP, mitochondria can carry out the oxidative phosphorylation reactions without any limitations, despite the structural associations of ANT with MtCK; on the other hand, the inhibition of oxidative phosphorylation does not result in inhibition of MtCK but only alters its apparent kinetic behavior [142, 148]. These facts clearly indicate that the structural associations of ANT with MtCK are flexible and do not result in formation of a completely isolated space between complexes. The metabolite molecules can leave this space, but they also can be arrested in it to realize functional coupling between the partners ANT and MtCK, if all substrates including creatine are present. Quantitative analysis of the experimental data on coupled MtCK and ANT by a complicated mathematical model, based on the analysis of the energy profile of the reaction, gave evidence for the direct transfer of ATP from ANT to MtCK, with significant changes in the energy level of complexes of ATP bound to ANT [149]. This interesting possibility merits further experimental studies.

The direct transfer of mitochondrial ATP to MtCK and the increased mitochondrial turnover of adenine nucleotides in the presence of creatine were confirmed by the isotopic method [150], by the thermodynamic approach [144, 151, 152], and by showing that inhibition of MtCK by monoclonal antibodies also inhibited

ADP–ADP exchange due to the proximity of MtCK to ANT [153]. An effective competitive enzyme method for studying the functional coupling phenomenon – namely, the pathway of ADP movement from MtCK back to ANT [154] – is to use the phosphoenolpyruvate (PEP)–pyruvate kinase (PK) to trap ADP and thus to compete with ANT for this substrate [155]. The use of this method is demonstrated in ▌Chapter 11▌.

3.3.3.2 Thermodynamic Evidence of Functional Coupling

Because of its importance, we reproduce this evidence again in Fig. 3.7. The creatine kinase reaction equilibrium is shifted in the direction of ATP production, the apparent $K'_{eq} = K_{eq} \times [H^+]$ in this direction:

$$K'_{eq} = ([ATP] \times [Cr])/([PCr] \times [ADP])$$

having the following values: pH 7.0, 38 °C, free $[Mg^{2+}] = 1$ mM equal to ~170, and the standard free energy change of the creatine kinase reaction in the direction of ATP synthesis $\Delta G^\circ = -13.4$ kJ mol^{-1} [156–158]. In the reverse direction, the apparent K'_{eq} is equal to 6×10^{-3} (see dotted line in Fig. 3.7B). In these experiments, reproduced from Ref. [144], the reaction conditions are such that $\Gamma = ([PCr][ADP])/([Cr][ATP])$ in the medium is higher than K'_{eq}; therefore, according to the laws of chemical thermodynamics, the reaction of phosphocreatine production should have proceeded under these conditions in the direction of lowering Γ to K'_{eq} by decreasing phosphocreatine concentration, as could be

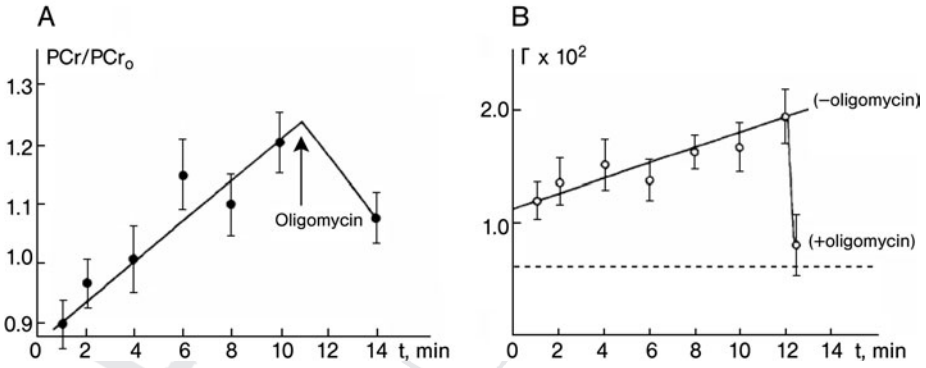


Fig. 3.7 (A, B) Thermodynamic evidence for functional coupling of MtCK with ANT in isolated heart mitochondria. The reaction medium contained all substrates and products of the MtCK reaction in relations, which gave $\Gamma = ([PCr][ADP])/([Cr][ATP])$ higher than the equilibrium constant (dotted line in Fig. 3.7B). Under conditions of oxidative phosphorylation, PCr/PCr_0 and Γ increased. Thus, Γ moved away from the equilibrium position, uphill on the curve shown in Fig. 3.2. Inhibition of oxidative phosphorylation by oligomycin reversed the direction of the reaction. (Data are reproduced from [144] with permission).

predicted from Fig. 3.2. Figure 3.7 shows, however, that in the presence of oxidative phosphorylation the concentration of PCr increases and Γ moves uphill (along the curve in Fig. 3.2) away from equilibrium. There should be a strong force and a significant amount of free energy change underlying this uphill movement, according to Fig. 3.2, meaning that oxidative phosphorylation is controlling the MtCK reaction. Most likely, the control occurs by direct supply of mitochondrial ATP to the active center of MtCK by ANT and by rapid removal of ADP by the latter – the functional coupling mechanism. When the oxidative phosphorylation reaction is inhibited by oligomycin, the MtCK can fully obey the thermodynamic rules and Γ decreases to the value of K'_{eq} (Fig. 3.7B).

It is interesting to recall again some facts from history. In 1939 Belitzer and Tsybakova observed in well-washed skeletal muscle homogenates a strong stimulation of respiration by Cr without addition of exogenous adenine nucleotides [159]. This was the earliest indication of the functional coupling of MtCK with oxidative phosphorylation. Much later, Kim and Lee showed the same effect for isolated pig heart mitochondria [160] and Dolder et al. for liver mitochondria from transgenic mice expressing active MtCK [161]. All these data show rapid recycling of endogenous ADP and ATP already present in mitochondria, due to the functional coupling of MtCK with ANT.

3.3.4
Heterogeneity of ADP Diffusion in Permeabilized Cells: Importance of Structural Organization

Studies on the regulation of mitochondrial respiration in permeabilized muscle cells and fibers have rendered important information about the structure–function relationships of cellular energetic systems, the role of creatine kinases, and calcium effects on respiration. In these experiments, the important role of the mitochondrial outer membrane in strengthening the functional coupling between MtCK and ANT (as predicted by Gellerich; see [155]) became evident.

Figure 3.8 illustrates this information. As mentioned above, in intact cardiac cells mitochondria are arranged very regularly at the level of A-bands of sarcomeres (Fig. 3.8A) and are functionally coupled to multiple intracellular ATP-consuming processes by energy transfer and metabolic feedback signaling networks within highly organized energetic units (see above). However, Claycomb and coworkers recently described the cultured (continuously dividing) HL-1 cell line with a differentiated cardiac phenotype [162, 163]. Pelloux et al. found that growing HL-1 cells with a different serum (GIBCO fetal bovine serum, batch 1147078) for five weeks (four passages) led to cells devoid of beating properties, which we classified as NB HL-1 cells [164]. Thus, the NB HL-1 cells represent an original phenotype displaying cardiac characteristics. Most remarkably, these cells are devoid of sarcomeric structures and possess randomly organized filamentous dynamic mitochondria [164, 165]. Unlike in adult rat cardiomyocytes, rapid movement, fission, and fusion of mitochondria (Y. Usson, personal communication) characterize filamentous mitochondria in NB HL-1 cells (Fig. 3.8B)

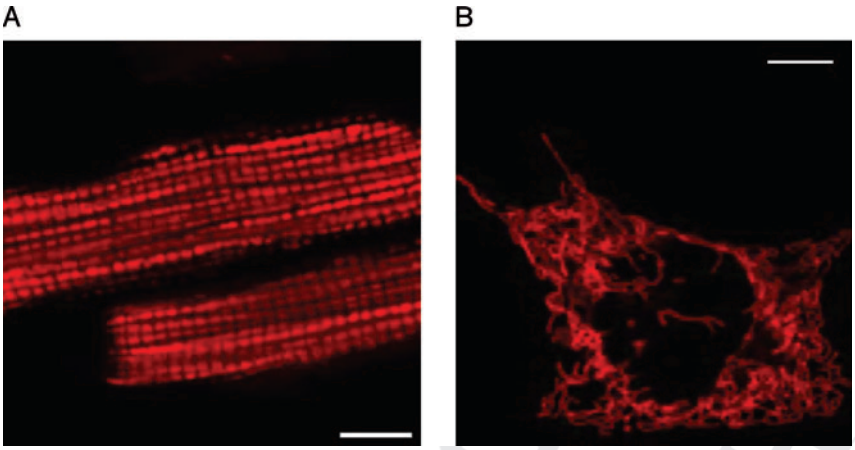


Fig. 3.8 Confocal fluorescent images of mitochondria in adult isolated cardiomyocytes (A) and cultured non-beating HL-1 cells (B) (scale bars: 10 μm). Mitochondrial arrangement was visualized by MitoTracker® Deep Red as described in [178]. (C, D) Different kinetics of regulation of the respiration in permeabilized adult cardiomyocytes and NB HL-1 cells. (C) ADP kinetic protocols and representative respiration traces for permeabilized cardiomyocytes recorded using a two-channel, high-resolution respirometer (Oroboros Oxygraph-2k, Oroboros, Innsbruck, Austria) in respirometry medium Mitomed [178]. Mitochondrial respiration increases with stepwise increasing concentrations of ADP in the range of 0.05 to 2 mM for cardiomyocytes to reach the saturated rate of respiration (V_{max}). At the end of the measure, cytochrome *c* (Cyt c, 8 μM) addition does not change the respiration, indicating that the outer membrane is intact. Atractyloside (Atr, 30 μM) results in a decrease in respiration back to V_o due to inhibition of adenine-dinucleotide translocase. (Reproduced from [178] with permission). (D) Comparison of respiration kinetics as normalized respiration rates versus [ADP] in permeabilized adult cardiomyocytes and in permeabilized HL-1 NB cells determined in experiments described in (C). Values of apparent K_m for exogenous ADP were equal to 360 ± 51 μM for isolated and permeabilized cardiomyocytes and 25 ± 4 μM for HL-1 NB cells [178]. For normalization, the respiration rates were expressed as fractions of the maximal rates, V_{max}, found by analysis of experimental data in double reciprocal plots. (E) Comparison of respiration regulation kinetics in isolated heart mitochondria and in permeabilized cardiomyocytes without and with creatine (20 mM). (Taken from [179, 180]).

[164]. Also, striking differences in the kinetics of respiration regulation by exogenous ADP are exhibited by these cells (Fig. 3.8C–F). In permeabilized adult cardiomyocytes, the apparent K_m for exogenous ADP is very high (360 ± 51 μM). This has been previously reported [166–176] and explained by local restrictions of ADP diffusion in the cells, including limitation of its diffusion across the mitochondrial outer membrane [33, 177]. In permeabilized cardiomyocytes, treatment with trypsin resulted in dramatic changes in intracellular structure that were associated with a threefold decrease in apparent K_m for ADP in the regulation of respiration [176, 178]. In contrast to permeabilized cardiomyocytes, in NB HL-1 cells the apparent K_m for exogenous ADP (25 ± 4 μM) was 14 times lower

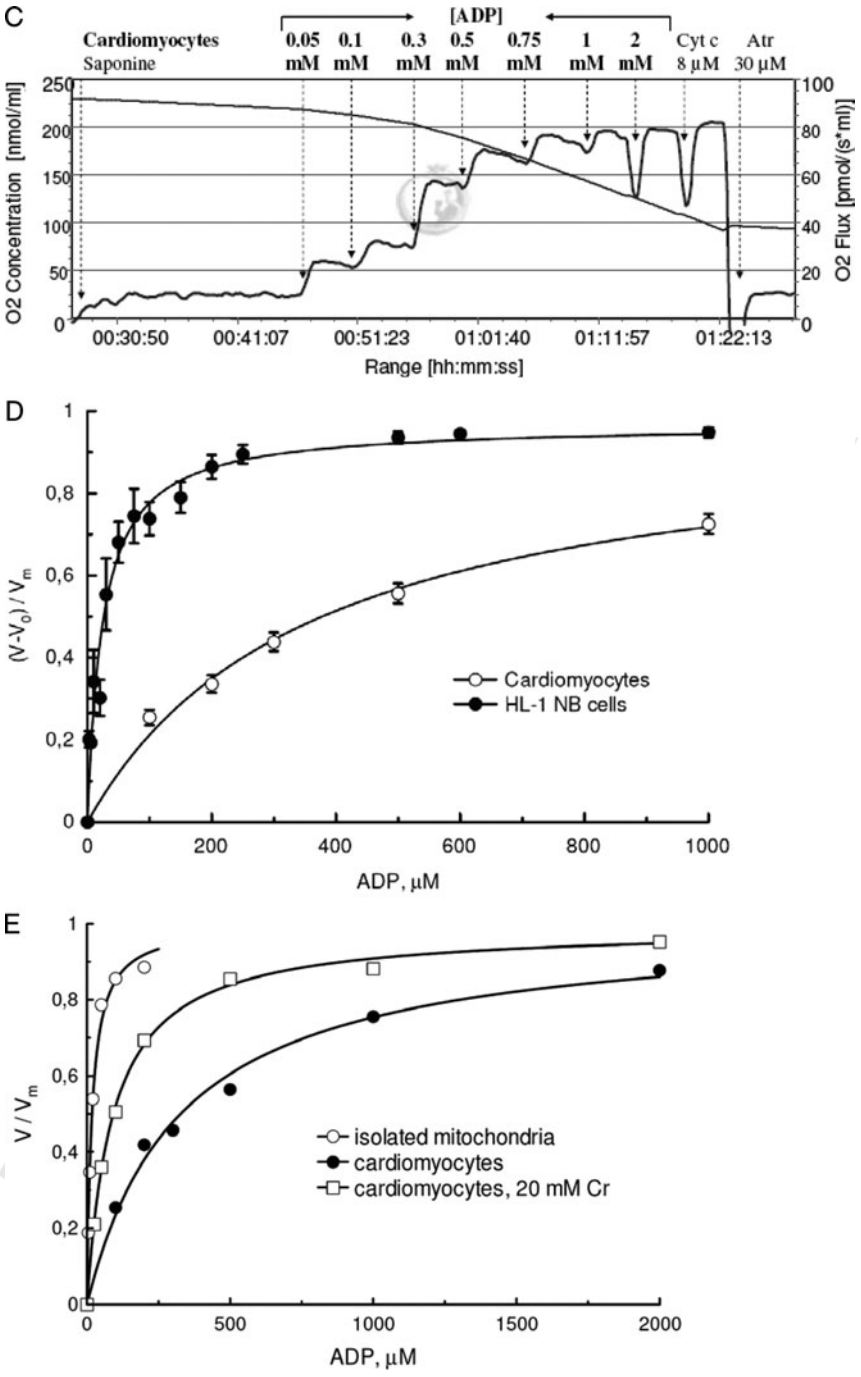

C
[ADP]
Cardiomyocytes
Saponine
0.05 mM
0.1 mM
0.3 mM
0.5 mM
0.75 mM
1 mM
2 mM
Cyt c 8 µM
Atr 30 µM
O2 Concentration [nmol/ml]
O2 Flux [pmol/(s*ml)]
Range [hh:mm:ss]
D
(V-V0) / Vm
Cardiomyocytes
HL-1 NB cells
ADP, µM
E
V / Vm
isolated mitochondria
cardiomyocytes
cardiomyocytes, 20 mM Cr
ADP, µM

Fig. 3.8 (C–E)

(Fig. 3.8C–E). The regulation of respiration by exogenous ADP in NB HL-1 cells is very close to that in isolated mitochondria (Fig. 3.8F). While in normal adult cardiomyocytes creatine significantly activates respiration and, as in the experiments described in Fig. 3.8E,F, decreases the apparent K_m for ADP (see also ▌Chapter 11▐), in HL-1 cells it exerts very little influence, if any, due to the down-regulation of creatine kinase [178]. These results show that in normal adult cardiomyocytes intracellular local restrictions of diffusion of adenine nucleotides and metabolic feedback regulation of respiration via phosphotransfer networks are related to the complex structural organization of these cells.

The high K_m for exogenous ADP has been shown to reflect the local restriction of diffusion of ADP and ATP, which in cardiac cells are bypassed by the creatine kinase system [177]. Most likely, these diffusion limitations are localized at or close to the outer mitochondrial membrane due to close connections to some cytoskeletal elements [33, 177, 179, 180]. In fact, simple logical analysis shows that the limited permeability of the mitochondrial outer membrane for ADP in cardiac cells *in vivo* should be expected. In the resting heart, or in the diastolic phase of contracting heart, when cytoplasmic creatine kinase is in the equilibrium state (see ▌Chapter 11▐), the cytoplasmic ADP concentration calculated from the CK equilibrium is in the range of 50–100 μM [181]. However, in isolated mitochondria, the apparent K_m of ANT for ADP is 10–20 μM [182]. If mitochondria behave in the cells *in vivo* as they do *in vitro*, the mitochondrial respiration rate should be maximal in resting heart or during the diastolic phase of the contraction cycle due to the saturation of ANT with ADP that is never observed. This means that the apparent K_m probably also is high for endogenous ADP, as was found in experiments with exogenous ADP (see Fig. 3.8), due to the decreased permeability of VDAC channels in the outer mitochondrial membrane for this substrate. This conclusion is further confirmed in experiments described in ▌Chapter 11▐, and that makes functionally coupled MtCK even more necessary for energy transfer and respiration regulation at high workloads.

Interestingly, the apparent K_m for exogenous ADP (which is very easy to measure in biopsy samples), by reflecting the complexity of intracellular interactions and organization in oxidative muscle cells, has become an important diagnostic parameter in studies of skeletal muscle bioenergetics and exercise physiology (see also ▌Chapter 14▐). In both experimental and clinical studies, endurance training has been shown to significantly increase (2–3 times) the apparent K_m for exogenous ADP in animal and human skeletal muscle fibers, and it correspondingly increases the stimulatory effect of creatine on respiration and thus the role of the creatine kinase system in intracellular communication [183–187]. Thus, this parameter has a clear practical and diagnostic value for muscle performance [182–188]. Identification of cell components responsible for specific organization of energy transfer systems and intracellular diffusion merits further investigation. For elucidation of the nature of cytoskeletal components responsible for specific organization of both mitochondrial arrangement and complex metabolic signaling and intracellular energy transfer pathways [178], further investigations on the proteome are needed.

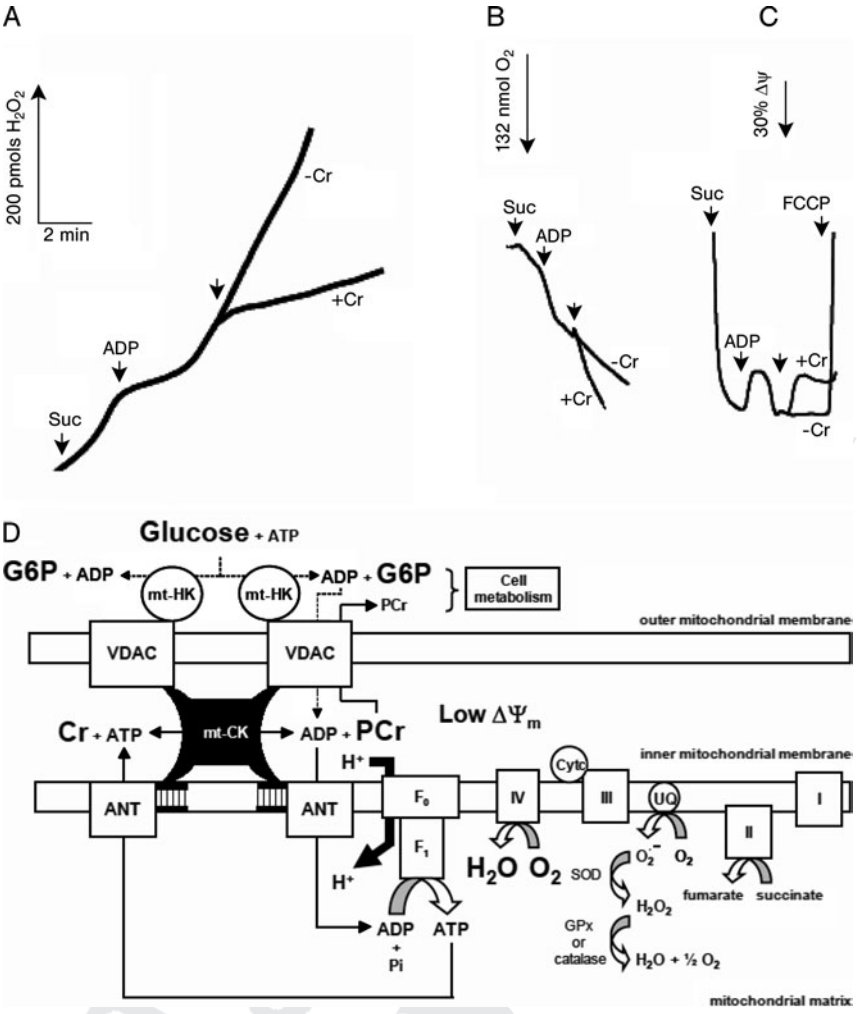


Fig. 3.9 H_2O_2 production (A), oxygen consumption (B), and membrane potential (C) in rat brain mitochondria are regulated by Cr phosphorylation by MtCK. Succinate was added to 10 mM; ADP, 0.15 mM; Cr, 10 mM; and FCCP 5 μM [194]. (D) Scheme of the reactions by which MtCK and hexokinase (Mt-HK) bound to the outer mitochondrial membrane regulate oxygen consumption and membrane potential, $\Delta\Psi m$, and inhibit ROS formation in the respiratory chain. SOD: superoxide dismutase; GPx: glutathione peroxidase; UQ; ubiquinone; cyt. C: cytochrome *c*. (Reproduced from [194] with permission).

3.3.5
Evidence for the Importance of Functional Coupling for Cell Life

The functional coupling mechanism is supported by the structural and functional studies of MtCK performed by Wallimann's group (see ▌Chapter 7▐). *In vivo*, the functional coupling between MtCK and ANT was verified recently in studies of heart energy metabolism performed *in vivo* by ^{31}P-NMR inversion transfer. This approach showed that in intact heart cells mitochondrial creatine kinase is strongly shifted in the direction of phosphocreatine production under aerobic conditions [189, 190]. It was also shown in mice with knockout of MtCK that, as predicted by the theory described above, these hearts had lower levels of PCr and reduced post-ischemic recovery [191–193].

A new, important role for MtCK's control over ANT is to hinder the opening of the mitochondrial permeability transition pore [161] and cell death. Important new data were published recently [194] showing that due to the active functional coupling between MtCK and ANT, MtCK-induced ADP recycling strongly decreases the production of reactive oxygen species (ROS) in brain mitochondria. Some of these important results are reproduced in Fig. 3.9. Taking into account that ROS production is now considered a major cause of many age-related diseases and aging itself [195, 196], the importance of these results is difficult to overestimate. It may soon become clear that our good health and longevity significantly depend upon the degree of functional coupling between MtCK and ANT in heart, skeletal and smooth muscles, brain, and many other tissues in which the creatine kinase network is important.

3.3.6
Myofibrillar Creatine Kinase

The myofibrillar end of the creatine–phosphocreatine cycle is represented by the MM isozyme of creatine kinase localized in the sarcomere and functionally coupled to the actomyosin Mg-ATPase [90, 93, 94, 197, 198]. Within the contraction cycle, ADP release is a necessary step for new binding of MgATP, dissociation of actomyosin cross-bridges, and muscle relaxation in order to start the contraction cycle anew [199–206]. This step is often found to be the slowest in the contraction cycle and therefore is rate controlling. MgADP may compete with MgATP for the substrate site on myosin and inhibit cross-bridge detachment by MgATP, the inhibition constant K_i being approximately 200 μM both in the Mg-ATPase reaction and in the sliding of fluorescent actin on myosin [200, 203–207]. Thus, MgADP should be rapidly removed from actomyosin, and the high local value of the MgATP:MgADP ratio along with the local phosphorylation potential should be maintained. The MM-CK is bound specifically to the M-line, and a significant portion of this isozyme is found in the I – band of sarcomeres [208–210]. An increasing amount of evidence points out that this MM creatine kinase is intimately involved in the contraction cycle at the level of ADP-release and ATP-rebinding steps by the mechanism of functional coupling, likely including

myofibrillar microcompartments of adenine nucleotides [93, 207, 211–213]. Ogut and Brozovich studied the kinetics of force development in skinned trabeculae from mice hearts and found that despite the presence of 5 mM MgATP, the rate of force development depended on the concentration of PCr. These authors concluded that there is a direct functional link between the creatine kinase reaction and the actomyosin contraction cycle at the step of ADP release in myofibrils [214]. This effective interaction probably occurs via small microcompartments of adenine nucleotides in myofibrils and is facilitated by anisotropic diffusion. When PCr is exhausted, the rate of local regeneration of ATP in myofibrils decreases, slowing the contraction cycle irrespective of high cytoplasmic ATP concentrations (see Fig. 3.3) [86, 212–214]. In agreement with this interpretation, it was recently shown that inhibition or knockout of the creatine kinase system by genetic manipulations results in a significant loss of work capacity [215, 216] (see ▌Chapter 11▌). The rigor state known as ischemic contracture appears only after complete exhaustion of all ATP in the cell [217] and can be significantly delayed by local production of ATP by the myofibrillar glycolytic system or accelerated by inhibition of this process (the so-called glycolytic ATP effect [74, 75, 86]). Under these conditions, the rate of glycolytic ATP production and its diffusion from the cytoplasm are too slow to sustain contraction.

3.3.7
Membrane-bound Creatine Kinases and Membrane Energy Sensing

Other ATP-consuming systems are found in both the membranes of the sarcoplasmic reticulum (SR) and in the plasmalemma (sarcolemma). Their function is to maintain the ionic homeostasis and, particularly, the regulation of the calcium cycle. The role of the MM-CK connected to the membrane of the SR and functionally coupled to the Ca/MgATP-dependent ATPase (SERCA) is to rapidly rephosphorylate local MgADP produced in the Ca-/Mg-ATPase reaction, thus maintaining a high local value of phosphorylation potential to keep a high efficiency of calcium pumping and avoid its reversal or inhibition due to accumulation of ADP [218–222].

In the control of the excitation–contraction coupling in the heart, a principal step is the sarcolemmal membrane metabolic sensor complex [34, 35]. Its main component is the sarcolemmal ATP-sensitive K^+ (K_{ATP}) channel that acts as an alarm system to adjust cell electrical activity to the metabolic state of the cell [34, 223–231]. The sarcolemmal MM-CK rephosphorylates the local ADP, maintaining a high ATP/ADP level in these microcompartments for coordination of membrane electrical activity with cellular metabolic status, notably with PCr levels. The K_{ATP} channel was discovered by Noma [227], and it was found that this channel has a high affinity for ATP, about 100 μM [227, 228]. Nevertheless, the channel is opened in the presence of millimolar ATP, as seen from rapid membrane repolarization and shortening of action potential in ischemic and hypoxic hearts or as shown directly in experiments with internally perfused cardiomyocytes [34, 35]. This is explained by strong diffusional restriction, and thus ATP

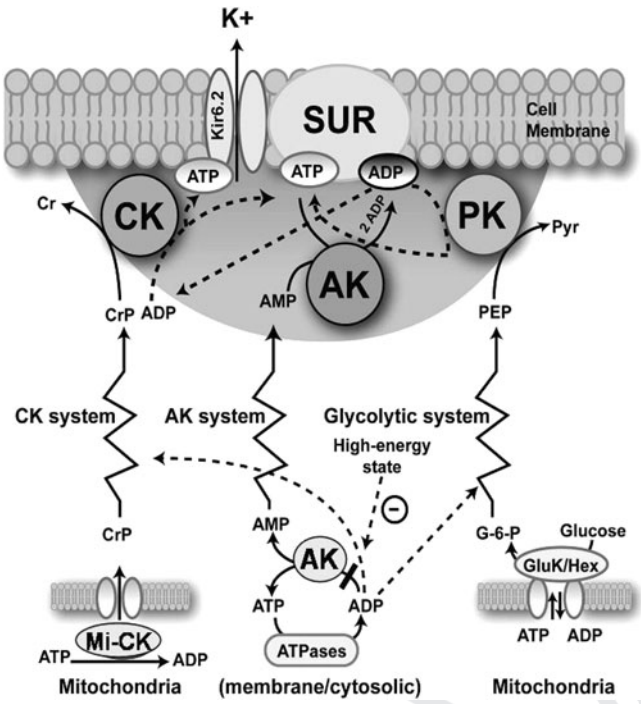


Fig. 3.10 Paradigm of phosphotransfer-mediated energetic signaling: coupling of cellular metabolic and electrical activities. Dynamic interaction between CK, AK, and glycolytic (represented by PK) phosphotransfer relays determines the behavior of a prototypic metabolic sensor – the K_{ATP} channel – and subsequent cellular responses, such as excitability, hormone secretion, intracellular calcium homeostasis, and vascular tone. The shadowed area represents a metabolic sensor ''sensing zone,'' where intimate local changes in nucleotide ratios are sensed and transduced into an appropriate cellular response. Phosphotransfer circuits connect the ''sensing zone'' with cellular processes. Dashed lines indicate pathways signaling the high-energy state, while solid lines represent low-energy state signal transmission. Kir6.2: potassium channel subunit; SUR: sulfonylurea receptor; GluK/Hex: glucokinase and hexokinase. (Reproduced from [102] with permission).

compartmentation, in the subsarcolemma and is linked to the cellular pool of PCr via CK reactions (Fig. 3.10). Pool exhaustion in the first minutes of ischemia certainly contributes to the cessation of contraction due to opening of the K_{ATP} channel and decreased calcium entry (see also ▌Chapter 11▌). These energy transfer and control functions are shared by the whole system, including the creatine kinase, adenylate kinase, and glycolytic systems, as was seen in experiments involving genetic manipulation [35, 225]. Similar cell membrane metabolic sensors also may be important in brain cells. The basic principles of phosphotransfer-mediated metabolic signaling to metabolic sensors at the sarcolemma are presented in Fig. 3.10. The premembrane area, where diffusion of molecules is

restricted due to the unstirred layer of structured water and molecular crowding [34, 35, 177, 229], represents the ''sensing zone'' of a metabolic sensor, where intimate changes in nucleotide ratios are sensed and transduced into appropriate cellular responses [35, 104–106]. Because cellular and even submembrane ATP concentrations are higher than required for half-maximal channel inhibition (between 10 μM and 100 μM) [34, 35, 226, 227], production of ADP by AK or membrane ATPases and ADP scavenging by CK and PK reactions are critical for channel activity. It may be assumed that dynamic interaction between CK, AK, and glycolytic enzyme (represented as PK) phosphotransfer relays determines the behavior of a prototypical metabolic sensor – the K_{ATP} channel – and subsequent cellular responses, such as excitability, hormone secretion, intracellular calcium homeostasis, and vascular tone [34, 35, 104, 225, 228–232]. These phosphotransfer relays communicate metabolic signals that originate in mitochondria or at cellular ATPases to metabolic sensors, conveying information about ''high'' or ''low'' cellular energy, oxygen supply, or hormonal states [34, 35, 104–106]. CK and AK deficiencies or their altered activity ratio compromises metabolic signaling to K_{ATP} channels [34, 225]. Decreases in CK activity, such as in CK knockouts or failing hearts, can be partially compensated by the increase in glycolytic phosphotransfer to alleviate cardiomyocyte electrical instability [106, 109].

3.3.8
The Mechanism of Acute Contractile Failure of the Ischemic Heart

Taken together, the results described in this section help us to explain why the heart stops contracting in the first minutes of total ischemia, when oxidative phosphorylation and thus PCr production in mitochondria are inhibited but the total ATP level is still very high. Clearly, the major contributors are the changes happening in the compartmentalized energy transfer. A rapid decrease in PCr concentration in the cytoplasm results in rapid exhaustion of the local ATP pools in myofibrils, which slows down the contraction cycle [175, 212–214], favors the opening of the sarcolemmal K_{ATP} channels, affects membrane repolarization, decreases the entry of calcium via L-type calcium channels, and inhibits Ca^{2+}-induced Ca^{2+} release (CICR) in the sarcoplasmic reticulum. As a result, contraction stops because of lack of fuel, but myofibrils stay relaxed (due to glycolytic ATP production in myofibrils) and also slow diffusion of ATP from the cytoplasm. The latter reveals itself as a protective mechanism that allows the cells to preserve a significant portion of the ATP and thus to survive an energetic crisis. Only total exhaustion of ATP results in development of ischemic contracture and irreversible damage of the heart [217]. Thus, the compartmentalized energy transfer mechanism permits an accurate explanation of the fundamental observations about the ischemic heart shown in Figs. 3.3 and 3.4. All these changes occur very rapidly, and there are no compensatory mechanisms available, in contrast to hypertrophy or in experimental animals with knockout of CK genes (see ▌Chapter 10▌). The initial proposal by Gudbjarnason was proved to be correct in general, but in the detailed investigations described above, several functionally very

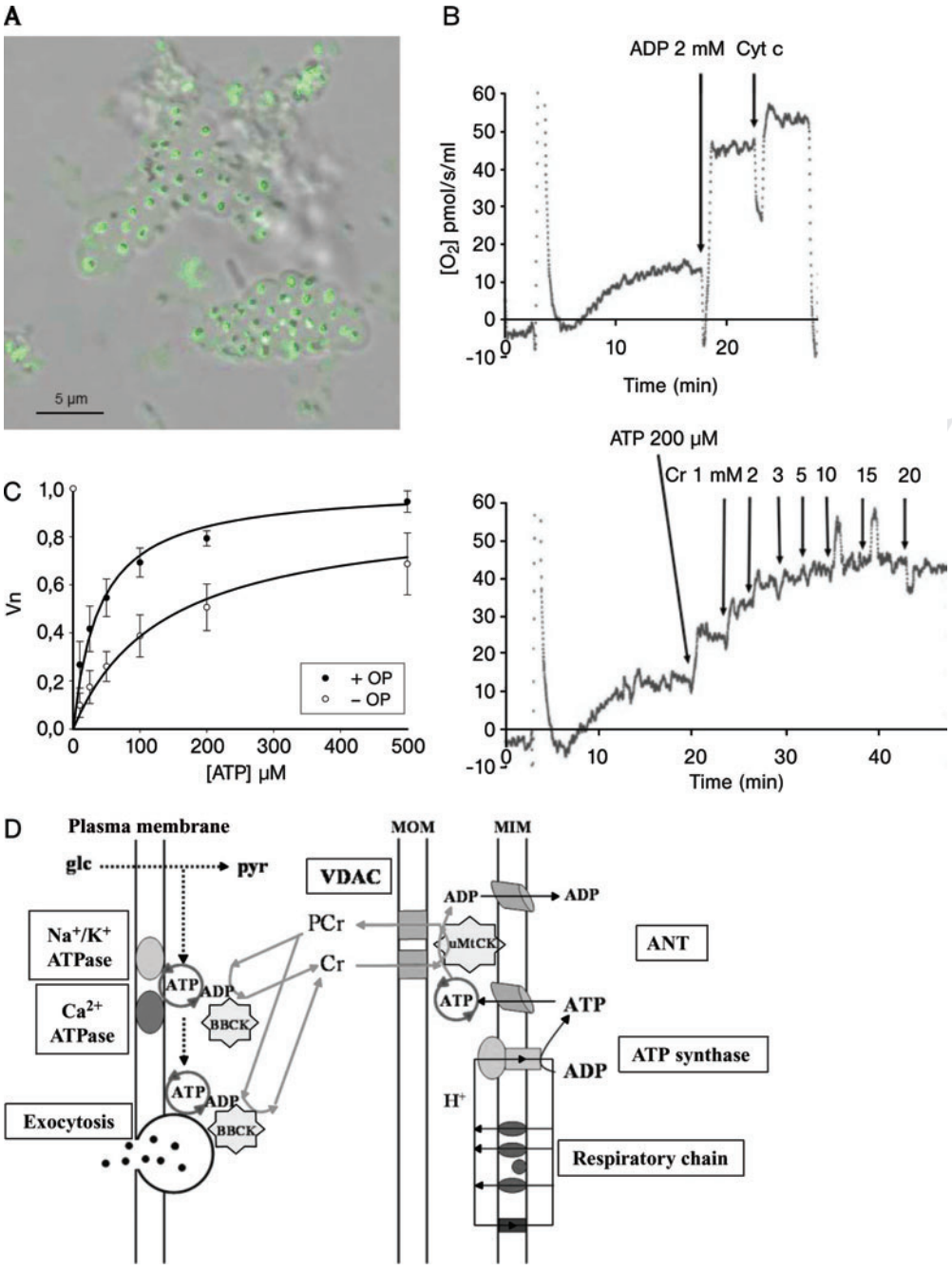


Fig. 3.11 (legend see p. 93)

important compartments of ATP were identified: in myofibrils and close to the subcellular membranes – the sarcoplasmic reticulum and the sarcolemma. All these functionally important pools of ATP are connected to the cytoplasmic phosphocreatine via compartmentalized CK isoenzymes (see Fig. 3.5).

In chronic heart failure the changes in the energy transfer system are of a structural and functional character and represent severe pathogenic mechanisms of contractile failure in both heart and skeletal muscle, as shown in recent reviews from Ventura-Clapier's group [175, 188, 233].

3.3.9
Creatine Kinase System in Brain Cells

Similar to cardiac cells, brain cells exhibit high levels of CK activity, represented in the mitochondria by the ubiquitous isoenzyme uMtCK and in the cytosol by BB-CK [234–238]. However, the role of the CK system in brain cells has been investigated to a much lesser extent than in cardiac cells [103, 105, 236–238], likely due to the high degree of tissue heterogeneity. In our recent studies we adopted the method of Clark for purification of rat brain synaptosomes and synaptosomal mitochondria. These purified preparations of synaptosomes contain a large amount of mitochondria (Fig. 3.11A) with high respiratory activity, which in the presence of ATP is well controlled by creatine due to the MtCK reaction (Fig. 3.11B). Figure 3.11C shows that the rate of CK for PCr production is enhanced with oxidative phosphorylation in synaptosomal mitochondria (Fig. 3.6). This shows that in the brain synaptosomes, the MtCK reaction is also tightly coupled to oxidative phosphorylation via ANT and the phosphocreatine–creatine kinase pathway is actively operative (Fig. 3.11D).

Fig. 3.11 Creatine kinase in brain synaptosomes. (A) Confocal imaging of mitochondria in rat brain synaptosomes. Mitochondria were visualized by the mitochondria-specific probe MitoTracker Green. Merging of transmission and fluorescent confocal images is shown. (B) Recordings of the rate of respiration of isolated rat brain mitochondria activated by exogenous ADP (left panel) and creatine at different concentrations in the presence of ATP (right panel). Respiration rates were recorded using a two-channel, high-resolution respirometer (Oroboros Oxygraph-2k, Oroboros, Innsbruck, Austria). (C) Comparison of the kinetics of regulation of the rate of CK reaction in isolated mitochondria of rat brain by exogenous ATP in the presence of 10 mM Cr in the presence or absence of oxidative phosphorylation (OP). (D) Schematic presentation of energy transfer in brain synaptosomes. Mitochondrial coupled reactions are very close to those in heart mitochondria (see explanation in the text). The energy-consuming reactions are related to the ion transport across the outer membrane and exocytose of glutamate (10) ▌AQ3▌ at the expense of ATP, produced locally by BB-CK from PCr. (Reproduced from [238] with permission).

3.3.10
Maxwell's Demon and Organized Cellular Metabolism

Vectorial metabolism, metabolic channeling, and functional coupling may well be explained and illustrated by Maxwell's demon, a general and important philosophical concept in the history of physical sciences. In 1994 Azzone and Mae-Wan Ho [239, 240] discussed the idea that Maxwell's demon is behind Schrödinger's principle of negentropy in living cells. This metaphor is useful for explaining general mechanisms that help the cell to keep entropy low through a very precise structural organization of all metabolic networks [101, 238].

In 1871 James Clerk Maxwell analyzed, in his book *Theory of Heat*, the nature of the second law of thermodynamics and described the following imaginary situation. In the state of thermodynamic equilibrium, all parameters of the system, such as temperature and pressure, have constant values and no work is possible. This is due to the constant average value of the kinetic energy. However, this average value is of a statistical nature due to the large number of molecules with different rates that distribute according to the Boltzmann function. Maxwell proposed consideration of the following situation: the homogenous system is divided into two parts separated by a small hole that can be closed or opened by a hypothetical being of intelligence but of molecular order. This hypothetical being, which was later nicknamed a "demon" by William Thomson, permits the molecules with a rate higher than the average to traverse the hole but closes it for the molecules with rates lower than the average. In this way Maxwell's demon disturbs the equilibrium by increasing the order, thus creating a temperature difference between the two compartments and making work possible without using an external energy supply. This imaginary experiment immediately initiated vivid philosophical discussions, which have lasted until today, has been particularly useful in information theory, and is often used for analysis of biological systems. It has been discussed by Szilard and Brillouin that the energy demanded for obtaining the information about molecules would be greater than that gained, and this could make Maxwell's demon's actions ineffective [241]. However, the information needed for the demon may be given by the proper system's organization [239]. In this case, its aim is not to break the second law of thermodynamics but to save the energy and help to avoid increasing the entropy. Most importantly, however, the Maxwell's demon metaphor is not in disagreement with either kinetic or thermodynamic aspects of metabolic channeling in organized multienzyme complexes and networks.

Equilibrium of an enzymatic reaction proceeding in a homogenous medium implies that the average concentrations of the substrates and products involved in the reaction are constant all over the cellular cytoplasm, i.e., determined by the equilibrium constant value and thus the value of the standard free energy change ΔG° of the reaction. It may be assumed that any given enzyme molecule catalyzes on average an equal number of direct and reverse reactions in a time unit (principle of microscopic reversibility) in a random manner.

At non-equilibrium steady states, the net flow through the reaction is steady but non-zero. Such a situation is kept through a continuous supply of substrate and removal of product. The multiple components of an enzymatic reaction system leave Maxwell's demon a much larger choice of parameters to play with than it had in the classical situation of Maxwell's time. The most interesting and important game could be to look at each enzyme molecule and decide in which direction it will catalyze the reaction, simply by giving it a necessary substrate and removing the product from it at the same time. If the demon wishes, it can constantly keep any given enzyme molecule working irreversibly in one direction, as in endergonic, energy-dependent reactions. Other enzyme molecules can be kept working in the reverse direction, to keep the metabolic system in the overall permanent steady state. In fact, it is the precise structural organization of the metabolic system that creates Maxwell's demon. This principle of intelligence, the concept of Maxwell's demon, is well realized in compartmentalized energy transfer pathways, such as creatine kinase systems with structurally fixed (bound) creatine kinase isoenzymes interacting with the adjacent ATP-producing, -transporting, or -consuming systems, as illustrated in Fig. 3.12. In this figure the Maxwell's

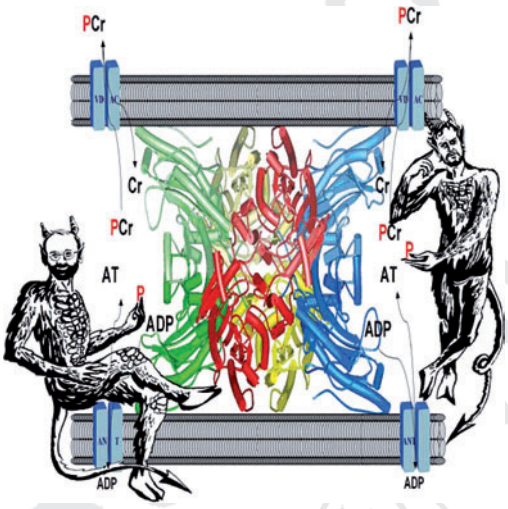


Fig. 3.12 Principle of Maxwell's demon as a metaphor for explaining and illustrating the functional coupling and metabolic channeling phenomena. The coupled MtCK reaction and ANT are shown. The hypothetical being of molecular size (Maxwell's demon) takes ATP molecules from ANT and puts them into the active site of creatine kinase for transfer of the terminal phosphate of ATP to creatine. In exchange, it takes ADP from CK and puts it back into the ANT. The intelligence of Maxwell's demon is realized by the precise structural arrangement of MtCK and ANT. For further explanation, see text. The structural image of the octameric form of the MtCK is courtesy of Theo Wallimann (Zurich, Switzerland) and Uwe Schlattner (Grenoble, France). (This figure was drawn by Tatiana Samoilova, Grenoble, France, and published with her permission).

demon metaphor is used to describe the functional coupling between MtCK and ANT, which is analyzed in detail in this chapter. ANT, which supplies the substrate ATP for MtCK and removes the product ADP from local microcompartments, fulfils the intelligent role of the demon directing the MtCK reaction uphill from equilibrium, as described in Figs. 3.2 and 3.7. At the same time, by using ATP and supplying ADP for ANT, MtCK fulfils the same role of the demon for ANT. For regulation of mitochondrial respiration in many cells with high-energy fluxes, this is the key mechanism (see also ▌Chapter 11▌). The demons shown in Fig. 3.12 are very good ones: by recycling ADP in mitochondria they help to avoid ROS production and protect the cells from unpleasant outcomes. Similar schemes may be used for description of the roles of other CK and AK isoenzymes (see Fig. 3.10) in other subcellular compartments and for the phenomena of metabolic channeling in general.

Thus, the intelligence of Maxwell's demon is realized in the proper structural organization of cellular metabolic systems. By controlling the direct supply of substrate to the enzyme and removing the product, the intelligence of Maxwell's demon helps to avoid an unnecessary increase in entropy and thus helps to increase the efficiency of energy transduction. In Schrödinger's days, the fine structural organization of metabolism was unknown, but it clearly contributes to the foundations of the basic law that he discovered [7].

Acknowledgments

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Articles 4 et 5

Découverte du facteur X

Tubulin binding blocks mitochondrial voltage-dependent anion channel and regulates respiration

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Regulation of mitochondrial outer membrane (MOM) permeability has dual importance: in normal metabolite and energy exchange between mitochondria and cytoplasm and thus in control of respiration, and in apoptosis by release of apoptogenic factors into the cytosol. However, the mechanism of this regulation, dependent on the voltage-dependent anion channel (VDAC), the major channel of MOM, remains controversial. A long-standing puzzle is that in permeabilized cells, adenine nucleotide translocase (ANT) is less accessible to cytosolic ADP than in isolated mitochondria. We solve this puzzle by finding a missing player in the regulation of MOM permeability: the cytoskeletal protein tubulin. We show that nanomolar concentrations of dimeric tubulin induce voltage-sensitive reversible closure of VDAC reconstituted into planar phospholipid membranes. Tubulin strikingly increases VDAC voltage sensitivity and at physiological salt conditions could induce VDAC closure at <10 mV transmembrane potentials. Experiments with isolated mitochondria confirm these findings. Tubulin added to isolated mitochondria decreases ADP availability to ANT, partially restoring the low MOM permeability (high apparent K_m for ADP) found in permeabilized cells. Our findings suggest a previously unknown mechanism of regulation of mitochondrial energetics, governed by VDAC and tubulin at the mitochondria–cytosol interface. This tubulin–VDAC interaction requires tubulin anionic C-terminal tail (CTT) peptides. The significance of this interaction may be reflected in the evolutionary conservation of length and anionic charge in CTT throughout eukaryotes, despite wide changes in the exact sequence. Additionally, tubulins that have lost significant length or anionic character are only found in cells that do not have mitochondria.

evolution | microtubules | oxidative phosphorylation | VDAC | tubulin C-terminal

Oxidative phosphorylation requires transport of metabolites, including cytosolic ADP, ATP, and inorganic phosphate, across both mitochondrial membranes for F_1F_0-ATPase to generate ATP in the matrix. Voltage-dependent anion channel (VDAC, also called mitochondrial porin) is the most abundant protein in mitochondrial outer membrane (MOM) and is known to be primarily responsible for ATP/ADP flux across the outer membrane (1, 2). Until recently, VDAC was generally viewed as a part of the pathway for release of cytochrome *c* and other apoptogenic factors from the mitochondrial intermembrane space into the cytosol at the early stage of apoptosis. The recent genetic studies undermined this view (3) but still left open a lot of questions concerning the role of VDAC in MOM permeabilization in apoptosis (4–6). A conserved property of VDACs in vitro is the ability to adopt a unique fully open state and multiple states with significantly smaller conductance (7). It was demonstrated that the latter, so called "closed states" are impermeable to ATP but still permeable to small ions (8), including Ca^{2+} (9).

In isolated mitochondria, respiration is characterized by an apparent K_m for exogenous ADP that is ≈10-fold lower than in permeabilized cells with oxidative metabolism (10, 11). The discrepancy is most likely caused by a lower permeability of the MOM (and hence VDAC) for ADP in the permeabilized cells and may be the result of the interaction of mitochondria with some cytoskeletal protein(s). Such a conjecture is supported by the action of mild trypsin treatment of permeabilized cells, which causes decrease in apparent K_m for ADP accompanied by mitochondrial rearrangement, implicating the existence of a protein factor(s) associated with the cytoskeleton (10). The decreased value of apparent K_m for ADP after mild proteolytic treatment was similar to K_m in isolated mitochondria and purified preparations of adenine nucleotide translocase (ANT) (10), thus showing that the kinetic values of ANT are similar in all preparations. Therefore, high values of apparent K_m for ADP in permeabilized cells are related to decreased permeability of MOM, possibly because of interaction with cytosolic proteins.

Tubulin, the subunit of microtubules, is a plausible candidate because this cytoskeletal protein is known to interact with mitochondria (12, 13). In addition to mitochondrial membranes, tubulin has been shown to interact with proteins associated with other membranes (14, 15). Tubulin is a heterodimer, composed of similar 50-kDa globular α and β subunits. Each subunit possess a negatively charged, extended C-terminal tail (CTT) that represents ≈3% of the subunit mass and ≈40% of the subunit charge (16, 17). Tubulin shows saturable, high-affinity binding to intact mitochondria (12) and hence to MOM, although the region of tubulin responsible for binding has not yet been identified. Immunoprecipitation experiments demonstrated a specific association of VDAC with tubulin (13), which indicates that VDAC could be a receptor for tubulin binding to MOM. Exploring this possibility, we studied the functional interaction between VDAC and tubulin to show that nanomolar concentrations of dimeric* tubulin induce highly voltage-sensitive reversible closure of VDAC reconstituted into planar phospholipid membranes.

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*Tubulin in all experiments was dimeric, not polymerized, because there was no GTP or Mg^{2+} added, and the total tubulin concentration was at least 100-fold below the critical concentration for polymerization in the presence of GTP and Mg^{2+}.

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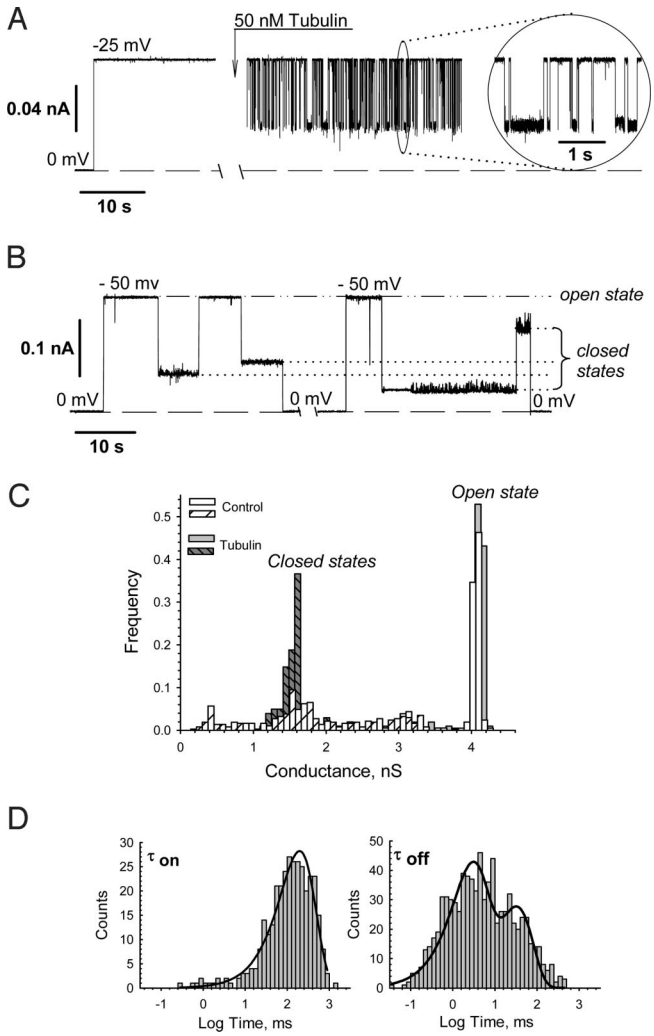

Fig. 1. Tubulin at nanomolar concentrations induces reversible partial blockage of VDAC. (*A*) Representative current record through a single channel before and after addition of 50 nM tubulin to the *cis* side at −25 mV applied voltage. Addition of tubulin induces time-resolved rapid events of partial channel blockages to a single well-defined conductance level, shown in the *Inset* at a finer scale. Here and elsewhere, dashed lines indicate zero-current level; current records were filtered by using averaging times of 10 ms (except for 1 ms in *Inset*). (*B*) Typical VDAC voltage gating in tubulin-free solution under periodically applied −50 mV voltage impulses. Under applied voltage, channel conductance moves from a single high-conducting open state to various low-conducting closed states. Relaxing the voltage to 0 mV reopens the channel. The dashed-and-dotted line indicates the conductance of the channel in its open state and dotted lines in its closed states. (*C*) Tubulin induces a single well-resolved closed state of VDAC, whereas in control voltage-induced gating a wide variety of closed states is observed. The distribution of conductances in control was collected from a series of −50 mV impulses of 30 s duration interrupted by 70 s periods of zero applied voltage to restore the open state. (*D*) Statistical analysis of the tubulin-induced closures of VDAC performed by logarithmic exponential fitting of the open times (τ_{on}) and closed times (τ_{off}). It is seen that open time is described by single exponent with characteristic time $\tau_{on} = 198.7 \pm 3.5$ ms. Closed-time histogram could be fitted by at least 2 exponents with characteristic times of $\tau_{off}^{(1)} = 2.4 \pm 0.1$ ms and $\tau_{off}^{(2)} = 30.4 \pm 5.7$ ms. The time histograms were collected from the current traces presented in *A*. Bilayer membranes were formed from the mixture of asolectin and cholesterol (10:1 wt/wt). VDAC was isolated from rat liver mitochondria. The medium consisted of 1 M KCl buffered with 5 mM Hepes at pH 7.4.

Results and Discussion

Tubulin Induces Reversible Blockage of VDAC Reconstituted into Planar Phospholipid Membranes. A representative experiment is shown in Fig. 1*A*, where addition of 50 nM tubulin to the *cis* side (the side of VDAC addition) of the membrane induced fast reversible blockage of VDAC conductance, markedly different from VDAC gating induced by voltage alone (Fig. 1*B*). Although voltage-induced and tubulin-induced VDAC closures are both voltage-dependent, the properties of these 2 processes are very different. First, tubulin greatly enhances VDAC voltage sensitivity, inducing channel closure at potentials as low as 10 mV. Without tubulin, VDAC remains open at the conditions of Fig. 1*A* for up to 1 h. Typical VDAC voltage gating without tubulin is shown in Fig. 1*B*, where application of 50 mV switches the channel from the open to low conducting, "closed" states. Once closed, VDAC will typically stay closed until the voltage is relaxed to 0 mV. Second, tubulin induces a single closed state of VDAC (0.4 of open-state conductance), seen in the distribution of conductances in Fig. 1*C*. In contrast, the control "normal" voltage gating exhibits a wide variety of closed states (Fig. 1 *B* and *C*). Open-state conductance is unaltered by tubulin addition.

This answers a long-standing quandary: how could VDAC voltage gating observed in planar lipid bilayer systems at relatively high potentials (>30 mV) be relevant to the situation in vivo, and in isolated mitochondria, where the most probable values of potential difference across MOM were estimated to be smaller than 10–20 mV (18)?

The binding parameters for tubulin-induced closure depend strongly on tubulin concentration, electrolyte concentration, and applied voltage (Fig. 2). The distributions of open time (τ_{open}) are satisfactorily described by single exponential fitting (Fig. 1*D Left*, continuous lines). As expected for simple bimolecular binding causing VDAC closure, τ_{open} decreases as tubulin concentration increases (Fig. 2*A a* and *d*). Open time decreases by 1 order of magnitude as tubulin concentration increases 10 times. Channel closed time, which is the residence time for the tubulin bound to VDAC pore, is independent of tubulin concentration, as expected for first-order dissociation of the tubulin–VDAC complex (Fig. 2*A e*, *b*, *f*, and *c*). Distributions of closed time require 2 exponentials for fitting (Fig. 1*D Right*, continuous line), indicating, most probably, that there are 2 forms of tubulin that differ in residence time. This could be the result of differential binding of the α- versus β-tubulin CTT. The β-CTT contains ≈40% more negative charge than α-CTT and may remain bound longer because of that. Further experiments will be required to resolve this point.

The involvement of electrostatic interactions in VDAC–tubulin binding is indicated by the strong dependence of τ_{open} on salt concentration (Fig. 2*A*). High-salt concentration screens charges on tubulin and VDAC, decreasing binding. The increased screening leads to a >100-fold rise in τ_{open} when KCl was increased 4-fold but does not affect either component of τ_{closed} (Fig. 2*A*).

With symmetrical tubulin addition, the closed time did not depend on the polarity of the applied voltage, at least within the measurement accuracy (Fig. 2*B*). After asymmetric addition, when tubulin was added from one side of the membrane only, the blockage events were observable only when potential on the side of tubulin addition was more negative than on the other side, with a strong exponential dependence of the on-rate on the applied voltage (Fig. 2*C*). Under these conditions, the closed time did not depend on the side of tubulin addition if the polarity of the applied voltage was reversed accordingly (Fig. 2*B*).

The tubulin dimer of 100-kDa molecular mass and approximate dimensions of 8 nm × 4.5 nm × 6.5 nm cannot permeate through the VDAC pore of 2.5- to 3.0-nm diameter. However, the CTT are long enough (17) to span the membrane channel and narrow enough to fit into the lumen of the channel, which permits us to suggest that the average time of tubulin-induced channel closure represents the average time of CTT residence in the channel lumen. Extrapolation of the on-rate and residence time dependencies to 0 voltage gives an estimate for the equilibrium constant of tubulin–VDAC binding as 0.5 and 2.5 nM^{-1} (for the 2 τ_{closed}). These results allow us to suggest that tubulin-binding site(s) are equally accessible

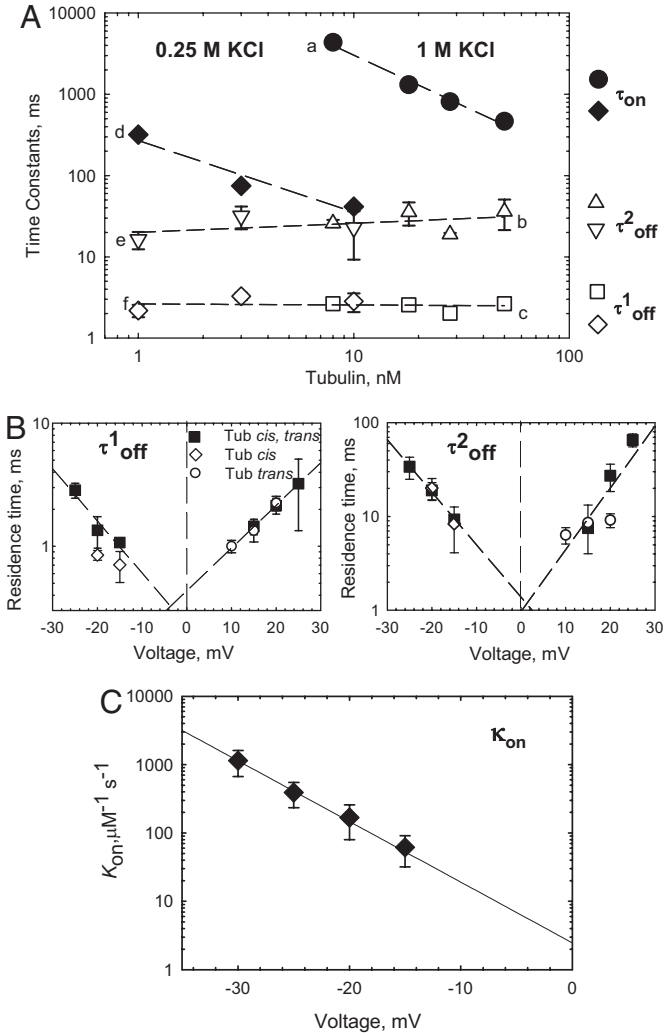

Fig. 2. The binding parameters for tubulin-induced VDAC closure depend on tubulin concentration, electrolyte concentration, and applied voltage. (*A*) (*a* and *d*) VDAC open time between successive blockages, τ_{on}, linearly decreases with tubulin concentration and depends on salt concentration. The medium consisted of 1 M KCl (*a*, filled circles) and 0.25 M KCl (d, filled diamonds). Both components of tubulin residence (closed) time, $\tau_{off}^{(1)}$and $\tau_{off}^{(2)}$, are independent of tubulin or salt concentration (*b*, *c*, *e*, and *f*, open symbols) and are equal to 2.8 ± 0.5 ms and 23.7 ± 6.3 ms, respectively. The applied voltage was −20 mV. (*B*) Voltage dependence of VDAC residence times, $\tau_{off}^{(1)}$and $\tau_{off}^{(2)}$, in the presence of 50 nM tubulin in *cis* (open diamonds), *trans* (open circles), or both sides (filled squares) of the membrane. Residence time in extrapolation to 0 voltage does not depend on 1-side (*cis* or *trans*) or 2-side tubulin addition. (*C*) Voltage dependence of the on-rate, κ_{on}, with 50 nM tubulin added to the *cis* side. The line is an exponential fit to $\kappa_{on} = \kappa_0 \exp(nVF/RT)$ with $n = 4.97$. Each time value presents the characteristic time of 9 different log probability fitting procedures ± SE. The medium consisted of 1 M KCl (*B* and *C*) and buffered with 5 mM Hepes at pH 7.4. VDAC was isolated from *N. crassa* mitochondria. Bilayer membranes were formed from DPhPC.

from both sides of the channel and that anionic CTT are mainly responsible for VDAC blockage (see Fig. 4).

Role of CTT in Tubulin–VDAC Interaction. To test the CTT-dependent mechanism, we studied the effect of tubulin with truncated C termini, tubulin-S, on VDAC conductance. Comparison of VDAC current traces in the presence of tubulin-S (Fig. 3*A*) and intact tubulin (*viz.*"tubulin") (Fig. 3*C*), obtained under the same experimental conditions, shows a striking difference. Tubulin-S did not induce reversible blockage typical for tubulin, but instead generated

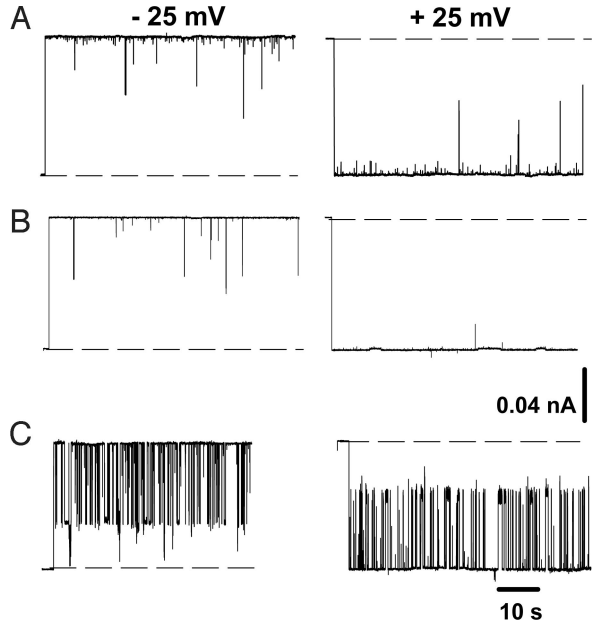

Fig. 3. Tubulin interaction with VDAC requires the presence of C-terminal tails of tubulin. Tubulin (50 nM) with truncated CTT, tubulin-S (*A*), or a mixture of 10 μM 2 synthetic peptides of mammalian α- and β-brain tubulin CTT (*B*) does not induce channel blockage characteristic for intact tubulin (10 nM), with fast reversible blockage to 1 closed state (*C*). Representative current traces through single VDAC were obtained at ±25 mV of applied voltage in 1 M KCl solutions buffered with 5 mM Hepes at pH 7.4. Tubulin, tubulin-S, and CTT peptides were added to both sides of the membrane. Other experimental conditions were as in Fig. 2 *B* and *C*.

shorter current interruptions with τ_{closed} <1 ms and rather broad distribution of conductances of the closed states [supporting information (SI) Fig. S1*a*]. An excess of current noise in the presence of tubulin-S indicates that the tubulin globular body interacts with VDAC even without CTT. However, these interactions most likely are of different nature than those with intact tubulin. Accordingly, experiments on multichannel membranes confirmed that tubulin-S does not affect VDAC voltage-gating parameters such as gating charge and the characteristic voltage at which half-channels are open and half-closed (Fig. S1*b* and Table S1). Results similar to tubulin-S (i.e., the absence of reversible channel blockage) were obtained with actin (Fig. S2), also an acidic protein but lacking CTT. This agrees with a previous report of actin interaction with VDAC from *Neurospora crassa* (19).

It was shown that a number of different synthetic and natural polyanions nonspecifically increase VDAC voltage gating (7). This raises a question about specificity of VDAC closure by tubulin, considering that CTT are highly negatively charged. To address this, we studied the effect of CTT synthetic peptides on VDAC properties. Two synthetic peptides with the sequences of mammalian α and β brain tubulin CTT did not induce detectable channel closure up to micromolar concentrations. In the experiment presented in Fig. 3*B*, a mixture of 2 synthetic peptides (10 μM each) corresponding in sequence to mammalian α and β brain tubulin CTT was added to both sides of the membrane, but the current traces through single channels were indistinguishable from control traces. This suggests that CTT peptides translocate through the channel too fast, in a <0.1 ms time scale, and do not block it in a time-resolved manner. Experiments on multichannel membranes confirmed that VDAC voltage-gating parameters were also not affected by CTT peptides (Fig. S3). Thus, the results with tubulin-S, actin, and CTT synthetic peptides support our model of tubulin CTT interaction with VDAC leading to the voltage-dependent channel closure.

When tubulin CTT penetrates into the VDAC lumen (Fig. 4), its

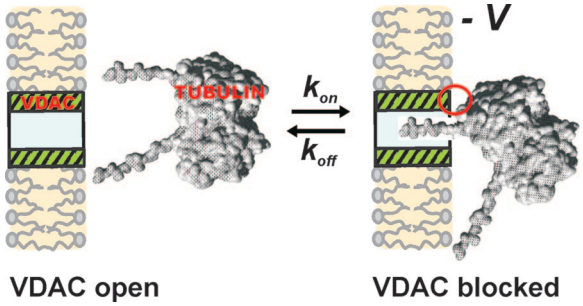

Fig. 4. Model of tubulin–VDAC interaction. One tubulin CTT partially blocks channel conductance by entering VDAC pore. This process is voltage-dependent and could be described by the 1st-order reaction of one-to-one binding of tubulin to VDAC. Some additional interaction between tubulin globular body and VDAC may be involved. The model of a tubulin dimer was redrawn from ref. 17.

negative charge changes channel ion selectivity. The residual conductance of the channel during tubulin block was cation-selective with the permeability ratio of $P_K/P_{Cl} = 2.4 \pm 0.7$ and reversal potential of (-14 ± 4) mV in 1.0 M *cis*/0.2 M *trans* gradient of KCl. This should be compared with the anion selectivity of VDAC open state, which is characterized by $P_K/P_{Cl} = 0.5 \pm 0.02$ and reversal potential of (12 ± 1) mV. The selectivity inversion further supports the proposed model where VDAC closure arises from interaction of negatively charged CTT with net positively charged channel interior (Fig. 4). A similar effect on VDAC selectivity was described when negatively charged phosphorothioate oligonucleotides blocked VDAC (20).

The effect of tubulin blockage of VDAC was confirmed with VDAC isolated from mitochondria from rat liver (see traces in Fig. 1*A*) and heart and from *N. crassa* (see traces in Fig. 3*C*), and with tubulin isolated from bovine and rat brain, which gave indistinguishable results. Tubulin isolated from *Leishmania tarentolae* and *Leishmania amazonensis* exhibited VDAC blockage qualitatively similar to mammalian tubulins, but quantitatively there were some differences, possibly because of the extensive posttranslational modifications (PTMs) in CTT of *Leishmania* tubulin.

Tubulin Decreases Respiration Rate of Isolated Mitochondria. Our results demonstrate that tubulin can induce reversible blockage of VDAC and hence should be able to regulate ATP/ADP flux across MOM. Can tubulin do so in the intact mitochondria? To answer this question, we conducted experiments on isolated mitochondria. Oxygen consumption of isolated brain and heart mitochondria in response to successive additions of ADP was measured by using oxygraph. The value of apparent K_m for exogenous ADP represents the availability of ADP for ANT to activate oxidative phosphorylation. The classic value of the K_m for ADP of isolated mitochondria is ≈10–20 μM (21), and the linearization of its kinetic of respiration is shown in Fig. 5*A*. Addition of 1 μM tubulin resulted in the appearance of a second component of mitochondria respiration kinetics with K_m of (169 ± 52) μM, >20 times higher than the first component $(7 \pm 2$ μM; Fig. 5*B* and Table 1). The high K_m component accounts for ≈30% of the total ADP flux in mitochondria. Similar results were obtained with mitochondria from heart (Table 1). Thus, there is tubulin binding to mitochondria as seen from the second component with high K_m. Furthermore, the absence of variation of the maximal rate of respiration, V_{max} (Table 1) between the control and the second component of the respiration kinetics in presence of tubulin, highlights the integrity of the respiratory chain (22). A possible explanation for the persistence of the first component (population of VDAC unaffected by tubulin) could be different affinity of tubulin to VDAC isomers 1, 2, and 3. There could also be other factors that compete with tubulin for VDAC binding, like hexokinases, which have been reported to bind

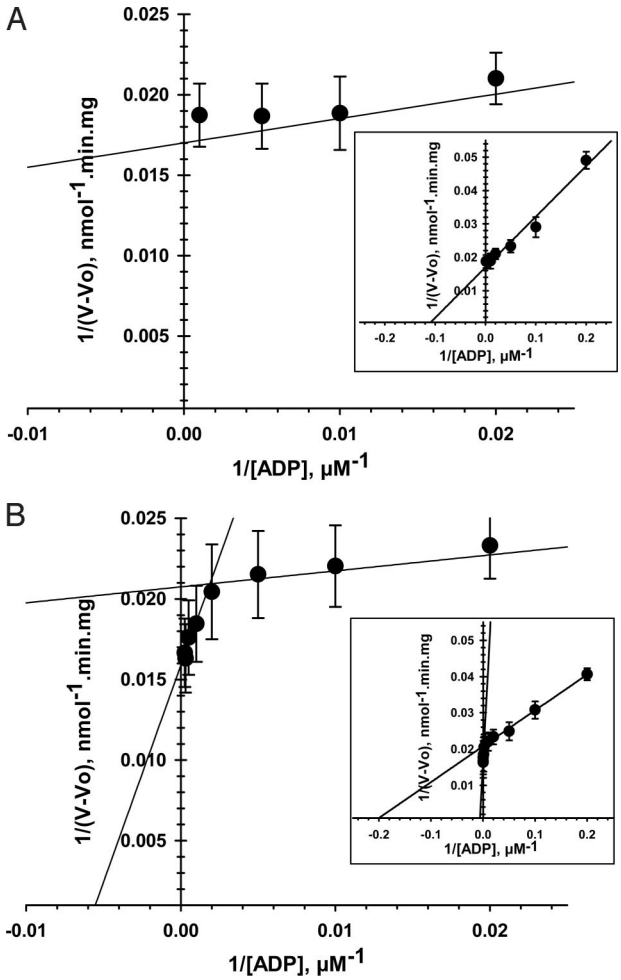

Fig. 5. Tubulin dramatically increases apparent K_m for ADP in regulation of respiration of isolated brain mitochondria. Shown are double-reciprocal representations of the respiration kinetics of brain mitochondria activated by ADP in control (*A*) and in the presence of 1 μM tubulin (*B*). The 2 straight lines represent 2 different respiration kinetics in the presence of tubulin (*B*). (*Insets*) Enlargements of *A* and *B*. Each data point is a mean of 6–9 independent experiments ±SE.

to MOM directly through VDAC or close by (23), possibly obstructing tubulin access to VDAC. However, the precise nature of the 2 components is not clear at this time and needs further careful studies.

The interaction of other cytosolic proteins with VDAC may be important for regulation of tubulin–VDAC binding. Tubulin is an abundant and stable protein, present in many cells at concentrations higher than those shown here to block VDAC. We suggest that tubulin–VDAC interaction may be modulated through competition with other proteins that bind to VDAC, preventing tubulin binding. Another possibility is that PTM of VDAC or tubulin may alter this interaction. This possibility is particularly intriguing because PTMs of tubulin mostly occur on the CTT (24), the locus of interaction with VDAC. Under physiological conditions, tubulin concentration does not change dramatically. However, it is worth mentioning here that in cells exposed to microtubule-targeting drugs such as colchicine or paclitaxel (Taxol), the balance between tubulin dimers and polymerized tubulin can change. This might affect cytosolic tubulin dimer concentration and alter tubulin–VDAC interaction.

Evolutionary Considerations. These results raise interesting evolutionary questions. Tubulin is a very old protein, found in all eukaryotes (25). This raises the question of whether the interaction

Table 1. Kinetic parameters of respiration in brain and heart isolated mitochondria with 1 μM tubulin and without it

	Apparent K_m (ADP), μM	V_{max}, nmol O_2/min/mg
Brain mitochondria (n = 6–9)		
Without tubulin	9 ± 1	55 ± 5
With tubulin		
First component	7 ± 2	49 ± 15
Second component	169 ± 52	61 ± 15
Heart mitochondria (n = 4–6)		
Without tubulin	11 ± 2	216 ± 11
With tubulin		
First component	9 ± 5	136 ± 9
Second component	330 ± 47	241 ± 10

Each value is a mean of n experiments ± SE.

we show here plays a role throughout the eukaryotic world. In support of this, we have shown that similar results are obtained with VDACs from rat or *Neurospora*, and with tubulin from rat or *Leishmania*. We can also approach this question by comparing the sequences of VDAC and tubulin from many species.

VDAC sequences (nearly 250) are known from plants, animals, fungi, stramenopiles, alveolates, rhodophyta, and charophyta, and functional data exist for VDAC from a number of organisms (26). Functional and structural properties of VDAC are preserved, although a great deal of sequence variation is found.

Tubulin sequences are known from throughout eukaryotes and reveal remarkable sequence conservation (27). A notable exception is the region of tubulin in the CTT, which shows significant sequence variation, both between isotypes in a given species and between species.

Although CTT sequences vary significantly, the functional properties that we found to be involved in VDAC–tubulin interaction are remarkably conserved throughout eukaryotic α- and β-tubuins, with a few significant exceptions to be discussed below. This is shown in alignments of β-CTT in Fig. 6*A* and in more extensive alignments of α- and β-CTT in Fig. S4. Our analysis shows that CTT length and charge are highly conserved in cells that contain mitochondria. Summary statistics for ≈100 α- and β-CTT sequences from our alignments and from ref. 27 are presented in Fig. 6*B*.

Tubulins with highly altered length or charge and that are the sole α or β gene in the organism are found only in organisms that do not have mitochondria (although not all organisms that lack mitochondria have altered CTT; see Fig. S4). Examples of altered tubulin CTT are from obligate intracellular eukaryotes that lack functional mitochondria and contain tubulin CTT much shortened in length and reduced in charge, including total loss of charge (Fig. 6 and Fig. S4). In addition, genes for α- and β-tubulins are found in bacteria (*Prosthecobacter* species) (28), likely the result of horizontal gene transfer from an unknown eukaryote (29). Although their function in the bacterial cell is unclear, these tubulins have essentially the same 3D structure as mammalian tubulin and exhibit GTP-dependent polymerization (29). However, the CTT differ radically from mammalian tubulin, perhaps in part because of the absence of mitochondria and hence loss of selection to maintain a CTT that can interact with VDAC. Both length and charge of the CTT, features that are highly conserved in eukaryotes, seem free to vary widely in *Prosthecobacter*, ranging from total loss to significant extension in length and acquisition of positive charges (Fig. 6*A*).

We suggest that the conservation of CTT length and charge in tubulins from organisms with functional mitochondria is caused in part to selective pressure to maintain tubulin CTT–VDAC interaction and thereby cytoplasmic regulation of mitochondrial energetics [although clearly other factors are also important, such as the evolutionarily conserved PTM that occur here (30)]. Conversely, the divergence in length and charge seen in some eukaryotes that lack functional mitochondria and in bacteria is because of the absence of this selective pressure.

Conclusions

We find that nanomolar concentrations of tubulin vastly increase VDAC sensitivity to voltage and could induce VDAC closure at low transmembrane potentials. Tubulin interaction with VDAC requires the presence of anionic CTT on the intact protein. This suggests a completely new role for dimeric tubulin and its charged CTT. Established roles for tubulin CTT have been dominated by interactions with microtubule-severing proteins (31), motor proteins (32), and plus-end-binding proteins (33), as well as being the

A **Sequence alignments of beta-tubulin CTT**

Eukaryotes with mitochondria	
Homo sapiens	ATADEQGEFEEEGEDEA
Gallus gallus	ATANDGEEAFEDDEEEINE
Danio rerio	ATADDEADFGEEGE
Xenopus laevis	ATADEQGEFEEEEDEA
Drosophila melanogaster	ATADEDAEFEEEQEAEVDEN
Homarus americanus	ATADDEAEFEEEGEVEGEYA
Zea mays	ATAEEYDEEEQDGEEEHD
Schizosaccharomyces pombe	AGIDEGDEDYEIEEEAEPLEY
Brugia malyi	ATADEEQDLQEGESEYIEQEE
Tetrahymena thermophila	ATAEEEGEFEEEEGEN
Leishmania tarentolae	ATVEEEGEYDEEEEPT
Eukaryotes lacking mitochondria	
Guillardia theta	AKMDNDAFEDQDLY
Enterocytozoon bieneusi	SQVGGY
Encephalitozoon cuniculi	ATIEDADDFLVN
Bacteria	
Prosthecobacter dejongeii (BTubB)	---
Prosthecobacter dejongeii (BTubA)	SQAAAAVQDSAGDTQMEAAAA-GVSDDAEGSMSLEDLVDRRF

B **Summary of alpha and beta CTT properties**

	Alpha (n = 86)	Beta (n = 132)
Charge [1]	-7.2 ± 1.4	-10.3 ± 1.5
Length (residues) [1]	9.9 ± 1.6	19.2 ± 2.8

1. Values are mean ± s.d.

Fig. 6. Analysis of tubulin CTT sequences. (*A*) Sequence alignments of β-tubulin CTT. Full sequences were aligned, but only the CTT, defined as the residues from C-terminal to the last residue in the crystal structure, are shown. Acidic residues are shown in red, basic residues in blue. More extensive alignments of both α-and β-CTT are in Fig. S4. (*B*) Summary of length and charge values for α- and β-CTT, taken from our alignments and from the data in ref. 27. Each value is a mean ± SD; *n*, number of species.

site of PTM (34). We propose a model for tubulin–VDAC interaction in which the tubulin CTT penetrates into the channel lumen, potentially reaching through the channel because of the length of the CTT, interacting with a positively charged domain of VDAC. Because of this interaction, VDAC has a high affinity for CTT, which partially blocks channel conductance (Fig. 4). Identification of which CTT, α, β, or both, blocks VDAC is a subject of future research.

The tubulin-induced increase of K_m for ADP in isolated mitochondria is evidence of a significant drop of the availability of ADP to ANT and partial restoration of the K_m found in permeabilized cells. Thus, a mechanism for increasing K_m by decreasing ATP/ADP permeability through MOM is closure of VDAC by tubulin. By this type of control, tubulin may selectively regulate metabolic fluxes between mitochondria and cytoplasm (35, 36).

Conservation of tubulin CTT length and charge and VDAC folding pattern throughout mitochondria-containing eukaryotes suggest that this interaction is widespread and ancient. Our experimental results with tubulins and VDAC from distantly related organisms demonstrate this. The loss of tubulin CTT properties only in organisms that lack mitochondria reinforces this view. Our results not only identify a previously unknown mechanism of regulation of MOM permeability and mitochondria respiration, but also identify a functional role for the cytoskeletal protein, dimeric tubulin.

Materials and Methods

Protein Purification. VDAC from rat liver or *N. crassa* mitochondrial outer membranes was isolated and purified as described in ref. 37 and were a generous gift of M. Colombini (University of Maryland, College Park, MD). Bovine brain tubulin and rabbit muscle actin were obtained from Cytoskeleton. Rat brain tubulin was purified as described in ref. 38 (see *SI Materials and Methods*). Tubulin-S was produced by subtilisin cleavage of rat brain tubulin as described in ref. 39. Peptides corresponding to the CTT of α- and β-tubulins were obtained from Sigma–Genosys. The sequence of the synthetic peptide corresponding to the CTT is that of the bα-1 isotype, EVGVHSVEGEGEEEGEEY, whereas the β-sequence is that of the βI isotype, DATAEEEEDFGEEAEEEA.

Electrophysiological Recordings. Planar lipid membranes were formed from monolayers made from 1% (wt/vol) of lipids in hexane on 70- to 80-μm-diameter orifices in the 15-μm-thick Teflon partition that separated 2 chambers as described in ref. 40. The lipid-forming solutions contained diphytanoyl phosphatidylcholine (DPhPC) or asolectin and cholesterol (10:1 wt/wt). DPhPC and asolectin (soybean phospholipids) were purchased from Avanti Polar Lipids, and cholesterol was purchased from Sigma. VDAC insertion was achieved by adding 0.1–1.5 μL of a 1% Triton X-100 solution of purified VDAC to the 1.2-mL aqueous phase in the *cis* compartment while stirring. Potential is defined as positive when it is greater at the side of VDAC addition (*cis*). After channels were inserted and their parameters were monitored, tubulin was added to one or both sides of the membrane under constant stirring for 2 min. Conductance measurements were performed as described in ref. 40 (see *SI Materials and Methods*). Amplitude, lifetime, and fluctuation analysis was performed by using Clampfit 9.2 (Axon Instruments).

Mitochondria Respiration Measurements. Brain and cardiac mitochondria were isolated as described (11, 41) (see *SI Materials and Methods*). All measurements of respiration rates were performed by using the high-resolution respirometry (Oroboros Oxygraph) with substrates of 10 mM succinate for brain mitochondria and 5 mM glutamate and 2 mM malate for heart mitochondria. Oxygen solubility in the Mitomed solution was taken to be 225 μM at 25 °C. Kinetics of activation of respiration in mitochondria was studied by increasing successively the final concentrations of ADP (5–10−20–50–100-200–500-1,000–2,000–3,000 μM) in the absence or in presence of 1 μM tubulin, with 0.2% of BSA and 1 UI apyrase for ADP regeneration. Mitochondria were incubated with 1 μM tubulin 30 min at room temperature (25 °C) before introduction in the oxygraphy chamber. Mitochondrial apparent K_m for ADP and proportion of each component of respiration kinetics were calculated as described in ref. 42.

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Study of possible interactions of tubulin, microtubular network, and STOP protein with mitochondria in muscle cells

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Abstract We studied possible connections of tubulin, microtubular system, and microtubular network stabilizing STOP protein with mitochondria in rat and mouse cardiac and skeletal muscles by confocal microscopy and oxygraphy. Intracellular localization and content of tubulin was found to be muscle type-specific, with high amounts in oxidative muscles, and much lower in glycolytic skeletal muscle. STOP protein localization and content in muscle cells was also muscle type-specific. In isolated heart mitochondria, addition of 1 μM tubulin heterodimer increased apparent K_m for ADP significantly. Dissociation of microtubular system into free tubulin by colchicine treatment only slightly decreased initially high apparent K_m for ADP in permeabilized cells, and diffusely distributed free tubulin stayed inside the cells, obviously connected to the intracellular structures. To identify the genes that are specific for oxidative muscle, we developed and applied a method of kindred DNA. The results of sequencing and bioinformatic analysis of isolated cDNA pool common for heart and *m. soleus* showed that in adult mice the β-tubulin gene is expressed predominantly in oxidative muscle cells. It is concluded that whereas dimeric tubulin may play a significant role in regulation of mitochondrial outer membrane permeability in the cells in vivo, its organization into microtubular network has a minor significance on that process.

Keywords Cytoskeleton · Mitochondria · Oxidative phosphorylation · Tubulin · Microtubular Network · STOP protein · Heart · Skeletal muscles

Abbreviations

STOP	Stabilizing tubule only polypeptide
CK	Creatine kinase
MtCK	Mitochondrial creatine kinase
ANT	Adenine nucleotide translocase
VDAC	Voltage-dependant anion channel
DTT	Dithiothreitol

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Introduction

In muscle cells, mitochondria functions within highly organized intracellular structures. Their intracellular arrangement is very regular and follows the crystal-like pattern, in accordance with the hypothesis of their organization into functional complexes with sarcoplasmic reticulum and myofibrils, the intracellular energetic units, ICEUs [1]. This specific arrangement can be changed by selective proteolytic treatment of the permeabilized cells that results also in the alteration of the parameters of mitochondrial respiration regulation, decreasing the value of apparent K_m for

exogenous ADP [2]. This phenomenon suggests that cytoskeletal network may participate in the regulation of mitochondrial function in muscle cells. However, this role of cytoskeletal network is still insufficiently documented and therefore requires further studies.

Our previous studies [3, 4] have demonstrated strongly expressed muscle type-specificity in regulation of mitochondrial respiration in vivo, as experiments on permeabilized muscle cells have revealed very low apparent affinity of mitochondrial respiration to exogenously added ADP in oxidative (myocardium, *m. soleus*) *versus* glycolytic muscles (*m. extensor digitorum longus*). Selective proteolysis resulted in increased affinity of mitochondria to ADP in oxidative muscles but exerted no effect to that parameter in glycolytic ones [3, 4]. It was hypothesized that the oxidative muscles, such as myocardium and *m. soleus*, likely express some identical proteins capable of exerting intracellular control over mitochondrial respiration, whereas the glycolytic muscles lack such proteinic compounds. In oxidative muscle cells, these proteins may participate in the organization of the intracellular energetic units, representing the functional and structural complexes of mitochondria with adjacent ATPases [5]. This type of organization likely confers specific regulatory properties of respiration to these muscles [5, 6]. Appaix et al. [7] studied the possible involvement of cytoskeletal proteins in mitochondrial respiration regulation. Several proteins (plectin, desmin, tubulin) were consider as serious candidates. Very recently, Rostotseva et al. [8] have shown that tubulin is able to bind to the voltage-dependant anion channel (VDAC) in the mitochondrial outer membrane and control its permeability [8, 9]. Tubulin, a heterodimer of α and β-isoform with a molecular weight of 55 kDa per monomer, occurs in cells as organized microtubules with a diameter of 25 nm that constantly turn over by polymerization and depolymerization, depending on temperature and ion force. In cardiomyocytes, only 30% of total tubulin is present in the polymerized form as microtubules whereas 70% occurs as non-polymerized cytosolic protein [10]. Microtubules are stable at physiological temperature (37°C) and even at 25°C but undergo a spontaneous cold-induced depolymerisation at 4°C. Microtubular network can be stabilized at cold temperatures in the presence of a MAP (microtubule-associated protein) called STOP for stabilizing tubule only polypeptide, a 145 kDa protein [11]. Recently, MAPs have been found to interact not only with microtubules but also with the actin network and various vesicular compartments [12, 13]. STOP protein is present in many tissues such as brain, heart, muscles, lung, and testis [14]. Studies of muscle physiology performed on STOP-deficient mice before have shown that there are no differences in body weight in comparison with wild-type mice, but some neurological characteristics are well established [15, 16]. The STOP−/− mice exhibit no detectable defects in brain anatomy, but they are devoid of cold-stable microtubules, and they show synaptic defects associated with schizophrenia-like behaviors [15].

With the aim of further investigation of the role of tubulin in regulation of the energetics of muscle cells in dependence of its structural organization, we studied in this work: (i) the connection of microtubules and STOP protein with mitochondria in cardiac and skeletal muscles and (ii) isolated genes that are expressed exclusively in oxidative muscles thereby conferring them specific type of regulation of respiration. For this, STOP-deficient mice and treatment with colchicine were used to better characterize the involvement of tubulin and micro-tubular network in mitochondrial arrangement and regulation.

Materials and methods

Materials

Wistar rats were used in experiments. Control B57/Bl6 mice and STOP-deficient mice were also used in experiments [15]. Wild-type mice from prof. H. Jockusch's group (University of Bielefeld) were used in gene expression studies. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the National Institute of Health (NIH Publication No 85-23, revised 1985).

Isolation of adult rat cardiac myocytes

Calcium-tolerant myocytes were isolated by perfusion with a collagenase medium as described earlier by Kay et al. [17].

Preparation of isolated mitochondria from rat heart and brain

The mitochondria from rat heart were isolated as described in Ref. [18]. Brain mitochondria were isolated from the forebrains as described by Booth and Clark [19].

Preparation of skinned muscle fibers

Skinned (permeabilized) muscle fibers were prepared from cardiac and skeletal muscles according to the method described earlier [20, 21].

Preparation of muscles homogenates

The muscles (heart, soleus, and gastrocnemius) from mice were homogenized with a polytron in a cell solubilizer

buffer (Tris-Triton buffer: Tris 10 mM, pH 7.4, NaCl 100 mM, EDTA 1 mM, EGTA 1 mM, Triton X-100 1%, glycerol 10%, SDS 0.1%, deoxycholate 0.5%). The homogenates were centrifuged for 10 min at 3,000*g* at 4°C.

Colchicine treatment

Permeabilized fibers were incubated for 2 h at 4°C in solution B in the presence of 1 or 10 μM colchicine. Then they were washed thrice in solution B before experiments.

Determination of the rate of mitochondrial respiration

The rates of oxygen uptake were recorded by using a two-channel respirometer (Oroboros oxygraph, Paar KG, Graz, Austria) in solution B containing (in mM) CaK_2EGTA 1.9, K_2EGTA 8.1, $MgCl_2$ 4.0, DTT 0.5, K-MES 100, imidazole 20, taurine 20, K_2HPO_4 3, pH 7.1 adjusted at 25°C or in Mitomed solution containing (in mM): EGTA 0.5 mM, $MgCl_2$ 3 mM, κ-lactobionate 60 mM, taurine 20 mM, KH_2PO_4 3 mM, sucrose 110 mM, DTT 0.5 mM, HEPES 20 mM, pH 7.1, in the presence of glutamate 5 mM and malate 2 mM and 2 mg ml^{-1} of bovine serum albumine (BSA). Determinations were carried out at 25°C, and the solubility of oxygen was taken as 215 nmol ml^{-1} [3].

Confocal microscopy: immunofluorescence labeling of β-tubulin, STOP protein, and desmin and mitochondrial imaging by autofluorescence of flavoproteins

Labeling of cytoskeletal proteins and mitochondrial imaging was performed on permeabilized mice fibers or rat cardiomyocytes in suspension. Cells were fixed for immunofluorescence labeling with 4% paraformaldehyde (PFA) for 20 min at room temperature under mild stirring. Cardiomyocytes or fibers were washed with PBS containing (mM): NaCl 56, KH_2PO_4 1.5, KCl 2.7, and Na_2HPO_4 8 (Biomedia) and incubated in PBS/BSA 2% with primary antibodies of cytoskeletal proteins overnight at 4°C. Monoclonal anti-β-tubulin (mouse IgG1 isotype) antibody (Sigma) at 1:200 and polyclonal anti-STOP (rabbit) 23C and 23N antibodies 1:400 were used. Monoclonal anti-(-tubulin was immunospecific for tubulin as determined by indirect immunofluorescence staining and immunoblotting procedures. After three washes in PBS, cells were incubated for 4 h in PBS/BSA 2% with secondary antibody rhodamine (TRITC)-conjugated AffiniPure $F(ab')_2$ fragment Donkey Anti-Mouse IgG or anti-rabbit IgG (excitation 503 nm, emission 530 nm) at 1/50 (Interchim). This time, cardiomyocytes or fibers were washed twice in PBS and once in distilled water. The labeled cells were deposited on glass coverships and mounted in a mixture of Mowiol and glycerol to which 1,4-diazobicyclo-[2]-octane (Acros Organics) was added to delay teh photobleaching. Samples were observed by confocal microscopy (DME IRE2, Leica) with ×40 oil immersion, NA 1.4, objective lens.

Western blot analysis

30 and 50 μg of muscular samples from b57/bl6 mice muscular homogenate or 30 μg of isolated mitochondria from rat brain and heart and were loaded onto 10% polyacrylamide gels, separated at 100 V under denaturing conditions (SodiumDodecylSulfate) and transferred with 120 mA/membrane for 45 min to nitrocellulose membranes by a semi-dry method [22]. For tubulin detection membranes were incubated with antibodies against α- or β-tubulin from rabbit (1:1000, overnight 4°C, Cell Signaling α-no 2144 and β-no 2146). The secondary anti-rabbit antibody coupled to peroxidase was purchased from GE Healthcare (1:5000, 1h 30 min at room temperature). Membranes were developed with the chemiluminescence detection kit from AppliChem and an autoradiographic film.

Preparation of total RNA

The total RNA was isolated from the ventricular myocardium, *m. soleus* and *m. extensor digitorum longus* of wild-type mice using total RNA isolating kit (Macherey-Nagel, Düren, Germany). All procedures were performed according to the manufacturer's protocol. The isolated RNA was ethanol-precipitated and resuspended in water in a final concentration 10 ng/μl.

Synthesis of total full-length cDNA

The total RNA was reverse transcribed to obtain the total cDNA using SMART cDNA synthesis protocol [23]. The resulting total cDNA was purified using NucleoSpin Extract Columns (Macherey-Nagel, Düren, Germany) and ethanol was precipitated and resuspended in water with concentration of 50 ng/μl. Subtractive hybridization and kindred DNA amplification techniques were performed as described in details Puurand et al. 2003 [24].

Agarose gel electrophoresis of full-length total cDNAs from different muscle tissues of mouse

1× TBE buffer was used for nucleic acid electrophoresis, diluted from 5× TBE buffer containing 0.445 M Tris-borate and 10 mM EDTA. Solid original for preparation of 5× TBE was obtained from NAXO Ltd.

Cloning of the kindred DNA amplification cDNAs

The kindred DNA amplification products were cloned by using the pTZ57R/T plasmid vector in the InsT/aclone PCR Product Cloning Kit according to the manufacturer's protocol (MBI Fermentas, Lithuania). The host strain used was JM109.

Preparation of DNA for hybridization

The cloned DNA inserts were amplified by PCR directly from the bacterial colonies.

A small amount of bacterial cells of a given colony was transferred into 25 μl of the reaction mixture containing 2.5 μM nested PCR primer 1, 2.5 μM nested PCR primer 2R, 0.2 mM dNTP, 0.5 μl Advantage 2^{TM} Polymerase Mix, 1× PCR reaction buffer, and sterile water. The PCR amplification was performed by 30 cycles at 94°C for 30 s, 68°C for 30 s, and 72°C for 1.5 min, and the PCR products were purified using NucleoSpin Extract Columns in an elution volume of 50 μl and analyzed by agarose/SYBR green gel electrophoresis.

Dot blot hybridization

Five microliters of amplified cDNA fragments were directly blotted onto three nylon filters with SHE clones keeping the same order and probed with total full-length cDNA from *m. soleus*, heart, and extensor. All the processes—DNA labeling, hybridization, and detection—were performed using DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions.

Analysis of cDNA sequences

The cDNA clones were sequenced at the MWG-Biotech sequencing laboratory (Ebersberg, Germany). Thirty sequences that passed the quality control standards of the MWG-Biotech sequencing laboratory were allowed to The Basic Local Alignment Search Tool (BLAST) by using the NCBI database (http://www.ncbi.nih.gov/BLAST) that finds regions of local similarity between sequences. The program compared nucleotide sequences obtained by us to sequence databases and calculates the statistical significance of matches. In our case, the program was optimized for searching highly similar sequences (Megablast).

Reagents

All reagents were purchased from Sigma (USA) except ADP, which was obtained from Boehringer (Germany).

Data analysis

The values in figures are expressed as means ± SD. The apparent K_m for ADP was estimated from a linear regression of double-reciprocal plots. Statistical comparisons were made using the one way Anova test using repeated measure comparisons (unpaired raw data). $P < 0.05$ was taken as the level of significance.

Results

Localization of microtubular network and tubulin in muscle cells

Figure 1 shows the results of immunolabeling of β-tubulin in different permeabilized muscle fibers of mouse when tissue fixation was carried out at 4°C. In spite of low temperature, the tubulin is organized into microtubular network in mouse cardiac muscle fibers (Fig. 1a) in the same way as in rat cardiomyocytes (see below). In soleus muscle, β-tubulin is localized with mitochondria both before and after knockout of STOP protein (Figs. 1b, e and 2d–f), which in skeletal muscle cells are close to the Z-line, but in heart cells occupy the level of A-band of sarcomeres between Z-lines [1, 25]. Very weak labeling of tubulin was seen in purely glycolytic EDL fibers (Fig. 1c). Similar pattern of mitochondria and tubulin distribution was also seen in mixed-type muscle gastrocnemius (Fig. 2a–c). In contrast, in the STOP protein knock-out mice, the network organization disappears in cardiomyocytes and tubulin is localized diffusely but still present inside the cell (Fig. 1d) suggesting that the microtubular network at 4°C in heart cells is maintained, most probably due to stabilizing action of STOP protein. Figure 3 shows that intracellular distribution of STOP protein is similar to that of tubulin.

The observed muscle type-specificity of the expression of tubulin in muscle cells was confirmed by the western blot analysis of muscle samples (Fig. 4). It shows that α- and β-tubulin proteins exist in both oxidative and glycolytic muscles, but in much larger quantity in oxidative muscles than in glycolytic ones. The amounts of α- and β-tubulin appear to be similar in two types of oxidative muscles, heart, and soleus.

Analysis of the role of microtubular network in regulation of mitochondrial respiration

Figure 5 shows the values of apparent K_m for exogenous ADP in regulation of mitochondrial respiration in the permeabilized fibers of heart muscle, soleus, white gastrocnemius, and EDL, in full accordance with earlier data demonstrating the muscle type-specific sensitivity of

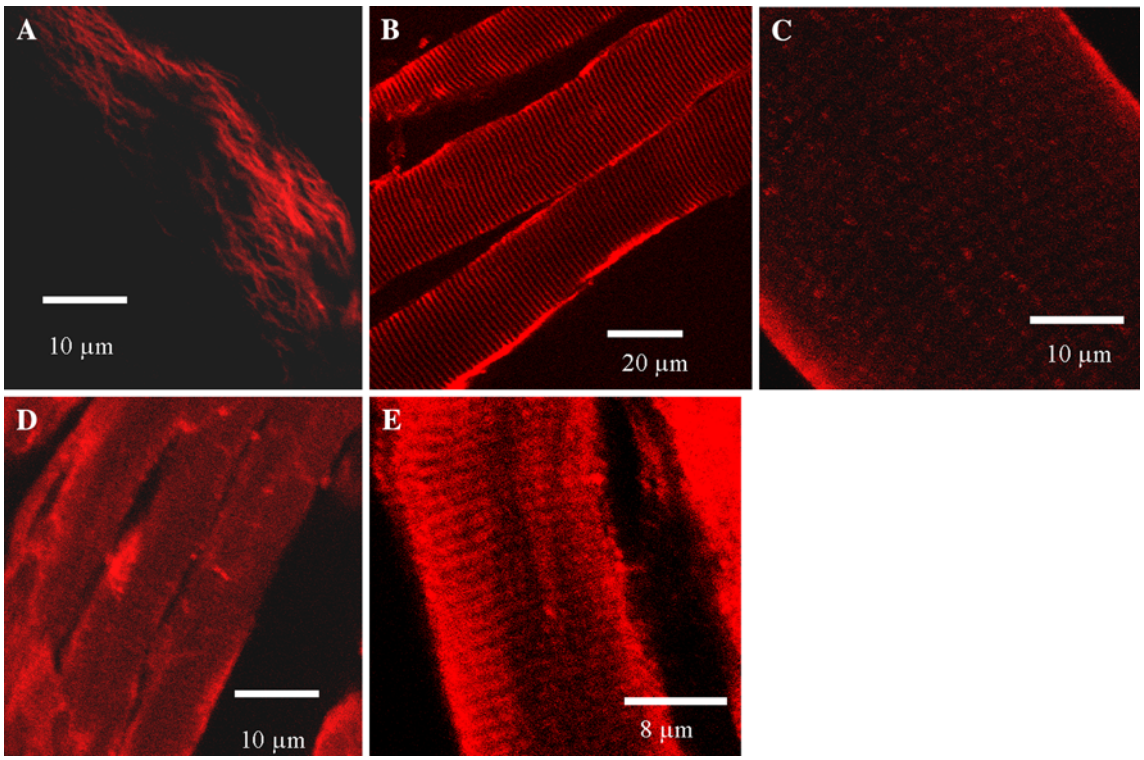


Fig. 1 Localisation of β-tubulin in different types of wild-type and STOP-deficient mice muscles. In wild-type mice muscles, microtubular network is present in heart (**a**), while there are regular striations in soleus (**b**), and a weak staining at the subsarcolemmal level in EDL (**c**). In STOP-deficient mice muscles, microtubular network is destroyed in heart, but tubulin is present and distributed diffusely (**d**). In soleus, there are no changes in the distribution of β-tubulin in control or STOP-deficient mice (**b** and **e**)

mitochondria to ADP [3, 17, 26]. Such a high specificity cannot be merely attributed to the differences in geometrical parameters of the muscle cells. Indeed, in spite of smaller diameters of heart and soleus muscle fibers as compared to the gastrocnemius and EDL, these oxidative muscles have a very high apparent K_m (ADP) which exceeds that for isolated mitochondria in vitro by more than order of magnitude, while in the fast glycolytic muscle fibers the apparent K_m (ADP) is close to that in mitochondria in vitro (Fig. 5). The thinnest oxidative muscle studied until now is the heart muscle of rainbow trout with cell diameter of 5 μm, but with high apparent K_m (ADP) [27]. These data confirm the earlier conclusion that the diffusion distance in bulk water phase is not a parameter upon which depends the value of the apparent K_m for exogenous ADP [28]. It is rather the peculiar intracellular organization and mitochondrial arrangement and their interaction with cytoskeletal proteins that causes high K_m value in oxidative muscle cells [29].

Lack of STOP protein did not associate with changes in apparent K_m for ADP in regulation of respiration (Fig. 5). The oxidative muscles heart and soleus exhibited similar to wild-type high levels of K_m for ADP, whereas the fast glycolytic muscles exhibited very low K_m values, typical to that in wild-type muscles. The muscles of STOP protein deficient mice also did not show alterations in Vmax of ADP-stimulated respiration (results not shown). Thus, it appears that it is not STOP protein but rather free tubulin that, by interacting with mitochondria, exerts a control over ADP diffusion to these organelles.

To further assess the importance of the structural organization of cytoskeleton in regulation of mitochondrial respiration, we treated the muscle specimens with colchicine and measured the changes in responses of mitochondria to exogenous ADP in permeabilized cells and fibers. Figure 6a shows the microtubular network to be similar to that for STOP protein, but without connection to the Z-lines in intact rat cardiomyocytes. At colchicine concentration of 10 μM, the microtubular network was totally disintegrated; however, free tubulin was present uniformly in the permeabilized cell (Fig. 6b), meaning that after total collapse of the microtubular network tubulin remains still attached to intracellular structures and does not leave the cell. A similar result was obtained with staining of tubulin in STOP-deficient mice (see above, Fig. 1a, d). In ghost cardiomyocytes, which represent the carcasses of cells after extraction of myosin with 800 mM KCl solution, the tubulin distribution also showed ordered striated pattern that followed the pattern of mitochondrial distribution (Fig. 6c) [7]. Disintegration of microtubular structure by

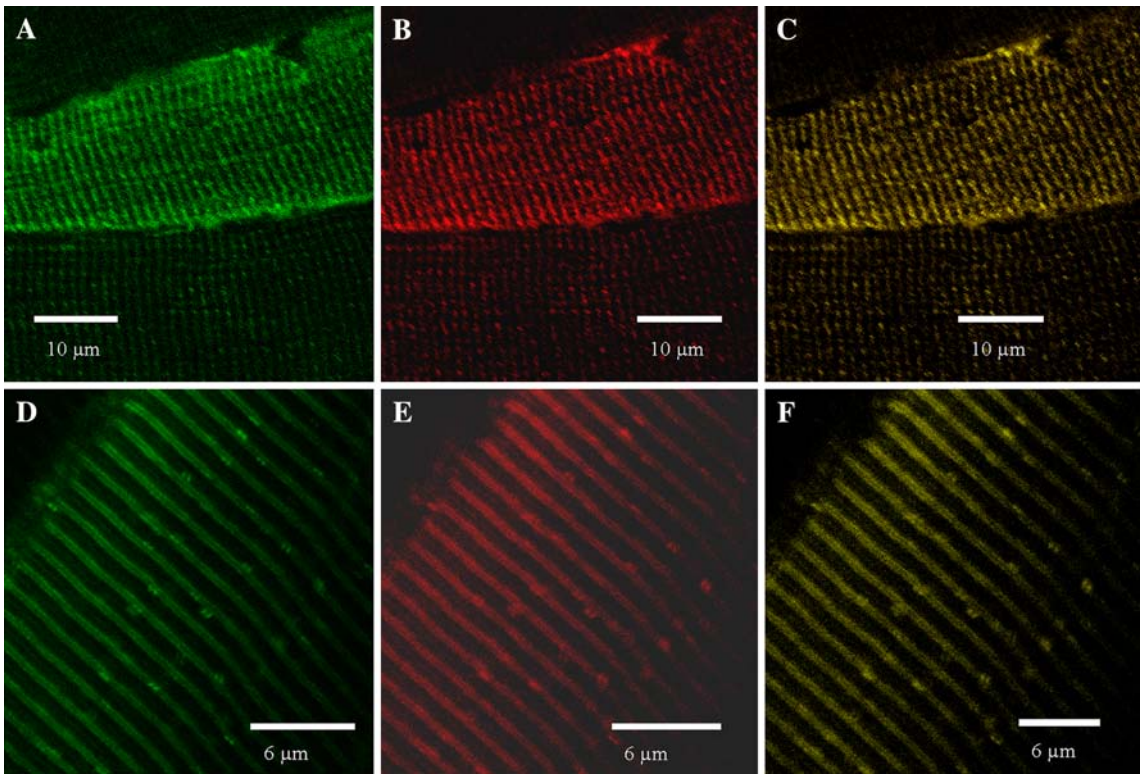


Fig. 2 Immunostaining of β-tubulin and mitochondrial flavoproteins autofluorescence in rat permeabilized fibers of skeletal muscles. The *upper panel* demonstrates the organization of mitochondria (**a**) and tubulin (**b**) in two neighboring fibers of white gastrocnemius, mixed-type muscle. The merge of the two pictures (**c**) reveals a complete colocalisation of both mitochondria and beta-tubulin in this mixed-type muscle permeabilized fibers. Soleus permeabilized fibers stainings (lower panel) of mitochondrial flavoproteins autofluorescence (**d**) and β-tubulin (**e**) show a striated pattern with complete colocalisation of both mitochondria and β-tubulin (**f**)

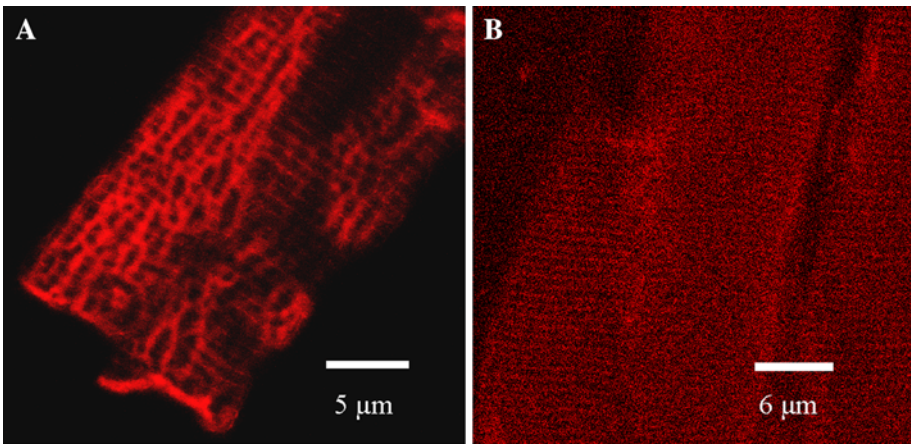


Fig. 3 Localisation of STOP protein in mouse heart fibers (**a**), soleus (**b**) at 4°C

colchicine only slightly affected the pattern of regulation of respiration in permeabilized cells. Two different populations of mitochondria became detectable in analysis of data in double reciprocal plot (solid lines in Fig. 6d), one with high K_m for ADP and another with low K_m for ADP in regulation of respiration. These mitochondrial populations represented 80 and 20% of total population, respectively. Determination of each population contribution was obtained from linear regression of all experimental data, as described earlier [20]. This gives an average apparent K_m for ADP about 170 μM corresponding to 20% of the content of mitochondrial population with low K_m for ADP [20]. These results are similar to those obtained for the STOP knock-out muscles (Fig. 5). Very interestingly, both these data show that in permeabilized cells, the heterodimeric tubulin molecules are not leaking out, but stay mostly fixed inside the cells attached to subcellular structures, including mitochondria. Only small part of tubulin

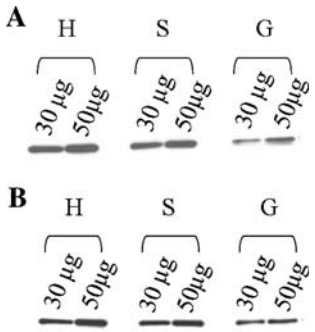


Fig. 4 Western blot analysis of α-tubulin (**a**) and β-tubulin (**b**) in rat heart (H), soleus (S), and gastrocnemius (G). 30 and 50 µg of protein of each tissue samples were loaded

seems to leak out from subsarcolemmal area leaving 20% of mitochondrial population without attached tubulin.

To test the role of non-polymerized heterodimeric tubulin in regulation of mitochondrial function, we studied the interaction of heterodimeric tubulin with isolated mitochondria. First, western blot analysis of isolated mitochondria from rat heart by using antibodies against α- and β-tubulins was performed which showed that neither isoform remained associated to mitochondria (Fig. 7). At the same time, both isoforms of tubulin were found to be bound to mitochondria isolated from rat brain. Since it is possible that cardiac mitochondria had lost their tubulins during isolation procedure, we checked whether tubulin can bind to mitochondria in reconstructed system in which 1 µM of heterodimeric tubulin was added to mitochondria. [8, 30]. The results shown in Table 1 demonstrate that tubulin induced an increase in apparent K_m for ADP (from 11 ± 2 to 330 ± 47) in heart muscle, in accordance with our earlier data [8]. The reconstructed system also became sensitive to creatine, which caused a decrease in apparent

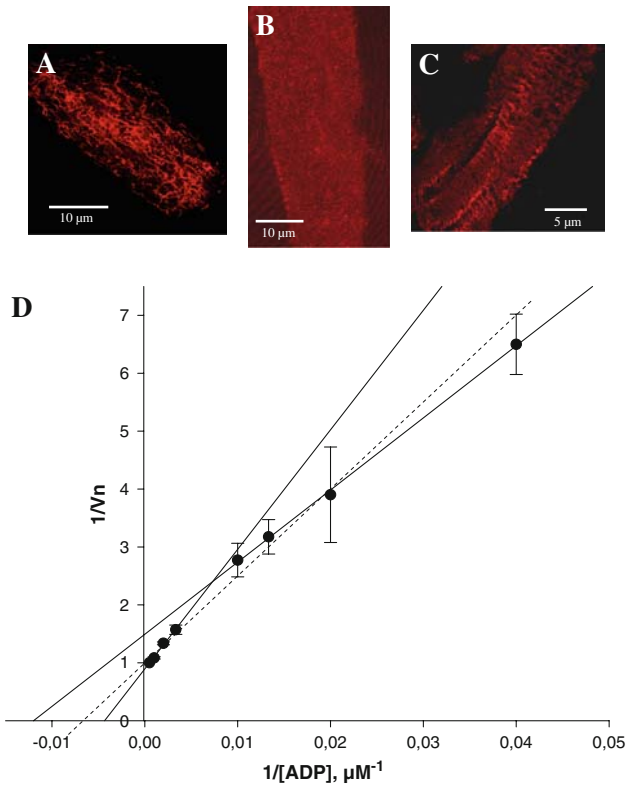
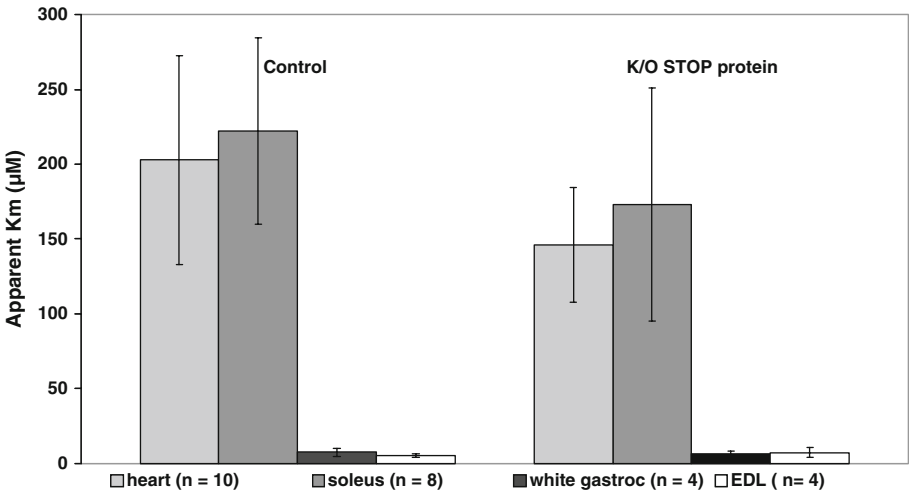


Fig. 6 Immunofluorescence of β-tubulin in rat permeabilized cardiomyocytes. **a** In the control, one can see the microtubular network. **b** Incubated for 2 h at 4°C with 10 µM colchicine. The collapse of the network seems complete, free tubulin is still present uniformly in the cell. **c** Immunostaining of tubulin in rat ghost cardiomyocytes. Tubulin has an ordered striated pattern. **d** Double reciprocal plot of the dependence of respiration rates of rat skinned cardiac fibers on ADP concentration in the medium after incubation for 2 h with 10 µM colchicine at 4°C. Two populations of mitochondria, with high and low K_m for ADP were detected. The *dashed line* represents the linear regression of all experimental data to find the % of population with low K_m as described in [20]

Fig. 5 Apparent K_m for exogenous ADP in regulation of mitochondrial respiration in the permeabilized fibers of heart muscle, soleus, white gastrocnemius, and EDL of control and transgenic mice. For heart, P was 0.05013 (quite not significant) and for the other muscles P was >0.05



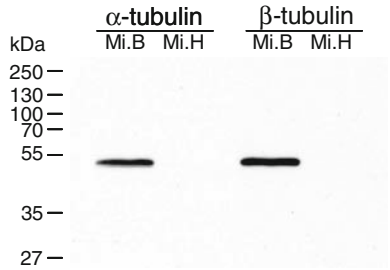


Fig. 7 Western blot analysis of α-tubulin and *β*-tubulin in isolated mitochondria from rat heart (Mi.H) and brain (Mi.B). 30 μg of protein of each sample were loaded

Table 1 Comparison of the apparent K_ms for exogenous ADP in presence of tubulin 1 μM and/or creatine 20 mM ($n = 5$)

	Apparent K_m (μM)
Control	11 ± 2
With tubulin first population	9 ± 5
With tubulin second population	330 ± 47
With tubulin and creatine	23 ± 6

K_m for ADP value. These results highlight the possible importance of tubulin in controlling mitochondrial respiration. It is conceivable that due to interactions with VDAC in the outer mitochondrial membrane, tubulin increases the diffusion constraints for ADP in the cytoplasm [8]. The activation of the mitochondrial creatine kinase (MtCK) allows overcoming such a diffusion limitation by switching in the cycling of adenine nucleotides due to the interaction of mitochondrial CK and ANT, this interplay resulting in high local ADP concentration near ANT thereby giving rise to decreased apparent K_m for ADP in the presence of creatine.

Differences in expression of tubulin isoforms in oxidative muscles

Observations that oxidative muscles differ from glycolytic counterparts by their common property, high apparent K_m for ADP, which, as the current study indicates, in turn can stem from specific interaction of tubulin with the MOM led us to an idea that oxidative muscles should possess a specific pattern of gene expression, leading to the synthesis of proteins capable to participate in regulation of mitochondrial function, whereas these proteins should not be expressed in glycolytic muscles. Based on this hypothesis, we established a new method for isolation of commonly expressed genes in different types of muscles [24], briefly outlined in Fig. 8. As a first step, the total RNA pools from mouse slow-twitch oxidative heart (H) and *m. soleus* (S), as well as fast-twitch glycolytic *m. extensor digitorum longus* (E) were isolated and reverse transcribed into

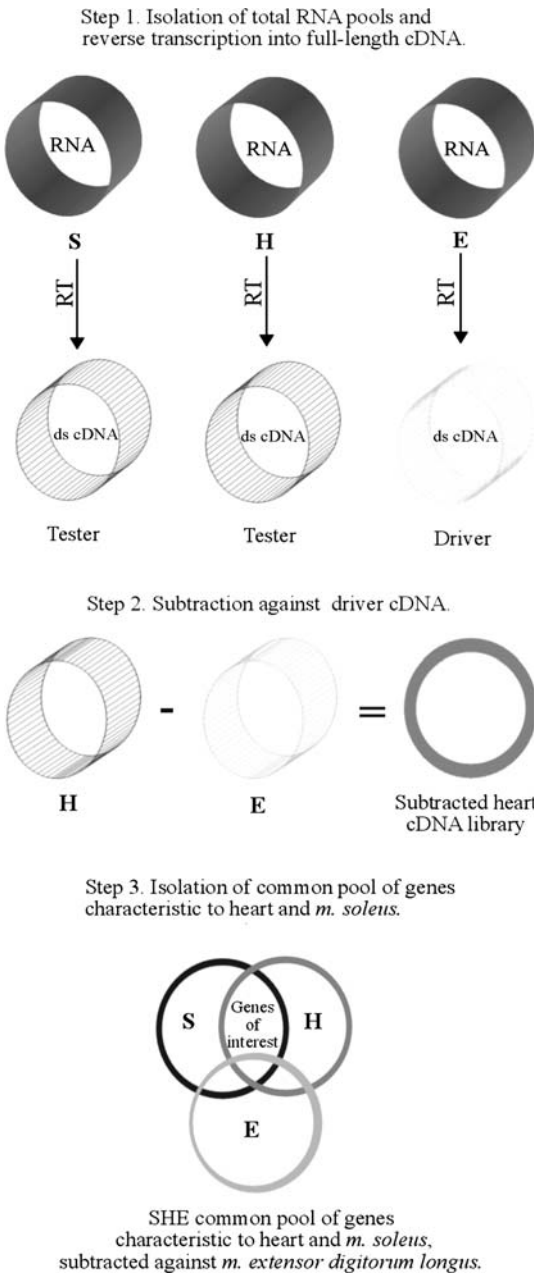


Fig. 8 The scheme for isolation of genes common between mouse heart and *m. soleus*, but not expressed in EDL. In step 1, total RNA was isolated from the tissues of interest and reverse transcribed into cDNA. The second step was the subtraction of *m. extensor* cDNA (tester) from heart cDNA (driver). The third step was to isolate the cDNAs that are commonly expressed in the heart and *m. soleus*. As a result of these three steps, the common pool of genes characteristic to myocardium and *m. soleus* (genes of interest), but not expressed in *m. extensor digitorum longus*, was obtained

double-stranded (ds) full-length cDNAs. The electrophoresis technique was applied to separate the fragments of three total cDNA libraries characteristic of *m. soleus* and heart (both oxidative) and *m. extensor digitorum longus* (glycolytic) (Fig. 9). As a second step, the cDNA libraries for ventricular myocardium (H) were subtracted against

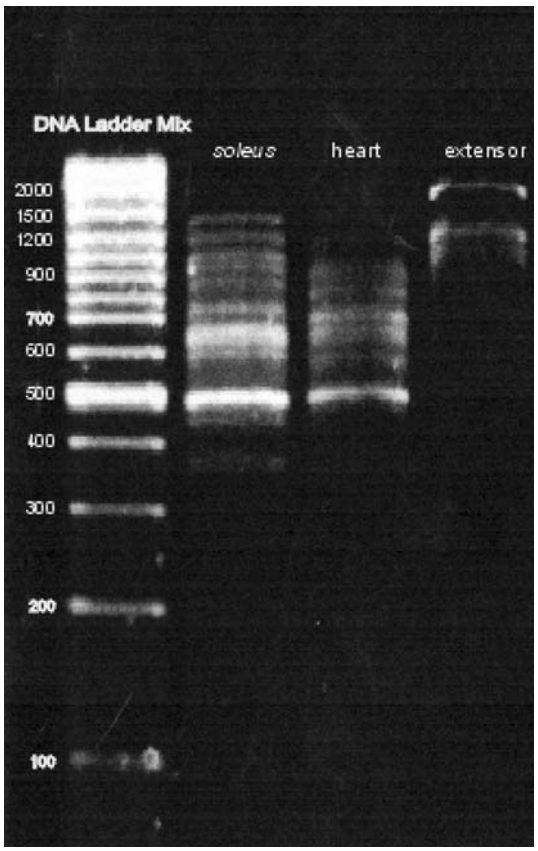


Fig. 9 Agarose gel electrophoresis of full-length total cDNAs from different muscle tissues of mouse: *m. soleus* (S), heart (H), and *m. extensor digitorum longus* (E). The common cDNAs of S and H are clearly visible, in contrast to those of E

that of *m. extensor digitorum longus* (E), this procedure yielding the heart-specific cDNA (Fig. 8). During the third step, the common cDNA fragments from heart-specific cDNA library and S (designated as "genes of interests") that do not express in EDL were obtained through kindred DNA amplification procedure [24]. To identify the genes of interest, their cDNA fragments were inserted into the plasmid vector, amplified for obtaining corresponding clones, verified in terms of muscle specificity using Southern blot [24], sequenced, and identified using BLAST algorithm provided by NCBI database that allows to find regions of local similarity between sequences [31]. As an outcome, we found that one of the cDNA nucleotide sequences isolated: GCAAATGCACAGTGGACATGGC TAGCAGACAGGCTGTGAAT GAATAAAGAGTTCAC ACTG, produced significant alignments for protein coding mouse β-tubulin gene (GeneID: 227613). Considering the principle of our method used, it may be therefore concluded that β-tubulin gene is expressed in mouse oxidative muscle (e.g., myocardium and *m. soleus*) but not in *m. extensor digitorum longus,* which is a representative of glycolytic muscle [32]. Obviously, this conclusion does not fit to observation that another glycolytic muscle, *m. gastrocnemius*, expressed both types of tubulins (Fig. 4). However, the discrepancy can be explained by the fact that *m. gastrocnemius* is a mixed-type of muscle, composed of glycolytic and oxidative fibres [33]. Thus, the results obtained in this work show that specific expression of β-tubulin in oxidative muscles enables this protein to take control over mitochondrial respiration in vivo, through binding to mitochondrial outer membrane and forming a regulatory network in the muscle cells.

Discussion

The results show that tubulin and STOP protein contents, as well as microtubular organization are tissue specific. Both tubulin and STOP protein are highly expressed in heart and oxidative muscle cells and almost absent in fast glycolytic skeletal muscles. Microtubular network can be dissociated by colchicine with minor changes in the apparent K_m values for exogenous ADP. Similar changes were observed in mice with knocked-out STOP protein. It is concluded that organization of microtubular network has only a minor role in regulation of mitochondrial function, but the connection of tubulin and other associated proteins to mitochondria may be important in the regulation of the VDAC channels permeability for adenine nucleotides.

The first interesting and novel observation in this work is the localisation of STOP protein and its connection both to the microtubular network and Z-line structures in the cardiomyocytes. The presence of this protein may explain why the microtubular system in cardiomyocytes in situ is stable at low temperatures. Indeed, in the heart muscle of mice deficient in STOP protein, the microtubular network was absent, and tubulin distribution was completely diffuse in these cells (Fig. 1). In soleus muscle, both STOP protein and tubulin were found in close connection to the Z-line area, where the mitochondria are also localized. Interestingly, both proteins were almost absent in the fast-twitch skeletal muscle (Figs. 1, 4, and 5).

The tissue-specificity of tubulin expression and organization into microtubular network is another novel observation. Indeed, we have shown different patterns of organization depending on the fiber type. Non-polymerized tubulin is seen in heart after colchicine treatment or in STOP K/O mice and also in permeabilized cells, indicating that it is attached to some intracellular structures.

We observed parallel changes in tubulin expression and localisation with alteration of the apparent K_m (ADP) values between heart, soleus, gastrocnemius, and EDL muscles. The intriguing question is whether we can explain the high values of apparent K_m (ADP) in oxidative muscles by these observations. Monge [30] and Rostovtseva [8]

have shown direct interaction between heterodimeric tubulin and VDAC. However, in intact cells, other proteins may modify tubulin binding to VDAC. Thus, Linden and Karlsson in 1996 [34] have shown that MAP-2 directly interacts with VDAC at the external mitochondrial membrane.

Lewis et al. [35] have cloned five mouse tubulin cDNAs, two (M alpha 1 and M alpha 2) that encode alpha-tubulin and three (M beta 2, M beta 4, and M beta 5) that encode beta-tubulin. The sequence of these clones reveals that each represents a distinct gene product. Two of the beta-tubulin isotypes defined by the cloned sequences are absolutely conserved between mouse and human, and all three beta-tubulin isotypes are conserved between mouse and rat. M alpha 1 and M beta 2 are expressed in an approximately coordinate fashion, and their transcripts are most abundant in brain and lung. M alpha 2 and M beta 5 are ubiquitously expressed and to a similar extent in each tissue, with the greatest abundance in spleen, thymus, and immature brain. In contrast, M beta 4 is expressed exclusively in brain. Whereas the expression of the latter isotype increases dramatically during the postnatal development, transcripts from all four other tubulin genes decline from maximum levels at or before birth. Moreover, presence or absence of tubulin is tissue specific as the high value or the low value of the apparent K_m for exogenous ADP in muscle cells. It is possible that probably only a mitochondria–MAP–tubulin interaction is important for the regulation of the external mitochondrial membrane permeability without a mitochondria-MAP-microtubule interaction. This hypothesis can be in agreement with the data from Carré et al. [36]. They have shown by electronic microscopy the presence of tubulin in mitochondrial membranes from human cancerous or normal cells. They have also analyzed different isotypes of tubulin present in these membranes. Mitochondrial tubulin is enriched in acetylated and tyrosinated α-tubulin and in β-tubulin 3 but contains a few of β-tubulin 4 in comparison with cellular tubulin. These results underline that the isotype β-tubulin 4 is mainly expressed in the cytoplasm that is in agreement with our study concerning its tissue specificity. Mitochondrial tubulin is probably organized in hétérodimères α/β (approximately 2% of cellular tubulin).

Immunoprecipitation experiments showed that mitochondrial tubulin is specifically associated with the mitochondrial VDAC [36]. One can submit the hypothesis that there is a transmembranar signal able to modify the VDAC permeability, thus to regulate mitochondrial respiration according to the cytoplasmic interaction of β-tubulin 4, tissue specific, with MAP and mitochondria. This hypothesis could be strengthened with the very recent paper of Rostovtseva et al. [8] and show the importance of the tubulin's anionic C-tail requirement for the tubulin–VDAC interaction.

The results of this study show that the microtubular network and STOP-proteins are organized in muscle cells in tissue-specific manner. In adult mice, the mouse β-tubulin gene M-beta-4 is muscle fiber-type specific. Gene expression analysis revealed that mouse β-tubulin gene M-beta-4 is expressed not ubiquitously and not exclusively in the (developing) brain as reported by Lewis group [35], but homological cDNA sequences have expressed in mouse myocardium and oxidative skeletal muscle *m. soleus*. The results show also that the presence of tubulin with associated proteins, but not its organization into micotubular network may be important for regulation of mitochondrial affinity for exogenous ADP in muscle cells.

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Article 6

Mise en évidence du couplage fonctionnel entre uMtCK et ANT dans les mitochondries de cerveau et caractérisation de la régulation de la respiration dans les synaptosomes

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Regulation of respiration in brain mitochondria and synaptosomes: restrictions of ADP diffusion in situ, roles of tubulin, and mitochondrial creatine kinase

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Abstract The role of ubiquitous mitochondrial creatine kinase (uMtCK) reaction in regulation of mitochondrial respiration was studied in purified preparations of rat brain synaptosomes and mitochondria. In permeabilized synaptosomes, apparent Km for exogenous ADP, Km (ADP), in regulation of respiration in situ was rather high (110 ± 11μM) in comparison with isolated brain mitochondria (9 ± 1 μM). This apparent Km for ADP observed in isolated mitochondria in vitro dramatically increased to 169 ± 52μM after their incubation with 1 μM of dimeric tubulin showing that in rat brain, particularly in synaptosomes, mitochondrial outer membrane permeability for ADP, and ATP may be restricted by tubulin binding to voltage dependent anion channel (VDAC). On the other hand, in synaptosomes apparent Km (ADP) decreased to 25 ± 1 μM in the presence of 20 mM creatine. To fully understand this effect of creatine on kinetics of respiration regulation, complete kinetic analysis of uMtCK reaction in isolated brain mitochondria was carried out. This showed that oxidative phosphorylation specifically altered only the dissociation constants for MgATP, by decreasing that from ternary complex MtCK.Cr.MgATP (K_a) from 0.13 ± 0.02 to 0.018 ± 0.007 mM and that from binary complex MtCK.MgATP (K_{ia}) from 1.1 ± 0.29 mM to 0.17 ± 0.07 mM. Apparent decrease of dissociation constants for MgATP reflects effective cycling of ATP and ADP between uMtCK and adenine nucleotide translocase (ANT). These results emphasize important role and various pathophysiological implications of the phosphocreatine–creatine kinase system in energy transfer in brain cells, including synaptosomes.

Keywords Brain · Creatine kinase · Functional coupling · Mitochondria · Synaptosomes · Tubulin

Abbreviations

CK	Creatine kinase
uMtCK	Ubiquitous mitochondrial creatine kinase
sMtCK	Sarcomeric mitochondrial creatine kinase
Cr	Creatine
PCr	Phosphocreatine
ANT	Adenine nucleotide translocase
VDAC	Voltage dependent anion channel
DTT	Dithiothreitol
SDS	Sodium dodecyl sulfate
PEP	Phosphoenol pyruvate
PK	Pyruvate kinase

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Introduction

Normal functioning of nerve and brain cells requires maintenance of gradients of ions and active transport across the plasma membrane by using the free energy released in the intracellular metabolic processes—in mitochondrial respiration and glycolysis [1, 2]. Integration of energy-producing and energy-consuming processes and their fine regulation involve energy supply and feedback metabolic regulation of respiration by phosphotransfer systems based on the mechanisms of metabolic channeling and functional coupling between different enzymes, multienzyme systems, and transporters [3–10]. It has already been established in classical studies by Hodgkin and coworkers on conduction of nerve impulse more than 40 years ago that both ATP and phosphagen (arginine phosphate) are needed for active transport of Na^+ and K^+ in squid giant axons [11–13]. Energy transfer via the kinase systems or localized glycolytic enzymes to membranes to support transport processes (e.g., glutamate loading of synaptic vesicles or Na^+,K^+-ATPase activity) is critical also for brain function [1, 14–17]. Brain cells, as cardiac cells, skeletal muscle, and many other cells contain a creatine kinase (CK) system [14, 15]. In the brain, the CK system is represented by the ubiquitous mitochondrial isoenzyme, uMtCK, which is co-expressed with the dimeric brain form, BBCK [14, 15]. Active role of creatine kinase and adenylate kinase reactions in energy transfer is essential for minimizing energy gradients, reducing energy dissipation, and directing energy to sites and pathways for specific processes [4, 8–10, 18, 19]. However, these processes and mechanisms of their regulation are best studied in great details in heart cells, allowing also their quantitative description and analysis by mathematical models [4, 5, 7–9, 18], but much less is known about the mechanisms of these processes in brain cells, mostly due to fine-grained cellular heterogeneity and technical difficulties of approaching the brain systems in vivo [1, 20]. In particular, the detailed mechanism of interaction of uMtCK with the oxidative phosphorylation provided by the ATP synthasome (a supercomplex of ATP synthase F_0F_1 with adenine nucleotide translocase and Pi-carrier) [21] in the inner mitochondrial membrane of brain mitochondria is not known. Functioning of the creatine kinase pathway is necessary for effective communications between intracellular microcompartments of adenine nucleotides resulting from localized restrictions of their diffusion [10, 22]. The precise mechanism behind these restrictions of the intracellular diffusion of adenine nucleotides is still to be investigated. It has been hypothesized that the restriction of the diffusion of adenine nucleotides may occur at the level of mitochondrial outer membrane due to the interaction of the voltage dependent anion channels (VDAC) in this membrane with some of the components of cytoskeletal network of the cells [4, 23, 24]. One of the candidates for this interaction may be tubulin which has been shown to be able to bind to the outer mitochondrial membrane [25, 26].

Therefore, the aim of this study was to investigate quantitatively the role of the uMtCK reaction in regulation of mitochondrial respiration in the purified rat brain synaptosomes in which mitochondria exist in their physiological intracellular surrounding [2, 16, 17, 27, 28]. Reconstitution of mitochondrial complexes with cytoskeleton was achieved by using isolated rat brain mitochondria and tubulin. The results emphasize the importance of effective production of phosphocreatine by uMtCK in brain mitochondria due to its functional coupling to ANT, leading to recycling of adenine nucleotides, and pointing therefore to the critical role of coupled creatine kinase system in energy transfer in brain synaptosomes.

Material and method

Isolation procedures

Rat brain synaptosomes and isolated mitochondria

Male Wistar rats (250–300 g) were used throughout the study. Synaptosomes and mitochondria were isolated from the forebrains as described by Booth and Clark [29].

The final synaptosomal and mitochondrial pellets were suspended in 2 ml of Mitomed solution (EGTA 0.5 mM, $MgCl_2$ 3 mM, K-lactobionate 60 mM, taurine 20 mM, KH_2PO_4 3 mM, sucrose 110 mM, DTT 0.5 mM, HEPES 20 mM, pH 7.1).

Isolation and purification of tubulin

Tubulin from rat brain and bovine brain was used with equivalent results. The bovine tubulin was obtained from Cytoskeleton (Boulter, CO, USA). The rat brain tubulin was purified as previously described [30, 31]. Frozen rat brains were thawed, homogenized in Assembly Buffer (0.1 M Mes, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.9), and centrifuged at 100,000*g*. Microtubule protein (tubulin plus microtubule associated proteins) was purified by several rounds of GTP-driven, temperature-dependent polymerization and depolymerization [30]. Tubulin was then purified from this material by selective polymerization in high buffer concentration, pelleted by centrifugation, redissolved in Assembly Buffer at 25 mg/ml, and drop frozen in liquid nitrogen [31]. In its final form the tubulin used was the $\alpha\beta$-heterodimer [30, 31].

Oxygraphy

All measurements of respiration rates were performed by using the high resolution respirometry (Oroboros oxygraph, Innsbruck, Austria). To achieve the maximal stability of the respiratory parameters of the permeabilized cellular preparations, the previously used medium [32] was replaced by the Mitomed solution [33, 34] supplemented with 2 mg/ml essentially fatty acid free bovine serum albumin. This solution allowed us to avoid using high EGTA concentrations and serious problems related to the contaminations in the commercial preparations of EGTA [35]. Respiratory substrates used were 5 mM glutamate + 2 mM malate, 5 mM pyruvate + 2 mM malate or 10 mM succinate. Oxygen solubility in the Mitomed solution was taken to be 225 μM at 25°C and 200 μM at 30°C [36].

Respiration in rat brain synaptosomes was analyzed after cell permeabilization with 50 μg/ml saponin directly in oxygraph chamber (2 ml). Kinetics of activation of respiration of brain mitochondria were studied by consecutively (step-wise) increasing the final concentrations of ADP (10–2,000 μM).

Activities of mitochondrial respiratory segments were measured as described previously [34]. Mitochondrial respiration was first activated by glutamate/malate (5 and 2 mM, respectively) via the complex I in the presence of ADP in saturating concentration (2 mM). Then, the complex I was inhibited by rotenone (10 μM) and the complex II was activated by succinate (10 mM), followed by inhibition of complex III by antimycin A (10 μM). Finally, the artificial substrates of the complex IV, TMPD/ascorbate (1 and 5 mM, correspondingly), were added.

Reconstitution studies

Isolated and purified rat brain mitochondria (5 mg/ml) were incubated in Mitomed solution (see above) with 1 μM tubulin for 30 min at room temperature (22°C). After that, the samples were injected into oxygraph chamber. Kinetics of activation of respiration were analyzed by successive addition of ADP (5–10–20–50–100–200–500–1,000–2,000–3,000 μM). Assay medium additionally contained 0.2% of serum bovine albumin and 1 IU/ml apyrase from potato (Sigma-Aldrich) as an ADP regeneration system. This isoenzyme of apyrase has an exceptionally high ATPase/ADPase ratio (10:1) and can be used for effective regeneration of ADP to maintain steady-state of respiration in the presence of limited amounts of ADP in kinetic studies.

Measurements of mitochondrial cytochromes content

The synaptosomal or mitochondrial samples were solubilized with 1% of sodium deoxycholate in phosphate buffer (KH_2PO_4 100 mM, pH 8). The differential spectrum (reduced by dithionite versus oxidized cytochromes) was obtained by scanning from 400 to 650 nm [37] using a Cary 50 Bio spectrophotometer (Varian, Palo Alto, USA). Cytochromes of the respiratory chain were reduced by addition of several crystals of sodium dithionite to 1 ml of suspension of mitochondria (final concentration 0.25 mg/ml) or synaptosomes (final concentration 2 mg/ml). The value of peak at 605 nm was used for quantification of respiratory chain cytochrome aa_3 contents (cytochrome *c* oxidase) both in isolated mitochondria and purified synaptosomes, using the extinction coefficient ε value equal to 24 mM^{-1} cm^{-1} [37].

Measurement of the creatine kinase activity

Creatine kinase activity was determined spectrophotometrically by using a coupled enzyme assay in the following buffer: 30 mM HEPES, 5 mM $MgCl_2$, 0.5 mM dithiothreitol. One milliliter of the assay medium contained 2 IU/ml glucose 6 phosphate dehydrogenase, 2 IU/ml hexokinase, 20 mM glucose, 1.2 mM ADP, 0.6 mM NADP, 20 mM phosphocreatine (PCr). First, baseline activity due to adenylate kinase was recorded, and after addition of PCr, a specific substrate of the creatine kinase, the activity of the latter was determined from the difference of rates of optical density changes after and before PCr addition. Synaptosomes were permeabilized with 50 μg/ml of saponin before activity measurements. Creatine kinase activities in isolated mitochondria and synaptosomes were measured at 30 and 25°C, respectively.

Confocal microscopy: mitochondrial imaging by TMRM

To analyze mitochondrial localization and inner membrane potential, permeabilized synaptosomes were incubated for 30 min at room temperature with 50 nM of specific agent tetramethylrodamine methyl ester (TMRM, Sigma), a fluorescent dye that accumulates in mitochondria on the basis of their membrane potential when added directly to the Mitomed medium. For imaging (colocalization) studies, mitochondria were also stained with MitoTracker® Green FM (100 nM, Molecular Probes, Eugene, OR, data not shown). The digital images of TMRM and MitoTracker® Green fluorescence were acquired with inverted confocal microscope (Leica DM IRE2) with a 63-x water immersion lens. The MitoTracker® Green fluorescence was excited with the 488-nm line of argon laser, using 490–516 nm for emission. TMRM fluorescence was measured using 543 nm for excitation (Helium–Neon laser) and greater than 580 nm for emission.

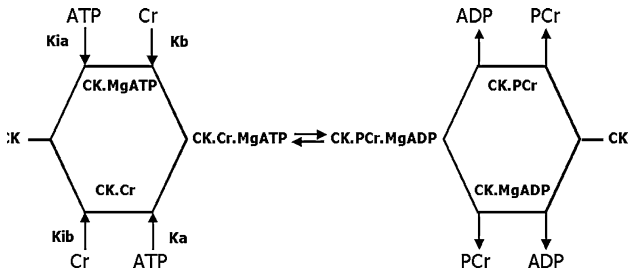
Kinetic study of the creatine kinase reaction

(a) Measurements. The creatine kinase reaction follows a BiBi random type quasi-equilibrium reaction mechanism (according to Cleland's classification) [38–45]:

In Scheme 1 the quasi-equilibrium dissociation constants of enzyme–substrate complexes for the forward reaction of phosphocreatine (PCr) production are shown. These are the constants which were experimentally determined in this study under conditions of the presence or absence of oxidative phosphorylation (the reverse reaction and its constants were not investigated). The constants K_{ia} and K_a are the constants of dissociation of MgATP into surrounding solution from binary complex CK.MgATP and ternary complex CK.Cr.MgATP, respectively, and K_{ib} and K_b are the dissociation constants of creatine, Cr, from the binary complex CK.Cr, and from the ternary complex CK.Cr.MgATP, correspondingly. For quasi-equilibrium binding and dissociation of substrates, the following relationship is valid (Eq. 1) [38, 41, 42, 45]:

$$K_{ia} \cdot K_b = K_{ib} \cdot K_a \quad (1)$$

The values of these constants were determined in the isolated mitochondria from rat heart and brain by two different methods, depending on the presence (case I) or absence (case II) of the activated oxidative phosphorylation as described before [38, 41, 43, 44]. In case I (see Scheme 2 below), when oxidative phosphorylation was activated in the presence of creatine and ATP in different concentrations, the respiration was maintained by production of ADP in the MtCK reaction. In these experiments mitochondria were added into respiratory Mitomed medium with 0.2% of serum bovine albumin and 10 mM of succinate at 30°C, and the kinetics of uMtCK activation in isolated mitochondria were studied by increasing concentration of ATP (10–25–50–100–200–500–1,000–2,000 μM) at different fixed concentrations of creatine (Cr) (3–5–10–15 mM).



Scheme 1 Creatine kinase reaction

The rate of the forward creatine kinase reaction was calculated from the respiration rate, according to Eq. 2:

$$v_{PCr} = 5.3 \times \Delta v_{O_2} \quad (2)$$

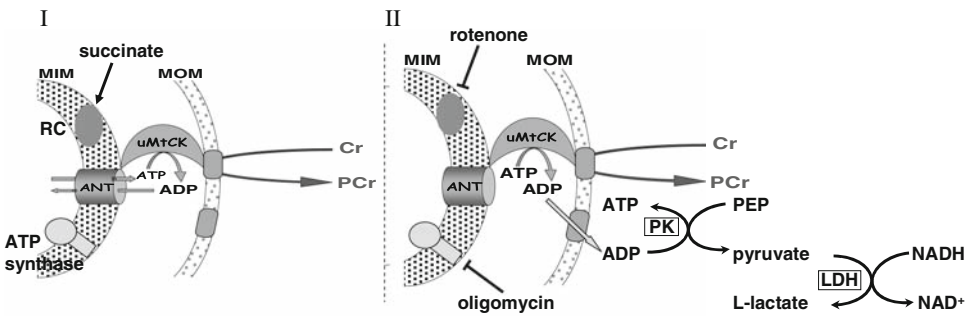
where $\Delta v_{O_2} = v_{O_2}(\text{ATP, Cr}) - v_{O_2}(\text{ATP})$, $v_{O_2}(\text{ATP})$ and $v_{O_2}(\text{ATP, Cr})$ being the respiration rates in the absence and in the presence of creatine, respectively, at the given ATP concentration in the medium. The coefficient 5.3 was found experimentally for both heart and brain mitochondria as described before [41].

In case II, the oxidative phosphorylation was inhibited by oligomycin (1 μM) and rotenone (10 μM), and the rate of the uMtCK reaction was measured spectrophotometrically by a coupled enzyme phosphoenolpyruvate–pyruvate kinase–lactate dehydrogenase (PEP–PK–LDH) system. Mitochondria were added into Mitomed medium with 0.2% BSA and a (PEP–PK) system (PEP 5 mM, PK 20 U/ml, LDH 5 U/ml, NADH 150 μM), successively increasing the concentration of ATP (10–25–50–100–200–500–1,000–2,000 μM) at fixed Cr concentrations (3–5–10–15 mM). The rate of NADH oxidation was recorded at 30°C using a Cary 50 Bio spectrophotometer. The creatine kinase reaction rates were found from differences in changes in optical density in the presence and absence of creatine (see above).

(b) Analysis of kinetic data. In both cases the experimental data were analyzed in a following way. The initial reaction rates of MtCK reaction with Bi–Bi random type quasi-equilibrium mechanism are described by the following equation (Eq. 3) [41]:

$$V = \frac{\text{Vm}[\text{Cr}][\text{MgATP}]}{K_{ia}K_b + K_a[\text{Cr}] + K_b[\text{MgATP}] + [\text{Cr}][\text{MgATP}]} \quad (3)$$

For determination of the dissociation constants, the classical primary and secondary analysis by a linearization method was used [38, 41, 43, 44, 46], instead of popular fitting methods [47, 48], which application lacks the potential to illustrate the important information of the mechanism of the effects studied (see section "Discussion"). For initial reaction rate measurements, the concentration of one substrate was fixed at different values



Scheme 2 The principles of kinetic studies of the effects of oxidative phosphorylation on the MtCK reaction in isolated brain mitochondria

and the rate determined as a function of the other substrate. For primary analysis, the measured reaction rates were expressed in double reciprocal plots as a function of the concentration of this substrate. If MgATP concentration was changed at fixed creatine concentration, in double reciprocal plots the reaction rate was expressed as

$$\frac{1}{V} = \frac{1}{[\mathrm{MgATP}]}\left(\frac{K_a}{\mathrm{Vm}}\left(\frac{K_{ib}}{[\mathrm{Cr}]}+1\right)\right)+\frac{1}{\mathrm{Vm}}\left(\frac{K_b}{[\mathrm{Cr}]}+1\right) \quad (4)$$

If the creatine concentration was changed at different fixed MgATP concentrations, the following rate equation in double reciprocal plots was used:

$$\frac{1}{V} = \frac{1}{[\mathrm{Cr}]}\left(\frac{K_b}{\mathrm{Vm}}\left(\frac{K_{ia}}{[\mathrm{MgATP}]}+1\right)\right)+\frac{1}{\mathrm{Vm}}\left(\frac{K_a}{[\mathrm{MgATP}]}+1\right) \quad (5)$$

From these primary linear plots, the ordinate intercepts (io) can be found as a linear function of the reciprocal of the concentration of the first substrate, which allows by the secondary analysis to find the values of the K_a, K_b and Vm, respectively, in the following way. From Eq. 4 we have

$$\mathrm{io} = \frac{1}{\mathrm{Vm}}\left(\frac{K_b}{[\mathrm{Cr}]}+1\right) \quad (6)$$

From Eq. 5 we have

$$\mathrm{io} = \frac{1}{\mathrm{Vm}}\left(\frac{K_a}{[\mathrm{MgATP}]}+1\right) \quad (7)$$

Secondary analysis of io versus 1/[Cr] gives an abscissa intercept in the first case equal to $-1/K_b$; in the second case the secondary analysis of io versus 1/[MgATP] gives an abscissa intercept equal to the value of $-1/K_a$. In both cases the ordinate intercept gives 1/Vm.

Accordingly, analysis of the slopes (s) from the primary linear analysis (Eqs. 4 and 5) allows finding the value of K_{ia} and K_{ib} in the following way. From Eq. 4 we have

$$\mathrm{slope} = \frac{K_a}{\mathrm{Vm}}\left(\frac{K_{ib}}{[\mathrm{Cr}]}+1\right) \quad (8)$$

From Eq. 5 we have:

$$\mathrm{slope} = \frac{K_b}{\mathrm{Vm}}\left(\frac{K_{ia}}{[\mathrm{MgATP}]}+1\right) \quad (9)$$

From the abscissa intercepts of these dependences, the values of $-1/K_{ib}$ and $-1/K_{ia}$ can be found.

It is important to note that these equations will be different when the reaction mechanism becomes the ordered one instead of random type [38]. Thus, the secondary analysis gives immediately the information of the reaction mechanism.

The last step in our analysis was to fit experimental data with kinetic equation describing creatine kinase kinetics using Eq. 1 as well as residual ATPase activity. The experimentally determined oxygen consumption rates were fitted by the following formula (Eq. 10):

$$V_{O_2}(\mathrm{ATP}, \mathrm{Cr}) = [V_{CK}(\mathrm{ATP}, \mathrm{Cr}) + V_{ATPase}(\mathrm{ATP})]/5.3 \quad (10)$$

where V_{CK} is the respiration rate controlled by uMtCK, which was calculated according to Eq. 3 and V_{ATPase} is the rate of respiration depending only on the ATPase reaction which was approximated by Michaelis–Menten kinetics. For fitting, a least squares method has been used and each experimental point was weighted by its standard deviation. During the optimization, the apparent kinetic constants K_{ia}, K_a, and K_b were optimized as well as apparent Km of ATPases and maximal rates of CK and ATPase dependent respiration.

Data analysis

All data are presented as means ± SD. Statistical analyses were performed using Student's t-test, and $P < 0.05$ was taken as the level of significance.

Reagents: protein determination

All reagents were purchased from Roche (Meylan, France) and Fluka (Buchs, Switzerland). Protein concentration was determined according to the BCA protein assay kit from Pierce [49].

Results

Confocal imaging, spectral, and respiratory characteristics of rat brain and synaptosomal mitochondria

Figure 1 shows a confocal image of mitochondria in isolated and purified rat brain synaptosomes as visualized by the mitochondria-specific probe TMRM. In synaptosomes, mitochondria are localized in random manner but rather immobilized in fixed positions as seen in long-term observations up to 20–30 min. Moreover, this mitochondrial localization was completely confirmed and colocalized with other mitochondria-specific probe MitoTracker® Green (data not shown). Isolated rat brain mitochondria showed high content of cytochromes of the respiratory chain (in particular cytochrome aa_3) determined from the difference spectra (reduced by dithionite minus oxidized) in the range 400–650 nm (Fig. 2). Table 1 shows the cytochrome aa_3 contents in both isolated brain mitochondria and synaptosomes. From these data one can calculate the content of mitochondrial protein in synaptosomes which was found to be about 0.26 mg/mg of the total synaptosomal protein. Table 1 shows

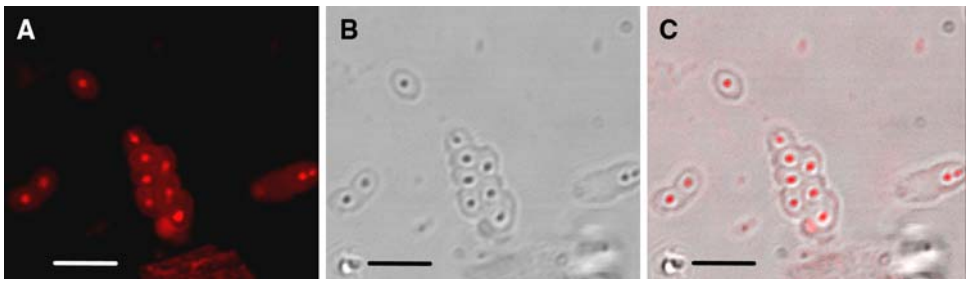


Fig. 1 Confocal fluorescent imaging of mitochondria in permeabilized synaptosomes. (**a**) Mitochondria were labeled by pre-incubation with mitochondrial inner membrane potential sensitive probe TMRM (50 nM, red); (**b**) light transmission imaging of rat synaptosomes; (**c**) overlay of TMRM fluorescence and transmission images. Scale bar, 5 μm

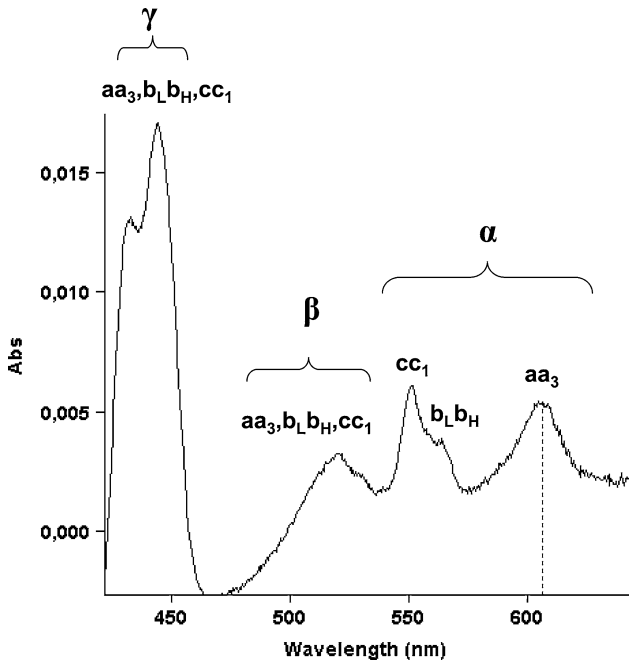


Fig. 2 Representative difference (reduced–oxidized) spectrum of mitochondrial cytochromes in rat brain mitochondria. The peaks α, β, and γ are light absorption peaks characteristic to all cytochromes of respiratory chain—cytochromes *a*, a_3, b_H, b_L, *c*, and c_1. The γ and β peaks of all cytochromes are very close to each other and thus superimposed. Only α peaks of the cytochromes aa_3 well apart from others and allows a precise measurement of respiratory chain content. Reduced (by sodium dithionite) minus oxidized differential spectra of mitochondrial cytochromes were obtained as described in section "Material and method" after solubilization of mitochondria in 1% sodium deoxycholate. Mitochondria were added to their final concentration of 0.25 mg/ml. The value of cytochrome aa_3 peak (at 605 nm) was used for quantification of mitochondrial respiration rates. Similar spectra but with much lower amplitudes of peaks were recorded also for synaptosomes (not shown)

also the respiratory parameters and substrate specificity of these two preparations determined by the approach presented in Fig. 3. Figure 3a demonstrates that glutamate + malate is rather poor substrate combination for brain mitochondria. Alternatively, the respiration was very active in the presence of succinate (Fig. 3a) or pyruvate + malate (Fig. 3b). With all substrates, the effect of the addition of exogenous cytochrome *c* on respiration rate was small, demonstrating the intactness of the outer mitochondrial membrane. Cytochrome *c* is attached to the outer surface of the inner mitochondrial membrane and has been shown to be capable for rapid three-dimensional diffusion and a high degree of collision efficiency in the inter-membrane space at high ionic strength [50]. The rapid three-dimensional diffusion at physiological ionic strength leads to a release of cytochrome *c* if the outer membrane is damaged, that limiting electron transport from complex III to complex IV and thus the respiration rate. Addition of exogenous cytochrome *c* up to 8–10 μM restores the respiration [32]. Therefore, the integrity of outer mitochondrial membrane can be checked by measuring the stimulatory effect of exogenous cytochrome *c* on the respiration rate. This property of cytochrome *c* has been found to be very useful for studies of the effects of pro- and antiapoptotic proteins on the outer mitochondrial membrane [51] as well as in ischemia reperfusion injury (cytochrome *c* test) [52]. This test is shown in Fig. 3b demonstrating that, in response to the addition of cytochrome *c*, almost no changes in the initially high respiration rate of isolated brain mitochondria were observed. Moreover, a specific inhibitor of translocase, atractyloside (Atr) inhibited the respiration back to the initial State 2 (V_0) level (Fig. 3b). Consequently, both the outer and inner mitochondrial membranes were shown to be intact and not damaged during isolation procedure. Table 1 shows also the respiration rates and enzymatic characteristics of both rat brain mitochondria and synaptosomes. In all further respiration experiments, succinate was used as mitochondrial substrate.

The uMtCK isozyme and respiration regulation

The exact values of the creatine kinase activities in the isolated brain mitochondria and synaptosomes are shown in Table 1. Interestingly, specific activities calculated per milligram of protein were not different, indicating the presence of extra-mitochondrial BBCK in synaptosomes, in concord with earlier data [53].

Table 1 Enzymatic and respiratory parameters of rat brain mitochondria and synaptosomes

Preparations	Parameters			
	V_{O_2} nmol O_2/min/mg of protein		[cytochrome aa_3] nmol/mg	Total activity of CK, Vm^{-1} μmol/min/mg
Isolated brain mitochondria substrates	V_0	V^3	0.35 ± 0.18	1.51 ± 0.07
Glutamate/malate	14 ± 4	36 ± 7		
Pyruvate/malate	15 ± 4	78 ± 11		
Succinate	16 ± 5	95 ± 13		
TMPD/ascorbate	14 ± 5	218 ± 8		
Synaptosomes substrates	V_0	V^3	0.09 ± 0.03	1.7 ± 0.08
Succinate	10 ± 3	43 ± 7		
Glucose	1 ± 0.01	1.5 ± 0.07		

Vo and V^3 are the respiration rates before and after addition of 2 mM ADP, correspondingly

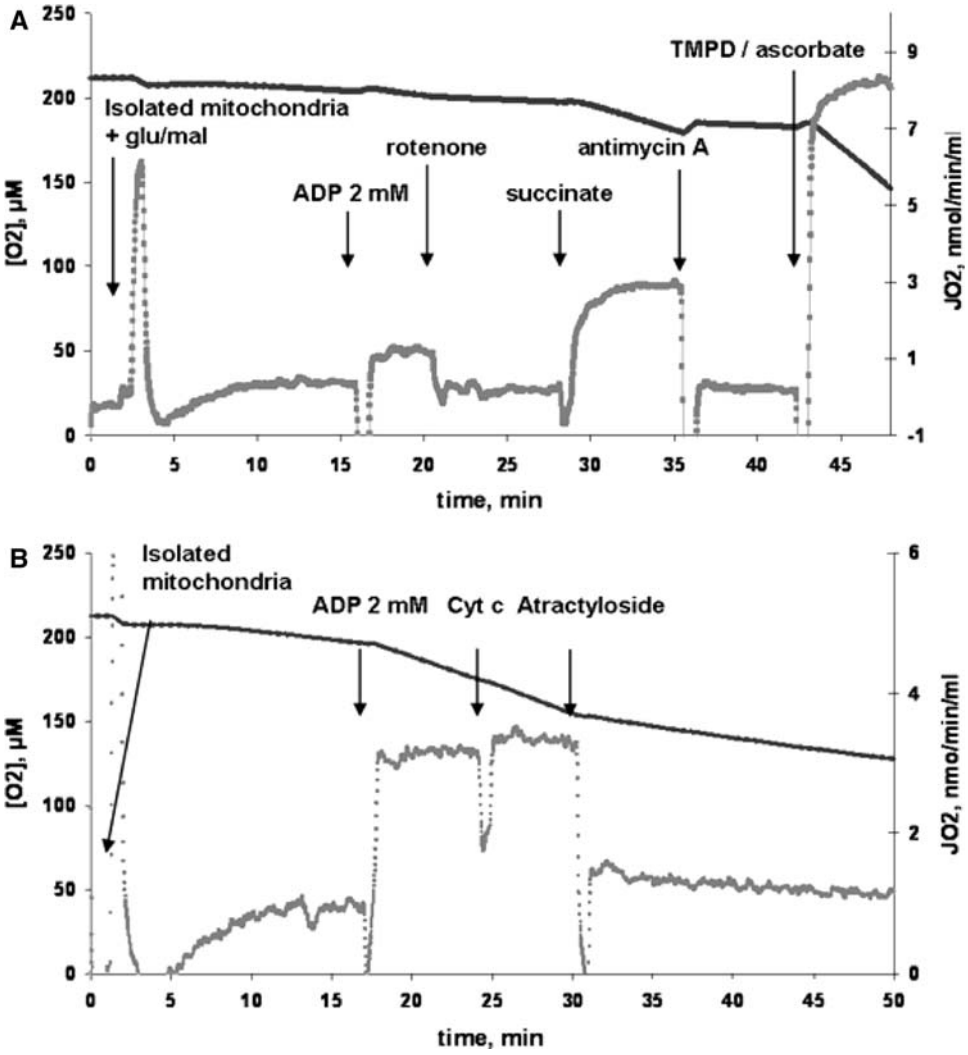


Fig. 3 (**a**) Analysis of the respiration chain complexes. Upper trace is the oxygen concentration and lower trace is the flux—the rate of oxygen consumption. (**b**) Oxygraph recording of the respiratory control ratio of isolated brain mitochondria. The respiration was activated with pyruvate (5 mM) and malate (2 mM). At the end of the measure, cytochrome *c* (cyt *c*, 8 μM) was added to check the integrity of the outer membrane. Next atractyloside (Atr, 30 μM) was added to inhibit the adenine nucleotide translocase (ANT), and the result is a decrease in respiration back to Vo. Upper trace is the oxygen concentration and lower trace is the flux—the rate of oxygen consumption. In this experiment the respiratory control index was 3.9. In all oxygraphy experiments, mitochondria were added to final concentration of about 0.03 mg of protein/ml

Mitochondrial creatine kinase content in synaptosomes was further analyzed by isoform-specific antibodies. A positive signal was obtained for the uMtCK isozyme (data not shown), in agreement with previously published data [14, 15]. As can be estimated from a comparison with pure recombinant uMtCK, this protein makes up about 1–2% of synaptosomal protein.

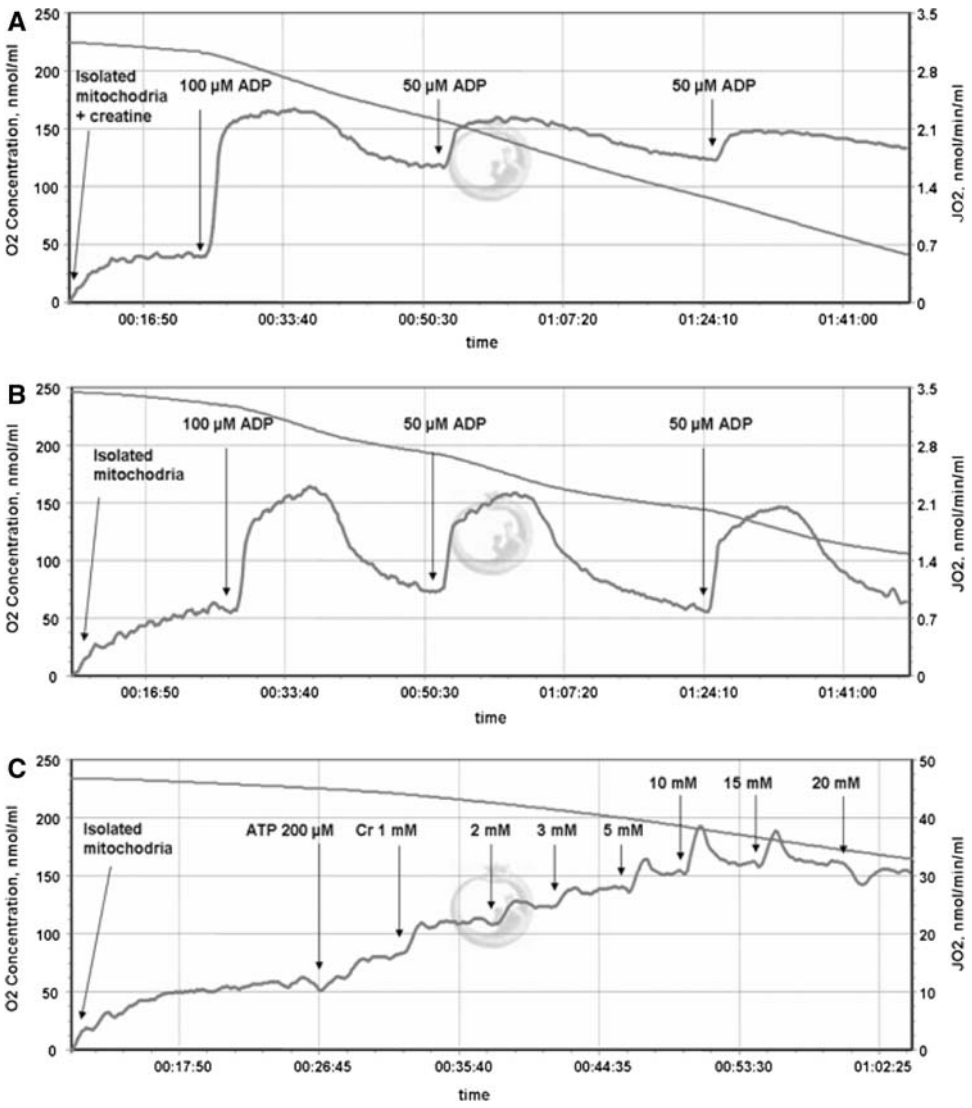


Fig. 4 (**a**, **b**) Oxygraph recording of respiratory control in isolated rat brain mitochondria by ADP (100–50–50 μM) in presence (**a**) or in absence (**b**) of 20 mM creatine (Cr), added at the beginning of the measurement. The respiration was activated with 10 mM of succinate. State 4 is never observed in presence of Cr, due to local ADP regeneration by uMtCK. (**c**) Kinetics of regulation of mitochondrial respiration by the uMtCK in isolated brain mitochondria. uMtCK was activated first by 10 mM succinate and 200 μM ATP and then by increased concentrations of Cr as indicated. Upper trace is the oxygen concentration and lower trace is the flux—the rate of oxygen consumption

uMtCK is localized at the outer surface of the inner mitochondrial membrane [54–56]. Figure 4 shows directly that this uMtCK in brain mitochondria effectively controls the respiration. Figure 4b represents the recordings of the well-established, classical phenomenon of respiratory control by the limited amounts of ADP, where respiration is activated by the addition of 50–100 μM ADP, but returns to the State 4, close to State 2 value after ADP exhaustion (rephosphorylation). In the presence of creatine (20 mM), however, State 4 level of respiration stays constantly high (increasing with increase in ADP concentration) due to continuous regeneration of local ADP by uMtCK in the vicinity of ANT and local recycling of ADP and ATP in mitochondria. This effect is in concord with many earlier findings shown for cardiac and brain mitochondria [57, 58]. Figure 4c shows the respiration regulation in the presence of ATP by the successive addition of creatine and step-wise increasing its final concentrations. Under these conditions, maximal activation of respiration was observed at creatine concentrations higher than 10 mM. Apparent Km for creatine in these experiments was found to be equal to 3–5 mM.

The kinetics of MtCK and functional coupling to the ANT: comparison of brain and cardiac mitochondria

To elucidate the mechanism of the interaction between the uMtCK reaction and oxidative phosphorylation in brain mitochondria, we carried out a complete kinetic analysis of the creatine kinase reaction in the absence and presence of oxidative phosphorylation, using approaches which were developed and described previously for cardiac mitochondria [41, 43, 44]. Reaction rates were measured at fixed various concentrations of one substrate, for example, creatine, changing the concentration of the second one MgATP, as shown in Fig. 5a. In this way, the complete

matrixes of reaction rates were obtained for the two following conditions: (1) oxidative phosphorylation was inhibited (by 10 μM of rotenone and 1 μM of oligomycin) and the uMtCK reaction kinetics was studied spectrophotometrically by coupled enzyme assay (Figs. 5 and 7) or (2) oxidative phosphorylation was activated and the kinetics of coupled reactions was studied by measuring the rate of oxygen consumption (Fig. 6). The data were analyzed in accordance with Eqs. 3–9 (see section "Material and method") to determine all kinetic constants.

The primary kinetic analysis of the uMtCK reaction for its substrates MgATP (Fig. 5a) and creatine (Fig. 5c) in absence of oxidative phosphorylation, and the corresponding linearized data in double reciprocal plots (Fig. 5b and d) give families of straight lines intercepting in one point. The latter is in accordance with the kinetic scheme of the Bi–Bi random type quasi-equilibrium mechanism [38].

The presence of oxidative phosphorylation (solid lines, Fig. 6) changed reaction rates and kinetic behavior of the MtCK reaction as compared to the absence of oxidative phosphorylation (dotted lines, Fig. 6). Primary kinetic data always yielded higher reaction rates and changed the kinetics of the direct creatine kinase reaction (Fig. 6a). This is also seen with linearized data in double reciprocal plots, giving two different families of straight lines (Fig. 6b).

Secondary analysis of all linearized primary data for both conditions studied is shown in Fig. 7. Plotting ordinate intercepts io and slopes of these straight lines shown in Figs. 5b and 6b versus 1/[Cr] gives the values of K_b and K_{ib} for creatine, correspondingly (Fig. 7b and d). When the

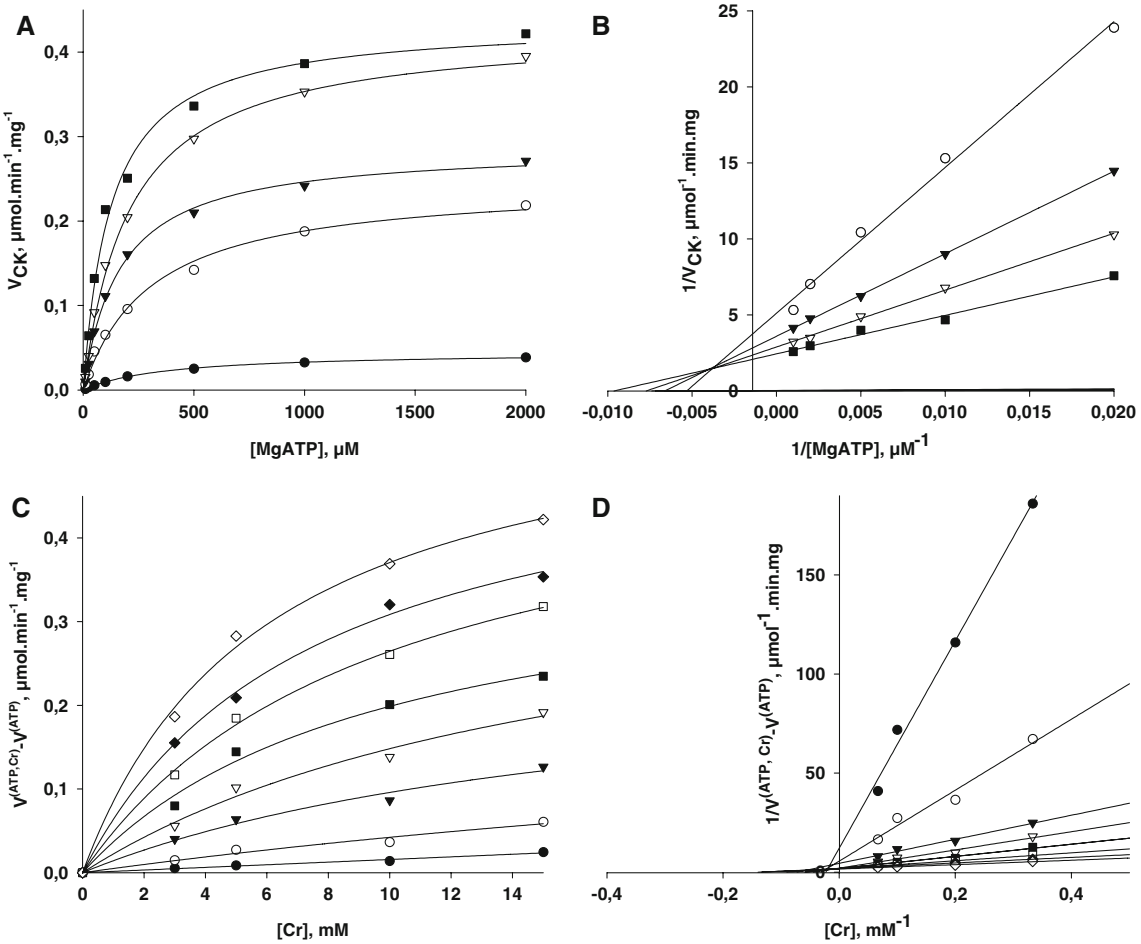


Fig. 5 The primary kinetic analysis of the uMtCK reaction in isolated rat brain mitochondria in the absence of oxidative phosphorylation. (**a**) The primary data: the dependence of the forward creatine kinase reaction rate on MgATP concentration in the presence of fixed different concentration of creatine (Cr). (**b**) Presentation of the dependences shown in Fig. 7a in double reciprocal plots for secondary analysis (see Fig. 9). (●) Without Cr, (○) 3 mM Cr, (▼) 5 mM Cr, (▽) 10 mM Cr, (■) 15 mM Cr. (**c**) The dependence of the forward creatine kinase reaction rates on the concentration of creatine for different fixed concentrations of MgATP. (**d**) Presentation of dependences given in Fig. 7c in double reciprocal plots for further secondary analysis as shown in Fig. 9: (●) 10 μM ATP, (○) 25 μM ATP, (▼) 50 μM ATP, (▽) 100 μM ATP, (■) 200 μM ATP, (□) 500 μM ATP, (◆) 1 mM ATP, (◇) 2 mM ATP. V_{CK} was obtained by calculating the rate of ATP production in presence of Cr minus the rate of ATP production in absence of Cr: $V_{CK} = V_{ATP}(Cr) - V_{ATP}(-Cr)$

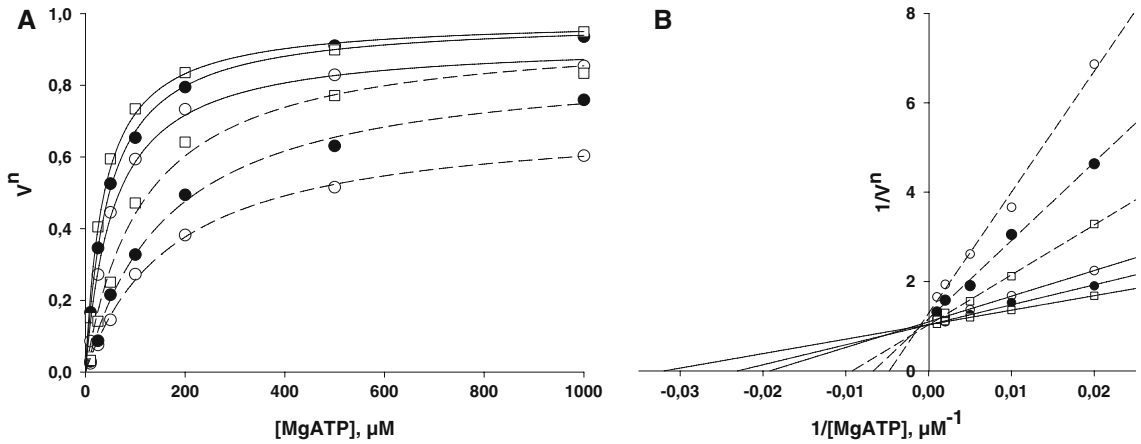


Fig. 6 Primary normalized rate of regulation of CK activity by exogenous MgATP and increased concentrations of creatine (5–10–15 mM) in presence (–) and in absence (- -) of oxidative phosphorylation. The reaction rates in presence of oxidative phosphorylation were obtained by oxygraphic measurements, and those obtained in absence of oxidative phosphorylation were measured by spectrophotometry in the same medium after inhibition of the respiratory chain and the ATP synthase by 10 μM rotenone and 1 μM oligomycin. (**a**) Michaelis–Menten representation of dependence of CK activity on exogenous MgATP and three concentration of creatine. (**b**) Double reciprocal representation of dependence of CK activity on exogenous MgATP and three concentrations of creatine. (○) 5 mM Creatine, (●) 10 mM creatine, (□) 15 mM creatine

matrix of the reaction rates was analyzed first for different fixed MgATP concentrations as functions of concentration of creatine, secondary analyses of io and slopes gave the values of K_a and K_{ia} for MgATP, correspondingly (Fig. 7a and c).

The significant advantage and importance of the use of secondary analysis is the possibility to identify and directly illustrate the reaction mechanisms [41] to quantitatively characterize and illustrate the mechanism of interaction between uMtCK, ANT, and oxidative phosphorylation. Indeed, formally in both cases the reaction rates are described by the same type of equations (Eqs. 1 and 2), and the secondary analysis of the slopes shows that the reaction mechanism was always of random type with respect to substrates in the surrounding medium and not converted into the ordered reaction mechanism [38, 46]. Active oxidative phosphorylation alters significantly only the dissociation constants of MgATP, most significantly, the dissociation constant K_a from ternary complex CK.Cr.MgATP, but also, to some extent, the dissociation constant K_{ia} from binary complex CK.MgATP (Fig. 7a and c), but does not change the dissociation constants for creatine (Fig. 7b and d). Quantitatively similar effects were found for heart mitochondria earlier and reproduced in this work under conditions used in our study (Table 2). Table 2 shows the values of all kinetic constants for the forward creatine kinase reaction both for uMtCK in brain mitochondria and sarcomeric sMtCK in heart mitochondria, both in the states of coupling and uncoupling of these reactions to oxidative phosphorylation. While the dissociation constants for creatine are somewhat lower for uMtCK as compared to sMtCK, as observed before in many investigations [59, 60], the effects of the oxidative phosphorylation on the MtCK were identical in both brain and heart mitochondria (see Table 2).

Figure 8 shows the results of computer analysis of the primary experimental data on the dependence of the oxygen consumption rate upon the substrates of the uMtCK reaction, when the concentration of MgATP was changed at different creatine concentration. Knowing from the secondary analysis described above that the reaction always follows the random type mechanism, the experimental data were analyzed by the least-squares method on the basis of Eqs. 2 and 3. The found apparent kinetic constants of CK reaction were as follows: $K_{ia} = 0.11$ mM, $K_a = 0.017$ mM, and $K_{ib} = 37.7$ mM, that giving $K_b = 5.8$ mM. The apparent Km of ATPase was 0.09 mM. The maximal respiration rates activated by uMtCK and ATPase corresponded to 73.4 and 13.8 nmol O_2/min/mg mitochondrial protein, respectively. These data are very close to those shown in Tables 1 and 2.

Regulation of respiration in permeabilized synaptosomes: effects of creatine

In cardiac and oxidative skeletal muscle cells, the intracellular diffusion of ADP is restricted, as it is evidenced by increased apparent Km for exogenous ADP in regulation of respiratory rate [22, 24, 61]. The phosphocreatine–creatine kinase system of intracellular energy transfer effectively overcomes these diffusion restrictions [7, 9, 10, 18, 20, 62]. Figure 9 shows the results of similar experiments performed using permeabilized synaptosomes. In comparison with isolated mitochondria where the apparent Km for exogenous

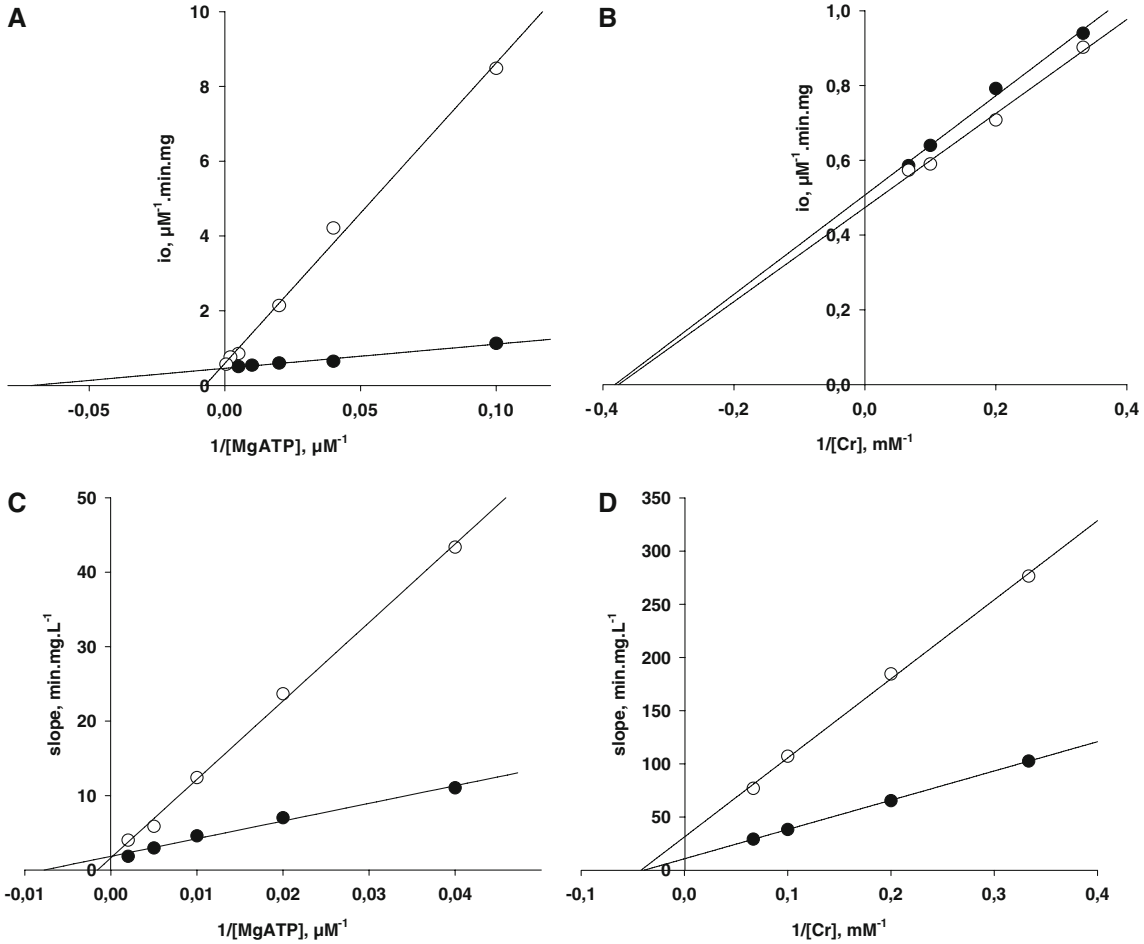


Fig. 7 Secondary analysis of the ordinate intercepts (io) and the slopes of data of the primary analysis (see Fig. 7). (**a**) The io of the lines were plotted versus ATP concentration, and the value of abscissa intercept allows to determine the dissociation constant for ATP (K_a) with and without oxidative phosphorylation according to Eq. 4. (**b**) The io of the lines were plotted versus Cr concentration, and the values of abscissa intercepts allow to determine the dissociation constant for Cr with and without oxidative phosphorylation, according to Eq. 5. (**c**) The slopes of the lines were plotted versus ATP concentration and allow the determination of the dissociation constant of ATP (K_{ia}) according to Eq. 6. (**d**) The slopes of the lines were plotted versus Cr concentration and allow the determination of the dissociation constant of Cr (Kib) according to Eq. 7. (●) With oxidative phosphorylation, (○) without oxidative phosphorylation

Table 2 Comparison of the kinetic constants of the forward creatine kinase reaction in brain and heart mitochondria: the effect of oxidative phosphorylation

Kinetic constants		Without oxidative phosphorylation	With oxidative phosphorylation
ATP constants			
K_{ia}, mM	Heart	0.85 ± 0.2	0.31 ± 0.1
	Brain	1.1 ± 0.29	0.17 ± 0.07
K_a, mM	Heart	0.16 ± 0.04	0.018 ± 0.004
	Brain	0.13 ± 0.02	0.018 ± 0.007
Creatine constants			
K_{ib}, mM	Heart	34.7 ± 11	28.5± 2.6
	Brain	24.8 ± 4	25.3 ± 5
K_b, mM	Heart	6.8 ± 3	4.4 ± 0.8
	Brain	2.9 ± 1.1	1.7 ± 0.4
Vm_1, mol/min/mol aa_3	Brain	504 ± 45	503 ± 69

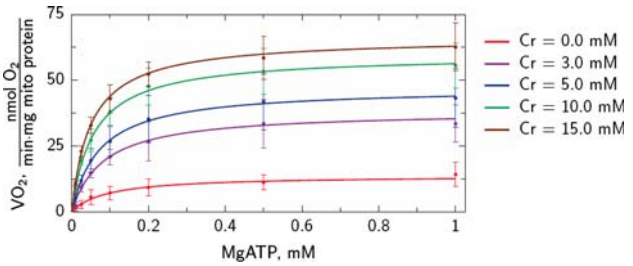


Fig. 8 Computer fitting of the brain mitochondrial respiration controlled by the creatine kinase reaction. The separate dots show the average values of the respiration rates and their standard deviations for five measurements. These rates were fitted by computer analysis based on Eq. 1 by changing the values of kinetic constants. Solid lines show the best fits of calculated dependences with experimental data

ADP is in the range of 10–20 μM, in the permeabilized rat brain synaptosomes, this Km was rather high, in the range of 110 ± 11 μM (Fig. 10). However, this latter value was decreased to 25 ± 1 μM in the presence of 20 mM creatine, showing the increased local recycling of ADP in the synaptosomal mitochondria under conditions of activated creatine kinase (Fig. 10), providing thus evidence of the important role of the uMtCK in the regulation of the mitochondrial respiration in rat brain synaptosomes. The Vm values were equal to 59 ± 11 nmol O_2/min/mg protein without creatine and 56 ± 13 nmol O_2/min/mg protein with creatine. When calculated for the cyt aa_3 content, the Vm was in the range of 600–650 nmol O_2/min/cyt *aa3*, and thus very close to the Vm of respiration in the isolated mitochondria (see Table 2).

Reconstitution experiments of isolated brain mitochondria with tubulin

One of the most possible explanation of the high apparent Km for ADP in regulation of respiration in permeabilized cells in situ is that some components (proteins) of the cytoskeleton (factor "X", [23]) are able to bind to the VDAC in the outer mitochondrial membrane and control (restrict) their permeability for adenine nucleotides. One suitable candidate protein is tubulin [24, 25, 63]. Therefore, we carried out the reconstitution experiments in which

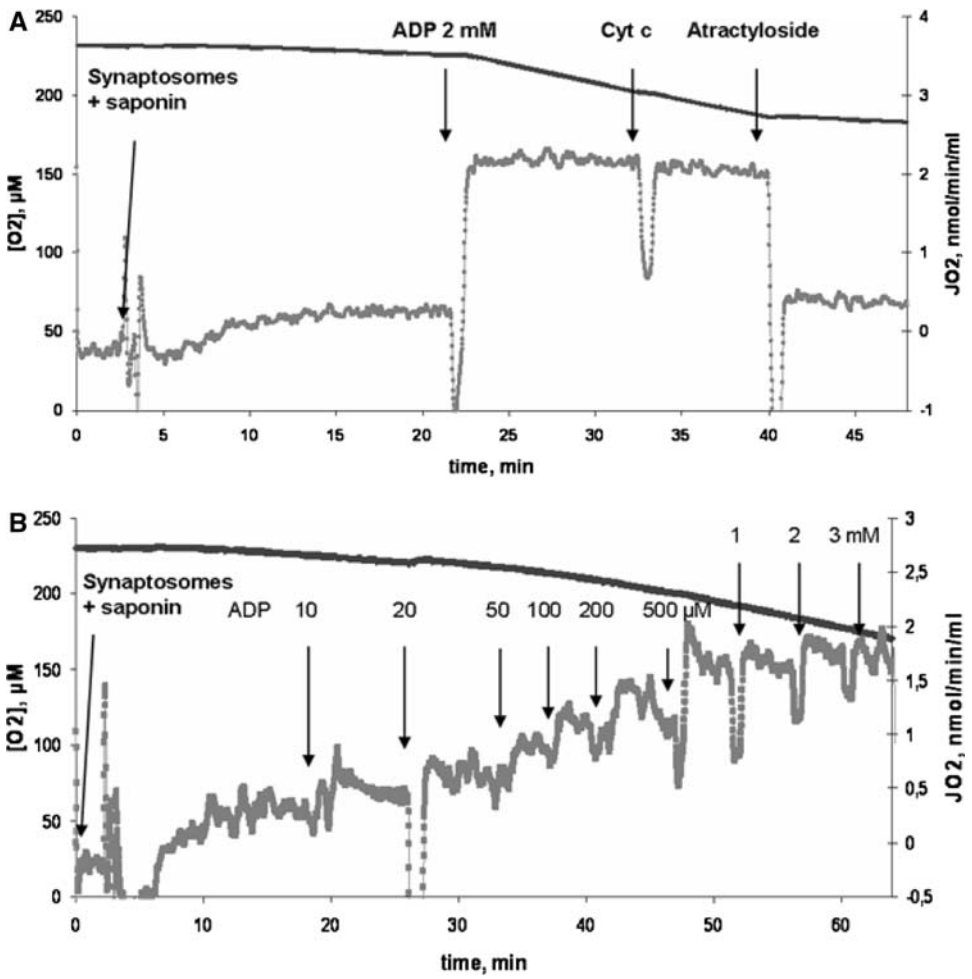


Fig. 9 (**a**) Oxygraph recording of the respiratory control ratio of permeabilized synaptosomes isolated from rat brain. Synaptosomes were permeabilized with 50 μM saponin. Upper trace is the oxygen concentration and lower trace is the flux—the rate of oxygen consumption. In this experiment, the respiratory control index was 4.6 ± 0.9. (**b**) Oxygraph recording of the regulation kinetic of synaptosomal respiration by increasing concentrations of exogenous ADP. Mitochondrial respiration was activated by 10 mM succinate. Synaptosomes were permeabilized with 50 μM saponin. Upper trace is the oxygen concentration and lower trace is the flux—the rate of oxygen consumption

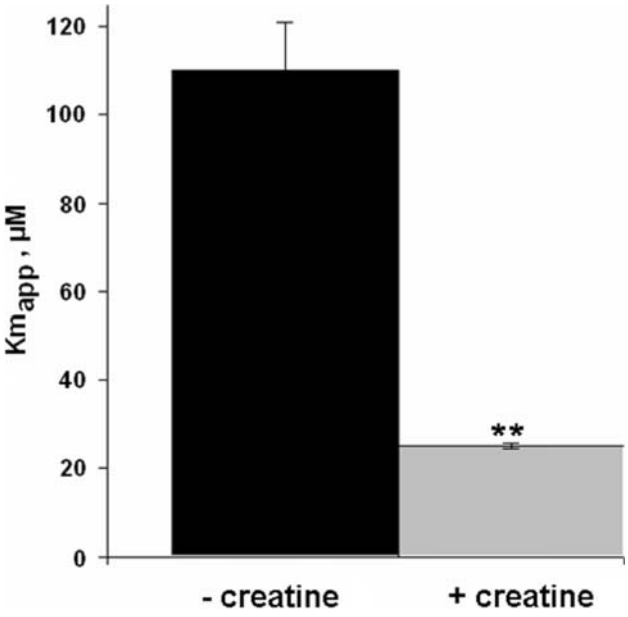


Fig. 10 Comparison of the apparent Km for exogenous ADP without and with creatine. The presence of creatine decreases the Km_{app} from 110 ± 11 to 25 ± 1 µM. Data are means ± SD ($n = 7$) and the two-tailed P value is <0.0001, considered extremely significant

isolated brain mitochondria were first incubated with tubulin (1 µM) from rat or cow brain and then respiration kinetics was analyzed. In this analysis, apyrase (1 IU/ml) was added to regenerate ADP and thus maintain steady state levels of respiration even at low ADP concentrations (see section "Material and method"). The results of these experiments are shown in Fig. 11, showing clear changes induced by tubulin in the kinetics of regulation of respiration of isolated brain mitochondria. While in experiments without tubulin, maximal respiration was achieved already at ADP concentrations higher than 100 µM (Fig. 11a); after incubation with tubulin the respiration rate continued to increase even after addition of ADP in millimolar concentrations (Fig. 11b). Analysis of these data showed that, while in the experiments without tubulin, there was only one population of mitochondria with low Km (ADP) equal to 9 ± 1 µM, addition of tubulin-induced biphasic behavior of the system, and appearance of the second population of mitochondria with high apparent Km (ADP) equal to 169 ± 52 µM (Fig. 11c). The Vm values were very close in both in absence (see Tables 1 and 2) and in presence of tubulin (not shown). These results of the reconstitution experiments show that tubulin binding to VDAC indeed induces a decrease of the VDAC permeability for ADP, in agreement with recent direct measurements [64]. These data are consistent with an assumption that one of the candidates responsible for the restricted VDAC permeability (factor "X") may be tubulin, probably in complex with some other cytoskeletal proteins.

Discussion

The results of this study show that effective production of phosphocreatine in the coupled uMtCK reaction permits to bypass the limited permeability of the VDAC in the outer mitochondrial membrane for adenine nucleotides, induced by interaction of these channels with tubulin and probably by other cytoskeletal proteins in rat brain synaptosomes. The detailed characterization of the kinetic behavior of the uMtCK in isolated and purified rat brain mitochondria performed first time in this work shows a tight functional coupling between this CK isoenzyme and ANT, and therefore the role of the coupled system in the control of oxidative phosphorylation. The mechanism of functional coupling between uMtCK in brain mitochondria is identical to that found between sMtCK and ANT in the heart [24, 42, 62, 65]. These results conform to earlier conclusions of the important role of the PCr–CK system in energy transfer in brain cells, and for the first time describe the presence of such system in the nerve endings–synaptosomes.

Energy metabolism of brain has been extensively studied for a long period of time, and the results were described in several review articles by Erecinska et al. [16, 17], Ames [1], Nicholls [2, 27]. The latest description of "state of art" in the field is available in the new, third edition of the Handbook of Neurochemistry and Molecular Neurobiology [20]. In particular, the energy metabolism of the synaptosomes was extensively studied by Erecinska and Nicholl's groups [2, 16, 27]. In these studies, the presence of PCr and CK system in synaptosomes was always noticed, but their roles were not fully investigated [16, 27]. Creatine kinase activity was found also to be associated with the synaptic plasma membrane and synaptic vesicles [53, 66]. The present study further develops these lines of investigation and describes the mechanism of the functioning of this system in brain, including synaptosomes.

In this study, we verified by kinetic methods the functional coupling mechanism of direct transfer of ATP from ANT to uMtCK, leading to rapid recycling of ADP and ATP and effective synthesis of phosphocreatine in mitochondria. As it is shown in this work, classical complete kinetic analysis of an enzymatic reaction is a very useful method for identification of both the mechanism and character of its interaction with other enzymes or transporters, especially if classical methods of the primary and secondary analysis by linearization are fully applied [38, 41–46]. Alternative approaches of directly fitting primary experimental data with proposed rate equation by using computer programs to find the values of kinetic constants [47, 48] are rapid and convenient. However, these methods usually leave researches in complete obscurity about real verification of reaction mechanisms (ordered, random type, etc.), for what the further use of complicated inhibitor

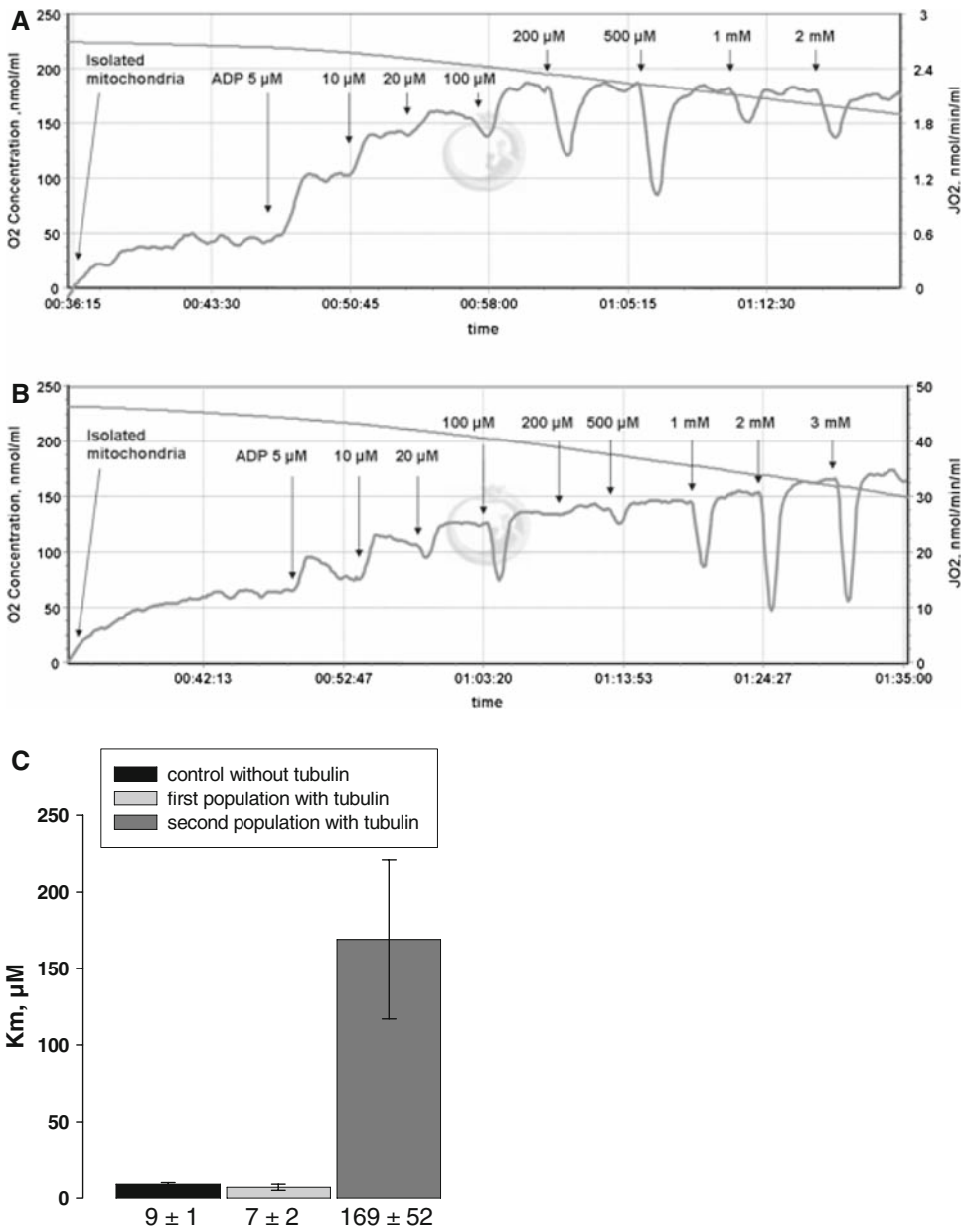


Fig. 11 Oxygraph recording of the regulation kinetic of mitochondrial respiration by exogenous ADP. Brain mitochondria were activated by 10 mM succinate and increasing concentrations of ADP (5–3,000 μM) in absence (**a**) and in presence (**b**) of tubulin 1 μM. (**c**) Comparison of the apparent Km values with or without tubulin. The upper trace is the oxygen concentration and the lower trace represents the flux—the rate of oxygen consumption

analysis with uncertain results is often needed and applied [47, 48]. At the same time, classical secondary analysis described in many textbooks [38, 46] illustrates these mechanisms immediately and shows the particularities introduced by interactions of enzymes with their partner proteins—in this case the mechanism of interaction of uMtCK with ANT. For example, the plots of slopes versus reciprocal concentration of the second substrate (Fig. 7c and d) are different for random type and ordered mechanism [38]. Most importantly, the secondary analysis shown in Fig. 7 directly and very clearly illustrates the mechanism of interaction between uMtCK and ANT—the specific and characteristic decrease of the dissociation constants for MgATP (apparent in this case for MgATP in the medium, for which it is calculated) clearly shows the direct channeling of ATP and rapid recycling of adenine nucleotides between ANT and MtCK under conditions of oxidative phosphorylation. In our case, the fitting of experimentally determined rates of respiration by variation of kinetic parameters of the MtCK reaction led to the same values of apparent constants as the secondary analysis. It has to be stressed that if fitting is used as an alternative to secondary analysis, it has to be complemented with the residual analysis to check whether the proposed equations describe the measurements adequately. However, after independent serious classical kinetic analysis (Figs. 6 and 7), the computer model of the uMtCK reaction can be confidently used for phenomenological mathematical modeling of the

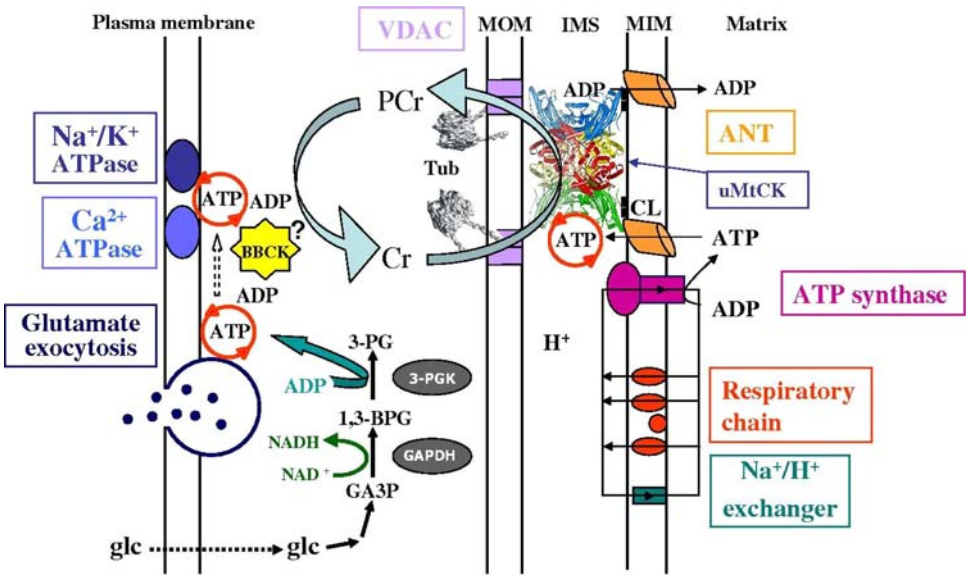


Fig. 12 Energetics of brain synaptosomes. Sites of ATP production (mitochondrial matrix) and sites of ATP consumption (ion transport across the plasma membrane and vesicle trafficking for neurotransmitter uptake and release, e.g., glutamate) are linked by an energy transfer pathway represented by the phosphocreatine/creatine kinase system. uMtCK bound to mitochondrial inner membrane (MIM) via cardiolipin (black squares). ATP consumed by the energy consuming reactions is reproduced locally by BBCK from PCr. GA3P, glyceraldehyde-3-phosphate; 1,3-BPG, 1,3 biphosphoglycerate; 3-PG, 3-phosphoglycerate; GAPDH, glycerate-3-phosphate-deshydrogenase; 3-PGK, 3-phosphoglycerate kinase; VDAC, voltage dependent anion channel; MOM, mitochondrial outer membrane; uMtCK, ubiquitous mitochondrial creatine kinase; Tub-$\alpha\beta$-heterodimer of tubulin interacting with the VDAC channels and limiting its permeability for adenine nucleotides (see text for explanation)

control of the respiration by creatine kinase in brain mitochondria, as it is shown in Fig. 8. In this way, the data shown in Fig. 8 may be taken as a first step of mathematical modeling of the compartmentalized energy transfer in brain.

It should be clearly stated that in the presence of oxidative phosphorylation the kinetic constants for MtCK are the apparent ones, since they can be influenced by many processes, such as oxidative phosphorylation, metabolite transport (in this case by ATP–ADP transporter), and by outer membrane permeability. Therefore, in many previous works the mechanism of functional coupling between MtCK and ANT has been carefully studied for heart mitochondria under different conditions [5, 10, 18, 19, 43, 44, 62]. Thus, it was also shown in previous works [43, 44] that this functional coupling was effective even in inner membrane-matrix preparation (mitoplast) in which the outer membrane was removed by digitonin [43]. Consequently, the coupling between sMtCK and ANT is independent of the permeability of the outer membrane but depends upon very close proximity of ANT and MtCK in and at the inner mitochondrial membrane [44, 62]. Indeed, this coupling can be interrupted by removing sMtCK from the membrane into the intermembrane space in KCl solution with high ionic strength [44]. The effect of oxidative phosphorylation on the kinetics of the creatine kinase reaction in heart mitochondria, particularly the decrease of K_a by more than an order of magnitude under these conditions, was recently analyzed by using the mathematical model based on thermokinetic analysis of free energy profiles of these reactions [10, 62]. This analysis showed that oxidative phosphorylation specifically alters the free energy profile of the MtCK reaction by increasing the energy level of ATP in the complex with ANT under conditions of oxidative phosphorylation, thus making the PCr production thermodynamically favorable, contrary to the thermodynamics of the CK reaction in isolated state [62]. The results of this study show that a similar mechanism is operative in the brain mitochondria. Thus, in heart, brain, skeletal and smooth muscle, and some other cells, both ANT and MtCK function within a real proteolipid supercomplex with the ATP-synthasome and VDAC, thus connecting mitochondrial ATP production with the cytoplasmic reactions of energy utilization via MtCK and VDAC [1–13]. This conclusion is in excellent concord with important new data showing that due to existence of the functional coupling between MtCK and ANT, the MtCK induced ADP recycling strongly decreases also the production of reactive oxygen species (ROS) in brain mitochondria [58].

The reconstitution experiments of isolated mitochondria with tubulin demonstrate that proteins actively involved in regulation of mitochondrial respiration are not restricted to those found in the inner and outer mitochondrial

membranes and the intermembrane space. Proteins that are thought of as purely cytoplasmic in function can also be important in regulation of respiration by altering the permeability of the outer membrane to ADP and ATP. Hexokinase is an example of a cytoplasmic protein long known to bind to VDAC in the outer membrane, but tubulin represents a completely new player in the interplay between the cytoplasm and the mitochondria. Additionally, the ability of tubulin to restrict exchange into the mitochondria represents a completely new role for this important and ubiquitous cytoplasmic protein. Indeed, both the studies of kinetics of regulation of respiration in permeabilized synaptosomes and reconstitution studies of isolated mitochondria with $\alpha\beta$-heterodimer of tubulin showed that most probably tubulin is one of the components of the cytoskeletal system which interacts with VDAC in the outer mitochondrial membrane in brain and muscle cells and limits its permeability for adenine nucleotides. Tight connections between mitochondria and cytoskeleton have been shown for many cells (reviewed by Leterrier [67], Capetenaki [68], Yaffe [69], Kuznetsov [70]). In different cells the roles of these connections are different; while in the muscle cells cytoskeleton fixes mitochondria in very precise position with almost crystal-like arrangement of the mitochondria in cardiomyocytes [71], in many other cells mitochondrial connections with cytoskeleton are the basis of their dynamic behavior [70, 72]. Bernier-Valentin and Rousset showed that tubulin purified from rat brain is able to bind with rat liver mitochondria with apparent K_d about 6×10^{-8} M, and this binding is decreased by mild treatment of mitochondria with trypsin [63]. Linden et al. discovered the microtubule associated protein MAP2 in the outer mitochondrial membrane in association with VDAC [73]. Carré et al. have found that tubulin is present in mitochondria isolated from different human cancerous and non-cancerous cell lines [25]. On the other hand, it has been shown in many studies that in permeabilized cardiac and oxidative muscle cells the apparent Km for exogenous ADP in regulation of respiration is increased by order of magnitude in comparison with isolated mitochondria, but mild treatment of these cells results in significant decrease of the apparent Km value for exogenous ADP, complete disarrangement of mitochondria in the cells and in disappearance of immunolabeling of the tubulin and microtubular network [4, 5, 10]. All these data are consistent with the assumption that tubulin plays a role in the control of the mitochondrial outer membrane permeability for ADP and ATP. Recently, it has been shown in direct measurements that tubulin induces reversible closure of VDAC channels at nanomolar concentrations and that tubulin–VDAC interaction requires the presence of the heavily negatively charged carboxy-terminal tails of tubulin [64]. Earlier, it has been shown in Colombini's laboratory that in the VDAC molecule a domain with net positive charge which forms part of the wall lining in an open state is driven out of the channel in closed state with reduced pore diameter [74, 75]. In the gating change mechanism, tubulin association with some proteins at the membrane surface may help to fix this domain in the outside position and thus decreases the channel permeability. An alternative mechanism is that tubulin enters by its negatively charged terminal tails directly into the channel lumen and thus decreases the permeability. It is an interesting problem to find out in future investigations whether in the tubulin-controlled state VDAC may be more permeable for smaller compounds (as creatine and for phosphocreatine) than for ATP and ADP. This selective permeability control could explain well the creatine-induced decrease of the apparent Km for exogenous ADP in permeabilized cells (cardiomyocytes and synaptosomes) at the unchanged values of Vm of respiration (see the text). Indeed, when calculated for the content of cytochrome aa_3, the maximal respiration values in permeabilized cells and isolated mitochondria are very close and not changed by tubulin binding.

All these results allow us to propose the following additions to the scheme of the processes of energy transduction in intact synaptosomes initially proposed by Nicholls [2]: (i) to add into this scheme the coupled uMtCK–ANT system, (ii) the binding of tubulin to VDAC, and then (iii) the whole energy transfer process by the PCr–CK system, as shown in Fig. 12. According to this scheme, mitochondrial ATP is used for phosphocreatine synthesis in the non-equilibrium uMtCK reaction after its direct transfer to the active center of this isoenzyme by ANT. At the plasma membrane of synaptosomes, PCr is used for local reproduction of the ATP. Another source of necessary ATP is the glycolytic system.

The presence of the active creatine kinase system in brain cells, particularly in synaptosomes, leads to important pathophysiological implications. It may well explain the numerous data on neuroprotective properties of creatine [6, 19, 76–82], importance of the creatine kinase system for the learning process [78–80], and usefulness of creatine as perspective agent against Parkinson's disease [81, 82].

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Articles 7 et 8

L´Interactosome Mitochondrial : mécanismes et bases structurales du couplage fonctionnel *in situ*

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Regulation of respiration controlled by mitochondrial creatine kinase in permeabilized cardiac cells in situ
Importance of system level properties

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ABSTRACT

The main focus of this investigation is steady state kinetics of regulation of mitochondrial respiration in permeabilized cardiomyocytes in situ. Complete kinetic analysis of the regulation of respiration by mitochondrial creatine kinase was performed in the presence of pyruvate kinase and phosphoenolpyruvate to simulate interaction of mitochondria with glycolytic enzymes. Such a system analysis revealed striking differences in kinetic behaviour of the MtCK-activated mitochondrial respiration in situ and in vitro. Apparent dissociation constants of MgATP from its binary and ternary complexes with MtCK, K_{ia} and K_a (1.94 ± 0.86 mM and 2.04 ± 0.14 mM, correspondingly) were increased by several orders of magnitude in situ in comparison with same constants in vitro (0.44 ± 0.08 mM and 0.016 ± 0.01 mM, respectively). Apparent dissociation constants of creatine, K_{ib} and K_b (2.12 ± 0.21 mM 2.17 ± 0.40 Mm, correspondingly) were significantly decreased in situ in comparison with in vitro mitochondria (28 ± 7 mM and 5 ± 1.2 mM, respectively). Dissociation constant for phosphocreatine was not changed. These data may indicate selective restriction of metabolites' diffusion at the level of mitochondrial outer membrane. It is concluded that mechanisms of the regulation of respiration and energy fluxes in vivo are system level properties which depend on intracellular interactions of mitochondria with cytoskeleton, intracellular MgATPases and cytoplasmic glycolytic system.

1. Introduction

In the beginning, the stimulating effect of creatine on respiration in muscle homogenates was recognised when studies in bioenergetics begun in 1930 with Engelhardt discovering ATP synthesis related to oxygen consumption [1–3], followed in 1939 by Belitzer and Tsybakova's discovery of the effects of creatine on oxygen consumption [4]. Belitzer and Tsybakova showed that creatine added to well washed homogenate of pigeon pectorals muscle strongly increased oxygen uptake and production of phosphagen (as phosphocreatine, PCr, was called at that time) without any added adenine nucleotides, which were present only in trace amounts. The efficiency the coefficient of aerobic synthesis of phosphagen, the PCr/O_2 ratio was between 5.2 and 7 [4]. Now we know that the high efficiency of the control of oxidative phosphorylation by creatine is due to the functional coupling of mitochondrial creatine kinase (MtCK) with adenine nucleotide translocase (ANT) [5–16]. However, the role of this coupling in the regulation of cardiac respiration and energetics is still not widely recognised [17–23].

Mitochondrial respiration and fine regulation of energy fluxes are of vital importance for normal cell life, especially for cells with high energy demand, such as cells of the heart, brain and skeletal muscle. In spite of the fundamental progress of knowledge of mitochondrial bioenergetics [24], the nature of respiratory control and in more general sense, the mechanisms of regulation of energy fluxes in the cardiac and other cells in vivo are still highly debated [11,12,18–20,25–30]. For further progress in this area and for elucidation of these complex mechanisms, application of newly developed Systems Biology approaches for analysis of complex integrated systems may be very helpful [31–34]. Systems Biology as a new paradigm provides novel concepts and approaches for the analysis of complex biological systems [35,36], including integrated energy metabolism of muscle and brain cells [12]. For these studies, the most useful and constructive concept of Systems Biology is that of system-level properties which are direct consequences of interactions between cellular components and not known for isolated components [28,32,33]. In the intact heart, the respiration rate is linearly dependent on the workload which itself is governed by the Frank–Starling law [11,37,38]. Remarkably, this

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occurs under conditions of metabolic stability (homeostasis), implying stable intracellular levels of metabolites such as phosphocreatine and ATP during workload and respiration rate changes [39,40]. Furthermore, under physiological conditions when workload is changed by alteration of left ventricular filling, the calcium transients in cytoplasm stay unchanged [41]. These fundamental observations evidently contradict the popular creatine kinase equilibrium theory in establishing cytoplasmic ADP concentration for respiration regulation (the latter does not change under conditions of metabolic stability), and exclude the explanation of respiration regulation by calcium ions only [25,42]. Calcium may help to activate respiration only under conditions of adrenergic stimulation which increases its entry into cells [43,44]. Further, in explaining respiration regulation the cell is often considered as a homogeneous reaction medium [18,21–23,45–48], thus ignoring the impact of the high degree of structural organization of the cell, in particular cardiomyocytes, macromolecular crowding phenomena, etc. (for a critical review see ref. [14]). However, multiple experimental studies of the kinetics of respiration regulation performed on permeabilized cells indicate the heterogeneity of ADP diffusion [49,50–69]. Local restrictions of intracellular diffusion of ADP and ATP are the basis for modular organization of cardiac energy metabolism [70–74]. Of major importance in bypassing these restrictions is the creatine kinase–phosphocreatine circuit (or shuttle) which includes both MtCK functionally coupled to the oxidative phosphorylation via ANT [74] and MM isoform of creatine kinase coupled to MgATPase reactions in myofibrils and in cellular membranes [5,6,11,12,28]. Under in vivo conditions oxidative phosphorylation is also influenced by the presence of other ADP utilizing systems within the cells such as glycolysis. System analysis of the regulation of mitochondrial respiration and oxidative phosphorylation taking into account all these complex interactions in organized intracellular medium in the cells is still absent. Our aim was to perform such a system analysis of the regulation of respiration experimentally in permeabilized cardiomyocytes in situ for further realistic developments of modelling of energy metabolism in the future. To achieve this aim, we investigated the kinetics of the regulation of MtCK-activated respiration both in isolated mitochondria and in permeabilized cardiomyocytes in the absence and presence of the glycolytic system (represented by pyruvate kinase and phosphoenolpyruvate) of trapping free ADP produced by MgATPases. The use of this protocol allowed us to study the kinetics of MtCK reaction in situ in permeabilized cardiomyocytes by excluding the influence of other ATP-consuming reactions. This molecular system analysis of a complex situation close to that in cardiac cells in vivo revealed novel aspects of the respiration regulation in cardiac cells not present in isolated mitochondria, showing that mechanisms of the regulation of respiration and energy fluxes in the cells are system-level properties dependent on the multiple interactions of mitochondria with cellular structures.

2. Materials and methods

2.1. Experimental protocols

The principles of this study are illustrated by four schemes of increasing complexity: Schemes 1 and 2 represent isolated mitochondrion as a reference system, and Schemes 3 and 4 illustrate permeabilized cardiomyocyte chosen as experimental study model. Experiments were performed first without or with activated MtCK reaction and then in the presence of ADP trapping system. This system, consisting of phosphoenolpyruvate (PEP) and pyruvate kinase (PK), traps extramitochondrial ADP produced by cytoplasmic isoforms of creatine kinases (MMCK) and MgATPase reactions and subsequently regenerates extramitochondrial ATP. Endogenous intramitochondrial ADP produced by MtCK forms a micro-domain within the intermembrane space (IMS) and is re-imported into the matrix via ANT due to its functional coupling with MtCK. A series of experiments were performed to check the properties of this model in order to make it useful for complete MtCK kinetic analysis.

2.2. Isolation of mitochondria from cardiac muscle

Heart mitochondria were isolated from adult white Wistar rats 300 g body weight, as described by Saks et al. [75]. The final pellet containing mitochondria was re-suspended in 1 ml of isolation medium containing 300 mM sucrose, 10 mM HEPES, pH 7.2, and 0.2 mM EDTA and kept in ice for no longer than 3 h.

2.3. Isolation of adult cardiac myocytes

Adult cardiomyocytes were isolated after perfusion of the rat heart with collagenase using the adaptation of the technique described previously [52]. Isolated cells were re-suspended in 1–2 ml of Mitomed solution [76] described below for respiration measurements and stored on ice during measurements. Isolated cardiomyocytes contained 70–90% of rod-like cells when observed under a light microscope.

2.4. Permeabilization procedure

In order to study the kinetics of the regulation of mitochondrial respiration in cardiomyocytes using different metabolites, the cell sarcolemma was permeabilized by saponin keeping the mitochondrial membranes intact [76,77]. The tests for intactness of the outer and inner mitochondrial membranes are described in the Results section. The permeabilization procedure was carried out directly in an oxygraph chamber with 25 μg/ml saponin during 10 min before starting the measurements of respiration rates at 25 °C and continuous stirring. To study the role of cytoskeleton in the regulation

Schemes 1–4.

Schemes 1 and 2 represent a system related to isolated heart mitochondrion. The respiratory chain (RC) complexes, ATPsynthase (F_1F_0) and Pi carrier PIC are integrated within the mitochondrial inner membrane (MIM). Mitochondrial creatine kinase (MtCK) is depicted as an octamer [15,16], located in the mitochondrial inter-membrane space (IMS) and attached to the inner membrane surface. In our experiments MtCK is activated by creatine (Cr) in the presence of ATP. The final products of MtCK-forward reaction are phosphocreatine (PCr) and endogenous ADP. The ADP phosphorylation is visualized by recording the oxygen consumption. In Scheme 1 endogenous intramitochondrial ADP produced by MtCK reaction forms a micro-domain within intermembrane space. The micro-compartmentalized ADP can either enter into mitochondrial matrix for phosphorylation or escape into the surrounding medium via voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane (MOM).

In Scheme 2 the model is supplemented with ADP-trapping system consisting of pyruvate kinase (PK) and phosphoenolpyruvate (PEP). This system utilizes all ADP leaving mitochondria to regenerate extramitochondrial ATP.

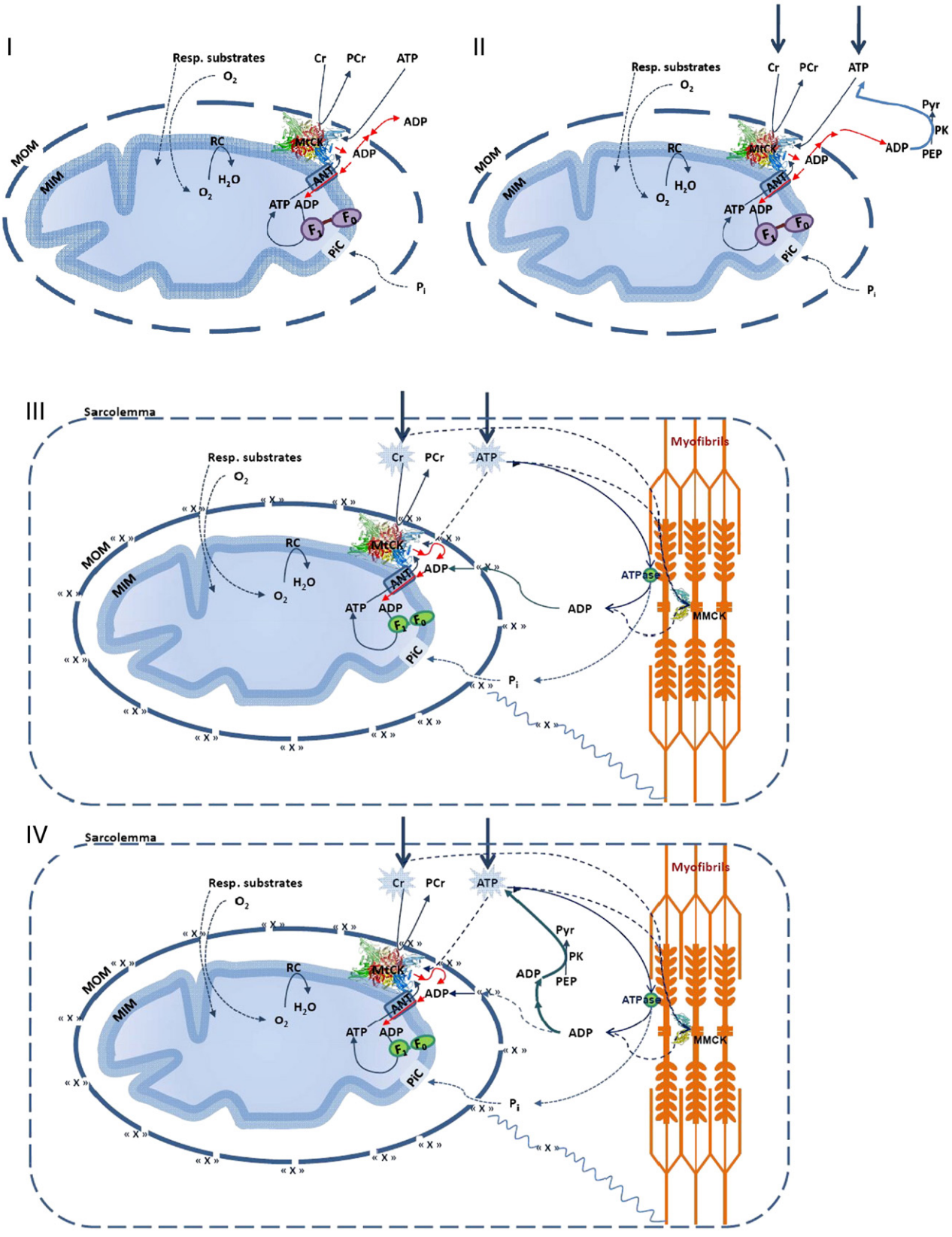
Scheme 3 represents mitochondrion in situ, in permeabilized cardiac cell, surrounded by cytoskeleton proteins (depicted as "x" factor) and myofibrils. The MOM is less permeable than in isolated mitochondrion, due to the interactions of VDAC with cytoskeleton proteins. Exogenous ATP is hydrolyzed by cellular ATPases into endogenous extramitochondrial ADP and inorganic phosphate (Pi). Mitochondrial (MtCK) and non-mitochondrial MM creatine kinases (cytosolic, myofibrillar, SERCA, sarcolemmal), activated by creatine in the presence of ATP, produce endogenous intra- and extramitochondrial ADP. Thus the oxidative phosphorylation is controlled by endogenous ADP produced by the MtCK, MMCK and ATPase reactions.

Scheme 4 represents system III supplemented with phosphoenolpyruvate (PEP) and pyruvate kinase (PK). PEP–PK system removes extramitochondrial ADP produced by intracellular ATP-consuming reactions and continuously regenerate extramitochondrial ATP. Endogenous intramitochondrial ADP produced by MtCK forms microcompartments within the IMS and is re-imported into the matrix via adenine nucleotide translocase (ANT) due to its functional coupling with MtCK. A series of experiments were performed to check the properties of this model in order to use it for complete MtCK kinetic analysis.

of mitochondrial respiration, permeabilized cardiomyocytes were treated with 0.3 μM trypsin, added into the oxygraph chambers. After 6 min of incubation, trypsin activity was inhibited by 2 μM Soybean trypsin inhibitor (STI) and 5 mg/ml fatty acid free BSA. Then the experiment was continued using these disorganized cells.

2.5. Measurements of oxygen consumption

The rates of oxygen uptake were determined with a high-resolution respirometer Oxygraph-2K (OROBOROS Instruments, Austria) in Mitomed solution [76] containing 0.5 mM EGTA, 3 mM $MgCl_2$,



60 mM K-lactobionate, 3 mM KH_2PO_4, 20 mM taurine, 20 mM HEPES, 110 mM sucrose, 0.5 mM dithiothreitol (DTT), pH 7.1, 2 mg/ml fatty acid free BSA, complemented with 5 mM glutamate and 2 mM malate as respiratory substrates.

Measurements were carried out at 25 °C; solubility of oxygen was taken as 240 nmol/ml [78].

In kinetic experiments with different fixed MgATP concentrations, stock solution of 100 mM MgATP was prepared by mixing equimolar amounts of $MgCl_2$ and ATP; pH was adjusted to 7.2.

2.6. Measurements of mitochondrial cytochrome content

For comparative quantitative analysis of the kinetics of the regulation of respiration in isolated mitochondria and permeabilized cardiomyocytes, the respiration rates were expressed in nmol of oxygen consumed per minute per nmol of cytochrome aa_3, but not per mg of protein (if not indicated differently). Cytochrome aa_3 content in both cases is representative of respiratory chain, while proteins contained in cardiomyocytes are not all present in mitochondria. The contents of mitochondrial cytochrome aa_3 in the isolated mitochondria and cardiomyocytes were measured spectrophotometrically according to the method described before [79,80]. The cells or mitochondria were solubilized with 1% of sodium deoxycholate in phosphate buffer (100 mM KH_2PO_4, pH 8). The differential spectrum (reduced by dithionite versus oxidized cytochromes) was obtained by scanning from 400 to 650 nm using a Cary 50 Bio spectrophotometer (Varian, Palo Alto, USA) or Evolution 600 spectrophotometer (Thermo Electron Scientific Instruments, UK). Cytochromes of the respiratory chain were reduced by addition of several crystals of sodium dithionite to 1 ml of suspension of mitochondria (4 mg/ml) or cardiomyocytes (2 mg/ml). The value of peak at 605 nm was used for quantification of respiratory chain cytochrome aa_3 contents (cytochrome *c* oxidase) both in isolated mitochondria and cardiomyocytes, using the extinction coefficient ε value equal to 24 mM^{-1} cm^{-1} [80,81]. Protein concentrations were determined using a BCA protein assay kit (Pierce, USA) as a standard.

2.7. Determination of the rate of PCr production in cardiomyocytes in situ by ion pair HPLC/UPLC

Determination of the rates of PCr synthesis in permeabilized cardiomyocytes in situ under conditions used in respirometry experiments was carried out using ion pair HPLC/UPLC by stopping the reaction typically at 3, 6 and 10 min. 100 µl aliquots of the reaction mixture were withdrawn and added to a 200 µl ice-cold 1 M $HClO_4$ solution, immediately supplemented with 5 µl of 100 mM EDTA and neutralized with 210 µl of 0.952 M KOH in 0.5–1 min. The samples were held on ice for additional 10–15 min for proper precipitate formation and centrifuged at 16,000 *g* and 4 °C for 2–3 min. The supernatants were immediately frozen (−40 °C) and analyzed within 5–6 h. Addition of EDTA (final 1 mM) proved to be useful in order to bind traces of Mg^{2+} to suppress any residual enzyme (particularly adenylate kinase, unpublished observations) activity and stabilize the preparations. Separations of Cr, PCr and adenine nucleotides were performed by ultraperformance ion-pair chromatography (UPLC) on a 2.1×100 mm ACQUITY UPLC HSS T3

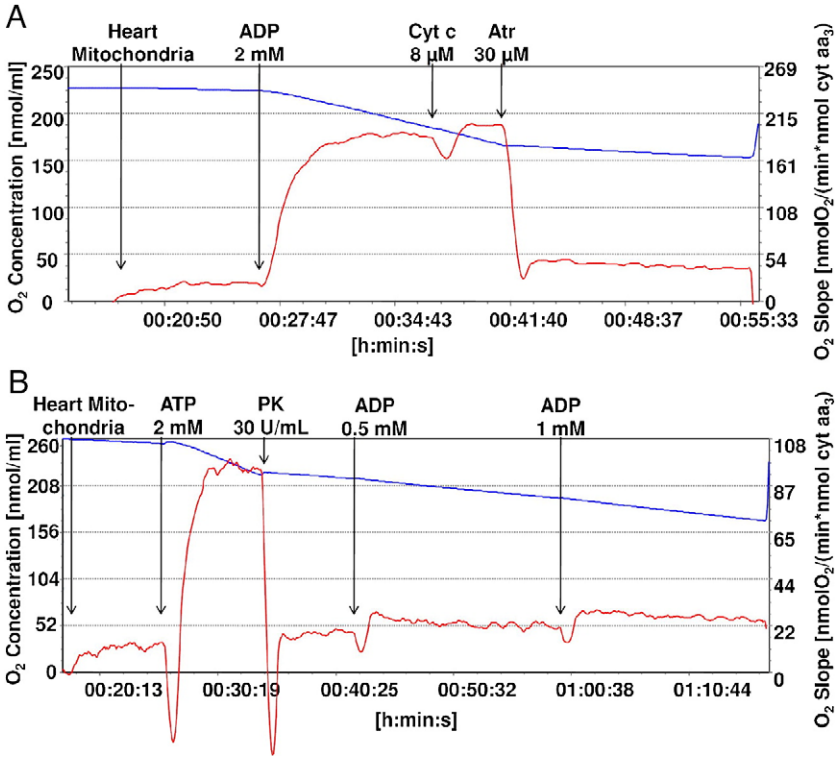


Fig. 1. (A) Representative respiration traces of isolated mitochondria recorded using a two-channel high resolution respirometer (Oroboros oxygraph 2k, Oroboros, Innsbruck, Austria). The left scale and the blue trace indicate the oxygen concentration ($nmolO_2$ ml^{-1}) in the experimental milieu. The right scale and the red trace show the rate of oxygen uptake expressed in $nmolO_2$ min^{-1} $nmol^{-1}$cyt. aa_3. The experiment was carried out in Mitomed solution with 5 mM glutamate/2 mM malate as respiratory substrates. State 3 of respiration rate (according to Chance) is achieved by adding 2 mM ADP. The integrity of the outer and inner mitochondrial membranes (MOM and MIM) was tested by addition of 8 µM cytochrome *c* and 30 µM atractyloside (Atr) respectively. Only samples with the respiratory control index ($RCI = V^3/V^2$) exceeding 7 and activation of respiration by exogenous cytochrome c less than 7% were used for experiments. (B) Effect of ADP-trapping system on respiration of isolated mitochondria stimulated by ATP. When the stable level of respiration is achieved 30 U/ml PK and 5 mM PEP are added. The PEP–PK system inhibits respiration using all free ADP. Subsequent addition of ADP in increasing amounts did not stimulate respiration.

C_{18} column packed with 1.7 μm particles (Waters) by recording optical density simultaneously at 210 nm and 254 nm for creatine and PCr, and adenine nucleotides, respectively. Sample volumes of 10 μl were injected by autosampler. The mobile phase consisted of buffer A (20 mM KH_2PO_4, 3 mM tetrabutylammonium bisulfate (TBAS)) and buffer B (200 mM KH_2PO_4, 10% (v/v) acetonitrile, 0.3 mM TBAS), both adjusted to pH 5.0 with 2 M phosphoric acid and filtered through a 0.2 μm membrane filter. The elution was performed at a flow rate 0.4 ml/min in buffer A for 2 min followed by 1:1 gradient elution with buffers A and B up to 8.5 min and additionally with buffer B up to 10 min. After the analysis, the column was re-equilibriated by washing for 1 min with water and buffer A for 9 min thus resulting in total time for an analysis of 20 min. The column was periodically cleaned by washing with 80% methanol. The retention time for the reagents were, in minutes, 0.63 (Cr), 1.70 (PCr), 6.33 (AMP, traces), 6.95 (ADP) and 7.29 (ATP), all within ±0.01 min. Stock solutions for calibration (0.1 M) were prepared in 0.2 M KH_2PO_4 at pH 7.0 and stored at −40 °C for not more than 2–3 days in order to minimize PCr and ATP degradation. Calibration solutions were prepared in supernatant solutions obtained after addition and precipitation of cardiomyocytes as described above.

2.8. Analysis of the steady state kinetics of the MtCK reaction coupled to respiration

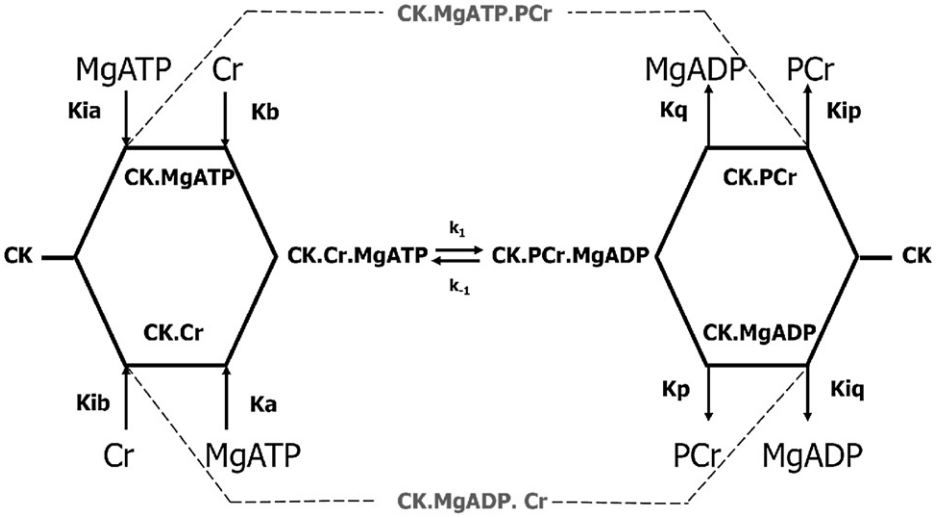
The steady state kinetics of MtCK reaction coupled to oxidative phosphorylation via ANT in permeabilized cardiomyocytes in situ was studied using the protocol shown in Scheme 4. Cardiac cells were injected into an oxygraph chamber and permeabilized with 25 μg/ml saponin by incubation for 10 min. Then respiration was activated by addition of MgATP at different fixed concentrations to initiate the endogenous MgADP production by MgATPases. Then 20 U/ml PK and 5 mM PEP were added for the trapping of MgADP and ATP regeneration. This significantly reduced the respiration rate. Then the MtCK reaction was activated by adding creatine in increasing concentrations. Under these conditions steady state kinetics of respiration follows the kinetics of MtCK reaction. For kinetic analyses only the respiration rates dependent on creatine were used; these rates were found by subtraction of the respiration rates after PK–PEP addition in the absence of creatine from the total respiration rates measured in the presence of ATP and creatine. Because of the constant PCr/O_2 ratio found in the experiments (see Results section), the steady state reaction rates were expressed as VO_2, since the main aim was to study the kinetics of the regulation of respiration dependent on MtCK. These rates can be easily converted into the MtCK reaction rates by using this PCr/O ratio according to the equation $V_{CK} = 5.7\ VO_2$. Experimental data were analyzed by applying the method of complete kinetic analysis of MtCK reaction described earlier for isolated mitochondria by W. Jacobus and V. Saks [82]. The methods of complete kinetic analysis of the creatine kinase reaction [75,83] are following. According to Cleland classification, the creatine kinase reaction mechanism is Bi-Bi random quasi-equilibrium type [83] (see Scheme 5).

Scheme 5 shows the interconversion of productive ternary enzyme–substrate (CK·Cr·MgATP) and enzyme–product (CK·PCr·MgADP) complexes in the presence of $MgATP^{2-}$, MgADP-, creatine and phosphocreatine. K_{ia}, K_{ib}, K_{iq}, and K_{ip} are the constants of dissociation from the binary and K_a, K_b, K_q, and K_p from the ternary complexes with creatine kinase. k_1 and $k_{-1}$are the rate constants of the forward and reverse reactions. The dead-end ternary complexes CK·MgATP·PCr and CK·MgADP·Cr are abortive complexes limiting enzyme activity [75].

Dissociation constants for MgATP K_{ia}, K_a and for Cr K_{ib}, K_b from their binary and ternary complexes with MtCK are:

$$K_{ia} = \frac{[CK] \cdot [MgATP]}{[CK \cdot MgATP]};\ K_b = \frac{[CK \cdot MgATP] \cdot [Cr]}{[CK \cdot Cr \cdot MgATP]};\ K_{ib} = \frac{[CK] \cdot [Cr]}{[CK \cdot Cr]};$$
$$K_a = \frac{[CK \cdot Cr] \cdot [MgATP]}{[CK \cdot Cr \cdot MgATP]}. \quad (1)$$

The limiting effect of the final product MgADP accumulation was avoided in experiments by using ATP regeneration system: oxidative phosphorylation or/and PEP–PK system. To study the kinetic properties of MtCK in situ, in the permeabilized cardiomyocytes, several conditions have to be fulfilled: firstly, MtCK must totally control oxidative phosphorylation, secondly, MgADP produced by MtCK should be compartmentalized within the intermembrane space without leaking into extramitochondrial medium but taken back by ANT into mitochondrial matrix. This has been shown to be the case in well-prepared cardiomyocytes with the content of rod-like Ca-tolerant cells higher than 70–90% and stable after permeabilization procedure. The last condition is to exclude maximally the stimulatory effect of extramitochondrial ADP on oxidative phosphorylation, and that was achieved by adding at least 20 U/ml of PK with 5 mM PEP.



Scheme 5. The kinetic mechanism of MtCK reaction.

2.8.1. Kinetics of the forward MtCK reaction

The rate of forward CK reaction in the presence of MgATP and creatine is defined by Eq. (2) if the formation of dead-end complexes is ignored:

$$v = \frac{V_m \cdot [\mathrm{MgATP}] \cdot [\mathrm{Cr}]}{K_{ia}K_b + K_b[\mathrm{MgATP}] + K_a[\mathrm{Cr}] + [\mathrm{MgATP}] \cdot [\mathrm{Cr}]}. \quad (2)$$

Primary analysis of data derived from Eq. (2) in double-reciprocal coordinates of 1/v versus 1/[S] is:

$$\frac{1}{v} = \left[\frac{K_b}{V_m}\left(\frac{K_{ia}}{[\mathrm{MgATP}]} + 1\right)\right]\frac{1}{[\mathrm{Cr}]} + \frac{1}{V_m}\left(\frac{K_a}{[\mathrm{MgATP}]} + 1\right) \quad (3)$$

for fixed [MgATP] and varying [Cr],and:

$$\frac{1}{v} = \left[\frac{K_a}{V_m}\left(\frac{K_{ib}}{[\mathrm{Cr}]} + 1\right)\right]\frac{1}{[\mathrm{MgATP}]} + \frac{1}{V_m}\left(\frac{K_b}{[\mathrm{Cr}]} + 1\right) \quad (4)$$

for fixed [Cr] and varying [MgATP].

This primary analysis provides the values of ordinate intercepts (i_1, i_2) and slopes (s_1, s_2) for secondary analysis (Eqs. (5) and (6)).

$$i_1 = \frac{1}{V_m}\left(\frac{K_a}{[\mathrm{MgATP}]} + 1\right);\ s_1 = \frac{K_b}{V_m}\left(\frac{K_{ia}}{[\mathrm{MgATP}]} + 1\right) \quad (5)$$

$$i_2 = \frac{1}{V_m}\left(\frac{K_b}{[\mathrm{Cr}]} + 1\right);\ s_2 = \frac{K_a}{V_m}\left[\frac{K_{ib}}{[\mathrm{Cr}]} + 1\right]. \quad (6)$$

By replotting the estimated regression parameters as functions of secondary substrates, one can obtain the values of dissociation constants for MgATP (K_{ia} and K_a,) and creatine (K_{ib} and K_b) from their binary and ternary complexes with MtCK.

At $i_1 = 0$ and $i_2 = 0$ the values for K_b and K_a, are acquired. Constants K_{ib} and K_{ia} are obtained in the case of $s_1 = 0$ and $s_2 = 0$.

2.8.2. Product inhibition of MtCK by PCr

For the reaction in the presence of PCr the total enzyme exists in five forms.

$$[\mathrm{CK}]_t = [\mathrm{CK}] + [\mathrm{CK} \cdot \mathrm{MgATP}] + [\mathrm{CK} \cdot \mathrm{Cr}] + [\mathrm{CK} \cdot \mathrm{MgATP} \cdot \mathrm{Cr}] + [\mathrm{CK} \cdot \mathrm{PCr}]. \quad (7)$$

Again, the formation of the dead-end complex CK·MgATP·PCr is ignored because of its very high dissociation constant [75]. The rate of the forward reaction in this case is defined by Eq. (8), where K_{ip} is the dissociation constant (Eq. (9)) from its binary complex with MtCK.

$$v = \frac{V_m \cdot [\mathrm{MgATP}] \cdot [\mathrm{Cr}]}{K_{ia}K_b + K_b[\mathrm{MgATP}] + K_a[\mathrm{Cr}] + \frac{[\mathrm{PCr}]K_{ia}K_b}{K_{ip}} + [\mathrm{MgATP}] \cdot [\mathrm{Cr}]} \quad (8)$$

$$K_{ip} = \frac{[\mathrm{PCr}] \cdot [\mathrm{CK}]}{[\mathrm{CK} \cdot \mathrm{PCr}]}. \quad (9)$$

The slopes s_1 of the series of straight lines, obtained from double reciprocal coordinates 1/v versus 1/[Cr] at varying [PCr] and [MgATP] concentrations are analyzed by Eq. (10).

$$s_3 = \frac{K_{ia}K_b}{V_m}\left(1 + \frac{[\mathrm{PCr}]}{K_{ip}}\right)\frac{1}{[\mathrm{MgATP}]} + \frac{K_b}{V_m}. \quad (10)$$

By plotting the resulting slopes $s(s_3)$ versus [PCr], according to Eq. (11), abscissa intercept directly provides the value of $-K_{ip}$.

$$s(s_3) = \frac{K_{ia}K_b}{V_m} + \frac{K_{ia}K_b}{V_m}\frac{[\mathrm{PCr}]}{K_{ip}}. \quad (11)$$

2.9. Data analysis

All data are presented as mean ± SEM. Statistical analysis were performed using Student's *t*-test and $p<0.05$ was taken as the level of significance.

2.10. Reagents

Ethylene glycol-bis(2-aminoethyl)-N,N,N′,N′ether-tetraacetic acid (EGTA), lactobionic acid, 1,4-dithio-DL-threitol (DDT), imidazole, potassium dihydrogen phosphate (KH_2PO_4), 2-aminoethanesulfonic acid (taurine), 2-morpholinoethanesulfonic acid monohydrate (MES), L(−)-malic acid, L-glutamic acid and creatine monohydrate, tetrabutylammonium bisulfate (TBAS)—Fluka; magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), sodium bicarbonate ($NaHCO_3$), potassium chloride (KCl), sodium chloride (NaCl), glucose, sucrose (cell culture tested) HEPES, BES pyruvate, BES, Mg acetate—Sigma; Na_2-ATP, leupeptine collagenase A, blendzyme 1, albumin, from bovine serum, essentially fatty acid free (BSA), STI, phosphocreatine disodium salt (PCr)—Roche.

3. Results

3.1. Reference systems I and II: regulation of respiration by MtCK in isolated mitochondria in the absence and presence of PEP–PK

Fig. 1A shows a recording of oxygen consumption by isolated heart mitochondria used in this work as a reference system for comparison with permeabilized cardiomyocytes. Addition of ADP in a saturating concentration of 2 mM to isolated mitochondria induced State 3 high respiration rate. The respiratory control index (RCI) usually exceeded 7 (Fig. 1A). Addition of exogenous cytochrome *c* only slightly increased the respiration rate showing the intactness of the outer mitochondrial membrane, and addition of atractyloside decreased the respiration rate close to the State 2 value showing the intactness of the inner mitochondrial membrane [76,77]. These characteristics show the quality of isolated mitochondrial preparation needed for kinetic analysis. Fig. 1B shows the experimental test of the ADP trapping power of the PEP–PK system. In the presence of such a system, exogenous ADP has no effect on respiration, since all added ADP is rapidly consumed by PEP–PK. However, when respiration of isolated heart mitochondria was stimulated by creatine in the presence of ATP (i.e. MtCK was activated), addition of PK and PEP decreases respiration rate only about 50% of its maximal value (Fig. 2A). The remnant rate of respiration (up to 50% of VO_2max) was due to the functional coupling between MtCK and ANT with the direct transfer of ADP into the matrix. Gellerich et al. [84–87] have proposed the hypothesis of the dynamic compartmentation of ADP in the mitochondrial intermembrane space, according to which ADP concentration gradients across the outer mitochondrial membrane may explain the respiration stimulated by creatine in the presence of the PEP–PK system. However, Vendelin et al. [8] showed by applying mathematical modelling that the direct ATP transfer between ANT and MtCK is more important for respiratory control by creatine in isolated mitochondria than the dynamic ADP compartmentation proposed by Gellerich [66]. Fig. 2B shows the kinetics of activation of respiration in isolated heart mitochondria by addition of increasing amounts of creatine up to 20 mM in the presence of ATP and ADP-trapping PEP–PK system. Again not more than 50% of V_{max} can be reached in this experiment due to the functional coupling of MtCK with ANT. That means that at least 50% of ADP produced locally by MtCK is accessible for the extramitochondrial PEP–PK system due to high permeability of VDAC for adenine nucleotides in isolated mitochondria, in accordance with classical data from Klingenberg's [88] and Colombini's laboratories [89]. At the same time, stimulation of respiration up to 50% of V_{max} by creatine in the presence of PEP–

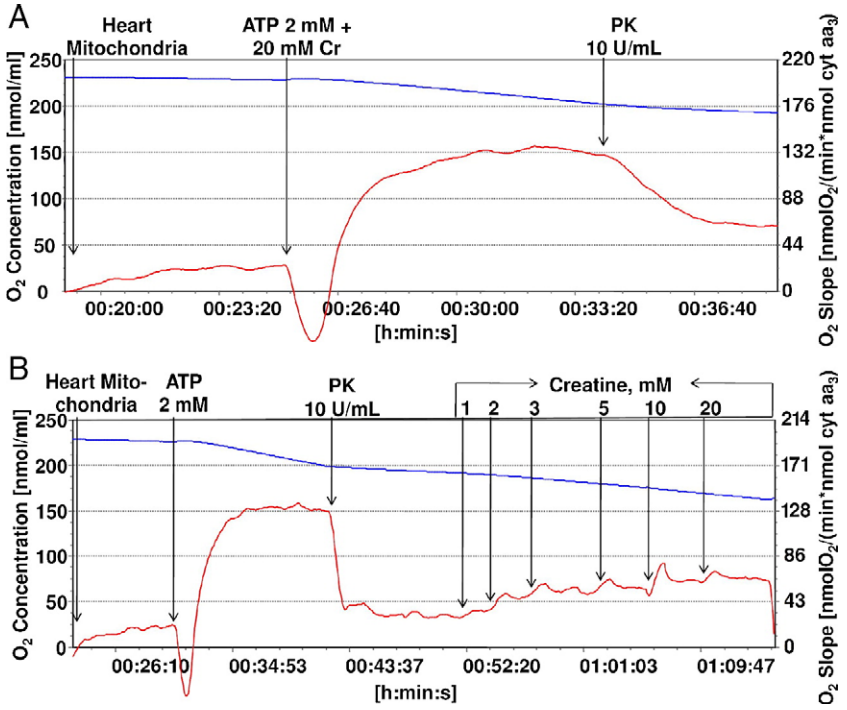


Fig. 2. (A) Stable respiration rate supported by MtCK activity in the presence of ATP and creatine. Under these conditions, approximately 50% of ADP, produced in the intermembrane space, can be trapped by powerful the PEP–PK system. The respiration is not completely inhibited because of the presence of direct ADP transfer from MtCK to ANT. (B) The kinetics of activation of respiration in isolated mitochondria stimulated by increasing amounts of creatine in the presence of ATP (i.e. activated MtCK reaction) and the absence of extramitochondrial ADP, which is efficiently consumed by the PEP–PK reaction.

PK system shows the existence of the functional coupling between MtCK and ANT.

3.2. Regulation of respiration in experimental system III—permeabilized cardiac cells without PEP–PK

Regular quality tests for isolated cardiomyocytes used in this work were similar to those reported for isolated mitochondria in Fig. 1A. The effect of the addition of exogenous cytochrome *c* on State 3 respiration was absent showing the intactness of MOM. Only preparations with these characteristics were used in the experiments reported in this work.

Table 1 summarizes the respiratory parameters of the isolated mitochondria and isolated cardiomyocytes. As it can be seen from Table 1, maximal respiration rates are equal to both isolated mitochondria and cardiomyocytes if calculated per nmol of cytochrome aa_3. Expressed in this way, the kinetic data reported in this work may be easily used for the quantitative analysis by mathematical modelling in the future.

Table 1
Basic respiration parameters of isolated rat heart mitochondria and of mitochondria in situ in permeabilized cardiomyocytes.

Parameter	Mitochondria in vitro	Mitochondria in situ (permeabilized cardiomyocytes)
V_0, $nmolO_2\ min^{-1}\ mg\ prot^{-1}$	26.37 ± 7.93	7.53 ± 1.61
V_3 (2 mM ADP), $nmolO_2\ min^{-1}\ mg\ prot^{-1}$	187.94 ± 40.68	84.45 ± 13.85
[Cyt aa_3], nmol mg $prot^{-1}$	1.00 ± 0.012	0.46 ± 0.09
V_3 (2 mM ADP), $nmolO_2\ min^{-1}$ nmol cyt aa_3^{-1}	187.94 ± 40.68	178.23 ± 33.96
$V_{Cr,ATP}$, $nmolO_2\ min^{-1}$ nmol cyt aa_3^{-1}	197.90 ± 31.86	162.63 ± 26.87

V_0—respiration rate in State 2 in the presence of substrates before addition of ADP or ATP.
V_3—respiration rate in the presence of 2 mM ADP.
$V_{Cr,ATP}$—respiration rate in the presence of activated MtCK by 2 mM ATP and 20 mM creatine.

In cells in vivo, endogenous ADP may be produced in MgATPase and in creatine kinase (MtCK and MMCK) reactions from ATP (see Scheme 3). In this scheme exogenous ATP added to permeabilized cardiomyocytes is hydrolyzed by cellular ATPases with the formation of endogenous extramitochondrial ADP which subsequently stimulates mitochondrial respiration. The apparent kinetics of activation of respiration by exogenous MgATP evidently follows the kinetics of activation of cellular ATPases by this substrate and is shown in Fig. 3A. In the absence of creatine, the apparent K_m (K_m^{app}) for exogenous MgATP is 157.75 ± 40.06 μM. In the presence of creatine the K_m^{app} for MgATP decreases to 24.89 ± 0.81 μM (Fig. 3A). Almost maximum activation of respiration in the presence of creatine and significant decrease of apparent K_m for MgATP under these conditions are related to the control of respiration by both intramitochondrial ADP locally produced in the MtCK reaction and extramitochondrial ADP produced in MMCK in MgATPases reactions. Functional coupling of MtCK with ANT is a powerful amplification mechanism of a regulatory ADP metabolic signal from cytoplasm, due to a manifold increase of ADP and ATP recycling rate in mitochondria. In the presence of both MgATP and creatine one can see the overall effects of all these mechanisms.

3.3. Regulation of respiration in experimental system IV—permeabilized cardiac cells supplemented with PEP–PK

To study the role of the coupled MtCK alone in the regulation of respiration in situ in the cells, the stimulatory effect of extramitochondrial ADP produced by MgATPases and MMCK can be extinguished by the PEP–PK system (Scheme 4). Remarkably, addition of the PEP–PK system helps to simulate the in vivo conditions in the cells, where the glycolytic system always is present and by consuming ADP competes with mitochondrial respiration. An interesting and important finding in these experiments was the fact that in the presence of fully activated MtCK the addition of competitive ADP-trapping PEP–PK system could not inhibit the mitochondrial respiration in permeabilized cardiac cells in situ (Fig. 3B), in contrast with the isolated

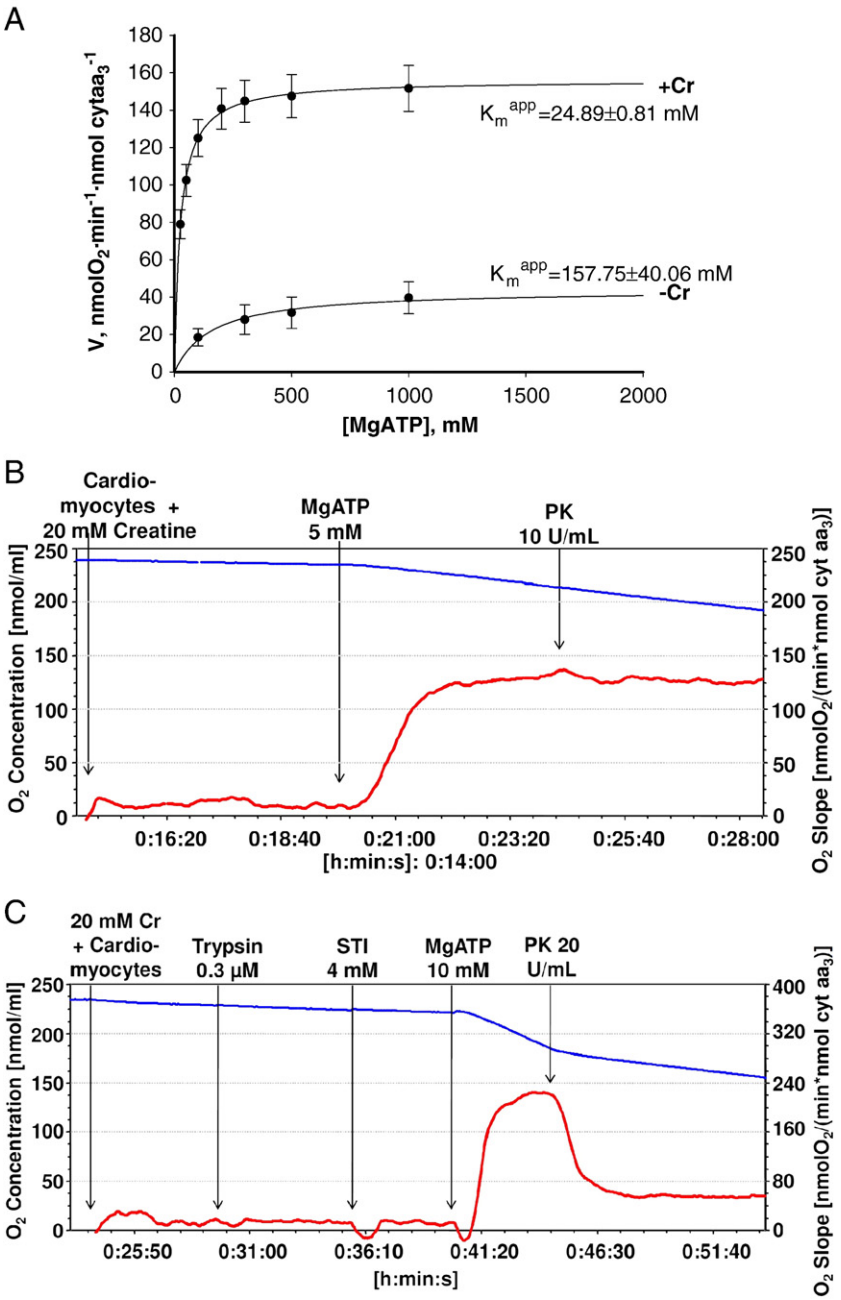


Fig. 3. (A) Rates of mitochondrial respiration in permeabilized cardiomyocytes as function of added exogenous MgATP in absence and in presence of 20 mM of creatine. After permeabilization, respiration rates were recorded after addition of different amounts of MgATP in absence and presence of creatine. Manifold increase in V_{max} and decrease in the apparent K_m for ATP in the presence of 20 mM creatine (i.e. under conditions of activated MtCK) is seen. (B) Full compartmentalization of intramitochondrial ADP produced by MtCK in the intermembrane space of mitochondria in permeabilized cardiomyocytes in situ. Instead of 2 mM ADP, respiration was activated by addition of MgATP (5 mM) and creatine (20 mM). Addition of PK in the presence of PEP (5 mM added into medium before) did not change significantly the respiration rate. In mitochondria in situ when respiration is controlled by the MtCK reaction, the powerful PEP–PK system is not able to inhibit respiration. The permeability of VDAC for ADP seems strongly decreased. (C) Proteolytic treatment of permeabilized cardiomyocytes eliminates the diffusion restrictions for endogenously produced ADP. Note, that after the treatment of isolated cardiomyocytes with trypsin in low concentration (0.3 μM) ADP becomes accessible to the PEP–PK trapping system. Rate of respiration in the presence of activated MtCK (by creatine and ATP) is due to the channelling of some part of ADP from MtCK to ANT within intermembrane space (see Fig. 2A).

mitochondria (Fig. 2A). Fig. 3B shows that the respiration rate of mitochondria in situ stimulated by 20 mM creatine and 5 mM MgATP did not decrease after addition of 10 U/ml PK in spite of the fact that the extramitochondrial ADP must have been significantly reduced. Under these conditions respiration is fully maintained by intramitochondrial ADP produced in MtCK reaction, which is not accessible for the PEP–PK system. In vivo and in situ the mitochondrial outer membrane in cardiomyocyte is most probably not easily permeable for ADP which is mainly transported by ANT back into the matrix for subsequent rephosphorylation (see Scheme 4). However, after treatment of permeabilized cardiomyocytes with trypsin, compartmentalized in the intermembrane space ADP becomes accessible for the PEP–PK system (Fig. 3C). In these experiments, cardiomyocytes were incubated in an oxygraph chamber in solution supplemented with trypsin (0.3 μM). After 6 min trypsin was inhibited by STI+ BSA, and mitochondrial respiration was stimulated by addition of 2 mM ATP and 20 mM creatine. The PK and PEP added under these conditions inhibit respiration by trapping almost all extramitochondrial ADP and a significant part of ADP produced by MtCK. A similar effect was observed in isolated mitochondria (Fig. 2A). These results

show that in the permeabilized cardiomyocytes ADP produced by MtCK in mitochondrial intermembrane space is not easily accessible for PEP–PK, most probably due to decreased permeability of VDAC in the mitochondrial outer membrane. The decrease of VDAC permeability for adenine nucleotides in mitochondria in situ as compared with mitochondria in vitro is caused probably by its interaction with cytoskeleton proteins [90–93], sensitive to trypsin treatment. Activation of respiration under these conditions directly follows the activation of MtCK by its substrates and the respiration rates can be used to study the steady state kinetics of MtCK activation by its substrates (MgATP and creatine). The micro-compartmentation of all intramitochondrial ADP produced by the MtCK reaction in the absence of its leak toward the extramitochondrial space is one of the most important properties of our experimental model—permeabilized cardiomyocyte (Scheme 4).

3.4. Regulation of respiration in experimental system IV—MtCK kinetic analysis

Such characteristics of permeabilized intact cardiomyocytes as complete micro-compartmentation of ADP produced in MtCK reaction in situ, the functional coupling of MtCK with ANT, and significant elimination of the stimulatory effect of extramitochondrial ADP by the PEP–PK system, has led us to elaborate the experimental protocol for studying in situ the role of MtCK in the regulation of mitochondrial respiration described in Materials and methods. The experimental protocol is shown in Fig. 4 and its principles are illustrated by Scheme 4. First, after permeabilization, mitochondrial respiration is stimulated by the addition of 2 mM ATP which is hydrolyzed by cellular ATPases with production of endogenous ADP. Secondly, when the stable level of respiration is achieved, the adequate quantity of PK and PEP are added to maximally uptake all free ADP and regenerate extramitochondrial ATP. Thus, the stable level of extramitochondrial ATP is maintained. *Finally*, the MtCK reaction is activated by increasing the amounts of creatine. As a result, oxidative phosphorylation is stimulated only by intramitochondrial ADP produced by the MtCK reaction (Fig. 4). To study the role of this ADP in the regulation of oxidative phosphorylation, we subtracted the oxygen consumption rate under PEP–PK (VO_{2PK}) before creatine addition from creatine-stimulated respiration rate (VO_{2Cr}).

This is the complete and the most precise protocol for studies of the MtCK functions in situ in permeabilized cells. Fig. 4 shows that the respiration rate determined by this protocol is very sensitive to changes in creatine concentration. It rapidly increases in response to the addition of creatine in rather low concentrations, 1–3 mM. To understand the reason for this high sensitivity to creatine, we performed the complete kinetic analysis of MtCK reaction under these conditions using different fixed concentrations of MgATP (0.5 to 10 mM) and varying creatine concentrations (1 to 10 mM). Graphical presentation of creatine activated respiration rates (VO_2) in function of varying concentrations of creatine and fixed MgATP and *vice versa*: in function of varying [MgATP] and fixed [Cr] are shown in Figs. 5A and 6A, respectively. The primary analysis of data in double reciprocal plots (1/V as a function of 1/[S]) according to Eqs. (3) and (4) are represented in Figs. 5B and 6B. This linearization gives the family of straight lines with one common point of interception, corresponding to the kinetic behaviour of the Bi-Bi random type quasi equilibrium reaction mechanism of creatine kinase (Figs. 5B and 6B). This primary analysis of kinetic data gives us values of ordinate intercepts and slopes for secondary linearization according to Eqs. (5) and (6) (Figs. 5C and 6C), which provides the dissociation constants for MgATP and creatine from their binary and ternary complexes with MtCK (Table 2).

This complete kinetic analysis of the MtCK reaction in cardiomyocytes in situ provides results remarkably different from MtCK kinetic properties of isolated mitochondria reported before. The kinetic analysis of the MtCK reaction of isolated mitochondria was performed applying the protocol described by Jacobus and Saks in 1982 and have been described in several previous publications [12,80,82,94]. The apparent constant of dissociation for MgATP from its binary complex with MtCK (K_{ia}) is 4 times higher (from 0.44 ± 0.08 to 1.94 ± 0.86 mM) and from ternary complex (K_a) is 100 times higher (from 0.016 ± 0.01 to 2.04 ± 0.14 mM) in mitochondria in situ than one in vitro. These data evidence strong decrease of mitochondrial affinity for free MgATP added into the medium, suggesting significant restriction of ATP diffusion in some local areas in cardiomyocytes, most probably at the level of MOM. On the other hand, we observed a strong decrease of the apparent constant of dissociation for creatine in situ. The K_{ib} for creatine (binary MtCK–substrate complex) was 15 times lower (from 28 ± 7 to 2.12 ± 0.21 mM) and K_bCr −2.5 times lower (from 5.0 ± 1.2 to 2.17 ± 0.40 mM) than in isolated mitochondria. These results show increased apparent affinity of MtCK in mitochondria in situ for creatine. Table 2 shows also the maximal steady state rates expressed as the maximal creatine-stimulated respiration rates. The rates of PCr production can be calculated from these respiration rates by using the PCr/O_2 ratio (see below). The maximal steady state rate of PCr production in isolated mitochondria calculated in this way is 1.1 μmol·min^{-1}·nmol cyt. aa_3^{-1} that is practically equal to the activity of MtCK measured in the direction of PCr production at saturating substrates' concentrations, and to the maximal activity of ATP synthesis reaction [75,95,96]. For cardiomyocytes, this rate

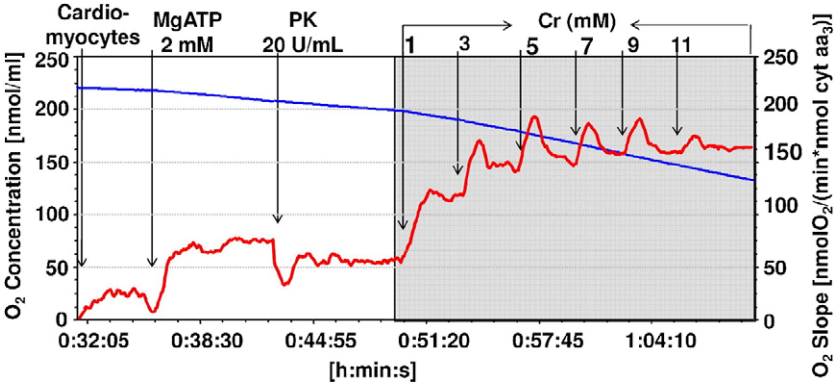


Fig. 4. The experimental procedure used for complete kinetic analysis of MtCK in mitochondria in situ (permeabilized cardiomyocyte). First, the respiration is activated by addition of MgATP after permeabilization of cardiomyocytes by saponin inducing production of endogenous ADP in MgATPase reaction (Schemes 3 and 4). Then PEP–PK is added to trap all extramitochondrial free ADP. This decreases the respiration rate, but not to initial level, due to structural organization of ICEU (see Schemes 3 and 4). Mitochondria are in privileged position to trap some of endogenous ADP. Under this conditions addition of creatine in different amounts activates MtCK reaction. The oxidative phosphorylation is stimulated mostly by intramitochondrial ADP, produced by MtCK reaction, which is not accessible for PEP–PK.

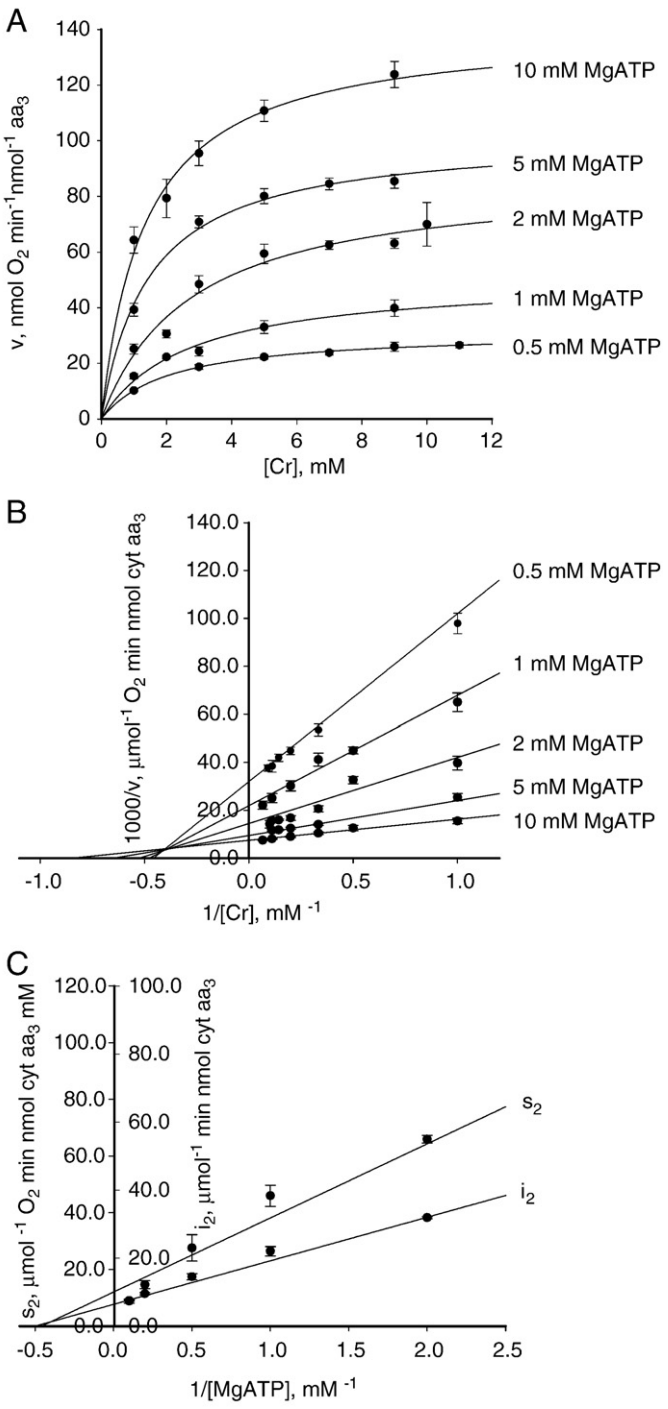


Fig. 5. (A) The rates of creatine activated respiration of permeabilized cardiomyocytes as functions of varying creatine concentrations at fixed MgATP concentrations. The respiration rates (VO_2) were calculated by subtraction of oxygen consumption rates in the presence of PEP–PK (VO_{2PK}) from the creatine activated respiration rates (VO_{2Cr}) ($VO_2 = VO_{2Cr} - VO_{2PK}$), (see Fig. 4 right panel). (B) The primary analysis of data (A) in double reciprocal coordinates of 1/v (1/respiration rate) versus 1/[Cr] (substrate with varying concentration) for different fixed MgATP concentration. (C) The secondary analysis of the primary plots from panel B. Slopes (s) and intercepts of y-axis (i) are plotted as a function of reciprocal coordinate of secondary substrate MgATP. The intercepts of x-axis provide directly the reciprocal values of dissociation constants of MgATP (K_{ia} and K_a) from the binary and ternary complexes of MtCK.

measured directly as described below (Kinetics of respiration in experimental system IV—MtCK kinetic analysis) is 0.51 ± 0.04 µmol min^{-1} mg^{-1}protein or again 1.1 µmol min^{-1} nmol cyt. aa_3^{-1}. That means that MtCK reaction coupled to ANT functions in steady state far from equilibrium, in good agreement with the results of mathematical modelling of this coupling [97] and confirmed by direct measurements with ^{31}P NMR inversion transfer technique [98].

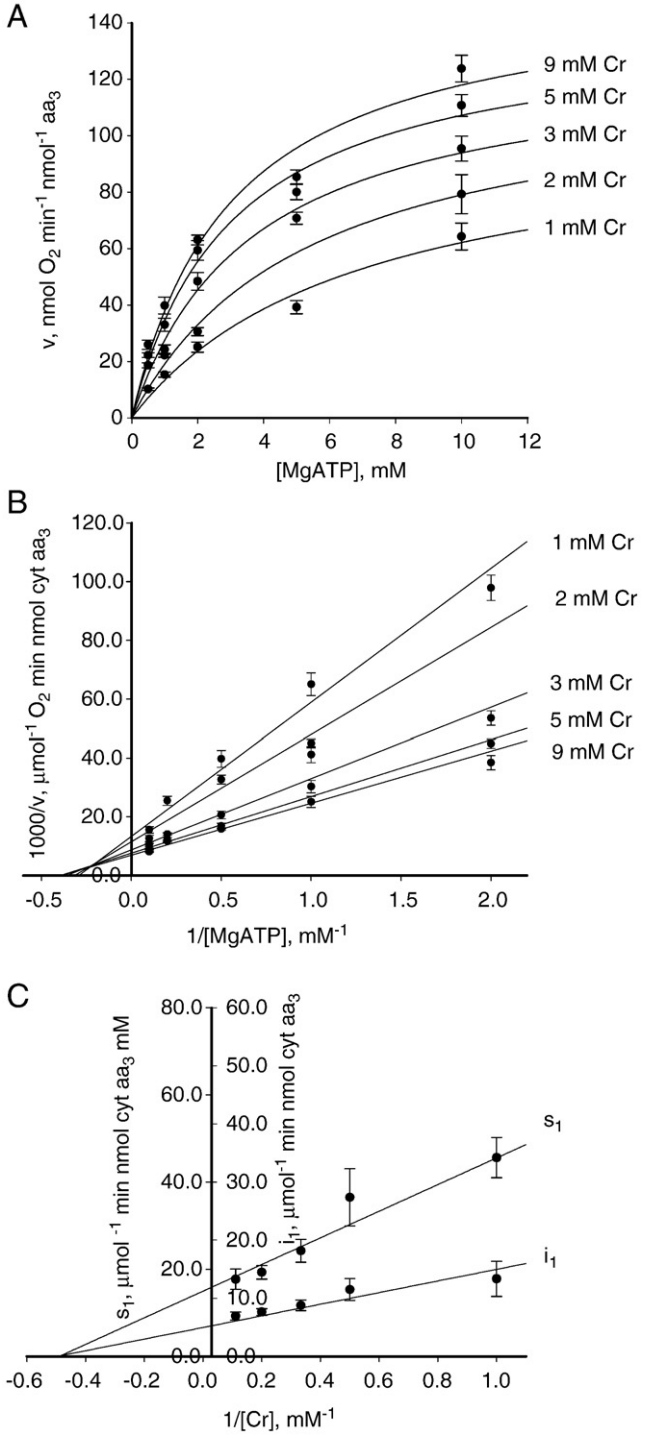


Fig. 6. (A) The rates of creatine activated respiration of permeabilized cardiomyocytes as functions of varying MgATP concentrations at fixed creatine concentrations. The respiration rates (VO_2) were calculated by subtraction of oxygen consumption rates in the presence of PEP–PK (VO_{2PK}) from the creatine activated respiration rates (VO_{2Cr}) ($VO_2 = VO_{2Cr} - VO_{2PK}$), (see Fig. 4 right panel). (B) The primary analysis of data (A) in double reciprocal coordinates of 1/v (1/respiration rate) versus 1/[MgATP] (substrate with varying concentration) for different fixed creatines concentration. (C) The secondary analysis of the primary plots from panel B. Slopes (s) and intercepts of y-axis (i) are plotted as a function of reciprocal coordinate of secondary substrate creatine. The intercepts of x-axis provide directly the reciprocal values of dissociation constants of creatine (K_{ib} and K_b) from the binary and ternary complexes of MtCK.

Table 2
Apparent kinetic constants of rat heart mitochondrial creatine kinase (MtCK) in mitochondria in vitro and in situ in permeabilized cardiomyocytes.

	K_{ia}MgATP (mM)	K_aMgATP (mM)	K_{ib} Cr (mM)	K_b Cr (mM)	V_{max}	K_{ip} (mM)
Mitoch. in vitro*	0.44 ± 0.08	0.016 ± 0.01	28 ± 7	5 ± 1.2	187.9 ± 40	0.84 ± 0.22
Mitoch. in situ (with PEP–PK)	1.94 ± 0.86	2.04 ± 0.14	2.12 ± 0.21	2.17 ± 0.40	161.65 ± 11.38	0.89 ± 0.17

K_{ia}MgATP and K_aMgATP—constants of dissociation of MgATP from its binary (K_{ia}) and ternary (K_a) complexes with mitochondrial creatine kinase (MtCK). K_{ib}Cr and K_bCr—constants of dissociation of creatine from its binary (K_{ib}) and ternary (K_b) complexes with MtCK. K_{ip}—constant of dissociation of phosphocreatine from its binary (K_{ip}) complex with MtCK. V_{max}—maximal oxygen consumption rates in nmol O_2/min/nmol cyt. aa_3 corresponding to the State 3 of respiration.
* Kinetic constants for isolated mitochondria are taken from references [12], [80] and [82].

It is most interesting to compare the kinetic behaviour of systems III and IV—the respiration rate in permeabilized cardiomyocytes in the presence of 10 mM creatine as a function of exogenous MgATP without and with added PEP–PK system. The data for these conditions were taken from Figs. 3A and 6A and their comparison is shown in Fig. 7. The PEP–PK system very strongly changes the kinetics from high apparent affinity to low apparent affinity for MgATP. In the case of system III there is the production of endogenous MgADP by MgATPases and extramitochondrial CK reactions; in the case of system IV the MgADP is trapped and respiration maintained only by mitochondrial MtCK reaction in the intermembrane space of mitochondria. An interesting observation was that pre-incubation with PK, before the addition of creatine, (see Fig. 3B) was needed to see the difference between systems III and IV—most probably this time was needed to allow penetration of PK molecules into the permeabilized cell interior and into the ICEUs. The presence of the PEP–PK system did not change the kinetic parameters of soluble MM creatine kinase (unpublished data). Therefore, the data in Fig. 7 show the importance of small endogenous MgADP fluxes from MgATPases to mitochondria where their effect on respiration is manifoldly amplified by the functionally coupled MtCK–ANT system. This is consistent with earlier findings from our and Theo Wallimann's laboratories showing high sensitivity of respiration of permeabilized cells in the presence of creatine to endogenous MgADP fluxes induced by addition of exogenous MgATP [71,72,99]. Under these conditions high respiration rates are observed when MgADP concentration in the medium is very low [99].

3.5. Kinetics of respiration regulation in system IV in the presence of PCr

To study the apparent affinity of MtCK in situ for PCr, we applied the protocol illustrated in Fig. 4. Experiments described in Fig. 5A were repeated in the presence of different fixed concentrations of PCr.

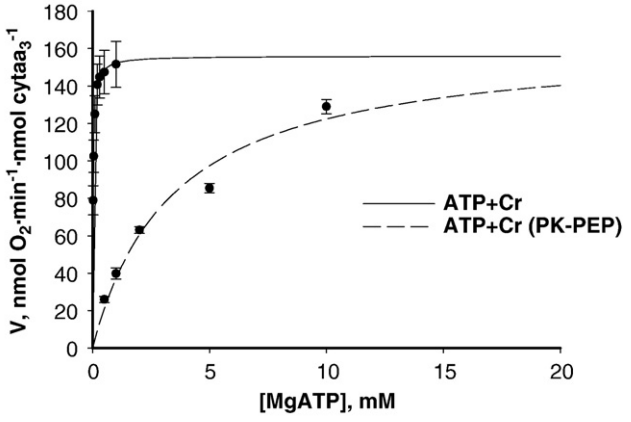


Fig. 7. Summary of the kinetics of regulation of mitochondrial respiration in situ in permeabilized cardiomyocytes by creatine in the presence of ATP without or with the PEP–PK system. The curve solid lines represents rate of respiration stimulated by endogenous ADP produced by ATPases from exogenous ATP and by creatine kinases via in the presence of 20 mM creatine (from Fig. 3A). The curve with dotted line represents rate of respiration stimulated by creatine via activated MtCK, while the endogenous ADP fluxes are excluded by the PEP–PK system (from Fig. 6A).

Phosphocreatine induced the decrease of respiration rate in a dose-dependent way in both models: isolated mitochondria [82] and permeabilized cardiomyocytes (Fig. 8). The primary and secondary kinetic analyses according to Eqs. (10) and (11), presented in Fig. 8A and B for cardiomyocytes, provide the value of dissociation constant for PCr from the binary complex MtCK·PCr, K_{ip}. Interestingly, the K_{ip} in isolated mitochondria and in mitochondria in situ are identical and thus independent of the change in mitochondrial outer membrane permeability; in both cases it was close to 0.9 mM (Table 2).

Decreased affinity of MtCK for exogenous MgATP in situ may be explained by the existence of strong restrictions of MgATP diffusion at the level of mitochondrial outer membrane, or within ICEU structures

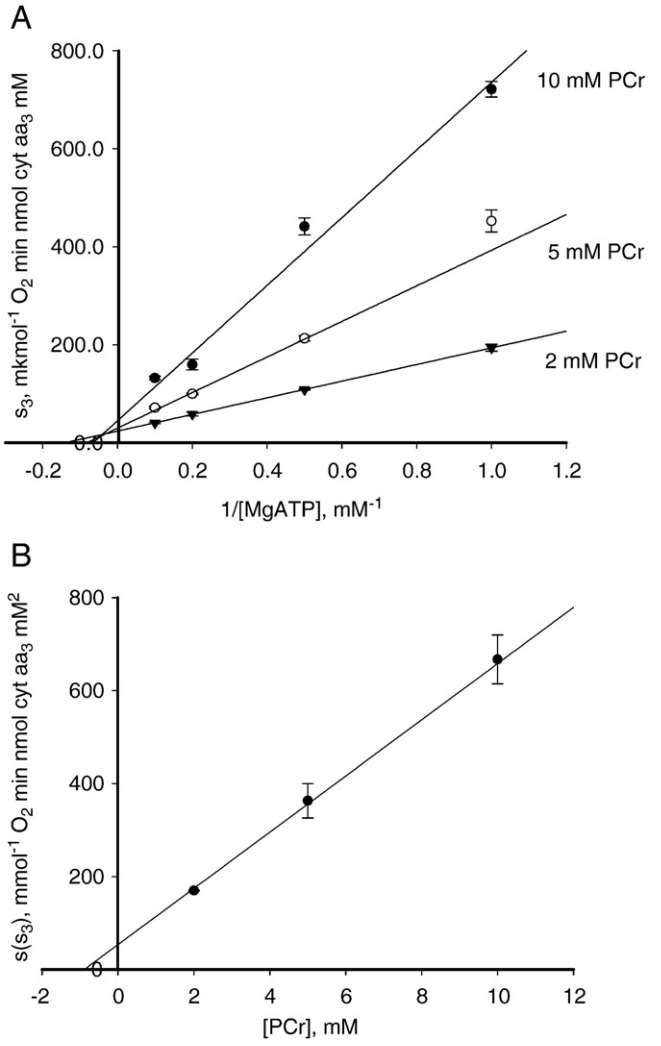


Fig. 8. (A) Competitive inhibition of MtCK in permeabilized cardiomyocytes by phosphocreatine. The slopes of straight lines obtained in double reciprocal plots of 1/v versus 1/[MgATP] for 9 mM of creatine (see Fig. 6B) for three different pfosphocreatine concentrations. (B) Secondary linearization of slopes of straight lines from panel A as a function of concentration of PCr provides value of K_{ip} (Eqs. 10 and 11 in Materials and methods).

near mitochondria. The unchanged constant of dissociation for PCr (K_{ip}) and decreased constant of dissociation for creatine (K_{ib} and K_b) evidence on the other hand the lack of these restrictions for creatine and PCr. Therefore it was interesting and important to verify that PCr produced by MtCK easily diffuses into cytoplasm. Therefore, we measured directly the rate of PCr production in mitochondria and its transfer into the cytoplasm (surrounding medium) under these experimental conditions in the presence of creatine and the PEP–PK system described in Fig. 4. Measurements were realised in the same conditions as kinetic studies on respiration: i.e. activation of mitochondrial respiration by the addition of creatine (20 mM) in the presence of fixed amounts of MgATP (5 mM) and the PEP–PK system. The samples were taken 3, 6 and 10 min after activation of the MtCK reaction by creatine. After the treatment described above (see Materials and methods) the concentrations of ATP, PCr and creatine in the medium were measured with UPLC. Concentrations of PCr increase with time, while ATP concentrations stay always constant (Fig. 9A). The PCr in cardiac cells can be produced by different isoforms of creatine kinase. To ascertain which amount of PCr content in the samples is of mitochondrial origin, the oxidative phosphorylation was inhibited by 10 mM rotenone (Fig. 9A). The difference of the rates of PCr synthesis in the absence and presence of rotenone is due exclusively to the activation of mitochondrial synthesis of PCr in MtCK reaction (Fig. 9B). From this rate and from V_{O2} measured in the parallel experiments, the PCr/O_2 ratio can be calculated. The PCr/O_2 ratio allows to quantitatively evaluate the efficiency of free energy conversion in the coupled MtCK–ANT-oxidative phosphorylation reactions. The high value of PCr/O_2 equal to 5.7 ± 0.7 is remarkably close to that found by Belitser and Tsybakova in 1939 and shows that all mitochondrial ATP is rapidly used up for PCr synthesis which does not accumulate in the intermembrane space but easily leaves mitochondria. This means that the VDAC permeability is high for PCr and changes selectively by mitochondrial–cytoskeleton interactions for adenine nucleotides.

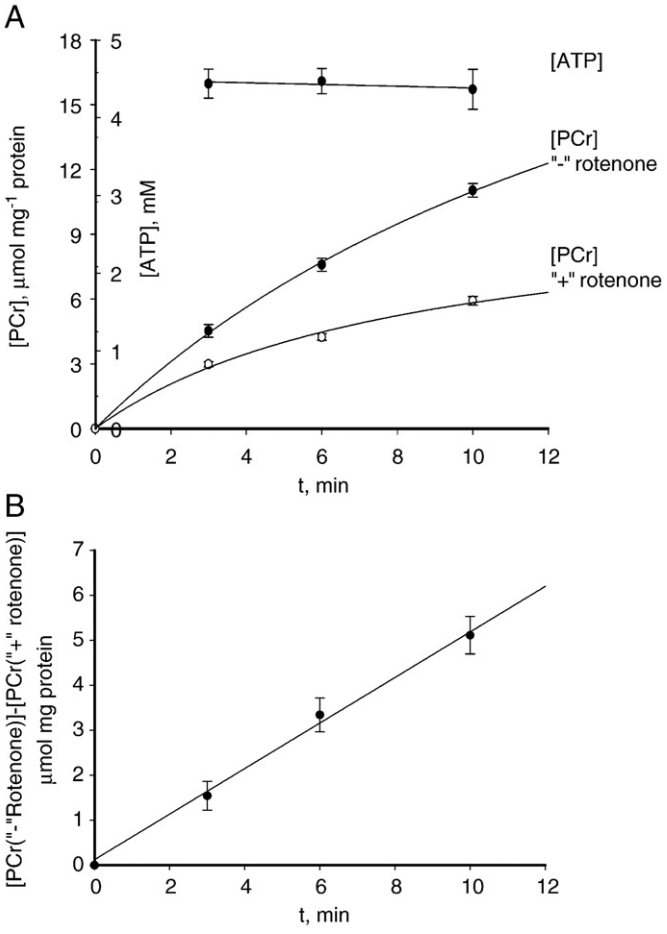


Fig. 9. (A) The rate of phosphocreatine production by mitochondrial and cytoplasmic creatine kinases in permeabilized non-inhibited cardiomyocytes (●). Analysis of samples of reaction mixture taken at 3, 6 and 10 min after activation of MtCK by creatine (20 mM) in permeabilized cardiomyocytes in the presence of MgATP (initially added to about 5 mM) and PEP (5 mM) and PK (20 U/ml) (see Fig. 4) were performed by using ion pair HPLC/UPLC as described in Materials and methods. The ATP level, continuously regenerated by the PEP–PK system, was stable during the experiment. When oxidative phosphorylation is inhibited by rotenone (○), the PCr can be produced only by cytoplasmic creatine kinases, MMCK. (B) The difference in phosphocreatine production rates under conditions of activated and inhibited (by rotenone) respiratory chain calculated from panel A. In separate experiments the oxygen consumption rates were measured, the creatine activated respiration rate in these experiments was 0.088 ± 0.007 $\mu molO_2\ min^{-1}\ mg^{-1}$protein. These data give the ratio of $PCr/O_2 = 5.7 \pm 0.7$.

4. Discussion

The results of this study show very clearly that the mechanisms of the regulation of mitochondrial respiration and energy fluxes in the cardiac cells are system-level properties dependent on the interaction of mitochondria with intracellular structures and functional interactions with metabolic systems including glycolysis, which are not predictable on the basis of properties of isolated mitochondria only. Cytoskeletal components like tubulin, and probably also desmin, plectin and others which are responsible for regular arrangement of mitochondria in cardiac cells and are sensitive to proteolysis most probably control also the permeability of VDAC in MOM [91–93]. The results of our study show that these proteins selectively limit the VDAC permeability, decreasing it mostly for ATP and ADP but not for creatine or phosphocreatine. Strongly decreased permeability of MOM for adenine nucleotides significantly enhances the functional coupling between MtCK and ANT increasing the rate of recycling of ADP and ATP in mitochondrial matrix-inner membrane space. Especially interesting and important is the significantly enhanced apparent affinity of MtCK for creatine in the cells in situ.

For some unknown reasons, numerous groups of investigators still consistently insist on the validity of the concept of the creatine kinase equilibrium in muscle cells which is taken as homogenous medium [18–20,22,23,26,27,45–47,100,101] and support the point of view that simple calculations of cytoplasmic ADP concentration and related parameters such as free energy of ATP hydrolysis in homogeneous intracellular medium are sufficient to explain the mechanisms of regulation of energy fluxes in the cells [23,47]. However, there are surprising and very obvious controversies in this point of view. It was already mentioned above that under conditions of metabolic stability the ADP concentration calculated from CK equilibrium is also constant, while the rate of oxygen consumption changes [37]. The calculated ADP concentration in resting heart cells is in the range of 50–100 µM [102]. If mitochondria in the cells behave as they do in vitro and ADP freely diffuses between cytoplasm and mitochondrial intermembrane space, ANT with its apparent K_m(ADP) around 10 µM [82] should be always almost saturated and respiration rate in the resting state almost maximal with glucose as substrate when Pi is also elevated [103]. However this is not true; heart respiration rate in the resting state is only 5% of V_{max} [37]. Low respiration rate of the heart in the resting state is well explained by decreased affinity (increased apparent K_m) for cytoplasmic ADP measured in experiments with permeabilized cells. This means that ADP diffusion across outer mitochondrial membrane is rather limited. The results of current study directly confirm this conclusion: while in isolated mitochondria the PEP–PK system decreases creatine-activated respiration rate by about 50% (Fig. 2A), in permeabilized cardiomyocytes the effect of PEP–PK is practically absent (Fig. 3B) but observed again after selective proteolysis (Fig. 3C).

Thus, the results of this work and many others show that the assumption of the creatine kinase equilibrium is an unnecessary

limitation [104]. Our data show that effective phosphocreatine production may occur in mitochondria with the rate close to maximal activity of MtCK and ATP synthesis, and it has been shown in direct measurements with ^{31}P NMR inversion transfer that in different cellular compartments in hearts the creatine kinase isoenzymes function in steady state in the direction dependent on their location and functional coupling either with oxidative phosphorylation via ANT in mitochondria (as MtCK), or with MgATPases in myofibrils and cellular membranes (as MMCK bound to these structures) [98]. Only in cytoplasmic compartment creatine kinase may approach quasi-equilibrium in the resting state, especially in resting glycolytic muscles with very high cytoplasmic CK activity. This conclusion is confirmed by the results of a mathematical model of compartmentalized energy transfer for analysis of experimental data (see below).

4.1. Peculiarities of the kinetics of regulation of respiration coupled to the MtCK reaction in mitochondria in situ

Classical works by Lardy and Wellman [105], Britton Chance [106,107] and many others [24] have established that the rate of mitochondrial respiration in isolated mitochondria in vitro is strictly regulated by availability of ADP for ANT in the mitochondrial inner membrane (MIM). In isolated mitochondria in vitro mitochondrial outer membrane is permeable for metabolites with molecular mass lower than 7 kDa due to the open state of the VDAC in MOM [88], and the efficiency of the regulation of mitochondrial functions in vitro by extramitochondrial ADP depending only upon the affinity of ANT for ADP, which is very high (the apparent K_m for ADP is in the range of 10–20 μM) [108].

However, in the cardiac and many other cells in vivo, mitochondria are involved in multiple structural interactions with other cellular structures, and functional interactions with other metabolic systems, such as the glycolytic system and cellular ATPases. All these interactions play important roles in the regulation of mitochondrial activities and energy fluxes in the cells, resulting in appearance of new, system-level properties. The interesting result is the strengthening of the role of functional coupling between MtCK and ANT in the regulation of energy fluxes and respiration.

Both the structure of MtCK and its interaction with ANT have been studied by using isolated and purified enzymes or isolated mitochondria. Classical studies by Theo Wallimann's group have given detailed structure of CK including MtCK and direct localisation of octameric MtCK close to ANT due to C-terminal ligand to cardiolipin negative charges [15,109–112]. The respiratory control by creatine was first discovered by Bessman and Fonio [113], Jacobus and Lehninger [114] and Vial [115]. All of these groups showed that in isolated heart mitochondria in the presence of ATP creatine exerts acceptor control of respiration by activating MtCK and supplying locally ADP for ANT. Quantitative analysis of this phenomenon showed that oxidative phosphorylation in mitochondria specifically accelerates the forward creatine kinase reaction of phosphocreatine production leading to an assumption of functional coupling between MtCK and ANT [75]. Jacobus and Saks have studied functional coupling between MtCK and ANT in isolated heart mitochondria by kinetic methods [82]. Then, the functional coupling has been shown repeatedly in kinetic [94] and thermodynamic experiments [96], directly confirmed by tracer and other methods by many independent groups [116–118]. One of the most important works was done by Barbour et al. who directly showed, using radioactive ADP, the recycling of ADP and ATP due to functional coupling of MtCK and ANT [116]. Bessman's laboratory showed the interaction of mitochondrially bound creatine kinase with oxidative phosphorylation by measuring the isotope incorporation into newly synthesized ATP and PCr [117,118] and thus also confirmed the functional coupling of MtCK with ANT. Detachment of MtCK from its binding site on mitochondrial membranes into intermembrane space results in loss of the effects of oxidative phosphorylation on the kinetics of MtCK reaction even in the presence of intact MOM [94]. Monoclonal inhibitory antibodies against MtCK have been shown to inhibit also the ADP/ATP exchange in mitoplasts [119]. Kim and Lee [120] showed that isolated pig heart mitochondria can form phosphocreatine continuously in the respiration medium without externally added adenine nucleotides, due to rapid recycling of their trace amounts in mitochondrial creatine kinase-oxidative phosphorylating system functionally coupled via the action of the adenine nucleotide translocase. Similar data were reported by Dolder et al. [10]. The functional coupling of MtCK and ANT has been found to be vital for protection of mitochondria from permeability transition pore (PTP) opening and from production of reactive oxygen species, ROS. Dolder et al. showed in experiments with the transgenic liver-MtCK mice that mitochondria are protected from PTP opening via functional coupling of the MtCK reaction to oxidative phosphorylation [10]. Meyer et al. demonstrated that in the presence of creatine, MtCK coupled to ANT increases recycling of adenine nucleotides in mitochondria, accelerates respiration, thus diminishing the reduced state of electron carriers and production of oxygen radicals, ROS, that are further converted to H_2O_2 by superoxide dismutase [121]. All these multiple works from different laboratories show the tight functional coupling of MtCK and ANT in heart and skeletal mitochondria. Similar coupling exists in brain mitochondria [80]. Vendelin et al. applying mathematical modelling, analyzed in details the mechanism of this functional coupling, showing that the direct ATP transfer from ANT to MtCK is more important for accelerated PCr production and respiratory control than dynamic ADP compartmentation in the intermembrane space proposed by Gellerich [8,85,86]. However, in cells in vivo this dynamic compartmentation of ADP may become an important additional factor of functional coupling between MtCK and ANT due to interaction of MOM with cytoskeletal elements which selectively limit VDAC permeability in cells in situ [91]. Numerous laboratories have recorded manifold differences in mitochondrial apparent affinity for free exogenous ADP in vitro (K_m^{app} for free ADP ~10 μM), in permeabilized cardiomyocytes in situ (K_m^{app} ~350 μM) and in cardiomyocytes pre-treated with trypsin (K_m^{app} ~70–100 μM) [50–53,55–65,77]. In the reconstituted complete system of mitochondria in situ in cardiac permeabilized cells complemented with glycolytic ADP-trapping system (Scheme 4) the apparent kinetics of the MtCK dependent respiration regulation is totally different from that seen in mitochondria in vitro. In fact, there are two remarkable differences. The first is the decrease in apparent affinity of MtCK for exogenous MgATP (apparent K_a increased more than 100 times, Table 2) in mitochondria in situ as compared to in vitro, most probably due the enhanced restriction of MgATP diffusion within organized structures of intracellular energetic units, ICEUs, most probably locally at the level of MOM. Due to the control of VDAC permeability by cytoskeleton, apparent affinity of MtCK to exogenous (cytoplasmic) MgATP is strongly decreased. In vivo the elements of cytoskeleton, most probably tubulin and some other proteins of this network, limit VDAC permeability. Rostovtseva et al., showed very recently that dimeric tubulin in nanomolar concentrations induce highly voltage sensible reversible closure of VDAC reconstituted into planar phospholipid membrane [92,93]. Added to isolated brain or heart mitochondria tubulin induces decrease of apparent affinity of ANT for free ADP (increase of the K_m^{app} ADP from ~10 μM to ~169 μM for isolated brain mitochondria and for sample supplemented with tubulin respectively) [80,92,93]. Strong diffusion restriction at the level of MOM in vivo increases the effective adenine nucleotides micro-compartmentation within intermembrane space and influences the respiratory control of oxidative phosphorylation.

Diffusion restrictions for ATP in permeabilized cardiac fibers have been registered also by Ventura-Clapier's group in studies of calcium uptake by sarcoplasmic reticulum (SR): they found that this uptake was much more effective when supported by mitochondrial oxidative phosphorylation or by PCr than in the presence of exogenous ATP

[122]. These data show the restrictions of diffusion of adenine nucleotides into some important areas of ICEUs. In their experimental protocol SR were preloaded by calcium during 5 min period of preincubations and caffeine-induced release of calcium seen as contraction transients were measured [122]. Since both parameters depend on local ATP/ADP ratios, both different rates of ADP removal and ATP supply in these local areas explain these results. The authors concluded by comparing the results of separate experiments with oxidative phosphorylation and exogenous ATP that ATP fluxes from mitochondria are equal to fluxes of PCr in energy supply to cytoplasm [122]. However, the kinetic studies of ATP fluxes were not performed in these works. These fluxes were measured in the present study as described in Fig. 9, and the measured PCr/O_2 ratio equal to 5.7 leaves little room for ATP fluxes in energy supply into cytoplasm in the presence of creatine when MtCK coupled to ANT is activated.

The second remarkable difference observed in this work is that apparent constant of dissociation of creatine from the binary complex with MtCK (K_{ib}) decreases about 10 times in mitochondria in situ, in permeabilized cardiomyocytes, as compared with isolated mitochondria (Table 2). At the same time, the apparent affinity of MtCK for phosphocreatine is similar in vitro and in situ (K_{ip} is about 2 mM, Table 2). Thus, there is no diffusion restriction for diffusion of these guanidino substrates across MOM into intermembrane space where MtCK is located. The remarkably high affinity of MtCK in mitochondria in situ for creatine may be a result of specific conformational state of the enzyme dependent of both configuration of cristae surface where MtCK is localized, and ATP supply by ANT. Recently Hornikova et al. have shown the importance of the binding of MM–CK to myofibrils for maintaining active conformation the presence of substrates [123]. The substrate-induced conformational changes are required in order to bring the substrates closer to each other for spontaneous catalysis [123]. We may assume that similar conformation changes may occur also in the MtCK in situ resulting in increased affinity for creatine when MtCK is continuously supplied by mitochondrially produced ATP. Vendelin et al. found that functional coupling of MtCK to ANT changes significantly the free energy profile of the MtCK reaction [8]. The precise mechanism of this important phenomenon needs, however, further detailed study by FRET and other available new methods.

As a result, creatine becomes one of the main regulators of the rate of coupled MtCK–oxidative phosphorylation reactions and of ADP/ATP turnover in mitochondria in cardiac cells in vivo, resulting in effective production of PCr. High $PCr/O_2 =$ 5.7 ratio found in experiments with permeabilized cells shows that all mitochondrially produced ATP is rapidly used up for PCr synthesis which easily leaves mitochondria (Fig. 9B). These results conform to the now commonly accepted theory of the role of PCr/CK phosphotransfer system [5–9,11,12,14–16,70,124,125] as the main pathway of energy transfer and feedback metabolic communication between mitochondria and cytoplasm in heart cells and further confirm its validity. All these are consistent with first classical observations by Belitzer and Tsybakova [4] and many other observations.

4.2. ADP flux amplified by coupled MtCK–ANT is a necessary component of metabolic feedback signaling

Our results allow us to make several important conclusions about the nature of metabolic feedback signaling between ATPases and mitochondria in the heart and probably skeletal muscle and the brain in vivo. This signaling seems to include three different components: Pi fluxes, changes in Cr/PCr ratios and fluxes of endogenous ADP strongly amplified by the functional coupling between MtCK and ANT [11,18,28,126,127].

The role of extramitochondrial MgADP fluxes in the regulation of mitochondrial respiration in the whole system (cardiac cell) can be seen from comparison of data from the difference of apparent kinetics of mitochondrial respiration controlled by activated MtCK in the presence and absence of free MgADP fluxes (Fig. 7, Schemes 3 and 4). In both situations endogenous extramitochondrial MgADP is produced by hydrolysis of exogenous MgATP in ATPase-reactions and by MM creatine kinases bound to myofibrils and sarcoplasmic reticulum. This MgADP is generated within ICEUs near mitochondria and diffuses into the intermembrane space even if VDAC permeability is restricted, and free ADP enters into the matrix for rephosphorylation. Creatine added under these conditions changes strongly the apparent affinity of mitochondrial respiration switching it from the control by endogenous extramitochondrial MgADP toward that produced in MtCK reaction (K_m^{app} for exogenous MgATP in the presence of creatine decreases from ~160 μM to ~24 μM), (Fig. 3A, Fig. 7). A similar tendency was observed when mitochondrial respiration was stimulated by exogenous ADP without and with creatine [49,71,91]. The apparent K_m for free ADP in the presence of creatine is typically ten times smaller than the K_m^{app} for ADP alone [49,71,91]. Kinetics becomes different when we eliminate extramitochondrial MgADP by trapping it in the PEP–PK reaction (Fig. 7, experimental model is represented in Scheme 4). One explanation could be that this difference is due to the inhibition of the MtCK reaction by its final product phosphocreatine, which as we can see in HPLC measurements (Fig. 9A) is accumulating in the experimental medium. The PCr production can be carried out by the forward MtCK and MMCK reactions. In our experiment, due to the PEP–PK system, the free MgADP is efficiently removed from the medium (Fig. 1B) and the extramitochondrial pool of free MgATP is maintained at a high stable level (Fig. 9A). Under these conditions and the stepwise increase of creatine concentrations, the PCr production by cellular creatine kinases increases (see Fig. 9B), showing that MOM is highly permeable to PCr. The constant of dissociation of PCr from MtCK·PCr complex (K_{ip}) is similar (~0.9 mM) for mitochondria in vitro and in situ in permeabilized cardiomyocyte, and thus does not depend on changes in MOM permeability (Table 2). At the same time, as we can see from Table 2 the apparent affinity of MtCK in situ for creatine increases manifoldly even in the presence of freely available PCr. Therefore the observed differences are not explained by inhibitory effects of PCr, which should result first in the increase of apparent dissociation constants for creatine, since they both have the same binding site in the active centre of MtCK. However, for future mathematical modelling we have to take into account the PCr production in the steady state by cellular creatine kinases, induced by our experimental conditions (Scheme 4).

Thus, the observed differences between systems III and IV (see Fig. 7) are most probably due to the presence of ADP fluxes in the absence of the PEP–PK system. The high respiration rate is maintained in situ in the absence of the PEP–PK system by endogenous MgADP but only if creatine is present to activate MtCK (Fig. 3A). Even under conditions of limited MOM permeability in vivo, in the presence of creatine the respiration is maximally activated due to amplification of stimulatory effect of MgADP from cytoplasm by the mechanism of metabolic channeling within coupled MtCK/ANT reactions [128]. Thus, the functional coupling is a powerful amplifier of the regulatory action of cytoplasmic MgADP. This conclusion is fully consistent with the results of the mathematical modelling of compartmentalized energy transfer [97,104,126,129]. This model quantitatively reproduced the linear dependence of oxygen uptake on workload (metabolic aspect of Frank–Starling mechanism) and showed firstly the remarkable stability of average values of ATP, phosphocreatine and creatine, (PCr/ATP ratio) when workload (MgATPase activity) was changed and secondly the significant workload (and VO_2) dependent variations of cytoplasmic ADP concentrations within the cardiac cycle. In the systolic phase the ADP level may be increased by a factor of 10 when VO_2 changed 10 times, induced by non-equilibrium behaviour of myofibrillar MMCK and MtCK [130]. These remarkable changes in MgADP concentrations are related to small cyclic changes (within the range of 5–10%) of creatine and PCr levels around their stable steady state levels [97,126]. These cyclic small scale changes in PCr and

creatine have been recorded in several careful experiments with the use of gated ^{31}PNMR [131,132]. Application of the Metabolic Control Analysis to this model showed that respiration rate may be controlled by Pi and by changes in creatine and ADP concentration, their flux control coefficients depending on workload and corresponding steady state values of respiration rates [133].

Our experimental results described in this work are in concord with the predictions of the mathematical model of compartmentalized energy transfer and may be used for further corrections of parameters of this model [126] when it will be included into future general mathematical model of heart energy metabolism which is still absent. The complete mathematical model of mitochondrial processes of energy conversion is also needed for further quantitative analysis of experimental data reported in this work and calculation of metabolic fluxes across the mitochondrial outer membrane in vivo. Further, in our experiments the ATPase reactions were limited by the lack of Ca^{2+} in solution. Further studies with different concentrations of Ca^{2+} to selectively activate MgATPases can better clarify the role of endogenous extramitochondrial ADP fluxes in regulation of mitochondrial respiration in situ. The oscillations of ADP concentration may be synchronized with localized changes of Ca^{2+}in cytoplasm and in mitochondria, modifying the activity of the enzymes of mitochondrial systems to increase the rate of ADP rephosphorylation and consecutively that of PCr production [126].

Our results are also consistent with important clinical observations described by Neubauer and others, who showed that in patients with heart failure myocardial ATP level remain normal (10 mM) until the advanced stage of disease, but the creatine and PCr levels decrease at earlier stages by 30–70% [125,134,135]. Thus the PCr/ATP ratio becomes a stronger predictor of mortality as a result of cardiovascular diseases than functional or clinical indexes [125]. All these changes can be explained by the important role of PCr in maintaining ATP in vitally important local cellular compartments, while the intracellular steady state concentration depend both on mitochondrial activities and intactness of functional coupling between MtCK and ANT.

Acknowledgements

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Direct measurement of energy fluxes from mitochondria into cytoplasm in permeabilized cardiac cells *in situ:* some evidence for mitochondrial interactosome

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Abstract The aim of this study was to measure energy fluxes from mitochondria in isolated permeabilized cardiomyocytes. Respiration of permeabilized cardiomyocytes and mitochondrial membrane potential were measured in presence of MgATP, pyruvate kinase – phosphoenolpyruvate and creatine. ATP and phosphocreatine concentrations in medium surrounding cardiomyocytes were determined. While ATP concentration did not change in time, mitochondria effectively produced phosphocreatine (PCr) with PCr/O_2 ratio equal to 5.68±0.14. Addition of heterodimeric tubulin to isolated mitochondria was found to increase apparent Km for exogenous ADP from 11±2 μM to 330±47 μM, but creatine again decreased it to 23±6 μM. These results show directly that under physiological conditions the major energy carrier from mitochondria into cytoplasm is PCr, produced by mitochondrial creatine kinase (MtCK), which functional coupling to adenine nucleotide translocase is enhanced by selective limitation of permeability of mitochondrial outer membrane within supercomplex ATP Synthasome-MtCK-VDAC-tubulin, Mitochondrial Interactosome.

Keywords Respiration · Cardiomyocytes · Mitochondria · Creatine kinase · Creatine · Phosphocreatine · Tubulin

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Introduction

Mitochondrial respiration, coupled to production of ATP and fine regulation of energy fluxes to the sites of ATP utilization are vital for normal cell life. In spite of the fundamental progress of knowledge of mitochondrial bioenergetics (Nicholls and Ferguson 2002), the nature of respiratory control and in more general sense, the mechanisms of regulation of energy fluxes during workload changes in the cardiac and other cells *in vivo* are still highly debated (Balaban 2009; Beard 2005, 2006; Guzun et al. 2009; Saks et al. 2007a, 2006, 2007c; Van Beek 2007, 2008; Vendelin et al. 2000; Wu et al. 2007, 2008). Intensive studies during several decades have accumulated an abundance of data showing compartmentation of adenine nucleotides and the role of phosphotransfer networks in energy transfer (Dzeja et al. 2007; Dzeja and Terzic 2003; Saks et al. 2008, 2007a, 2006, 2007c, 2004, Schlattner and Wallimann 2004; Schlattner et al. 2006; Vendelin et al. 2004a; Wallimann et al. 1992, 2007; Wyss et al. 1992). Of major importance are the creatine kinase – phosphocreatine circuit (or shuttle) which includes both mitochondrial creatine kinase (MtCK) functionally coupled to the oxidative phosphorylation via adenine nucleotide translocase (ANT) and MM isoform of creatine kinase coupled to MgATPase reactions in myofibrils and at cellular membranes, and the adenylate kinase shuttle (Dzeja et al. 2007; Dzeja and Terzic 2003; Saks et al. 2008, 2007a, 2006,

2007c, 2004, Schlattner and Wallimann 2004; Schlattner et al. 2006; Vendelin et al. 2004a; Wallimann et al. 1992, 2007; Wyss et al. 1992). However, in very many other works, while explaining respiration regulation the cell is often considered as a homogeneous reaction medium, thus ignoring the impact of the high degree of structural organization of the cell, in particular cardiomyocytes, macromolecular crowding phenomena etc. (for critical review see Saks et al. 2008). Usually, in these works the creatine kinase system is either totally ignored (Hom and Sheu 2009) or taken to be a cytoplasmic reaction in equilibrium providing researchers a simple method of calculation of ADP concentration and then free energy of ATP hydrolysis (Beard 2005; Wu et al. 2008). In many of these works, ATP utilization is taken to be matched to its production by changes in cytoplasmic calcium concentration (Balaban 2009; Hom and Sheu 2009). There are other works to propose the compromise that both pathways of energy transfer – by phosphotransfer networks and direct diffusion of ATP – play equal roles, both carrying 50 % of energy fluxes out of mitochondria (Joubert et al. 2008; Kaasik et al. 2001). To solve these controversies, in this work we measured directly the energy fluxes from mitochondria in permeabilized cardiomyocytes *in situ* under conditions close to those *in vivo* – in the presence of ATP, creatine and the glycolytic system (represented by pyruvate kinase and phosphoenolpyruvate) for trapping free ADP produced by MgATPases. Changes in ATP and phosphocreatine contents in the surrounding medium were measured by HPLC/UPLC technique and respiration rates were measured by oxygraphy. The rates of PCr production and respiration were used to determine the PCr/O_2 ratios to evaluate quantitatively the energy fluxes carried out in mitochondria *in situ* by PCr. The results show that at any initial ATP concentration high rates of respiration were maintained by MtCK reaction, the high PCr/O_2 ratios being close to the theoretically maximal value of P/O_2 equal to 6 (Nicholls and Ferguson 2002) showing directly that under physiological conditions the main carriers of energy into cytoplasm are phosphocreatine molecules.

Materials and methods

Experimental protocols

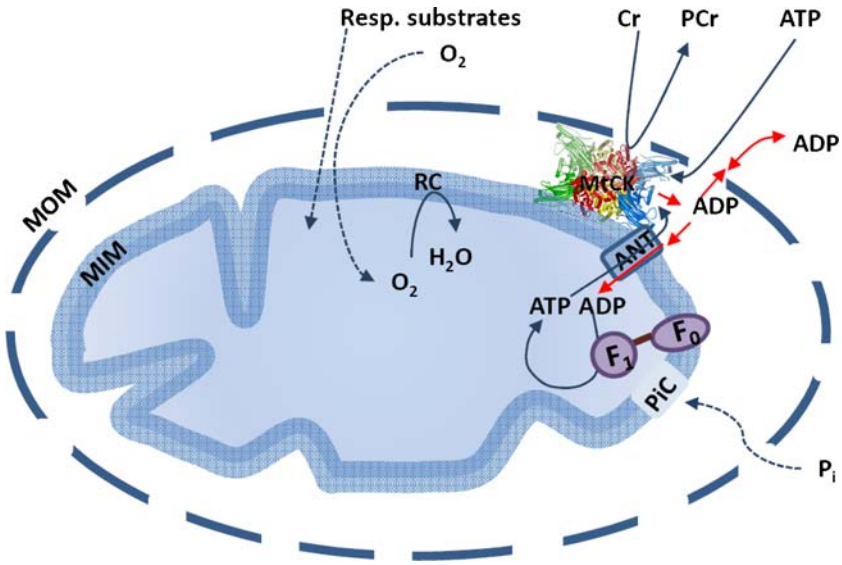
The principles of this study are illustrated by Schemes 1 and 2. Scheme 1 represents isolated mitochondria *in vitro* when mitochondrial creatine kinase is activated by addition of creatine, and Scheme 2 shows mitochondrion *in situ,* in permeabilized cardiac cells, surrounded by cytoskeleton proteins (depicted as "X" factor) and myofibrils. The respiratory chain (RC) complexes, ATP synthase (F_1F_0) and Pi carrier PIC are integrated within the mitochondrial inner membrane (MIM). Mitochondrial creatine kinase (MtCK) is depicted as an octamer, located in the mitochondrial intermembrane space (IMS) and attached to the inner membrane surface. In our experiments MtCK is activated by creatine (Cr) in the presence of ATP. The final products of MtCK-forward reaction are phosphocreatine (PCr) and endogenous ADP. The MOM is less permeable than in isolated mitochondrion, due to the interactions of VDAC with cytoskeleton proteins. Exogenous ATP is hydrolyzed by cellular ATPases into endogenous extramitochondrial ADP and inorganic phosphate (Pi). Mitochondrial (MtCK) and non-mitochondrial MM creatine kinases (cytosolic, myofibrillar, SERCA, sarcolemmal) activated by creatine in the presence of ATP, produce endogenous intra- and extramitochondrial ADP. Thus the oxidative phosphorylation is controlled by endogenous ADP produced by the MtCK, MMCK and ATPase reactions. The permeabilized cardiomyocytes were supplemented with phosphoenolpyruvate (PEP) and pyruvate kinase (PK). PEP-PK system removes extramitochondrial ADP produced by intracellular ATP consuming reactions and continuously regenerates extramitochondrial ATP. Endogenous intramitochondrial ADP produced by MtCK forms microcompartments within the IMS and is re-imported into the matrix via adenine nucleotide translocase (ANT) due to its functional coupling with MtCK.

Isolation of mitochondria from cardiac muscle

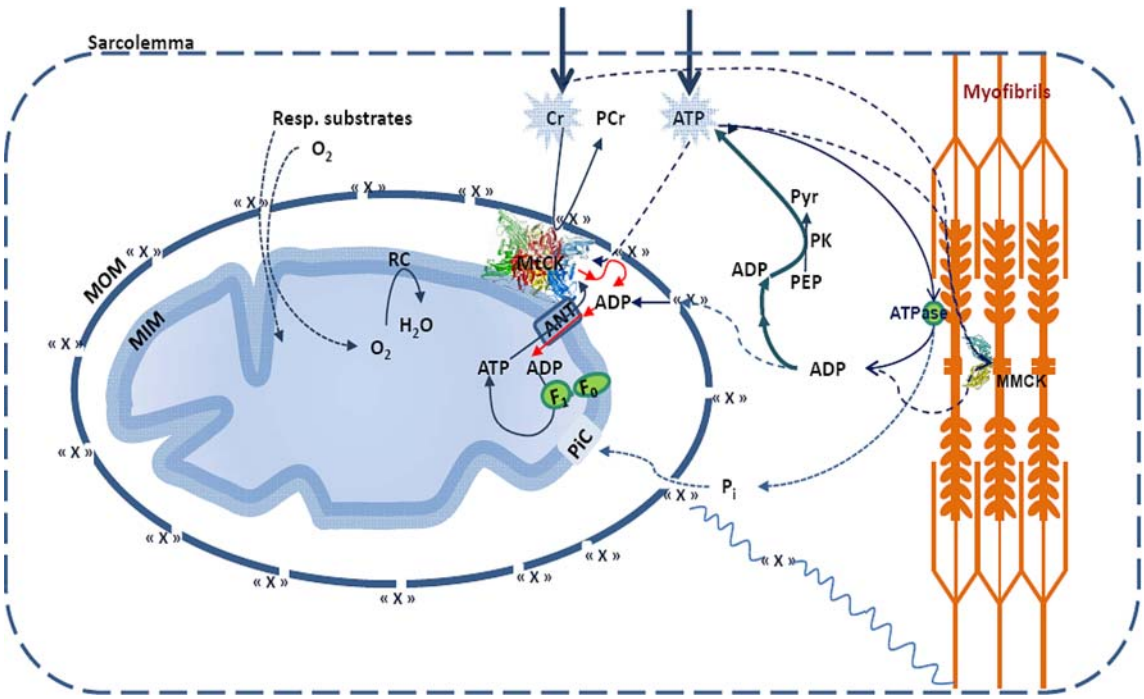
Mitochondria were isolated from adult rat hearts as described by Saks et al. 1975.

Isolation of adult cardiac myocytes

Adult cardiomyocytes were isolated after perfusion of the rat heart with collagenase using the adaptation of the technique described previously Saks et al. 1991. Wistar male rats (300–350 g) were anaesthetized with pentobarbital and de-coagulated using 500 U of heparin. The heart was quickly excised preserving a part of aorta and placed into isolation medium (IM) of the following composition: 117 mM NaCl, 5.7 mM KCl, 4.4 mM $NaHCO_3$, 1.5 mM KH_2PO_4, 1.7 mM $MgCl_2$, 11.7 mM glucose, 10 mM creatine, 20 mM taurine, 10 mM PCr, 2 mM pyruvate and 21 mM HEPES, pH 7.1. The excised rat heart was cannulated by aorta and suspended in Langendorf system for perfusion and washed for 5 min with a flow rate of 15–20 mL/min. The collagenase treatment was performed by switching the perfusion to circulating isolation medium supplemented with 0.03 mg/ml liberase Blendzyme I (Roche) and BSA 2 mg/ml at the flow rate of 5 ml/min for 20–30 min. The end of the digestion was determined



Scheme 1 represents a system related to isolated heart mitochondrion, used as a reference system in this work. The respiratory chain (RC) complexes, ATPsynthase (F_1F_0) and Pi carrier PIC are integrated within the mitochondrial inner membrane (MIM). Mitochondrial creatine kinase (MtCK) is depicted as an octamer, located in the mitochondrial inter-membrane space (IMS) and attached to the inner membrane surface. In the experiments MtCK is activated by creatine (Cr) in the presence of ATP. The final products of MtCK-forward reaction are phosphocreatine (PCr) and endogenous ADP. The ADP phosphorylation is visualized by recording the oxygen consumption. In scheme I endogenous intramitochondrial ADP produced by MtCK reaction forms a micro-domain within the intermembrane space. The micro-compartmentalized ADP can either enter into the mitochondrial matrix for phosphorylation or escape into the surrounding medium via voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane (MOM)



Scheme 2 represents a mitochondrion *in situ*, in a permeabilized cardiac cell, surrounded by cytoskeleton proteins (depicted as "X" factor) and myofibrils. The MOM is less permeable than in isolated mitochondrion, due to the interactions of VDAC with cytoskeleton proteins. Exogenous ATP is hydrolyzed by cellular ATPases into endogenous extramitochondrial ADP and inorganic phosphate (Pi). Mitochondrial (MtCK) and non-mitochondrial MM creatine kinases (cytosolic, myofibrillar, SERCA, sarcolemmal) activated by creatine in the presence of ATP, produce endogenous intra- and extramitochondrial ADP. The system is supplemented with phosphoenolpyruvate (PEP) and pyruvate kinase (PK). PEP-PK system removes extramitochondrial ADP produced by intracellular ATP consuming reactions and continuously regenerates extramitochondrial ATP. Endogenous intramitochondrial ADP produced by MtCK forms microcompartments within the IMS and is re-imported into the matrix via adenine nucleotide translocase (ANT) due to its functional coupling with MtCK

following the decrease in perfusion pressure measured by a manometer. After the digestion the heart was washed with IM for 2–3 min and transferred into IM containing 20 μM $CaCl_2$, 10 μM leupeptin, 2 μM STI and 2 mg/ml fatty acid free BSA. The cardiomyocytes were then gently dissociated using forceps and pipette suction. Cell suspension was filtered through a crude net to remove tissue remnants and let to settle for 3–4 min at room temperature. After 3–4 min the initial supernatant was discarded and the pellet of cardiomyocytes resuspended in 10 ml of IM containing 20 μM $CaCl_2$ and the protease inhibitors. This resuspension-sedimentation cycle with calcium-tolerant cells was performed twice. After that cardiomyocytes were gradually transferred from 20 μM Ca^{2+} IM into free calcium Mitomed (supplemented with protease inhibitors and BSA) and washed 5 times. Each time, slightly turbid supernatant was removed after 4–5 min of the cells' sedimentation. Isolated cells were re-suspended in 1 – 2 ml of Mitomed solution (Kuznetsov et al. 2008) described below for respiration measurements and stored on ice during measurements. Isolated cardiomyocytes contained 70–90% of rod-like cells when observed under the light microscope.

Permeabilization procedure

In order to study the kinetics of regulation of mitochondrial respiration in cardiomycytes using different metabolites, the cells sarcolemma was permeabilized by saponin keeping the mitochondrial membranes intact (Kuznetsov et al. 2008; Saks et al. 1998b). The tests for intactness of the outer and inner mitochondrial membranes are described in "Results" section. The permeabilization procedure was carried out directly in an oxygraph chamber with 25 μg/mL saponin during 10 min before starting measurements of respiration rates at 25°C and continuous stirring.

Measurements of oxygen consumption

The rates of oxygen uptake were determined with a high-resolution respirometer Oxygraph-2K (OROBOROS Instruments, Austria) in Mitomed solution (Kuznetsov et al. 2008) containing 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 3 mM KH_2PO_4, 20 mM taurine, 20 mM HEPES, 110 mM sucrose, 0.5 mM dithiothreitol (DTT), pH 7.1, 2 mg/mL fatty acid free BSA, complemented with 5 mM glutamate and 2 mM malate as respiratory substrates.

Measurements were carried out at 25°C; solubility of oxygen was taken as 240 nmol/ml (Gnaiger 2001).

In kinetic experiments with different fixed MgATP concentrations, a stock solution of 100 mM MgATP was prepared by mixing equimolar amounts of $MgCl_2$ and ATP, pH was adjusted to 7.2.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi$m) was measured by a spectrofluorimeter (F 2500 DIGILAB, HITACHI, Tokyo, Japan) with a fluorescent cationic dye tetramethylrodamine methyl ester (TMRM) according to the protocol decribed earlier (Freedman and Novak 1989). This indicator dye is a lipophilic fluorescent cation which passes cellular and mitochondrial membranes and accumulates within the mitochondrial matrix in a $\Delta\Psi m$-dependent manner, its fluorescence intensity decreases when the dye is accumulated by mitochondria (Nicholls and Ferguson 2002). The decrease of fluorescence intensity shows mitochondrial energization and is proportional to $\Delta\Psi$m. This property has been used to dynamically monitor $\Delta\Psi m$ in mitochondria *in situ* in permeabilized cardiomyocytes. The excitation wavelength was 548 nm and emission wavelength 574 nm. Data are reported as arbitrary fluorescence units (AFUs). TMRM was obtained from FluoProbes®, Interchim, France, dissolved in DMSO to a concentration of 1 mM. Aliquots of this stock solution were diluted in Mitomed solution described above and used in a final concentration of 0.2 μM.

The measurements of changes in $\Delta\Psi$ in isolated mitochondria and in mitochondria *in situ* in permeabilized cardiomyocytes induced by substrates, MgATP, PK-PEP system and creatine were performed in the same medium as described above for measurements of oxygen consumption (see above). Shortly, permeabilized cardiomyocytes (or isolated mitochondria) were incubated with 0.2 μM TMRM. This stage corresponds to zero polarization of mitochondrial inner membrane or to State 1 of respiration according to Chance (Chance and Williams 1956). Addition of respiratory substrates (5 mM Glutamate and 2 mM Malate) induces polarization of the mitochondrial inner membrane (decrease of AFU due to the accumulation of TMRM inside the matrix). This energy state corresponds to State 2 of respiration according to Chance. The subsequent addition of 2 mM ATP followed by 20 U/mL PK and 5 mM PEP (ADP-trapping system) should induce maximal energization of mitochondria, which corresponds to state 4 respiration. Creatine (10 mM) added in the presence of MgATP and the trapping system for free ADP activates MtCK reaction. Intramitochondrial ADP produced by activated MtCK is expected to decrease $\Delta\Psi$m due to its use for ATP synthesis respiration (State 3 according to Chance) and ADP/ATP translocation, both dependent on MtCK-ANT functional coupling. The experiment is terminated by addition of 5 μM uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) which provokes the $\Delta\Psi$m collapse and thus allows zero level of membrane potential.

Measurements of mitochondrial cytochromes content

For comparative quantitative analysis of the kinetics of regulation of respiration in isolated mitochondria and permeabilized cardiomyocytes, the respiration rates were expressed in nmoles of oxygen consumed per minute per nmoles of cytochrome aa_3, but not per mg of protein (if not indicated differently). Cytochrome aa_3 content in both cases is representative of the respiratory chain, while proteins contained in cardiomyocytes are not all present in mitochondria. The contents of mitochondrial cytochrome aa_3 in the isolated mitochondria and cardiomyocytes were measured spectrophotometrically according to the method described before (Fuller et al. 1985; Monge et al. 2008). The cells or mitochondria were solubilized with 1 % of sodium deoxycholate in phosphate buffer (100 mM KH_2PO_4, pH 8). The differential spectrum (reduced by dithionite *versus* oxidized cytochromes) was obtained by scanning from 400 to 650 nm using a Cary 50 Bio spectrophotometer (Varian, Palo Alto, USA) or Evolution 600 spectrophotometer (Thermo Electron Scientific Instruments, UK). Figure 1 shows the difference spectrum of cytochromes for isolated mitochondria. The value of peak at 605 nm was used for quantification of respiratory chain cytochrome aa_3 contents (cytochrome c oxidase) both in isolated mitochondria and cardiomyocytes using the extinction coefficient ε value equal to 24 $mM^{-1}.cm^{-1}$ (Monge et al. 2008; Van Gelder 1966). Protein concentrations were determined using a BCA protein assay kit (Pierce, USA) as a standard.

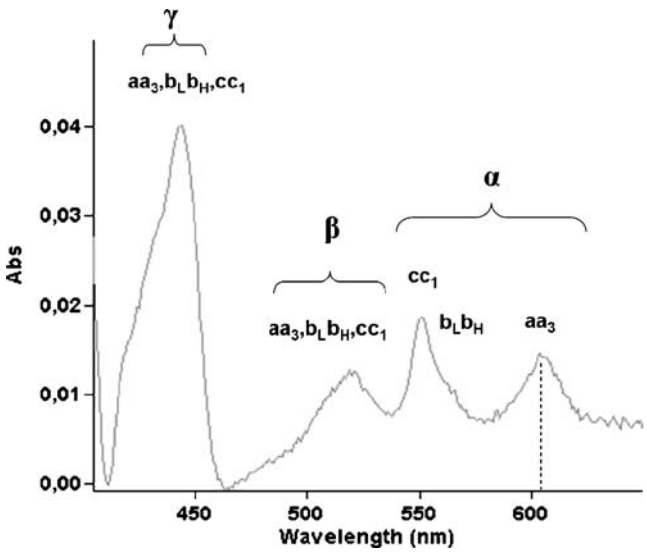


Fig. 1 The absorbance spectra of cytochromes (reduced versus oxidized), recorded by scanning the samples (isolated mitochondria or cardiomyocytes) from 530 to 650 nm in spectrophotometry

Isolation and purification of tubulin

Tubulin from rat brain and bovine brain was used with equivalent results. The bovine tubulin was obtained from Cytoskeleton (Boulter, CO, USA). The rat brain tubulin was purified as previously described (Sackett et al. 1991; Wolff et al. 1996). Frozen rat brains were thawed, homogenized in Assembly Buffer (0.1 M MES, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.9), and centrifuged at 100,000 g. Microtubule protein (tubulin plus microtubule associated proteins) was purified by several rounds of GTP-driven, temperature-dependent polymerization and depolymerization (Sackett et al. 1991). Tubulin was then purified from this material by selective polymerization in high buffer concentration, pelleted by centrifugation, redissolved in Assembly Buffer at 25 mg/ml, and drop frozen in liquid nitrogen (Wolff et al. 1996). In its final form the tubulin used was the αβ-heterodimer (Sackett et al. 1991; Wolff et al. 1996).

Reconstitution studies

Isolated and purified rat heart mitochondria (8 mg/ml) were incubated in Mitomed solution (see above) with 1 μM tubulin for 30 min at room temperature (22°C). After that, the samples were injected into an oxygraph chamber in presence or in absence of 20 mM creatine. Kinetics of activation of respiration were analyzed by successive addition of ADP (0.005–0.01–0.02–0.05–0.1–0.2–0.5–1–2–3 mM). Assay medium additionally contained 0.2% of serum bovine albumin and 1 IU/ml apyrase from potato (Sigma-Aldrich) as an ADP regeneration system. This isoenzyme of apyrase has an exceptionally high ATPase/ADPase ratio (10:1) and can be used for effective regeneration of ADP to maintain steady-state of respiration in the presence of limited amounts of ADP in kinetic studies.

Determination of the rate of PCr production in cardiomyocytes *in situ* by ion pair HPLC/UPLC

Determination of the rates of PCr synthesis in permeabilized cardiomyocytes *in situ* under conditions used in respirometry experiments was carried out using ion pair HPLC/UPLC by stopping the reaction typically at 3, 6 and 10 min. 100 μl aliquots of the reaction mixture were withdrawn and added to 200 μl ice-cold 1 M $HClO_4$ solution, immediately supplemented with 5 μl of 100 mM EDTA and neutralized with 210 μl of 0.952 M KOH in 0.5 – 1 min. The samples were held on ice for additional 10 – 15 min for proper precipitate formation and centrifuged at 16 000 g and 4ºC for 2 – 3 min. The supernatants were immediately frozen (−40˚C) and analyzed within 5 – 6 h.

Addition of EDTA (final 1 mM) proved to be useful in order to bind traces of Mg^{2+} to suppress any residual enzyme (particularly adenylate kinase, unpublished observations) activity and stabilize the preparations. Separations of Cr, PCr and adenine nucleotides were performed by ultra-performance ion-pair chromatography (UPLC) on a 2.1× 100 mm ACQUITY UPLC HSS T3 C_{18} column packed with 1.7 µm particles (Waters) by recording optical density simultaneously at 210 nm and 254 nm for creatine and PCr, and adenine nucleotides, respectively. Sample volumes of 10 µl were injected by autosampler. The mobile phase consisted of buffer A (20 mM KH_2PO_4, 3 mM tetrabutylammonium bisulfate (TBAS)) and buffer B (200 mM KH_2PO_4, 10% (v/v) acetonitrile, 0.3 mM TBAS), both adjusted to pH 5.0 with 2 M phosphoric acid and filtered through a 0.2 µm membrane filter. The elution was performed at a flow rate 0.4 ml/min in buffer A for 2 min followed by 1:1 gradient elution with buffers A and B up to 8.5 min and additionally with buffer B up to 10 min. After the analysis the column was re-equilibriated by washing for 1 min with water and buffer A for 9 min thus resulting in total time for an analysis 20 min. The column was periodically cleaned by washing with 80% methanol. The retention time for the reagents were defined/checked by measurements with the standard solutions prior to every test series Stock solutions for calibration (0.1 M) were prepared in 0.2 M KH_2PO_4 at pH 7.0 and stored at −40°C for not more than 2 – 3 days in order to minimize PCr and ATP degradation. Calibration solutions were prepared in supernatant solutions obtained after addition and precipitation of cardiomyocytes as described above.

Results

Figure 2a shows the classical respiratory control analysis. ADP in limited concentrations (0.2 – 0.4 mM) activates respiration but does not maintain the stable value of the rate of oxygen consumption by isolated mitochondria due to its rapid phosphorylation into ATP. As a result the fast transition of respiration from State 3 to the State 4 is observed (Fig. 2a). Addition of 20 mM creatine leads to stabilization of respiratory rate at the level close to the State 3 value (Fig. 2b). In this case a stable level of respiration is maintained by phosphorylation of endogenous ADP produced locally by activated MtCK. In accordance with many earlier data (Jacobus and Lehninger 1973; Meyer et al. 2006; Monge et al. 2008, Saks et al. 2007c, 1975, 2004), these results show that MtCK is able to maintain a maximal rate of respiration by supplying endogenous, locally produced ADP to ANT. Phosphocreatine produced in these coupled reactions leaves mitochondria via VDAC in the outer mitochondrial membrane. It was shown by Gellerich and Saks 1982 that part of ADP locally produced by MtCK in isolated mitochondria is equilibrated between intermembrane space and surrounding medium due to high permeability of the VDAC, but an equal amount of ADP is taken by ANT back to the mitochondrial matrix. This phenomenon can be easily revealed by addition of the ADP trapping system consisting of PK and PEP (Gellerich and Saks 1982; Gellerich et al. 1987, 2002, 2000). When respiration of isolated heart mitochondria was stimulated by creatine in the presence of ATP, addition of PK and PEP decreases the respiration rate to about 50 % of its maximal value. The remnant rate of respiration (up to 50% of VO_2max) was due to the functional coupling between MtCK and ANT with the direct transfer of ADP into the matrix (Vendelin et al. 2004a).

Movement of ADP across the outer membrane of isolated mitochondria can be limited by association of heterodimeric tubulin to VDAC (Monge et al. 2008; Rostovtseva and Bezrukov 2008; Rostovtseva et al. 2008). Under these conditions creatine effectively regulates respiration by increasing the rate of ADP-ATP recycling in the coupled MtCK-ANT system. The fact that tubulin in its dimeric form is able to interact directly with heart mitochondria was confirmed in experiments with isolated heart mitochondria (Monge et al. 2008), by a partial reconstruction of the cytoskeleton surrounding mitochondria. The results shown in Fig. 3 demonstrate that addition of tubulin (1 µM) to isolated heart mitochondria induce an increase in apparent Km for ADP from 11±2 µM to 330±47 µM. The Fig. 3a/b/c show oxygraph recordings of the activation of the mitochondrial respiration by exogenous ADP. Figure 3a is control kinetics of respiration regulation of isolated mitochondria. The maximal rate of respiration is observed in the presence of ADP at a concentration of 20 – 50 µM and the apparent Km is very low (11±2 µM), demonstrating the absence of diffusion barriers for ADP into the intermembrane space. Figure 3b shows the increase in diffusion constraints caused by tubulin obviously by direct interaction with VDAC in the outer mitochondrial membrane (Monge et al. 2008; Rostovtseva et al. 2008): respiration rate continues to increase even after addition of ADP at a concentration of 2 mM. In this case, kinetic analysis showed that in the presence of tubulin two populations of mitochondria with different apparent Km appear, one with very high apparent Km for exogenous ADP equal to 330±47 µM (Fig. 3d and e). In the presence of creatine (Fig. 3c) ADP again rapidly activated the respiration and only one population with an apparent Km equal to 23±6 µM was seen due to activation MtCK and increasing the recycling of ADP and ATP in mitochondrial matrix and inner membrane (Kim and Lee 1987; Meyer et al. 2006; Saks et al. 1998a, 1991, 1993). Evidently, creatine easily diffuses into the intermembrane

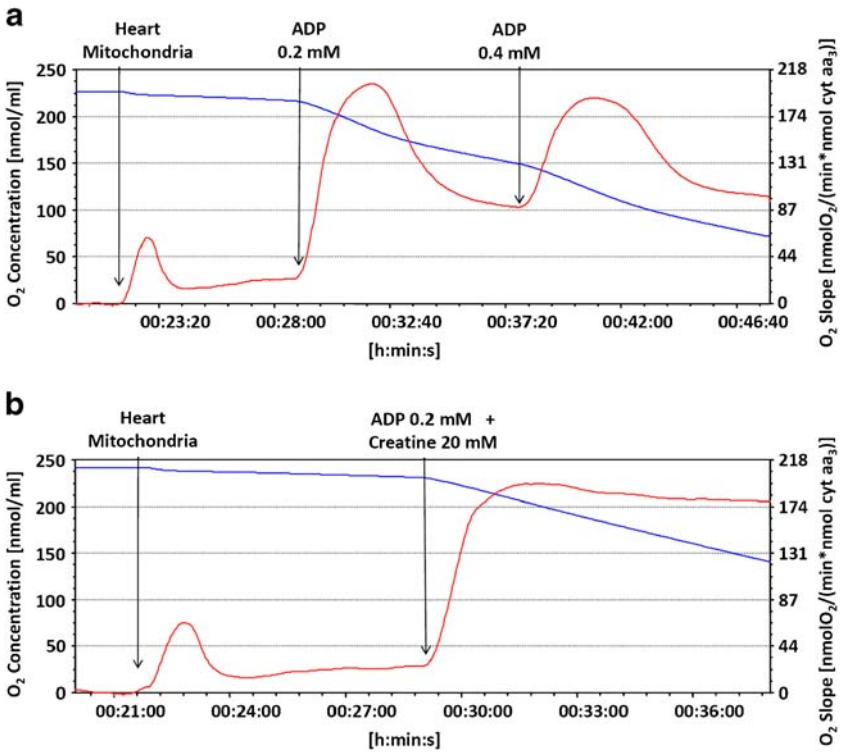


Fig. 2 **a** The classical respiratory control – transition between States 2, 3 and 4, according to Chance, in response to addition of limited amounts of ADP (0.2 mM; 0.4 mM) to isolated mitochondria, **b** Stable State 3 of respiration of isolated mitochondria stimulated by 0.2 mM ADP in the presence of 20 mM creatine. This stability is explained by the continuous production of endogenous ADP by mitochondrial creatine kinase (MtCK) reaction

space via the tubulin-VDAC complex (Rostovtseva et al. 2008) which in the mitochondrial membrane may include other cytoskeletal proteins (see "Discussion"). Thus, activation of the mitochondrial creatine kinase (MtCK) allowed overcoming the diffusion restriction for ADP provoked by the presence of tubulin in the vicinity of VDAC. The apparent Km for ADP in the control and in the presence of both tubulin and creatine are very close (Fig. 3d and e).

The experiments with isolated mitochondria and added tubulin shown in Fig. 3 reproduce well the kinetics of respiration regulation in permeabilized heart cells where the apparent Km for exogenous ADP is very high but decreased significantly by creatine (Appaix et al. 2003; Saks et al. 1998a, 1991, 1993, 1995).

To evaluate quantitatively the relative role of the phosphocreatine flux in energy transfer from mitochondria into cytoplasm in the cardiac cells *in vivo*, we used the permeabilized cardiac cells in combination with the added, exogenous PK-PEP system to simulate the interaction between mitochondria and glycolytic systems and their competition for extramitochondrial ADP. In permeabilized cardiac cells *in situ* in the presence of creatine and MgATP (see Scheme 2), MgADP is produced in the MgATPase reactions in myofibrils and sarcoplasmic reticulum (SR), in the MtCK reaction in the mitochondrial intermembrane space and in the MM creatine kinase reaction both in myofibrils and at SR membranes. If there is direct crosstalk between mitochondria and MgATPases by MgATP supply from mitochondria to ATPases and MgADP back as supposed in several studies (Joubert et al. 2008; Kaasik et al. 2001; Kuum et al. 2009), the PCr/O_2 ratio should be significantly less than the theoretically maximal P/O_2 ratio, which is equal to 6 (Nicholls and Ferguson 2002). Thus determination of PCr/O_2 ratio in a system described in Scheme 2 allows us to measure directly the energy fluxes between mitochondria and cytoplasm. Exogenous PK-PEP helps to keep extramitochondrial ADP concentration low and avoid rapid consumption of PCr in the coupled MMCK- MgATPase reactions.

Figure 4a shows a regular quality test for isolated cardiomyocytes used in this work. Addition of ADP in saturating concentration of 2 mM to permeabilized cardiomyocytes induced a State 3 high respiration rate. The respiratory control index usually exceeded 7 (Fig. 4a). Addition of exogenous cytochrome c did not increase the respiration rate, this showing the intactness of the outer mitochondrial membrane, and addition of atractyloside decreased the respiration rate close to the State 2 value, this showing the intactness of the inner mitochondrial membrane (Kuznetsov et al. 2008; Saks et al. 1998b). Only preparations with these characteristics were used in the experiments reported in this work.

Figure 4b shows the behaviour of the study system described by Scheme 2. Cardiomyocytes were permeabilized by saponin and State 2 respiration recorded. MgATP was added to a 2 mM final concentration to stimulate MgATPases, this increasing the respiration rate. This rate was decreased by addition of PK (20 IU/mL) in the

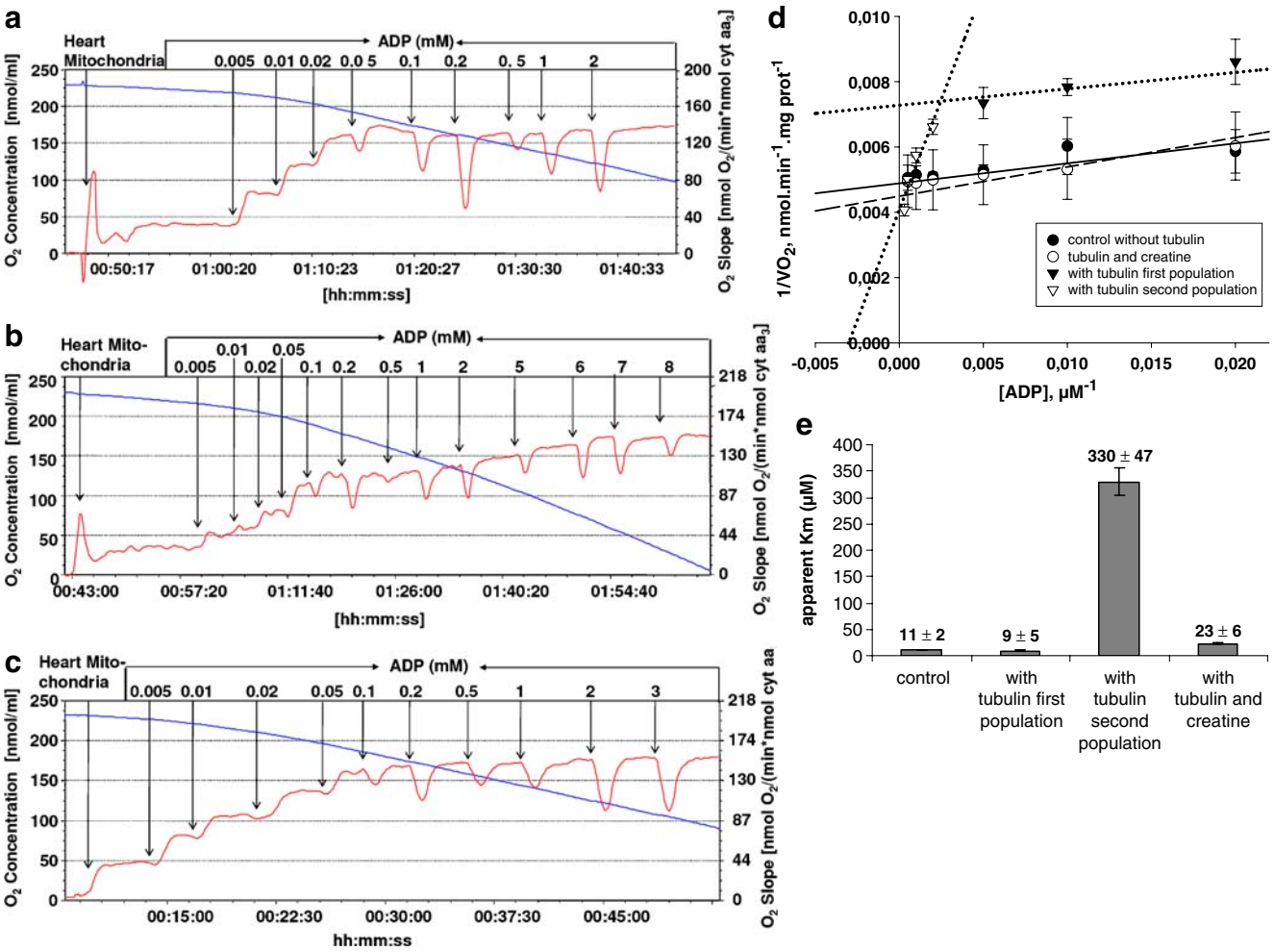


Fig. 3 **a** Oxygraph recording of the control kinetic of mitochondrial respiration activated by increasing concentrations of exogeneous ADP, **b** and **c** Oxygraph recording of the kinetics of mitochondrial respiration regulation in the presence of 1 μM tubulin (3B) and both 1 μM tubulin and 20 mM creatine (3C), **d** Double reciprocal representation (Lineweaver-Burk) of the kinetic of respiration regulation for isolated mitochondria (control, solid circles and straight line), with 1 μM tubulin (triangles and dotted lines) and with either 1 μM tubulin or either 20 mM creatine (empty circles and dashed line), **e** Comparison of the apparent Kms for exogenous ADP in the presence of tubulin and/or creatine. The values of the Kms indicated above the bars are in μM

presence of PEP (5 mM) due to trapping of a significant part of extramitochondrial MgADP. The respiration rate did not return to the State 2 level, this showing that some part of MgADP was channelled back to mitochondria. Subsequent addition of creatine rapidly increased the respiration rate. At a creatine concentration of 10 mM the maximal respiration rate was achieved; therefore, this concentration was used in further experiments. It was shown before that when respiration of mitochondria in permeabilized cardiomyocytes *in situ* is activated by creatine and MgATP (Guzun et al. 2009) and a high respiration rate is achieved, addition of PK – PEP does not result in a decrease of rate of oxygen consumption. That shows that ADP locally produced by MtCK in the intermembrane space is not accessible for exogenous ADP – trapping system, obviously, due to decreased permeability of VDAC as a result of binding of some cytoskeletal protein(s) to this channel. Selective treatment of permeabilized cardiomyocytes with trypsin to digest these proteins PK-PEP system again decreased the respiration rate, exactly as in isolated mitochondria in vitro (Guzun et al. 2009).

Table 1 summarizes the respiratory parameters of the isolated mitochondria and isolated cardiomyocytes. As it can be seen from Table 1, maximal respiration rates are equal both in isolated mitochondria and cardiomyocytes if calculated per nmol of cytochrome aa_3.

Since the mechanism of functioning of ANT is dependent upon and governed by the mitochondrial membrane potential $\Delta\Psi$ (Klingenberg 2008), it was important to record the changes in $\Delta\Psi$ under conditions described in Fig. 4b. The results of these measurements are shown in Fig. 5.

Figure 5 shows the recordings of changes in membrane potential in mitochondria *in situ* in permeabilized cardio-

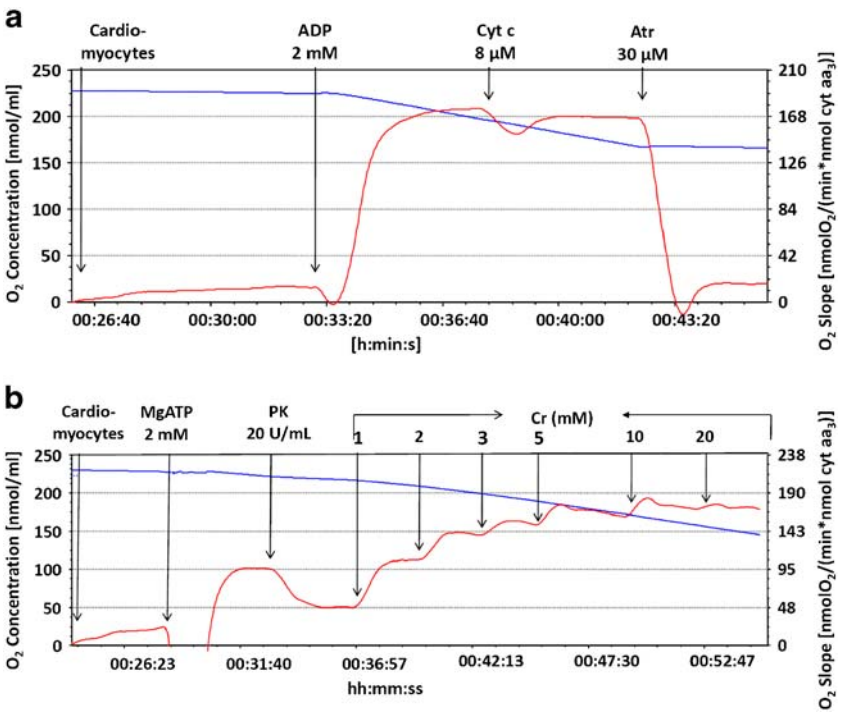


Fig. 4 **a** State 3 of mitochondrial respiration maintained in permeabilized cardiomyocytes. Cardiomyocytes were permeabilized with 25 μg/mL saponin in oxygraph cells during 10 min. Then respiration was activated in situ by addition of 2 mM exogenous ADP. Cytochrome c test shows intactness of MOM. Atractyloside test shows that respiration is totally controlled by ANT, **b** The respiration recording of the study system described by Scheme 2. Cardiomyocytes were permeabilized by saponin and State 2 respiration recorded. MgATP was added to 2 mM final concentration to stimulate MgATPases, this increasing the respiration rate. This rate was decreased by addition of PK (20 IU/mL) in the presence of PEP (5 mM) due to trapping of a significant part of extramitochondrial MgADP. Subsequent addition of creatine rapidly increased the respiration rate

myocytes. First, cardiomyocytes were permeabilized in fluorimeter cells into which TMRM and PEP were also added. Because of the presence of some endogenous substrates in cardiomyocytes, already some energization of the membrane was observed compared to the zero level at the end of experiments (Fig. 5). This energization was increased (fluorescence decreased) further after addition of glutamate and malate to induce the State 2 respiration. Subsequent addition of ATP did not lead to additional changes in membrane potential: energization of membrane due to the presence of ATP was equilibrated by ADP production in MgATPase reactions. Addition of PK induced transition into a true State 4 respiration and maximal energization of mitochondria due to effective removal of this extramitochondrial ADP. Addition of creatine in a final concentration of 10 mM induced a remarkable decrease in membrane potential and its transition to a new lower steady state level was observed. Addition of an uncoupler CCCP decreased the membrane potential to zero. These experiments show that MtCK in the presence of creatine effectively supplies local ADP to ANT which operates a in so called "productive" exchange mode (ADPin-ATPout) at a high value of $\Delta\Psi$ (as compared with the "unproductive" exchange ATPin-ADPout at low membrane potential) (Klingenberg 2008). These data directly show the effective functional coupling between ANT and MtCK.

Very interestingly, measurements of the respiration rates as a function of MgATP concentration at different steps of the experimental protocol described by Scheme 2 and in Figs. 4b and 5 gave remarkable and important results

Table 1 Basic respiration parameters of isolated rat heart mitochondria and of mitochondria in situ in permeabilized cardiomyocytes

Parameter	Mitochondria in vitro	Mitochondria in situ (permeabilized cardiomyocytes)
V_0,[a] $nmolO_2 \cdot min^{-1} \cdot mg\ prot^{-1}$	26.37±7.93	7.53±1.61
V_3[b], $nmolO_2 \cdot min^{-1} \cdot mg\ prot^{-1}$	187.94±40.68	84.45±13.85
[Cyt aa_3], $nmol \cdot mg\ prot^{-1}$	1.00±0.012	0.46±0.09
V_3, $nmolO_2 \cdot min^{-1} \cdot nmol\ cyt\ aa_3^{-1}$	187.94±40.68	178.23±33.96
$V_{Cr,ATP}$[c], $nmolO_2 \cdot min^{-1} \cdot nmol\ cyt\ aa_3^{-1}$	197.90±31.86	162.63±26.87

[a] V_0 respiration rate in State 2 in the presence of substrates before addition of ADP or ATP

[b] V_3 respiration rate in the presence of 2 mM ADP

[c] $V_{Cr,ATP}$ respiration rate in the presence of activated MtCK by 2 mM ATP and 20 mM creatine

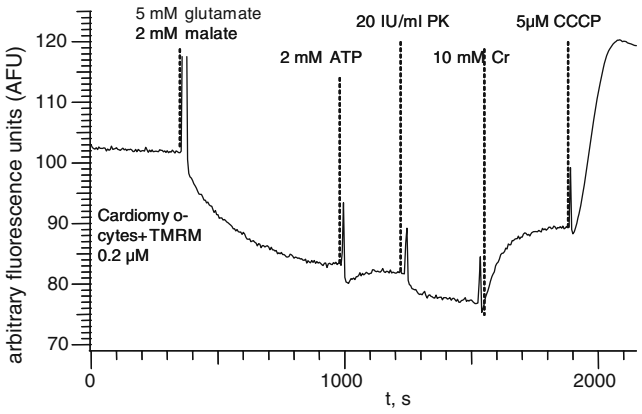


Fig. 5 Response of the TMRM fluorescence (excitation 548 nm, emission 574 nm) to mitochondrial respiration changes. Isolated cardiac cells were permeabilized with 25 μg/ml saponin and incubated in Mitomed solution supplied with 5 mM PEP and 0.2 μM TMRM in a thermostated fluorimeter cell. Addition of the substrates 5 mM glutamate, 2 mM malate caused a decrease in fluorescence indicating the accumulation of TMRM in mitochondrial matrix. 2 mM ATP produced a small change in mitochondrial membrane potential, ΔΨ. Activation of MtCK and mitochondrial respiration by addition of 10 mM creatine decreased ΔΨ to a lower steady state level. The uncoupling agent CCCP 5 μM was used to dissipate the membrane potential

concerning the role of ATP, ADP creatine and also selective restriction of diffusion of adenine nucleotides in cardiomyocytes and particularly across the mitochondrial outer membrane in regulation of mitochondrial respiration *in vivo*. First, these measurements were made in the absence and presence of creatine before addition of PEP-PK system. The MgATP concentrations added in these experiments are shown at the lower abscissa axis in Fig. 6. In the absence of creatine, the respiration rate was increased in response to addition of MgATP due to activation of extramitochondrial MgATPases with the apparent Km equal to 158±40 μM in accordance with many earlier determinations (Saks et al. 2001; Seppet et al. 2001), but the Vmax value was low due to absence of calcium ions in these experiments. In the presence of creatine, the addition of MgATP very rapidly increased the respiration rate to its maximal value and the apparent Km for MgATP decreased to 24±0.8 μM. Under these conditions, MgADP is produced both extramitochondrially in the MgATPase and MM-CK reactions and in the MtCK reaction coupled to ANT (see Scheme 2). To differentiate between these two sources of MgADP, PEP and PK were added. This completely changed the kinetics of respiration regulation: for activation of respiration, addition of much higher concentrations of MgATP was needed; these concentrations are shown on the upper abscissa axis in Fig. 6. In these experiments, the apparent Km for MgATP was increased to 2 mM, in accordance with our recent observations (Guzun et al. 2009). Under these conditions, almost all extramitochondrial ADP is trapped and mitochondrial respiration is exclusively dependent upon ADP supply by MtCK (as seen in Fig. 5b), which is only slowly activated by exogenous MgATP most possibly by limited permeability of VDAC in permeabilized cells *in situ* (Guzun et al. 2009). Thus, for maximal activation of respiration, some extramitochondrial MgADP is needed, and in the presence of creatine rapid recycling of this ADP in the coupled MtCK- ATP Synthasome system maintains a high respiration rate (Guzun et al. 2009).

Based on the results of experiments shown in Fig. 6, in the next experiments MgATP was used in concentrations of 1, 2 and 5 mM in the presence of creatine (10 mM) and the PEP-PK system for determination of the PCr/O_2 ratio in permeabilized cardiomyocytes.

Determination of PCr/O_2 ratio was performed with the use of HPLC/UPLC technique for detection and quantification of the compounds of interest, PCr and ATP, in the reaction mixture and with separate measurements of corresponding oxygen uptake with a high-resolution OROBOROS respirometer. Under experimental conditions described above mitochondrial respiration was activated by addition of 10 mM Cr in the presence of fixed MgATP (1, 2, 5 mM) concentration, and extramitochondrial ADP produced by MMCK and ATPase was trapped by the PEP (5 mM) – PK (20 IU/mL) system (Fig. 4b and 5). The product mixture samples were collected at 3, 6 and 10 min

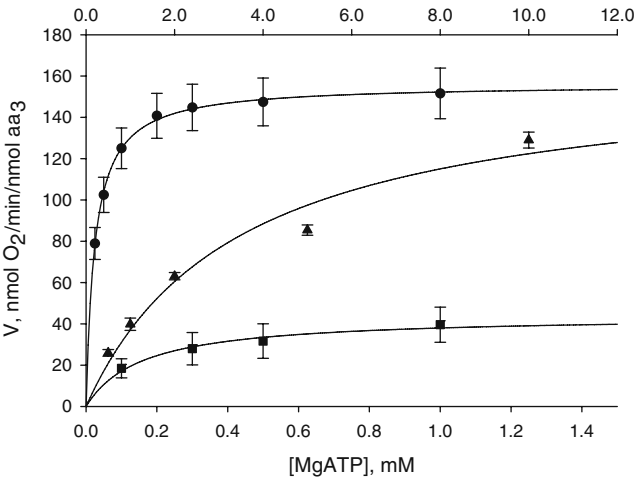


Fig. 6 Regulation of mitochondrial respiration as a function of the concentration of added exogenous MgATP in the absence (■) and the presence (●) of 20 mM Cr before addition of a PK-PEP system (bottom x-scale) and in the case of supplementation with PEP-PK system (▲, top x-scale). In the absence of PK-PEP system, the apparent affinity for exogenous MgATP without Cr (K_m^{app}=157,8± 40,1 μM), produced by hydrolysis in MgATPase reactions, is diminished due to addition of 20 mM Cr (K_m^{app}=24,9±0.8 μM). In the presence of PK-PEP system, apparent affinity for MgATP is significantly decreased (see the text). Maximum rate of respiration in all cases was similar. Addition of ADP-trapping PEP-PK system drastically changes the kinetics of regulation

after initializing MtCK reaction. Separation and analysis of the mixture of components were performed according to a standard HPLC/UPLC operating procedure (described in Materials and Methods section). The output presents a series of peaks located on the time axis, each corresponding to a compound in the test solution, which passed through UV detectors (Fig. 7a). Estimation of these substances was identified by the authentic samples. The concentration of the components was calculated from the area under corresponding peaks. By plotting peaks matching PCr it could be seen how its amount is increasing in time in the test medium (Fig. 7b).

ATP levels continuously regenerated by the PEP-PK system exhibited no significant change in the trial mixtures (Fig. 8a). However, PCr concentration in the surrounding medium increased rapidly in dependence on MgATP concentration (Fig. 8b).

Since PCr in permeabilized cardiac cells can be produced by different isoforms of creatine kinase (MtCK and non-mitochondrial creatine kinases (myofibrillar, SR and sarcolemmal), to ascertain which amount of PCr content in the samples is of mitochondrial origin, the oxidative phosphorylation was inhibited by 10 μM rotenone (Fig. 8b). The differences of the rates of PCr synthesis in the absence and presence of rotenone at defined MgATP concentrations estimate MtCK contribution in each case (Fig. 8c). Oxygen consumption rises with an increase of MgATP concentration (Table 2) in accordance with kinetic data shown in Fig. 6. The same tendency is observed for the rate of PCr production.

Table 2 summarizes the rates of PCr production and corresponding respiration rates at fixed ATP concentrations. From these data the PCr/O_2 ratio is calculated, the average value is equal to 5.68±0.14, which is close to the theoretical maximal value of 6 (Nicholls and Ferguson 2002).

Discussion

The results of this study show clearly the important role of the ANT - MtCK-VDAC- Tubulin system in regulation of respiration and energy fluxes in the cardiac cells (Fig. 9). ANT in the mitochondrial inner membrane is an integral part of the ATP Synthasome (Chen et al. 2004; Pedersen 2007a, b). Therefore, there seems to be a supercomplex of ATP Synthasome - MtCK – VDAC – Tubulin in contact sites (Brdizka 2007) in heart mitochondria which controls the regulation of respiration. This whole complex may be shortly named “Mitochondrial Interactosome,” MI (Fig. 9). This Mitochondrial Interactosome may in some cases

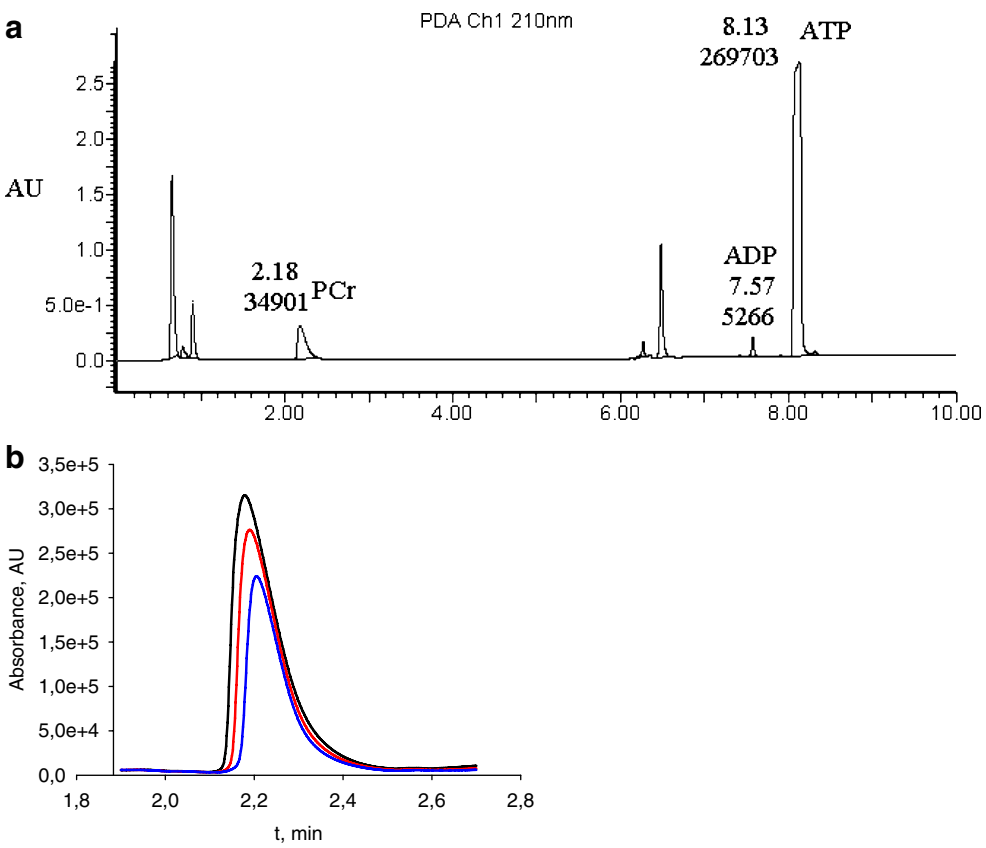


Fig. 7 **a** Chromatograms were obtained by and ACQUITY UPLC system from permeabilized cardiomyocyte incubations for 5 mM ATP. The samples of the reaction mixture were taken at specified time intervals after the initializing reaction. The positions of PCr and ATP traces on the retention time scale were detected at 2.2 and 8.1 min, respectively. Quantitative assessment of the concentrations of the mixture components was obtained from the peak area, **b** Replotted from original chromatograms graph with the peaks correponding to PCr appereance after 3, 6 and 10 min after activation MtCK reaction by adding 10 mM Cr into the medium

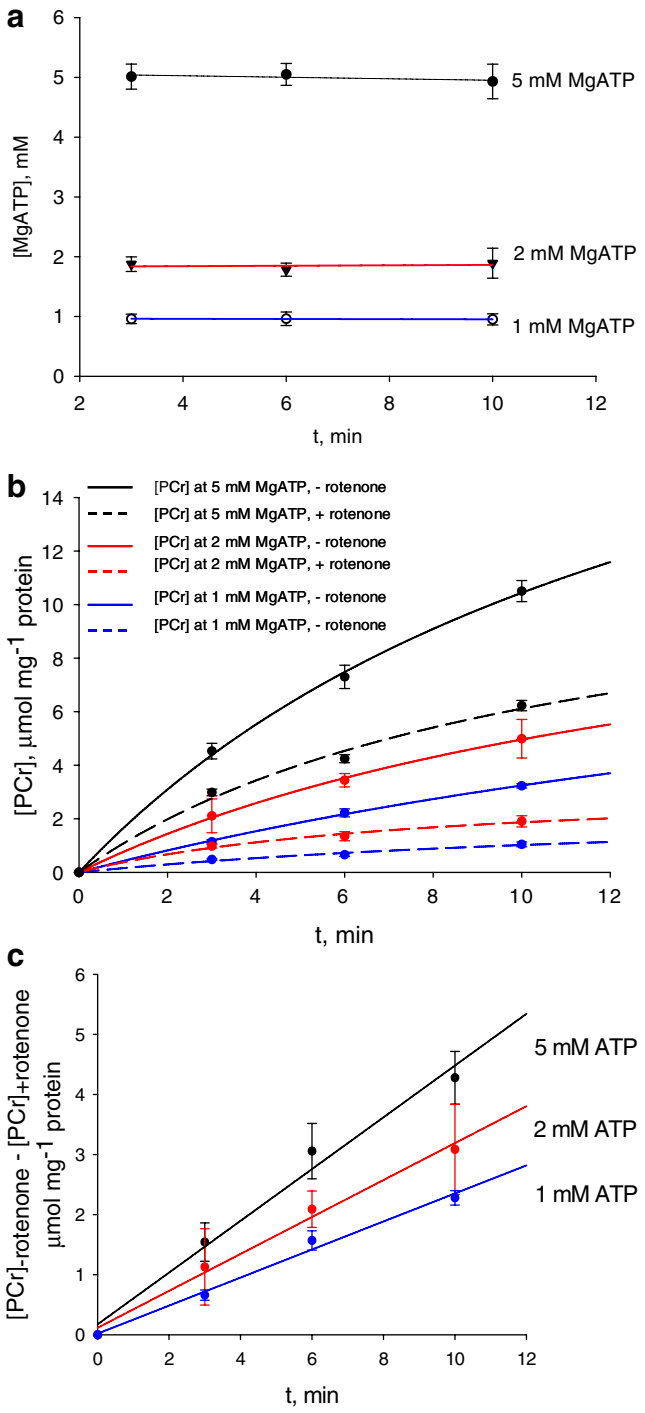


Fig. 8 **a** The ATP level, continuously regenerated by the PEP-PK system, was stable during the experimental procedure described in the Fig. 5, **b** The rate of phosphocreatine production by mitochondrial and cytoplasmic creatine kinases in permeabilized non-inhibited cardiomyocytes (solid lines). After activation of MtCK by creatine (10 mM) in permeabilized cardiomyocytes in the presence of MgATP (1, 2, 5 mM) and PEP (5 mM) and PK (20 U/ml) reaction was stopped after 3, 6 and 10 min. Analyses of the collected mixture were performed by using ion pair HPLC/UPLC as described in Materials and Methods. When oxidative phosphorylation is inhibited by rotenone, 10µM (dashed lines), the PCr can be produced only by cytoplasmic creatine kinases, MMCK, **c** The difference in phosphocreatine production rates under conditions of activated and inhibited (by rotenone) respiratory chain calculated from Fig. 6b. In parallel experiments corresponding oxygen consumption rates were measured. The creatine (10 mM) activated respiration rates rises with the increase of MgATP concentrations. For any MgATP concentrations PCr/O_2 is equal to 5.68±0.14

include also supercomplexes of the respiratory chain (Lenaz and Genova 2007; Vonck and Schafer 2009). Along the cristae membranes the MI contain only MtCK and ATP Synthasome. Direct measurements of energy fluxes from mitochondria into cytoplasm (surrounding medium in experiments with permeabilized cardiac cells) show PCr/O_2 ratios close to the theoretical maximal P/O_2 ratio under conditions similar to those *in vivo*. These high PCr/O_2 ratios are seen for all three MgATP concentrations up to 5 mM, which is close to physiological concentrations of ATP in cells. Thus, our data clearly show the effectiveness of the transmission of high energy bond from ATP to PCr within the MI. These data leave also little room for direct crosstalk between mitochondria and MgATPases by MgATP and MgADP channeling (Joubert et al. 2008; Kaasik et al. 2001; Kuum et al. 2009) this shows that energy is carried out of mitochondria by PCr fluxes and creatine effectively regulates the MtCK – ATP Synthasome complex due to selective restriction by heterodimeric tubulin of VDAC permeability only for adenine nucleotides but not for creatine or PCr (Guzun et al. 2009). Under these conditions, the amount of ATP and ADP diffusing through MOM is minimal but not zero. Kaasik et al. 2001 and Kuum et al. 2009 made their conclusion of the crosstalk between mitochondria and MgATPases by direct transfer of MgATP and MgADP on the basis of recordings in separate experiments of the amount of calcium in sarcoplasmic reticulum after a rather long period of incubation of permeabilized cells either with ATP, phosphocreatine and ATP, or ATP and respiratory substrates. No reaction rates or energy fluxes were recorded under physiological conditions – activation of MtCK and interaction of mitochondria with other cellular systems, including ATPases, cytoskeleton and the glycolytic system. The absence of such a system analysis does not allow conclusions to be made of the distribution of energy fluxes between mitochondria and cytoplasm *in vivo*.

In our experiments, both MtCK-controlled respiration and PCr production rates were dependent on MgATP concentration and increased with the elevation of the latter in the interval of 1 – 5 mM (Fig. 6 and 8c). This conforms to our recent kinetic data showing that in permeabilized cardiomyocytes *in situ* the diffusion of ATP into the intermembrane space is restricted (Guzun et al. 2009). At the same time PCr evidently rather easily diffuses through VDAC into the surrounding medium. Kinetic determina-

Table 2 Measured rates of PCr production, corresponding oxygen consumption and their calculated ratios for fixed ATP concentration in mitochondria in situ

	V_{PCr}[a] µmolmg−1 protein min−1	V_{O2}[b] µmolmg−1 protein min−1	V_{PCr}/V_{O2}[c]
1 mM ATP	0.23±0.02	0.041±0.001	5.80±0.45
2 mM ATP	0.31±0.02	0.056±0.02	5.44±0.44
5 mM ATP	0.43±0.04	0.074±0.003	5.81±0.48
		Average	5.68±0.14

[a] V_{PCr} rate of PCr production measured with the use of HPLC/UPLC

[b] V_{02} rate of oxygen consumption

[c] V_{PCr}/V_{O2} calculated ratio of PCr/O_2

tions showed that the affinity of MtCK for exogenous creatine in the permeabilized cardiomyocytes was even increased in comparison with isolated mitochondria, and not changed for phosphocreatine. Because of this rather selective control of VDAC permeability (Rostovtseva et al. 2008, Monge et al. 2008) and functional coupling between MtCK and ANT (Saks et al. 2004; Vendelin et al. 2004b), all ATP produced in oxidative phosphorylation is practically completely used for PCr production and ADP is rapidly channeled back through ANT to the mitochondrial matrix.

Our data are in line with an increasing understanding of the importance of the contacts of outer mitochondrial

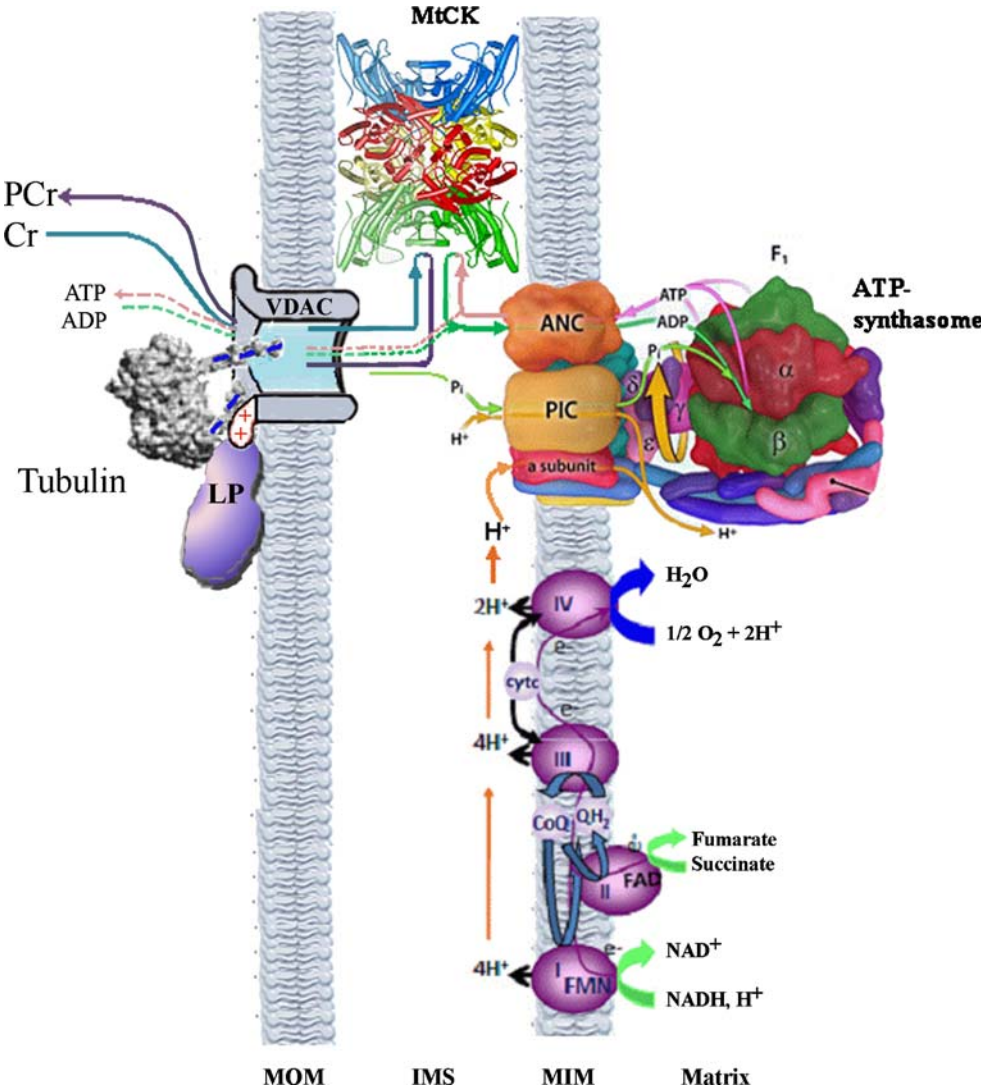


Fig. 9 Proposed model of regulation of respiration in a supercomplex named Mitochondrial Interactosome, consisting of ATP Synthasome - MtCK-VDAC- Tubulin. Macromolecular ATP synthase is represented as a part of the complex ATP Synthasome (*reprinted with kind permission from Peter L. Pedersen,* 2007a, b, 2008), with adenine nucleotides carriers (ANC) and phosphate carriers (PIC). Octameric mitochondrial creatine kinase (MtCK) (*the structure was kindly supplied by U.Schlattner*), located in the mitochondrial intermembrane space (IMS) and attached to mitochondrial inner (MIM) and in the contact sites to outer membranes (MOM). VDAC permeability is selectively regulated by heterodimeric tubulin, which binding to VDAC in intact mitochondrial membrane may be either direct or by some linker proteins (LP). This complex of VDAC with other proteins controls the outcome of adenine nucleotides and PCr fluxes into surrounding medium, and phosporylation by the ATP Synthasome system is effectivily regulated by creatine via MtCK

membrane VDAC with the cytoskeleton for regulation of energy fluxes and mitochondrial respiration in cardiac cells (Aliev and Saks 1997; Anflous et al. 2001; Boudina et al. 2002; Burelle and Hochachka 2002; Capetenaki 2002; Colombini 2004; Guerrero et al. 2005; Kummel 1988; Kuznetsov et al. 1989, 1996; Linden et al. 1989; Liobikas et al. 2001; Rostovtseva and Bezrukov 2008; Rostovtseva et al. 2008; Saks et al. 1998a, 2003, 1991, 1974, 1985, 1993, 1989; Veksler et al. 1995; Vendelin et al. 2004b; Zoll et al. 2003a, 2005, 2003b, 2002). Studies of permeabilized cells in many laboratories have shown an increased apparent Km for ADP for exogenous ADP in the regulation of respiration in comparison with isolated mitochondria (Anflous et al. 2001; Boudina et al. 2002; Burelle and Hochachka 2002; Guerrero et al. 2005; Kummel 1988; Kuznetsov et al. 1996; Liobikas et al. 2001; Saks et al. 1998a, 2007d, 2003, 1991, 1993, 1989; Veksler et al. 1995; Zoll et al. 2003a, 2005, 2003b, 2002). This was explained by local restriction of ADP diffusion in the cells due to binding of some cytoskeletal elements (called factor X) to the mitochondrial outer membrane (Appaix et al. 2003; Saks et al. 1995). Very recently, Rostovtseva et al. identified this factor X by showing direct interaction of heterodimeric tubulin with VDAC (Rostovtseva and Bezrukov 2008; Rostovtseva et al. 2008). In this work we show that kinetics of regulation of respiration of isolated mitochondria with added tubulin is similar to that in permeabilized cardiomyocytes. In both cases a high apparent Km for exogenous ADP is decreased when MtCK is activated by creatine (Fig. 3). In intact cells, other cytoskeletal proteins are also shown to form contacts with VDAC in the outer mitochondrial membrane, particularly desmin (Capetenaki 2002; Linden et al. 2001) and plectin (Reipert et al. 1999). Rostovtseva et al. 2008 have directly shown a strong interaction of purified tubulin with VDAC inserted into phospholipid membranes. In the cardiac cells *in vivo*, usually only about 30% of tubulin exists in the polymerized state within the microtubular system, the remaining part being in the free heterodimeric form (Tagawa et al. 1998). Therefore, the effects observed by Rostovtseva et al. 2008 and shown in Fig. 3 may well be valid for *in vivo* conditions. Interestingly, however, high apparent Km values are also characteristic for permeabilized cells (see above) from which dimeric tubulin may be thought to leak out. However, that does not happen: in experiments with use of colchicine (Guerrero 2005) to depolymerized tubulin in permeabilized cardiomyocytes, immunolabelling of tubulin by antibodies against β subunits and studies of its localization by confocal microscopy showed disappearance of the microtubular network but intensive labeling and diffused intracellular localization of tubulin, which diffusion may be limited due to its binding to other cytoskeletal elements and particularly to the outer mitochondrial membrane (Guerrero 2005). Correspondingly, only a minor decrease of apparent Km for endogenous ADP was seen (Guerrero 2005).

In the intact mitochondrial outer membrane some other proteins may also be associated with VDAC. These interactions may result in specific restriction of VDAC permeability only for adenine nucleotides, but not creatine or PCr. It has been shown that one of these proteins may be microtubule-associated protein 2 (MAP2) (Linden and Karlsson 1996; Linden et al. 1989) and cyclic nucleotide phosphodiesterase (Bifulco et al. 2002). Interestingly, similar association of tubulin via binding to linker proteins has been shown for the plasma membrane (Wolff 2009). These data allow supposing that in the contact sites between inner and outer mitochondrial membranes there is the supercomplex MI in the cells *in situ* where tubulin is associated either directly or via linker proteins to VDAC, which is associated with MtCK - ATP Synthasome complex (Fig. 9). Earlier, Pedersen et al. have shown the existence of a similar "supercomplex", i.e., the ATP Synthasome-VDAC-Hexokinase 2 in cancer cells that helps in explaining the Warburg effect (Chen et al. 2004; Pedersen 2007a, b, 2008). Our earlier studies of cancer cells of the cardiac phenotype - continuously dividing HL-1 cells are consistent with the explanation proposed by Pedersen. These studies have shown that in HL-1 cells apparent Km for exogenous ADP is very low, creatine kinase is downregulated and creatine has no effect on respiration (Anmann et al. 2006). On the contrary, in these cells hexokinase activity is increased manifold and glucose activates respiration via activation of membrane-bound hexokinase (not seen in normal cardiomyocytes) (Eimre et al. 2008). These results show that in the HL-1 cells tubulin is replaced by hexokinase 2 and creatine kinase is absent in the Mitochondrial Interactosome. Thus, alterations in the structure of MI may contribute in cancerogenesis. Another way to change the MI structure is knock-out MtCK by genetic manipulations resulting in increasing the energy transfer in the cells via adenylate kinase pathway (Dzeja et al. 2007).

The hypothesis of Mitochondrial Interactosome conforms to the fundamental theory of Peter Mitchell about vectorial metabolism (Mitchell 1979, 2004). According to this theory, an important consequence of the organization of the enzymes into multienzyme complexes is vectorial metabolism and ligand conduction which brings together "transport and metabolism into one and the same chemiosmotic molecular level - biochemical process catalyzed by group-conducting or a conformationally mobile group-translocating enzyme system" (Mitchell 1979). For enzymes and catalytic carriers that have spatially separated binding sites for donor and acceptor (as MgATP and creatine depicted for MtCK in Fig. 9), group transfer can be considered as vectorial group translo-

cation (Mitchell 1979). This is true also for movement of substrates and products from carrier to enzyme and via VDAC with selective permeability (see Fig. 9). In his latest reviews Peter Mitchell encouraged a wider use of the chemiosmotic principle and the biochemical concept of specific ligand conduction in explaining organization and operation of metabolic and transport processes within the cell (1979). Today this idea receives increased attention and is certainly another important insight of Peter Mitchell to the understanding of cellular energy conversion processes (Dzeja et al. 2007).

All data reported in this work and recently (Guzun et al. 2009) strongly support the theories of intracellular energy transport by phosphotransfer networks (Dzeja et al. 2007, Saks et al. 2008, 2007a, 2006, 2007c, 2004, Schlattner and Wallimann 2004; Schlattner et al. 2006; Vendelin et al. 2004a; Wallimann et al. 1992, 2007; Wyss et al. 1992). They also show that the popular theories of cells as homogenous medium (Barros and Martinez 2007; Meyer et al. 1984; Wu and Beard 2009) are not compatible with experimental data. An extreme case of these theories is an explanation, which from time to time appears in literature, perfect in its naïve simplicity and obviously based on observation of electron micrographs of cardiac cells saying that "mitochondria are "wrapped" partially around the myofilaments with certain degrees of variations. This close apposition of mitochondria to the contractile machinery strategically allows mitochondria to deliver ATP more efficiently to the sites where energy demands are high." (Hom and Sheu 2009). Others support this view, saying that "since the myofibrils generally have small diameters and are surrounded by tense mitochondria, it is possible that CK-facilitated transport does not play a significant role *in vivo*" (Wu and Beard 2009). This is some kind of "mechanical bioenergetics" when just looking at electron microscopic images is taken to replace careful biochemical research. Electron micrographs give useful information, but not enough. Our present study presents more clear evidence that the regulation of the cells' metabolism is a system level property dependent on the interactions of many intracellular structures and systems in the cell (Guzun et al. 2009) These interactions within MI, which lead to new, system level properties, occur within micro- and nanometer scales, much smaller than the diameter of myofibrils. From the point of view of Molecular System Bioenergetics (Guzun et al. 2009; Saks et al. 2007b), an important task is to clarify and describe quantitatively the regulatory mechanisms of the tubulin-VDAC-MtCK-ATP Synthasome supercomplex (Fig. 9), in the interaction with all the other metabolic systems in the cell. In this complex, the behaviour of MtCK cannot be described either by simple solution kinetics nor by even a more simple equilibrium equation (Vendelin et al. 2004b).

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Articles 9 et 10

Changements de structure de l´Interactosome Mitochondrial dans le cancer

Comparative analysis of the bioenergetics of adult cardiomyocytes and nonbeating HL-1 cells: respiratory chain activities, glycolytic enzyme profiles, and metabolic fluxes[1]

Claire Monge, Nathalie Beraud, Kersti Tepp, Sophie Pelloux, Siham Chahboun, Tuuli Kaambre, Lumme Kadaja, Mart Roosimaa, Andres Piirsoo, Yves Tourneur, Andrey V. Kuznetsov, Valdur Saks, and Enn Seppet

Abstract: Comparative analysis of the bioenergetic parameters of adult rat cardiomyocytes (CM) and HL-1 cells with very different structure but similar cardiac phenotype was carried out with the aim of revealing the importance of the cell structure for regulation of its energy fluxes. Confocal microscopic analysis showed very different mitochondrial arrangement in these cells. The cytochrome content per milligram of cell protein was decreased in HL-1 cells by a factor of 7 compared with CM. In parallel, the respiratory chain complex activities were decreased by 4–8 times in the HL-1 cells. On the contrary, the activities of glycolytic enzymes, hexokinase (HK), and pyruvate kinase (PK) were increased in HL-1 cells, and these cells effectively transformed glucose into lactate. At the same time, the creatine kinase (CK) activity was significantly decreased in HL-1 cells. In conclusion, the results of this study comply with the assumption that in contrast to CM in which oxidative phosphorylation is a predominant provider of ATP and the CK system is a main carrier of energy from mitochondria to ATPases, in HL-1 cells the energy metabolism is based mostly on the glycolytic reactions coupled to oxidative phosphorylation through HK.

Key words: cardiomyocytes, HL-1 cells, respiration, cytochromes, glycolysis, energy metabolism.

Résumé : On a effectué chez des rats adultes une analyse comparée des paramètres bioénergétique des cardiomyocytes (CM) et des cellules HL-1, qui ont une structure très différente mais un phénotype cardiaque similaire, pour montrer l'importance de la structure cellulaire pour la régulation de ses flux énergétiques. L'analyse par microscopie confocale a montré une répartition mitochondriale différente dans ces cellules. La teneur en cytochromes par mg de protéine cellulaire a diminué d'un facteur 7 dans les cellules HL-1 par comparaison à celles des CM. Parallèlement, les activités complexes de la chaîne respiratoire ont diminué de 4 à 8 fois dans les cellules HL-1. En revanche, les activités des enzymes glycolytiques, hexokinase (HK) et pyruvate kinase (PK), ont augmenté dans les cellules HL-1, lesquelles ont transformé efficacement le glucose en lactate. L'activité de la créatine kinase (CK) des cellules HL-1 a aussi diminué significativement. En conclusion, les résultats de cette étude viennent étayer à l'hypothèse que, contrairement aux CM où la phosphorylation oxydative est un fournisseur important d'ATP et le système CK, le principal transporteur d'énergie des mitochondries vers les ATPases, dans les cellules HL-1, le métabolisme énergétique repose principalement sur les réactions glycolytiques couplées à la phosphorylation oxydative par l'HK.

Mots-clés : cardiomyocytes, cellules HL-1, respiration, cytochromes, glycolyse, métabolisme énergétique.

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Introduction

Studies of energy metabolism in muscle cells have led to a conclusion that the metabolic compartmentalization and mechanisms of regulation of oxidative phosphorylation are system-level properties giving rise to the unitary organization of metabolic processes in the heart and in oxidative skeletal muscle cells (Seppet et al. 2001; Weiss et al. 2006; Saks et al. 2006; Saks 2007; Saks et al. 2008). The basis of this type of organization is the specific structure–function relationship characteristic of these cells. For example, in heart cells mitochondria are arranged regularly in a longitudinal lattice between the myofibrils and are located within the limits of the sarcomeres (Vendelin et al. 2005). Such a high level of structural organization results in the formation of complexes between ATPases and mitochondria that are termed intracellular energy units (ICEUs) (Saks et al. 2001; Seppet et al. 2001). Energy transfer and feedback metabolic regulation between mitochondria and ATPases occur via specialized pathways in the ICEUs that are mediated by creatine kinase (CK) and adenylate kinase (AK), and to some extent by direct adenine nucleotide channeling (Wallimann et al. 1992; Dzeja et al. 2007; Saks 2007). This organization is achieved by interaction of mitochondria with cytoskeletal structures (Seppet et al. 2001; Saks et al. 2001; Capetanaki 2002). Direct structural contacts between mitochondria and sarcoplasmic (and endoplasmic) reticulum (SR), mediated by cytoskeletal proteins and enabling the delivery of calcium from SR into mitochondrial intermembrane space and establishing local microdomains of high calcium concentration, have also been documented in many studies and are also consistent with the point of view of unitary organization of the cell metabolism (Sharma et al. 2000; Anmann et al. 2005; Csordás et al. 2006; Wilding et al. 2006). A useful reference system in these studies of the role of structure–function relationships in establishing the control over mitochondrial function has been the immortalized HL-1 mouse cardiomyocyte cell line (Seppet et al. 2006; Anmann et al. 2006). This cell line, first developed by Claycomb and colleagues, can continuously divide and spontaneously contract (beating, B HL-1 cells) while maintaining a differentiated cardiac phenotype through indefinite passages in culture (Claycomb et al. 1998; White et al. 2004). Recently, a novel subtype of HL-1 cells, the nonbeating (NB) cells, was developed by culturing B HL-1 cells in the medium with specific serum that led the cells to lose their beating properties without stopping their proliferation in normal culture medium (Pelloux et al. 2006). The NB HL-1 cells do not present the pacemaker current, spontaneous depolarization, or calcium oscillations of the original B HL-1 cells (Pelloux et al. 2006). Confocal imaging of mitochondria revealed very different structural organization of the HL-1 cells compared with adult cardiomyocytes (CM). Whereas the B HL-1 cells possess some residual sarcomeres, the NB HL-1 cells are devoid of these structures (Pelloux et al. 2006). Our previous studies have shown that these structural peculiarities are associated with striking differences in the kinetics of respiration regulation by exogenous ADP in the HL-1 cells compared with that of normal cardiomyocytes. We found that both the B and NB HL-1 cells exhibited much lower K_m for ADP in stimulating respiration than the cardiomyocytes did (Anmann et al. 2006). It was also observed that the HL-1 cells differ from normal cardiomyocytes by their gene expression profile of the AK and CK isoforms and by the absence of functional coupling of mitochondrial and cytosolic CK isoforms to oxidative phosphorylation and Ca^{2+}-Mg^{2+}-ATPases, respectively (Eimre et al. 2008). From these studies, it was concluded that contrary to CM in which mitochondria and Ca^{2+}-Mg^{2+}-ATPases are organized into the ICEUs, these complexes do not exist in HL-1 cells as a result of a less organized energy metabolism (Eimre et al. 2008).

However, the quantitative comparative analysis of different pathways of ATP production in the HL-1 cells and adult CM is still lacking. Therefore, in this study we investigated the differences in the activities of key glycolytic enzymes and respiratory chain complexes in adult rat CM, HL-1 cells, and m. gastrocnemius from rat (as a reference system for glycolytic muscles). The results show that in contrast to the adult CM, the HL-1 cells exhibit very low capacity of oxidative phosphorylation but high capacity of glycolytic system, which is the predominate source of ATP for energy-consuming reactions. The CK system that mediates energy transfer in CM cannot effectively operate in HL-1 cells owing to much lower activity compared with CM.

Materials and methods

Animals

Adult Wistar rats weighing 300–350 g were used in the experiments. The animals were maintained and studied in accordance with the US National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Preparation of isolated CM, HL-1 cell culture, and homogenates of m. gastrocnemius

The CM were isolated from adult rat ventricular myocardium as described previously (Anmann et al. 2006). The cells were washed and kept in Mitomed solution (Anmann et al. 2006) of the following composition (in mmol/L): EGTA 0.5, sucrose 110, potassium lactobionate 60, $MgCl_2$ 3, dithiothreitol 0.5, taurine 20, KH_2PO_4 3, and K-HEPES 20, pH 7.1, at 4 °C before the experiments.

The NB HL-1 cells were cultured as described previously (Pelloux et al. 2006). The cells were detached by trypsinization and the cell suspension was washed 3 times and centrifuged for 5 min at 1000*g* with Mitomed medium at 4 °C and stocked in this medium at 4 °C. The cells were cultured and kept in an atmosphere of 95% oxygen and 5% of air to avoid hypoxia.

The homogenates of glycolytic skeletal muscle, m. gastrocnemius as a reference tissue, were prepared in phosphate buffer (100 mmol/L KH_2PO_4, pH 8) from the muscle of adult rat by using a Polytron homogenizer. The suspensions were centrifuged at 6000*g* for 10 min and the supernatants were kept in this solution at 4 °C until the enzyme assays were started.

Laser confocal microscopy

For confocal microscopy, the HL-1 cells were detached by trypsin incubation and washed 5 times with Mitomed solution. These cells or CM were then incubated with a mitochondria-specific dye, MitoTracker Green FM (excitation

488 nm, emission 516 nm), which becomes fluorescent while accumulating in the lipid environment of mitochondria and is insensitive to membrane potential, and with a nucleus dye, Hoechst 342 (excitation 461 nm, emission 350 nm), which is a cell-permeable nucleic acid stain (DNA bound). The digital images of MitoTracker and Hoechst 342 fluorescence were acquired with a Leica DM IRE2 inverted confocal microscope with a 63× water immersion lens. The MitoTracker Green fluorescence was excited with the 488 nm line of argon laser, using 510–550 nm for emission.

Measurement of cytochrome content in different muscle cells

The HL-1 cardiac cells were thawed at 4 °C and rapidly disrupted in ice-cold PBS by vigorous vortex and Ultra-Turrax T25 homogenizator (Janke and Kunkel, Germany) at medium speed for 30 s. Insoluble material was removed by centrifugation at 2500*g* for 10 min. The supernatant was centrifuged at 15 000*g* for 30 min to remove myoglobin. The supernatant was discarded and the sediment was rehomogenized in 1.5 mL PBS. To obtain the cytochrome difference spectrum (reduced minus oxidized spectra), the mitochondria were solubilized by addition of 5% sodium deoxycholate (Sigma) into the cuvette to a final concentration of 0.3% (*v*/*v*), thereafter oxidized with potassium ferricyanide (reference spectrum), and then reduced with sodium dithionite in the same cuvette, as described previously (Fuller et al. 1985). Absorbance spectra of cytochromes were recorded by scanning the samples at 535–630 nm with a dual-beam spectrophotometer Lambda 900 (Perkin-Elmer) or with a Cary 50 Bio UV-Vis spectrophotometer (Varian, Palo Alto, USA). The cytochrome aa_3 content was calculated from the difference spectrum (reduced minus oxidized spectra) at the maximum absorption value in the range of 603–605 nm normalized for the absorbance of the isosbestic point at 630 nm; the content of cytochrome *c* was determined at the maximum absorption value of 550 nm normalized for the absorbance of the isosbestic point at 540 nm; the content of cytochrome *b* was calculated at the maximum absorption value of 562 nm normalized for the absorbance of the isosbestic point at 577 nm using the relevant extinction coefficient ε values according to Maguire et al. (1982). The same technique was applied to measure the cytochrome aa_3 content in CM and m. gastrocnemius. The data obtained were normalized for whole-cell or tissue protein content measured in the primary homogenates according to Bradford (1976) with bovine serum albumin (BSA) as a standard.

Determination of activity of the respiratory chain complexes

The activity of mitochondrial respiratory segments was measured in CM and B and NB HL-1 cells by using a high-resolution Oroboros oxygraph (Innsbruck, Austria). The cells were permeabilized in an oxygraph chamber containing the Mitomed solution with 25 μg/mL saponin and 2 mg/mL essential fatty acid-free BSA at 25 °C. Mitochondrial respiration was first activated by 5 mmol/L glutamate and 2 mmol/L malate via complex I in the presence of ADP in saturating concentration (2 mmol/L). Thereafter complex I was inhibited by rotenone (5 μmol/L) and complex II was activated by succinate (10 mmol/L), followed by inhibition of complex III by antimycin A (10 μmol/L). Finally the artificial substrates of complex IV, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) and ascorbate, were added (at 1 mmol/L and 5 mmol/L, respectively) to determine the activity of that complex.

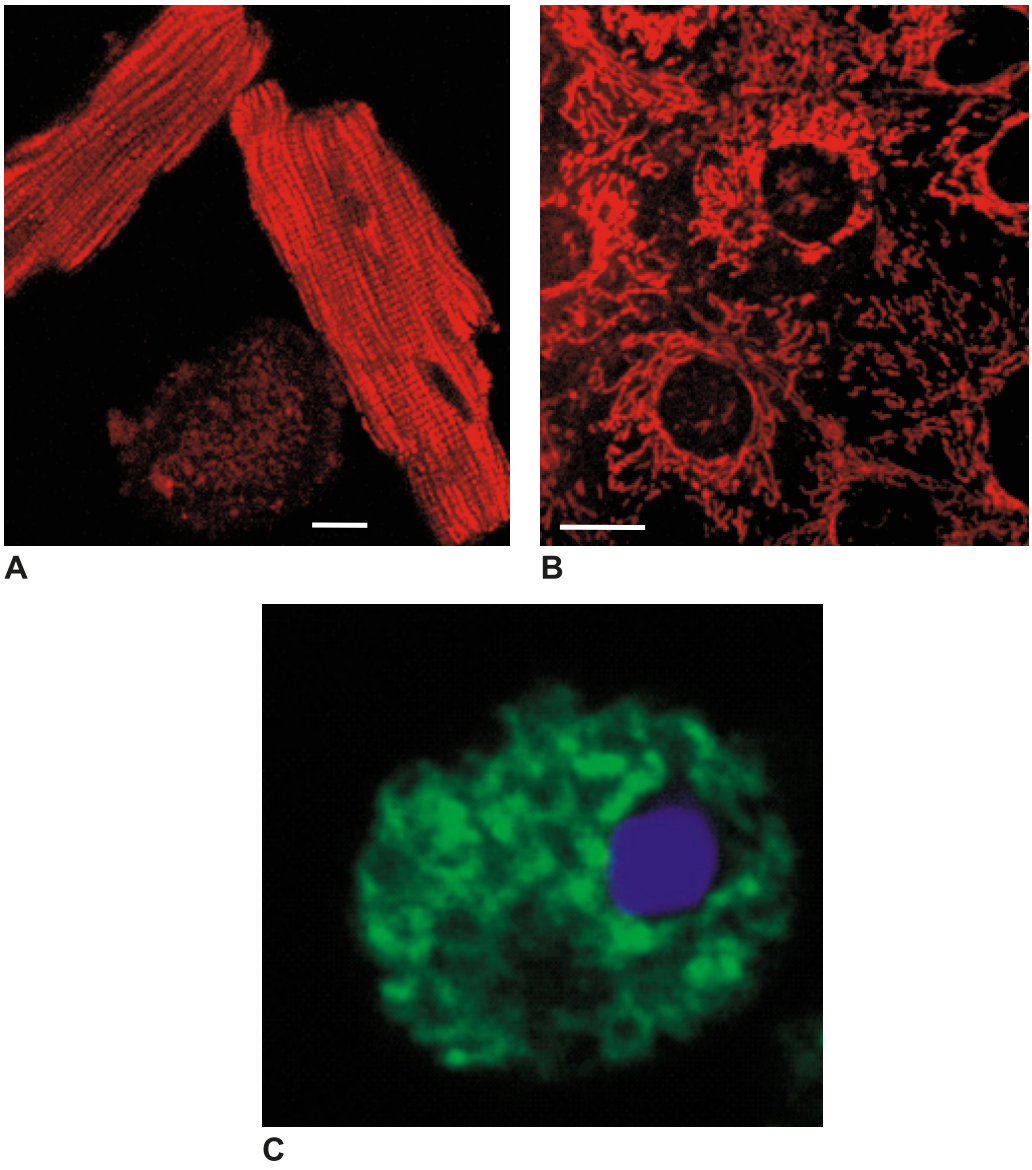
Assessment of enzyme activity

The enzyme activity in adult rat CM, NB HL-1 cells, and m. gastrocnemius homogenates was measured by the Cary 50 Bio UV-Vis spectrophotometer at 340 nm at 30 °C in the following basic buffer (BB) (in mmol/L): HEPES 50, magnesium acetate 5, postassium acetate 100, and EGTA 0.5; the pH was varied according to the optimum for the enzyme or coupled enzymes assessed, and 1% Triton X-100 was added to destroy the cellular membranes. The hexokinase (HK) activity was measured at pH 7.1 in BB supplemented with the following: 2 IU/mL glucose-6-phosphate dehydrogenase (G6PDH) and (in mmol/L) glucose 20, ATP 1.1, NADP 0.6, and P^1,P^5-di(adenosine-5′) pentaphosphate (Ap_5A) 0.01. Fructose-6-phosphokinase (FPK) activity was measured at pH 8.5 in BB supplemented with the following: 9.6 IU/mL lactate dehydrogenase (LDH), 5 IU/mL pyruvate kinase (PK), and (in mmol/L) phosphoenolpyruvate 0.4, fructose-1,6-diphosphate 0.64, fructose-6-phosphate 1.8, ATP 1.1, and NADH 0.2. The glyceraldehyde-3-phosphate dehydrogenase activity was assessed at pH 7.6 in BB additionally containing 15 IU/mL 3-phosphoglycerate kinase and (in mmol/L) 3-phosphoglycerate 6, ATP 1.1, and NADH 0.2. The PK activity was measured at pH 7.6 in BB containing 9.2 IU/mL LDH and (in mmol/L) phosphoenolpyruvate 6, ADP 1.2, and NADH 0.2. The LDH activity was assessed by registering the rate of NADH oxidation at pH 7.4 in BB containing (in mmol/L) pyruvate 10 and NADH 0.2. The CK activity was measured in the direction of ATP formation at pH 7.1 in BB supplemented with (in mmol/L) glucose 20, ADP 1.2, NADP 0.6, Ap_5A 0.01, and phosphocreatine 20, and with 2 IU/mL G6PDH and 40 IU/mL HK as the coupled enzymes.

Measurement of ATP and lactate/glucose ratio

For ATP measurement in cell lysates, the NB HL-1 cells were first incubated for 10 min in Tyrode's solution containing (in mmol/L) NaCl 150, KCl 5.4, NaH_2PO_4 0.9, $CaCl_2$ 1.8, $MgCl_2$ 1.2, HEPES 10, pH 7.2, adjusted with NaOH. This solution also contained 10 mmol/L glucose for control condition, or no glucose and 2 mmol/L 2-deoxyglucose to inhibit glycolysis. Then the cell plates were plunged into boiling water for 1.5 min, detergent solution was added (25 mmol/L Tris, 2 mmol/L EDTA, 0.5 mmol/L dithiothreitol (DTT), 0.1% Tween, pH 7.75 with acetic acid), and the plates were set to freeze at –80 °C. Thereafter the cells were thawed, sonicated, and scraped off on ice. The cell extracts were pipetted in precooled tubes, spun at 10 000*g* for 15 min at +4 °C. The ATP concentration was measured in supernatants using Prolux luciferase–luciferin kit (Euralam, France) in an Optocomp luminometer (MGM Instruments, Hamden, USA). For measurement of the lactate/glucose ratio, supernatants from 25 cm^2 culture flasks of NB HL-1 cells (10^7

Fig. 1. Confocal fluorescent imaging of mitochondria in normal CM in respiration medium (A) and in NB HL-1 cells in the culture medium (B). The mitochondria were visualized by cell incubation (20–30 min) with mitochondrial membrane potential-sensitive probe TMRM (100 nmol/L). Each fluorescent spot represents a mitochondrion. Scale bars, 10 μm. (C) NB HL-1 cells after detachment from the cell plates. Lighter grey shading is mitochondrial labelling with MitoTracker green (in the Web version, mitochondrial labelling appears as green fluorescence), and the darker grey shading is nuclear labelling with Hoechst 342 (in the Web version, this appears as blue fluorescence). CM, adult rat cardiomyocytes; NB HL-1, nonbeating mouse cardiomyocyte cell line; TMRM, tetramethylrhodamine methyl ester.



cells) kept for 24, 48, and 72 h of culturing in normal culture medium (supplemented Claycomb medium) were taken and incubated in the same medium containing 21 mmol/L glucose and 2 mmol/L lactate. The glucose and lactate concentrations were determined by using glucose-sensitive and lactate-sensitive CCX analyzer electrodes (Nova Biomedical, Waltham, USA). Glucose consumption and lactate production were calculated from the differences of their concentrations in samples and control medium.

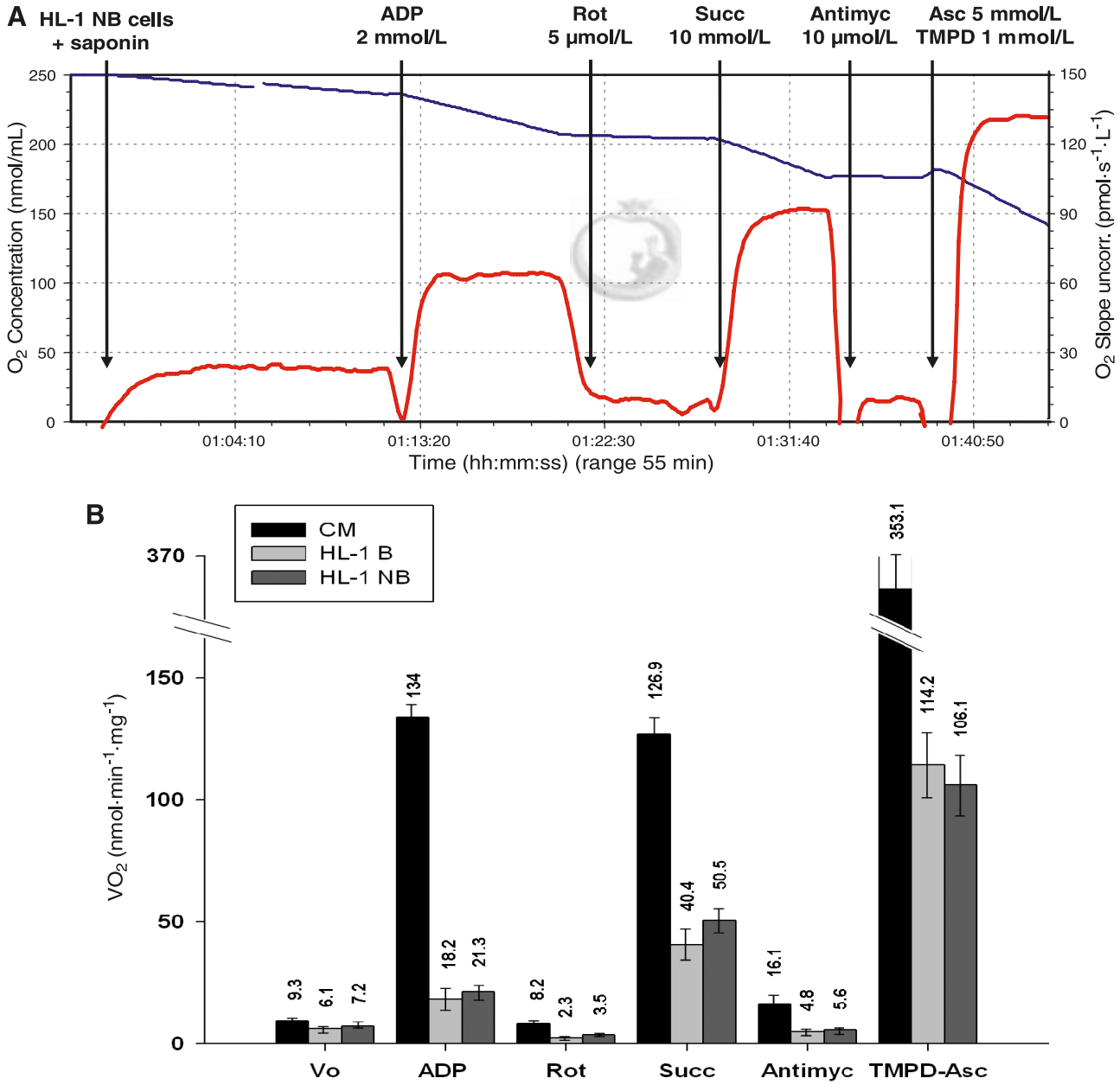
Statistical analysis

Means ± SE are presented. Statistical analysis of data was performed by one-way ANOVA with Bonferroni or Dunnett's post test.

Results and discussion

Figure 1 demonstrates that in contrast to CM, in which the mitochondria are regularly positioned between the myofibrils in a crystal-like manner, each at the level of the adjacent sarcomere (Fig. 1A), the mitochondria in NB HL-1 cells are chaotically organized within the cell interior, presenting the dynamically changing (from granular to filamentous) mitochondrial pattern (Fig. 1B). Figure 1C shows that after detachment of these cells by trypsinization they acquired a rounded shape. Previously we have shown that after saponin treatment to permeabilize the HL-1 cells, the mitochondria still remain within the cell interior despite marked deterioration of the cell membrane. From these experiments it was

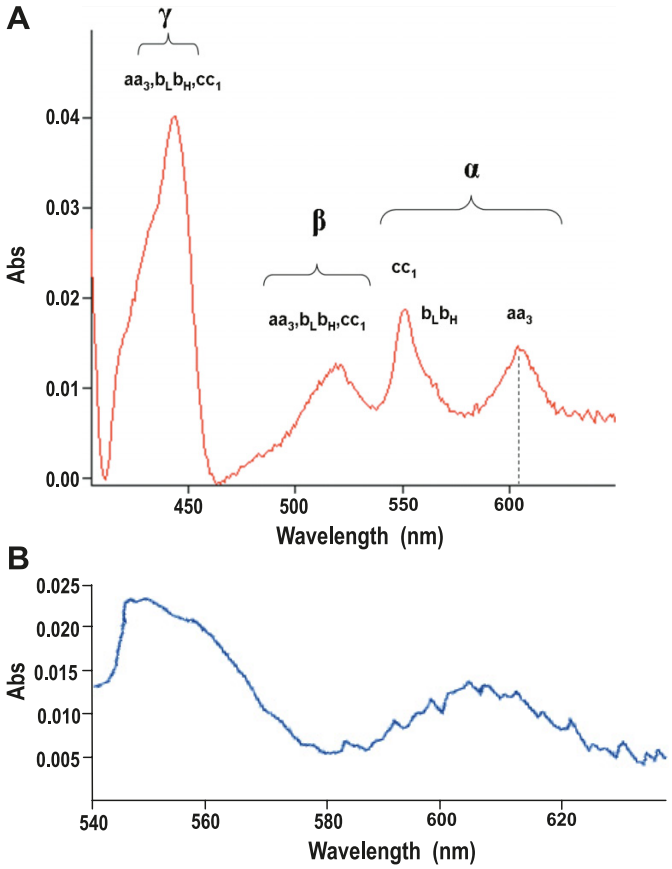
Fig. 2. (A) Original recording of the respiratory chain function in NB HL-1 cells permeabilized in Mitomed solution at 25 °C in the presence of 25 µg/mL saponin (plus 5 mmol/L glutamate and 2 mmol/L malate). Then 2 mmol/L ADP, 5 µmol/L rotenone (Rot), 10 mmol/L succinate (Succ), 10 µmol/L antimycin A (Antimyc), and 1 mmol/L TMPD with 5 mmol/L ascorbate (Asc+TMPD) were added to the oxygraph chamber. (B) The activity of the respiratory chain complexes in CM, B HL-1, and NB HL-1 cells, n = 5–7. B HL-1, beating HL-1 cell line.



also found that 15 min treatment of cells with saponin was optimal for selective permeabilization of the sarcolemma (Eimre et al. 2008).

Figure 2A shows the original recording of respirometric assessment of the activity of different complexes of the respiratory chain in NB HL-1 cells. Similar determinations for muscle and cardiac cells have been reported elsewhere (Eimre et al. 2008; Kuznetsov et al. 2008). Figure 2B demonstrates the mean levels of the respiratory data for CM and B and NB HL-1 cells. First, it can be seen that addition of 2 mmol/L ADP strongly activated respiration, thus showing effective control of oxidative phosphorylation by ADP in permeabilized HL-1 cells. Second, the respiration rate with complex I-dependent substrates was lower than with succinate, a complex II-dependent substrate, in NB HL-1 cells, whereas in CM the complex I-dependent respiration was close to that of the complex II-dependent one. These results, being in agreement with previous data (Eimre et al. 2008), suggest a relative deficiency in complex I in HL-1 cells, because in normal adult permeabilized cardiac cells the respiration rate with either glutamate or malate usually exceeds that with succinate (Seppet et al. 2005). Remarkably, the respiratory activities of the B and NB HL-1 cells were close to each other but about 4–8 times lower than in CM (Fig. 2B). Figure 3 demonstrates the cytochrome difference spectrum (reduced minus oxidized spectra) of isolated CM

Fig. 3. Original recordings of cytochrome spectra in the CM (A) and NB HL-1 cells (B).



(Fig. 3A) and HL-1 cells (Fig. 3B). Figure 3A shows the whole spectrum of cytochromes, while Fig. 3B shows only the α-peak area. From these spectra, the cellular content of cytochromes was calculated (Table 1). It can be seen that the content of cytochromes aa_3 is about 7 times less in HL-1 cells than in CM. Similarly, the cytochrome *c* content was much less in NB HL-1 cells than in adult CM (Table 1). Taken together these data allow us to conclude that the lower content of mitochondrial respiratory chains in HL-1 cells is responsible for their diminished respiratory capacity compared with CM (Fig. 2).

Determination of enzyme profiles (Table 2) revealed 5-fold elevated levels of the HK activity in the NB HL-1 cells. Also, the activity of PK was higher than in CM (Table 2). In general, the activities of glycolytic enzymes in HL-1 cells remained far less than in m. gastrocnemius cells. The activity of FPK was close to the activity of HK in the HL-1 cells, but lower than its activity in CM. However, FPK activity is known to follow sigmoid kinetics and is regulated allosterically by many intracellular factors (Drozdov-Tikhomirov et al. 1999), which is apparently favourable to keep the enzyme in HL-1 cells sufficiently active to maintain high glycolytic flux with glucose/lactate stoichiometry about 2, as shown in Fig. 4. Considering the effective transformation of glucose into lactate, the increased activity of several glycolytic enzymes, and the very low activity of oxidative phosphorylation, it appears that glycolysis may serve as the main energy-providing system in HL-1 cells. At the same time, the capacity of the glycolytic system to produce ATP is probably much lower in HL-1 cells than in glycolytic skeletal muscle (m. gastrocnemius), as indicated by comparison of the activity of glycolytic enzymes (Table 2). This difference may be explained by low energy requirements of HL-1 cells, since they do not contract because of the absence of sarcomeric structures (Pelloux et al. 2006).

Interestingly, our previous data have shown that unlike the case in adult CM, the HK in HL-1 cells effectively stimulates mitochondrial respiration, for it is bound to mitochondrial outer membrane that enables its coupling to oxidative phosphorylation (Eimre et al. 2008). The current study shows that this type of interaction between mitochondria and HK is ensured by very high activity of that enzyme. It is known that several cancer cell types overexpress HK (Mathupala et al. 2006; Gwak et al. 2005) and that in these cells HK is also coupled to oxidative phosphorylation so that mitochondrially produced ATP is preferably used for glucose phosphorylation by HK, whereas ADP liberated in that process is returned to mitochondria to stimulate ATP synthesis (Shinohara et al. 1997). It has been suggested that this mechanism allows the cancer cells to overcome potential inhibition of glycolysis due to lactate accumulation, particularly in conditions of hypoxia. Moreover, interaction of HK with mitochondria protects cells from apoptosis and excess reactive oxygen species production (Pastorino et al. 2002; da Silva et al. 2004) and provides cells with glycolytic intermediates as important components for different biosynthetic pathways, thus favouring proliferation of the cells and cancer growth (Mathupala et al. 2006, also reviewed by Seppet et al. 2007). In light of these data, our current results suggest that HL-1 are similar to cancer cells not only in their capacity for unlimited proliferation but also in their underlying mechanism: coupling glycolysis to oxidative phosphorylation, which helps to facilitate the glycolytic flux at the expense of mitochondrially produced ATP. These results are all consistent with and help to explain Warburg's original observation made in the 1920s that cancer cells are capable of increased lactate production under aerobic conditions (see Mazurek 2007 for a review).

Our earlier studies have revealed that permeabilized HL-1 cells exhibit much lower apparent K_m for ADP in regulation of oxidative phosphorylation (25 ± 4 μmol/L for NB and 47 ± 15 μmol/L for B) than that observed in adult permeabilized CM (360 ± 51 μmol/L) (Anmann et al. 2006). High K_m for ADP in CM stems from local restrictions of the ADP diffusion in the cells, including limited diffusion across the mitochondrial outer membrane (Saks et al. 2001; Seppet et al. 2001). This conclusion is substantiated by the observation that treatment of CM with trypsin leads to a 3-fold decrease in K_m value, along with dramatic changes in intracellular structure caused by this protease (Anmann et al. 2005, 2006). It is noticeable by their high apparent affinity to exogenous ADP that the HL-1 cells are similar not only to trypsin-treated adult CM but also to neonatal rat CM, which after permeabilization with saponin also exhibit very low K_m for ADP in stimulation of respiration (Tiivel et al. 2000). In addition, the neonatal CM are characterized by underdeveloped cellular structure, low capacity

Table 1. Cytochrome content in CM and in NB HL-1 cells.

Preparation	Cytochrome aa_3	Cytochrome b	Cytochrome cc_1
NB HL-1 cells	0.075±0.026*	0.096±0.036	0.051±0.01**
CM	0.5±0.16	0.45±0.17	0.49±0.1

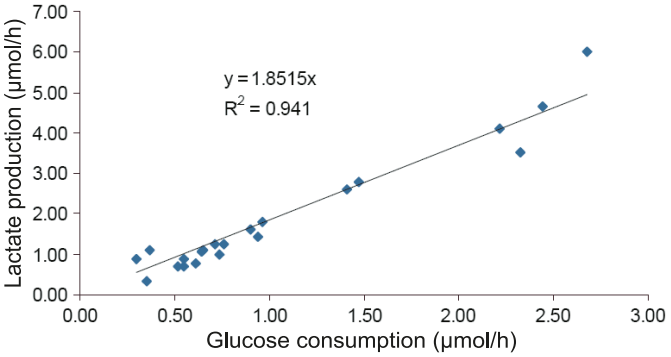
Note: Cytochrome content is given in nanomoles per milligram of cell protein (nmol/mg), n = 3–7. *, Significant at $p < 0.05$ and **, $p < 0.02$ compared with CM group.

Table 2. Enzyme activity of CM, NB HL-1 cells, and m. gastrocnemius homogenate (GH).

	CM	NB HL-1 cells	GH
Hexokinase	33±3*	148±12††	31±4
Fructose 6-phosphate kinase	698±23*	124±5††	1339±124
Creatine kinase	6327±724*	395±43††	83556±3866
Lactate dehydrogenase	4414±265	4069±449††	13985±800
Pyruvate kinase	637±69*	1375±187†	11571±722
Glyceraldehyde 3-phosphate dehydrogenase	2331±391	2693±490††	7511±701

Note: Activity is given in nanomoles per minute (nmol/min) per milligram of protein, n = 5–7. *, Significant at $p < 0.001$ vs. NB HL-1 cells. †, $p < 0.01$ and ††, $p < 0.001$ vs. GH.

Fig. 4. Lactate/glucose ratio represented by the slope of the correlation between the rates of lactate production and glucose consumption in NB HL-1 cells.



of oxidative phosphorylation, high rates of glycolysis, and absence of CK-mediated system of energy transfer compared with adult CM (Tiivel et al. 2000). Our recent data suggest that in the HL-1 cells, the CK-mediated energy transfer system is also not functional. Indeed, although creatine significantly activated the respiration and decreased the apparent K_m for ADP in normal adult CM, in HL-1 cells it exerted little, if any, influence because of the downregulation of mitochondrial CK and the absence of its coupling to oxidative phosphorylation (Anmann et al. 2006; Eimre et al. 2008). Likewise, the cytosolic CK isoforms were found not to be coupled to ATPases in HL-1 cells (Eimre et al. 2008). The current study explains these observations by extremely low activity of CK in these cells compared with CM (16-fold difference, as shown in Table 2), corresponding well to the marked downregulation of genes encoding mitochondrial and M and B isoforms of the CK at mRNA and protein levels in HL-1 cells (Eimre et al. 2008). On the basis of these data, it is evident that in contrast to CM in which the CK system represents the main mechanism of energy transfer, CK cannot play such a role in HL-1 cells.

Striking structural dissimilarities between the CM and HL-1 cells (Fig. 1) lead to the assumption that the mechanisms of interaction between the systems of producing and consuming ATP may be entirely different in these cells. Indeed, in contrast to normal CM, in which the intermyofibrillar mitochondria are strictly positioned at the level of sarcomeres, thus allowing formation of ICEUs, in HL-1 cells these complexes cannot be established because of the lack of sarcomeres (particularly in the NB subtype) and because of the continuous fusion and fission of mitochondria in HL-1 cells (Pelloux et al. 2006; Anmann et al. 2006). As a result of the less organized structure (loosely packed and even slowly moving mitochondria (Pelloux et al. 2006)), which leads to diminished restrictions for adenine nucleotides compared with those in CM, in HL-1 cells the mitochondria and ATPases may communicate via simple diffusion of adenine nucleotides, which is suggested by the low apparent K_m for ADP in regulation of respiration (Anmann et al. 2006). Another possibility is that the intracellular transfer of energy-rich phosphoryls is mediated by AK and HK, since the isoforms of these enzymes are strongly upregulated (this study and Eimre et al. 2008). The AK-mediated system of energy transfer is based on functional coupling of the mitochondrial isoform AK2 to adenine nucleotide translocase and the cytosolic isoform AK1 to ATPases (Dzeja and Terzic 2003). Regarding the role of HK, it is possible that because of its coupling to mitochondrial ATP synthesis, a situation is established in which glycolysis serves not only as a source of ATP, but also as a system of linear transfer of ATP from mitochondria to ATPases, with PK functioning as a terminal effector in local provision of ATP for ATPases (Dzeja and Terzic 2003). These modes of energy transfer may be sufficient to support the relatively low metabolic needs of HL-1 cells compared with those of CM.

In conclusion, the results of this study comply with the assumption that in contrast to CM in which oxidative phosphorylation is a predominant provider of ATP and the CK system is a main carrier of energy from mitochondria to ATPases, in HL-1 cells the energy metabolism is based primarily on the glycolytic reactions coupled to oxidative phosphorylation through HK.

Acknowledgements

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Review

Structure–function relationships in feedback regulation of energy fluxes in vivo in health and disease: Mitochondrial Interactosome

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ABSTRACT

The aim of this review is to analyze the results of experimental research of mechanisms of regulation of mitochondrial respiration in cardiac and skeletal muscle cells in vivo obtained by using the permeabilized cell technique. Such an analysis in the framework of Molecular Systems Bioenergetics shows that the mechanisms of regulation of energy fluxes depend on the structural organization of the cells and interaction of mitochondria with cytoskeletal elements. Two types of cells of cardiac phenotype with very different structures were analyzed: adult cardiomyocytes and continuously dividing cancerous HL-1 cells. In cardiomyocytes mitochondria are arranged very regularly, and show rapid configuration changes of inner membrane but no fusion or fission, diffusion of ADP and ATP is restricted mostly at the level of mitochondrial outer membrane due to an interaction of heterodimeric tubulin with voltage dependent anion channel, VDAC. VDAC with associated tubulin forms a supercomplex, Mitochondrial Interactosome, with mitochondrial creatine kinase, MtCK, which is structurally and functionally coupled to ATP synthasome. Due to selectively limited permeability of VDAC for adenine nucleotides, mitochondrial respiration rate depends almost linearly upon the changes of cytoplasmic ADP concentration in their physiological range. Functional coupling of MtCK with ATP synthasome amplifies this signal by recycling adenine nucleotides in mitochondria coupled to effective phosphocreatine synthesis. In cancerous HL-1 cells this complex is significantly modified: tubulin is replaced by hexokinase and MtCK is lacking, resulting in direct utilization of mitochondrial ATP for glycolytic lactate production and in this way contributing in the mechanism of the Warburg effect. Systemic analysis of changes in the integrated system of energy metabolism is also helpful for better understanding of pathogenesis of many other diseases.

1. General introduction

Quantitative analysis of complex systems of integrated energy metabolism needs the collection of vast amount of reliable experimental data and use of mathematical models for analysis and prediction of system behavior. Important data can be obtained by studies of intact cells and organs such as the heart, skeletal muscles or brain by using such methods as imaging, biochemical analysis, nuclear magnetic resonance, including saturation transfer and especially by isotope tracer method [1–3]. However, these methods usually give general information, not sufficient for revealing details of interactions between cellular components and for quantitative analysis of functional consequences of these interactions. This information can be easily obtained by permeabilized cell technique which application in combination with image analysis, kinetic methods and modeling is very useful and informative [4,5]. The aim of this review article is to describe and analyze experimental data obtained in our laboratories by this method in studies of the regulation of metabolic fluxes and respiration in muscle and brain cells, with main focus on the regulation of mitochondrial respiration in cardiac cells under normal physiological conditions when the heart function is governed by the Frank–Starling law [6,7]. We take advantage of the availability of the cells of cardiac phenotype with very different cellular organizations, such as adult isolated cardiomyocytes and cultured continuously dividing cancerous HL-1 cells [8]. Comparative studies of these cells gave us important information on the structure–function relationship in determining the mechanisms of regulation of respiration and integrated intracellular energy metabolism in cells [9,10]. Finally, we show that systemic analysis of the integrated

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cellular energy metabolism in the cells helps to understand pathogenetic mechanisms of several diseases, such as cancer and heart insufficiency.

2. Mitochondrial arrangement in adult cardiomyocytes versus HL-1 cells

By its nature, the contraction process needs very precise structural organization of sarcomeres of muscle cells [11]. Mitochondria in adult cardiomyocytes are located regularly at the level of A-band of sarcomeres, their positions are determined by their interactions with the cytoskeleton and the sarcoplasmic reticulum [12–17]. Recent studies have also shown that the precise, cell type-dependent organization of mitochondria, and numerous interactions between these organelles and other cellular structures, play a fundamental role in regulations of mitochondrial function [9,13,14,18–21]. In cardiac and skeletal muscles regularly arranged intermyofibrillar mitochondria [15] interact with other intracellular systems like the cytoskeleton and sarcoplasmic reticulum [12–14,17,22]. This type of organization provides a bioenergetic basis for contraction, recruiting cytoskeletal proteins, controlling both mitochondrial shape and arrangement in the cell within Intracellular Energetic Units [13,14]. Importantly, the mitochondrial interactions with various cytoskeletal proteins (desmin, vimentin, tubulin or plectin) have been suggested to be directly involved in the modulation of mitochondrial function [17–20,23–25]. Firm evidence for such a role has been found for tubulin (see below). These interactions control evidently also mitochondrial dynamics and movement of mitochondria in the living cells [26]. In our recent quantitative studies of these connections in adult rat cardiomyocytes and in cultured continuously dividing non-beating (NB) HL-1 cells with differentiated cardiac phenotype, mitochondria were stained with MitoTracker® Green and studied by fluorescent confocal microscopy [26]. High speed scanning (1 image every 400 ms) revealed very rapid fluctuation of positions of fluorescence centers of mitochondria but no mitochondrial fusion or fission in adult cardiomyocytes (Fig. 1F). These fluctuations followed the pattern of random walk movement within the limits of the internal space of mitochondria, probably due to transitions between condensed and orthodox configurational states of matrix and inner membrane [26]. In contrast, HL-1 cells with differentiated cardiac phenotype do not exhibit the strictly regular mitochondrial distribution typical for rat cardiac cells (Fig. 1A–E). In these cells, mitochondria can be heterogeneous, highly dynamic and motile, undergoing continual fission, fusion and fast intracellular displacements at a velocity of 0.1–0.2 μm/s [8,26]. Thus, mitochondrial fusion or fission was seen only in cancerous NB HL-1 cells (Fig. 1) but not in adult cardiomyocytes. The differences observed in mitochondrial dynamics are related to distinct specific structural organization and mitochondria–cytoskeleton interactions in these cells. It will be shown below that strikingly different intracellular organization and dynamics of mitochondria in adult cardiomyocytes and HL-1 cells are responsible for their remarkably different functional parameters.

3. Differences in the mechanisms of regulation of mitochondrial function in vitro and in vivo, factor X hypothesis

Rapid development of bioenergetics during the last 60 years was possible due to studies of mitochondria and their composition and components in an isolated state [27]. These studies resulted in establishing the Mitchell's chemiosmotic theory of oxidative phosphorylation and rotary mechanism of ATP synthesis, two fundamental bases of modern bioenergetics [27]. In the studies of metabolism in whole cells and organs, progress was made first by rapid freezing of tissue and subsequent biochemical analysis of extracts [28], and then by in vivo ^{31}P NMR spectroscopy [2,29]. These two methods when applied for studies of cardiac metabolism resulted in the discovery of the metabolic homeostasis of the heart, expressed as a constancy in concentrations of ATP, PCr and creatine, despite large variations in work load, in the myocardium [28,29]. Now the principal but unsolved question is whether one can explain the mechanisms of regulation of integrated energy metabolism of the cells in vivo by behavior of mitochondria in isolated state, in vitro? Very often, the answer a priori has been yes [30–35], leaving unanswered the question how intracellular organization and multiple interactions between cellular structures may influence the mechanisms of regulation of energy fluxes. Usually these interactions are simply ignored. However, theories based on simple extrapolation of data from studies on mitochondria in vitro fail in attempts to explain the metabolic homeostasis of cardiac cells under conditions of Frank–Starling law [7,10]. One of these simple theories, still very popular and actively used, assumes that mechanisms of regulation of mitochondrial respiration in vivo and in vitro by ADP are very similar; that mitochondria in vivo behave as in an homogenous medium and that cytoplasmic ADP is in equilibrium with the CK reaction [30–35]. Concentration of cytoplasmic ADP in equilibrium with CK reaction in heart cells under condition of metabolic stability is about 50–100 μM [36]. Taking into account that for isolated mitochondria the Km value for ADP is only 8–10 μM, one can see that no regulation of respiration by cytoplasmic ADP at its concentrations of 50–100 μM is possible, since adenine nucleotide translocator, ANT, is saturated by ADP and maximal respiration rate should always be observed. This is not the case, however, as it is known from classical studies in heart physiology that cardiac oxygen consumption increases linearly with elevations in workload (ATP hydrolysis) [6,37]. Moreover, under conditions of stable ATP, PCr and creatine concentrations, characteristic of energy metabolism of cardiac cells, the ADP concentration calculated from CK equilibrium should also be stable and should not respond to changes of respiration rate. In a similar way free Pi concentration and related parameters such as free energy of ATP hydrolysis (calculated from total metabolite contents and CK equilibrium) should not change under condition of metabolic stability. Thus, the main counter-arguments to the CK equilibrium theory – the phenomenon of metabolic stability of cardiac muscle and metabolic aspect of Frank–Starling law of the heart [29,37–40] – are uncontested. Veech et al. established the CK equilibrium experimentally only in the resting (noncontracting) muscle [41]. In the working muscle, creatine kinases within the PCr phosphotransfer network function mostly in a non-equilibrium state, especially at elevated workloads [7,42].

In order to explain the regulation of mitochondrial respiration under conditions of metabolic stability and at the same time assuming the CK equilibrium, the theory of parallel activation by Ca^{2+} was proposed and continues to be supported [43–47]. According to this theory, the increase of cytoplasmic Ca^{2+} during excitation–contraction coupling cycle activates ATP hydrolysis in myofibrils, and simultaneously three dehydrogenases of Krebs cycle in mitochondrial matrix increasing production of NADH and $FADH_2$ by push mechanism. The oxidation of the latter increases electron flow through the respiratory chain, generates the protonmotive force and drives ATP synthesis [27,48]. Ca^{2+} is thought also to activate directly F_1F_O-ATPase and complex I [43,49–52] However, the parallel activation theory still does not fit with the requirement for the main signal of coordination of energy metabolism in cardiomyocytes recently formulated by O'Rourke [48]. According to O'Rourke's principles, the variations of cytoplasmic [Ca^{2+}] have to correspond to changes in workload, ATP consumption and respiration. This condition is not fulfilled, because the intracellular Ca^{2+} transients do not change during the length-dependent activation of sarcomere (mechanism on which is based the Frank–Starling's law) [53–55]. Thus, the Frank–Starling mechanism puts into question the viability of the theory of parallel activation of contraction and respiration by Ca^{2+}. Regulation of respiration by Ca^{2+} seems to explain the adrenergetic activation of oxidative phosphorylation [43,47,49,52,56–

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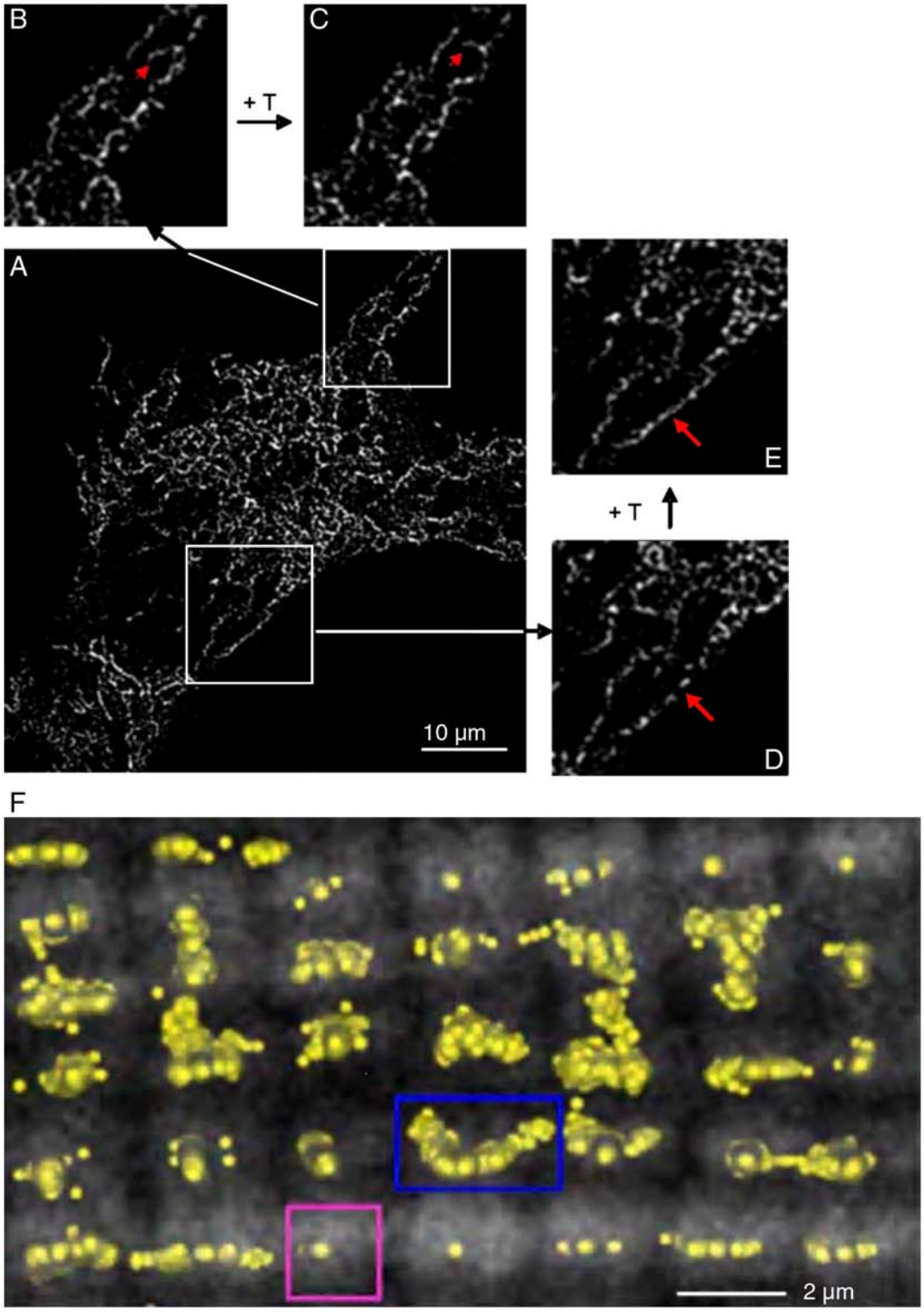

Fig. 1. A Enhanced confocal images of the mitochondrial network in NB HL-1 cells. Mitochondria were stained with MitoTracker® Green (in white). In details B, C, D and E, modifications of mitochondria network as a function of time *t* are depicted. Indeed, mitochondria are very dynamic undergoing continual fission and fusion events usually forming long and rapidly moving filament-like structures. (F) Visualization of the positions of mitochondrial fluorescent (mass) centers in a cardiomyocyte over a long time (total duration 100 s) of rapid scanning: movements of fluorescence centers are limited within internal space of mitochondria. Positions of the fluorescence centers were stacked as a function of time. These fluorescence centers (which are assimilated to the center of mitochondria in cardiomyocytes) are shown as small yellow spheres. The position of fluorescent centers was superimposed with a reference confocal image of MitoTracker® Green fluorescence (in grey) showing mitochondrial localization. Note that the fluorescence centers are observed always within the space inside the mitochondria, but from mitochondrion to mitochondrion the motion pattern may differ from very low amplitude motions (pink frame) to wider motions distributed over significant space but always within the internal space of a mitochondrion (blue frame). Reproduced from Beraud et al. with permission [26].

63], but not the feedback regulation of respiration by workload changes during cardiac contraction under physiological conditions of action of Frank–Starling law [7,64].

To find a solution to this important problem of metabolic studies, an application of the principles of Systems Biology is very helpful. One of the main principles of Systems Biology is that interactions between

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system's components lead to new system level properties which are absent when the components are isolated and which explain the mechanisms of functioning of the system, its biological function [10,65–67]. The permeabilized cell technique, in combination with kinetic analysis, mathematical modeling and whole cell and organ studies mentioned above is one of the principal methods of Molecular System Bioenergetics [10,67].

4. In vivo kinetics of regulation of respiration, central role of MtCK

Kümmel was first to apply the permeabilized cell technique for studies of cardiac energy metabolism [68] and to discover that the quantitative characteristics of regulation of mitochondrial respiration – apparent Km for exogenous ADP – are very different in vitro and in permeabilized cardiomyocytes in situ. The latter exceeds the former by order of magnitude, showing significantly decreased affinity of mitochondrial respiration for exogenous ADP in vivo. This result was then confirmed in very many laboratories (Table 1). Detailed studies of this phenomenon in our laboratories led to the conclusion that it is the result of an interaction of mitochondria with cytoskeletal components, resulting in restricted permeability of the voltage dependent anion channel VDAC in mitochondrial outer membrane for ADP and also related to the specific structural organization of the cell by the cytoskeleton resulting in regular arrangement of mitochondria with surrounding structures and MgATPases into Intracellular Energetic Units (ICEUs) in adult cardiomyocytes and oxidative muscle cells [13]. High apparent Km for exogenous ADP in permeabilized cells is tissue specific, observed for cardiac fibers and isolated cardiomyocytes even after extraction of myosin (ghost fibers), and in fibers from slow twitch oxidative (but not fast twitch) glycolytic skeletal muscle, its value is high also in permeabilized hepatocytes and synaptosomes (Table 1). This tissue specificity excludes any possibility of explaining high Km values for ADP by long diffusion distance or specific effects of saponin, a detergent used for permeabilization [4,5], sometimes proposed in literature and critically already analyzed before [69]. The value of this parameter decreases significantly after treatment of cells with a small amount of proteolytic enzymes in a low concentration, showing the role of some proteins sensitive to this treatment, in control of mitochondrial responses to ADP in vivo (Table 1). This hypothetical protein was given the name “Factor X” and assumed to be separated from mitochondria during isolation procedure [70], as shown in Fig. 2. The initial hypothesis was that this protein controls the permeability of outer mitochondrial membrane for ADP [70]. Thus, the high apparent Km for exogenous ADP in regulation of mitochondrial respiration is a system level property, depending on the interaction of mitochondria with other cellular structures. Comparison of isolated mitochondria, permeabilized cardiomyocytes and permeabilized HL-1 cells confirms this conclusion and shows directly the dependence of the mechanism of regulation of respiration upon structural organization of the cell. Fig. 3 shows that in isolated cardiomyocytes, where the mitochondria are fixed, with very regular “crystal-like” arrangement at the level of sarcomeres, and where no fusion into reticular structures is possible and only configuration of mitochondrial inner membrane changes rapidly (Fig. 1F) apparent Km for exogenous ADP is very high, in agreement with the data in Table 1. In contrast, in permeabilized HL-1 cells, where the mitochondria are very dynamic and undergo continuous fusion and fission (Fig. 1A–E), the apparent Km for exogenous ADP is very low and close to that in isolated mitochondria [9].

Table 1
Apparent K_m(ADP) for exogenous ADP in regulation of respiration in permeabilized cells and fibers from different tissues with or without creatine or trypsin treatment.

Preparation	K_m^{app}ADP, μM	K_m^{app}ADP (+Cr), μM	K_m^{app}ADP, μM, after treatment with trypsin	References
Heart tissue homogenate	228 ± 16		36 ± 16	[191]
Cardiomyocytes	329 ± 50			[9]
	250 ± 38	35.6 ± 5.6		[192]
	200–250			[23]
“Ghost” cardiomyocytes	200–250			[23]
Skinned cardiac fibers[a]	297 ± 35	85 ± 5		[193]
	260 ± 50	79 ± 8		[194]
	300 ± 23			[13]
	300–400			[14]
	277 ± 40	102 ± 35		[195]
	370 ± 70			[196]
	234 ± 24			[197]
	324 ± 25			[197]
	320 ± 36		83 ± 22	[191]
“Ghost” cardiac fibers	349 ± 24			[193]
	315 ± 45	85 ± 5		[198]
Permeabilized hepatocytes	275 ± 35			[199]
Synaptosomes	110 ± 11	25 ± 1		[88]
Skinned fast twitch skeletal muscle fibers	7.5 ± 0.5			[200]
	8–22			[198]
Rat heart isolated mitochondria	17.6 ± 1.0	13.6 ± 4.4		[194]
	13.9 ± 2.6		17.6 ± 1	[191]
Rat brain isolated mitochondria	9.0 ± 1.0			[88]

[a] The value of this parameter, apparent Km for exogenous ADP, is always equally high in permeabilized isolated cardiomyocytes and in skinned cardiac fibers. The equality of Km (ADP) in these two types of permeabilized preparations is always a necessary criterion for evaluation of quality of preparation when skinned fibers are used [4,5,9,191,193,194]. In opposite cases, as sometimes published in literature ([204], for critical review see [69]), the preparations are damaged, but there is no reason to blame the technique of skinned fibers so well used in many laboratories, for failures of authors to use it correctly [204].

Further important information was obtained when mitochondrial creatine kinase, MtCK was activated by creatine. These experiments confirm and clearly demonstrate that the role of mitochondrial outer membrane in adenine nucleotides compartmentation and the functional coupling between MtCK and ANT is much more important in vivo where the mitochondria interact with intracellular surrounding environment forming the unitary structure–functional organization of energy metabolism — Intracellular Energy Units (ICEUs) [13]. During permeabilization the cytoplasmic soluble enzymes not bound to the structures such as many glycolytic enzymes and MM-CK are released into the solution, but MtCK stays in the mitochondrial intermembrane space firmly fixed by lysine–cardiolipin interactions at the outer surface of inner membrane in the vicinity of ANT [71,72]. Table 1 shows that the addition of creatine significantly decreases the apparent Km for exogenous ADP by increasing the rate of recycling of ADP in mitochondria in MtCK and oxidative phosphorylation reactions functionally coupled via adenine nucleotide translocator, ANT.

One of the most fruitful approaches in these studies was a demonstration of the role of local ADP concentrations in activating oxidative phosphorylation by applying the powerful ADP trapping system consisting of high activities of pyruvate kinase, PK, and phosphoenolpyruvate, PEP, capable of capturing and phosphorylating all ADP in soluble phase of the cytoplasm or medium equilibrated with it. Fig. 4 demonstrates how this system was used in recent experiments [73]. In the presence of PK–PEP system ADP produced in MgATPase and myofibrillar MM-CK reactions is trapped and rephosphorylated into ATP, and respiration is activated only due to local ADP produced in the MtCK reaction (Fig. 4A). Fig. 4B shows that under these conditions MtCK maximally activates respiration and PK–PEP are not able to trap the ADP recycling in mitochondrial coupled reactions behind the outer mitochondrial membrane (MOM). It was shown in parallel experiments that in the in vitro system containing isolated mitochondria the PK–PEP system can trap significant amount of ADP produced by MtCK [73]. These remarkable

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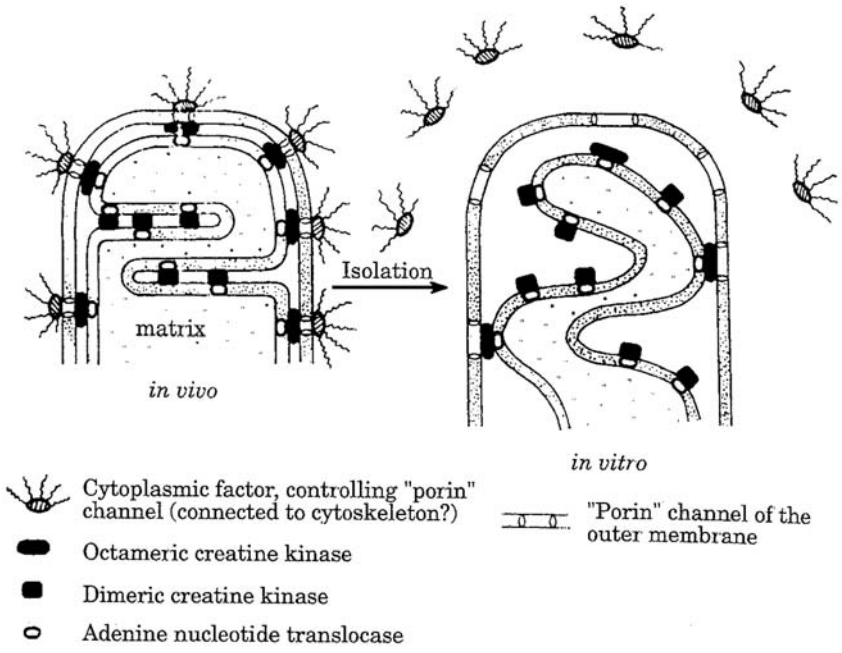

Fig. 2. Original hypothesis of the connection of mitochondrial outer membrane (MOM) and cytoskeleton in vivo, where the outer membrane VDAC channel ("porin") was assumed to be controlled by some cytoplasmic factor "X", which is lost during mitochondrial isolation, and therefore the (MOM) becomes in vitro absolutely permeable for ADP. Reproduced from Saks et al. with permission [70].

differences between mitochondria in vivo and in vitro were seen again in kinetic experiments described in Fig. 4C–E. After permeabilization, MgATP was first added to activate the intracellular MgATPases producing endogenous ADP that stimulates respiration (Fig. 4C). The PK–PEP system was then added to trap this endogenous ADP with an expected decrease in respiration rate. Finally creatine was added stepwise to study the role and kinetics of the MtCK reaction in regulation of respiration in vivo in a situation modeling some characteristics of intracellular milieu. Most remarkably, creatine addition to permeabilized cardiomyocytes rapidly activates respiration up to a maximal value (Fig. 4C). Results of the kinetic analysis of this action are described below. However, when isolated heart mitochondria were used, creatine addition increased respiration rate to only about half of its maximal value (Fig. 4D) meaning half of ADP produced by MtCK leaked out through the mitochondrial outer membrane and was trapped by the PEP–PK system. Table 2 shows that the respiratory parameters — maximal respiration rates in the presence of ADP or MgATP + creatine are similar in isolated heart mitochondria and permeabilized cardiomyocytes when calculated per cytochrome aa_3 content. Thus, the effect of PEP–PK on the respiration of isolated mitochondria is due to the leak of ADP via mitochondrial outer membrane. Experimental studies of kinetic properties of MtCK in isolated cardiac mitochondria and mathematical modeling of these properties [74–77] showed that they are dependent on functional coupling of MtCK with oxidative phosphorylation via ANT. The apparent constant of dissociation of ATP from its tertiary complex with MtCK decreases 10 times in the presence of activated oxidative phosphorylation [78]. This strong affinity of MtCK for ATP disappears when MtCK is detached from mitochondrial membranes [79]. Mathematical modeling of these effects showed that it is explained by direct channeling of ATP from ANT to MtCK [74,80]. However, ADP produced by MtCK can either be taken back into the matrix by ANT or leave intermembrane space if the outer membrane is easily permeable (see Fig. 4A). This is observed in isolated mitochondria (Fig. 4D) but not in permeabilized cardiomyocytes (Fig. 4B and C). Fig. 4E shows that in permeabilized fibers from human skeletal muscle m. vastus lateralis MtCK also effectively regulates respiration. In similar experiments with permeabilized fibers from biopsy samples of human m. vastus lateralis the apparent Km for exogenous ADP was found to be high but decreased significantly in the presence of creatine [81,82], supporting the conclusion that in oxidative skeletal muscles the ADP diffusion into mitochondrial intermembrane space is restricted. These reliable experimental results contradict the theoretical conclusion of mitochondrial ADP ultrasensitivity in these muscles made by Jeneson et al. [32] from analysis of PCr recovery after exercise on the basis of the assumption of CK equilibrium: experimental value of apparent Km for ADP was close to 200 μM [81] while theoretical calculations gave 22 μM [32]. Once again, the assumption of the CK equilibrium is not sufficient to explain the experimental data.

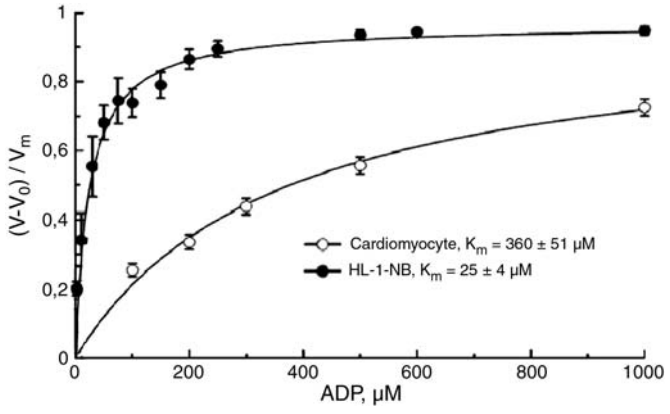

Fig. 3. Different kinetics of regulation of the respiration in permeabilized adult cardiomyocytes and non-beating (NB) cardiac tumoral HL-1 cells. For normalisation, the respiration rates were expressed as fractions of the maximal rates, Vmax, found by analysis of experimental data in double-reciprocal plots. Reproduced from Anmann et al. with permission [9].

To assess the role of structural organization in determining the mechanisms of regulation of oxidative phosphorylation, we have taken advantage of the comparison of cardiomyocytes from an adult heart with HL-1 cell culture developed from mouse atrial cardiomyocytes and expressing cardiac phenotype [8,83]. The HL-1 cells are characterized by entirely different kinetics of regulation of respiration

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by ADP (see Fig. 3). Moreover, in the permeabilized HL-1 cells the creatine effect on respiration was not observed (Fig. 4F). It was recently shown that MtCK is not expressed in these cells, but only the BB isoform of CK can be seen, in contrast to rat heart cells, where BB isoform is in trace amounts and the major forms are MtCK and MM-CK isoforms, and also some amount of hybrid form MB is seen [84]. Remarkably, in both cells two hexokinase isoforms are present [84]. Measurements of enzyme activities in both these cells showed that CK activity is manifold decreased in HL-1 cells as compared to cardiomyocytes, while the activity of hexokinase is significantly increased in HL-1 cells (Fig. 5). Significance of these findings for cancer cell bioenergetics is discussed below.

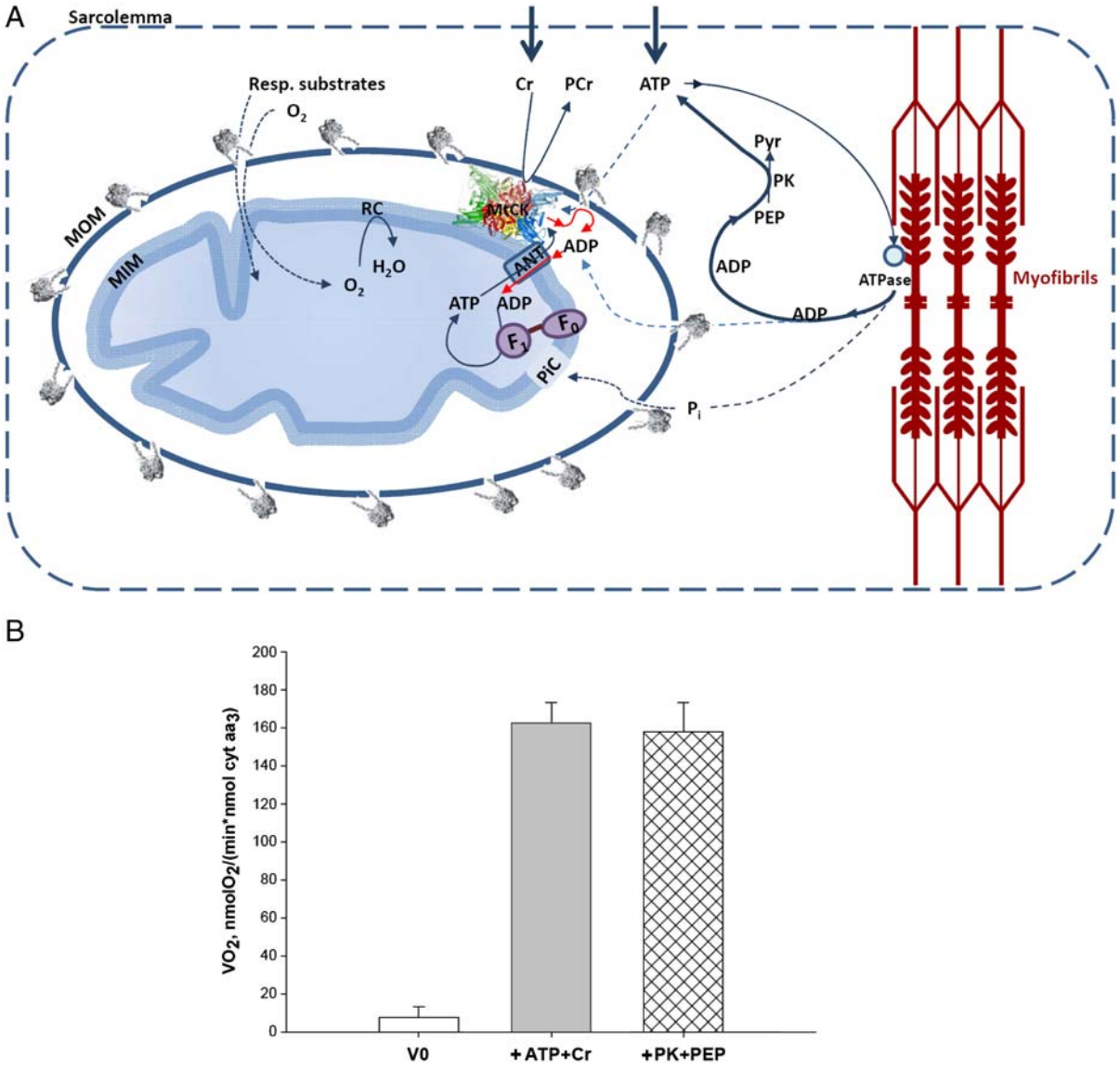

Fig. 4. (A) The scheme represents a mitochondrion in situ, in a permeabilized cardiac cell. The mitochondrial outer membrane (MOM) is less permeable than in isolated mitochondrion, due to the interactions of VDAC with cytoskeleton protein-tubulin. Exogenous ATP is hydrolyzed by cellular ATPases into endogenous extramitochondrial ADP and inorganic phosphate (Pi). Mitochondrial (MtCK) and non-mitochondrial MM creatine kinases (cytosolic, myofibrillar, SERCA, and sarcolemmal) activated by creatine in the presence of ATP, produce endogenous intra- and extramitochondrial ADP. The system is supplemented with phosphoenolpyruvate (PEP) and pyruvate kinase (PK) which remove extramitochondrial ADP and continuously regenerates extramitochondrial ATP. Intramitochondrial ADP produced by MtCK forms microcompartments within the intermembrane space (IMS) and is re-imported into the matrix via adenine nucleotide translocase (ANT) due to its functional coupling with MtCK. Reproduced from Guzun et al. [73] with permission. (B) Respiration rates of permeabilized cardiomyocytes. Respiration was activated by addition of MgATP (5 mM) and creatine (20 mM) resulting in activation of the MtCK reaction with local production of intramitochondrial ADP. Addition of 20 IU/mL PK in the presence of PEP (5 mM added into medium before) did not change significantly the respiration rate because of the inaccessibility of compartmentalized, in mitochondrial intermembrane space, ADP for PK–PEP system. (C) The experimental procedure used for complete kinetic analysis of MtCK in mitochondria in situ (permeabilized cardiomyocyte). First, addition of MgATP induces production of endogenous ADP in MgATPase reaction. Secondly added PEP–PK trap all extramitochondrial free ADP inducing decrease of respiration rate, but not to initial level, due to structural organization of ICEU. Under this conditions addition of creatine in different amounts rapidly activates the MtCK reaction. The oxidative phosphorylation is stimulated mostly by intramitochondrial ADP, produced by MtCK reaction, which is not accessible for PEP–PK. Adapted from [73]. (D) Respiration rates of isolated mitochondria stimulated by increasing amounts of creatine in the presence of ATP (i.e. activated MtCK reaction) and the absence of extramitochondrial ADP (consumed by the PEP–PK reaction). Adapted from [73]. (E) Respiration of permeabilized fibers from human skeletal m. vastus lateralis prepared from biopsy samples of healthy volunteers in the presence of 2 mM malate and 5 mM glutamate as substrates. Addition of 2 mM MgATP activates respiration due to production of endogenous MgADP in ATPase reaction. Pyruvate kinase (PK) in the presence of 5 mM phosphoenolpyruvate (PEP) decreases respiration rate due to removal of extramitochondrial MgADP. Creatine in the presence of MgATP activates mitochondrial creatine kinase (MtCK) reaction of production of endogenous intramitochondrial MgADP which rapidly activates respiration up to the maximal rate, that showing that mitochondrial ADP is not accessible for PK–PEP system due to the limited permeability of mitochondrial outer membrane in the cells in situ. Reproduced from Cherpec thesis [201] with permission. (F) The absence of the stimulatory effect of creatine on the respiration rate of non-beating (NB) cardiac tumoral HL-1 cells in the presence of PEP–PK system under conditions described above. Recorded respiratory rate is due to the stimulatory effect. Adapted from [9].

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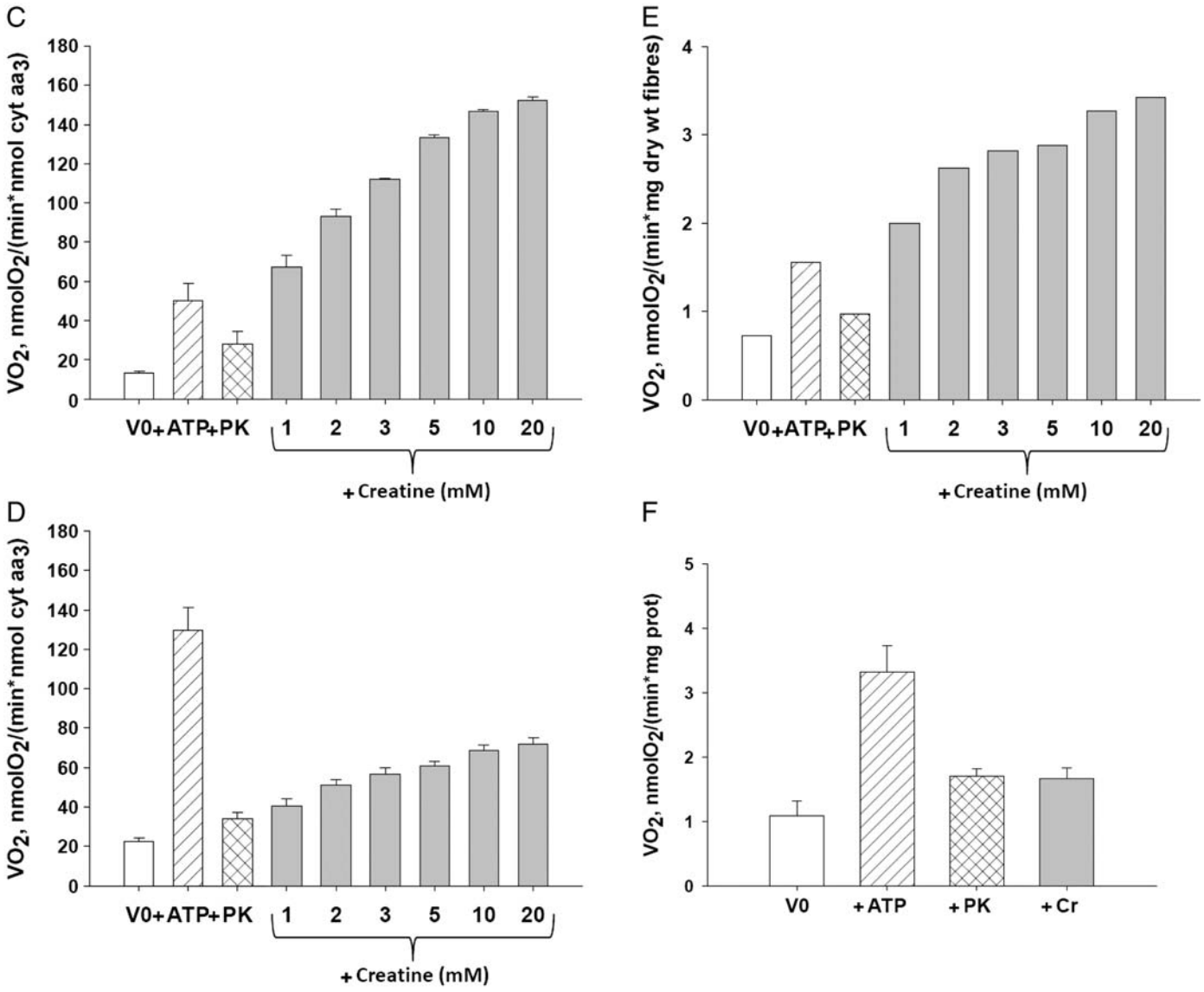

Fig. 4 (*continued*).

5. Mitochondrial–cytoskeletal interactions, heterodimeric tubulin as factor X

Many experimental evidences discussed above point to the role of some cytoskeleton protein, factor "X" in regulating MOM permeability for adenine nucleotides. One of the real candidates for this role is tubulin. Carre et al., by using the immunoprecipitation method showed the association of tubulin with VDAC [85]. Appaix et al. demonstrated that selective proteolytic treatment of permeabilized cardiomyocytes by trypsin in low concentration, which decreases apparent Km for exogenous ADP, also results in the almost complete disappearance of immunolabeling of tubulin [19]. These results are reproduced in Fig. 6. Very recently, functional interaction of tubulin with VDAC was revealed by applying biophysical and oxygraphic methods by Rostovtseva et al. [86,87] and Monge et al. [88]. In experiments with VDAC reconstituted into planar phospholipid membranes the reversible voltage dependent partial blockage of channel by dimeric tubulin (in nanomolar concentration insufficient for polymerization and in the absence of GTP and Mg^{2+}) was observed. Under similar experimental conditions but without tubulin VDAC remains open up to 1 h [87]. Rostovtseva et al. proposed the model for tubulin–VDAC interaction in which the negatively charged C-terminal tail of tubulin penetrates into the channel lumen due to the interaction with a positively charged domain of VDAC close it [87].

The role of interaction of tubulin with VDAC in regulation of mitochondrial oxidative phosphorylation was studied directly by recording the respiration rates of isolated brain and heart mitochondria stimulated by exogenous ADP in the absence and presence of heterodimeric tubulin (Fig. 7). As was expected, the apparent Km for free ADP in isolated mitochondria was about 10–20 μM. In the presence of 1 μM tubulin the sensitivity of mitochondria for free ADP decreased: apparent K_m^{app} increased to ~170 μM for the brain and ~330 μM for the heart mitochondria, respectively [87,88]. Creatine addition effectively decreased the apparent Km for ADP again (Fig. 8).

These studies allowed the factor X to be finally identified as the heterodimeric tubulin [86,87].

The effect of creatine and kinetic analysis of system described in Fig. 4C showed that VDAC cannot be completely closed in vivo. We analyzed the kinetics of respiration regulation by creatine and ATP in permeabilized cardiomyocytes in situ when respiration was stimulated by intramitochondrial ADP produced in MtCK reaction and extramitochondrial ADP was continuously consumed by the PEP–PK system (see Fig. 4A–C). These experiments modeled an interaction of mitochondria with glycolytic system in vivo. The apparent kinetics of

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Table 2
Basic respiration parameters of isolated rat heart mitochondria and of mitochondria in situ in permeabilized cardiomyocytes. V_0 — respiration rate in State 2 in the presence of substrates before addition of ADP or ATP; V_3 — respiration rate in the presence of 2 mM ADP; $V_{Cr,ATP}$ — respiration rate in the presence of activated MtCK by 2 mM ATP and 20 mM creatine. Reproduced from [73] with permission.

Parameter	Mitochondria in vitro	Mitochondria in situ (permeabilized cardiomyocytes)
V_0, nmol O_2 min^{-1} mg $prot^{-1}$	26.37 ± 7.93	7.53 ± 1.61
$V_{3\ (2\ mM\ ADP)}$, nmol O_2 min^{-1} mg $prot^{-1}$	187.94 ± 40.68	84.45 ± 13.85
[Cyt aa_3], nmol mg $prot^{-1}$	1.00 ± 0.012	0.46 ± 0.09
$V_{3\ (2\ mM\ ADP)}$, nmol O_2 min^{-1} nmol cyt aa_3^{-1}	188 ± 39.93	178.23 ± 33.96
$V_{Cr,ATP}$, nmol O_2 min^{-1} nmol cyt aa_3^{-1}	197.90 ± 31.86	162.63 ± 26.87

the MtCK dependent respiration regulation was found to be totally different from that seen in mitochondria in vitro [73]. In fact, there are three remarkable differences. First is the decrease in apparent affinity of MtCK for exogenous MgATP (apparent Ka increased more than 100 times, Table 3) in mitochondria in situ as compared to in vitro. Second, the apparent constant of dissociation of creatine from the binary complex with MtCK (Kib) decreases about 10 times in mitochondria in situ, in permeabilized cardiomyocytes, as compared with isolated mitochondria. Third, the apparent affinity of MtCK for PCr is similar in vitro and in situ in permeabilized cells (Kip is about 1 mM) (Table 3). The decreased apparent affinity of MtCK in situ for extramitochondrial MgATP can be most probably due to the enhanced restriction of diffusion at the level of MOM (i.e. limited VDAC permeability) which induces the increase of adenine nucleotide micro-compartmentation within mitochondrial intermembrane space influencing the respiratory control of oxidative phosphorylation. The remarkably high affinity of MtCK in mitochondria in situ for creatine and PCr points to the absence of restriction of diffusion of these guanidino substrates across MOM into intermembrane space where MtCK is located. Direct measurements of energy fluxes from the mitochondria into the surrounding medium (measured using high performance liquid chromatography (HPLC) for the same experiments) showed stable MgATP concentration though the experiment and progressive increase of PCr concentration was dependent on a stepwise increase in creatine concentration. The PCr/O_2 ratio was equal to 5.7 and close to the theoretical maximal P/O_2 ratio under conditions similar to those in vivo [89]. These results show that PCr is the main energy flux carried out from mitochondria in permeabilized cardiomyocytes.

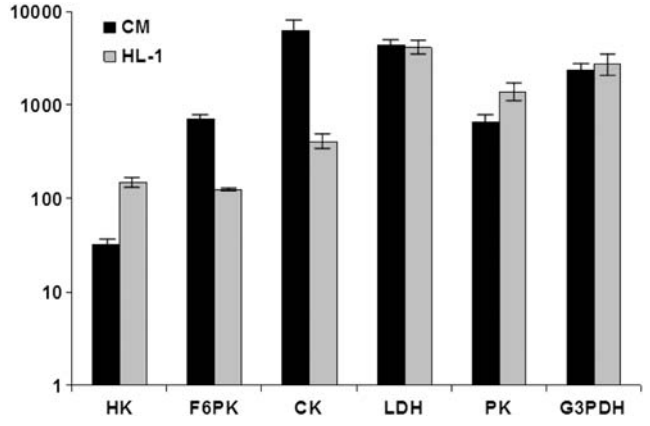

Fig. 5. Enzyme activity profile in rat adult cardiomyocytes (CM), in non-beating HL-1 cells. Hexokinase (HK), fructose-6-phosphokinase (F6PK), lactate dehydrogenase (LDH), pyruvate kinase (PK), glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and creatine kinase (CK) activities were measured and represented on a logarithmic scale. The means are presented ± SD. Adapted from [202].

The selective regulation of barrier functions of MOM by cytoskeleton and functional coupling of MtCK with ANT is highly important for the structural and functional organization of energy metabolism and regulation of effective exchange of phosphoryl groups between two systems of recycling metabolites. ATP/ADP recycling is restricted mostly to mitochondrial intermembrane and matrix spaces, while Cr and PCr are recycled between mitochondrial intermembrane and cytoplasmic spaces, resulting in energy transport from mitochondria into cytoplasm by freely diffusible PCr molecules via the system of compartmentalized CK reactions (Fig. 8). This figure shows the scheme of PCr/CK shuttle or circuit in the brain, heart and skeletal muscle cells, in details described elsewhere [7,10,21,42,70–73,90]. The coupling between two metabolic cycles is realized in mitochondria by a supercomplex which we called Mitochondrial Interactosome, MI (Fig. 9). This complex is formed by ATP synthasome (a term proposed by Pedersen [91–94] and constituted by ATP synthase, ANT and Pi carrier) functionally coupled to MtCK [10,42,75,76,86,89,95–97], and VDAC with tubulin and some other regulatory proteins (Fig. 9) [86,89]. This unit can also include the super complex formed by the respiratory chain [98,99]. The role of Mitochondrial Interactosome is to ensure continuous recycling of adenine nucleotides in mitochondria, their transphosphorylation and metabolic channeling of ATP via ANT to MtCK, and back ADP, resulting in the export of free energy from mitochondria into cytoplasm as flux of PCr. The functioning of this complex structure is best explained by the theory of vectorial metabolism and the vectorial ligand conduction, proposed by P. Mitchell [100]. Initially, this theory was developed to explain the organization of enzymes in supercomplexes allowing the scalar transport of electrons and the vectorial conduction of protons through the mitochondrial inner membrane to create the electrochemical potential [100,101]. Later, this concept was applied to the functioning of the phosphotransfer shuttle PCr/CK, AK [1,102,103], and to the transmission of [ADP] feedback signal from myofibril towards mitochondria [10,70,80,90,104].

Mitochondrial Interactosome is an integral part of the creatine kinase phosphotransfer network (shuttle) [10,42,90,105–108]. This network explains metabolic aspects of Frank–Starling law of the heart [7,108] and is quantitatively described by the mathematical model of compartmentalized energy transfer which describes non-equilibrium kinetics of creatine kinases functioning in opposite directions in mitochondria and myofibrils and takes into account the limited permeability of mitochondrial outer membrane for adenine nucleotides [104]. The model shows that cytosolic free ADP concentration may reach the levels of even 400 μM [7,36,104]. This value markedly exceeds the free ADP levels calculated from the CK equilibrium constant (50–100 μM) [36]. This mathematical model also helps to explain the importance of the limitation of the permeability of the outer mitochondrial membrane in cardiac cells within the Mitochondrial Interactosome for the effective control of oxidative phosphorylation by one of intracellular factors — the cytoplasmic ADP. As we have seen above, the apparent Km for free ADP in mitochondria in situ (in permeabilized cardiac cells and fibers) is about 20 times higher than in isolated mitochondria. When MOM is permeable, as in isolated mitochondria, the respiration regulation by cytoplasmic ADP is impossible because of saturating ADP concentrations (50–400 μM) in Fig. 10. Under these conditions cytoplasmic ADP has no role in respiration regulation. According to O'Rourke's principle mentioned above [48], changes in metabolic regulator should correlate with changes in workload and respiration rates. When ADP diffusion is restricted at the level of MOM, as it is in mitochondria in situ in permeabilized cardiomyocytes, the respiration rates become almost linearly dependent on cytoplasmic ADP concentrations when the latter changes in the range of physiologic concentrations up to 200–400 μM (shown by shaded area in Fig. 10). When MtCK is activated as it is in MI in vivo, the linear relationship between respiration rates and increase in free [ADP] is amplified and displaced towards the left region of Henri–

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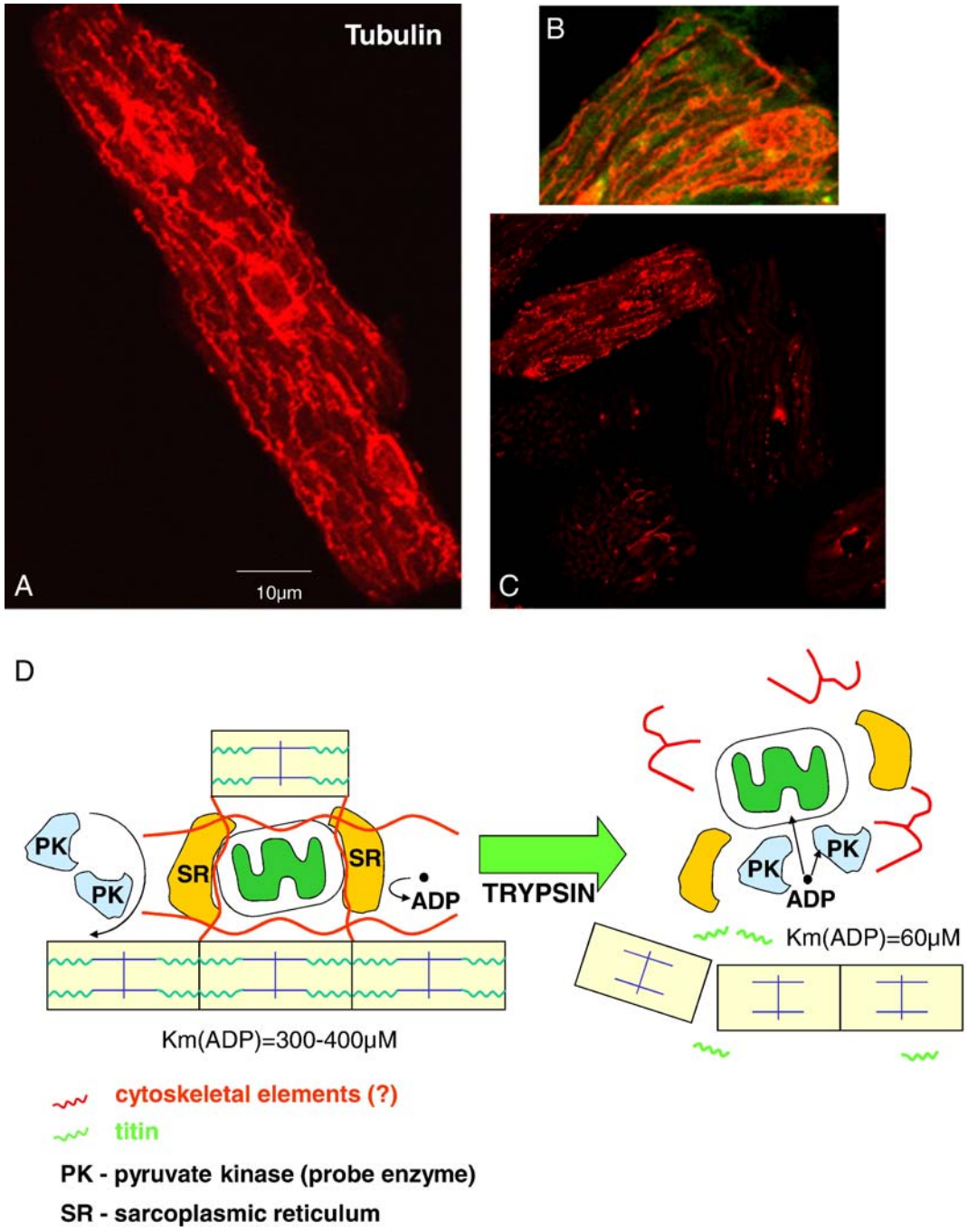

Fig. 6. (A, B, C) Confocal imaging immunofluorescence of microtubular network in cardiomyocytes. A, Microtubule network in control cardiomyocyte. B, Double labelling immunofluorescence of mitochondria and tubulin in control cardiomyocyte. The green colour is that of MitoTracker Green FM associated with mitochondrial membranes, and the red colour is the staining for tubulin. C, Effect of trypsin treatment (5 min, 1 mM at 4 °C) on the intracellular organization of microtubular network of cardiomyocyte: tubulin labelling disappears. Lower panel: schematic presentation of the role of cytoskeleton in the organization of mitochondria into functional complexes with sarcoplasmic reticulum (SR) and sarcomeres, i.e. into intracellular energetic units, ICEUs [13]. PK, pyruvate kinase. Proteolytic treatment with trypsin results in the collapse of the cytoskeleton and disorganization of the regular arrangement of mitochondria within the cells. For further explanation see text. Reproduced from with permission from Apaix et al. [19].

Michaelis–Menten representation due to recycling of ADP in coupled reactions in MI (see Fig. 10). In other words, for effective regulation of respiration in dependence of workload in cardiac cells in vivo, changes in concentrations of cytosolic ADP (as calculated by the model) are necessary and sufficient only when MtCK is actively functioning within the coupled systems in Mitochondrial Interactosome. An interesting and important task will be to apply the methods of Metabolic Control Analysis in experiments with permeabilized cells with fully activated MtCK in the presence of the PEP–PK system (see Fig. 4) to measure the flux control coefficients of different components of Mitochondrial Interactosome to quantitatively characterize their role in control of respiration and energy fluxes. Calculation of flux control coefficients from the mathematical model of compartmentalized energy transfer [109] showed that other important signals in metabolic feedback regulation of respiration may be cyclic changes in Pi and PCr/Cr ratio. Possible role of Pi in regulation of respiration was confirmed experimentally [110,111]. Both mathematical modeling and direct experimental determination of energy fluxes from mitochondria in permeabilized cardiomyocytes showed that under these conditions energy is carried into the cytoplasm mostly by phosphocreatine

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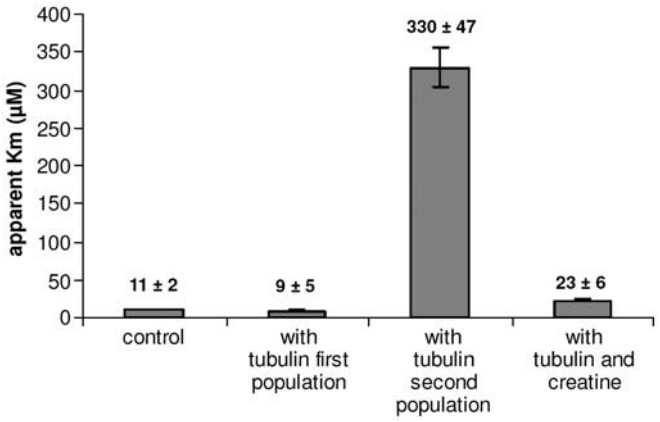

Fig. 7. Comparison of apparent Km for exogenous ADP in isolated heart mitochondria in three different conditions: control (mitochondria without tubulin and creatine), with tubulin 1 μM (two populations of mitochondria appeared) and with tubulin 1 μM and creatine 20 mM. The incubation with 1 μM tubulin was performed for 30 min at room temperature. The means are presented ± SD. Adapted from [203].

molecules [73,89,112]. Thus, there is clear separation of mass and energy transfer (by PCr and Cr) and information transfer (feedback metabolic signaling) [89].

The very remarkable observation is that physical activity changes the regulation of mitochondrial respiration via Mitochondrial Interactosome by increasing the value of apparent Km for exogenous ADP by a factor of 3 [81,82,113], while at the same time increasing the activity of MtCK [81,114]. As a result, the effect of creatine on respiration rate will be more significant [81,82,113]. The elucidation of the nature of changes induced in MI by physical exercise needs further experimental studies.

Mitochondrial Interactosome, as it is shown in Fig. 9, helps us to explain also another classical observation in the history of bioenergetics. Belitzer and Tsybakova showed in 1939 [115] that creatine added to a well washed homogenate of pigeon pectoral muscle strongly increased oxygen uptake and production of phosphagen (as phosphocreatine, PCr, was called at that time) without any added adenine nucleotides present only in trace amounts. The efficiency coefficient of aerobic synthesis of phosphagen, the PCr/O_2 ratio was between 5.2 and 7 [115]. This was one of the first determinations of

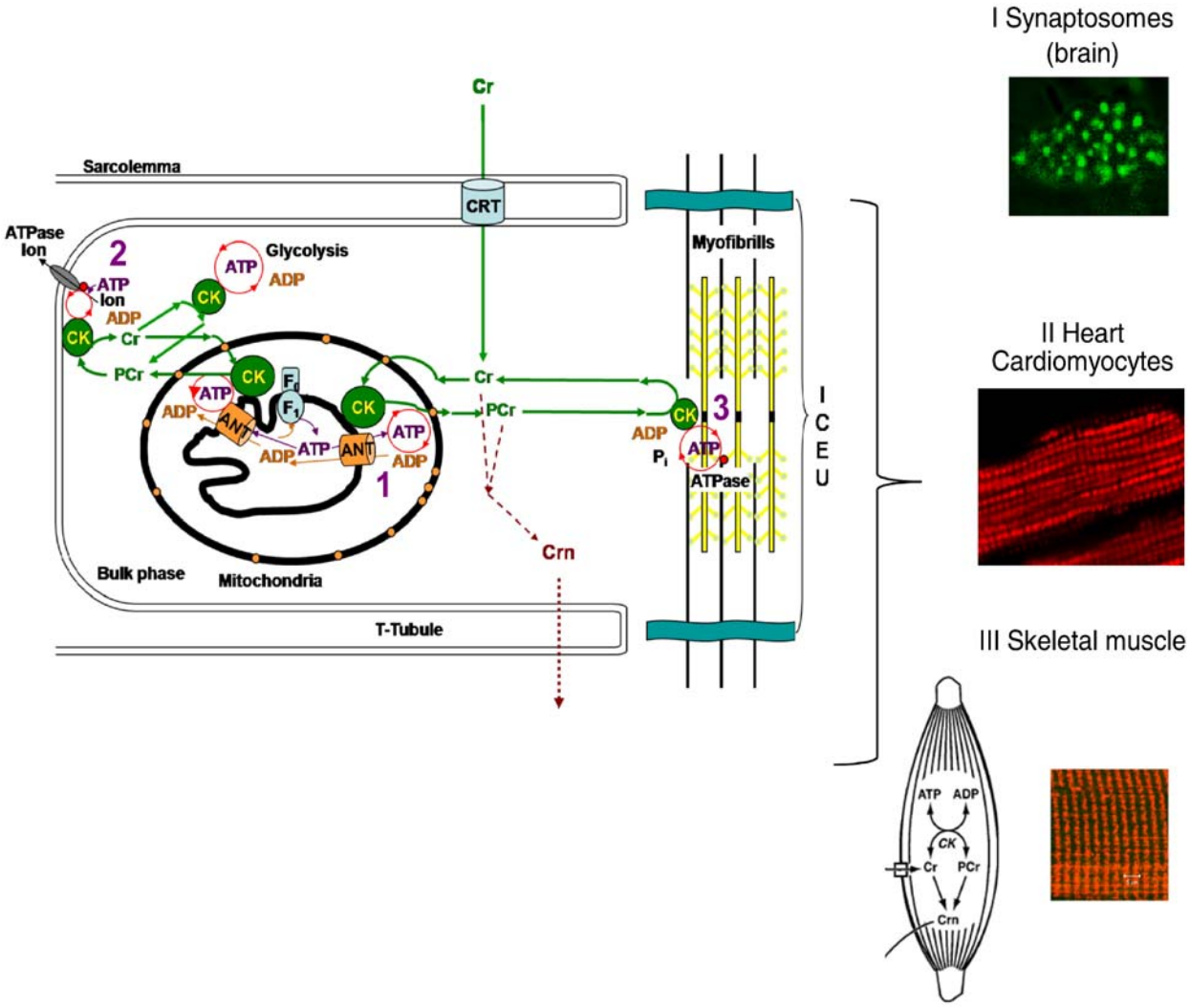

Fig. 8. Organization of compartmentalized energy transfer and metabolism in cardiac, skeletal muscle and brain cells and major routes of Cr metabolism in the mammalian body. The scheme shows the structural organization of the energy transfer networks of coupled creatine kinase (CK) reactions in mitochondria (1), at sarcolemmal membrane (2) and in myofibrils (3). The mitochondria, ATP-sensitive systems in sarcolemma and MgATPase of myofibrils are interconnected by creatine (Cr) and phosphocreatine (PCr) and energy transfer by the creatine kinase–phosphocreatine system. In brain cell systems energy transfer reactions are presented only by coupled reactions (1) and (2). Adenine nucleotides within local compartments 1, 2 and 3 do not equilibrate rapidly with adenine nucleotides in the bulk water phase. Right panels show confocal images of rat brain synaptosomes, cardiac cells and m. soleus. Mitochondria were labelled by MitoTracker Red or MitoTracker Green (50 nM). Very regular arrangement of mitochondria in striated muscles and fixed granular mitochondria in synaptosomes is seen. Creatine is not synthesized in these cells, but transported into cells by creatine transporter, CRT.

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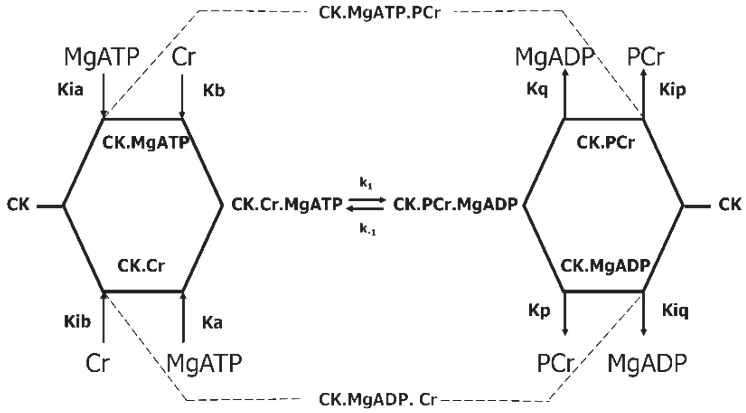
Table 3
Kinetic properties of MtCK in situ in cardiomyocytes. The MtCK reaction mechanism, BiBi quasi equilibrium random type is characterized by two dissociation constants for each substrate as shown in the following scheme [73,88]:



Values of constants for isolated mitochondria are taken from the literature [78,137]. In isolated mitochondria the oxidative phosphorylation decreases dissociation constants of MgATP from MtCK-substrate complexes suggesting the privileged uptake of all ATP by MtCK. In mitochondria in situ in permeabilized cardiomyocytes the increase of apparent constants of dissociation of MgATP compared with in vitro mitochondria shows the decrease of apparent affinity of MtCK in situ for extramitochondrial MgATP. The decrease of apparent constants of dissociation of creatine from MtCK-substrate complexes suggests the increase of the apparent affinity of MtCK for creatine in situ. The apparent constant of dissociation for PCr did not change in situ compared with isolated mitochondria. Reproduced from Guzun et al. with permission [73].

		K_{ia} (MgATP), mM	K_a (MgATP), mM	K_{ib} (Cr), mM	K_b (Cr), mM	K_{ip} (PCr), mM
Isolated mitoch.	−OxPhosph	0.92 ± 0.09	0.15 ± 0.023	30 ± 4.5	5.2 ± 0.3	
	+OxPhosph	0.44 ± 0.08	0.016 ± 0.01	28 ± 7	5 ± 1.2	0.84 ± 0.22
Mitoch. in situ (PEP–PK)		1.94 ± 0.86	2.04 ± 0.14	2.12 ± 0.21	2.17 ± 0.40	0.89 ± 0.17

stoichiometric coefficients in oxidative phosphorylation. Now we can easily explain this observation by the recycling of catalytic amounts of ADP and ATP within MI activated by creatine and coupled to phosphocreatine synthesis in skeletal muscle (Fig. 9).

6. Pathogenic mechanisms related to changes in organization of integrated energy metabolism

Dysfunction of integrated energy metabolism may be among leading mechanisms of pathogenesis of many diseases. A classical example of the importance of cellular organization of a complex system of energy metabolism is the Warburg effect: increase of lactate production in tumor cells in the presence of oxygen [116–119] first reported in the 1920s by Otto Warburg [118]. The conversion of glucose to lactate yields 2 mol of ATP per mole of glucose in comparison to 38 mol of ATP when glucose is completely degraded to CO_2 and H_2O. Thus, degradation of glucose to lactate yields only 5% of the energy available from glucose. This apparently senseless waste of energy prompted Warburg to postulate a defect in respiration in tumor cells as a cause for the increased "aerobic glycolysis" [116,118]. These and many other diseases are caused by cellular pathologies related to changes in mitochondrial structure and function, known as mitochondrial pathologies [119,120].

In normal cells competition between glycolysis and oxidation of fatty acids leads to their coordinated function with the aim to extract more free energy into the adenylate system (ΔG_{ATP}) from catabolic reactions [21] mostly to maintain cellular work, ion transport and biosynthesis. When workload increases, ATP production and respiration are increased due to feedback regulation via the CK system [7,21]. These pathways occur under aerobic conditions. Among the mechanisms limiting the glycolytic rate in normal cells are the intrinsic kinetic properties of soluble isoforms of hexokinase, HK, the inhibition of HK by the reaction product glucose-6-phosphate (G-6-P), and finally the inhibition by citrate via phosphofructokinase (PFK).

In cancer cell this mechanism of regulation is lost and substituted by other mechanisms of interaction between glycolysis and oxidative phosphorylation that result in the parallel activation of both systems [121]. Our studies on HL-1 cells, which are derived from mice atrial cardiomyocytes but also carry some properties of cancer cells (such as unlimited proliferation) have been useful for understanding the metabolic changes associated with the development of tumor cell phenotype. As we have seen in Fig. 3, mitochondria in NB HL-1 cells exhibit very high apparent affinity for exogenous ADP similar to that of isolated mitochondria. In addition, creatine is unable to stimulate respiration of NB HL-1 cells (Fig. 4F) due to downregulation of mitochondrial MtCK and cytosolic MM-CK [84]. The only CK isoform in cytosol of NB HL-1 cells was found to be BB-CK isoform [84] At the same time, metabolic profile of NB HL-1 cells is characterized by the prevalence of glycolytic enzyme activity, especially by that of HK and PK (Fig. 5). Total HK activity in NB HL-1 cells homogenate was increased by a factor of 5 in comparison with adult cardiomyocytes (Fig. 5) [122]. High activity of hexokinase is seen even after permeabilization of cells when only the activity of membrane-bounded enzymes can be measured [84]. Fig. 11 shows that glucose exerted a remarkable stimulatory effect on mitochondrial respiration in NB HL-1 cells, in comparison with a negligible effect on respiration of permeabilized heart fibers [84]. Lack of stimulatory effect of glucose on oxygen uptake in the presence of ATP in permeabilized heart cells means that while hexokinase is expressed in cardiomyocytes [84], it is not bound to mitochondrial membrane where tubulin occupies binding sites near VDAC within Mitochondrial Interactosome. In cancerous HL-1 cells the Mitochondrial Interactosome structure is significantly modified (Fig. 12): tubulin has evidently given place to HK, and the absence of MtCK allows all mitochondrial ATP to be captured for phosphorylation of glucose and stimulation of glycolytic lactate production. Thus, the Mitochondrial Interactosome typical for normal cardiomyocytes is replaced by another type of Mitochondrial Interactosome, in which mitochondrially bound HK substitutes for the role of MtCK. These structural rearrangements result in a specific metabolic phenotype characterized by the replacement of creatine control of respiration, characteristic of adult cardiac cells, by that of glucose in NB HL-1, thus underlying the so-called "Warburg effect". As

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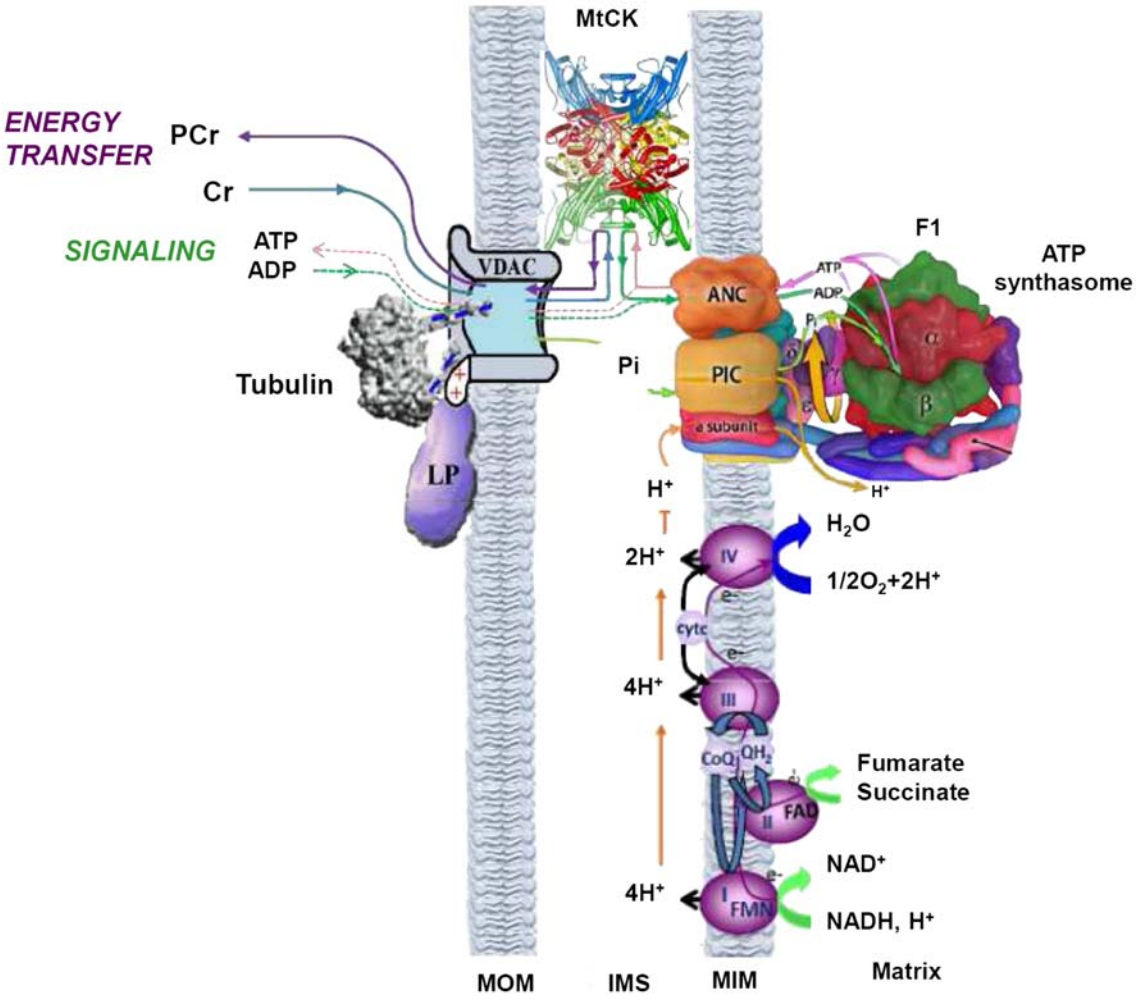

Fig. 9. Mitochondrial Interactosome (MI) in cardiac, oxidative skeletal muscle and brain cells consisting of ATP synthasome (formed by ATP synthase, adenine nucleotide carrier (ANC) and inorganic phosphate carrier (PIC) as proposed by Pedersen [92], mitochondrial creatine kinase (MtCK) functionally coupled to ATP synthasome and voltage dependent anion channel (VDAC) with regulatory proteins (tubulin and linker proteins(LP)). ATP regenerated by ATP synthase is transferred to MtCK due to its functional coupling with ATP synthasome. MtCK catalyses transfer of phosphate group from ATP to creatine producing phosphocreatine (PCr) which leaves mitochondria as a main energy flux. ADP is returned to and recycled in ATP synthasome due to highly selective permeability of VDAC. VDAC permeability is regulated by heterodimeric tubulin and by some linker proteins (LP). Small signaling amounts of cytosolic ADP enter the intermembrane space and increasing production of the PCr within MI due to the functional coupling of ATP synthasome with MtCK which amplifies cytosolic ADP signal. MOM, mitochondrial outer membrane; MIM, mitochondrial inner membrane; IMS, mitochondrial intermembrane space. Reproduced from Timohhina et al. with permission [89].

it was described by Pederson et al., in cancer cells the over-expressed HK2 is bound to VDAC in the MOM [91–93,121,123–126] and via this coupling can exert control over oxidative phosphorylation. The apparent Km of membrane-bound HK2 for glucose is about 250 times lower than for soluble isoenzymes [127] and it is protected from the G-6-P product inhibition by its connection with VDAC. Glycolytic ATP synthesized during lactate production is used in biosynthetic pathways for growth and proliferation [125,126]. In this way, it seems that the "energetic" role of mitochondria in cancer cells is reduced to maintain glycolysis. However, oxidative phosphorylation, although suppressed due to decreased biogenesis of mitochondria is still preserved, in order to facilitate glycolysis. Currently, it is not clear how the transformation from normal to cancer metabolism proceeds, how normal mechanisms of coordination between glycolytic and oxidative networks degrade into the most primitive model of glycolytic energy metabolism. Groof et al., in 2009 tried to answer this question by studying the mechanisms of Warburg phenotype development during immortalization of mouse fibroblast cells (H-RasV12/E1A). This process was shown to be dependent on the number of culture-passages and characterized by the increase in glucose-to-lactic flux and cellular oxygen consumption associated with the decreased TCA and oxidative phosphorylation activity [128].

Most of the other pathologies are related to defects in the respiratory chain complexes and lead to decreased ATP production as a result of either inhibition of respiration or uncoupling of phosphorylation and respiration [120]. Among pathogenic mechanisms related to changes in mitochondrial functions is the opening of mitochondrial permeability transition pore, PTP, induced by calcium overload of cells and accelerated by production of reactive oxygen species, ROS [129,130]. PTP opening leads to cell death by necrosis, and this is the most common mechanism related to ischemic and reperfusion injuries [129,130]. ROS production by chemical (non-enzymatic) reaction of molecular oxygen with reduced electron carriers in the complexes I and III of the respiratory chain is considered also as the major mechanism of ageing and cancerogenesis [131]. ROS production consumes from 1 to 5 % of oxygen supplied to the cells and cannot be avoided, but can be controlled by means of controlling the red-ox state of the respiratory chain [131]. Both ROS production and PTP opening can be effectively controlled and inhibited by the MtCK reaction which is functionally coupled to the adenine nucleotide translocase, controls respiration and ATP–ADP recycling in mitochondria and by this way controls the red-ox state of the respiratory chain and also the conformational state of mitochondrial carriers important for PTP opening [97,132].

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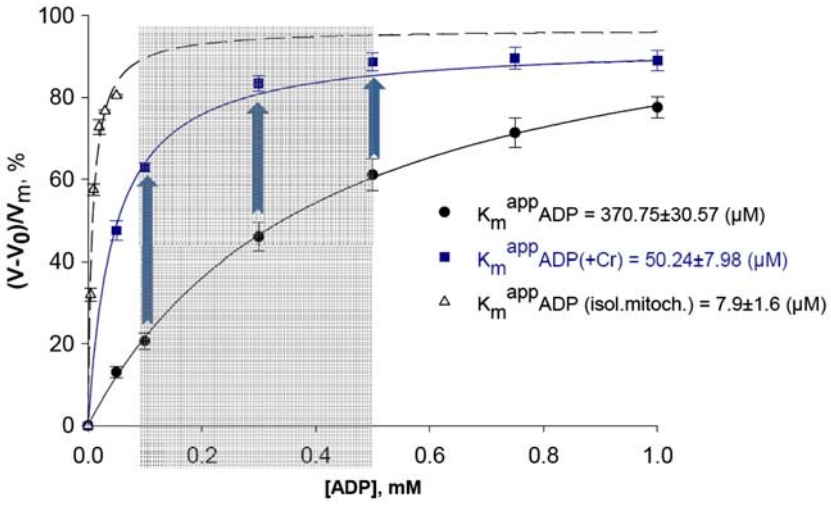

Fig. 10. Henri–Michaelis–Menten hyperbolic representation of kinetics of respiration regulation by free ADP in isolated heart mitochondria and permeabilized cardiomyocytes (in the absence or presence of creatine). The grey area delimits physiologic range of changes in cytosolic [ADP] taken from the model of compartmentalized energy transfer in cardiomyocytes [104]. In isolated mitochondria (curve (Δ)), no regulation of respiration is possible because of the saturating free [ADP] for the minimal workload. When the ADP diffusion is restricted as in mitochondria in situ in permeabilized cardiomyocytes (curve (●)), the respiration rates become linearly dependent on ADP concentrations in their physiological range. In this interval of quasi-linear dependence under physiological conditions the activating effect of ADP can be amplified by creatine (curve (■)), due to activation of coupled MtCK. The resulting apparent Km for cytoplasmic ADP is significantly decreased and respiration rate increased.

7. Parameters of PCr/Cr system as diagnostic means

In the cells with high energy demand the creatine kinase system is responsible for facilitating energy supply to local pools of the ATP near ATPases and ATP-sensitive channel in sarcolemma (see Fig. 8), thus controlling both contraction and excitation–contraction coupling [1,7,10,42,88,105,108,133–143]. Changes in this central system of energy supply either due to alteration of creatine kinase isoenzyme composition, activity and mechanisms of interaction with ATP producing and consuming systems, or decrease of total creatine content may lead to serious pathology of the heart, skeletal muscle and nervous system [133–135,143]. Clinical studies of patients with severe cardiomyopathy by non-invasive nuclear magnetic resonance imaging and spectroscopy revealed a very high diagnostic value of parameters of functioning of creatine kinase system — the PCr/ATP ratio for prognosis of patient's mortality rate and thus survival [133]. Alternatively, dietary supplementation of creatine has been shown to be an effective mean of pharmacological treatment and protection of patients with muscular and neurodegenerative diseases [105,135,136, 139–141]. Systems biology approaches to studies of these integrated processes of energy metabolism in normal cell life and in their pathology may have an utmost importance for clinical medicine in the near future [7,144,145].

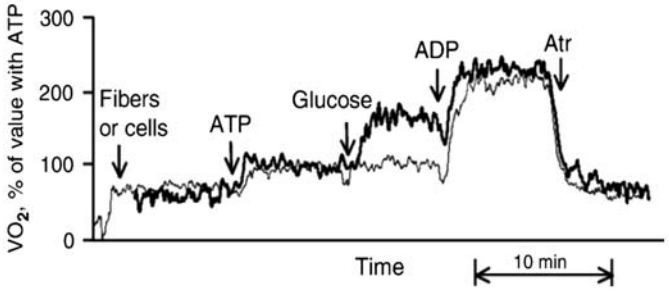

Fig. 11. Oxygraphic analysis of coupling of hexokinase (HK) to OxPhos in permeabilized HL-1 cells and rat left ventricular fibers. The thin line corresponds to respiration rates of permeabilized rat left ventricular fibers or cardiomyocytes; the thick line shows respiration rates of HL-1 cells. Ten millimolars of glucose activates HL-1 cell respiration in the presence of 0.1 mM MgATP due to the endogenous ADP production in HK reaction. Similar amount of glucose cannot stimulate respiration rate of permeabilized fibers or cardiomyocytes. ADP — 2 mM MgADP, Atr — 0.1 mM atractyloside. Reproduced from Eimre et al. with permission [84].

In myocardial infarction and in heart failure, a rapid decrease of PCr content occurs due to lack of oxygen supply and pathological changes in the creatine kinase system [108,133–137]. Total ATP content usually changes very slowly and its changes, as well as changes in the free energy of ATP hydrolysis calculated from total metabolites' contents, are dissociated from the rapid fall of the cardiac contractile force [28,146], total depletion of ATP resulting in contracture of the heart muscle [147]. A rapid decline in heart contractile function in hypoxia and ischemia is most likely to be related to changes in compartmentalized energy transfer systems, leading to decreased regeneration of ATP in functionally important cellular compartments, as shown in the scheme in Fig. 8. Firstly, the rapid decline in ATP regeneration in subsarcolemmal area results in changes of ion currents across this membrane and thus in shortening of action potential [1,137], and secondly, the rapid decline in ATP regeneration in myofibrillar microcompartments due to lack of phosphocreatine slows down the contraction cycle [108,137]. Similar but slower changes are observed in chronic cardiac and skeletal muscle diseases [148–157]. In concord with this conclusion are the results published by Weiss et al. [154] showing that cardiac ATP flux through CK is reduced by 50% in cases of human heart failure in the absence of reduction of ATP stores. Local phosphotransfer networks in the subsarcolemmal area are an important part of the membrane sensors of the cellular energy state also in brain cells [136,139–141], explaining the dependence of functional state of these cells on phosphocreatine supply, and thus the central importance of the PCr/Cr system. In brain cells ubiquitous mitochondrial creatine kinase is co-expressed with BB isoenzyme localized both in cytoplasm and at the cell membrane (coupled creatine kinases 1 and 2 in the Fig. 8). Alterations of these systems are observed in many neurodegenerative diseases [139,140].

The content of phosphocreatine, central energy carrier in the cells, depends both on its continuous regeneration in mitochondrial creatine kinase reaction coupled to oxidative phosphorylation, and on the total creatine content which in significant part depends on the import of creatine (not produced in muscle or brain cells) from blood by creatine transporters in the cell membrane. This explains the beneficial effects of dietary supplementation of creatine on the energy metabolism and functional state of skeletal muscle and brain cells,

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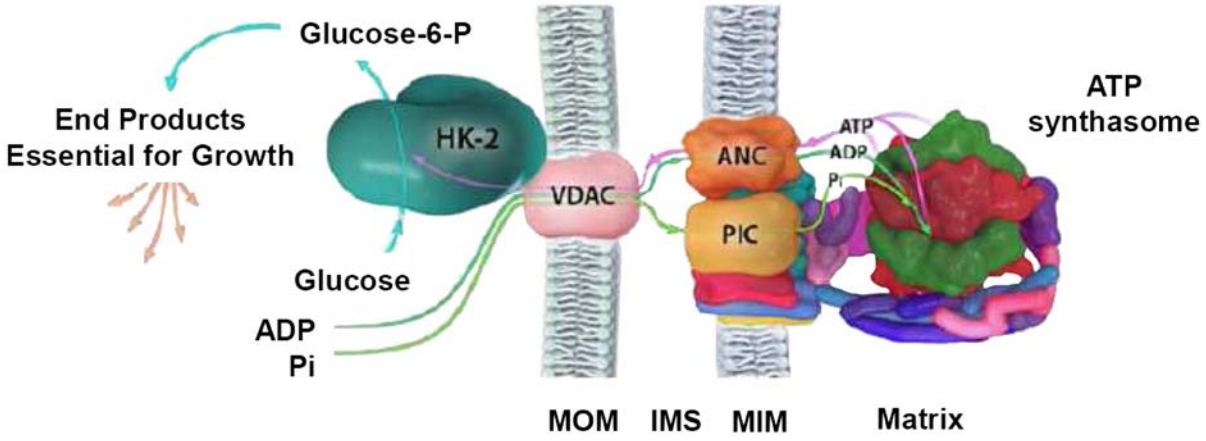

Fig. 12. Current view showing how HK2 bound to VDAC in the mitochondrial outer membrane in HL-1 cells has preferred access to ATP synthesized on the inner membrane by the ATP synthasome, a complex between the ATP synthase and carriers (transporters) for ADP and phosphate (Pi). The structural integrity of this entire network is essential for the survival of those cancer cells that exhibit the "Warburg" effect. Thus, HK2 while preventing apoptosis by binding to VDAC, also supports cancer cell growth by receiving preferred access to ATP newly synthesized by the ATP synthasome. Reproduced from Pedersen with permission [91]. This Figure and ATP synthasome in Fig. 9 are artworks by David Blume, Johns Hopkins University, USA.

especially in patients with neurodegenerative diseases [139–141,158–160].

Dissociation of total content of ATP from cell function was recently clearly demonstrated by O'Connor et al. [142], who showed that it is the PCr/CK system which sustains localized ATP-dependent reactions during actin polymerization in myoplast fusion. Myoplast treated with exogenous creatine showed enhanced intracellular PCr stores without any effect on ATP levels. This increase in PCr induced the myoplast fusion and myotube formation during the initial 24 h of myogenesis. During this time BB-CK became localized and after 36 and 48 h was found close to the ends of the myotubes [142]. Actin polymerization is critical for myoplast fusion and occurs with involvement of ATP both during the addition of actin monomers to the growing ends of filaments and the dissociation of monomeres at the tail. It is this localized ATP which is rapidly regenerated by BB-CK at the expense of PCr, and it seems that the formation of these ATP microdomains is a dynamic process during actin cytoskeleton remodeling. Local injection of creatine into injured skeletal muscle increased the growth of regenerating myofibers from satellite cells via differentiation and fusion of myoplasts [142]. All these results add new insight into the functioning of the PCr/CK system in muscle cells, showing its new role in energy supply for cytoskeletal remodeling. These results may help to better explain the therapeutic effects of creatine supplementation [105,135,136,143,158–160].

Intensive and numerous studies have been carried out on transgenic mice with knockout of different CK isoenzymes, or enzymes responsible for creatine metabolism and transport (reviewed in [149,152,161,162]). In spite of multiple adaptive mechanisms — activation of alternative phosphotransfer pathways, such as adenylate kinase shuttle [161,163], structural changes in the cells and increase of oxidative capacity of skeletal muscle [152,161], and many others [164], significant functional and metabolic changes especially related to calcium metabolism and contractile performance have been observed in these experiments [162,164–167]. Thus, Momken et al. have reported that double knockout of MtCK and MM-CK very significantly impairs the voluntary running capacity of mice [165]. Knockout of enzymes of creatine biosynthesis in mice resulted in significantly reduced responses to inotropic stimulation [162]. Similarly, the hearts of rats treated with guanidinopropionic acid performed much less pressure-volume work [168]. Most interestingly, recent works from Neubauer's laboratory have shown that overexpression of creatine transporter and supranormal myocardial creatine contents lead to heart failure [169,170]. In these hearts creatine content is increased by more than a factor of 2 [169]. Most interestingly, these experiments put into evidence the importance of the PCr shuttle: heart failure may be due to the formation of dead-end complex CK.MgADP.Cr formation [171] and inhibition of PCr utilization for local ATP regeneration.

In summary, data obtained in experiments with CK knockout mice are in concordance with our conclusion made in this work and before [7,10,42,107,136,137] that muscle (and other) cells are viable without MtCK and other CK isoenzymes, as HL-1 cells, but PCr–CK and other phosphotransfer pathways are necessary for effective energy transfer and metabolic regulation at higher energy demand, and thus for survival under stress conditions. An important observation is that exercise training results in cytoskeleton remodeling, including changes in Mitochondrial Interactosome and increased efficiency of energy transfer via PCr–CK pathway [81,82,113,114]. By analogy, PCr–CK pathway is as an efficient highway connecting ATP production and consumption sites. Without this highway, cells have to find other ways of ATP and energy transfer, but the efficiency of communication and regulation is lost and energy may be wasted (as in HL-1 cells). Under these conditions, muscle and brain cells degrade into pathological state.

Two important large-scale clinical studies of changes in the PCr/CK system in the myocardium of patients with heart failure have been performed [133,134,148]. The first large-scale international study was organized by Ingwall and Allen [148]. In this study, myocardium was sampled from subjects who underwent heart transplant, from subjects maintained in an intensive care unit before heart harvesting, from accident victims, and patients undergoing heart surgery. Since the characteristics of myocardium of potential organ donors differed from those of myocardium of accident victims, data are presented for three groups: failing, donor, and control. MM-CK and the mitochondrial isoenzyme activities were lower in failing and donor LV, and MB-CK activity and B-CK content were higher in failing and donor hearts. Creatine contents were 64 ± 25 and 56 ± 18.6 nmol/mg protein in LV and RV of failing, 96 ± 30 and 110 ± 24 nmol/mg protein in LV and RV of donor, and 131 ± 28 nmol/mg protein in LV of control hearts [148]. Thus, in the failing hearts the total creatine content is significantly decreased, as compared to the control heart. Also, failing hearts showed much lower creatine kinase activity than those of the control [148].

The second important clinical study was performed by Neubauer's group in United Kingdom [133,134]. By using ^{31}P-NMR spectroscopy in combination with imaging for investigation of cardiac muscle energy metabolism in patients, the authors showed that in patients with cardiac disease — dilated cardiomyopathy (DCM) the decreased

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PCr/ATP ratio (lower than 1.6) is very clear and strong diagnostic index of increased mortality. In the heart of patients with DCM the ATP content remained the same as in healthy control patients, but PCr decreased by 70% as compared to control. This shows the vital importance of the phosphocreatine–creatine kinase energy transfer network for the cardiac muscle normal function and life [133,134].

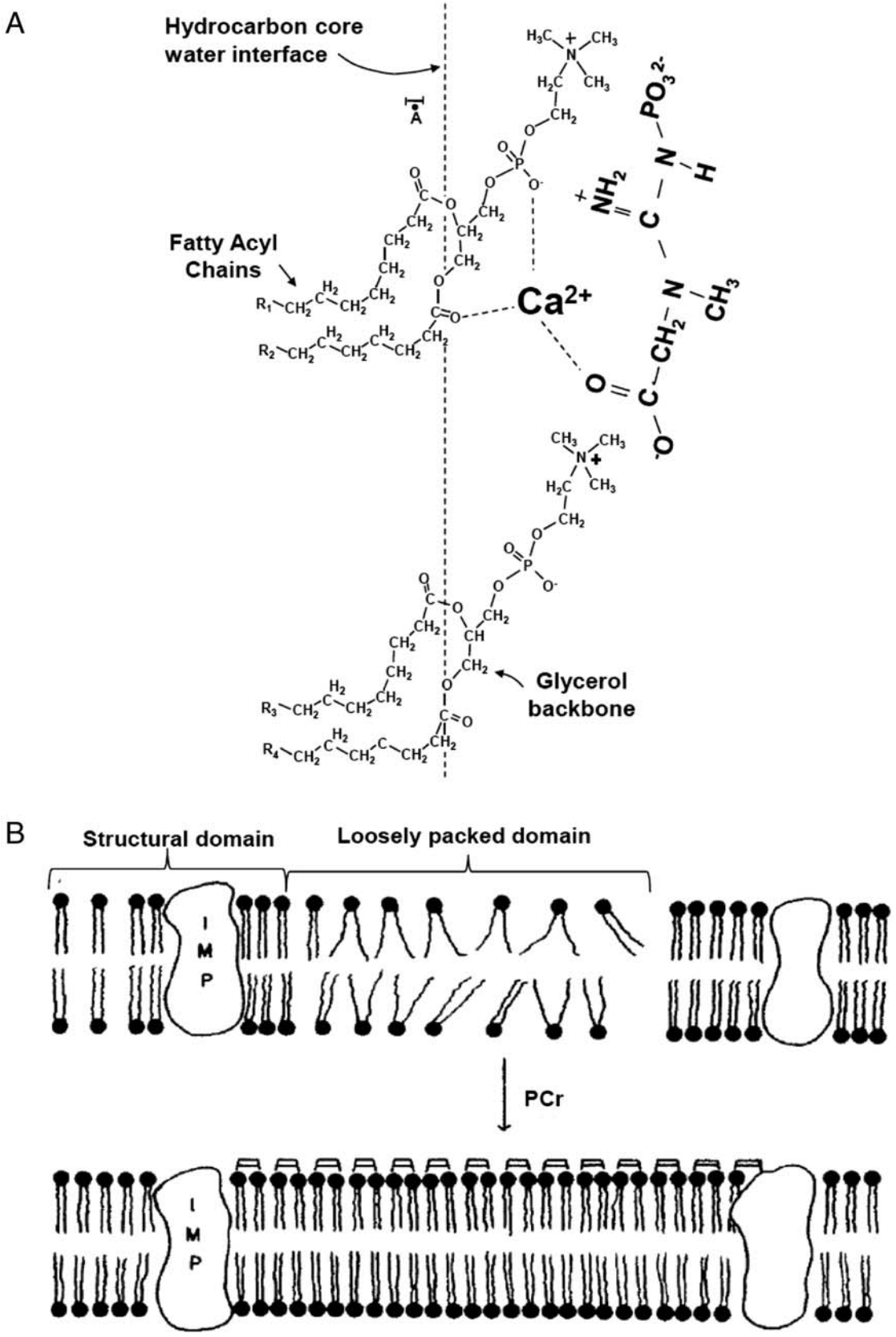

Fig. 13. Zwitterionic interaction of phosphocreatine with bipolar heads of phospholipid molecules in the membrane surface interphase. Adapted from Saks and Strumia [176] with permission.

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It has been found in multiple clinical investigations of biopsy samples of skeletal muscle of patients with heart failure, that it has induced very clear changes of the creatine kinase system in these muscles most probably by decreasing oxygen supply and altering circulation (reviewed in [155,156,161,172]).

Thus, the PCr/ATP ratio is an important diagnostic parameter of heart disease, as is the total creatine content. Low PCr concentrations and low PCr/ATP ratios mean decreased regeneration of ATP by the PCr/CK system in microdomains (compartments) which are critically important for the function of the heart, skeletal muscle and brain. These microdomains are localized in myofibrils, near the sarcolemma and the membrane of sarcoplasmic reticulum in muscle cells and near cellular membrane in brain cells (see Fig. 8). There is a general consensus now among the researchers in muscle and brain energy metabolism that the further challenge and urgent need is to develop better bioprobes to image metabolic microdomains of ATP and functional proteomics to identify physical interactions between key proteins responsible for their formation [133,137,144].

In addition to its important role in supporting regeneration of local ATP pools as a substrate for MM-CK reactions in myofibrils and cellular membranes, the PCr molecule appears to have another very useful property — membrane stabilizing action (Fig. 13). This was revealed in long series of clinical use of extracellular phosphocreatine injection with clear protective effect on ischemic myocardium, and in detailed experimental studies [173–175]. In all these studies extracellular phosphocreatine was used and shown to decrease the ischemic damage of the heart muscle by multiple mechanisms. Among others, there is a clear membrane stabilizing effect of PCr [176] which may be explained by interaction of its zwitterionic molecule carrying positive and negative charges with opposite charges of phospholipid polar heads in the membrane surface interphase (Fig. 13A), resulting in the transition of the mobile domain (fluid phase) of membranes into a structured domain (gel phase) as shown in Fig. 13B, leading to the decrease of the rate of phospholipid degradation into lysophospholipids and lipid peroxidation [176]. Rapid fall of the intracellular PCr pool in hypoxia and ischemia may thus be a significant factor of destabilization of cellular membranes.

In patients with peripheral skeletal muscular diseases, very informative non-invasive diagnostic methods of assessment of the energy state by recording the parameters of the PCr/CK system have been developed due to rapid progress in NMR imaging and spectroscopic technologies [2,133,134,177–180]. Four quantitative parameters of the PCr/CK system which can be measured in patients by ^{31}P and ^{1}H NMR spectroscopy in combination with imaging [133] are: the contents of PCr and ATP and PCR/ATP ratio measured by ^{31}P NMR spectroscopy, the content of creatine measured by ^{1}H NMR spectroscopy [2,154,177–180], the ATP flux through creatine kinase system measured by saturation transfer method [2,154,180], and the kinetics of PCr recovery in skeletal muscle after exercise measured by ^{31}P NMR spectroscopy [2,180]. In combination with the biochemical analysis of biopsy samples taken from skeletal muscles of patients [5,113,181] these methods give an exhaustive diagnostic means for clinical analysis of the energy transfer networks in patients in health and disease [182].

8. General conclusion

Living cells and organisms are thermodynamically opened systems which exchange material and energy with its environment, since they must "avoid the decay into the inert state of equilibrium to keep alive by continually drawing from its environment negative entropy" as it was discovered by Schrödinger [183]. This basic property allows a living system to maintain the "stability of its internal milieu" or homeostasis described already by Claude Bernard [184,185]. Therefore, the cellular life is governed by laws of non-equilibrium irreversible thermodynamics and non-equilibrium steady state kinetics [10,186–190]. A decrease of internal entropy in such systems is achieved by free energy extraction from environment and energy dissipation to realize cellular work [183,188–190]. It is increasingly understood now that in living cells the effective regulation of metabolism is realized through formation of "dissipative metabolic networks" [189] due to complex intracellular interactions within the inhomogeneous intracellular medium. These interactions lead to new system level properties and mechanisms such as intracellular metabolic compartmentation, functional coupling, downward causation including higher level control of gene expression, retrograde response between mitochondria and nuclei etc. which are the topics of studies in Molecular System Bioenergetics [10,67], part of Systems Biology [65–67].

Results reviewed in this article show the importance of structural and functional organization of intracellular phosphotransfer networks interconnecting ATP-utilization and ATP regeneration processes into intracellular energy units (ICEU). The role of the ICEUs is not reduced only to the high efficiency of coordination of energy metabolism. Their role is more fundamental: in conformity with the theory of dissipative metabolic structures [189,190], formation of the ICEUs helps to extract Gibbs free energy and negentropy from the environment. ICEU can be seen as a "dissipative metabolic network" which functions in the non-equilibrium state made up of the dissipative enzymatic sub-networks (glycolysis, the Krebs cycle, fatty-acid's oxidation, the electrons transport chain, the shuttles creatine kinase/phosphocreatine, malate/aspartate, etc) structured and connected together by flows and regulating signals. Association of various enzymes within big multienzyme complexes allows the direct transfer of intermediate metabolites (vectorial ligand conduction). One of such complexes is Mitochondrial Interactosome which regulates the interaction between mitochondrial cycles of adenine nucleotides and PCr/Cr cycles in the cytoplasm of the heart, skeletal muscle and brain cells. Mitochondrial Interactosome and PCr pathway of intracellular energy transfer explain well the metabolic aspects of Frank–Starling law of the heart and classical observation of Belitzer and Tsybakova on effective coupling of PCr production and oxidative phosphorylation in muscles. Changes in Mitochondrial Interactosome lead to severe pathology and may contribute in the mechanism of Warburg effect in cancer cells. Systemic analysis of changes in phosphotransfer networks helps to explain many pathogenic mechanisms in numerous diseases.

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Article 11

Mesure directe de l´hétérogénéité de la diffusion de l´ATP par Spectroscopie à Corrélation de Fluorescence.

ATP DIFFUSION IS HETEROGENOUS IN CARDIOMYOCYTES

Evidence from Fluorescence Correlation Spectroscopy

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List of abbreviations: AC - autocorrelation, CM - cardiomyocyte, Cr - creatine, FCS - fluorescence correlation spectroscopy, Fluo-ATP - Alexa Fluor® 647 adenosine 5´triphosphate, ANT - adenine nucleotide translocator, DTT - dithiothreitol, CK – creatine kinase, MMCK – dimeric muscular creatine kinase, MtCK – mitochondrial dreatine kinase, Atr - atracyloside

Abstract:

The aim of this study was to investigate diffusion of ATP in permeabilised cardiomyocytes, and suspension of isolated mitochondria and solutions by using a fluorescent ATP analogue (ATP-Alexa 647, Fluo-ATP) and Fluorescence Correlation Spectroscopy (FCS). The preferential accumulation of Fluo-ATP in mitochondria is demonstrated by confocal microscopy both in isolated mitochondria and in cardiomyocytes. The multicomponent analysis of the FCS data reveals the presence of multiple correlation times likely associated with cytosolic ATP diffusion, enzymatic reactions, restricted intramitochondrial diffusion and organelle movements, and which depend on mitochondrial functional state. These results give one of the first experimental evidence of existence of multiple intracellular binding sites of ATP resulting in heterogeneity of its concentration and diffusion properties in the cells.

Introduction

Functioning of heart, skeletal muscles and brain depends on aerobic mitochondrial ATP production and ATP consumption by ATPases (in myofibrills or ion pumps). While the mechanism of ATP synthesis in mitochondria and the biochemistry of MgATPases [1] are well understood, the question of how ATP diffuses from mitochondria to all sites of ATP consumption is still open. Here, the opinions are sharply divided. One group of investigators still considers that the diffusion of ATP in cytosol is a homogeneous process which can be described quantitatively by using the same high diffusion coefficient as in diluted aqueous solution [2,3]. The second group of investigators considers that the intracellular medium is highly compartmentalised in these cells, that ATP diffusion is heterogeneous and that ATP pools in mitochondria and in sites of ATP consumption are interconnected by phosphotransfer networks [4]. A precise description of the ATP diffusion process within cardiomyocytes is clearly needed for correct understanding of the mechanisms of regulation of mitochondrial respiration and energy fluxes in the cells in vivo. The aim of this work is to study the diffusion of ATP using its fluorescent analogue (ATP-Alexa 647, Fluo-ATP) and Fluorescence Correlation Spectroscopy (FCS) in experimental systems with increased complexity: first in solution, then in isolated mitochondria and finally in cardiomyocytes. FCS yields information about probe dynamics from the statistical analysis of its fluorescence fluctuations. This sensitive and non-invasive technique can measure nanomolar concentrations of fluorophore in a sub-femtoliter volume chosen directly inside a living cell. The fit of resulting autocorrelation (AC) curves to biophysical models revealed the presence of multiple diffusion times in cardiomyocytes and isolated mitochondria in opposite to the pure medium. The results give an evidence for the heterogeneity of the ATP analogue accumulation and diffusion in highly organised biological systems.

Material and methods

Isolation of mitochondria and cardiomyocytes. Heart mitochondria were isolated from adult Wistar rats 300 g body weight, as described earlier [5]. The final pellet containing mitochondria was resuspended in 1 ml of Mitomed medium composed of: 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 3 mM KH_2PO_4, 20 mM taurine, 20 mM HEPES, 110 mM sucrose, 0,5 mM dithiothreitol (DTT), pH 7.1.
Adult cardiomyocytes were isolated from Wistar male rats (300-350g) after perfusion of the rat heart with collagenase using an adaptation of the technique described previously [6] The final pellet is resuspended in Mitomed medium.

Cell incubation and labelling for confocal microscopy. For the study of ATP localisation, the intact or permeabilised cardiomyocytes were incubated with 200 nM of ATP-Alexa and 1 µM of MitoTracker® Green FM (Molecular Probes) for 30 min at 37°C. The imaging was performed under conditions of oxidative phosphorylation: in presence of 1mM ADP (saturating concentration to reach the maximal rate of mitochondrial respiration), 5 mM glutamate, and 3 mM malate (mitochondrial complex I respiration substrates). The plasma membrane of isolated cardiomyocytes were permeabilised by addition of 25 mg/ml saponin.

Fluorescence correlation spectroscopy. All confocal microscopy and FCS measurements were performed using a LSM 510 – Confocor 2 Combi microscope (Carl Zeiss, Jena, Germany) based on an inverted Axiovert 135M stage equipped with a C-Apochromat 40x/1.2 W Korr water immersion objective.

The localisation and mobility of the ATP in cardiomyocytes was assessed with a fluorescent analog of ATP: Alexa Fluor® 647 adenosine 5´triphosphate (Fluo-ATP, Molecular Probes). The plasma membrane of isolated cardiomyocytes was permeabilised by addition of 25 mg/ml saponin. For the study of ATP localisation, the intact or permeabilised cardiomyocytes were incubated with 200 nM of ATP-Alexa and 1 µM of MitoTracker® Green FM (Molecular Probes) for 30 min at 37°C. The imaging was performed under conditions of oxidative phosphorylation: 1mM ADP, 5 mM glutamate and 3 mM malate. Creatine and atractloylside were added at the end of the measurements at concentrations of 20 mM and 30 µM, respectively.

For FCS measurements, the Fluo-ATP dye was excited by 633 nm laser line and fluorescence detected by the avalanche photodiode using LP650 filter. The laser power on the sample was kept below 15 kW/cm^2, unless specially indicated. The measurements were performed at room temperature (20° C) in 8-well chambered coverslips LabTek I (Nalge Nunc Int., NY, USA) after sedimentation of cardiomyocytes or isolated mitochondria or in a drop of solution of at least 40 µl volume. The well preserved rod-like CMs and the spot position for FCS measurements were routinely selected using confocal and transmitted light images. Each FCS acquisition of 30 s was repeated 5 to 10 times. Data series containing significant photon bursts or constant variation of the count rate were excluded from analysis. The average result was fit using Zeiss LSM510 4.2 software module to the following autocorrelation equation:

$G(\tau) = 1 + A * G_d * G_b$, where A is amplitude, G_d is a diffusive term and G_b is a fast bunching:

$$A = \frac{1}{N}, \qquad G_d(\tau) = \sum_{i=1}^{n} \frac{F_i}{\left(1 + \frac{\tau}{\tau_{d,i}}\right)\sqrt{1 + \frac{\tau}{\tau_{d,i}} SP^2}}, \qquad G_b(\tau) = 1 + \frac{K \cdot e^{-\left(\frac{\tau}{\tau_b}\right)^{\beta}}}{1 - K}, \text{ where}$$

N is the number of fluorescent molecules in the confocal detection volume, F_i and $\tau_{d,i}$ are the fraction and the diffusion time of the component i, SP is the ratio of the axial (ω_z) to the radial (ω_{xy}) waist of the Gaussian detection volume, K is the effective fraction of molecules in all non fluorescent states, τ_b is the characteristic bunching time and β is the stretch parameter. The number of diffusion components n was fixed to 1 in solutions and to 2 or 3 in CMs and mitochondria measurements. Other fit parameters were unconstrained unless specially indicated.

In homogeneous solution the diffusion coefficient was calculated as follows: $D_{diff} = \frac{\omega_{xy}^2}{4\tau_{d,1}}$.

Results and Discussion

Fluo-ATP repartition in cardiomyocytes under phosphorylative conditions

Figure 1 shows the confocal microscopic image of isolated intact cardiomyocyte in the presence of Fluo-ATP. Under our experimental conditions (30 min incubation at 37°C with 1µM MitoTracker green and 200 nM Fluo-ATP) the ATP analogue does not enter non permeabilised cells. We observed a strong red fluorescence of Fluo-ATP in the surrounding medium of the cardiomyocyte but only the green fluorescence of MitoTracker inside the cell. Figure 2 shows the results of another experiment in which saponin (25 mg/ml) was used to permeabilise the plasma membrane in the presence of 1 mM ADP and respiratory chain substrates. In this case the uptake of Fluo-ATP was very rapid (few seconds). The distribution of Fluo-ATP was not uniform showing regular pattern with preferential accumulation in the

areas of mitochondrial localization at the level of sarcomeres but absence at the level of Z-lines (Fig 2A). A similar distribution was observed for the mitochondrial dye MitoTracker green (Fig 2B), as already shown in previous work [7,8]. The double staining Fluo-ATP /MitoTracker green allowed to obtain merge images (Fig. 2C) where the yellow color (representing an overlap between the two fluorescent probes) was preponderant. The signal overlap was confirmed by a colocalisation scatter plot (Fig. 2D) and an intensity plot (Fig. 2E) which clearly show that there is a strong overlap and a positive correlation of the MitoTracker Green and the Fluo-ATP signals under conditions of active oxidative phosphorylation. However, we observed also some diffuse Fluo-ATP signal, outside of mitochondrial areas.

Fluorescence correlation spectroscopy study of Fluo-ATP in solution

The Alexa Fluor 647 conjugate of ATP is a probe well suited for the *in situ* FCS measurements, due to its (i) long wavelength excitation/emission far from the cellular autofluorescence, (ii) important photon count rate per molecule and (iii) very good photostability [9]. However, like some other far-red fluorophores (e.g. Cy5) Alexa 647 was reported to possess a strong environment-dependent µs-scale bunching kinetics [10] that may in some cases interfere with the translational diffusion kinetics measured from fluorescence AC, but can also provide rich information on microenvironment. It has been shown recently that one can take advantage of this property of the fluorophore for development of fluorescence lifetime-based probe for kinases inhibitors [11].

The AC curve of Fluo-ATP in vitro shows two distinct parts (Fig 3B): a short time-scale (~10 µs) bunching part and longer time-scale part corresponding to translational diffusion. The fast fluorescence bunching is thought to include the variable contributions of a triplet state transit, a photoinduced reversible cis-trans isomerisation and other environmental effects dependent on the irradiation intensity.

To differentiate between fluorescence bunching and translational diffusion the AC curves of Fluo-ATP were measured as a function laser power (0.4 – 72 kW/cm^2, 633nm) in water, Mitomed solution and in supernatant of mitochondrial extract (Supplementary data, Fig. S2) The observed shortening of the bunching time-scale with the increase in laser power was consistent with the previous observations on cyanine derivatives [12,13] and confirmed the non-diffusive nature of this part of the AC curve.

As expected, the longer time scale part of AC curves was almost insensitive to the variation of the excitation power. The only moderate shortening of translational diffusion was observed at the highest excitation intensities due to the photobleaching of the dye molecules (data not shown).

Due to the complex nature of AC curves, they were fit with a model including stretched-exponential bunching and a multicomponent 3D translational diffusion (see Material and methods). The high degree of freedom of the stretched exponentials allowed better fit of the bunching thus minimising the possibility of its cross-talk with rapid diffusion components. Hereafter only the results of fit of the diffusion part will be discussed.

In vitro, the AC curves were successfully fit with the mono-exponential diffusion model. The resulting Fluo-ATP diffusion coefficient in water is $2.0\pm0.1*10^{-6}$ cm^2/s (τ_{diff} = 66±4 µs) at 20°C. This value coincide well with that reported elsewhere ($1.83\pm0.27*10^{-6}$ cm^2/s at 22°C, [14]) and is consistent with the slightly faster diffusion of unconjugated ATP published previously ($1.75\pm0.09*10^{-6}$ cm^2/s at 5°C and $3.68\pm0.14*10^{-6}$ cm^2/s at 25°C, [15]). In Mitomed and in the supernatant of mitochondrial extract the diffusion coefficients of Fluo-ATP slowed down to $1.4\pm0.1*10^{-6}$ cm^2/s (τ_{diff} = 92±6 µs) and $0.76\pm0.09*10^{-6}$ cm^2/s (τ_{diff} = 170±11 µs), respectively. The 1.4 times slower diffusion in Mitomed compared to water was

also observed by FCS using another fluorophore TMRM (data not shown) and is probably due to the higher viscosity of this medium.

Fluo-ATP AC curves in isolated mitochondria and cardiomyocytes

Surprisingly complex, multicomponent AC curves of Fluo-ATP were obtained in permeabilised CMs and in suspension of isolated mitochondria (Figures 3B, 4). Figure 3A highlights the differences in the Fluo-ATP count rate trace in CM versus Mitomed solution showing a presence of longer photon bursts and higher amplitude of fluctuations in the former case.

The analysis of the obtained AC curves by fitting them to different diffusion models (LSM510 FCS 4.2 software) clearly showed the presence of several ranges of correlation in 10^{-4} to 10^{-1} s time scale (Table 1). We used here the simplest Brownian diffusion approximation of Fluo-ATP kinetics in the aim to quantitatively compare their relative contribution to the total AC under different physiological conditions. In some cases, an individual component was found to be close to a free diffusion time measured in model conditions and was then fixed to this value during fit.

The choice of FCS models always relies on the prior hypothesis on the type of kinetic behaviour, and, of course, may not be reduced here to a simple sum of several 3D Brownian components. Tested separately, the *anomalous* diffusion model with two components was also able to fit AC curves resulting however in sub- and "super-" diffusion factors [16] that were difficult to interpret. Strictly speaking, the term 'diffusion' is inappropriate to describe the AC in such a complex system. One should employ the more general term 'correlation', which accounts for as many diverse processes as: passive diffusion, active transport, organelle traffic, mitochondria inner membrane remodelling, enzymatic reactions and all rapid changes of the microenvironment resulting in modification of the brightness of Fluo-ATP sub-populations. Moreover, the supposed presence of ATP pools with locally restricted diffusion (either isolated by membranes or by the functional channelling) may lead to the effects of ergodicity breaking, memory and aging of the system and generally needs a revisiting of the basis of FCS theory [17,18].

In both isolated mitochondria and cardiomyocytes, most of fits were three-exponential, as shown in Fig. 3B. The mean values of correlation time of diffusion components and their relative contribution are listed in Table 1. The two-exponential model was only used in the cases where the contribution of one of three components was negligible (e.g. under conditions of oxidative phosphorylation).

The value of short correlative component in isolated mitochondria was systematically found to be close to Fluo-ATP diffusion time in the supernatant solution (170 μs) and was then fixed during a new fit to this value (Table 1). The presence of this component seems to be logic when one compares the axial size of the confocal detection volume (~ 2.5 μm) to the typical size of individual mitochondrion (~ 1 μm). Due to this size difference as well as imperfect alignment of the laser spot and the organelle and its natural movement, a part of photon counts was collected outside of mitochondrion.

Contrary to the isolated mitochondria, no particular correlation time could be fixed during the fit procedure in CMs since the cell to cell variations were rather important.

The second component in isolated mitochondria is in the millisecond range (Table 1). It may correspond to the restricted diffusion of Fluo-ATP in the intermembrane space, shuttling between this space and the extraorganelle medium, cycling of Fluo-ATP/ADP in the enzymatic reactions or binding to a mobile carrier. The penetration of the dye through the inner mitochondrial membrane to the matrix via the ANT is highly improbable due to the high specificity of the carrier.

The third component is about tens of milliseconds in isolated mitochondria. This slow component reflects the existence of Fluo-ATP pools and probably corresponds to the membrane delimited pools. In the assumption of free Brownian motion, this diffusion time should correspond to the diffusing spheres of hydrodynamic radius 0.3 to 1 μm. Thus, the value of 20 ms was obtained in a control FCS experiment using a water suspension of 0.17 μm fluorescent beads. However, FCS measurements performed with mitochondria labelled with Mitotracker red, the membrane potential insensitive dye, resulted in a slower AC decay with a characteristic time between 150 and 300 ms.

Finally, in permeabilised cardiomyocytes, a fourth component in the range of seconds was detected in non-phosphorylative conditions (data not shown). The relative contribution of this component did not exceed a few percents. We assume that this correlation time corresponds to the Fluo-ATP tightly associated to intracellular structures and cell movements.

Effects of mitochondrial activators or inhibitors on Fluo-ATP dynamics

The Figure 4 shows normalised AC curves of Fluo-ATP measured on CMS in phosphorylative and non-phosphorylative conditions or in the presence of ANT inhibitor atractyloside or creatine. For comparison, the 'reference' AC curve of Fluo-ATP in Mitomed solution is overlaid (blue AC curve). The red AC curve represents Fluo-ATP diffusion in permeabilised CMs. The important curve shift to the right indicates an increase in diffusion time comparing to aqueous and homogeneous solution (blue AC curve). Under conditions of oxidative phosphorylation (respiration substrates and ADP), the AC curve shifts back to the left (green AC curve) and tends to come closer to the curve in solution. Consequently Fluo-ATP diffusion becomes globally faster when oxidative phosphorylation is activated in CM. However, a drastically slower diffusion is observed in the same phosphorylative conditions after addition of ANT inhibitor atractyloside (brown AC curve). Here the general tendency is the curve shift to the right, i.e. closer to the AC curve in non-phosphorylative conditions. Similar results were obtained with isolated mitochondria (Supplementary data, Fig. S2), expect for atractyloside effect which was much apparent than in CM.

The Table 1 resumes the correlation times and relative contributions of different components after multiexponential fit as described earlier. It is remarkable that in non-phosphorylative conditions there is almost no "extramitochondrial" free diffusion of Fluo-ATP in isolated mitochondria. Indeed, the first component (170 μs) represents only 8% of total Fluo-ATP AC while most of correlation is in the ms and tens of ms ranges. Similar Fluo-ATP behaviour is observed in CMs where the shortest component is simply absent. The addition of respiration substrates (5 mM glutamate and 3 mM malate) does not change the Fluo-ATP diffusion behaviour both in isolated mitochondria and in cardiomyocytes. On the other hand, the addition of 1 mM ADP accelerates the diffusion of Fluo-ATP both in mitochondria and CMs until 66 % and 80 % respectively of Fluo-ATP diffusion is in the first correlation range.

We suppose that, this modification is due to mitochondrial production of non-fluorescent ATP leading to a rapid ADP/ATP turnover and a competition with Fluo-ATP for binding sites. This rapid diffusion was slowed down again upon addition of the ANT inhibitor, atractyloside which inhibited the mitochondrial respiration. The effect was most pronounced in isolated mitochondria (only 27 % of the rapid component and 20% of the slower one), and in CM one can notice also appearance of 6 % of slow component. Atraclyloside thus probably leads to recovery of Fluo-ATP pools bound to intramitochondrial or intracellular structures.

Effect of creatine kinase activation on compartmentation of Fluo-ATP

The addition of creatine to CMs in phosphorylative conditions leads to the slowing down Fluo-ATP diffusion time. 41 % of Fluo-ATP exhibit a correlation time in the range of tens of ms, whereas such a slow diffusion is found only in absence of ADP. Both creatine kinase (CK) isoforms cytosolic and mitochondrial are indeed able to generate locally ATP or ADP pools [18]. In the cytosol, the presence of MMCK (cytosolic muscle isoform) allows a local turnover of ATP in the sites of ATP consumption (membrane or myofibril ATPases). In the mitochondrial inter membrane space, MtCK (mitochondrial isoform of creatine kinase), by functional coupling with ANT [19], supplies a local regeneration of ADP (ADP turnover) for the oxidative phosphorylation (ATP synthase). This increase in correlation time of Fluo-ATP indicates that actively functioning CK-system is able to influence the heterogeneity of diffusion of AT/DP within the mitochondrion and the cell.

Conclusion

Investigation of Fluo-ATP diffusion by Fluorescence Correlation Spectroscopy reveals heterogeneity of its diffusion in mitochondria and within CM that is related to its restricted mobility and compartmentation.

Further studies are needed to identify the sites of ATP localisation and binding in the cells, and for directly visualizing the microcompartments of ATP.

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Figure legends

Fig.1. Impermeability of the plasma membrane to ATP-Alexa by confocal miscoscopy. The cardiomyocytes were incubated 30 min at 37°C with 1 μM of MitoTracker green before addition of 200 nM of ATP-Alexa. The red fluorescent dye does not enter the cell in these conditions. In the next experiments 25 mg/ml of saponin will be add to permeabilised the plasma membrane.

Fig. 2. Confocal image of permeabilised cardiomyocyte labelled with the MitoTracker green and incubated in the presence of 0.5 nM Fluo-ATP in the Mitomed solution. The distribution of Fluo-ATP shows the preferential localisation in the nodules of the regular pattern (**A**).

Mitochondria distribution (**B**) overlaps that of Fluo-ATP as shown by the merge in the panel **C**. The scatter plot (**D**) of the whole image witnesses a strong positive correlation and overlap between both signals (right-hand table). The intensity plot (**E**) along a white line indicated in panel **C** shows a concomitant variation of Fluo-ATP (red line) and MitoTracker (green line) fluorescence with a mean period about 2 μm. Scale bar 10 μm, SD – standard deviation.

Fig. 3. Typical fluorescence intensity fluctuations (**A**) and normalized AC curves (**B**) of the Fluo-ATP measured in aqueous buffer Mitomed (blue) and in permeabilised cardiomyocyte under conditions of oxidative phosphorylation (red). The insert in panel **A** zooms in a 20 s interval of count rate record highlighting the long-range fluctuations in case of cardiomyocyte. The corresponding red autocorrelation curve in panel **B** presents much slower decay. The curve fitting in this case was performed using a three component model (–). For comparison a one-component fit (---) is also shown and the residuals of both fits are .superposed in panel **C**.

Fig. 4. Shift of auto-correlation curves of ATP-Alexa diffusion in function of the functional state of cardiomyocytes. The blue curve (□) is the AC curve of the dye in solution (Mitomed medium). The red curve (○) is the AC curve for permeabilised cardiomyocyte, the green curve (+) is the AC curve in phosphorylative conditions (1 mM ADP, 5 mM glutamate and 3 mM malate), the brown curve (Δ) is the AC curve in phosphorylative conditions in presence of 30 μM atractyloside and the black curve (-) is in presence of both respiration substrates and 20 mM creatine.

Table 1 Correlation times of Fluo-ATP in isolated mitochondria and cardiomyocytes under different conditions. The values are completed by their standard deviations and their respective contribution in the model. CM-cardiomyocytes, Mt-mitochondria, sub-substrates, Atr-atractyloside, Cr-creatine.

System studied	Correlation time 1 (µs)	SD	%	Correlation time 2 (µs)	SD	%	Correlation time 3 (µs)	SD	%
extramitochondrial medium	**170**	-	100	-	-	-	-	-	-
Mt Alexa (+/- sub)	**170**	-	8	**1879**	1015	41	**16619**	6076	51
Mt Alexa sub ADP	**170**	-	66	**3700**	1279	34	-	-	-
Mt Alexa sub ADP Atr	**170**	-	27	**2751**	1367	53	**47762**	17868	20
CM Alexa (+/- sub)	-	-	-	**746**	435	26	**17607**	7990	68
CM Alexa sub ADP	**161**	60	80	**2910**	511	20	-	-	-
CM Alexa sub ADP Atr	**370**	77	94	-	-	-	**39872**	20278	6
CM Alexa sub ADP Cr	**271**	118	59	-	-	-	**9988**	2121	41

Fig 1

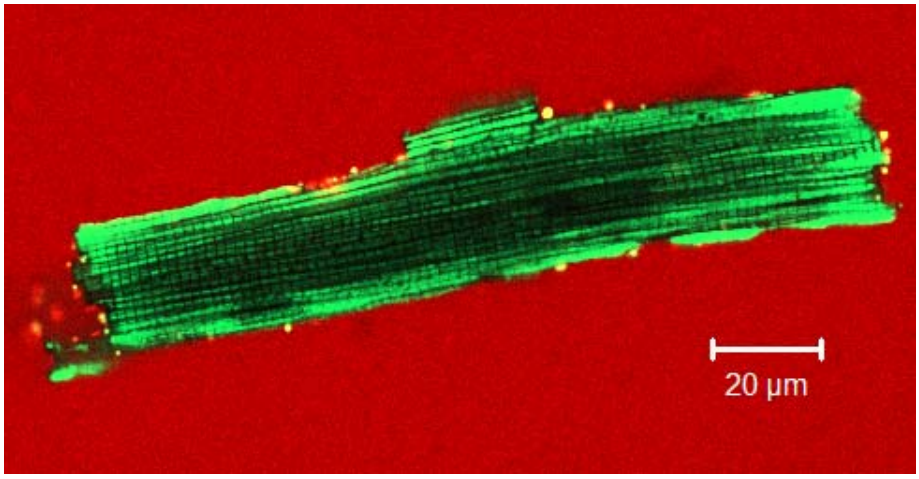
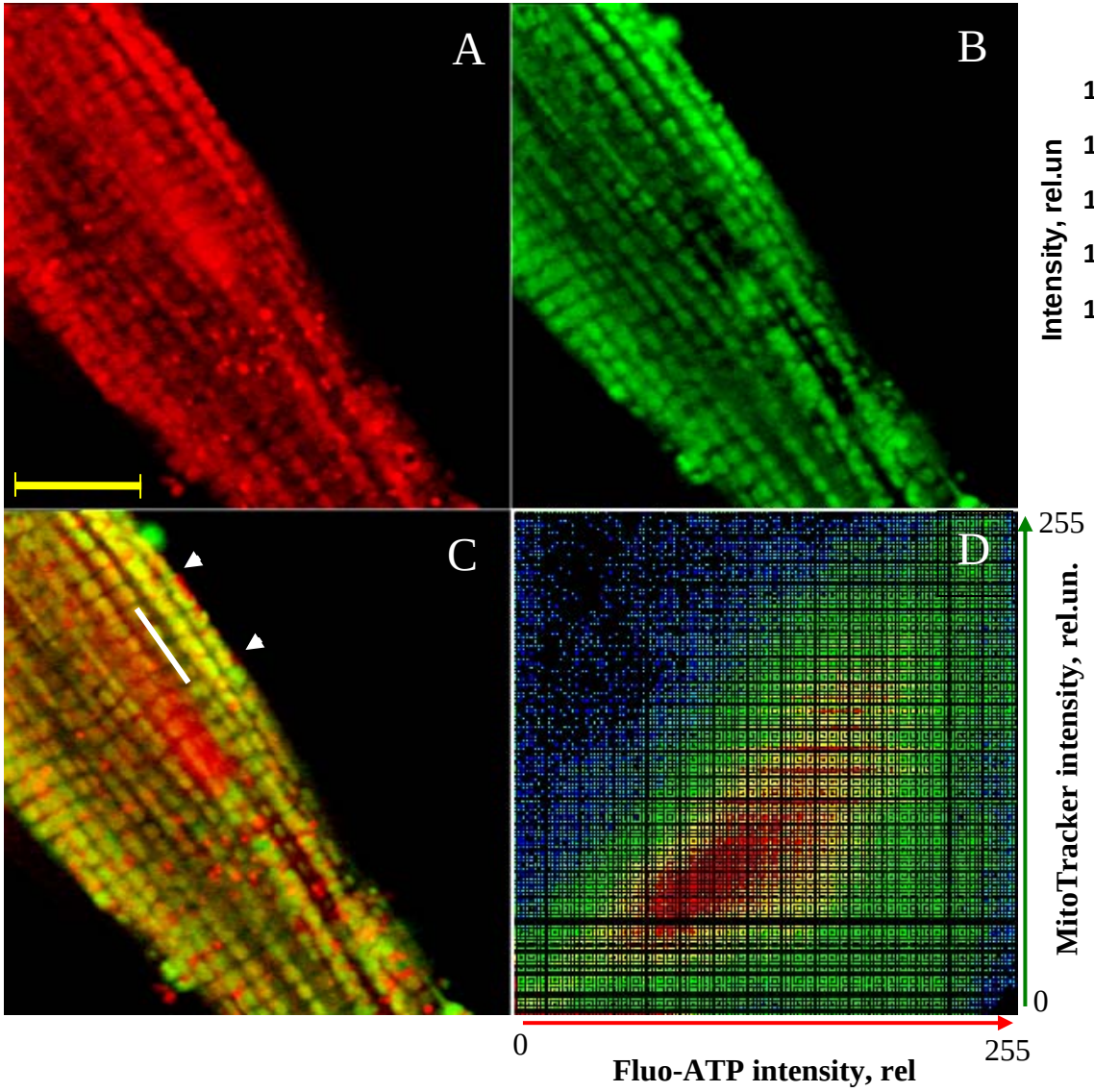
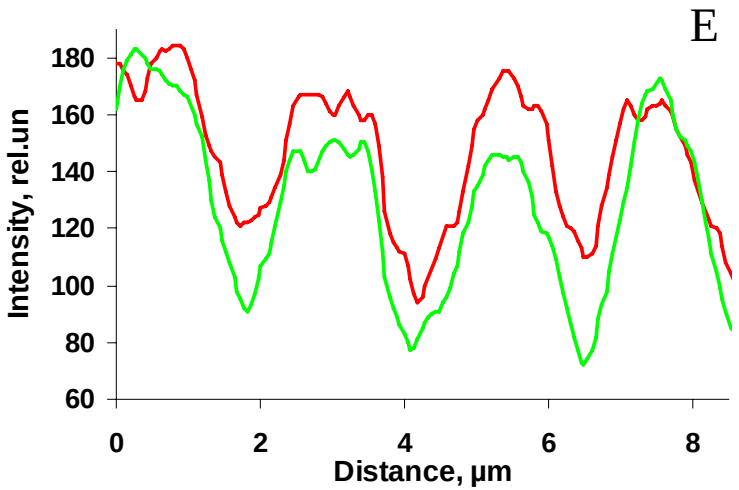


Fig 2





SD MitoTracker green	SD Fluo-ATP	Overlap Coefficient	Correlation Coefficient
75	**60**	**0.90**	**0.85**

Absolute pixel frequency

Fig 3

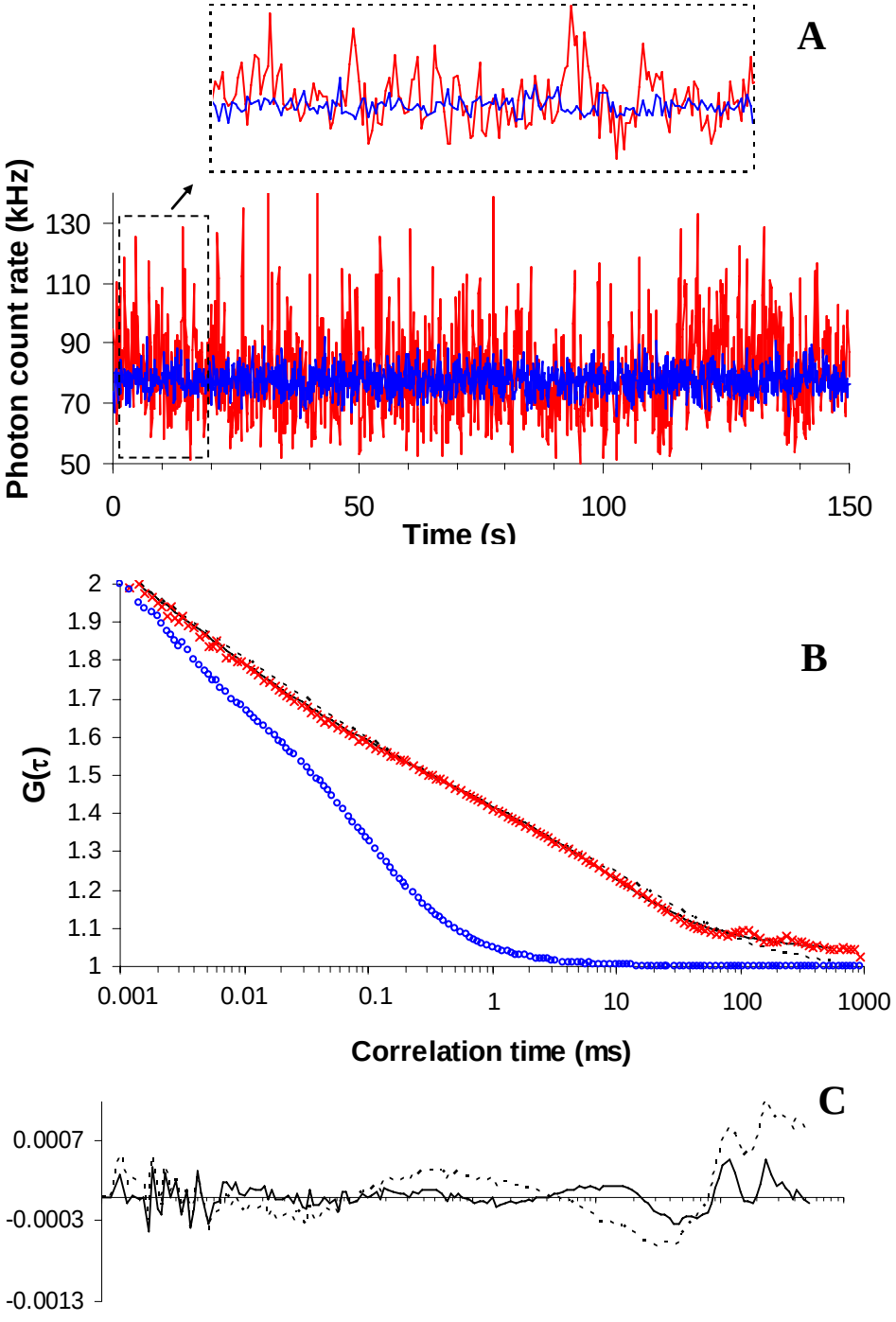
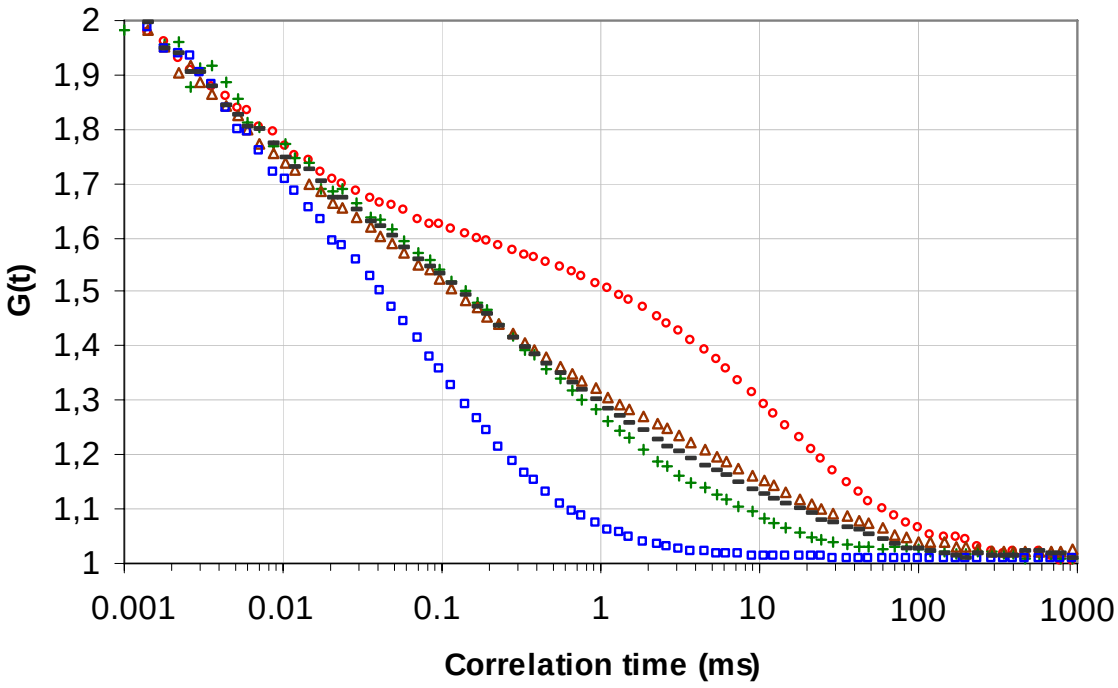

A
Photon count rate (kHz)
130
110
90
70
50
0
50
100
150
Time (s)
B
G(τ)
2
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0.001
0.01
0.1
1
10
100
1000
Correlation time (ms)
C
0.0007
-0.0003
-0.0013

Fig 4


G(t)
2
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1,8
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1
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1
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Correlation time (ms)

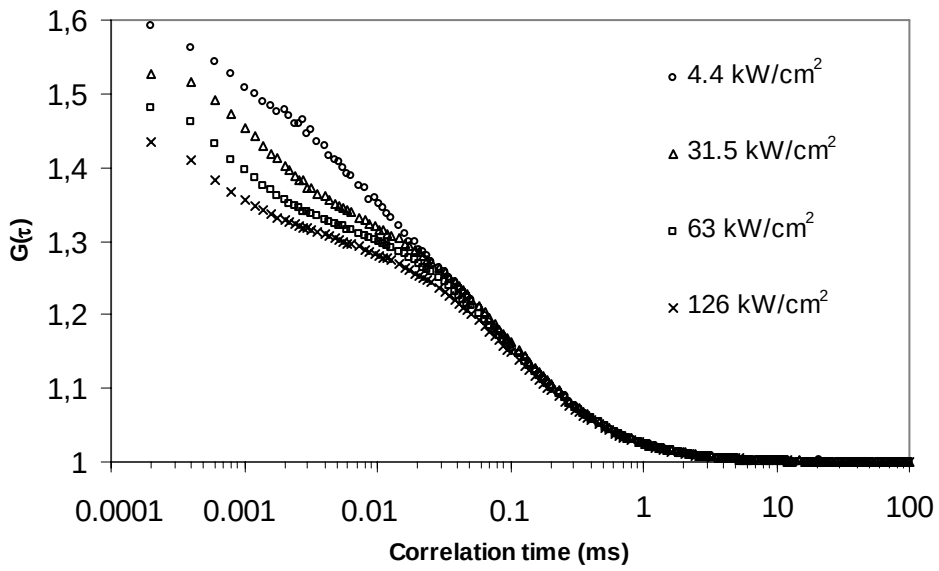

Fig. S1. Fluorescence autocorrelation curves measured in Mitomed solution using various powers of the laser spot on the sample.

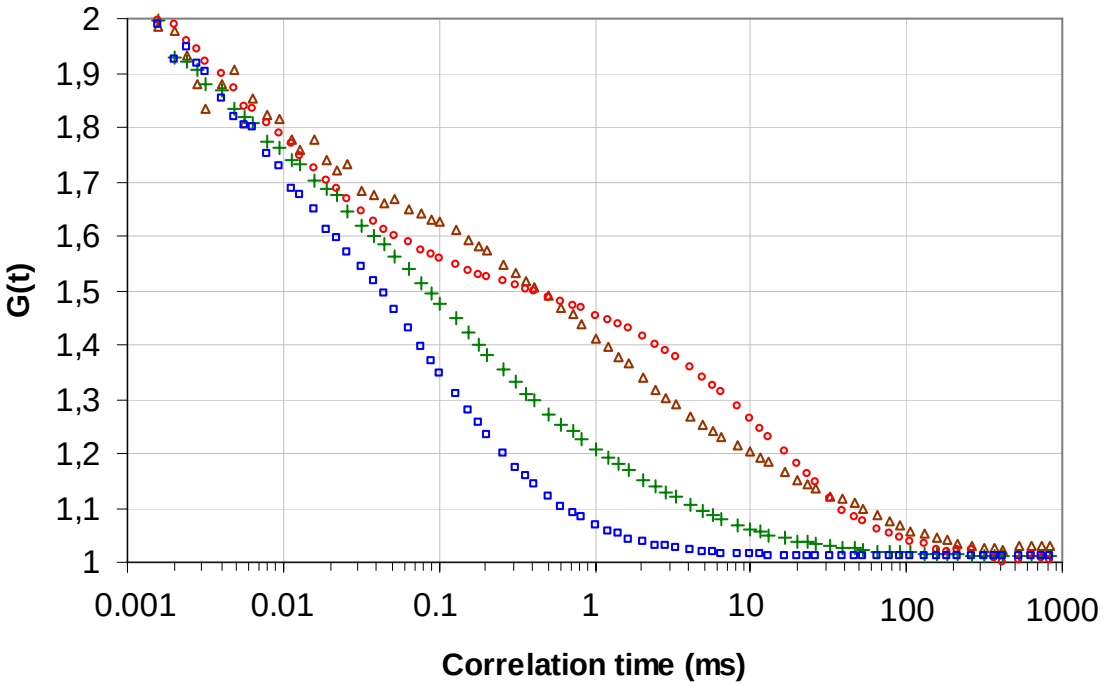

Fig. S2. Shift of auto-correlation curves of ATP-Alexa diffusion in function of the functional state of isolated mitochondria. The blue curve (□) is the AC curve of the dye in solution (Mitomed medium). The red curve (○) is the AC curve for suspension of isolated mitochondria, the green curve (+) is the AC curve in phosphorylative conditions (1 mM ADP, 5 mM glutamate and 3 mM malate), the brown curve (Δ) is the AC curve in phosphorylative conditions in presence of 30 µM atractyloside.