

## REVIEW

**THE CREATINE KINASE ISOENZYMES IN ORGANIZED METABOLIC  
NETWORKS AND REGULATION OF CELLULAR RESPIRATION :  
*A NEW ROLE FOR MAXWELL'S DEMON***

by

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*“Research is to see what everybody has seen  
and think what nobody has thought”*

Albert Szent-Gyorgyi: Bioenergetics  
Academic Press, New York, 1957

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## SUMMARY

In this review we analyse the existing data on the cellular mechanisms of functioning of the creatine kinases and their role in the regulation of mitochondrial respiration in muscle cells. From large amount of experimental data some general conclusions can be made. The functionally coupled creatine kinases may be taken to play the intelligent role of Maxwell's demon in the cell, selectively displacing the creatine kinase reaction out of equilibrium in different directions in various compartments of the cells to ensure effective functioning of the energy transfer networks. This metabolic feedback regulatory mechanism is the only one explaining the wide range of changes in the rate of respiration at constant levels of high energy phosphates in the working heart muscle - the phenomenon of metabolic stability - under physiological conditions of regulation of heart function by Frank-Starling mechanism. The new methodological approaches – mathematical modeling in combination with careful experimental analysis of data on regulation of respiration in permeabilized muscle cells – are analyzed and used for quantitative description of intracellular restrictions of ADP and ATP diffusion and effective metabolic signaling by creatine kinases.

*Key words:* heart, skeletal muscle, creatine kinase, respiration, mitochondria, regulation.

## 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). Originally, the creatine kinase pathway, or “shuttle” theory was proposed in the early works by Bessman (6,7), by Klingenberg (8), and later confirmed in very multiple studies of large number of laboratories, and detailed reviews of all these works are available (1 - 5, 9 - 13). Their important role in the intracellular energy transfer puts creatine kinase systems into central position in cellular regulation of the energy metabolism, of the respiration and of the physiological functions of the cell, particularly muscular contraction and membrane excitability. The huge amount of information on the intracellular mechanisms of functioning of creatine kinases allow us to make some important general conclusions of their roles in the cell’s life, as it will be shown in this review. Among the other energy transfer networks, as adenylate kinase and glycolytic systems (1-5), the creatine kinase network is the most active one and represents probably the best studied examples of the phenomenon of functional coupling, which appears to be a general one in cellular metabolism. The nature and significance of this phenomenon is well illustrated by its analogy with the 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.

## **Part 1. Functional coupling phenomenon**

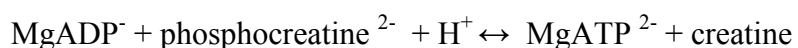
### Regulation of mitochondrial respiration *in vitro* versus *in vivo*.

The mitochondrial respiration is intimately regulated by the availability of the ADP to the adenine nucleotide translocase, ANT (14 -17). 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* (15,16). Further, mitochondrial respiration can be modulated by agents which influence the mitochondrial membrane potential, such as uncouplers, and by availability of substrates, inorganic phosphate etc. (13,14,17). The regulation of respiration in isolated mitochondria by ADP is a very well elucidated mechanism, the structure of the ATPsynthase ( $F_0F_1$  complex), of the ANT and its conformational changes related to the ADP-ATP translocation being resolved at atomic level (18,19).

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 other, the ADP necessary for regulation of respiration is supplied via energy transfer networks, mainly via the creatine kinase system (1-13). In this review we describe the general principles and mechanisms of functioning of these coupled creatine kinase systems.

### The classical creatine kinase equilibrium approach.

After findings by the Davies group in 1962 (20) that the physiological function of phosphocreatine in muscle cells is to rephosphorylate ADP in the creatine kinase (CK) reaction and thus to produce ATP for muscular contraction (an excellent description of the earlier history of studies of muscle cells' energetics was given by Mommaerts in 1969 (21)), the creatine kinase reaction:



was considered almost immediately as an equilibrium one in muscle cells *in vivo* with the purpose of effective buffering of ATP concentration, and thus just the “free energy reserve”. This was found by Veech et al. (22) to be consistent with experimental data on determination of the cytoplasmic phosphorylation potential in cardiac cells. On the basis of these findings, it is still accepted in many investigations that the CK equilibrium allows to calculate the free ADP concentration from known values of total creatine, ATP and phosphocreatine concentrations (the two latter ones being usually found by <sup>31</sup>P-NMR technique) according to the equation (23-25):

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

where the  $K'_{\text{eq}} = K_{\text{eq}} \times [\text{H}^+]$ . The equilibrium is shifted strongly in direction of ATP synthesis, the value of the apparent equilibrium constant for pH 7.0, 38°C and free  $[\text{Mg}^{2+}] = 1 \text{ mM}$  being equal to ~170, and respectively, the standard free energy change of the creatine kinase reaction in the direction of ATP synthesis is - 13.4 kJ/mol (26-28).

Meyer et al. (29) have analyzed the consequences of the possible equilibrium relationship of the creatine kinase in cell cytoplasm by showing, interestingly, that due to the equilibrium constant value, the steady state energy flux in contracting muscle is presented by the flux of the phosphocreatine, and thus is consistent with the phosphocreatine pathway of the energy transfer in the muscle cell cytoplasm. However, many parallel and further studies showed that this is not the main mechanism of functioning of the creatine kinase systems in muscle cells.

In fact, the problems with the equilibrium creatine kinase theory started to accumulate almost immediately after the works of Davies group, especially when this simple equilibrium

concept was used to explain the regulation of cell energy metabolism, particularly of mitochondrial respiration.

First, two years after the Davies work, Heldt et al. in Klingenberg's laboratory showed the existence of the mitochondrial form of creatine kinase, MtCK (30). Now it is known that this isoenzyme is encoded by two genes and expressed as sMtCK (sarcomeric) in muscle cells and uMtCK (ubiquitous) in non-muscle cells (31 -34), and the structure of MtCK is now known to atomic resolution (35). During the evolution, these MtCK appeared long before BB and MM families (36,37).

Second, it was found soon that 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, 10, 38 - 42 ). For what?

Third, and most important, it was found in studies of the mitochondrial ADP-ATP carrier, a key transporter of ADP into mitochondria from cytoplasm that this carrier has very high affinity to this substrate:  $K_m$  for ADP is in the range of 7 – 10  $\mu\text{M}$  (43). Accordingly, in studies of the isolated mitochondria the apparent  $K_m$  value for ADP in regulation of respiration was always found to be 15 – 20  $\mu\text{M}$  (15,16). Klingenberg (44) explained this observation by showing that the outer mitochondrial membrane permeability for metabolites was high *in vitro*. Now it is clear that this is because of the open state of VDAC in isolated mitochondria (45). In resting cardiac cells, the free cytoplasmic ADP concentration calculated from CK equilibrium is always around 40 – 60  $\mu\text{M}$  (46 - 49), that meaning that the respiration rate should be always very high, at the level of about 75 – 80 % of its maximal value, if calculated from simple Michaelis – Menten hyperbolic relationship. But this is not what is found in the experiments on intact heart, the respiration rate in resting state being very low, 15 – 20 times lower than  $V_{\text{max}}$ , and the rate of oxygen consumption by cardiac tissue increasing

linearly with the augmentation of workload induced by the physiological Frank-Starling mechanism (50,51). Situation became even more complicated when the phenomenon of metabolic stability of the heart, that meaning practically constant levels of phosphocreatine and ATP in the cells at any workload (52-54), was discovered. This was an important observation, meaning that the cytoplasmic ADP levels are dissociated from respiration rate and workload levels, and *vice versa*.

To solve the conflict between these two physiological observations – linear dependence of the respiration rate upon the workload and metabolic stability of the heart - and the convenient and simple equilibrium CK reaction theory, it was proposed that the respiration rate is not at all regulated *in vivo* by ADP, but by  $\text{Ca}^{2+}$  in parallel with regulation of contraction (“«parallel activation» theory”) (55-60). This may indeed save the situation: if creatine kinase reaction is in rapid equilibrium and maintains relatively high cytoplasmic ADP concentrations which practically saturate the ANT, the creatine kinase reaction will indeed not be a regulatory one and simply keeps mitochondria prepared for regulation by  $\text{Ca}^{2+}$  ions, mostly by alteration of substrate (NADH) supply for respiratory chain (60). Many experimental and theoretical studies have been performed to support this rather popular theory (57-65). And indeed, multiple complex interactions between mitochondria and cellular  $\text{Ca}^{2+}$  cycle have been identified (57,63,66,67). However, the principal question is whether the basic physiological mechanism of regulation of cardiac energetics and respiration by Frank-Starling mechanism involves or not any increase in calcium release from intracellular stores and correspondingly, the changes in calcium transients or calcium sparks’ amplitudes during workload elevation, as predicted by the “parallel activation “ and the equilibrium creatine kinase theories. As it will be shown below in the next section, this is not the case.

*The Frank-Starling law of the heart, the crisis of the «parallel activation» theory and of the creatine kinase equilibrium approach.*

Thus, to explain the role of the creatine kinase system in regulation of mitochondrial respiration, it is necessary to analyse first the validity of the theory of «parallel activation» of contraction and respiration by calcium ions in heart cells, together with the equilibrium (or quasi-equilibrium) theory of creatine kinase functioning. Interestingly enough, this question has its origin in long series of classical physiological experiments dating back to the beginning of the last century, to the period of 1912 - 1926. First, Evans and his co-workers showed, by using the dog's heart-lung preparation, that any increase of the cardiac work, whether by increasing the arterial resistance or by augmentation the preload (inflow into the heart) caused a corresponding increase in gaseous metabolism of the organ, whether measured by oxygen intake or CO<sub>2</sub> output, and Starling and Visscher showed further that both the oxygen uptake and cardiac work were linearly increased with the increase of the left ventricle volume (68). The results of Starling and Visscher were published in *Journal of Physiology* in 1926 in an article entitled "The regulation of the energy output of the heart". This was long before the discovery of the oxidative phosphorylation. The connection between ATP and PCr was understood in 1934, when Lohmann discovered the creatine kinase reaction (69). 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 (70). The latter result was the earliest indication of the functioning of the mitochondrial creatine kinase coupled to oxidative phosphorylation (see below). Taken into account that in 1926 the contraction was mostly related to lactate production ("lactate theory of contraction") (21), the article by Starling and Visscher, which related oxygen consumption to the cardiac energetics, was indeed a genial insight into the nature of the phenomenon and future studies of cardiac cells' energy metabolism. As were the results of this series of investigations, which led to establishing of the Frank-Starling mechanism as a basic law of heart physiology (Otto Frank had shown earlier that increase in the diastolic volume of the left



ventricle results in increase of the force of contraction (71)). As it has been discussed by Opie, the Frank-Starling law may be stated in many forms, but the most general and classical one is the following: “Within physiological limits, the larger the volume of the heart, the greater the energy of its contraction and the amount of chemical change at each contraction” (72). Notably, the chemical change includes the oxygen consumption. Now we know that this law reflects the force-length relationship of heart muscle cells and is related to the cross-bridge mechanism of sarcomere contraction (73-75), an increased venous pressure stretches the fibers more at the end of diastole, and systolic contraction is more vigorous with an increased stroke volume and force of contraction due to the increased number of active crossbridges (72). The important conclusion from these works is that the physiological mechanisms of regulation of mitochondrial oxygen consumption in heart *in vivo* are the changes in cardiac pre- and afterloads, both resulting in the changes in the sarcomere length due to alterations of the left ventricle end – diastolic volume. The alteration of the sarcomere length results in the changes in the calcium sensitivity of myofibrils and in changes of the number of active crossbridges involved in the contraction cycle in the cells (74, 75). In 1926 Starling and Visscher foresighted also the much later discussions on the «parallel activation» mechanism of respiration regulation in the future. They wrote: "There is evidence that adrenaline increases oxygen consumption at a given fiber length, without however altering the general correspondence between changes in diastolic volume and in oxygen consumption" (68). Now we know that adrenaline increases the calcium cycling in cardiac cells (76), but this does not mean “parallel regulation” of respiration by calcium ions, it means simply the modification of metabolic fluxes and feedback mechanism of regulation of respiration (see below).

In sixties and seventies of the last century the research in cardiac energetics advanced rapidly due to the development of molecular and cellular cardiology. In this area, two investigators - J.R. Neely and J.R. Williamson - made the most important contributions into our understanding of cellular mechanisms of the regulation of cardiac energy metabolism and

mitochondrial respiration. J.R. Neely developed the experimental model of isolated working heart preparation (often called Neely's preparation) (51) and confirmed the linear relationship between the rate of oxygen consumption and cardiac work, earlier observed by Evans, Starling and Visscher (68). In these studies he discovered the new phenomenon of metabolic stability – the constant levels of high energy phosphates, ATP and PCr, which are independent of workload and oxygen consumption (52). This observation was later confirmed in several laboratories, particularly by Balaban et al. (53) and Wan et al. (54). Neely and Morgan gave also an elegant and detailed explanation of the intracellular mechanisms of the interplay of the fatty oxidation and glycolysis in regulation of respiration and explained why the free fatty acids are the main substrates for the cardiac cells (77). J.R. Williamson et al. used, in one of their classical studies, the Neely's working heart preparation to establish quantitatively the interval of the rates of respiration in dependence of the workload regulated by the changes in the rate of left ventricular filling rate, by the means of the physiological Frank-Starling mechanism (50). They found that the rate of oxygen consumption by cardiac tissue changes about 15-20 times, from 6 - 12  $\mu\text{mol/g dw/min}$  in the rest up to 170  $\mu\text{mol/g dw/min}$  at the maximal workload under conditions of metabolic stability (50). In the first investigation on the metabolic stability in 1972 Neely et al. used glucose and glucose+ acetate as substrates for respiration (52), and Williamson et al. used as the substrates both octanoate and glucose (50). While glucose maintains lower levels of the steady state concentration of phosphocreatine in the cells than pyruvate or fatty acids (78), the metabolic stability is always observed independently from the nature of the substrate of oxidation.

These are the classical experimental data on the regulation of mitochondrial respiration in cardiac cells *in vivo* which should be explained by any correct and valid cellular or molecular theory.

As mentioned above, the equilibrium creatine kinase theory does not explain these observations, since in this case metabolic stability means stable and relatively high

cytoplasmic levels of ADP, in comparison with the affinity of mitochondrial ANT for this substrate, which therefore should activate respiration already at the rest, and needs the help of the “parallel activation” theory of simultaneous regulation of contraction and respiration by calcium ions to explain the physiological phenomena. Together, these two theories predict that in the rest there should be very low free calcium available in cytoplasm (low peak values of calcium transients), an increase in workload should increase these peak values of calcium transient and calcium, after the entry into mitochondria, will activate the substrate supply for respiratory chain. In principle, this is plausible, since among the multiple mechanisms of Ca-mitochondrial interactions, there are the effects of activation of the mitochondrial dehydrogenases by calcium discovered and described in details by Hansford, Denton and McCormack (57,58,61-63). It is clear that rapid uptake of calcium by mitochondria (57,58,61-67,79) is necessary for activation of the key dehydrogenases of the Krebs cycle and others, usually by the mechanism of increasing the affinity of these enzymes for their substrates (57,63). If this is indeed the main regulatory mechanism, the calcium concentration in the microdomains around the mitochondria should increase with an increase of the workload, and mitochondria should also respond to these changes, that meaning that the calcium concentration should change around its apparent  $K_m$  (or  $K_d$ ) value of the calcium uniport system. Could the predicted changes in calcium transients be observed and can these mechanisms explain the 15 – 20 fold changes in respiration rate under conditions of Frank-Starling law and metabolic stability *in vivo*?

The answer came from studies with the use of intracellular calcium probes injected into cardiac cells of intact heart muscle, and the answer is a clear “No”. Kentish and Wrzosek studied the changes in force and cytosolic  $[Ca^{2+}]_i$  after length changes in isolated rat ventricular trabeculae by micro-injecting of calcium specific probe fura-2 (80). A step increase in length of the muscle produced a rapid potentiation of twitch force but not the

calcium transient (80). Since direct release of calcium from sarcoplasmic reticulum (SR) into the microdomains close to mitochondria, due to their proximity, is a well-known phenomenon (66,67) and may mask the effects of length changes on calcium transients in cytoplasm, the authors used ryanodine and cyclopiazonic acid to inhibit the release of calcium from SR and its uptake by SR, respectively. While both the twitch and the calcium transients were reduced and prolonged by SR inhibition, the rise of the force after muscle re-lengthening was not affected (80). The authors concluded that the rapid increase in the twitch force after muscle stretch was caused by length dependent properties of myofibrils, mostly due to increase in myofibrillar  $\text{Ca}^{2+}$  sensitivity. Earlier, similar conclusions were made by Allen and Kentish (81). Very recently, Shimizu et al. measured calcium and ventricular pressure transients on isovolumic and ejecting contractions in isolated, blood - perfused physiologically after loaded canine hearts at different left ventricle volumes (82). While there was an approximately linear relationship between peak pressure and left ventricle diastolic volume, as expected by Frank-Starling law, no detectable influence of the volume change on the intracellular calcium transients was observed, and the peak value of  $[\text{Ca}^{2+}]_i$  was always close to 800 nM (82).

Thus, the sarcomere length increase and corresponding elevation of cardiac work and energy consumption are observed at unchanged calcium transients within the contraction cycle.

These experimental data can be complemented by the results of the studies of Balaban's group on the effects of calcium on the respiration of isolated heart mitochondria *in vitro* (64,65). These authors studied the effects of calcium on mitochondrial respiration,  $\text{F}_0\text{F}_1$  ATPase and  $\Delta\Psi$ , and found that in the isolated and  $\text{Ca}^{2+}$  - depleted mitochondria, the respiration rate was already relatively high (196.6  $\text{nmolO}_2/\text{min}/\text{nmol cyt}_a$ ), but was rapidly increased 2 times (to 307  $\text{nmolO}_2/\text{min}/\text{nmol cyt}_a$ ) in response to the addition of calcium into

the medium to the final concentration 535 nM. At the calcium concentration of 600 nM the respiration rate was maximal, showing the phenomenon of saturation (64,65). Similarly, the State 4 rate was increased also by factor of 2 (64). This increase in respiration was observed in parallel to the increase of production of NADH in mitochondria (64,65). Since no uncoupling of oxidative phosphorylation by calcium under these conditions was seen, these observations indicated also the activation of ATP production in mitochondria, but the direct effect of  $\text{Ca}^{2+}$  on  $F_0/F_1$  remains unclear (64). Changes in mitochondrial calcium are rapid enough to participate in regulation of respiration, but can increase the respiration rate only up to 2 times with an increase of the free cytoplasmic calcium concentration up to 600 nM. Elevation of  $[\text{Ca}^{2+}]_i$  in this range in resting muscle can be easily achieved after adrenergic activation of the  $\beta$  - receptors by the activating calcium-induced calcium release (CICR) via increasing calcium influx through the slow calcium channel in cardiac cells' plasma membrane (76). This is totally consistent with one of the conclusion regarding the effects of adrenaline in Starling and Visscher work mentioned above.

However, if applied to interpret the physiological data described by Shimizu et al., these data by Balaban's group mean that mitochondrial respiration should always proceed with the constant rate rather close to  $V_{\text{max}}$  under *in vivo* conditions, since the peak value of Ca transient of 800 nM clearly exceeds the saturation level of the mitochondrial Ca-sensitive system, if we take into account that calcium transient is the composed of multiple calcium sparks next to mitochondria (66). This conclusion is in sharp contrast with all classical observations described above, starting with those by Evans, Starling and Visscher, then Neely et al. (52,68), Wan et al. (54), and notably with those by Williamson et al. (50). Further, the physiological range of changes in the mean cytoplasmic calcium concentration may extend up to 1 - 3  $\mu\text{M}$  (57), and the calcium concentration in the local areas (calcium sparks) may be even much higher. Indeed, there are good evidences of direct channelling of calcium

from sarcoplasmic (endoplasmic) reticulum to mitochondria (66). In these ranges of calcium concentrations, however, no effects of calcium on respiration rates could also be expected, since mitochondrial respiratory system *in vitro* is already saturated at 600 nM free calcium concentration (64,65). Thus, on the one hand both the kinetics of the action of calcium on mitochondrial enzymes *in vitro* and on the other hand *in vivo* studies of the effects of Frank-Starling mechanism on both of respiration and calcium cycle do not support the «parallel activation» theory. This theory, and with it the theory of creatine kinase equilibrium fail to explain the main physiological phenomenon, the 15 – 20 fold changes in respiration rate in cardiac cell induced by Frank-Starling mechanism under conditions of metabolic stability *in vivo* at saturating peak values of calcium transients (50,82).

Further, the rapid increase in workload was found not to increase the mitochondrial NADH content as predicted by the parallel activation theory, but, on the contrary, to cause rapid oxidation of NADH and electron carriers in the respiratory chain (83-86). Thus, these experiments directly showed the failure of both theories.

It should be mentioned, nevertheless, that mitochondrial calcium uptake and release cycle which is necessary for maximally activating the Krebs cycle dehydrogenases and thus the mitochondrial respiration (for that, 600 nM concentration in cytoplasm is sufficient) is also a necessary condition for the metabolic stability and metabolic feedback regulation of cellular respiration. Indeed, inhibition of the mitochondrial calcium uniporter by ruthenium red results in immediate loss of this phenomenon: in the presence of this inhibitor, workload increase always results in rapid decrease of the phosphocreatine levels (87). Thus, this is not ADP which, being in the excess, prepares mitochondria for regulation by calcium, but on the contrary, this is the calcium which keeps the mitochondrial systems in the activated state and ready for regulation by metabolic signals.

Therefore, we need to look for alternative non-equilibrium mechanisms of functioning of the creatine kinase system in the muscle cells *in vivo*. This alternative approach has indeed been very fruitful and led to discoveries of important phenomena and mechanisms, which give quantitative explanation of regulation of mitochondrial respiration in cardiac cells. One of these is the phenomenon of functional coupling, which leads us to another general concept in the history of science – to the concept of Maxwell's demon.

### *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,  $\Delta G_0$  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 realised 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.

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" (88). 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 (89).

The general theory of the metabolic channelling can be found in several monographs, notably in that by Ovadi (90- 92). Its application for the muscle cells, particularly for cardiac muscle, has been recently reviewed (92). Below we analyse the data relevant to the cellular mechanisms of regulation of respiration in the cells with high energy fluxes.

#### *The coupled creatine kinase reactions.*

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 chapters of this volume and in many excellent earlier reviews (1,3,31-35). 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 by the mechanisms of functional coupling, to fulfil the intelligent function of Maxwell's demons, in particular in the regulation of the mitochondrial respiration and oxidative phosphorylation in intact adult muscle cells, to achieve the maximal efficiency of regulation and metabolic stability.

*a) Mitochondrial creatine kinases.*

Most of information of functional role of the mitochondrial creatine kinase has been obtained in studies of heart mitochondria, sMtCK and in some lesser extent in skeletal muscle sMtCK and also for brain and smooth muscle mitochondria, in both cases *μ*MtCK (31-34). The work of Belitser and Tsybakova on muscle homogenates showing constant PCr/O<sub>2</sub> ratio was first to describe the activation of respiration by creatine, due to creatine kinase reaction, as it was already mentioned above (70). Bessman and Fonyo showed in isolated heart muscle mitochondria that addition of creatine increased the respiration rate in the State 4 (presence of ATP) (93). Similar data were reported by Vial et al. (94). 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 (95). 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 (39) 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 (96). The result of the use of the modeling, then the new approach was the conclusion 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 (39,96). 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 (96). 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 channelling possible (96). In this way the shadow of the Maxwell's demon was first seen in this field. 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 (97). 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 (97). The Haldane relationship for the creatine kinase reaction was no more valid, showing the involvement of some other processes – oxidative phosphorylation and ANT (97). 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 (95-97). 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 (98) and by the thermodynamic approach by De Furia (99), Saks et al. (100), and Sobol et al. (101). 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. (102-105). 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 (102). 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 (103-106). 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 (see below).

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 (107).

Structural aspects of the MtCK and its functioning have been extensively studied in excellent experimental investigations by Theo Wallimann group (35,108-110). Among their achievements is description of a spatial structure of this enzyme at 2.8 Å resolution (35). The peculiarity of the MtCK, in contrast with other dimeric CK isoenzymes (MM and BB), is that it forms octameres (1,109). It is still not yet completely clear whether in intact mitochondria there are both forms (dimeric and octameric) of the MtCK present (111), but the existence of MtCK in mitochondria 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 (112). 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 (113-115). This is well described in the chapter by Schlattner and Wallimann in this volume. Kuznetsov et al. have used the labeled oxidized dialdehyde analogs of ATP or ADP to determine the number of ADP binding sites of MtCK (protected by substrates) and in ANT (carboxyatractyloside sensitive) in isolated rat heart mitochondria and found that the ratio of ADP binding centers of MtCK to carboxyatractyloside binding sites of ANT was found to be 1:1 in many types of mitochondria (116). Since each monomere of

MtCK contains an active center (108) and carboxyatractyloside binds to a dimer of ANT (43), the stoichiometry found by Kuznetsov means that each dimer in one octamere of MtCK may be functionally interacting with a cluster of 4 ANT monomers, a tetramere. Further, it was found in studies with mitoplasts (mitochondria without the outer membrane) that binding of the inhibitory monoclonal antibodies to MtCK inhibits simultaneously both the MtCK and ANT activities (117), this conforming to the idea of the close spatial proximity of the MtCK active centers and ANT nucleotide binding sites.

In the case if the MtCK is bound to the membrane in its dimeric form (111), the stoichiometric data by Kuznetsov (116) and inhibition of ANT by antibodies to MtCK mean that most probably this dimer may cooperate with the tetrameric cluster of the ANT (118). The existence of the tetrameric form of ANT in the mitochondrial inner membrane was confirmed by Vignais data (118,119). In fact, there is no difference for the structural relationship between the ANT and MtCK whether dimers of MtCK are separately fixed at the membrane or organized into octameres at the outer side of the inner mitochondrial membrane (120). In the latter case ANT clusters should also be organized in more ordered manner, but the ratio of MtCK/ANT and structural proximity may stay unchanged.

The structure of the ANT was recently resolved at 2.2 Å resolution by Brandolin group in Grenoble (19). The translocation of both ATP and ADP in the Mg-free forms is related to the conformation changes of pore-forming monomers (19). Klingenberg's group thinks that both monomers within a dimer 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") (121-124). This conformation change ("pore") mechanism leads in its simplest version to the Ping-Pong reaction mechanism of transport (123). On the other hand, the kinetics of ATP-ADP exchange conforms to sequential mechanism of the simultaneous binding of nucleotides on both sides

(125). 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 dimers 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 (126,127).

This well coordinated transmembrane transport of ATP and ADP by the ANT monomers within di- or tetramers by conformation changes of transporter proteins in heart, brain, skeletal muscle and many other types of mitochondria is the Maxwell's demon mechanism which obviously controls the functioning of the MtCK. This control mechanism was recently quantitatively described and analyzed recently by Vendelin et al. on the basis of the thermodynamically consistent approach (128). In this study, two alternative mechanisms were studied: (1) dynamic compartmentation of ATP and ADP, which assumes the differences in concentrations of the substrates between intermembrane space and surrounding solution due to some diffusion restriction; (2) direct transfer of the substrates between MtCK and ANT. The mathematical models based on these possible mechanisms were composed and simulation results were compared with the following experimental data: (a) changes in the apparent kinetic properties of the MtCK reaction when coupled to oxidative phosphorylation (97,100); (b) competition between MtCK-activated mitochondrial respiration by competitive ATP-regenerating system (102); and (c) studies of radioactively labelled adenine nucleotide uptake by mitochondria in presence of MtCK activity (98). According to the analysis, dynamic compartmentation hypothesis is not sufficient to reproduce the measured values of apparent dissociation constants of MtCK reaction coupled to oxidative phosphorylation. Regardless to the used diffusion restriction between microcompartment and surrounding solution for ATP and ADP as well as other parameters of the model, the measured apparent dissociation constants of ATP both from ternary and binary complex with MtCK were not

reproduced with the model simultaneously (Fig 1). Situation is different, if the direct transfer of ATP and ADP between ANT and MtCK is assumed. In this case, all the analyzed experiments can be reproduced. For this, several changes in free energy profile of MtCK-ANT interaction are required. Namely, the free energy of ANT state with ANT binding site directed towards the intermembrane space and ATP attached has to be changed. The changes are shown in example of free energy profile of MtCK reaction coupled to ANT (Fig 2). In this free energy profile, the free energies of MtCK-ANT complex before the transfer of ATP<sup>4-</sup> to MtCK are considerably elevated and the free energies after transport of ADP<sup>3-</sup> to ANT are slightly dropped. Due to such changes, the synthesis of PCr from ATP which is transferred from mitochondrial matrix by ANT becomes energetically advantageous. The net free energy change during the transfer of ATP<sup>4-</sup> from ANT to MtCK, MtCK reaction, and the transfer of ADP<sup>3-</sup> from MtCK back to ANT, is negative and ranges from -3.7 kJ mol<sup>-1</sup> to -20.7 kJ mol<sup>-1</sup> depending on the states of MtCK-ANT complex at the beginning and the end of coupled reaction along the main pathway (the thick lines in the scheme). Note, that if the free energies of MtCK-ANT states would be kept the same as in the uncoupled case, then the corresponding net free energy difference would be from -0.3 kJ mol<sup>-1</sup> to +16.7 kJ mol<sup>-1</sup> (see boxes with the dashed borders in Fig. 2).

Thus, both structural and functional data available now show convincingly that the oxidative phosphorylation controls, as a Maxwell's demon, 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 (70). Much later Kim and Lee



showed the same effect for isolated pig mitochondria (129). 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 (130,131). 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 (132), this preventing from the cell death by inhibiting apoptosis and necrosis. This again illustrates the vital importance of the functional coupling phenomenon and Maxwell's demon principle for the cell life.

#### *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 (133-150). In spite of the very high activity of the glycolytic enzymes and of total creatine 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 than in heart cells, and no metabolic stability is observed (133-136). Instead, the muscle fatigue is a common phenomenon if the PCr pool is exhausted (137-140). The differences in the organization of the creatine kinase shuttles in different muscles have recently been extensively analyzed (141). In the cytoplasmic compartment, the MM creatine kinase seems to be indeed 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 (*133,142*), which is the substrate for the coupled creatine kinase in the myofibrillar compartment. This equilibrium position shifting is, however, a slow and difficult job to do in the absence of any aid from Maxwell's demon, and therefore the net rate of phosphocreatine production in the cytoplasm is never adequate to the rapid contraction, this leading to the loss of the metabolic stability and muscle fatigue.

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 (*143,144*). As it has been described in the early works by Michael Mahler (*144*) and later by Kent Sahlin group (*143*) and by many others (*141,145*), 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 (*146*).

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 (*147-154*). This is another example of the functioning of the principle of the Maxwell's demon in muscle cells, and this coupling is necessary for smooth running of the contraction cycle. 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 (*137,139,140,150-156*). 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 (*140,155,156*). Indeed, because of the structural similarity with MgATP, MgADP binds easily to the actomyosin with inhibition constant  $K_i$  being in the range of 200  $\mu\text{M}$  both in MgATPase reaction and in sliding of fluorescent actin on myosin (*140,155,156*). Thus, accumulation of MgADP fixes the crossbridges in their rigor states and

by inhibiting the contraction contributes in muscle fatigue (137-139). 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 (20), 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 (141,148,157-159). 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, and does the Maxwell's demon has anything to do with that system? Wallimann group has shown that the MM CK is bound specifically to the M-line due to (150,158,159), and significant part of this isozyme is found in the space of I - band of sarcomeres (147). *In vitro*, the interactions of myosin and CK have been known for a long time (149). 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 (141,157). 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  $K_m$  value (148). In accordance with this, Sata et al. found that sliding velocity of fluorescently labeled actine on a cardiac myosin layer coimmobilized with cardiac myosin showed significantly smaller apparent  $K_m$  for MgATP than in the absence of CK (160). 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 (161). 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 (162-164). The results of <sup>31</sup>P-NMR inversion transfer studies by Joubert et al. directly confirmed this conclusion (165).

Thus, an increase of the number of active crossbridges due to the Frank-Starling phenomenon during workload changes results in the rapid use of the phosphocreatine and liberation of creatine as an initial metabolic signal in the feedback regulation of respiration. This induces a small-scale cyclic changes in the cytoplasmic MgADP concentration, and both signals are strongly amplified in the coupled mitochondrial creatine kinase reaction, as it was revealed by mathematical modeling (164), explaining the linear dependence of the rate of oxygen consumption upon the workload and the famous phenomenon of metabolic stability.

### *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) and the Maxwell's demon principle 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 and intracellular membranes are the mechanism of intracellular signalling and 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 (166-169). 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<sup>2+</sup> content, while the exogenous ATP regenerating system (phosphoenol pyruvate and pyruvate kinase) was less effective (167). 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<sup>2+</sup> sparks (170). Thus, despite the presence of millimolar levels of cytosolic ATP, depletion of phosphocreatine impairs the Ca<sup>2+</sup> uptake (167-170). All these data clearly show the importance of the MM CK, bound to the membrane of SR, in rapid regeneration of local MgADP produced in the Ca,MgATPase reaction, independently from cytoplasmic situation and thus clearly in non-equilibrium manner, again in accordance with the Maxwell's demon principle. 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 (171).

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<sup>+</sup> (K<sub>ATP</sub>) channel acting as an alarm system to adjust cell electrical activity to the metabolic state of the cell (172-174). 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 (174,175). 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 (176,177). The MM creatine kinase was first described in the purified rat heart sarcolemmal preparations by Saks et al. already in 1977 (178). 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  $K_{ATP}$  subunit SUR2 (175). Abraham et al. (174) and Selivanov et al. (176) 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  $K_{ATP}$  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. (179) showing the 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  $\beta$ -cells (180).

These very detailed experimental data show that the Maxwell's demon principle is 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.

Thus, it seems that the Maxwell's demon principle is a central one in covering the cardiac cell's energy metabolism and both electrical and contractile functional activities.

## **Part 2. 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 (181,182), 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 (183), for which the Maxwell demon is a stranger. However, we have seen above the serious failure of this simple theory and its inconsistency with the experimental observations in the field of cardiac physiology and metabolism. What is wrong with this theory of the homogenous cell metabolism?

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 a homogenous solution is at least a naïve and a very rough approximation to the reality. Fig. 3A 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 (184, unpublished experiments). Fig. 3B shows the immunolabeling of the microtubular system of these cells after their fixation and permeabilization. The microtubular system wraps the mitochondria in the cells, and to these systems made visible by confocal imaging and immunolabelling, one should add the other important components of cytoskeleton, such as desmin, plectin, etc. (185), and of course the sarcomere structures. 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 (89,186,187). The studies of the mitochondrial function and its regulation *in situ*, in the intracellular medium have led to the conclusion of the unitary nature of the muscle cell metabolism, and to the understanding that one of the important regulatory factors of energy metabolism is the structural organization.

First, it is important to find out how the structure influences the diffusion of ATP and ADP, since this is the structure that is responsible for their compartmentation. Jacobus was one of the first authors to think and analyze the role of the diffusion and concentration gradients in feedback regulation of the metabolism (188). He analyzed the possible diffusional interactions between a mitochondrion and a myofibril and found that the concentration gradient of creatine is much more favorable for the feedback regulation of respiration than that of ADP (188). Kammermeier came to very similar conclusion (189). Intensive further experimental investigations in the area of cellular bioenergetics have been in great details in three special volumes of Molecular and Cellular Biochemistry (190-192). An important information was obtained when regulation of mitochondrial respiration in permeabilized cardiac cells was studied. These studies clearly revealed the heterogeneity of the intracellular diffusion of adenine nucleotides (193). Unusually high values of the apparent  $K_m$  for



exogenous ADP in permeabilized cardiac cells have been found in many laboratories since 1988 (194-206). Similar high values of this parameter were found in several other oxidative muscles (199,200), in hepatocytes (207), but not in fast skeletal muscle (202,203,206). 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 (199). Rupture of the outer mitochondria membrane by hypo-osmotic shock reduces the apparent  $K_m$  for exogenous ADP to the level of that for isolated mitochondria *in vitro*, that leading to the conclusion that the phenomenon is related to the decreased permeability of the mitochondrial outer membrane for ADP (196-198). The high values of apparent  $K_m$  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 (193,208). 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 (209-211). That means that all processes of energy metabolism may take place in a small space of the dimensions of several  $\mu\text{m}$ , 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. And this is the space within the ICEUs where the mitochondrial respiration regulation mostly by the creatine kinase system takes place. 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. Indeed, it can be seen from Fig.4 that the

regular arrangement and thus the structural and functional complexes of mitochondria with SR and myofibrils can be extremely easily destroyed by short proteolytic treatment. Treatment of permeabilized cells or fibers with 1  $\mu$ M trypsin for 5 minutes results in total disorganization of mitochondrial regular arrangement and structure of cell interior (Fig. 4). In line with previous data (203,209), this treatment significantly decreases the value of apparent  $K_m$  for exogenous ADP. Moreover, this disorganization of the cell structure and mitochondrial arrangement in the cells evidently destroys the direct channeling of endogenous ADP from MgATPases to mitochondria and significantly decreases the extent of compartmentation of ADP which becomes more accessible for externally added pyruvate kinase (Fig.4C). Fig. 4C shows that 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). This activation of respiration is due to the endogenous ADP production which was not maximally activated at the  $pCa = 7$ . This respiration was decreased only by 40 % after addition of a very powerful ADP consuming system of phosphoenol pyruvate – pyruvate kinase. The activation of the MtCK reaction by 20 mM creatine resulted in maximal activation of the respiration up to the real State 3 level (observed in experiments only in the presence of exogenous ADP in high concentration), in spite of the presence of the 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 (128), and the mtCK reaction exerted its central role in the control of respiration. 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. Since the outer membrane was not broken by this treatment (193), the explanation is the increase of the 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 (193).

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 (193). Hypothetically, this interaction may involve the interaction of cytosolic (cytoskeletal) proteins with the unusually long loop of VDAC molecule facing the cytosol (45). 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. (102-106) and shown in Fig. 5. 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. This is observed in isolated mitochondria (102) and in trypsin – treated fibers (193): in both cases the PEP-PK system decreases the respiration rate by about 50 % showing that ADP fluxes are distributed equally between ANT and outward fluxes (Fig. 5A). However, in intact permeabilized fibers, all ADP is taken up by the ANT and not available for the external PK-PEP system, this most probably showing the decreased and controlled permeability of the outer mitochondrial membrane under these conditions (Fig.5B).

Fig. 6 illustrates the roles of the coupled creatine kinases functioning in the non-equilibrium steady state within the possible structure of ICEUs in cardiac cells according to the Maxwell's demon principle. The microcompartments within ICEUs are formed due to the specific structural organization of the cell resulting in the local restriction of the diffusion of adenine nucleotides. In these microcompartments the CK functions mostly by the mechanisms of functional coupling. In fact, there is an interplay of many Maxwell's demons. All these coupled creatine kinases are united into effective metabolic system by phosphocreatine and

creatine as mobile energy carrier and feedback signal molecules, respectively. Due to effective functional coupling mechanisms, the effects of the creatine flux on respiration are reinforced by parallel flux of the Pi from ATPases to mitochondria (see also next section). The ATP in the bulk water phase of the cell represents most obviously some important reserve of the high energy compounds, and due to the heterogeneity of diffusion and its local restrictions, this ATP may be relatively slowly mixed with metabolically important ATP and ADP pools in these microcompartments, which take part in the functional coupling (193).

The local restrictions of the diffusion of ATP and ADP (193, 208), and the necessity of maintaining high local values of the phosphorylation potential in microcompartments near all ATPases, especially near the reversible ion pumps under conditions of high energy fluxes explain the existence of the organized creatine kinase and adenylate kinase networks of energy transfer and feedback signalling within the ICEUs, by now well and in great details described by the methods of the *in vivo* kinetic studies (2,5).

These conclusions were confirmed by Hoerter's group in *in vivo* studies of cardiac cell energetics by using NMR inversion transfer methods in combination with mathematical analysis (165,212,213). The results of NMR inversion experiments were analyzed by a model of steady state kinetic exchange between different compartments inside the cell, and the best fit was obtained for the three compartment system of both ATP and creatine kinase compartmentation (165). These are the mitochondrial, cytoplasmic and myofibrillar compartments, where the creatine kinase reactions function in different states and directions and their substrate concentrations are different (212). Remarkably, mitochondrial creatine kinase was shifted in the working heart in the steady state direction of aerobic phosphocreatine (and ADP) production, out of equilibrium, and in myofibrils – in reverse direction of ATP production (165,212). This is consistent with all biochemical data and with

the studies on permeabilized fibers, and with the results of our mathematical modeling, which is based on reaction – diffusion kinetics inside the cells (162,214).

And this is the direct experimental evidence of the validity of the Maxwell's demon principle in regulation of the cellular respiration, and of the non-equilibrium state of the creatine kinases.

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 (215,216). 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 a new quantitative methods of research – the mathematical modeling.

### **Part 3. Mathematical models of metabolic regulation.**

#### General principles.

Computational modeling of biological systems has gained great interest during the last decade. This modeling has been given the name of studies *in silico*. This concerns many different areas, but its perspective and importance are well formulated, for example, in the Physiome Project (217). *In silico* models are characterized (218) as a tool to perform computational experiments that should be accompanied by the validation against *in vivo* or *in vitro* experiments. The main aim of computations is then to predict the biological behavior. Also, using mathematical language, modeling describes the processes in the most

economical way and gives a new insight to possible interactions or limitations of the process in general. In other words, modeling may clearly say what depends on what.

There is a special feature in biological systems - the hierarchy. One has to distinguish between two types of hierarchy: the hierarchy of biological structures and the hierarchy of metabolic processes. The first is related to the architecture of living tissues. In case of cardiovascular systems it means the sequence : heart - cardiac muscle - fibers - myofibrils - sarcomeres - actin and myosin filaments. The second is related to the successive processes. The same case is then arranged as the following sequence: oxygen consumption - energy transfer -  $\text{Ca}^{2+}$  signals - cross-bridge activation - contraction. Whatever is the complexity of a biological system, its behavior is governed by thermodynamical laws (219). An important concept of modeling - the concept of internal variables (220) means distinguishing between macroscopic and microscopic behavior. Macroscopic behavior is observable and the corresponding variables are called observable. Microscopic behavior is hidden to the external observer but nevertheless depends on corresponding variables called internal. This formalism (220-222) introduces free energy (or Lagrangian) and a dissipation potential and permits so to derive all governing equations and satisfy the thermodynamical restrictions. In this context, the main novel idea is to introduce the concept of hierarchical internal variables. For example, in case of cardiac contraction, the observable variable describing the macroscopic behavior is stress which characterizes contraction. This is influenced by first-level internal variables - the amounts of cross-bridges producing force. These first-level internal variables are influenced by second-order internal variables - the activation parameter. This is influenced by the  $\text{Ca}^{2+}$  signal - the third-level internal variable.

The main difficulty of *in silico* modeling is involved in the complexity of governing mathematical system including various types of equations (for example, hyperbolic and parabolic). In modeling of metabolic regulation, two alternative approaches are commonly

used: based either on kinetic equations or on a (or several) biological objective function (223). The kinetic approach, which will be covered mainly here, is based on detailed description of enzyme reactions. Usually, this approach is used for description of relatively small-scale systems. Alternatively, one can model the metabolic systems by considering all possible intracellular fluxes which are consistent with the stoichiometry of reactions, i.e. flux balance analysis. By restricting the solution space through application of thermodynamic laws, thermodynamically consistent fluxes can be obtained (223-225). Together with the optimization of proposed biological objective functions, such as metabolic stability, for example, one can obtain the distribution of the fluxes in rather large and complex biochemical systems. As it was pointed out by Qian et al. (223), the main challenge of this approach "is to discover cellular principles in terms of optimalities, if they exist". Obviously, one can mix the both approaches by incorporating kinetic equations for some of the reactions and apply flux balance analysis with the thermodynamic constraints to study the system. Here, we will cover some of the results obtained by applying kinetic approach to study metabolic regulation of the heart muscle cells and outline the influence of creatine kinase system to the regulation. Our modelling is based on principles and concepts described above even when these are not explicitly shown.

#### *The models of regulation of mitochondrial respiration.*

One of the first models of regulation of mitochondrial respiration was developed by Bohnensack (226). This model was developed further by Korzeniewski (56) 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 (227). In one of

the versions of this model, the CK reaction in its equilibrium state was included (228). Neither realistic CK kinetics nor functional coupling between CK and ANT were considered (227-229). 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 in this review, the  $\text{Ca}^{2+}$  cannot pretend for the role of the parallel activator, and the mysterious alternative chemical compounds proposed by Korzeniewski, simultaneously activating both ATP production and utilisation have never been found (in this respect, the Korzeniewski's proposal recalls the failed "high energy chemical intermediate" theory of coupling of electron transfer in the respiratory chain to the phosphorylation, which was popular before the Michell's chemiosmotic theory and now has only an historical interest). No direct and real effects of the calcium on mitochondrial enzymes, such as Krebs cycle dehydrogenases, were included into the Korzeniewski's model.

Much more complete and probably the best of the existing mathematical models of mitochondrial respiration was recently described by Cortassa et al. (230). 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 (230) in good accordance with the experimental data by Balaban group (64,65), 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 (50-52,68). 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 Bers and Brandes (83-85), the supply of ADP from cytoplasm was beeded, in accordance with the theory of metabolic feedback regulation of respiration.

*Modeling the feedback metabolic regulation of mitochondrial respiration.*

We have seen above that the mitochondrial respiration rate *in vivo* may vary 20 fold, from 8-10  $\mu\text{mol min}^{-1} \text{g}^{-1}$  dry weight in resting (KCl-arrested) aerobic hearts to at least 170  $\mu\text{mol min}^{-1} \text{g}^{-1}$  dry weight in rat hearts (50). As it has been shown by Neely et al. and Williamson et al. (50-52), the oxygen consumption of the heart muscle is linearly dependent on the heart workload under conditions when the heart is metabolically stable (52,53). 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 (162) and then adapted by Vendelin et al. (193,214). This model describes the metabolic events within intracellular energetic units, ICEUs (89,193,209). The spatially inhomogenous reaction-diffusion model of energy transfer considers the reactions in three main compartments of cardiac cells: the myofibril together with the myoplasm, the mitochondrial intermembrane (IM) space, and the mitochondrial inner membrane-matrix space. This corresponds to the main components of the intracellular energetic units, ICEUs (193,209). The metabolites described by the model in the myofibrils and IM space are ATP, ADP, AMP, phosphocreatine (PCr), creatine (Cr), and Pi. All these metabolites diffuse between the cytosolic and IM compartments, where the metabolites are involved in the creatine kinase (CK) and adenylate kinase (AK) reactions. In addition, the ATP is hydrolyzed in the myofibrils. In the IM space the mitochondrial CK reaction is coupled to the adenine nucleotide translocase (ANT); the coupling is moderated by a diffusional leak of the intermediates. The metabolites described by the model in the matrix compartment and in the inner membrane are NADH, coenzyme Q, cytochrome c, protons,

ATP, ADP, and Pi. Three coupled reactions representing the production of protonmotive force by complexes I, III, and IV are included in the model, as originally described by Korzeniewski (56). Protonmotive force is consumed by ATP synthase and membrane leak. The ANT rate is considered to depend on membrane potential. Pi is transported by a phosphate carrier. The description of respiratory chain processes was adapted from model by Korzeniewski (56,214). By this model, the linear relationship between workload and oxygen consumption was reproduced in the simulations (214). The model describes quite satisfactorily the stable levels of PCr, ATP, and Cr at oxygen consumption rates up to 100  $\mu\text{mol min}^{-1} \text{g}^{-1}$  dry weight, in accordance with the experimental data (214). The metabolic stability is reduced together with maximal achievable  $\text{vo}_2$  if the level of total creatine content is reduced (231) or functional coupling between CK and ANT is not considered (89).

The model (214) showed that linear dependence of the respiration rate upon the workload under conditions of metabolic stability may be explained by small-scale oscillations of the cytoplasmic concentration of ADP and other metabolites during the cardiac cycle () predicted by the simulations of Aliev and Saks (162). This is due to manyfold amplification of the metabolic signals in the coupled creatine kinase reaction in mitochondria (165). The amplitude of ADP oscillations was increasing with an increase of the workload (214). The average level of computed inorganic phosphate  $\text{P}_i$  was low at low to moderate workloads (214) in accordance with NMR measurements performed on pyruvate perfused hearts (232). Thus, by this model it is possible to reproduce the measurements of PCr, ATP, and Cr levels at different workloads by simple feedback regulation mechanism.

Our model predicts an interesting feature of the regulation mechanism: the mitochondrial respiration is regulated by different cytoplasmic metabolites depending on the workload and CK activity. At low and moderate workloads, the oxidative phosphorylation may be regulated mostly by cytoplasmic  $\text{P}_i$  providing the required feedback signal and

possibility for constant PCr-to-ATP ratio (231). In the coupled creatine kinase – actomyosin reactions in myofibrils, the metabolic end-products are free creatine and Pi and the feedback signal for mitochondria is represented both by parallel Pi and creatine (resulting in oscillating cytoplasmic ADP) fluxes. Due to much lower initial concentrations of Pi, its regulatory effect on mitochondrial respiration will be stronger. By entry into mitochondria by a phosphate carrier, Pi increases the rate of ATP production coupled to oxidation, on the one hand, and on the other hand, to the phosphocreatine production due to functional coupling between ANT and MtCK. The final result is the increased respiration and constant level of phosphocreatine – metabolic stability. This prediction is in concord with the recent experimental works of (233). Our simulations predict that at higher workloads the regulation is shared among the participating metabolites. If CK is inhibited, the oxidative phosphorylation is mainly regulated by cytoplasmic ADP level (231). In any cases, it is the feedback signalling of different nature and by parallel metabolic fluxes which precisely matches the energy requirements of the cell to the ATP production coupled to the mitochondrial respiration.

If all these processes occur inside the space limited by the ICEUs structure, the next most important but still unclear question is that of the nature of the signal which synchronizes the redox state and respiration of mitochondria in different ICEUs. Some of indications for this kind of synchronization have already been obtained by O'Rourke and Aon (234).

### 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. 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. During decades of the research in this area, we have made also an interesting psychological observation. It has always been hard to explain and discuss these principles of compartmentation and metabolic channelling, and why the creatine kinase is out of equilibrium, with investigators who use in their research big instruments, such as NMR. These big instruments may obviously influence the "macroscopic" way of thinking of their users: first, the equilibrium concept of creatine kinase is the simplest and easiest way to interpret the  $^{31}\text{P}$ -NMR spectra, and the second, by recording the spectra of the whole tissue, there is no direct way to see the need for these compartmentation theories. On the contrary, in the field of electrophysiology where the object of a study is usually a single cell, the patch-clamp and microelectrode techniques are used to study the single channels and limited areas of the cells, the subcellular compartmentation theories are the usual ways of thinking. The best results are obtained when these two approaches and ways of thinking are combined, as evidenced by elegant results of the group of Jacqueline Hoerter,

who has always been working in close contacts with electrophysiologists and with experts in the field of creatine kinase compartmentation (see their chapter in this volume). The combination of two approaches can be supported by the Prigogine's view on holistic systems – interactions between parts of a big system may explain new qualities not evident from the properties of single parts. At the present, when the optical techniques of imaging as confocal microscopy allow to visualise the events in subcellular microdomains, the compartmentation theories are becoming even trivial.

What is needed most now is the development of the methods of the quantitative studies of these phenomena and their roles in the integrated metabolism of the living cell and its regulation, and mathematical modelling, if combined with firm experimental evidence, is one of the promising further studies. The mathematical modelling when based on well-elaborated energetic principles and permitting large-scale *in silico* experiments is a powerful tool for understanding the structures of Nature.

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## LEGENDS FIGURES

**Figure 1:** Calculated apparent dissociation constants of ATP both from ternary and binary complex ( $K_a$  and  $K_{ia}$ , respectively) of MtCK reaction in the presence of oxidative phosphorylation. Coupling between MtCK and oxidative phosphorylation was modelled according to the dynamic compartmentation hypothesis. Here, apparent dissociation constants  $K_a$  and  $K_{ia}$  (represented by small dots in the figure) were computed in case of different combinations of the values of ATPase activity in the solution  $v_{ATPase}$  and exchange constants for ATP and ADP. In the figure, the measured values are shown by open circles in the right upper corner (no oxidative phosphorylation) and in the left lower corner (with oxidative phosphorylation). When taking the maximal ANT activity equal to the maximal rate of MtCK reaction, all combinations of computed  $K_a$  and  $K_{ia}$  are aligned along the line with index 1 (indexes are shown within larger empty circles in the figure). By increasing the maximal activity of ANT by 10 or 100 times, this line can be shifted to the left (lines with indexes 2 and 3, respectively). When instead of increasing the maximal activity of ANT, the apparent dissociation constant of ADP is increased, the line shifts to the right (line with index 4). Note that regardless of the used values of ANT kinetic constants, all computed combinations of  $K_a$  and  $K_{ia}$  were considerably adrift from the measured values of these constants in the presence of oxidative phosphorylation. This means that the dynamic compartmentation mechanism is insufficient to explain the oxidative phosphorylation induced changes in MtCK kinetics. Good fitting was observed only in the case of direct transfer mechanism (128). Experimental data from (Jacobus and Saks, 1982). The figure is reproduced from ref.128 with permission.

**Figure 2:** Partial free energy profile of MtCK reaction functionally coupled to ANT by a direct transfer mechanism. The free energies of the coupled system (boxes with solid

border) are compared with the free energies of uncoupled MtCK and ANT (boxes with the dashed border). In the first column, three states of two-protein complexes are shown with ATP (T in the scheme) attached to ANT directed to intermembrane space (Ni). The total free energy of ANT-MtCK complex depends on the state of the coupled MtCK, as it is shown in the first column. ATP, attached to ANT, is either released to solution (the second column) or directly transferred from ANT to MtCK (the third column). After MtCK reaction, ADP (D in the scheme) is transferred to ANT (the last column) and then either released to solution (the fourth column) or transported to mitochondrial matrix (not shown). In the scheme, all reactions which are in the pathway leading to synthesis of PCr after the transfer of ATP from ANT to MtCK are shown by thick lines. Thus, direct transfer mechanism makes the functional coupling between MtCK and ANT energetically favorable. Note that there are several simplifications made to keep the profile as simple as possible. First, the free energies changes indicated in the profile are induced by differences of the free energies of the complex states as well as changes in solution due to binding and release of the substrates and magnesium. However, in the scheme, release and binding of ATP and ADP are indicated only. Second, possible binding of ATP and ADP by ANT during MtCK reaction is not indicated in this profile. The figure is reproduced from ref.128 with permission.

**Figure 3.** A. Confocal image of mitochondria in isolated cardiomyocytes.

Imaging of mitochondria using MitoTracker(R) Red CMXRos. Cardiomyocytes were incubated with MitoTracker<sup>®</sup> Red CMXRos for 45 min at 37°C in Flexiperm<sup>®</sup> chambers (from Vivascience, Hanau, Germany) in a solution with 5 mM glutamate and 2 mM malate. The fluorescence of this dye was measured (excitation and emission maxima at 579 nm and 599 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. Immunolabelling of microtubular network in the permeabilized cardiac cells.

Beta-tubulin was labeled with a monoclonal primary antibody and a TRITC-conjugated secondary antibody. Labeling was performed after fixation and permeabilization. One can observe a very stable microtubular network. Tubulin is not specifically associated with mitochondria.

Cells were first fixed in paraformaldehyde (PFA) 4% for 15 min at room temperature before being permeabilized with Triton X100 1%, 5 min at room temperature. Then, cardiomyocytes were washed with phosphate-buffered saline (PBS, Biomedica, Boussens, France) and incubated in 2% (w/v) bovine serum albumine in PBS overnight at 4°C with primary monoclonal anti-tubulin antibody (Sigma) at a 1/200 dilution. After washes in PBS, cells were incubated for 4h in 2% (w/v) PBS/BSA with secondary antibody rhodamine tetramethyl rhodamine isothiocyanate (TRITC)-conjugated AffiniPure F(ab')<sub>2</sub> fragment donkey anti-mouse IgG at a dilution of 1/50 (Interchim, Montluçon, France). Cardiomyocytes were then washed three times in PBS and three times in water. The labelled cells were deposited on glass cover slips and mounted in a mixture of Mowiol<sup>®</sup> and glycerol to which 1,4-diazabicyclo-[2,2,2]octane (Acros Organics, Pittsburgh, PA, USA) was added to delay photobleaching. Samples were observed by confocal microscopy (DM IRE2, Leica, Leica microsystems, Heidelberg, Germany) with a 40X oil immersion objective lens (NA 1.4).

**Figure 4.** Alterations of regular arrangement of mitochondria and regulation of respiration in permeabilized cardiac fibers by trypsin treatment.

A and B. Confocal imaging of mitochondria using Mitotracker<sup>®</sup> Green FM fluorescence. Permeabilized cells were preloaded with Mitotracker<sup>®</sup> Green FM and fixed as described in “Methods” section. A. Control fibers; B. After incubation with 1 μM of trypsin for 5 min at 4°C.

C. Representative oxygen consumption traces showing changes of metabolic channeling of endogenous ADP from Ca,MgATPases to mitochondria. For visualization of metabolic channeling, PK-PEP competitive enzyme method was used before and after treatment of permeabilized cardiac fibers with trypsin, leading to the disorganization of regular arrangement of mitochondria: the T-test. (The first derivative of oxygraph recordings of oxygen consumption are shown, directly showing the values of respiration rate). ATP was added to the final concentration of 2 mM. Thin line – control fibers. Thick line – fibers treated with 5  $\mu$ M of trypsin for 15 min at 4°C. *I(-)* and *I(+)* indicate inhibition of mitochondrial respiration by competing PK-PEP system without (-) and with (+) trypsin treatment. At the end of each experiment, creatine (20 mM) was added. Note that creatine activates the respiration in non-treated permeabilized fibers by 130% (to the State 3 level) in spite of the presence PEP-PK. After trypsin treatment the maximal creatine-activated respiration decreased by 50 %. For explanations see the text. Reproduced from ref.193 with permission.

**Figure 5.** The participation of the outer mitochondrial membrane in the mechanism of functional coupling between ANT and MtCK in the cells in vivo.

A. In isolated mitochondria or in trypsin-treated fibers the contacts of mitochondria with cytoskeleton is lost and the VDAC in the outer mitochondrial membrane in open configuration. ATP is directly channeled from ANT to MtCK and used to produce phosphocreatine PCr and ADP which is partially channelled 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 (102,193).

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 6.** The Maxwell's demons in action: the coupled CK and AK reactions within the intracellular energetic units – ICEUs. 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 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.

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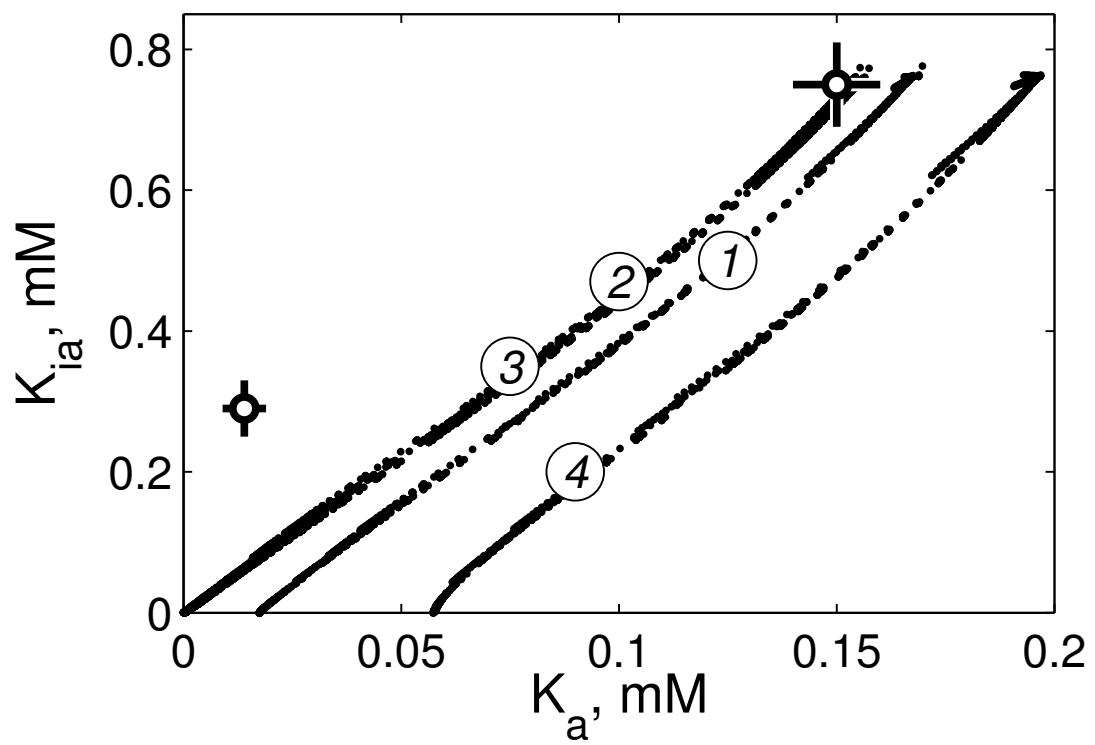


Figure 1:

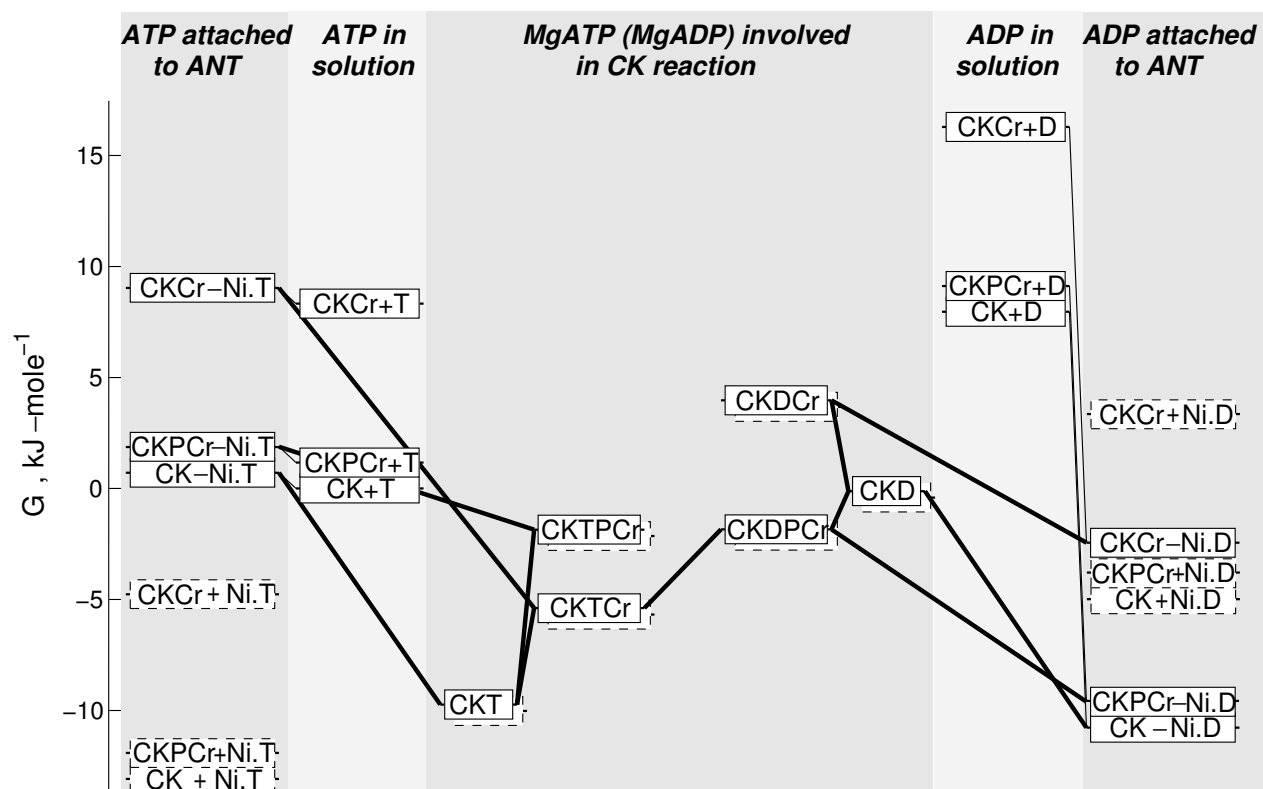
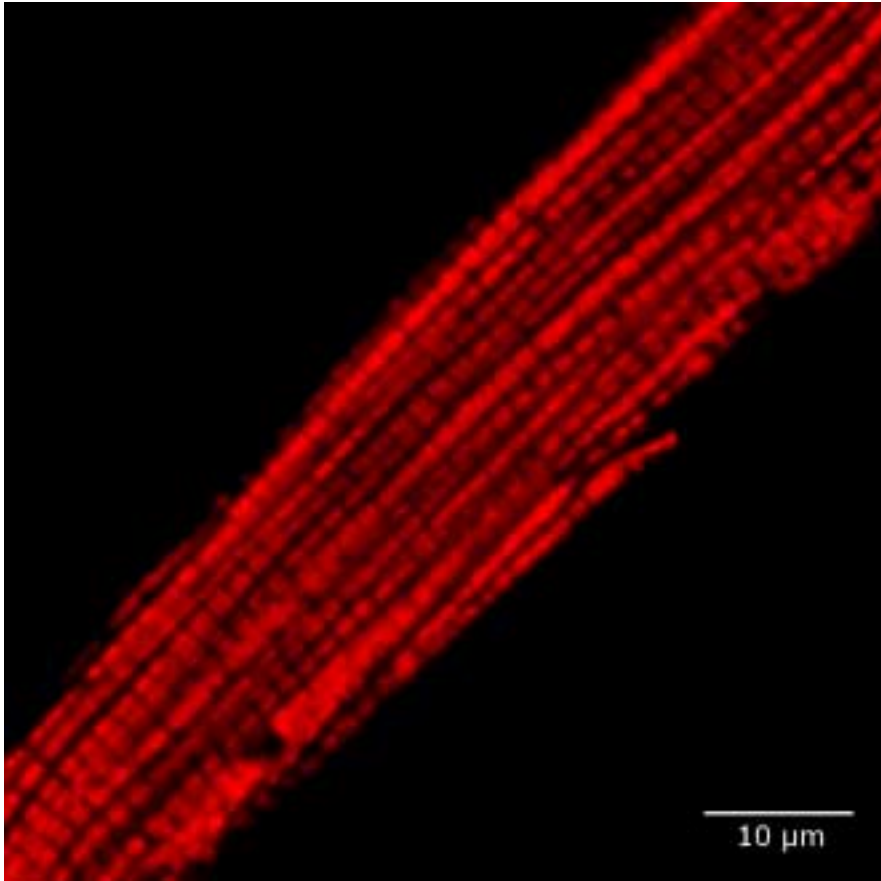


Figure 2:

**A**



**B**

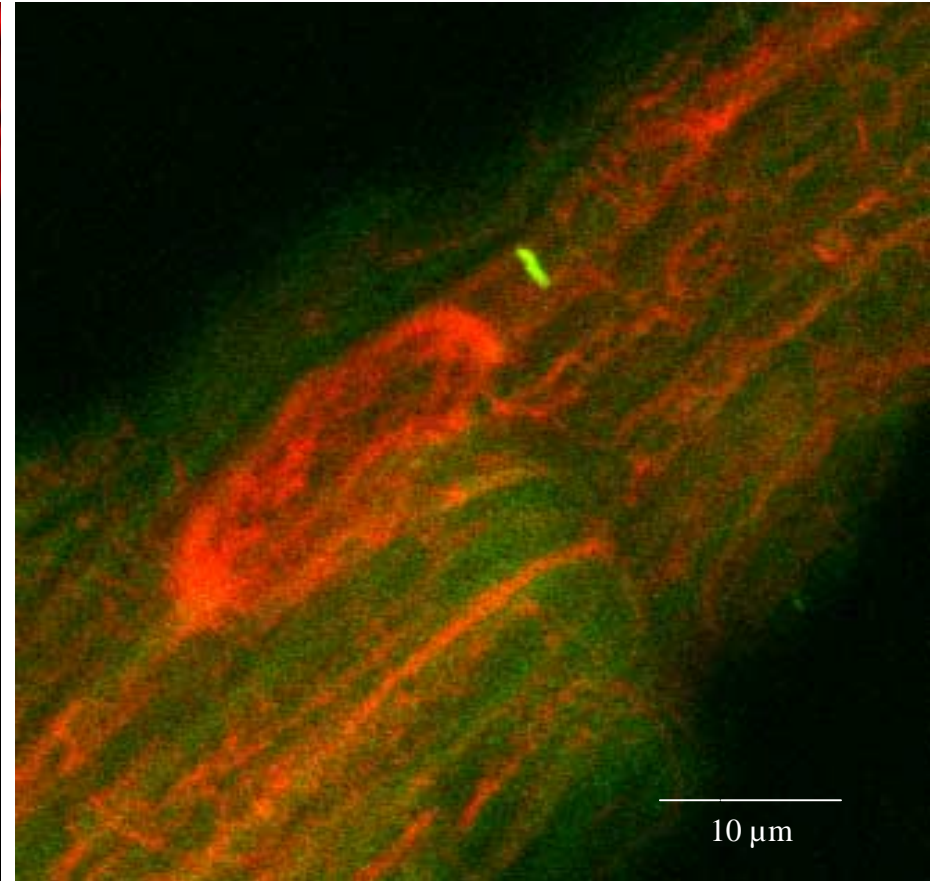


Fig.3



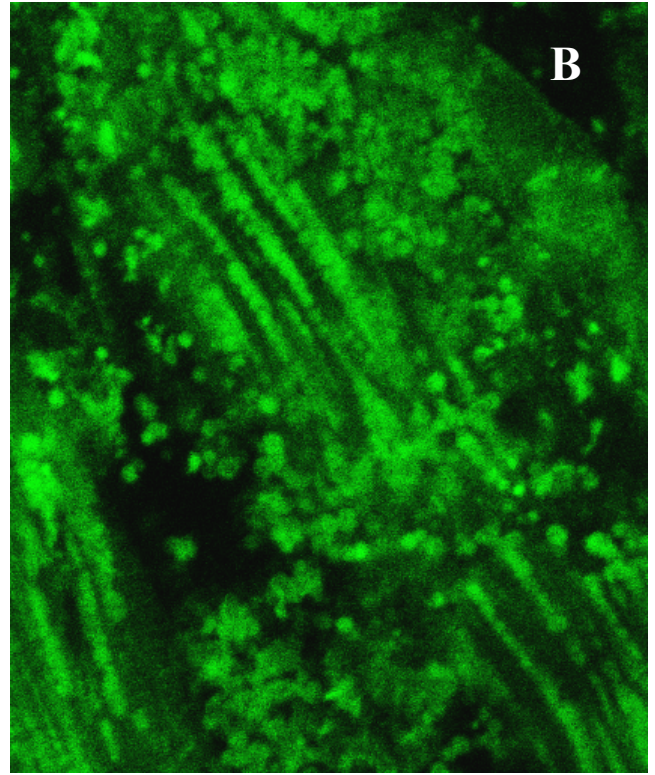
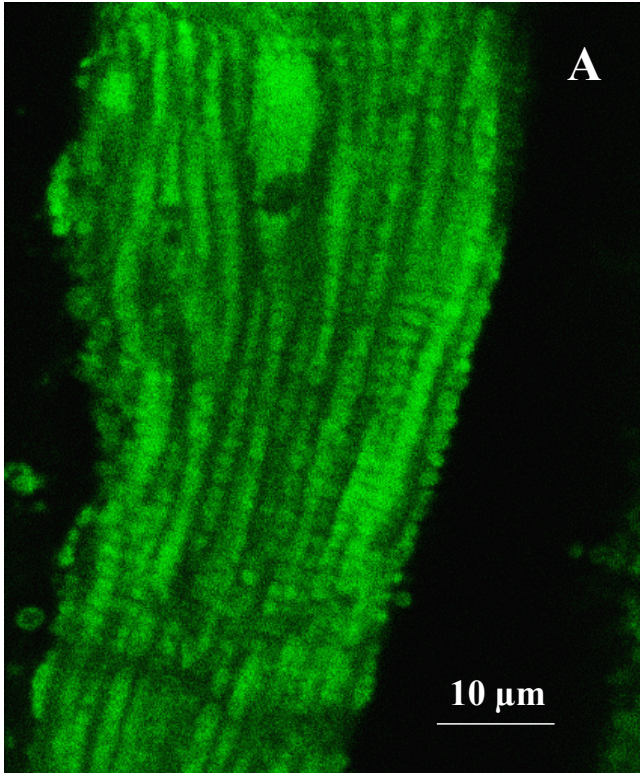


Fig.4

C

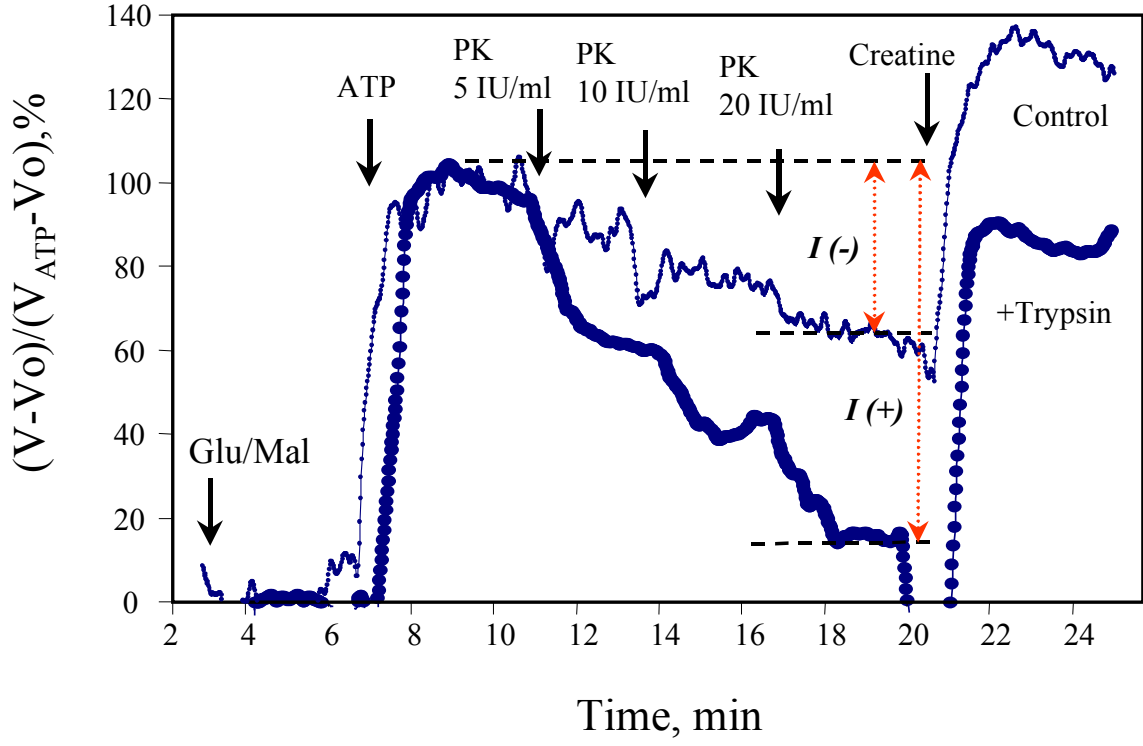


Fig.4

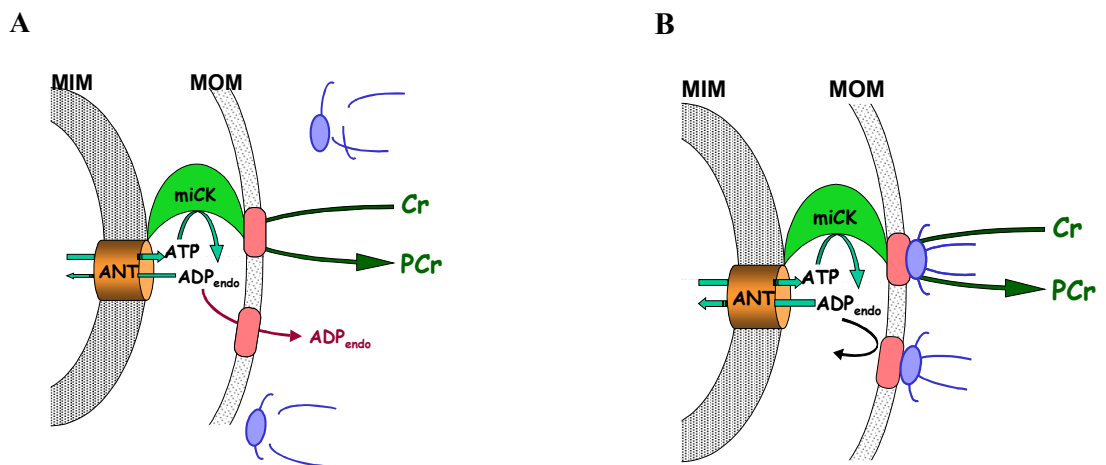


Fig.5.

# Intracellular Energy Units (ICEU)

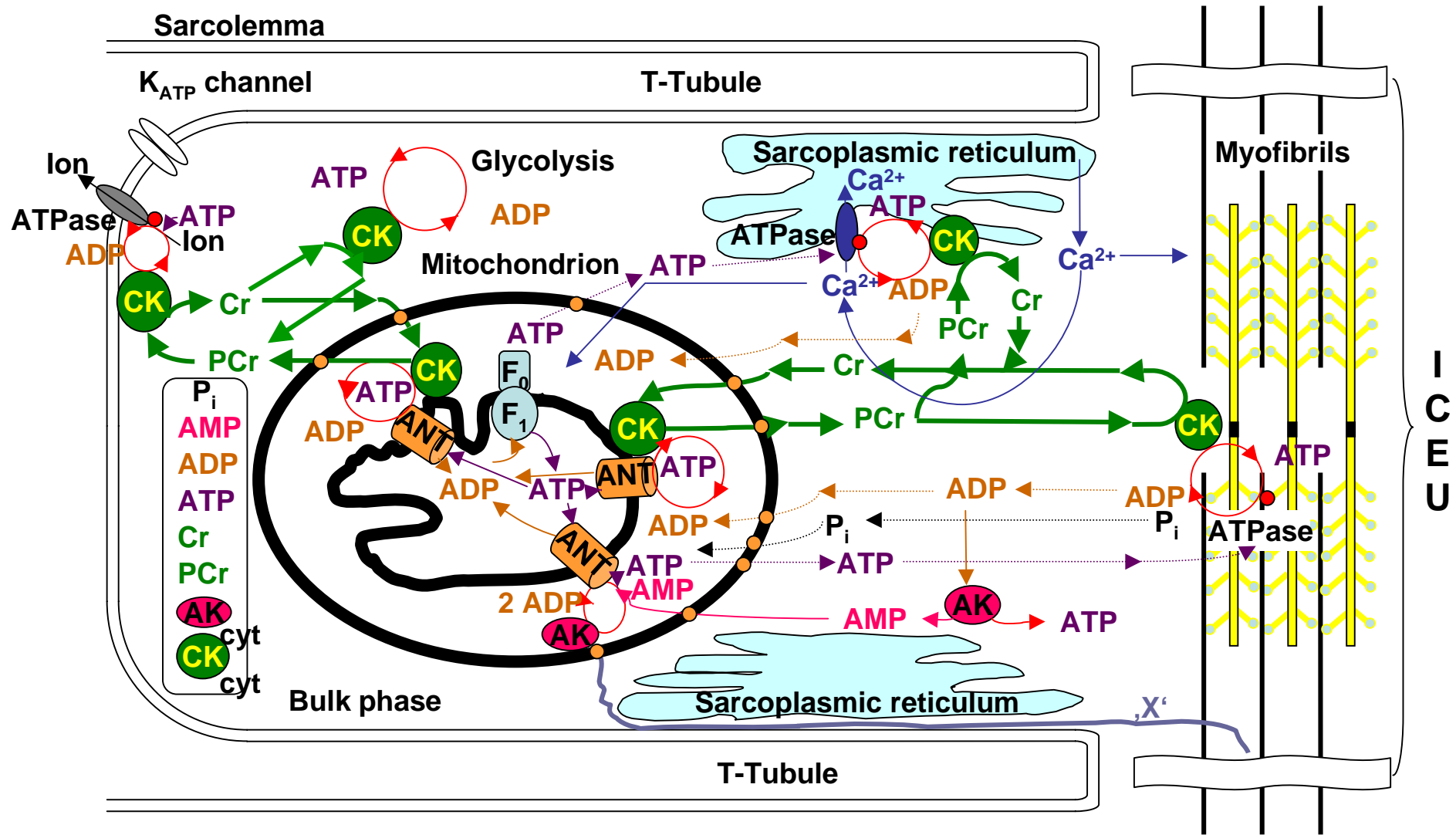


Fig.6