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Review

Structure–function relationships in feedback regulation of energy fluxes in vivo in health and disease: Mitochondrial Interactosome

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ABSTRACT

The aim of this review is to analyze the results of experimental research of mechanisms of regulation of mitochondrial respiration in cardiac and skeletal muscle cells in vivo obtained by using the permeabilized cell technique. Such an analysis in the framework of Molecular Systems Bioenergetics shows that the mechanisms of regulation of energy fluxes depend on the structural organization of the cells and interaction of mitochondria with cytoskeletal elements. Two types of cells of cardiac phenotype with very different structures were analyzed: adult cardiomyocytes and continuously dividing cancerous HL-1 cells. In cardiomyocytes mitochondria are arranged very regularly, and show rapid configuration changes of inner membrane but no fusion or fission, diffusion of ADP and ATP is restricted mostly at the level of mitochondrial outer membrane due to an interaction of heterodimeric tubulin with voltage dependent anion channel, VDAC. VDAC with associated tubulin forms a supercomplex, Mitochondrial Interactosome, with mitochondrial creatine kinase, MtCK, which is structurally and functionally coupled to ATP synthasome. Due to selectively limited permeability of VDAC for adenine nucleotides, mitochondrial respiration rate depends almost linearly upon the changes of cytoplasmic ADP concentration in their physiological range. Functional coupling of MtCK with ATP synthasome amplifies this signal by recycling adenine nucleotides in mitochondria coupled to effective phosphocreatine synthesis. In cancerous HL-1 cells this complex is significantly modified: tubulin is replaced by hexokinase and MtCK is lacking, resulting in direct utilization of mitochondrial ATP for glycolytic lactate production and in this way contributing in the mechanism of the Warburg effect. Systemic analysis of changes in the integrated system of energy metabolism is also helpful for better understanding of pathogenesis of many other diseases.

1. General introduction

Quantitative analysis of complex systems of integrated energy metabolism needs the collection of vast amount of reliable experimental data and use of mathematical models for analysis and prediction of system behavior. Important data can be obtained by studies of intact cells and organs such as the heart, skeletal muscles or brain by using such methods as imaging, biochemical analysis, nuclear magnetic resonance, including saturation transfer and especially by isotope tracer method [1–3]. However, these methods usually give general information, not sufficient for revealing details of interactions between cellular components and for quantitative analysis of functional consequences of these interactions. This information can be easily obtained by permeabilized cell technique which application in combination with image analysis, kinetic methods and modeling is very useful and informative [4,5]. The aim of this review article is to describe and analyze experimental data obtained in our laboratories by this method in studies of the regulation of metabolic fluxes and respiration in muscle and brain cells, with main focus on the regulation of mitochondrial respiration in cardiac cells under normal physiological conditions when the heart function is governed by the Frank–Starling law [6,7]. We take advantage of the availability of the cells of cardiac phenotype with very different cellular organizations, such as adult isolated cardiomyocytes and cultured continuously dividing cancerous HL-1 cells [8]. Comparative studies of these cells gave us important information on the structure–function relationship in determining the mechanisms of regulation of respiration and integrated intracellular energy metabolism in cells [9,10]. Finally, we show that systemic analysis of the integrated

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cellular energy metabolism in the cells helps to understand pathogenetic mechanisms of several diseases, such as cancer and heart insufficiency.

2. Mitochondrial arrangement in adult cardiomyocytes versus HL-1 cells

By its nature, the contraction process needs very precise structural organization of sarcomeres of muscle cells [11]. Mitochondria in adult cardiomyocytes are located regularly at the level of A-band of sarcomeres, their positions are determined by their interactions with the cytoskeleton and the sarcoplasmic reticulum [12–17]. Recent studies have also shown that the precise, cell type-dependent organization of mitochondria, and numerous interactions between these organelles and other cellular structures, play a fundamental role in regulations of mitochondrial function [9,13,14,18–21]. In cardiac and skeletal muscles regularly arranged intermyofibrillar mitochondria [15] interact with other intracellular systems like the cytoskeleton and sarcoplasmic reticulum [12–14,17,22]. This type of organization provides a bioenergetic basis for contraction, recruiting cytoskeletal proteins, controlling both mitochondrial shape and arrangement in the cell within Intracellular Energetic Units [13,14]. Importantly, the mitochondrial interactions with various cytoskeletal proteins (desmin, vimentin, tubulin or plectin) have been suggested to be directly involved in the modulation of mitochondrial function [17–20,23–25]. Firm evidence for such a role has been found for tubulin (see below). These interactions control evidently also mitochondrial dynamics and movement of mitochondria in the living cells [26]. In our recent quantitative studies of these connections in adult rat cardiomyocytes and in cultured continuously dividing non-beating (NB) HL-1 cells with differentiated cardiac phenotype, mitochondria were stained with MitoTracker® Green and studied by fluorescent confocal microscopy [26]. High speed scanning (1 image every 400 ms) revealed very rapid fluctuation of positions of fluorescence centers of mitochondria but no mitochondrial fusion or fission in adult cardiomyocytes (Fig. 1F). These fluctuations followed the pattern of random walk movement within the limits of the internal space of mitochondria, probably due to transitions between condensed and orthodox configurational states of matrix and inner membrane [26]. In contrast, HL-1 cells with differentiated cardiac phenotype do not exhibit the strictly regular mitochondrial distribution typical for rat cardiac cells (Fig. 1A–E). In these cells, mitochondria can be heterogeneous, highly dynamic and motile, undergoing continual fission, fusion and fast intracellular displacements at a velocity of 0.1–0.2 μm/s [8,26]. Thus, mitochondrial fusion or fission was seen only in cancerous NB HL-1 cells (Fig. 1) but not in adult cardiomyocytes. The differences observed in mitochondrial dynamics are related to distinct specific structural organization and mitochondria–cytoskeleton interactions in these cells. It will be shown below that strikingly different intracellular organization and dynamics of mitochondria in adult cardiomyocytes and HL-1 cells are responsible for their remarkably different functional parameters.

3. Differences in the mechanisms of regulation of mitochondrial function in vitro and in vivo, factor X hypothesis

Rapid development of bioenergetics during the last 60 years was possible due to studies of mitochondria and their composition and components in an isolated state [27]. These studies resulted in establishing the Mitchell's chemiosmotic theory of oxidative phosphorylation and rotary mechanism of ATP synthesis, two fundamental bases of modern bioenergetics [27]. In the studies of metabolism in whole cells and organs, progress was made first by rapid freezing of tissue and subsequent biochemical analysis of extracts [28], and then by in vivo ^{31}P NMR spectroscopy [2,29]. These two methods when applied for studies of cardiac metabolism resulted in the discovery of the metabolic homeostasis of the heart, expressed as a constancy in concentrations of ATP, PCr and creatine, despite large variations in work load, in the myocardium [28,29]. Now the principal but unsolved question is whether one can explain the mechanisms of regulation of integrated energy metabolism of the cells in vivo by behavior of mitochondria in isolated state, in vitro? Very often, the answer a priori has been yes [30–35], leaving unanswered the question how intracellular organization and multiple interactions between cellular structures may influence the mechanisms of regulation of energy fluxes. Usually these interactions are simply ignored. However, theories based on simple extrapolation of data from studies on mitochondria in vitro fail in attempts to explain the metabolic homeostasis of cardiac cells under conditions of Frank–Starling law [7,10]. One of these simple theories, still very popular and actively used, assumes that mechanisms of regulation of mitochondrial respiration in vivo and in vitro by ADP are very similar; that mitochondria in vivo behave as in an homogenous medium and that cytoplasmic ADP is in equilibrium with the CK reaction [30–35]. Concentration of cytoplasmic ADP in equilibrium with CK reaction in heart cells under condition of metabolic stability is about 50–100 μM [36]. Taking into account that for isolated mitochondria the Km value for ADP is only 8–10 μM, one can see that no regulation of respiration by cytoplasmic ADP at its concentrations of 50–100 μM is possible, since adenine nucleotide translocator, ANT, is saturated by ADP and maximal respiration rate should always be observed. This is not the case, however, as it is known from classical studies in heart physiology that cardiac oxygen consumption increases linearly with elevations in workload (ATP hydrolysis) [6,37]. Moreover, under conditions of stable ATP, PCr and creatine concentrations, characteristic of energy metabolism of cardiac cells, the ADP concentration calculated from CK equilibrium should also be stable and should not respond to changes of respiration rate. In a similar way free Pi concentration and related parameters such as free energy of ATP hydrolysis (calculated from total metabolite contents and CK equilibrium) should not change under condition of metabolic stability. Thus, the main counter-arguments to the CK equilibrium theory – the phenomenon of metabolic stability of cardiac muscle and metabolic aspect of Frank–Starling law of the heart [29,37–40] – are uncontested. Veech et al. established the CK equilibrium experimentally only in the resting (noncontracting) muscle [41]. In the working muscle, creatine kinases within the PCr phosphotransfer network function mostly in a non-equilibrium state, especially at elevated workloads [7,42].

In order to explain the regulation of mitochondrial respiration under conditions of metabolic stability and at the same time assuming the CK equilibrium, the theory of parallel activation by Ca^{2+} was proposed and continues to be supported [43–47]. According to this theory, the increase of cytoplasmic Ca^{2+} during excitation–contraction coupling cycle activates ATP hydrolysis in myofibrils, and simultaneously three dehydrogenases of Krebs cycle in mitochondrial matrix increasing production of NADH and $FADH_2$ by push mechanism. The oxidation of the latter increases electron flow through the respiratory chain, generates the protonmotive force and drives ATP synthesis [27,48]. Ca^{2+} is thought also to activate directly F_1F_O-ATPase and complex I [43,49–52] However, the parallel activation theory still does not fit with the requirement for the main signal of coordination of energy metabolism in cardiomyocytes recently formulated by O'Rourke [48]. According to O'Rourke's principles, the variations of cytoplasmic [Ca^{2+}] have to correspond to changes in workload, ATP consumption and respiration. This condition is not fulfilled, because the intracellular Ca^{2+} transients do not change during the length-dependent activation of sarcomere (mechanism on which is based the Frank–Starling's law) [53–55]. Thus, the Frank–Starling mechanism puts into question the viability of the theory of parallel activation of contraction and respiration by Ca^{2+}. Regulation of respiration by Ca^{2+} seems to explain the adrenergetic activation of oxidative phosphorylation [43,47,49,52,56–

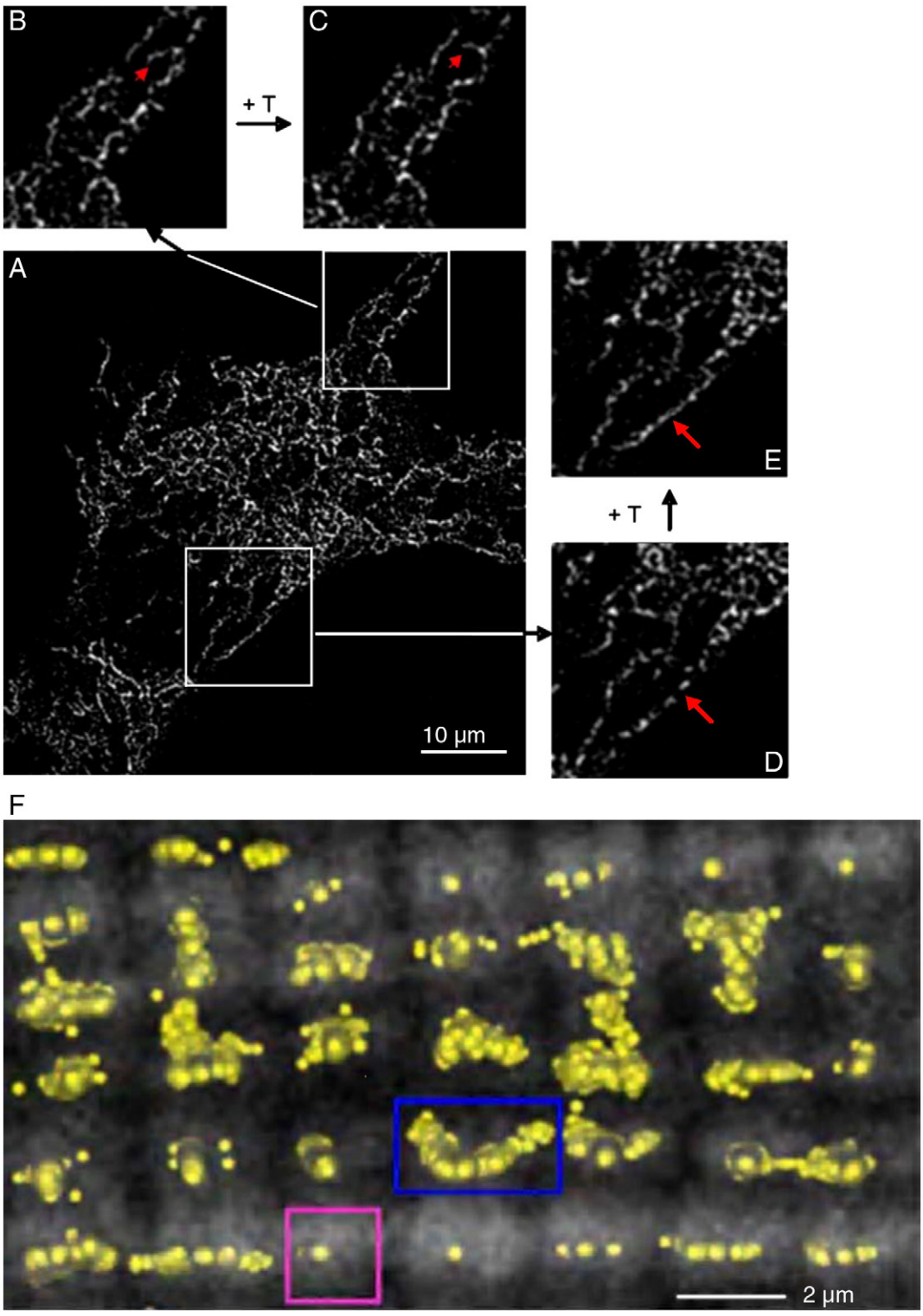


Fig. 1. A Enhanced confocal images of the mitochondrial network in NB HL-1 cells. Mitochondria were stained with MitoTracker® Green (in white). In details B, C, D and E, modifications of mitochondria network as a function of time *t* are depicted. Indeed, mitochondria are very dynamic undergoing continual fission and fusion events usually forming long and rapidly moving filament-like structures. (F) Visualization of the positions of mitochondrial fluorescent (mass) centers in a cardiomyocyte over a long time (total duration 100 s) of rapid scanning: movements of fluorescence centers are limited within internal space of mitochondria. Positions of the fluorescence centers were stacked as a function of time. These fluorescence centers (which are assimilated to the center of mitochondria in cardiomyocytes) are shown as small yellow spheres. The position of fluorescent centers was superimposed with a reference confocal image of MitoTracker® Green fluorescence (in grey) showing mitochondrial localization. Note that the fluorescence centers are observed always within the space inside the mitochondria, but from mitochondrion to mitochondrion the motion pattern may differ from very low amplitude motions (pink frame) to wider motions distributed over significant space but always within the internal space of a mitochondrion (blue frame). Reproduced from Beraud et al. with permission [26].

63], but not the feedback regulation of respiration by workload changes during cardiac contraction under physiological conditions of action of Frank–Starling law [7,64].

To find a solution to this important problem of metabolic studies, an application of the principles of Systems Biology is very helpful. One of the main principles of Systems Biology is that interactions between

system's components lead to new system level properties which are absent when the components are isolated and which explain the mechanisms of functioning of the system, its biological function [10,65–67]. The permeabilized cell technique, in combination with kinetic analysis, mathematical modeling and whole cell and organ studies mentioned above is one of the principal methods of Molecular System Bioenergetics [10,67].

4. In vivo kinetics of regulation of respiration, central role of MtCK

Kümmel was first to apply the permeabilized cell technique for studies of cardiac energy metabolism [68] and to discover that the quantitative characteristics of regulation of mitochondrial respiration – apparent Km for exogenous ADP – are very different in vitro and in permeabilized cardiomyocytes in situ. The latter exceeds the former by order of magnitude, showing significantly decreased affinity of mitochondrial respiration for exogenous ADP in vivo. This result was then confirmed in very many laboratories (Table 1). Detailed studies of this phenomenon in our laboratories led to the conclusion that it is the result of an interaction of mitochondria with cytoskeletal components, resulting in restricted permeability of the voltage dependent anion channel VDAC in mitochondrial outer membrane for ADP and also related to the specific structural organization of the cell by the cytoskeleton resulting in regular arrangement of mitochondria with surrounding structures and MgATPases into Intracellular Energetic Units (ICEUs) in adult cardiomyocytes and oxidative muscle cells [13]. High apparent Km for exogenous ADP in permeabilized cells is tissue specific, observed for cardiac fibers and isolated cardiomyocytes even after extraction of myosin (ghost fibers), and in fibers from slow twitch oxidative (but not fast twitch) glycolytic skeletal muscle, its value is high also in permeabilized hepatocytes and synaptosomes (Table 1). This tissue specificity excludes any possibility of explaining high Km values for ADP by long diffusion distance or specific effects of saponin, a detergent used for permeabilization [4,5], sometimes proposed in literature and critically already analyzed before [69]. The value of this parameter decreases significantly after treatment of cells with a small amount of proteolytic enzymes in a low concentration, showing the role of some proteins sensitive to this treatment, in control of mitochondrial responses to ADP in vivo (Table 1). This hypothetical protein was given the name “Factor X” and assumed to be separated from mitochondria during isolation procedure [70], as shown in Fig. 2. The initial hypothesis was that this protein controls the permeability of outer mitochondrial membrane for ADP [70]. Thus, the high apparent Km for exogenous ADP in regulation of mitochondrial respiration is a system level property, depending on the interaction of mitochondria with other cellular structures. Comparison of isolated mitochondria, permeabilized cardiomyocytes and permeabilized HL-1 cells confirms this conclusion and shows directly the dependence of the mechanism of regulation of respiration upon structural organization of the cell. Fig. 3 shows that in isolated cardiomyocytes, where the mitochondria are fixed, with very regular “crystal-like” arrangement at the level of sarcomeres, and where no fusion into reticular structures is possible and only configuration of mitochondrial inner membrane changes rapidly (Fig. 1F) apparent Km for exogenous ADP is very high, in agreement with the data in Table 1. In contrast, in permeabilized HL-1 cells, where the mitochondria are very dynamic and undergo continuous fusion and fission (Fig. 1A–E), the apparent Km for exogenous ADP is very low and close to that in isolated mitochondria [9].

Table 1
Apparent K_m(ADP) for exogenous ADP in regulation of respiration in permeabilized cells and fibers from different tissues with or without creatine or trypsin treatment.

Preparation	K_m^{app}ADP, μM	K_m^{app}ADP (+Cr), μM	K_m^{app}ADP, μM, after treatment with trypsin	References
Heart tissue homogenate	228 ± 16		36 ± 16	[191]
Cardiomyocytes	329 ± 50			[9]
	250 ± 38	35.6 ± 5.6		[192]
	200–250			[23]
“Ghost” cardiomyocytes	200–250			[23]
Skinned cardiac fibers[a]	297 ± 35	85 ± 5		[193]
	260 ± 50	79 ± 8		[194]
	300 ± 23			[13]
	300–400			[14]
	277 ± 40	102 ± 35		[195]
	370 ± 70			[196]
	234 ± 24			[197]
	324 ± 25			[197]
	320 ± 36		83 ± 22	[191]
“Ghost” cardiac fibers	349 ± 24			[193]
	315 ± 45	85 ± 5		[198]
Permeabilized hepatocytes	275 ± 35			[199]
Synaptosomes	110 ± 11	25 ± 1		[88]
Skinned fast twitch skeletal muscle fibers	7.5 ± 0.5			[200]
	8–22			[198]
Rat heart isolated mitochondria	17.6 ± 1.0	13.6 ± 4.4		[194]
	13.9 ± 2.6		17.6 ± 1	[191]
Rat brain isolated mitochondria	9.0 ± 1.0			[88]

[a] The value of this parameter, apparent Km for exogenous ADP, is always equally high in permeabilized isolated cardiomyocytes and in skinned cardiac fibers. The equality of Km (ADP) in these two types of permeabilized preparations is always a necessary criterion for evaluation of quality of preparation when skinned fibers are used [4,5,9,191,193,194]. In opposite cases, as sometimes published in literature ([204], for critical review see [69]), the preparations are damaged, but there is no reason to blame the technique of skinned fibers so well used in many laboratories, for failures of authors to use it correctly [204].

Further important information was obtained when mitochondrial creatine kinase, MtCK was activated by creatine. These experiments confirm and clearly demonstrate that the role of mitochondrial outer membrane in adenine nucleotides compartmentation and the functional coupling between MtCK and ANT is much more important in vivo where the mitochondria interact with intracellular surrounding environment forming the unitary structure–functional organization of energy metabolism — Intracellular Energy Units (ICEUs) [13]. During permeabilization the cytoplasmic soluble enzymes not bound to the structures such as many glycolytic enzymes and MM-CK are released into the solution, but MtCK stays in the mitochondrial intermembrane space firmly fixed by lysine–cardiolipin interactions at the outer surface of inner membrane in the vicinity of ANT [71,72]. Table 1 shows that the addition of creatine significantly decreases the apparent Km for exogenous ADP by increasing the rate of recycling of ADP in mitochondria in MtCK and oxidative phosphorylation reactions functionally coupled via adenine nucleotide translocator, ANT.

One of the most fruitful approaches in these studies was a demonstration of the role of local ADP concentrations in activating oxidative phosphorylation by applying the powerful ADP trapping system consisting of high activities of pyruvate kinase, PK, and phosphoenolpyruvate, PEP, capable of capturing and phosphorylating all ADP in soluble phase of the cytoplasm or medium equilibrated with it. Fig. 4 demonstrates how this system was used in recent experiments [73]. In the presence of PK–PEP system ADP produced in MgATPase and myofibrillar MM-CK reactions is trapped and rephosphorylated into ATP, and respiration is activated only due to local ADP produced in the MtCK reaction (Fig. 4A). Fig. 4B shows that under these conditions MtCK maximally activates respiration and PK–PEP are not able to trap the ADP recycling in mitochondrial coupled reactions behind the outer mitochondrial membrane (MOM). It was shown in parallel experiments that in the in vitro system containing isolated mitochondria the PK–PEP system can trap significant amount of ADP produced by MtCK [73]. These remarkable

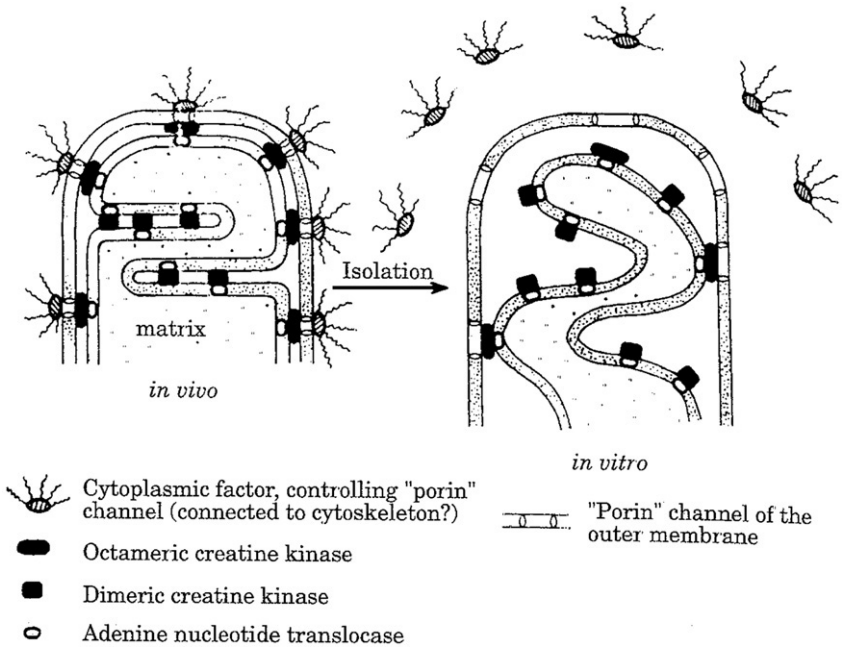


Fig. 2. Original hypothesis of the connection of mitochondrial outer membrane (MOM) and cytoskeleton in vivo, where the outer membrane VDAC channel ("porin") was assumed to be controlled by some cytoplasmic factor "X", which is lost during mitochondrial isolation, and therefore the (MOM) becomes in vitro absolutely permeable for ADP. Reproduced from Saks et al. with permission [70].

differences between mitochondria in vivo and in vitro were seen again in kinetic experiments described in Fig. 4C–E. After permeabilization, MgATP was first added to activate the intracellular MgATPases producing endogenous ADP that stimulates respiration (Fig. 4C). The PK–PEP system was then added to trap this endogenous ADP with an expected decrease in respiration rate. Finally creatine was added stepwise to study the role and kinetics of the MtCK reaction in regulation of respiration in vivo in a situation modeling some characteristics of intracellular milieu. Most remarkably, creatine addition to permeabilized cardiomyocytes rapidly activates respiration up to a maximal value (Fig. 4C). Results of the kinetic analysis of this action are described below. However, when isolated heart mitochondria were used, creatine addition increased respiration rate to only about half of its maximal value (Fig. 4D) meaning half of ADP produced by MtCK leaked out through the mitochondrial outer membrane and was trapped by the PEP–PK system. Table 2 shows that the respiratory parameters — maximal respiration rates in the presence of ADP or MgATP + creatine are similar in isolated heart mitochondria and permeabilized cardiomyocytes when calculated per cytochrome aa_3 content. Thus, the effect of PEP–PK on the respiration of isolated mitochondria is due to the leak of ADP via mitochondrial outer membrane. Experimental studies of kinetic properties of MtCK in isolated cardiac mitochondria and mathematical modeling of these properties [74–77] showed that they are dependent on functional coupling of MtCK with oxidative phosphorylation via ANT. The apparent constant of dissociation of ATP from its tertiary complex with MtCK decreases 10 times in the presence of activated oxidative phosphorylation [78]. This strong affinity of MtCK for ATP disappears when MtCK is detached from mitochondrial membranes [79]. Mathematical modeling of these effects showed that it is explained by direct channeling of ATP from ANT to MtCK [74,80]. However, ADP produced by MtCK can either be taken back into the matrix by ANT or leave intermembrane space if the outer membrane is easily permeable (see Fig. 4A). This is observed in isolated mitochondria (Fig. 4D) but not in permeabilized cardiomyocytes (Fig. 4B and C). Fig. 4E shows that in permeabilized fibers from human skeletal muscle m. vastus lateralis MtCK also effectively regulates respiration. In similar experiments with permeabilized fibers from biopsy samples of human m. vastus lateralis the apparent Km for exogenous ADP was found to be high but decreased significantly in the presence of creatine [81,82], supporting the conclusion that in oxidative skeletal muscles the ADP diffusion into mitochondrial intermembrane space is restricted. These reliable experimental results contradict the theoretical conclusion of mitochondrial ADP ultrasensitivity in these muscles made by Jeneson et al. [32] from analysis of PCr recovery after exercise on the basis of the assumption of CK equilibrium: experimental value of apparent Km for ADP was close to 200 μM [81] while theoretical calculations gave 22 μM [32]. Once again, the assumption of the CK equilibrium is not sufficient to explain the experimental data.

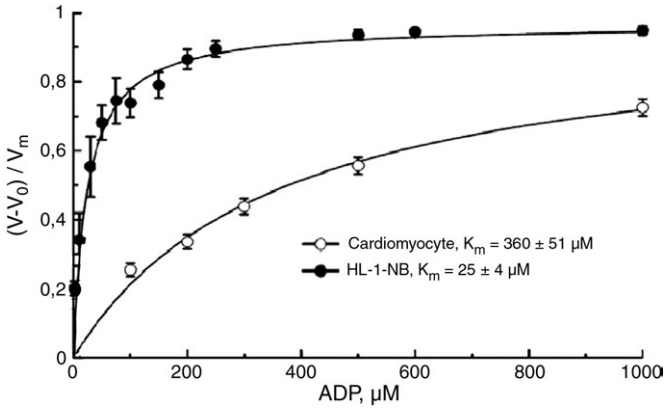


Fig. 3. Different kinetics of regulation of the respiration in permeabilized adult cardiomyocytes and non-beating (NB) cardiac tumoral HL-1 cells. For normalisation, the respiration rates were expressed as fractions of the maximal rates, Vmax, found by analysis of experimental data in double-reciprocal plots. Reproduced from Anmann et al. with permission [9].

To assess the role of structural organization in determining the mechanisms of regulation of oxidative phosphorylation, we have taken advantage of the comparison of cardiomyocytes from an adult heart with HL-1 cell culture developed from mouse atrial cardiomyocytes and expressing cardiac phenotype [8,83]. The HL-1 cells are characterized by entirely different kinetics of regulation of respiration

by ADP (see Fig. 3). Moreover, in the permeabilized HL-1 cells the creatine effect on respiration was not observed (Fig. 4F). It was recently shown that MtCK is not expressed in these cells, but only the BB isoform of CK can be seen, in contrast to rat heart cells, where BB isoform is in trace amounts and the major forms are MtCK and MM-CK isoforms, and also some amount of hybrid form MB is seen [84]. Remarkably, in both cells two hexokinase isoforms are present [84]. Measurements of enzyme activities in both these cells showed that CK activity is manifold decreased in HL-1 cells as compared to cardiomyocytes, while the activity of hexokinase is significantly increased in HL-1 cells (Fig. 5). Significance of these findings for cancer cell bioenergetics is discussed below.

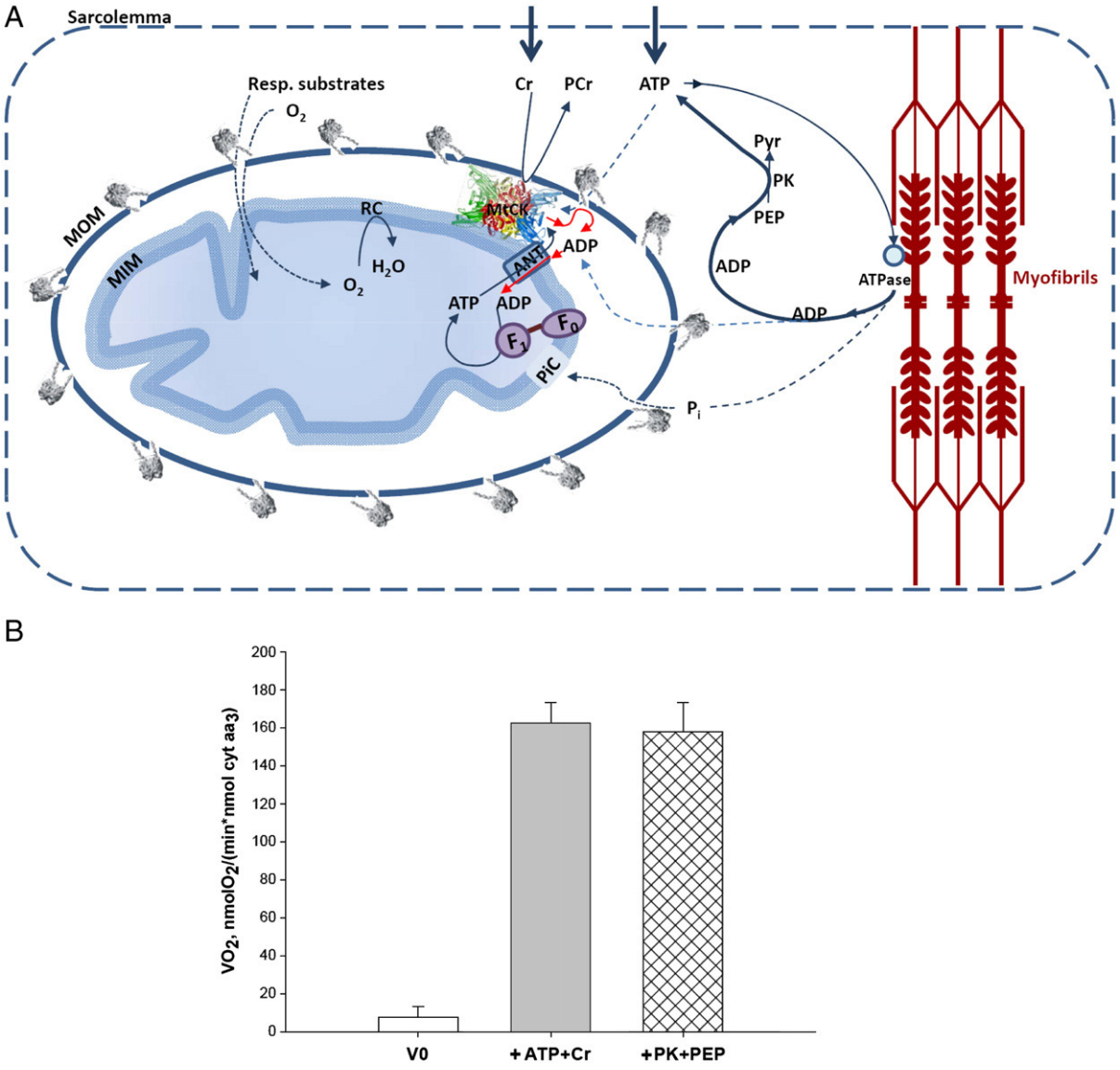


Fig. 4. (A) The scheme represents a mitochondrion in situ, in a permeabilized cardiac cell. The mitochondrial outer membrane (MOM) is less permeable than in isolated mitochondrion, due to the interactions of VDAC with cytoskeleton protein-tubulin. Exogenous ATP is hydrolyzed by cellular ATPases into endogenous extramitochondrial ADP and inorganic phosphate (Pi). Mitochondrial (MtCK) and non-mitochondrial MM creatine kinases (cytosolic, myofibrillar, SERCA, and sarcolemmal) activated by creatine in the presence of ATP, produce endogenous intra- and extramitochondrial ADP. The system is supplemented with phosphoenolpyruvate (PEP) and pyruvate kinase (PK) which remove extramitochondrial ADP and continuously regenerates extramitochondrial ATP. Intramitochondrial ADP produced by MtCK forms microcompartments within the intermembrane space (IMS) and is re-imported into the matrix via adenine nucleotide translocase (ANT) due to its functional coupling with MtCK. Reproduced from Guzun et al. [73] with permission. (B) Respiration rates of permeabilized cardiomyocytes. Respiration was activated by addition of MgATP (5 mM) and creatine (20 mM) resulting in activation of the MtCK reaction with local production of intramitochondrial ADP. Addition of 20 IU/mL PK in the presence of PEP (5 mM added into medium before) did not change significantly the respiration rate because of the inaccessibility of compartmentalized, in mitochondrial intermembrane space, ADP for PK–PEP system. (C) The experimental procedure used for complete kinetic analysis of MtCK in mitochondria in situ (permeabilized cardiomyocyte). First, addition of MgATP induces production of endogenous ADP in MgATPase reaction. Secondly added PEP–PK trap all extramitochondrial free ADP inducing decrease of respiration rate, but not to initial level, due to structural organization of ICEU. Under this conditions addition of creatine in different amounts rapidly activates the MtCK reaction. The oxidative phosphorylation is stimulated mostly by intramitochondrial ADP, produced by MtCK reaction, which is not accessible for PEP–PK. Adapted from [73]. (D) Respiration rates of isolated mitochondria stimulated by increasing amounts of creatine in the presence of ATP (i.e. activated MtCK reaction) and the absence of extramitochondrial ADP (consumed by the PEP–PK reaction). Adapted from [73]. (E) Respiration of permeabilized fibers from human skeletal m. vastus lateralis prepared from biopsy samples of healthy volunteers in the presence of 2 mM malate and 5 mM glutamate as substrates. Addition of 2 mM MgATP activates respiration due to production of endogenous MgADP in ATPase reaction. Pyruvate kinase (PK) in the presence of 5 mM phosphoenolpyruvate (PEP) decreases respiration rate due to removal of extramitochondrial MgADP. Creatine in the presence of MgATP activates mitochondrial creatine kinase (MtCK) reaction of production of endogenous intramitochondrial MgADP which rapidly activates respiration up to the maximal rate, that showing that mitochondrial ADP is not accessible for PK–PEP system due to the limited permeability of mitochondrial outer membrane in the cells in situ. Reproduced from Cherpec thesis [201] with permission. (F) The absence of the stimulatory effect of creatine on the respiration rate of non-beating (NB) cardiac tumoral HL-1 cells in the presence of PEP–PK system under conditions described above. Recorded respiratory rate is due to the stimulatory effect. Adapted from [9].

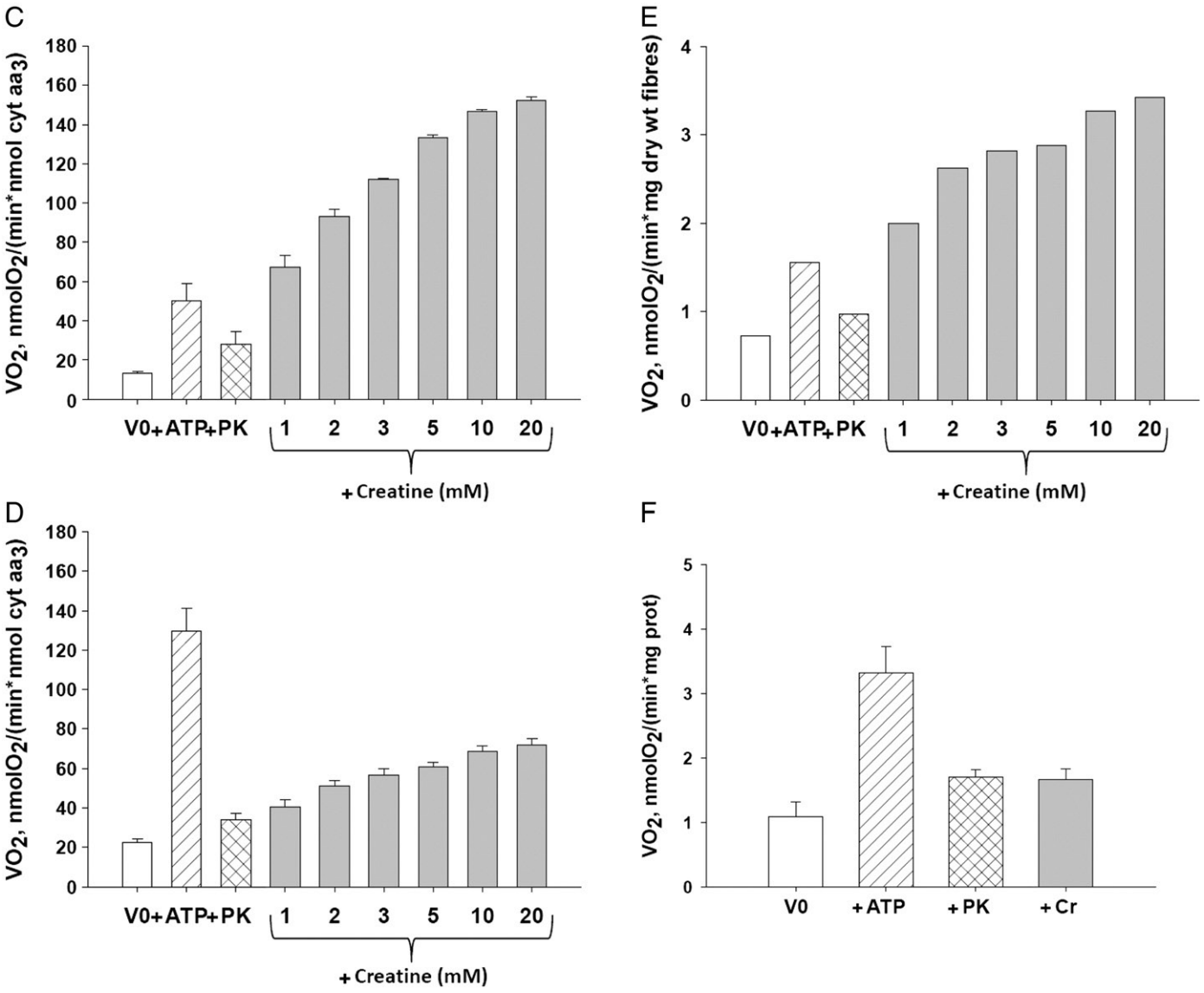


Fig. 4 (*continued*).

5. Mitochondrial–cytoskeletal interactions, heterodimeric tubulin as factor X

Many experimental evidences discussed above point to the role of some cytoskeleton protein, factor "X" in regulating MOM permeability for adenine nucleotides. One of the real candidates for this role is tubulin. Carre et al., by using the immunoprecipitation method showed the association of tubulin with VDAC [85]. Appaix et al. demonstrated that selective proteolytic treatment of permeabilized cardiomyocytes by trypsin in low concentration, which decreases apparent Km for exogenous ADP, also results in the almost complete disappearance of immunolabeling of tubulin [19]. These results are reproduced in Fig. 6. Very recently, functional interaction of tubulin with VDAC was revealed by applying biophysical and oxygraphic methods by Rostovtseva et al. [86,87] and Monge et al. [88]. In experiments with VDAC reconstituted into planar phospholipid membranes the reversible voltage dependent partial blockage of channel by dimeric tubulin (in nanomolar concentration insufficient for polymerization and in the absence of GTP and Mg^{2+}) was observed. Under similar experimental conditions but without tubulin VDAC remains open up to 1 h [87]. Rostovtseva et al. proposed the model for tubulin–VDAC interaction in which the negatively charged C-terminal tail of tubulin penetrates into the channel lumen due to the interaction with a positively charged domain of VDAC close it [87].

The role of interaction of tubulin with VDAC in regulation of mitochondrial oxidative phosphorylation was studied directly by recording the respiration rates of isolated brain and heart mitochondria stimulated by exogenous ADP in the absence and presence of heterodimeric tubulin (Fig. 7). As was expected, the apparent Km for free ADP in isolated mitochondria was about 10–20 μM. In the presence of 1 μM tubulin the sensitivity of mitochondria for free ADP decreased: apparent K_m^{app} increased to ~170 μM for the brain and ~330 μM for the heart mitochondria, respectively [87,88]. Creatine addition effectively decreased the apparent Km for ADP again (Fig. 8).

These studies allowed the factor X to be finally identified as the heterodimeric tubulin [86,87].

The effect of creatine and kinetic analysis of system described in Fig. 4C showed that VDAC cannot be completely closed in vivo. We analyzed the kinetics of respiration regulation by creatine and ATP in permeabilized cardiomyocytes in situ when respiration was stimulated by intramitochondrial ADP produced in MtCK reaction and extramitochondrial ADP was continuously consumed by the PEP–PK system (see Fig. 4A–C). These experiments modeled an interaction of mitochondria with glycolytic system in vivo. The apparent kinetics of

Table 2
Basic respiration parameters of isolated rat heart mitochondria and of mitochondria in situ in permeabilized cardiomyocytes. V_0 — respiration rate in State 2 in the presence of substrates before addition of ADP or ATP; V_3 — respiration rate in the presence of 2 mM ADP; $V_{Cr,ATP}$ — respiration rate in the presence of activated MtCK by 2 mM ATP and 20 mM creatine. Reproduced from [73] with permission.

Parameter	Mitochondria in vitro	Mitochondria in situ (permeabilized cardiomyocytes)
V_0, nmol O_2 min^{-1} mg $prot^{-1}$	26.37 ± 7.93	7.53 ± 1.61
$V_{3\ (2\ mM\ ADP)}$, nmol O_2 min^{-1} mg $prot^{-1}$	187.94 ± 40.68	84.45 ± 13.85
[Cyt aa_3], nmol mg $prot^{-1}$	1.00 ± 0.012	0.46 ± 0.09
$V_{3\ (2\ mM\ ADP)}$, nmol O_2 min^{-1} nmol cyt aa_3^{-1}	188 ± 39.93	178.23 ± 33.96
$V_{Cr,ATP}$, nmol O_2 min^{-1} nmol cyt aa_3^{-1}	197.90 ± 31.86	162.63 ± 26.87

the MtCK dependent respiration regulation was found to be totally different from that seen in mitochondria in vitro [73]. In fact, there are three remarkable differences. First is the decrease in apparent affinity of MtCK for exogenous MgATP (apparent Ka increased more than 100 times, Table 3) in mitochondria in situ as compared to in vitro. Second, the apparent constant of dissociation of creatine from the binary complex with MtCK (Kib) decreases about 10 times in mitochondria in situ, in permeabilized cardiomyocytes, as compared with isolated mitochondria. Third, the apparent affinity of MtCK for PCr is similar in vitro and in situ in permeabilized cells (Kip is about 1 mM) (Table 3). The decreased apparent affinity of MtCK in situ for extramitochondrial MgATP can be most probably due to the enhanced restriction of diffusion at the level of MOM (i.e. limited VDAC permeability) which induces the increase of adenine nucleotide micro-compartmentation within mitochondrial intermembrane space influencing the respiratory control of oxidative phosphorylation. The remarkably high affinity of MtCK in mitochondria in situ for creatine and PCr points to the absence of restriction of diffusion of these guanidino substrates across MOM into intermembrane space where MtCK is located. Direct measurements of energy fluxes from the mitochondria into the surrounding medium (measured using high performance liquid chromatography (HPLC) for the same experiments) showed stable MgATP concentration though the experiment and progressive increase of PCr concentration was dependent on a stepwise increase in creatine concentration. The PCr/O_2 ratio was equal to 5.7 and close to the theoretical maximal P/O_2 ratio under conditions similar to those in vivo [89]. These results show that PCr is the main energy flux carried out from mitochondria in permeabilized cardiomyocytes.

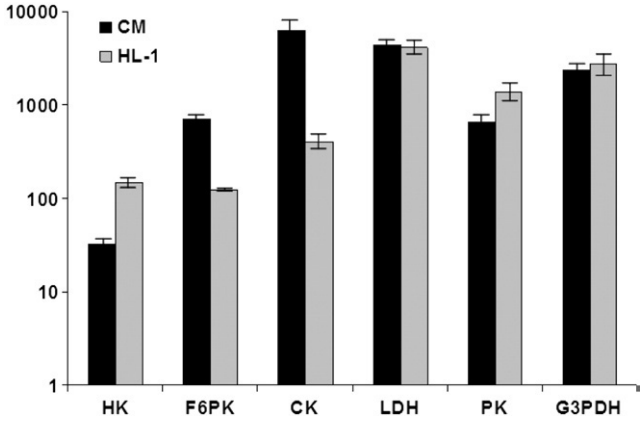


Fig. 5. Enzyme activity profile in rat adult cardiomyocytes (CM), in non-beating HL-1 cells. Hexokinase (HK), fructose-6-phosphokinase (F6PK), lactate dehydrogenase (LDH), pyruvate kinase (PK), glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and creatine kinase (CK) activities were measured and represented on a logarithmic scale. The means are presented ± SD. Adapted from [202].

The selective regulation of barrier functions of MOM by cytoskeleton and functional coupling of MtCK with ANT is highly important for the structural and functional organization of energy metabolism and regulation of effective exchange of phosphoryl groups between two systems of recycling metabolites. ATP/ADP recycling is restricted mostly to mitochondrial intermembrane and matrix spaces, while Cr and PCr are recycled between mitochondrial intermembrane and cytoplasmic spaces, resulting in energy transport from mitochondria into cytoplasm by freely diffusible PCr molecules via the system of compartmentalized CK reactions (Fig. 8). This figure shows the scheme of PCr/CK shuttle or circuit in the brain, heart and skeletal muscle cells, in details described elsewhere [7,10,21,42,70–73,90]. The coupling between two metabolic cycles is realized in mitochondria by a supercomplex which we called Mitochondrial Interactosome, MI (Fig. 9). This complex is formed by ATP synthasome (a term proposed by Pedersen [91–94] and constituted by ATP synthase, ANT and Pi carrier) functionally coupled to MtCK [10,42,75,76,86,89,95–97], and VDAC with tubulin and some other regulatory proteins (Fig. 9) [86,89]. This unit can also include the super complex formed by the respiratory chain [98,99]. The role of Mitochondrial Interactosome is to ensure continuous recycling of adenine nucleotides in mitochondria, their transphosphorylation and metabolic channeling of ATP via ANT to MtCK, and back ADP, resulting in the export of free energy from mitochondria into cytoplasm as flux of PCr. The functioning of this complex structure is best explained by the theory of vectorial metabolism and the vectorial ligand conduction, proposed by P. Mitchell [100]. Initially, this theory was developed to explain the organization of enzymes in supercomplexes allowing the scalar transport of electrons and the vectorial conduction of protons through the mitochondrial inner membrane to create the electrochemical potential [100,101]. Later, this concept was applied to the functioning of the phosphotransfer shuttle PCr/CK, AK [1,102,103], and to the transmission of [ADP] feedback signal from myofibril towards mitochondria [10,70,80,90,104].

Mitochondrial Interactosome is an integral part of the creatine kinase phosphotransfer network (shuttle) [10,42,90,105–108]. This network explains metabolic aspects of Frank–Starling law of the heart [7,108] and is quantitatively described by the mathematical model of compartmentalized energy transfer which describes non-equilibrium kinetics of creatine kinases functioning in opposite directions in mitochondria and myofibrils and takes into account the limited permeability of mitochondrial outer membrane for adenine nucleotides [104]. The model shows that cytosolic free ADP concentration may reach the levels of even 400 μM [7,36,104]. This value markedly exceeds the free ADP levels calculated from the CK equilibrium constant (50–100 μM) [36]. This mathematical model also helps to explain the importance of the limitation of the permeability of the outer mitochondrial membrane in cardiac cells within the Mitochondrial Interactosome for the effective control of oxidative phosphorylation by one of intracellular factors — the cytoplasmic ADP. As we have seen above, the apparent Km for free ADP in mitochondria in situ (in permeabilized cardiac cells and fibers) is about 20 times higher than in isolated mitochondria. When MOM is permeable, as in isolated mitochondria, the respiration regulation by cytoplasmic ADP is impossible because of saturating ADP concentrations (50–400 μM) in Fig. 10. Under these conditions cytoplasmic ADP has no role in respiration regulation. According to O'Rourke's principle mentioned above [48], changes in metabolic regulator should correlate with changes in workload and respiration rates. When ADP diffusion is restricted at the level of MOM, as it is in mitochondria in situ in permeabilized cardiomyocytes, the respiration rates become almost linearly dependent on cytoplasmic ADP concentrations when the latter changes in the range of physiologic concentrations up to 200–400 μM (shown by shaded area in Fig. 10). When MtCK is activated as it is in MI in vivo, the linear relationship between respiration rates and increase in free [ADP] is amplified and displaced towards the left region of Henri–

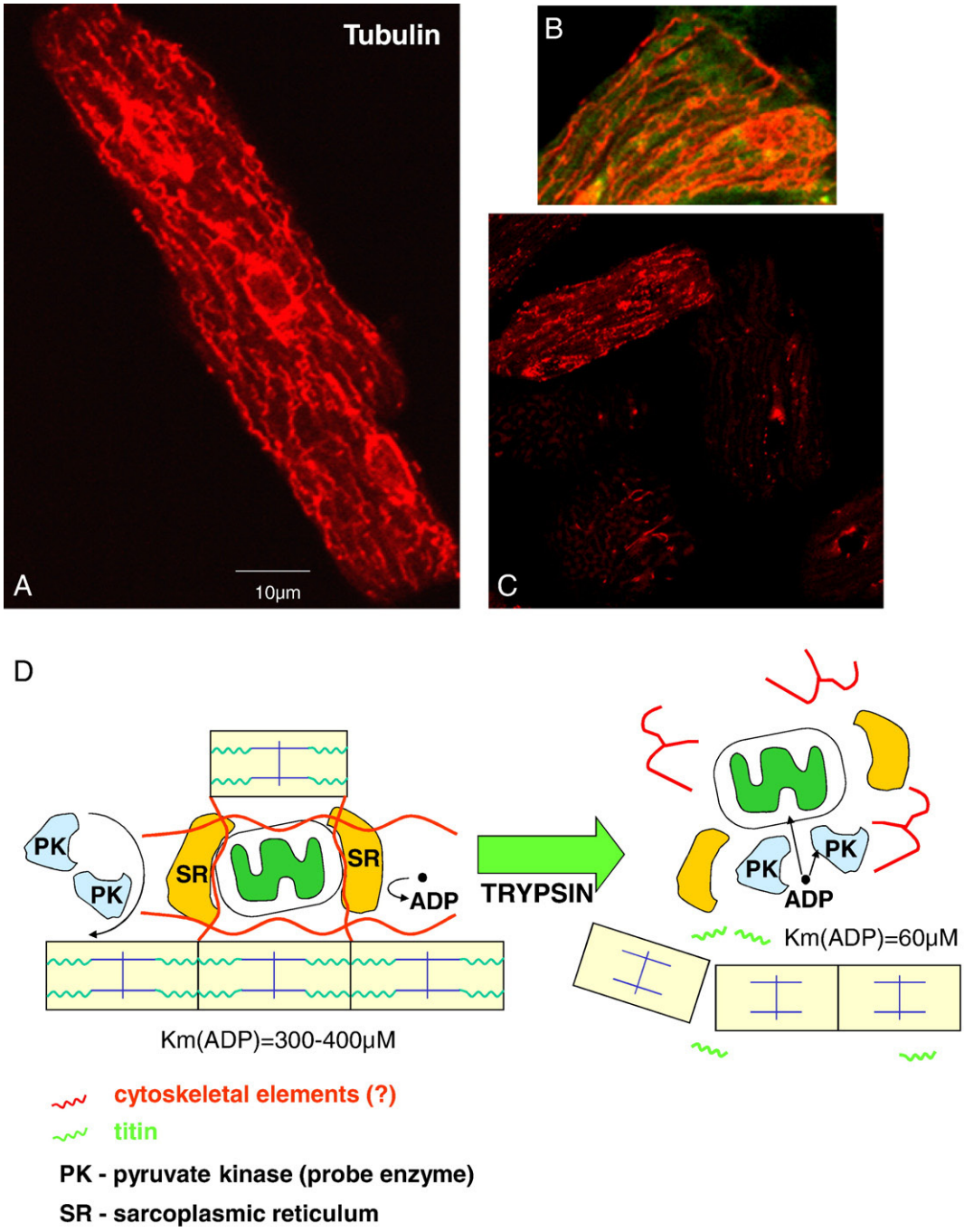


Fig. 6. (A, B, C) Confocal imaging immunofluorescence of microtubular network in cardiomyocytes. A, Microtubule network in control cardiomyocyte. B, Double labelling immunofluorescence of mitochondria and tubulin in control cardiomyocyte. The green colour is that of MitoTracker Green FM associated with mitochondrial membranes, and the red colour is the staining for tubulin. C, Effect of trypsin treatment (5 min, 1 mM at 4 °C) on the intracellular organization of microtubular network of cardiomyocyte: tubulin labelling disappears. Lower panel: schematic presentation of the role of cytoskeleton in the organization of mitochondria into functional complexes with sarcoplasmic reticulum (SR) and sarcomeres, i.e. into intracellular energetic units, ICEUs [13]. PK, pyruvate kinase. Proteolytic treatment with trypsin results in the collapse of the cytoskeleton and disorganization of the regular arrangement of mitochondria within the cells. For further explanation see text. Reproduced from with permission from Apaix et al. [19].

Michaelis–Menten representation due to recycling of ADP in coupled reactions in MI (see Fig. 10). In other words, for effective regulation of respiration in dependence of workload in cardiac cells in vivo, changes in concentrations of cytosolic ADP (as calculated by the model) are necessary and sufficient only when MtCK is actively functioning within the coupled systems in Mitochondrial Interactosome. An interesting and important task will be to apply the methods of Metabolic Control Analysis in experiments with permeabilized cells with fully activated MtCK in the presence of the PEP–PK system (see Fig. 4) to measure the flux control coefficients of different components of Mitochondrial Interactosome to quantitatively characterize their role in control of respiration and energy fluxes. Calculation of flux control coefficients from the mathematical model of compartmentalized energy transfer [109] showed that other important signals in metabolic feedback regulation of respiration may be cyclic changes in Pi and PCr/Cr ratio. Possible role of Pi in regulation of respiration was confirmed experimentally [110,111]. Both mathematical modeling and direct experimental determination of energy fluxes from mitochondria in permeabilized cardiomyocytes showed that under these conditions energy is carried into the cytoplasm mostly by phosphocreatine

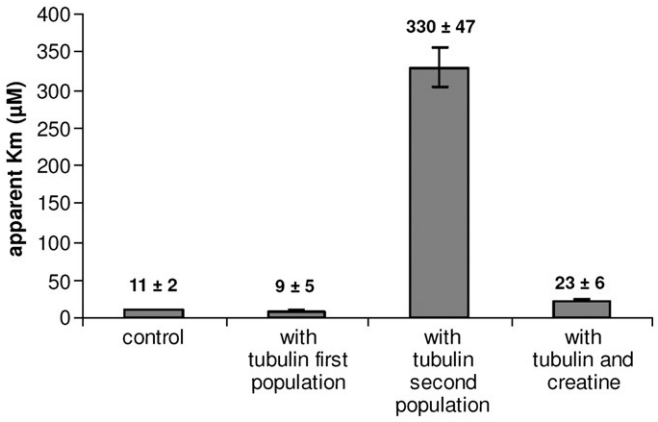


Fig. 7. Comparison of apparent Km for exogenous ADP in isolated heart mitochondria in three different conditions: control (mitochondria without tubulin and creatine), with tubulin 1 μM (two populations of mitochondria appeared) and with tubulin 1 μM and creatine 20 mM. The incubation with 1 μM tubulin was performed for 30 min at room temperature. The means are presented ± SD. Adapted from [203].

molecules [73,89,112]. Thus, there is clear separation of mass and energy transfer (by PCr and Cr) and information transfer (feedback metabolic signaling) [89].

The very remarkable observation is that physical activity changes the regulation of mitochondrial respiration via Mitochondrial Interactosome by increasing the value of apparent Km for exogenous ADP by a factor of 3 [81,82,113], while at the same time increasing the activity of MtCK [81,114]. As a result, the effect of creatine on respiration rate will be more significant [81,82,113]. The elucidation of the nature of changes induced in MI by physical exercise needs further experimental studies.

Mitochondrial Interactosome, as it is shown in Fig. 9, helps us to explain also another classical observation in the history of bioenergetics. Belitzer and Tsybakova showed in 1939 [115] that creatine added to a well washed homogenate of pigeon pectoral muscle strongly increased oxygen uptake and production of phosphagen (as phosphocreatine, PCr, was called at that time) without any added adenine nucleotides present only in trace amounts. The efficiency coefficient of aerobic synthesis of phosphagen, the PCr/O_2 ratio was between 5.2 and 7 [115]. This was one of the first determinations of

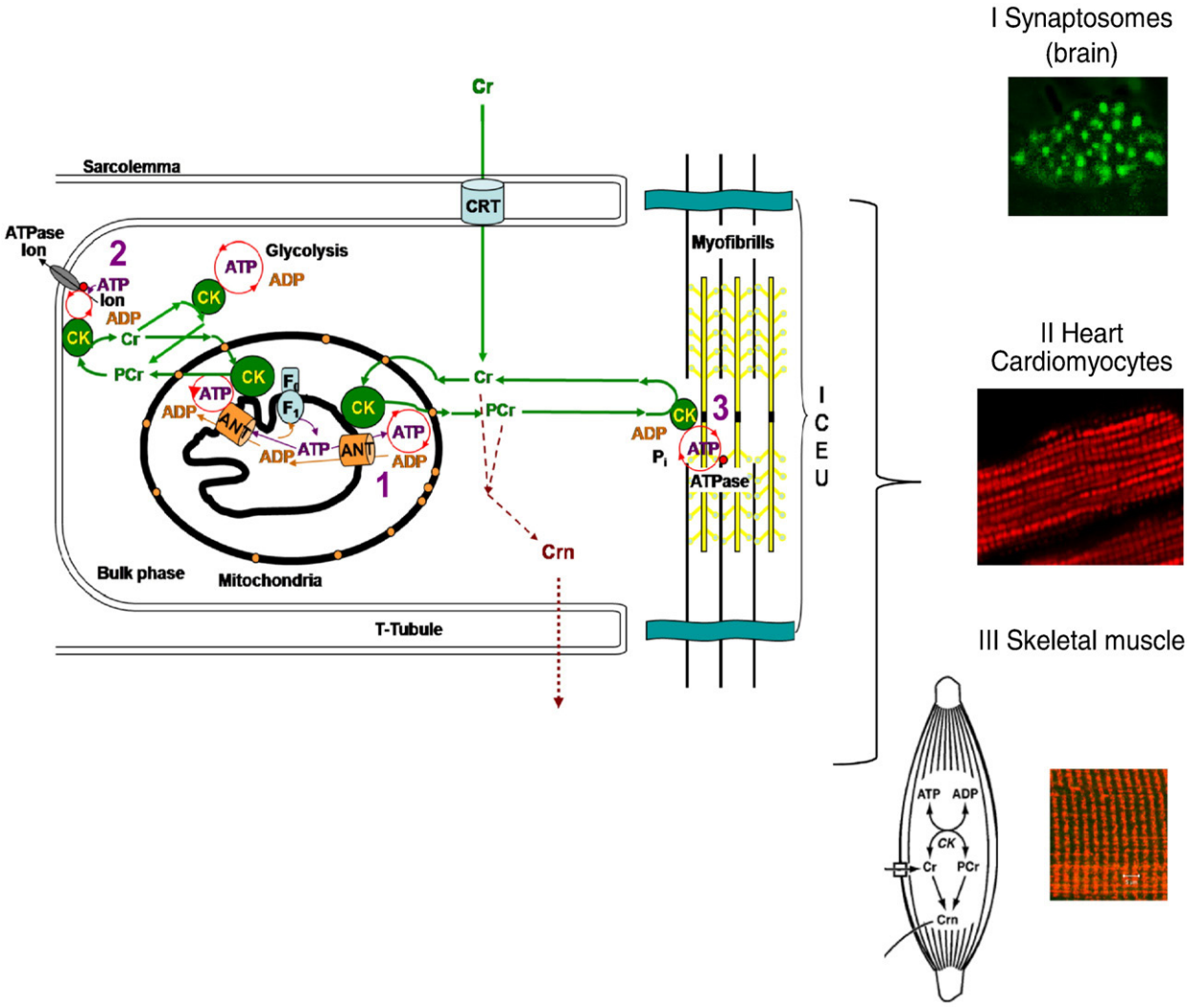
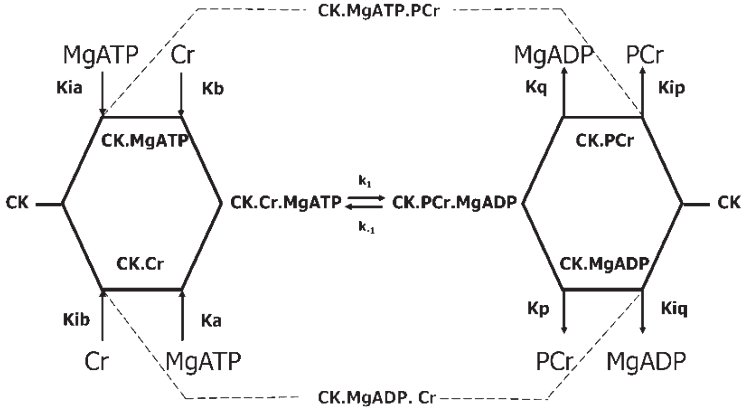


Fig. 8. Organization of compartmentalized energy transfer and metabolism in cardiac, skeletal muscle and brain cells and major routes of Cr metabolism in the mammalian body. The scheme shows the structural organization of the energy transfer networks of coupled creatine kinase (CK) reactions in mitochondria (1), at sarcolemmal membrane (2) and in myofibrils (3). The mitochondria, ATP-sensitive systems in sarcolemma and MgATPase of myofibrils are interconnected by creatine (Cr) and phosphocreatine (PCr) and energy transfer by the creatine kinase–phosphocreatine system. In brain cell systems energy transfer reactions are presented only by coupled reactions (1) and (2). Adenine nucleotides within local compartments 1, 2 and 3 do not equilibrate rapidly with adenine nucleotides in the bulk water phase. Right panels show confocal images of rat brain synaptosomes, cardiac cells and m. soleus. Mitochondria were labelled by MitoTracker Red or MitoTracker Green (50 nM). Very regular arrangement of mitochondria in striated muscles and fixed granular mitochondria in synaptosomes is seen. Creatine is not synthesized in these cells, but transported into cells by creatine transporter, CRT.

Table 3
Kinetic properties of MtCK in situ in cardiomyocytes. The MtCK reaction mechanism, BiBi quasi equilibrium random type is characterized by two dissociation constants for each substrate as shown in the following scheme [73,88]:



Values of constants for isolated mitochondria are taken from the literature [78,137]. In isolated mitochondria the oxidative phosphorylation decreases dissociation constants of MgATP from MtCK-substrate complexes suggesting the privileged uptake of all ATP by MtCK. In mitochondria in situ in permeabilized cardiomyocytes the increase of apparent constants of dissociation of MgATP compared with in vitro mitochondria shows the decrease of apparent affinity of MtCK in situ for extramitochondrial MgATP. The decrease of apparent constants of dissociation of creatine from MtCK-substrate complexes suggests the increase of the apparent affinity of MtCK for creatine in situ. The apparent constant of dissociation for PCr did not change in situ compared with isolated mitochondria. Reproduced from Guzun et al. with permission [73].

		K_{ia} (MgATP), mM	K_a (MgATP), mM	K_{ib} (Cr), mM	K_b (Cr), mM	K_{ip} (PCr), mM
Isolated mitoch.	−OxPhosph	0.92 ± 0.09	0.15 ± 0.023	30 ± 4.5	5.2 ± 0.3	
	+OxPhosph	0.44 ± 0.08	0.016 ± 0.01	28 ± 7	5 ± 1.2	0.84 ± 0.22
Mitoch. in situ (PEP–PK)		1.94 ± 0.86	2.04 ± 0.14	2.12 ± 0.21	2.17 ± 0.40	0.89 ± 0.17

stoichiometric coefficients in oxidative phosphorylation. Now we can easily explain this observation by the recycling of catalytic amounts of ADP and ATP within MI activated by creatine and coupled to phosphocreatine synthesis in skeletal muscle (Fig. 9).

6. Pathogenic mechanisms related to changes in organization of integrated energy metabolism

Dysfunction of integrated energy metabolism may be among leading mechanisms of pathogenesis of many diseases. A classical example of the importance of cellular organization of a complex system of energy metabolism is the Warburg effect: increase of lactate production in tumor cells in the presence of oxygen [116–119] first reported in the 1920s by Otto Warburg [118]. The conversion of glucose to lactate yields 2 mol of ATP per mole of glucose in comparison to 38 mol of ATP when glucose is completely degraded to CO_2 and H_2O. Thus, degradation of glucose to lactate yields only 5% of the energy available from glucose. This apparently senseless waste of energy prompted Warburg to postulate a defect in respiration in tumor cells as a cause for the increased "aerobic glycolysis" [116,118]. These and many other diseases are caused by cellular pathologies related to changes in mitochondrial structure and function, known as mitochondrial pathologies [119,120].

In normal cells competition between glycolysis and oxidation of fatty acids leads to their coordinated function with the aim to extract more free energy into the adenylate system (ΔG_{ATP}) from catabolic reactions [21] mostly to maintain cellular work, ion transport and biosynthesis. When workload increases, ATP production and respiration are increased due to feedback regulation via the CK system [7,21]. These pathways occur under aerobic conditions. Among the mechanisms limiting the glycolytic rate in normal cells are the intrinsic kinetic properties of soluble isoforms of hexokinase, HK, the inhibition of HK by the reaction product glucose-6-phosphate (G-6-P), and finally the inhibition by citrate via phosphofructokinase (PFK).

In cancer cell this mechanism of regulation is lost and substituted by other mechanisms of interaction between glycolysis and oxidative phosphorylation that result in the parallel activation of both systems [121]. Our studies on HL-1 cells, which are derived from mice atrial cardiomyocytes but also carry some properties of cancer cells (such as unlimited proliferation) have been useful for understanding the metabolic changes associated with the development of tumor cell phenotype. As we have seen in Fig. 3, mitochondria in NB HL-1 cells exhibit very high apparent affinity for exogenous ADP similar to that of isolated mitochondria. In addition, creatine is unable to stimulate respiration of NB HL-1 cells (Fig. 4F) due to downregulation of mitochondrial MtCK and cytosolic MM-CK [84]. The only CK isoform in cytosol of NB HL-1 cells was found to be BB-CK isoform [84] At the same time, metabolic profile of NB HL-1 cells is characterized by the prevalence of glycolytic enzyme activity, especially by that of HK and PK (Fig. 5). Total HK activity in NB HL-1 cells homogenate was increased by a factor of 5 in comparison with adult cardiomyocytes (Fig. 5) [122]. High activity of hexokinase is seen even after permeabilization of cells when only the activity of membrane-bounded enzymes can be measured [84]. Fig. 11 shows that glucose exerted a remarkable stimulatory effect on mitochondrial respiration in NB HL-1 cells, in comparison with a negligible effect on respiration of permeabilized heart fibers [84]. Lack of stimulatory effect of glucose on oxygen uptake in the presence of ATP in permeabilized heart cells means that while hexokinase is expressed in cardiomyocytes [84], it is not bound to mitochondrial membrane where tubulin occupies binding sites near VDAC within Mitochondrial Interactosome. In cancerous HL-1 cells the Mitochondrial Interactosome structure is significantly modified (Fig. 12): tubulin has evidently given place to HK, and the absence of MtCK allows all mitochondrial ATP to be captured for phosphorylation of glucose and stimulation of glycolytic lactate production. Thus, the Mitochondrial Interactosome typical for normal cardiomyocytes is replaced by another type of Mitochondrial Interactosome, in which mitochondrially bound HK substitutes for the role of MtCK. These structural rearrangements result in a specific metabolic phenotype characterized by the replacement of creatine control of respiration, characteristic of adult cardiac cells, by that of glucose in NB HL-1, thus underlying the so-called "Warburg effect". As

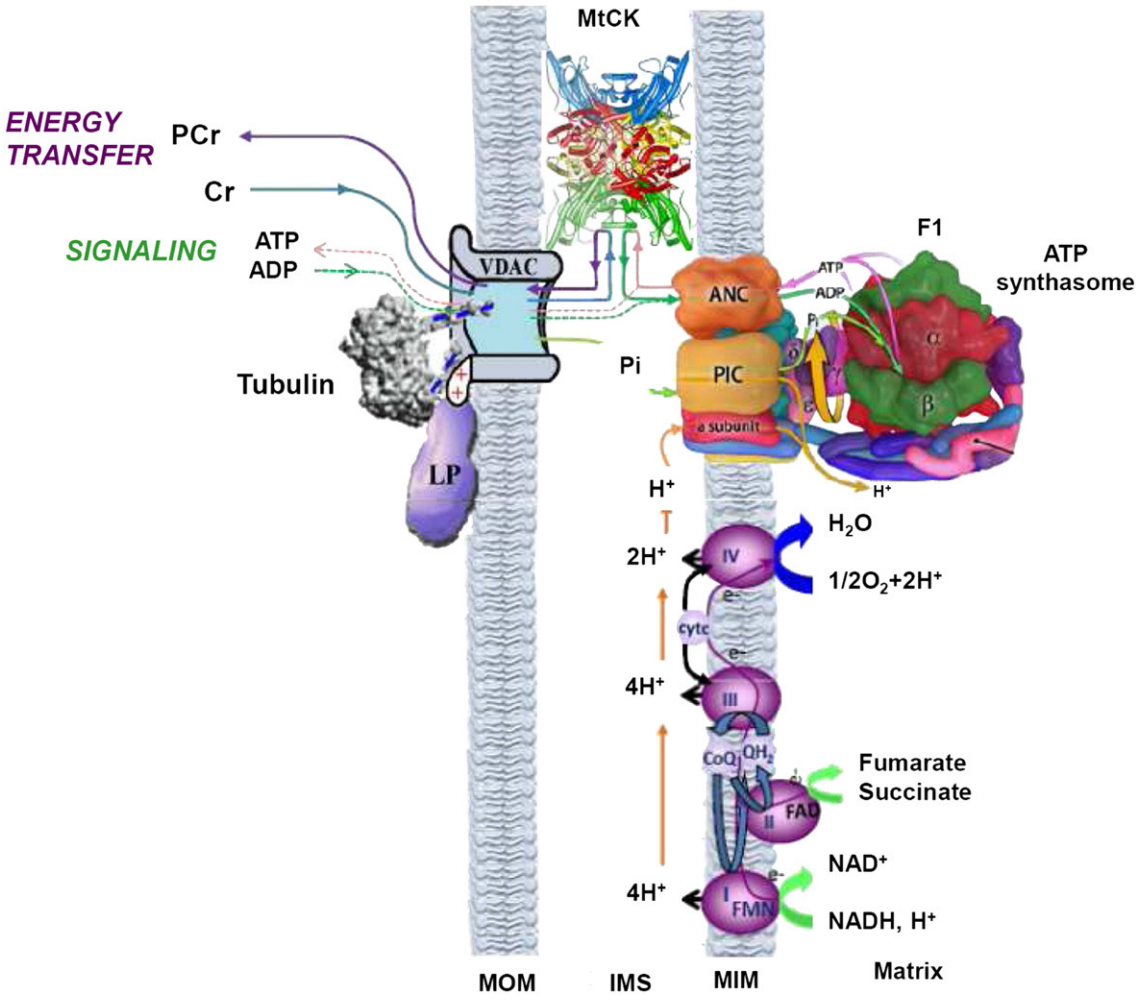


Fig. 9. Mitochondrial Interactosome (MI) in cardiac, oxidative skeletal muscle and brain cells consisting of ATP synthasome (formed by ATP synthase, adenine nucleotide carrier (ANC) and inorganic phosphate carrier (PIC) as proposed by Pedersen [92], mitochondrial creatine kinase (MtCK) functionally coupled to ATP synthasome and voltage dependent anion channel (VDAC) with regulatory proteins (tubulin and linker proteins(LP)). ATP regenerated by ATP synthase is transferred to MtCK due to its functional coupling with ATP synthasome. MtCK catalyses transfer of phosphate group from ATP to creatine producing phosphocreatine (PCr) which leaves mitochondria as a main energy flux. ADP is returned to and recycled in ATP synthasome due to highly selective permeability of VDAC. VDAC permeability is regulated by heterodimeric tubulin and by some linker proteins (LP). Small signaling amounts of cytosolic ADP enter the intermembrane space and increasing production of the PCr within MI due to the functional coupling of ATP synthasome with MtCK which amplifies cytosolic ADP signal. MOM, mitochondrial outer membrane; MIM, mitochondrial inner membrane; IMS, mitochondrial intermembrane space. Reproduced from Timohhina et al. with permission [89].

it was described by Pederson et al., in cancer cells the over-expressed HK2 is bound to VDAC in the MOM [91–93,121,123–126] and via this coupling can exert control over oxidative phosphorylation. The apparent Km of membrane-bound HK2 for glucose is about 250 times lower than for soluble isoenzymes [127] and it is protected from the G-6-P product inhibition by its connection with VDAC. Glycolytic ATP synthesized during lactate production is used in biosynthetic pathways for growth and proliferation [125,126]. In this way, it seems that the "energetic" role of mitochondria in cancer cells is reduced to maintain glycolysis. However, oxidative phosphorylation, although suppressed due to decreased biogenesis of mitochondria is still preserved, in order to facilitate glycolysis. Currently, it is not clear how the transformation from normal to cancer metabolism proceeds, how normal mechanisms of coordination between glycolytic and oxidative networks degrade into the most primitive model of glycolytic energy metabolism. Groof et al., in 2009 tried to answer this question by studying the mechanisms of Warburg phenotype development during immortalization of mouse fibroblast cells (H-RasV12/E1A). This process was shown to be dependent on the number of culture-passages and characterized by the increase in glucose-to-lactic flux and cellular oxygen consumption associated with the decreased TCA and oxidative phosphorylation activity [128].

Most of the other pathologies are related to defects in the respiratory chain complexes and lead to decreased ATP production as a result of either inhibition of respiration or uncoupling of phosphorylation and respiration [120]. Among pathogenic mechanisms related to changes in mitochondrial functions is the opening of mitochondrial permeability transition pore, PTP, induced by calcium overload of cells and accelerated by production of reactive oxygen species, ROS [129,130]. PTP opening leads to cell death by necrosis, and this is the most common mechanism related to ischemic and reperfusion injuries [129,130]. ROS production by chemical (non-enzymatic) reaction of molecular oxygen with reduced electron carriers in the complexes I and III of the respiratory chain is considered also as the major mechanism of ageing and cancerogenesis [131]. ROS production consumes from 1 to 5 % of oxygen supplied to the cells and cannot be avoided, but can be controlled by means of controlling the red-ox state of the respiratory chain [131]. Both ROS production and PTP opening can be effectively controlled and inhibited by the MtCK reaction which is functionally coupled to the adenine nucleotide translocase, controls respiration and ATP–ADP recycling in mitochondria and by this way controls the red-ox state of the respiratory chain and also the conformational state of mitochondrial carriers important for PTP opening [97,132].

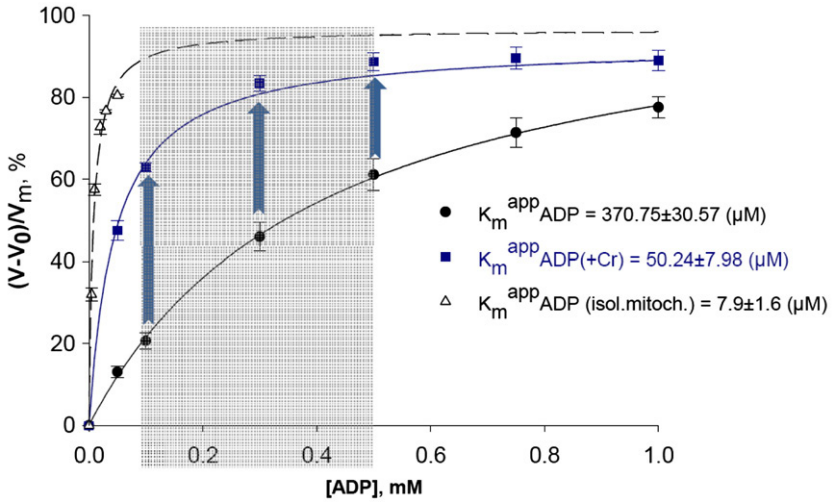


Fig. 10. Henri–Michaelis–Menten hyperbolic representation of kinetics of respiration regulation by free ADP in isolated heart mitochondria and permeabilized cardiomyocytes (in the absence or presence of creatine). The grey area delimits physiologic range of changes in cytosolic [ADP] taken from the model of compartmentalized energy transfer in cardiomyocytes [104]. In isolated mitochondria (curve (Δ)), no regulation of respiration is possible because of the saturating free [ADP] for the minimal workload. When the ADP diffusion is restricted as in mitochondria in situ in permeabilized cardiomyocytes (curve (●)), the respiration rates become linearly dependent on ADP concentrations in their physiological range. In this interval of quasi-linear dependence under physiological conditions the activating effect of ADP can be amplified by creatine (curve (■)), due to activation of coupled MtCK. The resulting apparent Km for cytoplasmic ADP is significantly decreased and respiration rate increased.

7. Parameters of PCr/Cr system as diagnostic means

In the cells with high energy demand the creatine kinase system is responsible for facilitating energy supply to local pools of the ATP near ATPases and ATP-sensitive channel in sarcolemma (see Fig. 8), thus controlling both contraction and excitation–contraction coupling [1,7,10,42,88,105,108,133–143]. Changes in this central system of energy supply either due to alteration of creatine kinase isoenzyme composition, activity and mechanisms of interaction with ATP producing and consuming systems, or decrease of total creatine content may lead to serious pathology of the heart, skeletal muscle and nervous system [133–135,143]. Clinical studies of patients with severe cardiomyopathy by non-invasive nuclear magnetic resonance imaging and spectroscopy revealed a very high diagnostic value of parameters of functioning of creatine kinase system — the PCr/ATP ratio for prognosis of patient's mortality rate and thus survival [133]. Alternatively, dietary supplementation of creatine has been shown to be an effective mean of pharmacological treatment and protection of patients with muscular and neurodegenerative diseases [105,135,136, 139–141]. Systems biology approaches to studies of these integrated processes of energy metabolism in normal cell life and in their pathology may have an utmost importance for clinical medicine in the near future [7,144,145].

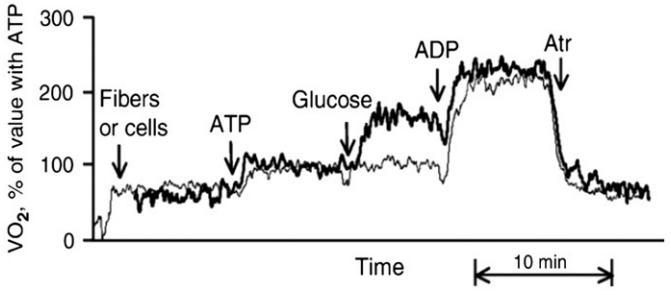


Fig. 11. Oxygraphic analysis of coupling of hexokinase (HK) to OxPhos in permeabilized HL-1 cells and rat left ventricular fibers. The thin line corresponds to respiration rates of permeabilized rat left ventricular fibers or cardiomyocytes; the thick line shows respiration rates of HL-1 cells. Ten millimolars of glucose activates HL-1 cell respiration in the presence of 0.1 mM MgATP due to the endogenous ADP production in HK reaction. Similar amount of glucose cannot stimulate respiration rate of permeabilized fibers or cardiomyocytes. ADP — 2 mM MgADP, Atr — 0.1 mM atractyloside. Reproduced from Eimre et al. with permission [84].

In myocardial infarction and in heart failure, a rapid decrease of PCr content occurs due to lack of oxygen supply and pathological changes in the creatine kinase system [108,133–137]. Total ATP content usually changes very slowly and its changes, as well as changes in the free energy of ATP hydrolysis calculated from total metabolites' contents, are dissociated from the rapid fall of the cardiac contractile force [28,146], total depletion of ATP resulting in contracture of the heart muscle [147]. A rapid decline in heart contractile function in hypoxia and ischemia is most likely to be related to changes in compartmentalized energy transfer systems, leading to decreased regeneration of ATP in functionally important cellular compartments, as shown in the scheme in Fig. 8. Firstly, the rapid decline in ATP regeneration in subsarcolemmal area results in changes of ion currents across this membrane and thus in shortening of action potential [1,137], and secondly, the rapid decline in ATP regeneration in myofibrillar microcompartments due to lack of phosphocreatine slows down the contraction cycle [108,137]. Similar but slower changes are observed in chronic cardiac and skeletal muscle diseases [148–157]. In concord with this conclusion are the results published by Weiss et al. [154] showing that cardiac ATP flux through CK is reduced by 50% in cases of human heart failure in the absence of reduction of ATP stores. Local phosphotransfer networks in the subsarcolemmal area are an important part of the membrane sensors of the cellular energy state also in brain cells [136,139–141], explaining the dependence of functional state of these cells on phosphocreatine supply, and thus the central importance of the PCr/Cr system. In brain cells ubiquitous mitochondrial creatine kinase is co-expressed with BB isoenzyme localized both in cytoplasm and at the cell membrane (coupled creatine kinases 1 and 2 in the Fig. 8). Alterations of these systems are observed in many neurodegenerative diseases [139,140].

The content of phosphocreatine, central energy carrier in the cells, depends both on its continuous regeneration in mitochondrial creatine kinase reaction coupled to oxidative phosphorylation, and on the total creatine content which in significant part depends on the import of creatine (not produced in muscle or brain cells) from blood by creatine transporters in the cell membrane. This explains the beneficial effects of dietary supplementation of creatine on the energy metabolism and functional state of skeletal muscle and brain cells,

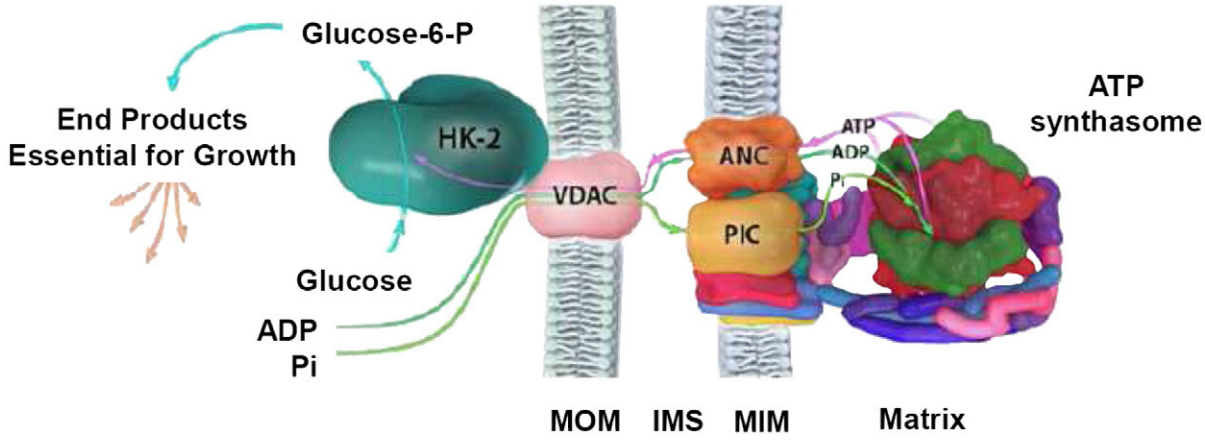


Fig. 12. Current view showing how HK2 bound to VDAC in the mitochondrial outer membrane in HL-1 cells has preferred access to ATP synthesized on the inner membrane by the ATP synthasome, a complex between the ATP synthase and carriers (transporters) for ADP and phosphate (Pi). The structural integrity of this entire network is essential for the survival of those cancer cells that exhibit the "Warburg" effect. Thus, HK2 while preventing apoptosis by binding to VDAC, also supports cancer cell growth by receiving preferred access to ATP newly synthesized by the ATP synthasome. Reproduced from Pedersen with permission [91]. This Figure and ATP synthasome in Fig. 9 are artworks by David Blume, Johns Hopkins University, USA.

especially in patients with neurodegenerative diseases [139–141,158–160].

Dissociation of total content of ATP from cell function was recently clearly demonstrated by O'Connor et al. [142], who showed that it is the PCr/CK system which sustains localized ATP-dependent reactions during actin polymerization in myoplast fusion. Myoplast treated with exogenous creatine showed enhanced intracellular PCr stores without any effect on ATP levels. This increase in PCr induced the myoplast fusion and myotube formation during the initial 24 h of myogenesis. During this time BB-CK became localized and after 36 and 48 h was found close to the ends of the myotubes [142]. Actin polymerization is critical for myoplast fusion and occurs with involvement of ATP both during the addition of actin monomers to the growing ends of filaments and the dissociation of monomeres at the tail. It is this localized ATP which is rapidly regenerated by BB-CK at the expense of PCr, and it seems that the formation of these ATP microdomains is a dynamic process during actin cytoskeleton remodeling. Local injection of creatine into injured skeletal muscle increased the growth of regenerating myofibers from satellite cells via differentiation and fusion of myoplasts [142]. All these results add new insight into the functioning of the PCr/CK system in muscle cells, showing its new role in energy supply for cytoskeletal remodeling. These results may help to better explain the therapeutic effects of creatine supplementation [105,135,136,143,158–160].

Intensive and numerous studies have been carried out on transgenic mice with knockout of different CK isoenzymes, or enzymes responsible for creatine metabolism and transport (reviewed in [149,152,161,162]). In spite of multiple adaptive mechanisms — activation of alternative phosphotransfer pathways, such as adenylate kinase shuttle [161,163], structural changes in the cells and increase of oxidative capacity of skeletal muscle [152,161], and many others [164], significant functional and metabolic changes especially related to calcium metabolism and contractile performance have been observed in these experiments [162,164–167]. Thus, Momken et al. have reported that double knockout of MtCK and MM-CK very significantly impairs the voluntary running capacity of mice [165]. Knockout of enzymes of creatine biosynthesis in mice resulted in significantly reduced responses to inotropic stimulation [162]. Similarly, the hearts of rats treated with guanidinopropionic acid performed much less pressure-volume work [168]. Most interestingly, recent works from Neubauer's laboratory have shown that overexpression of creatine transporter and supranormal myocardial creatine contents lead to heart failure [169,170]. In these hearts creatine content is increased by more than a factor of 2 [169]. Most interestingly, these experiments put into evidence the importance of the PCr shuttle: heart failure may be due to the formation of dead-end complex CK.MgADP.Cr formation [171] and inhibition of PCr utilization for local ATP regeneration.

In summary, data obtained in experiments with CK knockout mice are in concordance with our conclusion made in this work and before [7,10,42,107,136,137] that muscle (and other) cells are viable without MtCK and other CK isoenzymes, as HL-1 cells, but PCr–CK and other phosphotransfer pathways are necessary for effective energy transfer and metabolic regulation at higher energy demand, and thus for survival under stress conditions. An important observation is that exercise training results in cytoskeleton remodeling, including changes in Mitochondrial Interactosome and increased efficiency of energy transfer via PCr–CK pathway [81,82,113,114]. By analogy, PCr–CK pathway is as an efficient highway connecting ATP production and consumption sites. Without this highway, cells have to find other ways of ATP and energy transfer, but the efficiency of communication and regulation is lost and energy may be wasted (as in HL-1 cells). Under these conditions, muscle and brain cells degrade into pathological state.

Two important large-scale clinical studies of changes in the PCr/CK system in the myocardium of patients with heart failure have been performed [133,134,148]. The first large-scale international study was organized by Ingwall and Allen [148]. In this study, myocardium was sampled from subjects who underwent heart transplant, from subjects maintained in an intensive care unit before heart harvesting, from accident victims, and patients undergoing heart surgery. Since the characteristics of myocardium of potential organ donors differed from those of myocardium of accident victims, data are presented for three groups: failing, donor, and control. MM-CK and the mitochondrial isoenzyme activities were lower in failing and donor LV, and MB-CK activity and B-CK content were higher in failing and donor hearts. Creatine contents were 64 ± 25 and 56 ± 18.6 nmol/mg protein in LV and RV of failing, 96 ± 30 and 110 ± 24 nmol/mg protein in LV and RV of donor, and 131 ± 28 nmol/mg protein in LV of control hearts [148]. Thus, in the failing hearts the total creatine content is significantly decreased, as compared to the control heart. Also, failing hearts showed much lower creatine kinase activity than those of the control [148].

The second important clinical study was performed by Neubauer's group in United Kingdom [133,134]. By using ^{31}P-NMR spectroscopy in combination with imaging for investigation of cardiac muscle energy metabolism in patients, the authors showed that in patients with cardiac disease — dilated cardiomyopathy (DCM) the decreased

PCr/ATP ratio (lower than 1.6) is very clear and strong diagnostic index of increased mortality. In the heart of patients with DCM the ATP content remained the same as in healthy control patients, but PCr decreased by 70% as compared to control. This shows the vital importance of the phosphocreatine–creatine kinase energy transfer network for the cardiac muscle normal function and life [133,134].

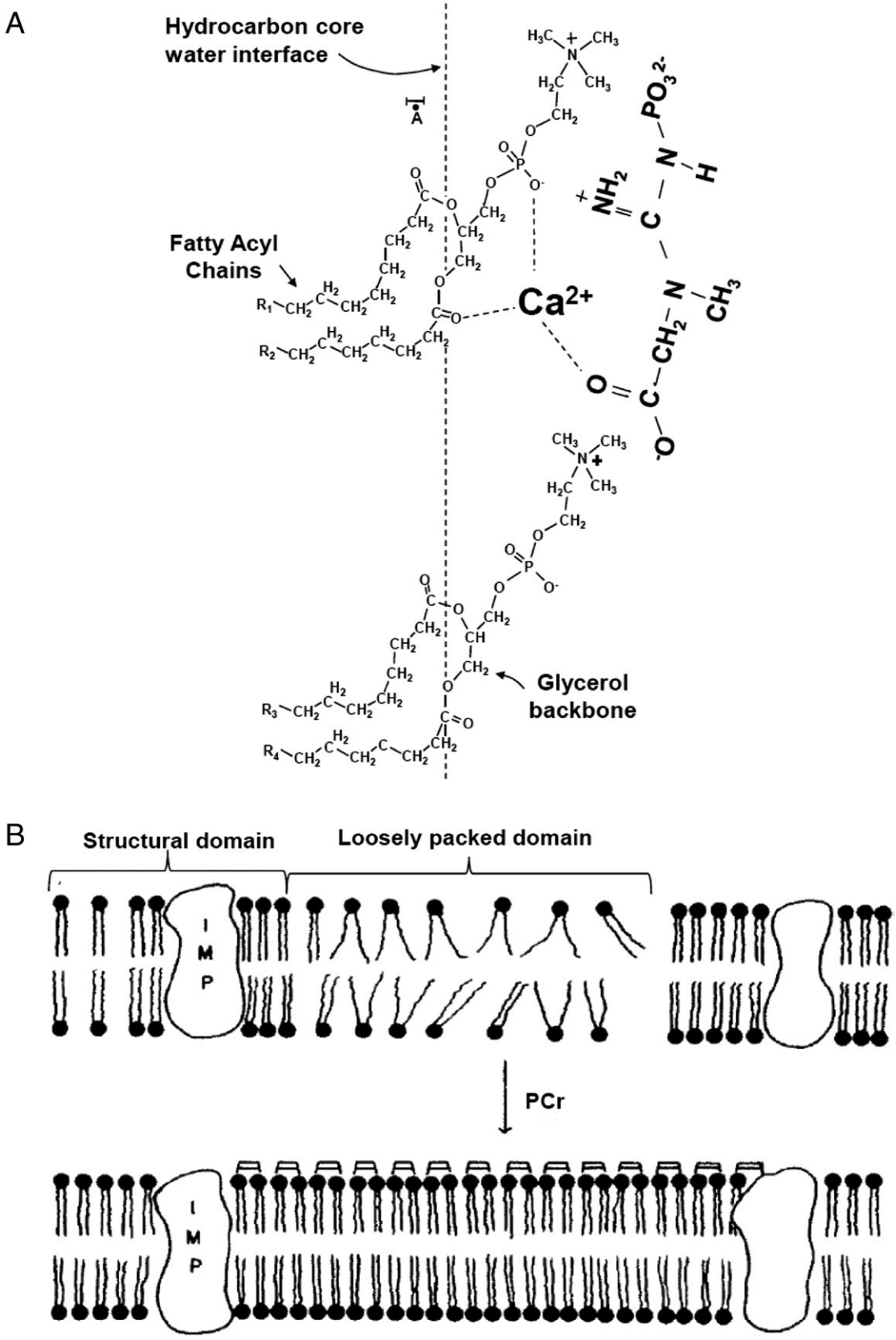


Fig. 13. Zwitterionic interaction of phosphocreatine with bipolar heads of phospholipid molecules in the membrane surface interphase. Adapted from Saks and Strumia [176] with permission.

It has been found in multiple clinical investigations of biopsy samples of skeletal muscle of patients with heart failure, that it has induced very clear changes of the creatine kinase system in these muscles most probably by decreasing oxygen supply and altering circulation (reviewed in [155,156,161,172]).

Thus, the PCr/ATP ratio is an important diagnostic parameter of heart disease, as is the total creatine content. Low PCr concentrations and low PCr/ATP ratios mean decreased regeneration of ATP by the PCr/CK system in microdomains (compartments) which are critically important for the function of the heart, skeletal muscle and brain. These microdomains are localized in myofibrils, near the sarcolemma and the membrane of sarcoplasmic reticulum in muscle cells and near cellular membrane in brain cells (see Fig. 8). There is a general consensus now among the researchers in muscle and brain energy metabolism that the further challenge and urgent need is to develop better bioprobes to image metabolic microdomains of ATP and functional proteomics to identify physical interactions between key proteins responsible for their formation [133,137,144].

In addition to its important role in supporting regeneration of local ATP pools as a substrate for MM-CK reactions in myofibrils and cellular membranes, the PCr molecule appears to have another very useful property — membrane stabilizing action (Fig. 13). This was revealed in long series of clinical use of extracellular phosphocreatine injection with clear protective effect on ischemic myocardium, and in detailed experimental studies [173–175]. In all these studies extracellular phosphocreatine was used and shown to decrease the ischemic damage of the heart muscle by multiple mechanisms. Among others, there is a clear membrane stabilizing effect of PCr [176] which may be explained by interaction of its zwitterionic molecule carrying positive and negative charges with opposite charges of phospholipid polar heads in the membrane surface interphase (Fig. 13A), resulting in the transition of the mobile domain (fluid phase) of membranes into a structured domain (gel phase) as shown in Fig. 13B, leading to the decrease of the rate of phospholipid degradation into lysophospholipids and lipid peroxidation [176]. Rapid fall of the intracellular PCr pool in hypoxia and ischemia may thus be a significant factor of destabilization of cellular membranes.

In patients with peripheral skeletal muscular diseases, very informative non-invasive diagnostic methods of assessment of the energy state by recording the parameters of the PCr/CK system have been developed due to rapid progress in NMR imaging and spectroscopic technologies [2,133,134,177–180]. Four quantitative parameters of the PCr/CK system which can be measured in patients by ^{31}P and ^{1}H NMR spectroscopy in combination with imaging [133] are: the contents of PCr and ATP and PCR/ATP ratio measured by ^{31}P NMR spectroscopy, the content of creatine measured by ^{1}H NMR spectroscopy [2,154,177–180], the ATP flux through creatine kinase system measured by saturation transfer method [2,154,180], and the kinetics of PCr recovery in skeletal muscle after exercise measured by ^{31}P NMR spectroscopy [2,180]. In combination with the biochemical analysis of biopsy samples taken from skeletal muscles of patients [5,113,181] these methods give an exhaustive diagnostic means for clinical analysis of the energy transfer networks in patients in health and disease [182].

8. General conclusion

Living cells and organisms are thermodynamically opened systems which exchange material and energy with its environment, since they must "avoid the decay into the inert state of equilibrium to keep alive by continually drawing from its environment negative entropy" as it was discovered by Schrödinger [183]. This basic property allows a living system to maintain the "stability of its internal milieu" or homeostasis described already by Claude Bernard [184,185]. Therefore, the cellular life is governed by laws of non-equilibrium irreversible thermodynamics and non-equilibrium steady state kinetics [10,186–190]. A decrease of internal entropy in such systems is achieved by free energy extraction from environment and energy dissipation to realize cellular work [183,188–190]. It is increasingly understood now that in living cells the effective regulation of metabolism is realized through formation of "dissipative metabolic networks" [189] due to complex intracellular interactions within the inhomogeneous intracellular medium. These interactions lead to new system level properties and mechanisms such as intracellular metabolic compartmentation, functional coupling, downward causation including higher level control of gene expression, retrograde response between mitochondria and nuclei etc. which are the topics of studies in Molecular System Bioenergetics [10,67], part of Systems Biology [65–67].

Results reviewed in this article show the importance of structural and functional organization of intracellular phosphotransfer networks interconnecting ATP-utilization and ATP regeneration processes into intracellular energy units (ICEU). The role of the ICEUs is not reduced only to the high efficiency of coordination of energy metabolism. Their role is more fundamental: in conformity with the theory of dissipative metabolic structures [189,190], formation of the ICEUs helps to extract Gibbs free energy and negentropy from the environment. ICEU can be seen as a "dissipative metabolic network" which functions in the non-equilibrium state made up of the dissipative enzymatic sub-networks (glycolysis, the Krebs cycle, fatty-acid's oxidation, the electrons transport chain, the shuttles creatine kinase/phosphocreatine, malate/aspartate, etc) structured and connected together by flows and regulating signals. Association of various enzymes within big multienzyme complexes allows the direct transfer of intermediate metabolites (vectorial ligand conduction). One of such complexes is Mitochondrial Interactosome which regulates the interaction between mitochondrial cycles of adenine nucleotides and PCr/Cr cycles in the cytoplasm of the heart, skeletal muscle and brain cells. Mitochondrial Interactosome and PCr pathway of intracellular energy transfer explain well the metabolic aspects of Frank–Starling law of the heart and classical observation of Belitzer and Tsybakova on effective coupling of PCr production and oxidative phosphorylation in muscles. Changes in Mitochondrial Interactosome lead to severe pathology and may contribute in the mechanism of Warburg effect in cancer cells. Systemic analysis of changes in phosphotransfer networks helps to explain many pathogenic mechanisms in numerous diseases.

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