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Molecular System Bioenergetics of Cardiac Cells: Quantitative Analysis of Structure-Function Relationship

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Declaration:

Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree or examination.

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Molekulaarne süsteemibioenergeetika: südameraku struktuuri ja funktsiooni vaheliste suhete kvantitatiivne analüüs

KERSTI TEPP

TTÜ KIRJASTUS

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PUBLICATIONS

I. Monge, C., Beraud, N., **Tepp, K**., Pelloux, S., Chahboun, S., Kaambre, T., Kadaja, L., Roosimaa, M., Piirsoo, A., Tourneur, Y., Kuznetsov, A., Saks, V., Seppet, E. (2009), Comparative analysis of the bioenergetics of the adult cardiomyocytes and non-beating HL-1 cells Respiratory chain activities, glycolytic enzyme profiles and metabolic fluxes. Canadian Journal of Physiology and Pharmacology, 87(4), 318-326.

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IV. Saks, V., Guzun, R. Timohhina, N., **Tepp, K.,** Varikmaa, M., Monge, C., Beraud, N., Kaambre, T., Kuznetsov, A., Kadaja, L., Eimre, M. Seppet, E.(2010) Structure-function relationships in feedback regulation of energy fluxes *in vivo* in health and disease: Mitochondrial Interactosome. Biochimica et Biophysica Acta, 1797, 678 - 697.

V. Tepp, K., Chekulayev, V., Shevchuk, I., Timohhina, N., Kaambre, T., Saks, V. (2010) Metabolic control analysis of integrated energy metabolism in permeabilized cardiomyocytes - experimental study. Acta Biochimica Polonica, 57(4), 421 - 430.

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VII. Saks, V., Kuznetsov, A.V., Gonzalez-Granillo, M., **Tepp, K.,** Timohhina, N., Varikmaa-Karu, M., Kaambre, T., Dos Santos, P., Guzun, R. (2011) Intracellular Energetic Units regulate metabolism in the cardiac cells. *JMCC* doi:10.1016/j.yjmcc.2011.07.015.

VIII. Gonzalez–Granillo, M., Grichine, A., Guzun, R., Usson, Y., **Tepp, K.**, Chekulayev, V., Shevchuk, I., Karu-Varikmaa, M., Kuznetsov, A. V., Grimm, M. Saks, V., Kaambre, T. (2011) Studies of the role of tubulin beta II isotype in regulation of mitochondrial respiration in intracellular energetic units in cardiac cells. JMCC doi:10.1016/j.yjmcc.2011.07.027.

The author`s contribution to the publications:

I – III, VIII	Author has carried out part of the experiments and participated in writing the manuscript.
V – VI	Author was responsible for planning and performing the experimental part. She carried out experiments with her coworkers and wrote major part of the manuscript.
IV, VII	Author participated in writing the manuscript in collaboration with coworkers.

ABBREVATIONS

ADP	adenosine 5´-diphosphate
AK	adenylate kinase
AMP	adenosine monophosphate
ANT	adenine nucleotide translocase
ATP	adenosine 5´-triphosphate
BSA	bovine serum albumin
CAT	carboxyatractyloside
Ca^{2+}	calcium ion
CK	creatine kinase
CM	cardiomyocytes
Cr	creatine
DDT	dithiothretol
DNFB	2,4-dinitrofluorobenzene
FCC	flux control coefficient
FAD	flavin adenine dinucleotide
$FADH_2$	1,5-dihydro- flavin adenine dinucleotide
Fe-S	ferrum-sulfur center
GGG	triglycine
ICEU	Intracellular Energetic Unit
IMS	mitochondrial intermembrane space
IM	isolation medium
LDH	lactate dehydrogenase
MCA	Metabolic Control Analysis
MCC	metabolic control coefficient
MI	Mitochondrial Interactosome
MtCK	mitochondrial creatine kinase
MMCK	muscle creatine kinase
MIM	mitochondrial inner membrane
MOM	mitochondrial outer membrane
NAD	nicotineamine adenine dinucleotide
NADH	dihydronicotineamine adenine dinucleotide
PCr	phosphocreatine
PEP	phosphoenolpyruvate
Pi	inorganic phosphate
PIC	inorganic phosphate carrier
PK	pyruvate kinase
SERCA	sarcoplasmic reticulum Ca^{2+} release channel
SR	sarcoplasmic reticulum
TnC	troponin C
TnI	troponin I
TnT	troponin T
VDAC	voltage dependent anion channel

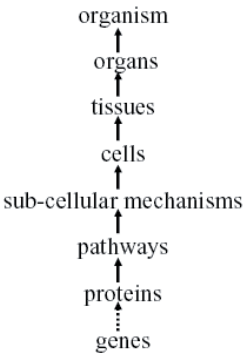
REVIEW OF LITERATURE

1. Philosophical background

1.1. Systems Biology – historical overview

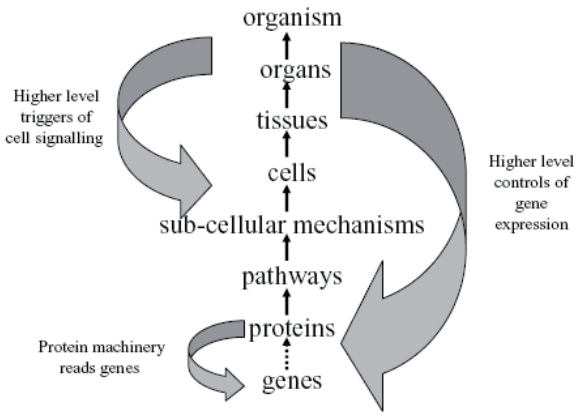
In the last two decades a new paradigm has been established in biological sciences: Systems Biology or Integrative Biology. The aim of the Systems Biology is to describe quantitatively, at different levels, complex biological systems [1-3]. As defined by Westerhoff, it is “the science that aims to understand how biological function absent from macromolecules in isolation, arises when they are components of their system” [4]. As it follows from the definition, the properties of such integrated system result from the interactions between individual components, and depend on the level of organization, “system level properties” [1, 3, 5]. The main aim of each level is to maintain the life of the whole organism.

This methodology is opposed by a more ancient approach called “reductionism”, which explores objects in their isolated state. Reductionism is a stage absolutely needed in order to determine characteristic intrinsic properties of objects, which are not manifested or cannot be explored in their native fundamental state. For example, due to one of important representatives of reductionism, structural biology could determine the structure of proteins or protein complexes. Also, genetics during several decades permitted to obtain phenomenal success in understanding cellular processes. In the late fifties of the last century, after the description of the structure of the DNA double helix by Watson and Crick in 1953 [6], DNA became considered as a factor of heredity. Genetics has long been predominant and omniscient in biological sciences so that the gene-centrism, placing the DNA as the "cause" of life, has quickly emerged. This concept of the "selfish" gene, now a bit disturbing, stipulates that all depends on genes, their mutations and their translation, and that a gene could egoistically ensure its survival, its transmission providing a selective advantage for an organism [7]. Denis Noble in his book “The Music of Life” schematized this situation: Scheme 1 is the representation of a reductionist causality initiated by DNA in linear direction within an organism. It is true that the basis of our constitution is encoded in our genetic heritage handed down by our parents, but that genetic determinism is a negation of the Rest. “It is systems (organisms) that live or die, not genes” [2].



Scheme 1. A bottom-up reductionist causality. This chain starts with the genes and the flow progresses linearly to the body. According to [2].

Denis Noble described perfectly that the Rest (across Scheme 2) represents the evolution of views on the role of the gene in biology. The Scheme 2 is the Scheme 1, supplemented by regulatory loops, such as cell signaling loops and loops of gene regulation, which provide fine control and a high degree of cellular organization.



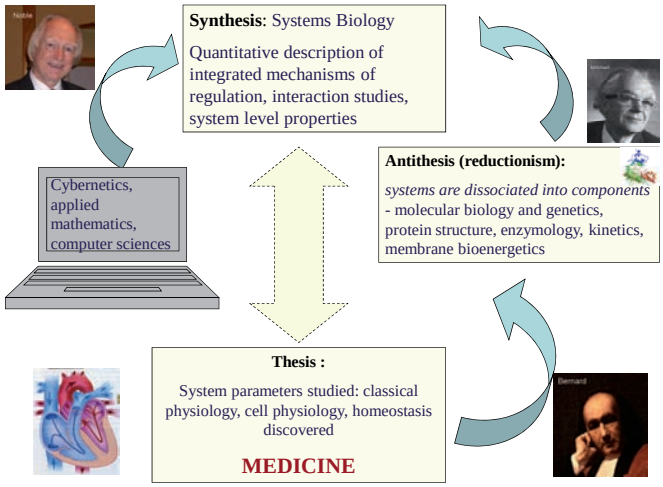
Scheme 2. A downward causality. The scheme 1 has been supplemented by a representation of cell signaling and regulation of retrograde gene expression. According to [2].

We finally see that the gene function is rather to serve an organism via interacting with its environment, and it must act together with other genes or proteins to provide a physiological function.

Besides the strict study of biological processes, the power of systems biology lies in use of mathematical modeling. The systems approach to biology took shape when the concepts of dynamics, regulation and control of biological functions were combined with well-established mathematical models (obtained

starting from the reductionism). The origins of principles of systems biology can be found in the works by French physiologist Claude Bernard published about 150 years ago [8]. He developed the idea of the constancy of internal environment homeostasis, due to integrated self-regulative mechanisms. That is “the condition for free and independent life: the mechanism that makes it possible is that which assured the maintenance, with the internal environment, of all the conditions necessary for the life of the elements” [8]. In fact, he has introduced the term "general equation” in order to describe the mechanisms of life by mathematical modeling. However, at the time of Claude Bernard science had only very little experimental data. Now, systems biology has experimental data, which are needed in order to send a challenge to “the general equation” [9]. Later, this idea was further extended in the E. Schrödinger’s book “What is Life?” - book on principles of organization of cellular processes and negentropy extraction from the surroundings [10] and N.Wiener work on the theory of feedback regulation on the mechanism of homeostasis [11]. In order to describe the work of the whole system the multilevel and multiscale analysis should be applied. The modern tool of scientific research, mathematical modeling, is a promising perspective for understanding the function of complex biological systems. Another approach gaining popularity is Metabolic Control Analysis. It is aimed to explain how and at what extent biological systems functions are derived from molecular interactions.

The developments of biological sciences from the time of Claude Bernard to our days follow the logic of Hegelian dialectic – succession from thesis to antithesis to synthesis [12]. The Scheme 3 illustrates these developments. Georg Wilhelm Friedrich Hegel, German philosopher of the late XVIII and early XIX century, in his search for the Absolute Idea [13], determined the methodology based on logic to understand the whole rather than isolated parts. The famous Hegelian dialectic has three stages: thesis, antithesis, synthesis, and can be easily adapted to requirements of Systems Biology to regroup what might appear to be opposites on a methodological level. Medicine (thesis), via its phenomenological observations, followed the reductionism (antithesis), whereas Systems Biology (synthesis) uses the data obtained by reductionism to explore the properties of systems (Scheme 3). The latter (systems biology) itself serves medicine, especially due to application of mathematics and informatics tools, in order to model physiological and pathophysiological processes [12].



Scheme 3. Hegelian logic of reflection and an approach of the biological sciences. Reprinted from [12] with permission.

1.2.Molecular System Bioenergetics and thermodynamic laws

Bringing principles of systems biology to cellular bioenergetics gives rise to the new field of research, Molecular System Bioenergetics. It describes and accounts not only energy metabolism as integrated networks but also its spatial (organization) and temporal (dynamic) aspects [3].

Molecular System Bioenergetics is one of the new scientific directions and an integral part of the Systems Biology that describes cellular bioenergetics starting from molecular processes up to a system level. The Molecular System Bioenergetics considers that the intracellular organization is dynamic and its` structure carries information on regulation of cellular metabolism. Therefore, the study of properties at a system level is essential for understanding cellular phenomena [13].

Cells can be considered as open systems that operate far from the thermodynamic equilibrium and exchange energy and matter with the external environment [14]. A process can only occur if it results in an increase in the entropy of the universe [14], at the same time, systems ability to perform work, chemical potential or Gibbs free energy depends on entropy:

$$dG = dH - TdS \quad (1)$$

Therefore, the increase of entropy at the constant temperature and pressure decreases the free energy the capacity of the system to perform work. Shrödinger, in his book “What is life“, explained the controversy of cells` ability to function in a steady state, far from the equilibrium and defined the fundamental principle of the bioenergetics, the principle of negentropy. "It is by avoiding the rapid decay into the inert state of `equilibrium' that an organism

appears so enigmatic...What an organism feeds upon is negative entropy." [10]. The total entropy change can be split into two contributing parts [15].

$$dS = dS_{ext} + dS_{int} \qquad (2)$$

where dS_{ext} is the entropy of catabolism and dS_{int} is the entropy of anabolism.

Shrödinger showed that a cell maintains low entropy, high level of organization by exporting of entropy to surrounding. “The essential thing in integrated metabolism is that the organism succeeds in freeing itself from all entropy it cannot help producing while alive” [10].

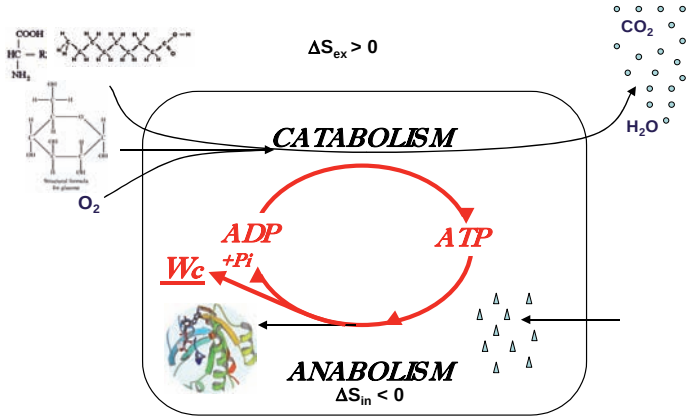


Figure 1. **The cell as an open thermodynamic system.** It exchanges matter and energy with surrounding medium. Exchange of matter is metabolism and involves two general processes: catabolism, which increases entropy in the surrounding medium and anabolism, which decreases internal entropy. These two processes are coupled by reactions of cellular energetics, which are thus in the center of cellular life. Wc - cellular work. Reproduced from [9] with permission.

A part of the energy inflow is used to lower entropy, emerging highly ordered macro structures and complex functional dynamic behaviors which allow diminishing the number of chemical entities [9].

Quantitatively, free energy change in chemical and biochemical processes is described by the key equation relating Gibbs free energy change to changes of the concentrations of reactants [14, 16]:

$$\Delta G \text{ (kJ /mol)} = 2{,}3\ RT \log_{10} (\Gamma/K) \qquad (3)$$

where Γ is mass action ratio and K equilibrium constant [14].

K is related to the standard change of free energy ΔG_0 by the van`t Hoff equation

$$\Delta G_0 = -\ 2{,}3\ RT \log_{10} K \qquad (4)$$

These equations give:

$$\Delta G = \Delta G_0 + 2{,}3\ RT \log_{10}\Gamma \qquad (5)$$

To ensure the enrgy demand of the cell mitochondria have to maintain high value of the Γ of the reaction of ATP synthesis: ATP+Pi ⇄ ATP

$$\Gamma = \frac{[ATP]}{[ADP]\bullet[Pi]} \qquad (6)$$

usually at the value of 10^5. Since K of this reaction is about 10^{-5} [14], the amount of free energy available in ATP system in cells, called phosphorylation potential, is equal to

$$\Delta G = \Delta G_0 + 2{,}3\ RT \log_{10} \frac{[ATP]}{[ADP]\bullet[Pi]} \cong 60\ \text{kJ/mol} \qquad (7)$$

This energy is used in cellular ATPase reactions for movement (contraction), ion transport and biosynthesis (anabolism). In our work we apply Molecular System Bioenergetics for investigation of the problems of regulation of cardiac cell respiration and energy fluxes *in vivo*.

2. Energy metabolism in the muscle cells.

2.1. The mitochondria

The discovery by Kennedy and Lehninger in 1948 that mitochondria (Figure 2) are the site of oxidative phosphorylation is the beginning of modern studies of biological energy transductions [17]. Aerobic organisms gain ATP mainly by glycolysis in cytosol and by oxidative phosphorylation in the mitochondria. In cardiac cells mitochondria transform over 90% of energy used. Besides ATP synthesis in the respiratory chain complexes and processes directly connected with it (citric acid cycle and β-oxidation of fatty acids – see Figure 3), the mitochondria have also important role in thermogenesis, apoptosis, signaling and regulation of ion homeostasis [17].

The content of mitochondria in the skeletal muscles can vary from approximately one percent of cellular volume in the glycolytic muscles up to 50% in the oxidative muscles. In the cardiac cells with high and varying energy demand, the mitochondria occupy approximately 30-40% of the volume [18]. By using scanning electron microscopy it was found that mitochondrial morphology varied significantly in white, red and intermediate muscle fibers [19]. In the oxidative muscle cells the mitochondria have a regular arrangement between myofibrils, comparable to a crystal.

The internal structure of the mitochondria is formed of two membranes (inner membrane and outer membrane), delimiting intermembrane space. The inner membrane of mitochondria forms protuberances into the mitochondrial interior known as cristae, attached to the inner membrane by small tubes called pediculi to increase the surface of membrane [20]. Enclosed by inner membrane is the mitochondrial matrix - very concentrated aqueous solution of enzymes and intermediates of energy metabolism, mitochondrial DNA genome and ribosomes (Figure 2).

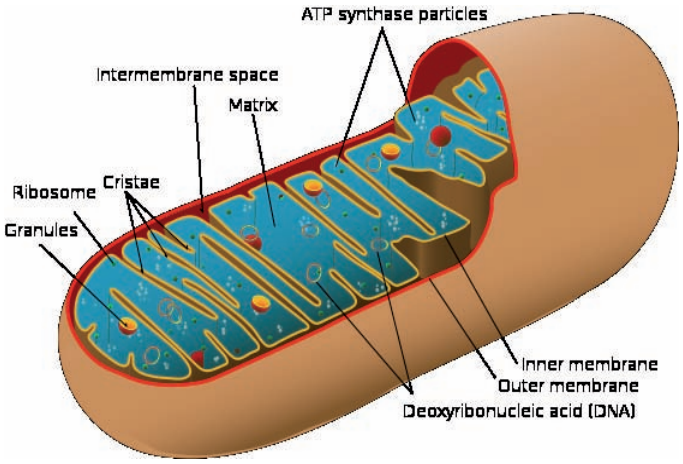


Figure 2. Mitochondrion. Cristae are protuberances of inner membrane of mitochondria into the mitochondrial interior. These are the regions in mitochondria where most of the ATP synthesis is taking place in respiratory chain in mitochondria. The mitochondrial matrix is concentrated aqueous solution of enzymes and intermediates of energy metabolism, mitochondrial DNA genome and ribosomes.

2.2. Respiratory chain

The main oxidative system in the cell, the respiratory chain with the ATP production sites is located in the cristae part of the membrane of mitochondria [21-22]. Figure 3 shows the main processes, which occur in the mitochondrial inner membrane and matrix space.

The inner membrane is impermeable for most small molecules, including H^+. It contains 30% phospholipids, 70% of protein which includes protein complexes of the respiratory chain and various transporters to ensure the transfer of metabolites and proteins coming from outside the mitochondria [17]. Respiratory chain in the mitochondrial inner membrane (MIM) comprises of three proton pumps: NADH dehydrogenase-complex I, cytocrome *c* reductase or cytochrome *bc1*- complex III, cytochrome c oxidase-complex IV and an enzyme complex not pumping protons (succinate dehydrogenase-complex II). The four protein complexes sequentially funnel electrons through prosthetic groups capable of accepting or donating one or two electrons. [14, 17, 23].

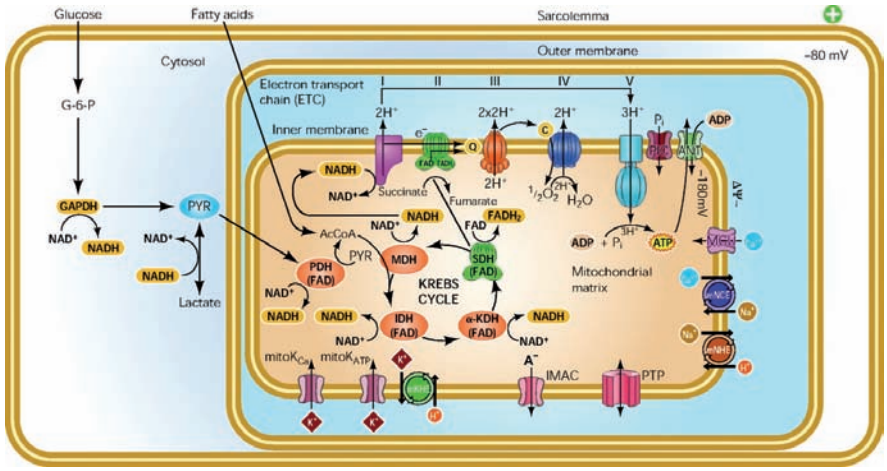


Figure 3. Overview of oxidative phosphorylation in the cardiac cell. The sequential oxidation of fuels (e.g., fatty acids and glucose) leads to the common substrate for the Krebs cycle, acetyl-CoA, which drives the production of the reducing equivalents NADH and $FADH_2$. Electrons are passed to the electron-transport chain, where coupled redox reactions mediate proton translocation across the inner membrane to establish an electrical potential and pH gradient (proton-motive force) that drives ATP synthesis by the mitochondrial ATP synthase. Ion-selective or nonselective mitochondrial ion channels dissipate energy and alter the ionic balance and volume of the mitochondrial

matrix, which is partly compensated by antiporters coupled to H^+ movement. ANT - adenine nucleotide translocase, G-6-P - glucose-6-phosphate. IMAC - inner-membrane anion channel, MCU - mitochondrial Ca^{2+} uniporter, mitoK_{Ca}, - mitochondrial Ca^{2+}-activated K^+ channel, mitoK_{ATP} - mitochondrial ATP-sensitive K^+ channel, PIC - phosphate carrier, PTP - permeability transition pore, PYR- pyruvate, KHE- K^+/H^+ exchanger, NHE - Na^+/H^+ exchanger, NCE - Na^+/Ca^{2+} exchanger, IDH - isocitrate dehydrogenase, KDH - α-ketoglutarate dehydrogenase, MDH- malate dehydrogenase, PDH - pyruvate dehydrogenase, SDH - succinate dehydrogenase. From [23] with permission

Complex I of the respiratory chain called NADH-ubiquinone oxidoreductase is a large L-shape enzyme. It consists of prosthetic flavinmononucleotide (FMN) redox group and six Fe-S centers. Complex I catalyzes two simultaneous processes: the transfer of two electrons from NADH to coenzyme Q:

$$NADH + H^+ + Q \rightarrow NAD^+ + QH_2$$

and pumping of four protons from the matrix side to the intermembrane space forming the proton gradient.

Complex II: Succinate – dehydrogenase comprises flavin adenine dinucleotide redox prosthetic group and several Fe-S centers. It catalyzes the transfer of electrons from succinate via FAD and Fe-S centers to ubiquinone.

Fatty acids and glycerol 3-phosphate also direct electrons into the ubiquinone via FAD. The β-oxidation of fatty acids is directly linked with the respiratory chain. The β-oxidation of acyl-CoA catalyzed by the flavoprotein acyl-CoA dehydrogenase in mitochondrial matrix involves the transfer of electrons from the substrate to the FAD of the dehydrogenase to the electron transfer flavoprotein (ETF), which transfers them to ETF-ubiquinone oxidoreductase [14] (Figure 4). Ubiquinol (QH_2) diffuses in the mitochondrial inner membane from complex I to Complex III.

Complex III called cytochrome bc_1 complex or Ubiquinone-cytochrome c oxidoreductase is a dimer of identical monomers, each with 11 different subunits, consisting of Rieske iron-sulfur protein with 2Fe-2S center, cytochrome b and cytochrome c_1. It catalyzes the transfer of electrons from ubiquinone to cytochrome c. The net equation of the cycle is

$$QH_2 + 2\ \text{Cyt c (oks)} + 2\ H^+_N \rightarrow Q + \text{Cyt c (red)} + H^+_P$$

where H^+_N is proton in the matrix side and H^+_P in the intermembrane space.

Complex IV: Cytochrome oxidase catalyzes oxidation of reduced Cyt c by molecular oxygen, which is the final acceptor of electrons from the electron transport chain.

$$4\text{Cyt c (red)} + 8\ H^+_N + O_2 \rightarrow \text{Cyt c (oks)} + 4\ H^+_P + H_2O$$

The reduction of O_2 must occur without release of incompletely reduced intermediates (hydrogen peroxide, hydroxyl free radical) that would damage cellular components.

The redox potential of the respiratory chain complexes increases gradually along the chain.

The displacement of the protons across the mitochonrial inner membrane creates a positive charge on the external surface of the internal membrane and a negative charge on the side of the matrix. It forms gradient of electrochemical potential of protons ($\Delta\mu_{H+}$) [24-25] consisting of two components - an electric potential ($\Delta\Psi$) and a chemical component – concentration gradient (ΔpH). $\Delta\mu_{H+}$, the gradient of electrochemical potential of protons across the inner mitochondrial membrane represents the intermediate form of energy in the oxidative phorphorylation.

$$\Delta\mu_{H+} = F\,\Delta\Psi - 2{,}3RT\Delta pH \qquad (8)$$

Mitchell defined the term proton motive force as

$$\Delta p\ (mV) = -(\Delta\mu_{H+})/F = \Delta\Psi - 59\Delta pH, \text{ when } T = 25^{o}C \qquad (9)$$

This proton motive force makes possible the ATP synthesis as described below.

In intact well-coupled mitochondria the MIM is relatively impermeable for backflow of the proton. The stoichiometric efficiency of oxidative phosphorylation is defined by the P/O ratio, or the amount of inorganic phosphate incorporated into ATP per amount of consumed oxygen molecule. Maximal P/O ratio for NAD-linked substrates is 2,5–3 and 1,5–2 for succinate [26-27].

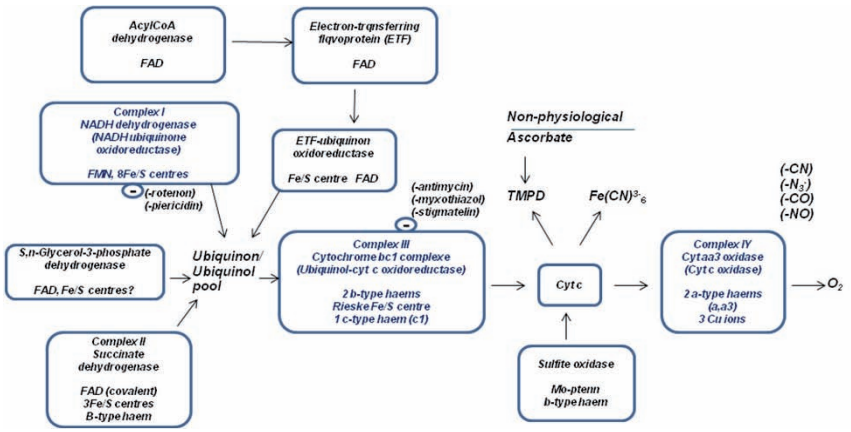


Figure 4. Electron transfer in respiratory chain with important inhibitors. Reprinted from [28] with permission.

2.3. ATP synthase

The ATP synthase (F_O, F_1) is an enzyme complex, located in the inner mitochondrial membrane, where it catalyses the reaction of ATP synthesis from the ADP and Pi in the presence of Mg^{2+} (Figure 5) driven by electrochemical gradient of protons generated by electron transfer [29-30]. The ATP synthase is composed up to fifteen sub-units [31]: five different subunits ($\alpha_3\beta_3\gamma\delta\varepsilon$) form the F_1 where each of three β subunit serves as an enzymatic center of the ATP synthesis (Figure 6). F_O complex contains three different subunits: $abc_{10\text{-}14}$. The c subunits, incorporated in the internal membrane form the engine with proton channel (subunit a), subunits b form the "stator" which extends from the segment incorporated in the internal membrane until the top of F_1 [31].

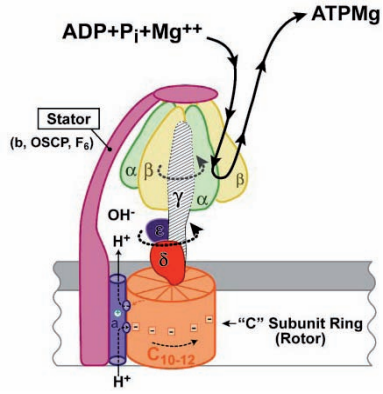


Figure 5. **Mitochondrial ATP synthase** F_1 consists of α, β, γ, δ, ε subunits, where in enzymatic center of β the synthesis of ATP is taking place. In F_O complex c subunits are forming rotor with proton pump, subunit b with other complexes forms stator for F_1**.** Reprinted from [32] with permission**.**

Proton gradient, generated by electron transport chain in oxidative phosphorylation drives the rotation of the central rotor located in nanomotor within F_O leading to conformation changes and the sequential release of bound ATP from the catalytic site of β subunits in F_1 (Figure 6)

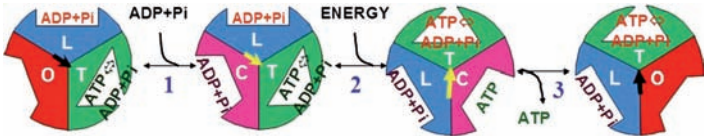


Figure 6. Mechanism of ATP-Synthase. The reaction of synthesis of the ATP proceeds recurrently from left to right by stages 1, 2, and 3. Each circle represents a conformation of the F1- unit of ATP-synthase. The sectors in each circle represent the three αβ pairs: the conformation of the subunit β in each pair is coloured in: green (T, tight), blue (L, loose), red (O, open), pink (C, closed). Two other steps in making ATP in mitochondria are: entry of the substrates by phosphate carrier (PIC) and release and exit of ATP by nucleotide carrier (ANT) are very localized and highly coordinated, forming together an ATP synthasome complex. Reprinted from [33-34] with permission.

2.4. Adenine nucleotide transporter

Adenine nucleotide transporter (ANT), an antiporter, transporting ADP/ATP across the mitochondrial inner membrane in eukaryotic cells (Figure 3), is carrying out a critical metabolic step in the supply of ATP for intracellular processes [35]. ANT is a principal member of the family of membrane transporters, which carry the anionic substrates through the membrane.

The Km of ANT for ADP is about 1 to 10 μM and approximately 20 mM for ATP [36]. There are three isoforms of ANT: ANT-1 (muscle) and ANT-3 (ubiquitous) exporting ATP produced by mitochondrial oxidative phosphorylation and ANT-2 expressed during the glycolytic cancer cell proliferation that is supposed to import the glycolytic ATP in the mitochondria to maintain the potential gradient of the inner membrane and prevent apoptosis [37-38]. The carrier-substrate complex changes conformation between two basic configurations: c (cytosolic state) and m (matrix state), via the transition state (partial opening and closing doors for entry and exit). [35].

There are strong evidences that to overcome the diffusion restrictions of ATP and ADP, the entry of the substrates ADP and Pi into mitochondria, the synthesis of ATP on F(1), and the release and exit of ATP are very localized and highly coordinated events. Several studies confirm existence of ANT-ATP synthase-PIC complex, ATP Synthasome complex [33-34].

2.5. The contractile module

The contractile units of the cell are sarcomeres of myofilaments consisting of thick (myosin) and thin (actin) filaments, where the chemical energy is transformed to the mechanical energy and work. In striated muscle sarcomeres are delimited by Z lines (Figure 7).

In each sarcomere dark band (A band) containing myosin and actin and I band containing only the thin filaments (actin) can be discerned. The myosin is fixed at the M-line in the centre of the sarcomere. The actin is fixed at Z-lines (discs) with alpha-actinin. The titin, forming the third filament system, is spread out from Z- disc towards the M band where it interacts with the myosin. It maintains the myosin spacing, centres the thick filaments in the sarcomere and acts as an elastic spring element during muscle contraction by limiting its length. The length of the A band (anisotropic band) is determined by the length of thick filament.

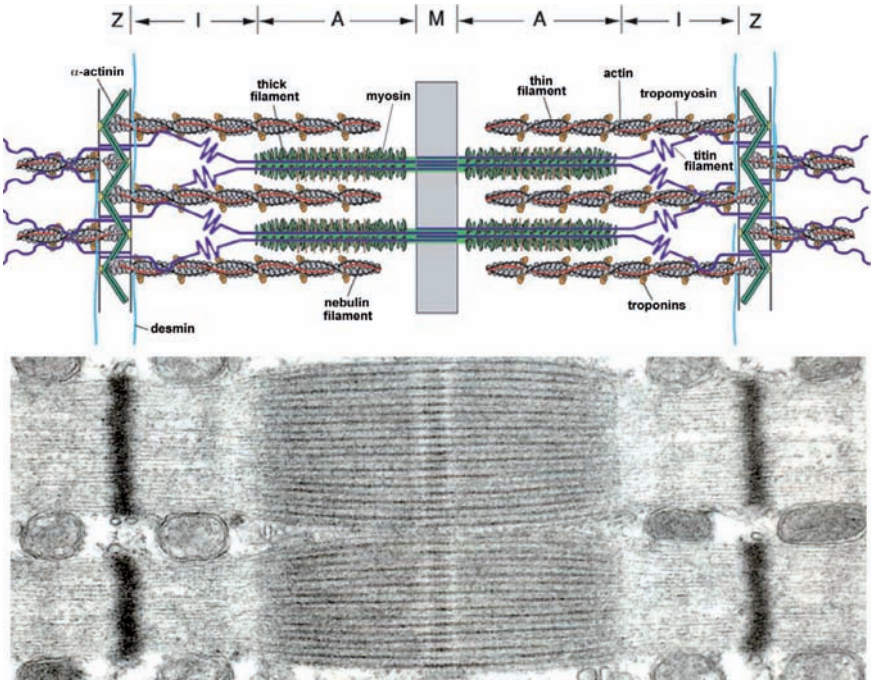


Figure 7. Major compartments of cardiac muscle sarcomere and electron-microscopic image. A band - myosin and actin, I band – actin. Myosin is fixed in M band, actin in Z disc with alphe actinin. Reprinted from [39] with permission.

Myosin is a molecular engine where the Gibbs free energy of ATP is transformed into mechanical energy. It consists of two heavy chains (200 kDa), twisted one around the other, and of 2-3 light chains (20 kDa). The chains comprise an ATP activated globular head (myosin ATPase) and an alpha helical tail. Myosine heads create the cross-bridges that interact with actin to generate the contraction. Each thick filament consists of 300 myosin molecules. During contraction actin combines with myosin and ATP (Figure 9).

The rate-limiting step of the contraction (cross-bridge) cycle is the release of products of ATP hydrolysis (Figure 8). The physical model, called sliding filament theory, was introduced by Huxley [40-41]; the chemical background of the process was explained by Goldman [42] and Brenner [43] (see Figure 8). In muscle contraction the Ca^{2+} activation is needed. It has been known for a long time that different types of muscles have different contractile properties. According to the classical theory [44] myosin from the fast twitch muscle has higher ATPase activity and therefore catalyses a more rapid breakdown of ATP. Myosin from slow-twitch muscle such as heart has lower ATPase activity, a slower rate of ATP breakdown and a slower rate of contraction. Increasing the sarcomere length is accompanied by a rise in sensitivity to Ca^{2+} [45-46]. Gomes et. al. [47] showed that the transients of Ca^{2+} remained stable during the length-dependent activation of sarcomere. In this case, an increase in the myocyte length induces decrease in the space between the filaments and increases the

number of actin-myosin bridges that promote the force of contraction without changing the level of Ca^{2+} [48-49]. The Moss team was the first to propose the role of filaments (lattice spacing) in length-dependent activation of cardiac muscle [50].

Granzier and Labeit showed that there are connections between titin and actin at the level of Z disk, which induce radial and longitudinal forces (Figure 7). These radial forces contribute to a very tight approach of thick and thin filaments thereby reducing the space between them [51-52]. The technique of synchrotron X-ray diffraction showed that the lattice spacing in intact heart fiber decreases with sarcomere length [53]. Thus, titin is responsible for the increased tension of cardiac muscle related to an increase in the ventricular volume through modulation of the lattice spacing between the filaments (Figure 7).

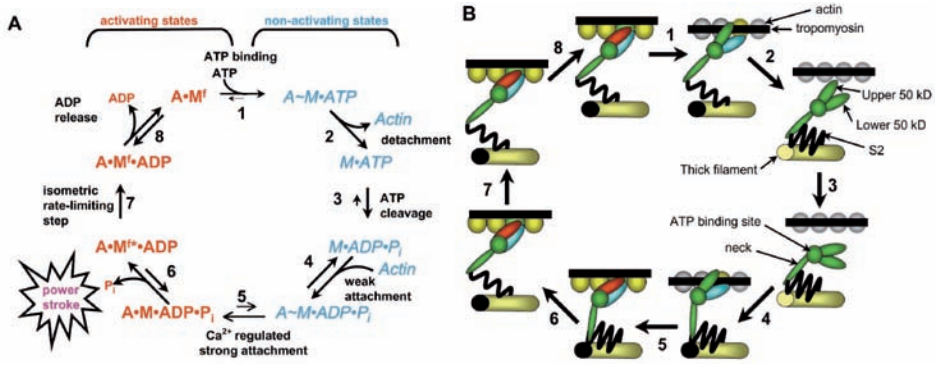


Figure 8. The mechanism of the cross-bridge cycle. Formation of actin-myosin cross-bridges (AM) in terms of various reagents and products (A) and the corresponding structural changes (B).

In figure 8A strong connection is marked by "•" and a weak connection by "~".

ATP binding to myosin (step 1) is very rapid and irreversible. The subsequent detachment of actin from the actin-myosin·ATP (A~M·ATP) complex (step 2) is similarly rapid and is caused by an opening between myosin's upper and lower 50-kDa regions (Fig. 8A) like the opening of jaws. A "flexing" or bending of the myosin neck region (step 2 in Fig. 8B) is followed by step 3, the hydrolytic cleavage of ATP. Following ATP cleavage, myosin again binds weakly to actin at a high rate, but in the absence of Ca^{2+} Tm sterically blocks access of the myosin head to strong binding sites on actin (Fig. 8B). However, when Ca^{2+} is bound to TnC, TnI detaches from actin, allowing the Tm/Tn complex to roll or slide over the thin filament surface. Consequently, the rate of strong cross-bridge attachment, the flux through step 5 is dependent on [Ca^{2+}] and Tm position. Strong binding of myosin to actin (Fig. 8B) is associated with movement of the upper and lower 50 kDa sub-domains toward each other (or closing the jaws). In any event, myosin neck extension related to the release of Pi, step 6, is the power stroke that, in isometric muscle, stretches an elastic element (represented here as the S2 segment) by some 10 nm and produces a force of ~2 pN/cross-bridge (Molloy, 1995). In nonisometric conditions, shortening of the neck extension causes the thick and thin filaments to slide past each other. Step 7 is an irreversible isomerization and is the rate-limiting step for the cross-bridge cycle. Finally,

ADP is released from A·M ADP (where f is a cross-bridge exerting force) in the reversible step 8 to form the rigor state, A·M. Non-interacting cross-bridge-actin pairs are shown as gray actin and green myosin; weak interactions as yellow actin and light blue myosin, and strong interactions as green and red myosin, elastic element is represented as the S2 segment (From [54] with permission).

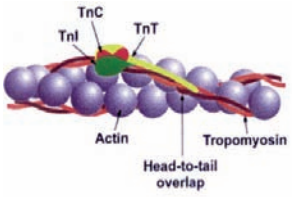


Figure 9. Thin filament-actin–tropomyosin-troponin interaction. By Ca^{2+} attaching to troponin C conformal change exposes binding sites for myosin (active sites) on the actin filament. TnC troponin C -, TnI-troponin I, TnT-troponin T. (From [55] with permission)

The passive force created by titin can strain the thick filament, allowing a shift of myosin heads of thick filaments relative to thin filament [56]. The elongation of sarcomere induces an increase in the number of actin-myosin (AM) bridges available in the region of single lap increasing the level of activation. This augmentation of a level of activation induces an increase in the number of cross bridges and, via the feedback control mechanism, increases cooperatively the affinity of troponin for Ca^{2+} [57].

2.6. The Excitation-Contraction coupling

The concept of excitation-contraction coupling was formulated by Sandow: "When a stimulus is applied to muscle the response is indicated first by excitation, which is set up in membrane of each reacting fiber, and then by contraction, which is a function of the substance within the membrane" [58]. The general mechanism of the excitation-contraction coupling is an action potential formation by neurons, which propagates down by its axon to the neuromuscular junction. In cardiac muscle an action potential is induced by pacemaker cells and conducted to cells through gap junctions. The action potential is sensed by a voltage-dependent calcium channel, which causes influx of Ca^{2+} ions. Sacolemma, sarcoplasmatic reticulum (SR) and mitochondria play crucial role in control of concentration changes of the cytoplasmic Ca^{2+}. In cardiac cell the excitation-contraction coupling mechanism involves cyclic changes in intracellular free Ca^{2+} ion concentration. The phenomenon called calcium induced calcium release [59-60] involves influx of Ca^{2+} ions into the cytosol causing further release of Ca^{2+} from the SR. In heart the action potential induced by pacemaker cells in Sinoatrial node activates L-type Ca^{2+} channels; Ca^{2+} enters the cell via Ca^{2+} current and also a much smaller amount via Na^{+}/ Ca^{2+} exchanger [61]. The increase of intracellular Ca^{2+} level triggers ryanodine receptors (RyR) that release Ca^{2+} from sarcoplasmatic reticulum. In the contractile module Ca^{2+} binds to Troponin C moving the troponin complex off the actin binding site (Figure 9).

The cytoplasmic free Ca^{2+} concentration in muscle and other cells at rest is around 20–50 nM; this is by two orders of magnitude lower than the free Ca^{2+} concentration in the extracellular space (usually millimolar) or in the lumen of SR (0.1–2.0 mM). The large Ca^{2+} gradients across cellular boundaries are established and maintained by powerful Ca^{2+} pumps located in the cell surface membranes and in the SR [62-63] and with contributions by the mitochondria [64-65]. During the cardiac action potential RyR controls Ca^{2+} influx from SR Ca^{2+} [66]. Ca^{2+} is removed from the myofilaments by the SR Ca-ATPase pump modulated by phospholamban (PLB), sarcolemmal Ca-ATPase pump, Na/Ca exchanger (NCX) and mitochondrial uniporter.

2.7. Integrated energy metabolism. The intracellular phosphotransfer network

2.7.1. Mitochondrial creatine kinase (MtCK)

Creatine kinase (CK) belongs to the family of guanidine kinases that catalyzes reversible transfer of a phosphate moiety (Pi) from ATP to the nitrogen center of the guanidine group [67]. This enzyme is present in the cells with high and variable energy needs. The CK catalyses reversible transfer of Pi of MgATP to creatine, releasing PCr, MgADP and a proton:

$$PCr^{2-} + MgADP^{-} + H^{+} \rightleftarrows Cr + MgATP^{2-}$$

The CK reaction plays a central role in the intracellular energy transfer, being specifically located in the places of production and utilization of ATP and also part of Cr/PCr circuit.

There are four isoenzymes of CK: two cytosolic forms MCK (muscular) and BCK (brain) present in dimeric form, and two mitochondrial forms sMtCK (sarcomeric) and uMtCK (ubiquitous) present in dimeric or octameric form [68]. Cytosolic isoforms of CK mainly use PCr to reproduce ATP at sites of energy consumption. MtCK is responsible for the use of ATP for production of PCr in the intermembrane space of mitochondria. In this work our interest will be in the kinetic properties of the sarcomeric mitochondrial CK (sMtCK), exclusively present in in heart and skeletal muscle. In mitochondria mitochondrial creatine kinase (MtCK) is strongly attached to the external side of the inner mitochondrial membranes by cardiolipin in close association with ANT [67, 69]. Octameric MtCK forms proteolipidic complexes with VDAC [70] crosslinking mitochondrial inner and outer membrane (Figure 10). Cardiolipin is situated in the mitochondrial inner membrane, binds ANT and is considered to serve as contact site for MtCK [71-74].

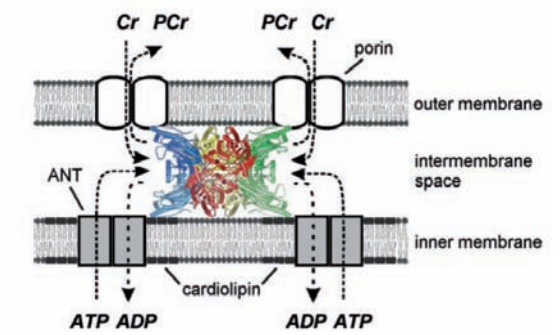


Figure 10. Mitochondrial creatine kinase. Octameric MtCK binds with cardiolipin in inner membrane and could also form complexes with mitochondrial porin channel (VDAC) in intermembrane space. From [75] with permission.

Several studies showed that MtCK (and/or the HK II), ANT and VDAC could form micro-compartments for the transfer of adenine nucleotides between the mitochondrial matrix and MtCK and also export of PCr via VDAC to cytosol without release them into the medium [75-80]. In the MtCK-ANT functional coupling, the ANT channels MgATP (ATP in the presence of Mg^{2+}) to MtCK and immediately transports the produced ADP into the mitochondrial matrix. Thus, the reaction never reaches the equilibrium state [81], the functional coupling MtCK-ANT directs the reaction toward production of PCr (Fig.10).

By calculating the constants of dissociations of the ATP and creatine from the complexes with MtCK, Jacobus and Saks [36] showed that the affinity of MtCK for the ATP increases significantly in the presence of oxidative phosphorylation. These experiments allowed concluding that MtCK controls oxidative phosphorylation. Moreover, it was shown that functional coupling of MtCK and ANT prevents formation of permeability of transition pore in mitochondria and having a protective role from the excessive formation of reactive oxygen species (ROS). [68, 82-83].

2.7.2. The Phosphotranspher network

The role of creatine kinase in cytosole is not limited by the regulation of concentrations of ADP and ATP in the sites of energy demand. Energy [ΔG (ATP)] is transfered towards the sites of energy consumption in the form of PCr via the Cr/PCr “shuttle” [68, 72, 84-85] (Figure 11). In this role PCr is thought to function as an “energy carrier“, connecting sites of mitochondrial OxPhos with sites of energy utilization [86-87].

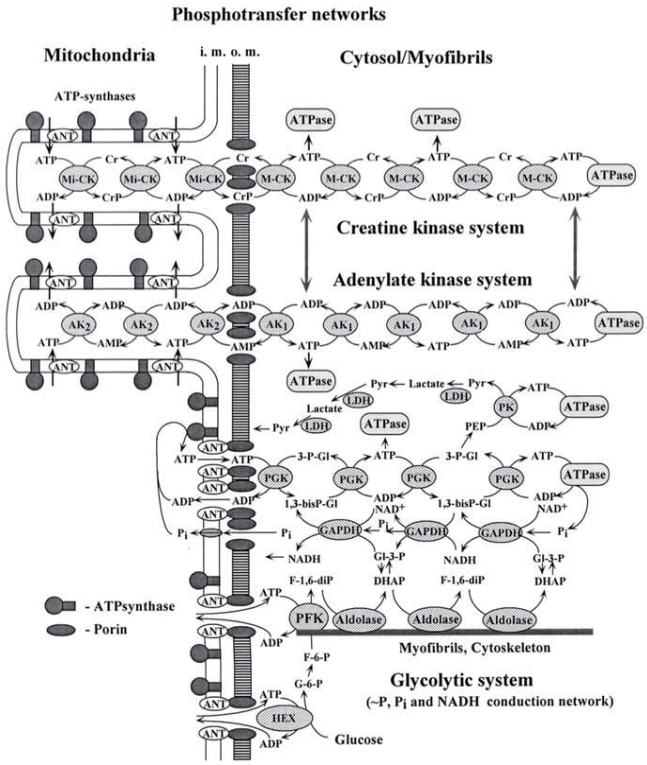


Figure 11. Proposed functional arrangement of the intracellular phosphotransfer network. Mitochondrial creatine kinase and adenylate kinase (AK2) serve the spatially and diffusionally restricted intra-cristae and intermembrane space to provide ADP for oxidative phosphorylation and to facilitate ATP export. Exported ATP molecules are relayed/conducted to ATPases through sequential chains of reactions catalyzed by the cytosolic isoforms M-CK and AK1. The glycolytic system could serve a similar intracellular energy transfer and signal communication function by transferring mitochondrially-produced high-energy phosphoryls through hexokinase (Hex), phosphofructokinase (PFK), and near-equilibrium glyceraldehyde-3-phosphate dehydrogenase/3-phosphoglycerate kinase (GAPDH/PGK) enzymes, present in the vicinity or bound to the mitochondrial outer membrane, as well as by generating additional ATP molecules as products of glycolysis. Near-equilibrium reactions catalyzed by the GAPDH/PGK chains could also facilitate transfer of P*i* and NADH from remote cellular locales to mitochondria, while chains of reversible lactate dehydrogenase (LDH) reactions could facilitate intracellular lactate/pyruvate and coupled NAD+/NADH movement.(From [88] with permission)

The Cr/PCr circuit helps surpass the diffusion barriers in the cell [72, 75, 87, 89-91], avoid the dissipation of energy due to the transport of the ATP and, in addition, to avoid accumulation of ADP (and thus the inhibition of the activity of ATPases) and accumulation of the AMP due to the reaction of the AK [88, 92-93]. The sites of energy production and consumption are connected by a series

of the reactions of the CK are resulting the almost instantaneous transfer of Pi towards the sites of demand and the metabolic signal towards mitochondria. The compartmentation of the reactions catalysed by the different CK isoenzymes ensures a high ATP/ADP ratio in the vicinity of ATPases and low ratio in the vicinity of ANT. Local availability of ADP to ANT is a potential key regulator of respiration [85, 94]. The CK knockout mice are viable but lose the exercise capacity [89, 95-96]. In the MtCK and MMCK knockout mice a cellular reorganization is observed, characterized by an increase of mitochondrial volume and a bringing together mitochondria to ATP consumption sites to increase the effectiveness of the energy metabolism [86, 97]. The resynthesis of PCr and its transfer via the CK-PCr system decrease at the time of cardiac insufficiency [98-100].

2.8. Concept of ICEU

Figure 11 shows that besides PCr/CK system, phosphotranspher network includes also adenylate kinase and glycolytic system [101]. The structure of cardiac cells is highly organized and plays important role in regulation of energy metabolism [102]. It was shown in 2001 that in cardiomyocytes mitochondria are organized into Intracellular Energetic Units, ICEUs [9, 103-104]. The concept of ICEUs (Figure 12) was developed on the basis of the information of cardiac cell structure and experimental data obtained in the studies of permeabilized cardiac cells and fibers, which revealed the major importance of structure-function relationship in regulation of cardiac cell metabolism. ICEU is considered as structural and functional unit of striated muscle cells consisting of distinct mitochondria localized at the level of sarcomeres between Z-lines and interacting with surrounding myofibrils, sarcoplasmic reticulumn, cytoskeleton and cytoplasmic enzyme systems [105-106], as shown in Figure 12. In adult cardiac cells ICEUs interact with other dissipative metabolic structures - calcium release units, CRUs, shown in upper right corner in Figure 12 by shaded area [107-108]. Electron microscopic studies have always shown very regular arrangement of major part of mitochondria in cardiomyocytes at the level of A-bands of sarcomeres of myofibrils, which are surrounded also by the network of sarcoplasmic reticulum [109-110].

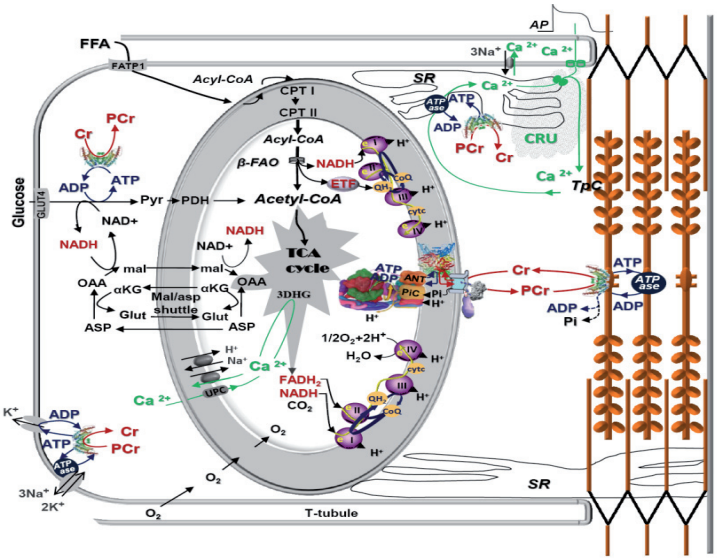


Figure 12. Functional scheme of the Intracellular Energetic Unit of adult cardiac muscle cell. Free fatty acids (FFA) taken up by a family of plasma membrane proteins (FATP1), are esterified to acyl-CoA which are entering further the β-fatty acids oxidation (β-FAO) pathway resulting in acetyl-CoA production. Electron-transferring flavoprotein (ETF)-ubiquinon oxidoreductase delivers electrons from β-FAO directly to complex III of the respiratory chain (RC). NADH produced by β-FAO is oxidized in the complex I of the RC passing along two electrons and two protons, which contribute to the polarization of mitochondrial inner membrane (MIM). Glucose (GLU) is taken up by glucose transporter-4 (GLUT-4) and oxidized, pyruvate produced from glucose oxidation is transformed by the pyruvate dehydrogenise complex (PDH) into acetyl-CoA. The NADH redox potential resulted from glycolysis enters mitochondrial matrix via malate–aspartate shuttle. Malate generated in the cytosol enters the matrix in exchange for α-ketoglutarate (αKG) and can be used to produce matrix NADH. Matrix oxaloacetate (OAA) is returned to the cytosol by conversion to aspartate (ASP) and exchange with glutamate (Glut). Acetyl-CoA is oxidized to CO_2 in the tricarboxylic acids (TCA) cycle generating NADH and $FADH_2$, which are further oxidized in the RC (complexes I, II) with final ATP synthesis. G6P inhibits HK decreasing the rate of glucolysis. The complexes to transfer energy from mitochondria to cytoplasm are adenine nucleotides translocase (ANT), mitochondrial creatine kinase (MtCK) and voltage-dependent anion channel (VDAC) with bound cytoskeleton proteins. MtCK is responsible for phosphorylation of creatine into phosphocreatine. PCr is then used to regenerate ATP locally by CK with ATPases (actomyosin ATPase, sarcoplasmic reticulum SERCA and ion pumps ATPases). The rephosphorylation of ADP in MMCK reaction increases the Cr/PCr ratio, which is transferred towards MtCK via CK/PCr shuttle. Calcium liberated from local intracellular stores during excitation–contraction coupling through calcium-induced calcium release mechanism, (1) activates contraction cycle by binding to troponin C in the troponin–tropomyosin complex of thin filaments and (2) enters the mitochondria mainly via the mitochondrial Ca^{2+} uniporter (UPC) to activate Krebs cycle dehydrogenases: PDH, αKG, isocitrate dehydrogenase. CRU - Calcium Release Unit. (From [111] with permission)

3. Regulation of respiration in mitochondria in the cardiac cells

3.1. The Frank-Starling law

Phenomenological aspects of intracellular feedback regulation in cardiomyocytes were initially recognized by the Starling laboratory, already before ATP was discovered [112]. In his works he discovered the capacity of ventricle to vary its force of contraction as a function of preload. The heart maintains normal blood circulation under a wide range of workload, the cardiac performance increases with an increase in end-diastolic ventricular volume. In modern terms, the contractile function of myocardium is described by the Frank-Starling law (or the Frank-Starling mechanism) stating that the force of contraction (active tension) increases, if the pre-load (passive tension) increases. If the ventricular end-diastolic volume (preload, ventricular filling) is increased, the length of sarcomere of cardiac muscle is increased, resulting increase of the number of force generating cross-bridges [55] and the amplification of muscle tension. In this way, cardiac output is directly related to venous return, the factor determining the most important pre-load [18, 48, 94, 113].

The respiration of isolated perfused heart measured depending on the left ventricular filling shows that mitochondrial respiration is a linear function of workload and may increase by 20 times (V_{O2} = 170 μmol min^{-1} (g dry wt)$^{-1}$) as compared with the basal state (no load, V_{O2} = 8-12 μmol min^{-1} (g dry wt)$^{-1}$) [114]. This relationship is the basis of the Frank-Starling law (Figure 13).

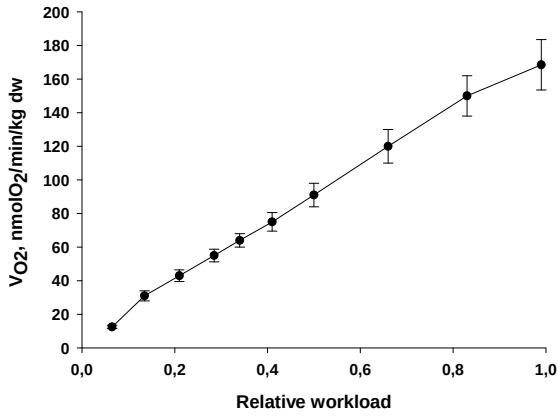


Figure 13. Metabolic aspect of the Frank-Starling's law of the heart. Linear increase of oxygen consumption rates as a function of increased relative workload (which is a fraction of maximal workload). Experimental data are taken from [114].

The main cellular mechanism, on which the Frank-Starling law is based, is the length-dependent activation of sarcomere discovered by Hibberd and Jevell [115], already discussed above. Thus cardiac work regulates energy fluxes and mitochondrial respiration in large scale. The main question for cellular

bioenergetics is what is the mechanism of the regulation of respiration under physiological conditions of Frank-Starling law. In spite of clarifying the mechanism of the synthesis of ATP and membrane bioenergetics [14, 25, 29], the mystery of regulation of cardiac energy metabolism remains. The experimental data obtained in studies of isolated mitochondria showed that in this case the control of respiration is by ADP and Pi. As shown below, in physiological condititons the regulation is more complicated involving regulation at the system level. Therefore, data obtained from studies with isolated mitochondria are not sufficient to explain the fundamental Frank-Starling law in physiological conditions, in cardiomyocytes.

3.2. Importance of intracellular organization in regulation of metabolism – diffusion problems

During more than three decades the intensive studies have shown that the content of cell is not a homogeneous diluted aqueous medium, but, particularly in muscle and brain cells, the specific organization of the intracellular structure which causes heterogeneity of diffusion, metabolite compartmentation and metabolic channeling [68, 116-119]. All the classical studies of diffusion have been investigating weakly interacting rigid particles at low concentrations. In the real cell, to begin with the cellular water, the intracellular mobility is significantly reduced, leading to the partitioning of the metabolites between different water phases [120-122]. Cytoskeletal structures and macromolecular crowding - high concentration of macromolecules in the cells, decrease the available volume for free diffusion of substrates [123-125]. The density of enzymes and other proteins in mitochondria is up to 60% of the matrix volume [126]. It is also shown that diffusion of ATP and PCr are anisotropic in muscle cells [127-128].

To overcome the problem of diffusion restriction, mechanisms are based on protein-protein interactions - metabolic channeling and functional couplingare present, the movements of individual molecules become co-coordinated and vectorally directed due to the organization of enzymes into multi-enzyme complexes, there is no randomness in the molecular mobility.

In analysis of diffusion of ADP and ATP the term apparent diffusion coefficient is used

$$D^{app} = D_{Fx}D_0$$

where D_0 is the diffusion coefficient in bulk water phase and D_{Fx} is a diffusion factor accounting for all intracellular mechanisms locally restricting particles movement [9, 80, 117]. Ridgway and his coworkers showed that due to the phenomenon of diffusion restriction, the apparent diffusion coefficient might be decreased by several orders of magnitude [129]. The results of different studies show that in some areas of cell, for example in regions close to

sarcolemma and mitochondrial outer membrane values of D_{Fx} may be even in the range of 10^{2}-10^{-5} [89-90, 119, 130].

3.3. Theories of the regulation of mitochondrial respiration

The cardiomyocytes are cells with very high and varying energy demand. The metabolic aspect of Frank-Starling law states the linear dependence of the rate of oxygen consumption upon cardiac work. The problem, how on the metabolic level the ATP demand difference is met and the workload is regulated is still highly debated. O'Rourke stated requirements the metabolic signal controlling respiration *in vivo* must meet: 1) be able to regulate ATP production; 2) its change must correlate with workload [131]. Alas, the theories developed till now and described below could not meet these criteria.

For isolated mitochondria, control of respiration by ADP and Pi is indisputable [14, 132-133]. Also, activation of Krebs cycle dehydrogenases by Ca^{2+} ions has been well established [134-135]. However, as is shown by several authors [136-140], regulation of mitochondrial respiration involves also interaction of mitochondria with proteins of cytoskeleton and other cellular complexes. Therefore experimental data obtained in studies of isolated mitochondria and other cellular structures are valuable but not sufficient to explain the central phenomenon in cardiac bioenergetics. Two principal observations have led to this conclusion. Firstly, the increase of cardiac work and mitochondrial respiration rate in aerobic hearts occurs at unchanged cellular contents of both ATP and PCr – a phenomenon called metabolic stability or homeostasis, discovered by Neely et al. [141] and confirmed by Balaban and others [142-143] (Figure 14A). Also, it is not possible to explan linear dependence of workload of cardiac oxygen consumption by a mechanism involving the control of mitochondrial respiration by ADP, or by Pi as they are in equilibrium with PCr and ATP by the CK reaction. [144-147].

Secondly, multiple and detailed physiological experiments using optical methods for measurement of the intracellular Ca^{2+} concentrations showed that workload and oxygen consumption changes induced by alteration of left ventricle filling – referred to as the Frank-Starling phenomenon - are observed at unchanged calcium transients [45] (Figure 14B). This second set of essential observations exclude any explanation of the metabolic aspect of Starling's law by a mechanism involving the control of mitochondrial respiration by intracellular calcium, as it was proposed by several authors [143, 148], Ca^{2+} mechanism may be important only in the case of adrenergic activation of the heart [149].

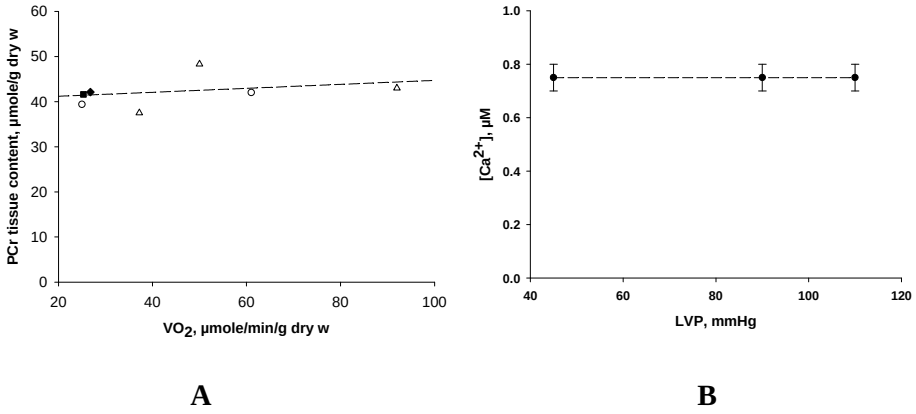


Figure 14. Metabolic homeostasis for increasing respiration rates. (A) Cardiac intracellular PCr homeostasis for different respiration rates. Experimental data were summarized from studies [141, 150-151] Intracellular calcium homeostasis (**B)** for different heart workloads (different left ventricular pressure, LVP) of canine's heart. Experimental data are adapted from [45].

Obviously, the two very popular hypotheses described above do not meet criteria posted by O´Rourke and leave the mechanism of the metabolic aspect of Starling's law in complete obscurity. The problem of regulation of oxygen consumption and substrate uptake by cardiac work evidently demands for its solution application of complex methods developed in the framework of Systems Biology. The physiological phenomena could be explained by investigation of intracellular interactions and resulting regulatory mechanisms using both experimental methods of research and computer modeling [4, 12, 116, 152]. Revealing the mechanisms of regulation of integrated energy metabolism *in vivo* in the framework of Systems Biology are the aims of Molecular System Bioenergetics, which basic concept includes studies of system level properties arising from intracellular interactions, such as metabolic compartmentation, channeling and functional coupling [3, 152-153]. The mechanism of feedback metabolic regulation of cellular metabolism linking the cardiac work with mitochondrial respiration under conditions of metabolic stability can be understood on the basis of quantitative analysis of experimental data describing intracellular interactions, the structure-function relationships and non-equilibrium steady state kinetics of reactions in the cardiac cells.

3.4. The role of the external mitochondrial membrane: selective diffusion barrier in the regulation of respiration - the role of the cytoskeleton

3.4.1. Differences in regulation of oxidative phosphorylation in mitochondrial *in vitro* and *in vivo* - the theory of factor X

Long-time studies have shown connection of cell structure and regulation of oxidative phosphorylation. It was shown that some components of cytoskeleton may have regulatory role in oxidative phosphorylation in mitochondria [103, 154-157]. In the beginning of bioenergetics studies oxygen consumption measurements were carried out with isolated mitochondria. The apparent Km for ADP value in mitochondria *in vitro* - 10-20 μM is very close to the parameter for isolated adenine nucleotide translocator [158], showing high permeability of ADP by the mitochondrial outer membrane in isolated mitochondria. In these conditions the efficiency of the regulation of mitochondrial functions *in vitro* by extramitochondrial ADP depending only upon the affinity of ANT for ADP, which is very high. After the development and introduction of the technique of permeabilized fiber and cardiomyocytes, the kinetic studies showed that the apparent Km for ADP in permeabilized cardiac muscle fibers is 20 times higher (250-300 μM) compared to isolated mitochondria. [69, 103-104, 159-161]. At the same time, Saks, Kuznetsov, and others showed that after trypsin treatment of cardiomyocytes apparent Km for exogenous ADP drops drastically to 80-40 μM, whereas the apparent Km for isolated mitochondria is not changed by this treatment (about 15 μM) [69, 162-163]. This effect of trypsin on mitochondrial respiration regulation *in situ* suggested that cytoskeletal proteins, then called Factor X, could be involved in the control of MOM permeability [69, 162]. These were the first evidence of the existence of a protein factor or complex regulating strongly mitochondrial respiration, as the outer membrane permeability becomes higher in the case of isolated mitochondria (*in vitro*) compared to permeabilized cardiac fibers or cells (*in situ*). The protein, responsible for the regulation of oxidative phosphorylation is highly sensitive to trypsin. Appaix et al. showed in permeabilized cardiomyocytes that tubulin and plectin are very sensitive to proteolytic digestion [157].

Comparable measurements of affinity of exogenous ADP to oxidative phosphorylation were also made with HL-1 cells. HL-1 cells developed by Claycomb and his coworkers [164] represent qualities of an adult myocyte with less organized ultrastructure similar to embryonic myocyte. There are two subtypes of the HL-1 cell line: beating (BHL-1) and non-beating (NBHL-1) cells. In beating HL-1 cells presence of highly ordered myofibrils and cardiac-specific junctions in the form of intercalated discs could be seen; they have the ability to undergo spontaneous contractions while remaining in a mitotic state, typical of normal *in vivo* immature mitotic cardiomyocytes [164]. Kinetic studies have revealed that permeabilized HL-1 cells exhibit much lower apparent Km for ADP in regulation of oxidative phosphorylation (25 ± 4 μM for NB and 47 ±

15µM mmol/l for B) than that observed in CM (370 ± 31µM [140]. High apparent affinity to exogenous ADP is also characteristic to neonatal rat CM [165].

A theory was proposed of existence of a intracellular, cytoplasmic protein or protein complexes which were controlling the permeability of the mitochondrial outer membrane for ADP in the muscle cell *in vivo*. The factor "X", which could be represented by one or more proteins of the cytoskeleton, could influence the properties of the mitochondrial outer membrane permeability [157, 162]. Rostovtseva and her coworkers tested participation of several proteins in the modification of the properties of VDAC, the MOM porin channel [166-167]. First candidate for the role of "factor X" is αβ heterodimeric tubulin, which upon binding to VDAC reconstructed into a planar lipid membrane strongly modulated the channel's conductance.

3.4.2. Voltage Dependent Anion Channel, Tubulin and Mitochondrial Interactosome

The permeability of MOM is regulated by the voltage dependent anion channel (VDAC) – or mitochondrial porin, which is positioned in the mitochondrial outer membrane and is the major channel in the transport of metabolites between mitochondria and cytosol [168-169] (Figure 15).

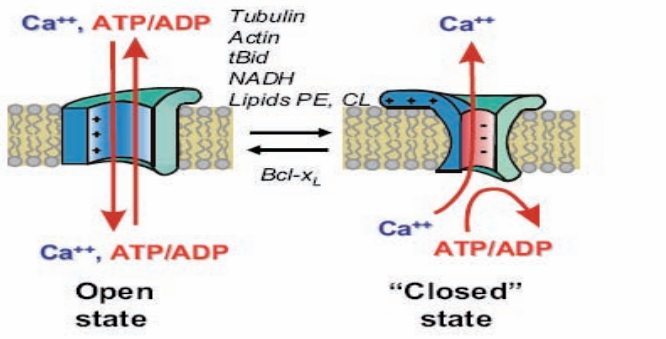


Figure 15. Voltage Dependent Anion Channel and possible candidates to cause VDAC closure. From [166-167] with permission.

VDAC is a monomeric ß-barrel protein with molecular weight ca 32kDa. Three isoforms of VDAC have been characterized in mammals: VDAC 1, VDAC 2 and VDAC 3. Exact functional and physiological differences between VDAC isoforms *in vivo* are not still clarified. VDAC forms a large pore 2.5-3 nm diameter [169], permeable to uncharged polymers up to 3.4-6 kDa. It was shown that the effective size of the cannel depends upon charge of the solute, 3D structure and could even distinguish molecules of similar size and charge, based on their structure [171-173].

The selectivity of porins toward anionic species depends on their size, charge and membrane potential. It is shown with reconstituted channels that the most conserved property of VDAC is its ability to switch between open (membrane potential V ~0mV) when the pore had diameter of 4nm and channel is open for anionic compounds such as Pi and adenine nucleotides and multiple "closed" states. The diameter of the pore is reduced to 2nm at membrane potential higher than 30mV [168, 172, 174]. It was thought for a long time that the role of porin channels in the mitochondrial outer membrane is only to limit the dislocation of high molecular weight compounds (3-10Da) [175]. However, by now it has been shown in many studies that the VDAC is primarily responsible for the transport of energy flux through the outer membrane of mitochondria [138, 176].

The closure of VDAC allows transport of small (positively charged) ions but it greatly reduces the diffusion of adenine nucleotides and therefore with the switching from open to closed states VDAC could regulate the ATP/ADP flux from mitochondrial intermembrane space to cytosol. The question remains, how this voltage gating is functioning *in vivo*, where the potential across the membrane is significantly lower than 30mM The cytosolic regulators, associated with VDAC which are physiologically relevant candidates for permeability regulation were proposed as follows: the actine G [177], Bcl-XL [176], dynein [178], protein mtHSP70 [178] and tubulin [166-167, 179].

Several recent works have proved the role of tubulin as a possible regulator protein of the VDAC. In cytosol heteromeric tubulin dimer, composed of two subunits, α and β tubulin are in a dynamic equilibrium with microtubular network [180-181]. Each tubuline isomer has an acid C-terminal end (called tail), which define different isoformes of tubuline (Figure 16). It has been shown already 30 years ago that dimeric tubulin binds to mitochondrial membranes [182]. Rostovtseva et. al demonstrated functional interaction between dimeric tubulin and VDAC [166-167]. They found that dimeric tubulin in nanomolar concentrations induced highly voltage dependent closure of VDAC reconstituted into planar lipid membranes. The tubulin induces fast well time resolved events in a 1-100 ms range leading to the partial channel blockage. The anionic C-terminal tails (CTT) of tubulin are the possible candidates for the regulators of the permeability of VDAC. The effect of tubulin without CCT was studied in comparison with intact tubulin and it was shown that tubulin with detached CTT did not induce the VDAC closure [167].

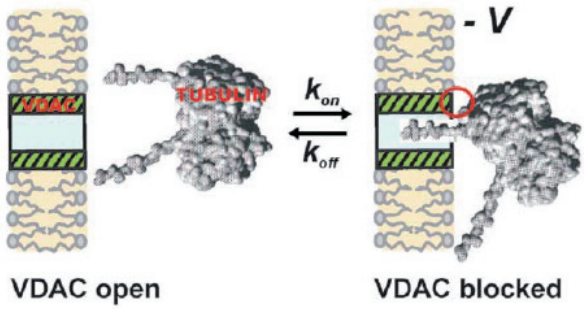


Figure 16. Possible regulation mechanism of VDAC by tubulin. Closure of VDAC could be caused by anionic C-terminal tails of tubulin, which enter physically into the channel. From [163] with permission.

The detailed nature of the complexes and metabolic regulation mechanisms of energy transfer in physiological conditions are open questions arised from the studies.

Recently Guzun et al. have shown by immunofluorescence confocal microscopy that in cardiac muscle cells the prevalent isoform of tubulin is βeta II, missing in HL-1 cells [183].

As a result of studies of our and other laboratories described above the hypothesis of existence of supercomplex of Mitochondrial Interactosome (MI) was developed (Figure 17). It was proposed that MI consists of ATP synthasome with respiratory chain, ANT, MtCK and VDAC with proteins regulating its permeability. According to this theory, in mitochondria *in situ*, diffusion of adenine nucleotides is impeded. Complexes of MI regulate oxidative phosphorylation in mitochondria. To overcome the diffusion restrictions direct channeling is taking place between the complexes of MI. The aim of this work was to prove the hypothesis.

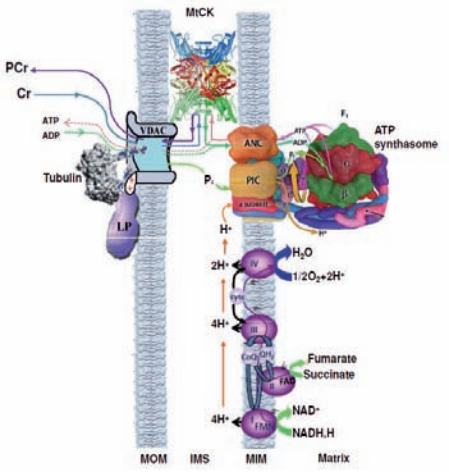


Figure 17. The hypothetical model of Mitochondrial Interactosome. This supercomplex consists of adenosine nucleotide translocator (here ANC), mitochondrial creatine kinase (MtCK), ATP synthasome, respiratory chain complexes and voltage dependent anion channel (VDAC) with proteins regulating its permeability.

4. Metabolic Control Analysis

Some decades ago the problem of metabolic regulation was studied by attempting to formulate qualitative descriptions of the systems behavior or identify the molecular details of the mechanisms. The general concept was that there is a regulatory enzyme or a rate-limiting step responsible for the regulation. One of the suppositions, following from the concept was an expectation that an enzyme catalyzing reaction most displaced from the equilibrium might affect the flux. From this point of view the possible solutions to discover candidates for the regulatory enzyme is to measure the near-disequilibria ratio of all the complexes in the pathway. Kashiwaya and his coworkers measured displacement of reactions from equilibrium in glucose utilization in working heart [184]. From these results it is visible that on the most pathways there is always significant number of enzymatic reactions that are potential candidates for being regulatory. Also the definition of the rate limiting step theory states, that the regulatory step should be the slowest in the pathway. In metabolic steady state flux rate is constant in linear pathway, therefore the slowest reaction could not be determined.

In 1970s Kacser & Burns [185] and Heinrich & Rapoport [186-187] simultaneously developed concept of Metabolic Control Theory. The aim of the theory was not to find the regulatory enzyme of the pathway, but to describe how strong the relative regulatory role of the enzymes of the pathway is. In order to quantify the amount of control exerted by a step in a pathway on flux through the pathway they introduced the concept of control strength. The control strength (Ci) of a step in a metabolic pathway is defined as the fractional change in flux through the pathway induced by a fractional change in the enzyme under consideration. Control coefficients allow the identification of system components that are crucial in the control of pathway flux or metabolite concentration.

The control structure of a pathway is determined by the values of flux control coefficient, elasticity coefficients and concentration control coefficient. Flux control coefficient (C^J_{vi} *or FCC*) shows the degree of control that the rate (v) of a given enzyme i exerts on flux J [188-189]:

$$C^J_{vi} = \left(\frac{\partial J}{J}\right) \Big/ \left(\frac{\partial v_i}{v_i}\right) = \frac{\partial \ln J}{\partial \ln v_i} \tag{10}$$

The concentration control coefficient (C^X_{vi}) shows the degree of control that a given enzyme i exerts on the concentration of a metabolite (X):

$$C^X_i = \left(\frac{\partial X}{X}\right) \Big/ \left(\frac{\partial v_i}{v_i}\right) = \frac{\partial \ln X}{\partial \ln v_i} \tag{11}$$

The elasticity coefficient (ε_i) shows the properties of an individual enzyme how temperature, pH, substrate concentrations and other effectors influence on reaction rate.

$$\varepsilon_i = \left(\frac{\partial |v_i|}{v_i}\right) \Big/ \left(\frac{\partial S}{S}\right) = \frac{\partial \, ln|v_i|}{\partial \, ln \, S} \qquad (12)$$

When Kackser and Burns developed MCA they showed that sum of all flux control coefficients of enzymes influencing the linear pathway comes to 1, called summation theorem [185]

$$C^J_1 + C^J_2 \ldots + C^J_{vi} = 1 \qquad (13)$$

It was also shown by Kackser and Burns that if we choose an metabolite X and find all enzymes whose rates influence the concentration of the metabolite, the sum of flux control coefficients of the enzymes on the flux multiplied by its elasticity with respect to X is zero – the connectivity theorem.

$$C^J_i \varepsilon^i_X + C^J_k \varepsilon^k_X + C^J_l \varepsilon^l_X \ldots + C^J_n \varepsilon^n_X = 0 \qquad (14)$$

Theoretical aspects of MCA have been subsequently analysed in many works by Kholodenko, Westerhoff, Cascante and others [4, 190-193]. As said, the laws of MCA postulated that the sum of the MCC in a linear pathway should be close to one. Kholodenko, Westerhoff and their coworkers investigated theoretically the problem of "simple" metabolic pathways *versus* channeled pathways and showed that in channeled pathways the responsiveness to an external signal is enhanced and corresponding coefficients are larger than in non-channeled pathways, [194-195]. They showed in these studies that the sum of the flux control coefficients depend on the degree of channelling in the pathway [4, 190]; it can vary from less than unity to two depending on the ratio of the channeled and bulk-phase fluxes and the kinetic properties of the enzymes involved. Therefore, if we compare a system in two conditions and in the one of them the sum of the FCC of the pathway is increased, we could conclude that there is direct channeling in the pathway in this condition.

Many authors have applied MCA for investigation of the respiration regulation in different tissues using mitochondria *in vitro* [196-201]. Croen et. al. [200] made experiments to quantify the amount of control (control strength) exerted by different steps in oxidative phosphorylation on the rate of mitochondrial oxygen uptake and concluded that there is no one limiting step in mitochondrial oxidative phosphorylation, the control is distributed among different steps, including the adenine nucleotide translocator and cytochrome *c* oxidase. It was found in the works with isolated mitochondria that the sum of flux control coefficients of respiratory chain complexes, ATP synthase and metabolite carriers is close to 1 that corresponding to the behaviour of a linear metabolic system [192-193, 196, 202]. Analogous works with similar results were carried out also in case of permeabilized cardiac fibers when respiration was activated by direct addition of ADP [203-204]. Interestingly, already in

studies of isolated mitochondria it was shown by Gellerich et al. and Groen et al. [198, 200] that the flux control coefficient value might change upon the presence of ADP regenerating system. In our work we compare FCC of complexes of MI with and without activated MtCK. In physiological conditions Cr is always present and therefore the first condition represents situation *in vivo*.

AIM OF THE STUDY

The aim of this study was to investigate the mechanism of control of energy fluxes and the role of structure-function relationship in metabolic regulation in cardiomyocytes in physiological conditions *in vivo*.

In order to achieve this aim the objectives of this study were:

1. Comparison of respiration regulation dependance on cell structure in permeabilized cardiomyocytes versus HL-1 cells of cardiac phenotype.
2. Kinetic analysis of the regulation of respiration by mitochondrial creatine kinase within MI in permabilized cardiomyocytes (mitochondria *in situ*).
3. Direct measurements of energy fluxes from mitochondria to cytosol in isolated permeabilized cardiomyocytes.
4. Metablic Control Analysis of reactions in Mitochondrial Interactosome in permeabilized cardiomyocytes.

MATERIALS AND METHODS

1. Preparation of biological materials

1.1.Animals

Male Wistar rats weighing 300-350 g were used. The animals were housed five per cage at constant temperature (22°C) in environmental facilities with a 12:12 h light-dark cycle and were given standard laboratory chow *ad libitum*. Animal procedures were approved by the Estonian National Committee for Ethics in Animal Experimentation (Estonian Ministry of Agriculture).

1.2.Preparation of skinned fibers of rat heart

Skinned fibers were prepared according to the method described earlier [205]. The animals were anaesthetized, chest opened and heart exertized and put into cooled solution. Muscle strips (5-10 mg wet weight) from endocardium of left ventricles along fiber orientation were cut to avoid mechanical damage. By using sharp forceps the muscle fibers were separated from each other leaving only small contact. The fibers were permeabilized with the 50 mg/ml saponin in A solution [205].

1.3.Isolation of adult cardiomyocytes

Adult cardiomyocytes were isolated after perfusion of the rat heart with collagenase A (Roche) using the adaptation of the technique described previously [161]. Isolated cardiomyocytes contained 70–90 % of rod-like cells when observed under light microscope. In order to study the regulation of mitochondrial respiration in cardiomycytes, the sarcolemma was permeabilized by saponin keeping the mitochondrial membranes intact [205-206]. The permeabilization procedure was carried out at 25°C with 20 μg/mL saponin.

1.4.Isolation of mitochondria from cardiac muscle

Mitochondria were isolated from adult rat hearts as described by Saks et al. [207].

2. Methods

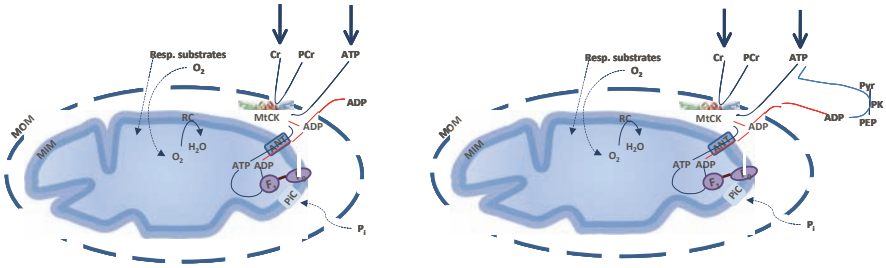
2.1. Oxygraphic measurements

2.1.1. Principle

The rates of oxygen uptake were determined with high-resolution respirometer (Oxygraph-2K, from OROBOROS Instruments, Austria) in Mitomed solution [205]. Measurements were carried out at 25ºC; solubility of oxygen was taken as 240 nmol/ml [208]. The respiration rates were expressed in nmol of oxygen consumed per minute per nmol of cytochrome *aa3* or in nmol of oxygen consumed per second per mg of protein.

2.1.2. Experimental models

The principles of this study are illustrated by four schemes of experiment of increasing complexity (Figure 18): Schemes 18A and 18B represent isolated mitochondrion as a reference system, and Schemes 18C and 18D illustrate permeabilized cardiomyocytes chosen as experimental study model. Experiments were performed in four conditions: (1) without or with activated MtCK reaction and then (2) in the presence of ADP trapping system. This system, consisting of phosphoenolpyruvate and pyruvate kinase (PK), traps extramitochondrial ADP produced by cytoplasmic isoforms of creatine kinases (MMCK) and MgATPase reactions and subsequently regenerates extramitochondrial ATP. Endogenous intramitochondrial ADP produced by MtCK forms a micro-domain within the intermembrane space (IMS) and is re-imported into the matrix via ANT due to its functional coupling with MtCK. A series of experiments were performed to check the properties of this model in order to make it useful for complete MtCK kinetic analysis.



A **B**

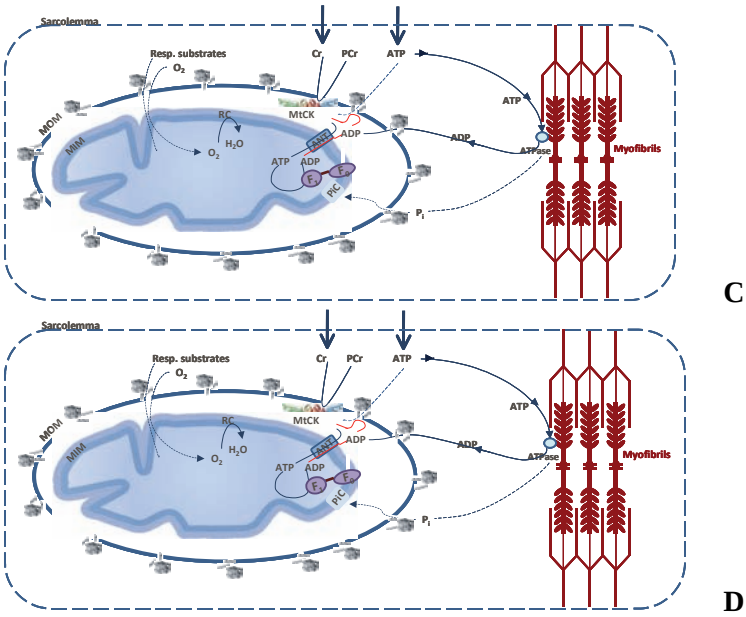


Figure 18. Experimental models. Figures 18A and 18B represent a system related to the isolated heart mitochondria. The respiratory chain (RC) complexes, ATPsynthase (F_1F_0) and Pi carrier PIC are integrated within the mitochondrial inner membrane. Mitochondrial creatine kinase (MtCK) is depicted as an octamer [68, 71], located in the mitochondrial inter-membrane space (IMS) and attached to the inner membrane surface. In our experiments MtCK is activated by creatine (Cr) in the presence of ATP. The final products of MtCK-forward reaction are phosphocreatine (PCr) and endogenous ADP. The ADP phosphorylation is visualized by recording the oxygen consumption.

In Figure 18A endogenous intramitochondrial ADP produced by MtCK reaction forms a micro-domain within intermembrane space. The micro-compartmentalized ADP can either enter into mitochondrial matrix for phosphorylation or escape into the surrounding medium via voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane (MOM).

In Figure 18B the model is supplemented with ADP-trapping system consisting of pyruvate kinase (PK) and phosphoenolpyruvate. This system utilizes all ADP leaving mitochondria to regenerate extramitochondrial ATP.

Figure 18C represents mitochondrion *in situ*, in permeabilized cardiac cell, surrounded by cytoskeleton proteins and myofibrils. The MOM is less permeable than in isolated mitochondrion, due to the selective permeability of VDAC which is caused of interactions of the channel with cytoskeleton proteins. Exogenous ATP is hydrolyzed by cellular ATPases into endogenous extramitochondrial ADP and inorganic phosphate (Pi). Mitochondrial (MtCK) and non-mitochondrial creatine kinases (present in cytosol, myofibrills, SERCA, sarcolemma); activated by creatine in the presence of ATP, produce endogenous intra- and extramitochondrial ADP. Thus the oxidative phosphorylation is controlled by endogenous ADP produced by the MtCK, MMCK and ATPase reactions.

Figure 18D represents system 18C supplemented with phosphoenolpyruvate and pyruvate kinase (PK). PEP–PK system removes extramitochondrial ADP produced by

intracellular ATP-consuming reactions and continuously regenerates extra-mitochondrial ATP. Endogenous intramitochondrial ADP produced by MtCK forms microcompartments within the IMS and is re-imported into the matrix via adenine nucleotide translocase (ANT) due to its functional coupling with MtCK. A series of experiments were performed to check the properties of this model in order to use it for complete MtCK kinetic analysis. This protocol is called Gellerich–Guzun protocol. From [137] with permission, Article II.

2.1.4. Quality tests of cardiomyocytes.

In order to to ensure reliable results, regular quality tests of CM and isolated mitochondria preparation were performed. Only the mitochondria and cardiomyocytes meeting the requirements described below were used in kinetic experiments.

Addition of ADP in a saturating concentration of 2 mM to CM induced high State 3 respiration rate (Figure 19). The respiratory control index (RCI) usually exceeded 6. The effect of the addition of exogenous cytochrome c on State 3 respiration was absent showing the intactness of MOM. After an addition of carboxyatractyloside CAT respiration rate decreased back to the State 2 (V_0) level showing the intactness of the inner membrane [205-206].

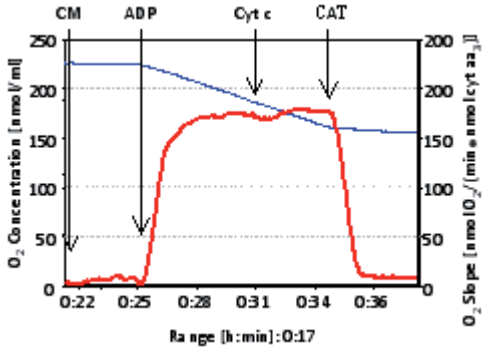


Figure 19. Quality test of CM.Respiration was activated with 2mM ADP. Addition of cytochrome c did not change respiration rate indicating intactness of MOM, addition of CAT decreased respiration to the V_0 level due to the shutting of ANT and intactness of MIM.

In cells *in vivo*, endogenous ADP may be produced by MgATPase and by creatine kinase reactions from ATP by both MtCK and MMCK (see Figure18C). In this scheme exogenous ATP added to permeabilized cardiomyocytes is hydrolyzed by cellular ATPases with the formation of endogenous extramitochondrial ADP which subsequently stimulates mitochondrial respiration.

To study the role of the coupled MtCK alone in the regulation of respiration *in situ* in the cells the stimulatory effect of extra-mitochondrial ADP produced by MgATPases and MMCK can be extinguished by the PEP–PK system (Figure 18D). The addition of the PEP–PK system helps to simulate the *in vivo* conditions in the cells where the glycolytic system is present and it competes with mitochondrial respiration through consuming ADP. Figure 20A shows that the respiration rate of mitochondria *in situ* stimulated by 10 mM creatine and 5

mM MgATP did not decrease after the addition of 10 U/ml PK in spite of the fact that the extramitochondrial ADP must have been significantly reduced. Under these conditions respiration is fully maintained by intramitochondrial ADP produced in MtCK reaction which is not accessible for the PEP–PK system. Mitochondrial outer membrane in cardiomyocyte is not permeable for ADP, which is transported back into the matrix by ANT for subsequent rephosphorylation. (Figure 18D, Figure 20A).

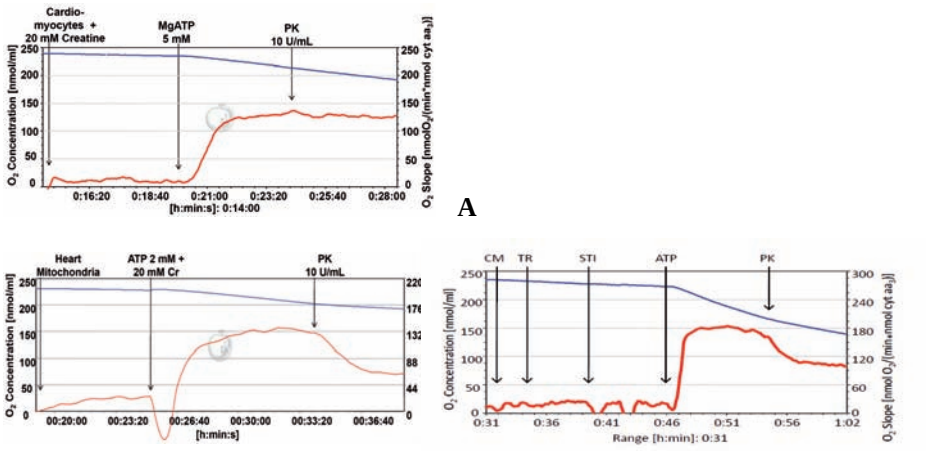


Figure 20 Quality tests with PK-PEP trapping system. (A) Permeabilized CM, **(B)** isolated mitochondria and **(C)** CM after trypsin treatment (by 0.2 µM trypsin for 5 min at 25 °C).

In isolated mitochondria (Figure 20B**)** the influence of PK–PEP trapping system on the oxygen consumption rate could be observed: when the respiration of the isolated heart mitochondria was stimulated by creatine in the presence of ATP (i.e. MtCK was activated), the addition of PK and PEP decreases respiration rate for about 50% from its maximal value The gained rate of respiration (up to 50% of V_{O2max}) was due to the functional coupling between MtCK and ANT with the direct transfer of ADP into the matrix. After the treatment of permeabilized cardiomyocytes with trypsin, compartmentalized in the intermembrane space ADP becomes accessible for the PEP–PK system (Figure 20C). The PK and PEP added under these conditions inhibit respiration by trapping significant part of the extramitochondrial ADP and a significant part of ADP produced by MtCK. The effect is similar to the one observed in isolated mitochondria (Figure 20B). These results show that ADP produced by MtCK in mitochondrial intermembrane space is not accessible for PEP–PK in the permeabilized cardiomyocytes, due to the regulated impermeability of mitochondrial outer membrane. The regulatory effect is lost in isolated mitochondria and therefore the PK/PEP system is the most sensitive control mechanism of the quality of cardiomyocytes outer membrane. These basic

quality tests of permeability of mitochondrial outer and inner membrane were routinely used in experiments described here. Only preparations with the characteristics described were used in the experiments reported in this work.

2.3. Spectrophotometric measurements

2.3.1. Measurements of concentration of cytochrome *aa3*

For comparative quantitative analysis of the kinetics of the regulation of respiration in isolated mitochondria and permeabilized cardiomyocytes, the respiration rates were expressed in nmol of oxygen consumed per minute per nmol of cytochrome *aa3*, but not per mg of protein. Cytochrome *aa3* content in both cases is representative of respiratory chain, while proteins contained in cardiomyocytes are not all present in mitochondria. The contents of mitochondrial cytochrome *aa3* in the isolated mitochondria and cardiomyocytes were measured spectrophotometrically according to the method described in [209]. The differential spectrum (reduced versus oxidized cytochromes) was obtained by scanning from 400 to 650 nm using a Cary 100 spectrophotometer (Varian, Palo Alto, USA) or Evolution 600 spectrophotometer (Thermo Electron Scientific Instruments, UK).

2.3.2. Measurements of MtCK activity

For calculation of inhibition curve of MtCK, the activity of CK was measured at different concentrations of inhibitor with 2.4 dinitrofluorobenzene (DNFB) in a Cary 100 Bio spectrophotometer according to the method described before [209]. The MtCK activity was measured at the same conditions as was the oxygen consumption change in the Oxygraph at temperature 25^{o}C. Isolated mitochondria were used to measure selectively the activity of MtCK. A 5 min inhibition period of CK was allowed after the addition of DNFB and then triglycine (GGG) was added to stop the inhibition.

2.4. Determination of protein content

Protein concentrations were determined by using a BCA protein assay kit (Pierce, USA) as a standard.

2.5. Determination of the rate of ATP and PCr production in CM by ion pair HPLC

Determination of the rates of PCr synthesis in permeabilized cardiomyocytes *in situ* under conditions used in respirometry experiments was carried out using ion pair HPLC/UPLC according the method described before [210]. Separations of Cr, PCr and adenine nucleotides were performed by ultraperformance ion-pair

chromatography (UPLC) on a 2.1×100 mm ACQUITY UPLC HSS T3 C18 column packed with 1.7 μm particles (Waters) by recording optical density simultaneously at 210 nm and 254 nm for creatine and PCr, and adenine nucleotides, respectively. The retention time for the reagents were, in minutes, 0.63 (Cr), 1.70 (PCr), 6.33 (AMP, traces), 6.95 (ADP) and 7.29 (ATP), all within ±0.01 min.

2.6. Immunofluorescence

A method for the study of arrangement of tubulin in cardiomyocytes method was used as described in [183].

For the study of arrangement of mitochondria in cardiomyocytes and HL-1 cells, freshly isolated or cultured cells were preloaded with mitochondria-specific fluorescent probe 0.2 μM MitoTracker Red™ and Green™ (Molecular Probes, Eugene, OR) for 2 h at 4 °C for cardiomyocytes and 15 min at 37° for HL-1 cells. Images were then analyzed using Volocity software (Improvision, France).

2.7. Confocal microscopy

The fluorescence images were acquired with a Leica TCS SP2 AOBS inverted laser scanning confocal microscope (Leica, Heidelberg, Germany) equipped with a 63×water immersion objective (HCX PL APO 63.0×1.20W Corr). Laser excitation was 488 nm for FITC and MitoTracker™ Green, 543 nm for Mito-ID™, 633 nm for Cy5, MitoTracker™ Red and 461 nm for Hoechst 342, nucleus dye.

3. Kinetic analysis of CK reaction

In Figure 21 a scheme of the reaction catalyzed by CK is presented, which is Bi-Bi type random quasi-equilibrium reaction in accordance with the classification of Cleland. K_{ia} and K_a are the dissociation constants of MgATP from its binary and ternary complexes (CK-MgATP and CK-Cr.MgATP, respectively); K_{ib} and K_b are the dissociation constants of creatine from its binary and ternary complexes (CK-Cr and CK-Cr-MgATP, respectively). K_{ip} is the dissociation constant of PCr from its binary complex with CK (CK-PCr) and k_1 and k_{-1} are the rate constants of direct and reverse reactions. Complexes of CK-MgATP-PCr and CK-MgADP were neglected in our work because they have very high dissociation constants [211].

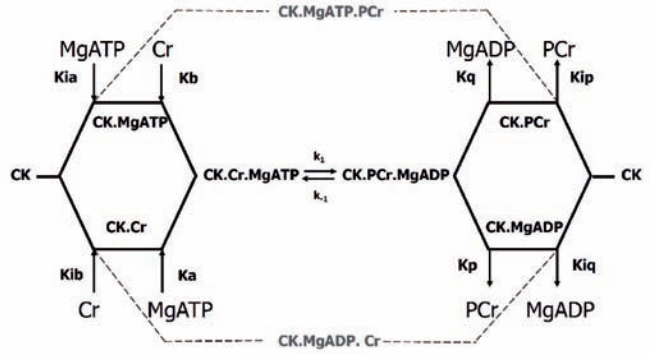


Figure 21. The kinetic mecanism of MtCK reaction. The reaction is Bi-Bi random quasi-equilibrium type according to Cleland classification [212].

Dissociation constants for MgATP Kia, Ka and for Cr Kib, Kb from their binary and ternary complexes with MtCK are [36]:

$$K_{ia} = \frac{[CK]\cdot[MgATP]}{[CK\cdot MgATP]} \quad K_b = \frac{[MgATP\cdot CK]\cdot[Cr]}{[CK\cdot MgATP\cdot Cr]}$$
$$K_{ib} = \frac{[CK]\cdot[Cr]}{[CK\cdot Cr]} \qquad K_a = \frac{[CK\cdot Cr]\cdot[MgATP]}{[CK\cdot MgATP\cdot Cr]} \tag{15}$$

$$v = \frac{Vm\cdot[Cr]\cdot[MgATP]}{K_{ia}K_b + K_b[MgATP] + K_a[Cr] + [MgATP]\cdot[Cr]} \tag{16}$$

Primary analysis of data derived from Eq. (16) in double-reciprocal coordinates of 1/v versus 1/[S for fixed [MgATP] and varying [Cr] is:

$$\frac{1}{v} = \left[\frac{K_b}{V_m}\left(\frac{K_{ia}}{[MgATP]}+1\right)\right]\frac{1}{[Cr]} + \frac{1}{V_m}\left(\frac{K_a}{[MgATP]}+1\right) \tag{17}$$

and for fixed [Cr] and varying [MgATP]:

$$\frac{1}{v} = \left[\frac{K_a}{V_m}\left(\frac{K_{ib}}{[Cr]}+1\right)\right]\frac{1}{[MgATP]} + \frac{1}{V_m}\left(\frac{K_b}{[Cr]}+1\right) \tag{18}$$

This primary analysis provides the values of ordinate intercepts (i_1, i_2) and slopes (s_1, s_2) for secondary analysis - Eqs. (19) and (20). By replotting the

estimated regression parameters as functions of secondary substrates one can obtain the values of dissociation constants for MgATP (Kia and Ka,) and creatine (Kib and Kb) from their binary and ternary complexes with MtCK (Figure 22).

$$i_1 = \frac{1}{V_m}\left(\frac{K_a}{[MgATP]}+1\right) \quad s_1 = \frac{K_b}{V_m}\left(\frac{K_{ia}}{[MgATP]}+1\right) \tag{19}$$

$$i_2 = \frac{1}{V_m}\left(\frac{K_b}{[Cr]}+1\right) \qquad s_2 = \frac{K_a}{V_m}\left(\frac{K_{ib}}{[Cr]}+1\right) \tag{20}$$

By replotting the estimated regression parameters as functions of secondary substrates, one can obtain the values of dissociation constants for MgATP (Kia and Ka,) and creatine (Kib and Kb) from their binary and ternary complexes with MtCK. At i_1=0 and i_2=0 the values for Kb and Ka, are acquired. Constants Kib and Kia are obtained in the case of s_1=0 and s_2=0.

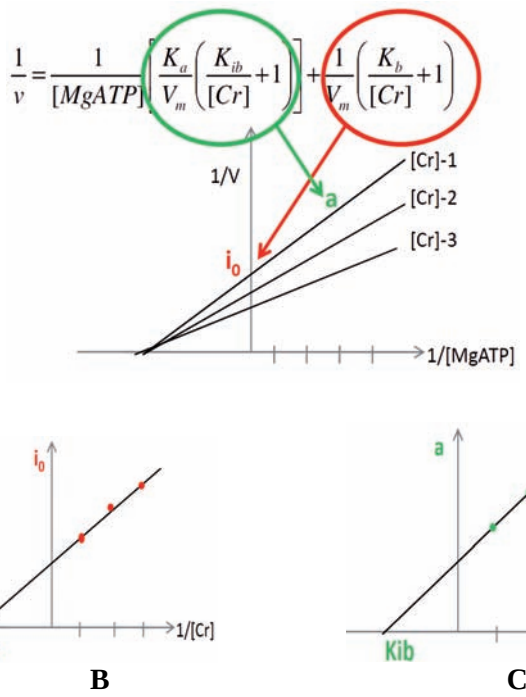


Figure 22A The primary analysis of data in double reciprocal coordinates of 1/v (1/respiration rate) versus 1/[MgATP] (substrate with varying concentration) for different fixed creatine concentrations. **B,C** The secondary analysis of the primary plots from (A). Slopes (a) and intercepts of y-axis (i_0) are plotted as a function of reciprocal coordinate of secondary substrate Cr. The intercepts of x-axis provide directly the reciprocal values of dissociation constants of MgATP (Kib and Kb) from the binary and ternary complexes of MtCK.

4. Metabolic Control Coefficient (MCC) determination

There are two different methods to estimate the value of flux control coefficients. In this work both of the two methods were used: (1) method developed by Groen et. al. for irreversible inhibitor [200], and (2) equation developed by Gellerich [198] to estimate the flux control coefficients in linear pathway more precisely. To estimate the flux control coefficients of oxidative phosphorylation in mitochondria the method of inhibition titration has been widely used [160, 189, 196, 200-203]. It has been shown that inhibitors, used also in this study could be considered pseudo-irreversible under these conditions, which allow using the equation of irreversible inhibition.

The enzyme is stepwise titrated with a specific inhibitor, as the amount of inhibitor tends to zero, the response of the flux to the inhibitor can be expressed in MCA terms. The flux control coefficient were defined as the variations in flux (*J*) when an infinitesimal change in the enzyme *i* concentration or activity takes place [188]. In practice, the infinitesimal changes in *vi* are undetectable, and hence measurable noninfinitesimal changes are analyzed. Groen and his coworkers have shown that for the case of irreversible specific inhibitor an estimate of the value of the flux control coefficient defined by equation (7) is given by:

$$C_E^J = (\Delta J / \Delta I) * (I_{\max} / J_0) \qquad (21)$$

Where (ΔJ/ ΔI) is initial slope of the flux/inhibition graph. [189].

The inhibitors used were: rotenone for Complex I of respiratory chain, antimycin for Complex III, sodium cyanide for Complex IV, oligomycin for Complex V (ATP synthase), carboxyatractyloside (CAT) for ATP/ADP transporter, mersalyl for PIC, and DNFB for MtCK.

We used also equation developed by Gellerich [198] who introduced non-linear function calculations to reduce the possible effect of experimental and calculation errors influencing results in so-called graphic method. The results of the use of these two methods were compared.

5. Chemicals and Solutions

Enzymes and other chemicals were obtained from Sigma, Fluka and Roche.

6. Data analysis

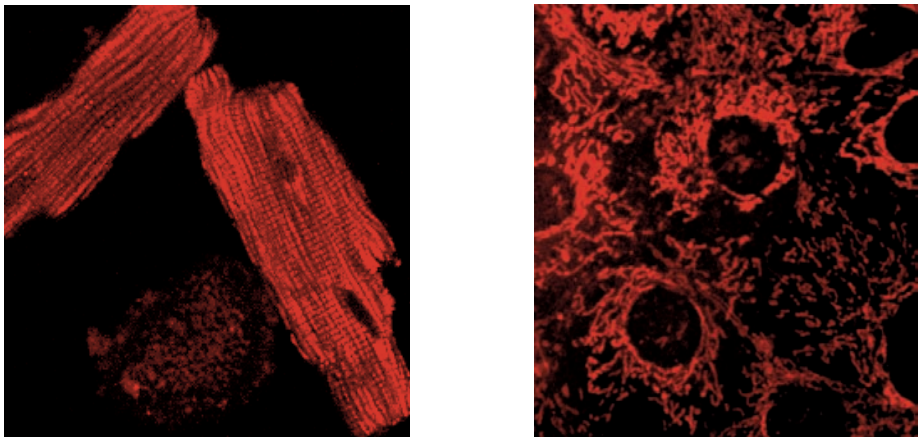
To reduce the possibility of random error the experiments were repeated seven to twenty times. All data are presented as mean ± SEM (standard error of the mean). Statistical analysis were performed using Student's t-test and $p<0.05$ was taken as the level of significance.

RESULTS AND DISCUSSION

1. Structure-funcion relationship in regulation of energy fluxes in cardiomyocytes and HL-1 cells. (Article I)

Structure-function relationship in the regulation of energy fluxes was studied in this work. In cardiac cells the concept of ICEUs [103] was developed on the basis of information of cardiac cell structure and experimental data obtained in the studies of permeabilized cardiac cells and fibers. To reveal the significance of structure-function relationship in energy metabolism regulation, comparative analysis of the bioenergetics parameters (structure, kinetic properties of ATP synthesis by mitochondria etc.) of adult rat cardiomyocytes and both subtypes of HL-1 cells (see 3.4.1) was carried out.

Confocal microscopic analysis showed very different mitochondrial arrangement in non-beating (NB) HL-1 cells in comparision with CM (Figure 23). The mitochondria are chaotically organized in NBHL-1 cell interior; their shape was dynamically changing from granular to filamentous (Figure 23B). In CM the mitochondria are regularly positioned between the myofibrils in a crystal-like structure (Figure 23A), each beside of the adjacent sarcomere thus. thus forming of dissipative metabolic structures, ICEUs, in which mitochondria are precisely juxtapositioned with ATP consuming systems [9, 103] see also Figure 12.



A **B**

Figure 23. Confocal fluorescent imaging of mitochondria. (A) in normal CM in respiration medium and (B) in NBHL-1 cells in the culture medium. The mitochondria were visualized by cell incubation (20–30 min) with mitochondrial membrane potential-sensitive probe TMRM (100 nmol/L). Each fluorescent spot represents a mitochondrion. Scale bars, 10 μm.

In HL-1 cells ICEUs cannot be formed because of missing of sarcomeres (particularly in the non-beating subtype) and because of the continuous fusion and fission of mitochondria in HL-1 cells [140, 213].

In the result of the less organized structure with loosely packed and even slowly moving mitochondria, adenine nucleotides have less restricted diffusion in HL-1 cells compared with that in CM. Low apparent Km for ADP in regulation of respiration [140] is supporting the idea that the mitochndria and ATPases in HL-1 cells may communicate via simple diffusion of adenine nucleotides. In HL-1 cells the oxygen consumption rate with complex I dependent substrates (glutamate and malate) was lower than that with succinate, a complex II dependent substrate (Figure 24). These results, being in agreement with previous data [140, 214], suggest a relative deficiency in complex I in HL-1 cells. In normal adult permeabilized cardiac cells the respiration rate with either glutamate or malate usually exceeds that with succinate [205]. Remarkably, the respiratory activities of respiratory chain complexes of the B and NBHL-1 cells were close to each other but about 4–8 times lower than in CM (Figure 24).

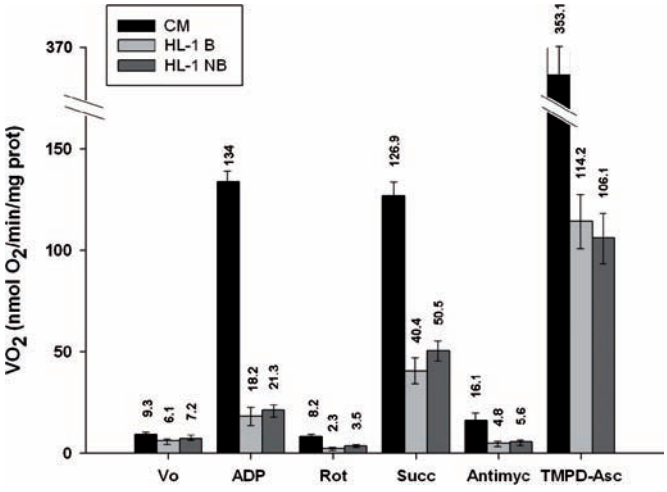


Fig. 24. The activity of the respiratory chain complexes in CM, B HL-1, and NBHL-1 cells. Oxygen consumption rates were measured in Mitomed solution at 25^{o}C in the presence of 25 mg/mL saponin (plus 5 mM glutamate and 2 mM malate). Then 2 mM ADP, 5 mM rotenone (Rot), 10 mM succinate (Succ), 10 mM antimycin A (Antimyc), and 1 mM TMPD with 5 mM ascorbate (Asc+TMPD) were added to the oxygraph chamber. n = 5–7.

The oxygen consumption rates are usually calculated in units nmol O_2 consumed in minute per mg cell protein, however, number of respiratory chain complexes per mg cell protein varies remarkably in different cell types. Content of cytochromes, hemoproteins of the respiratory chain is spectrophotometrically easily measureable; therefore the relative cytochrome content in cell could be used as a measure of maximal oxidative phosphorylation capacity of a cell. Our

study showed that the cytochrome content per milligram of cell protein in HL-1 cells was by a factor of 7 lower than in CM (see Table 1). This could be one of the reasons of the low respiration rate in HL-1 cells compared to CM.

Table 1. Cytochrome content in CM and in NBHL-1 cells.

	Cytochrome aa^3	Cytochrome b	Cytochrome cc^1
NBHL-1 cells	0.075±0.026*	0.096±0.036	0.051±0.01**
CM	0.5±0.16	0.45±0.17	0.49±0.1

Note: Cytochrome content is given in nanomoles per milligram of cell protein (nmol/mg)
n = 3–7. *differences significant at $p < 0.05$ and ** differences significant at $p < 0.02$

Activities of glycolytic enzymes, hexokinase (HK), and pyruvate kinase (PK) were higher in HL-1 cells and at the same time the creatine kinase (CK) activity was significantly lower in HL-1 cells in comparison with CM (Table 2). The results comply with the assumption that in contrast to CM in which oxidative phosphorylation is a predominant provider of ATP and the CK system is a main carrier of energy from mitochondria to ATPases, in HL-1 cells the energy metabolism is based primarily on the glycolytic reactions coupled to oxidative phosphorylation through HK.

Table 2. Enzyme activity of CM, NBHL-1 cells, and gastrocnemius muscle homogenate (GH).

	CM	NB HL-1	GH
Hexokinase	33±3*	148±12	31±4
Fructose 6-phosphate kinase	698±23*	124±5	1339±124
Creatine kinase	6327±724*	395±43	83556±3866
Lactate dehydrogenase	4414±265	4069±449	13985±800
Pyruvate kinase	637±69*	1375±187	11571±722
Glyceraldehyde 3-phosphate dehydrogenase	2331±391	2693±490	7511±701

Note: Activitias of the enzymes are presented in nanomoles per minute (nmol/min) per milligram of protein, n = 5–7. * Differences significant at $p < 0.001$ CM vs. NBHL-1 cells, differences significant at $p < 0.01$ CM vs. NBHL-1 cells and at $p < 0.001$ CM vs. GH.

Mitochondria have a crucial role in apoptosis: release of SMAC (small mitochondrial activator of caspases) into the cytosol, where it binds to inhibitor proteins of apoptosis proteins, induces higher permeability of MOM, and formation of mitochondrial outer membrane permeabilization pore, release of cytochrome c and other apoptotic signaling proteins. As is shown in the study, in tumor cells like HL-1 the switch from CK to HK regulation in mitochondria is taking place. HK is bound to mitochondrial outer membrane that enables its coupling to oxidative phosphorylation [214]. It is visible from the results that interaction between mitochondria and HK is ensured by the very high activity of

that enzyme. It can be considered that HK regulated energy transfer from mitochondria have an important role in tumor cells to evade apoptosis.

It is known that several cancer cell types over-express HK [215-217] and that in these cells HK is coupled with oxidative phosphorylation so that ATP produced in mitochondria is preferably used for glucose phosphorylation by HK, whereas ADP liberated in that process is returned to mitochondria to stimulate ATP synthesis [218]. It has been suggested that this mechanism allows the cancer cells to overcome potential inhibition of glycolysis due to lactate accumulation, particularly in conditions of hypoxia. Moreover, interaction of HK with mitochondria protects cells from excess of reactive oxygen species [219-220] and provides cells with glycolytic intermediates as important components for different biosynthetic pathways, thus favoring proliferation of the cells and cancer growth [215]. From these results we could even assume that disruption of mitochondrion–HK complex in cancer cells could be used as a very specific anti-cancer treatment.

Summarizing, the results of our study support the theory that regular arrangement of the mitochondria in cell supported by cytoskeleton and a probable regulation by its components (like tubulin dimer) is an inseparable aspect of the energy transfer regulation in cardiomyocytes. As the irregular arrangement of mitochondria and kinetic properties of ATP production by mitochondria visible in HL-1 cells are similar to that in CM after trypsin treatment (Km^{app} 50.2± 8 μM) [111], it could be assumed that the probable candidate of the regulatory protein is hydrolyzed by trypsin.

2. Regulation of respiration controlled by mitochondrial creatine kinase in permeabilized cardiac cells in situ – the importance of system level properties (Article II, VIII)

The aim of the next step of the work was to clarify the regulatory role of the mitochondrial outer membrane in the mitochondrial creatine kinase (MtCK) kinetics. MtCK is one of the most important complexes in the regulation of energy fluxes in cardiomyocytes [9, 68]. Kinetic analysis of the MtCK was performed in previous works using the results of the measurements of isolated mitochondria [36, 211]. However, it could be assumed that regulation of the mitochondrial outer membrane channels by interaction with cytoskeletal proteins is lost during the isolation process probable. Regulation of respiration by mitochondrial creatine kinase was performed in this study in mitochondria *in situ* in permeabilized cardiomyocytes in the presence of pyruvate kinase (PK) and phosphoenolpyruvate (PEP) to simulate the interaction of mitochondria with glycolytic enzymes. It is possible to study the kinetic properties of MtCK on system level in these conditions, including regulation of mitochondrial outer membrane channels through possible interactions with regulatory proteins.

Table 3. Basic respiration parameters of isolated rat heart mitochondria and of mitochondria *in situ* in permeabilized cardiomyocytes

Parameter	Mitochondria *in vitro*	Mitochondria *in situ* in permeabilized CM
V_0, nmol O_2 min^{-1} mg $prot^{-1}$	26.37±7.93	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 aa3] nmol mg prot−1	1.00±0.012	0.46±0.09
V_3 (2 mM ADP), nmol O_2 min^{-1} nmol aa_3^{-1}	187.94±40.68	178.23±33.96
$V_{Cr,ATP}$, nmol O_2 min^{-1} nmol aa_3^{-1}	197.90±31.86	162.63±26.87

V_0 - respiration rate in State 2 in the presence of substrates before addition of ADP or ATP.
V_3 - respiration rate in the presence of 2 mM ADP.
$V_{Cr,ATP}$ - respiration rate in the presence of activated MtCK by 2 mM ATP and 20 mM creatine.

Two main experimental models were studied in this work: isolated cardiac mitochondria (*in vitro*) as a reference model and permeabilized cardiomyocytes (mitochondria *in situ*) as a model of the study. In order to compare respiration rates of cardiomyocytes and isolated mitochondria in kinetic studies we used oxygen consumption rate per nmol of cytochrome aa^3 content of cell in claculations. As is seen from the results (Table 3) the maximal respiration rates

are the same in the same conditions in isolated mitochondria as well as in CM. Therefore, we can compare isolated mitochondria with mitochondria *in situ* to reveal kinetic differences due to the mitochondrial outer membrane regulation of energy fluxes.

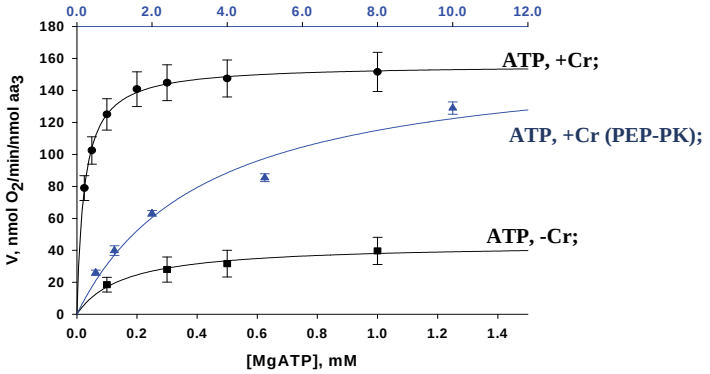


Figure 25. Respiration rates of CM at different concentrations of ATP with and without activated MtCK. ■ – CM without MtCK activation. In ● and ▲ CM with activated MtCK (10mM Cr) ▲- with Cr in the presence of PK/PEP system – repiration activated by intramitochondrial ADP only.

In the first part of experiments carried out in this study respiration of permeabilized cardiomyocytes was stimulated stepwise by increasing the concentrations of ATP in the absence and presence of creatine. ADP produced by ATPases stimulated mitochondrial oxidative phosphorylation, measured as respiration rate in oxygraph (Figure 25). These recordings show an important difference in the behavior of the experimental model. In the presence of creatine, when MtCK is activated, the apparent K_m for exogenous ATP decreases by 6-fold compared to K_m^{app} in the absence of Cr (from 157.7μM to 24.9μM). Respiration of permeabilized cardiomyocytes in the presence of exogenous ATP and activated MtCK is stimulated by two sources: either *via* the reaction of hydrolysis of exogenous ATP or by endogenous ADP produced locally by activated MtCK. In the presence of PK/PEP system exogenous ADP is missing and increase of the respiration rate is slower (K_m^{app} 2.1 mM). The very rapid activation of respiration of permeabilized cardiomyocytes stimulated by creatine in the presence of exogenous ATP compared to stimulation by exogenous ATP only is mediated by the preferential control of oxidative phosphorylation by MtCK coupled with ANT.

The principles of the next stage of the study were illustrated by the scheme described previously in Methods (Scheme 18D) and by oxygen consumption trace in Figure 26. Experiments were performed with activated MtCK reaction in the presence of PEP-PK system, which traps extramitochondrial ADP produced by cytoplasmic isoforms of creatine kinases (MMCK) and MgATPase reactions and subsequently regenerates extramitochondrial ATP. Endogenous

intramitochondrial ADP produced by MtCK forms a micro-domain within the intermembrane space (IMS) and is re-imported into the matrix via ANT due to its functional coupling with MtCK. To measure the kinetic constants of the MtCK reaction, the endogenous ADP resulting from the hydrolysis of exogenous ATP, initially stimulated respiration, then the PEP-PK system was added to an oxygraph chamber. Under the conditions of extra-mitochondrial ATP regeneration by the PEP-PK system, creatine, added in gradually increasing concentrations, activates MtCK and stimulates the respiration (Figure 26). This Gellerich – Guzun protocol [137, 197] allows linking the consumption of O_2 to phosphorylation of endogenous ADP recycled by MtCK locally in the mitochondrial intermembrane space.

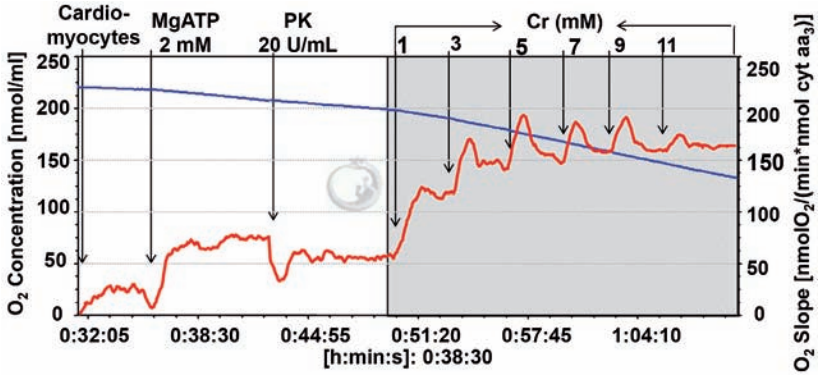
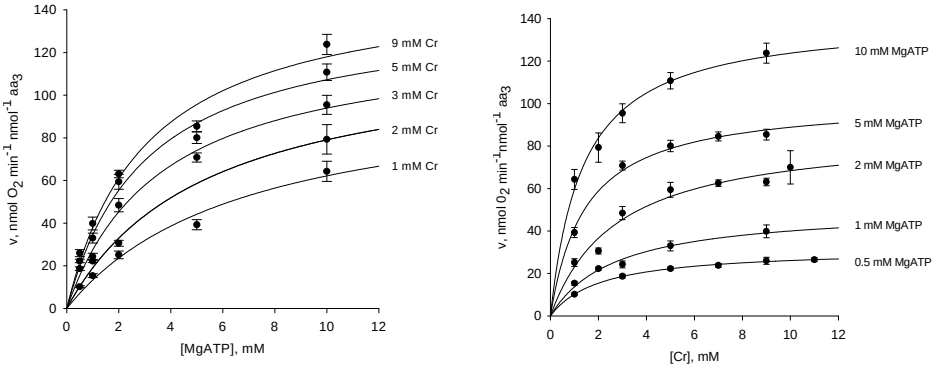


Figure 26. Gellerich-Guzun protocol. After activation of respiration with 2mM ATP, addition of PK in the presence of 5mM PEP in the solution decreses respiration rate due to the trapping of exstramitochondrial ADP. Activation of MtCK increases oxygen consumption rate to the maximal level.



A **B**

Figure 27. Oxygen consumption rates of CM (A) in case of different ATP concentration with fixed Cr and (B) in case of different Cr concentration at fixed ATP**.**

Taking into account that MtCK catalyzes the reaction of two substrates, dissociation constants of binary and ternary complexes of both substrates with MtCK were determined (see Methods) Oxygen consumption measurements were

performed in the presence of a fixed concentration of one substrate and varying concentrations of the other, and vice versa (Figure 27A and B). This stimulated by creatine respiration was calculated by subtracting V_{O2} PK from the V_{O2} (Cr) value to avoid the residual stimulatory effect of extra-mitochondrial ADP. The kinetic constants of MtCK reaction were calculated according to the method described in Methods and in [36, 137].

Table 4. Apparent dissociation constants of ATP, Cr and PCr with MtCK

	K_{ia}MgATP (mM)	K_aMgATP (mM)	K_{ib}Cr (mM)	K_bCr (mM)	K_{ip}PCr (mM)
Mitochondria *in vitro**	0.44±0.08	0.016±0.01	28.0±7.0	5.0±1.2	0.84±0.22
Mitochondria *in situ* (with PEP-PK)	1.94±0.86	2.04±0.14	2.12±0.21	2.17±0.40	0.89±0.17

We compared the kinetic parameters of MtCK determined *in situ* in our experiments with dissociation constants of MtCK obtained from the measurements of isolated mitochondria published in the literature [36]. An analysis of the *in situ* obtained kinetic parameters of MtCK showed an increase (by 10-100 times) of the apparent dissociation constants of MgATP in binary and ternary complex are compared with typical values for the isolated mitochondria (Table 4). This *in situ* decrease in the affinity of MtCK for exogenous MgATP is probably mediated by a restricted diffusion of ATP at the level of outer mitochondrial membrane in permeabilized cells. The apparent dissociation constants of creatine (Kib, Kb) were decreased by 2-15 times in comparison with the isolated mitochondria (Table 4), reflecting an increase of the affinity of MtCK for creatine. The study of the apparent affinity of MtCK for PCr was performed by using the analogous model presented in Figure 25 where concentrations of PCr were varied in the presence of fixed concentrations of ATP and creatine. The affinity of MtCK for PCr in permeabilized cardiac cells is unchanged as compared with isolated mitochondria (Kip is approximately 0.9 mM in both cases) (Table 4). This finding indicates that *in situ* PCr and creatine circulate freely through the outer membrane of mitochondria in permeabilized cells, and that energy flow in a cardiac cell is presented by the flux of PCr rather than by flow of ATP, which is limited at the level of the membrane. These data indicate the selective restriction of diffusion of metabolites at the level of mitochondrial outer membrane. It could be concluded from these data that mechanisms of the regulation of respiration and energy fluxes *in vivo* are system level properties, which depend on intracellular interactions of mitochondria with cytoskeleton, intracellular MgATPases and cytoplasmic glycolytic system. The kinetic data described in section confirm the hypothesis of Mitochondrial Interactosome and describe its functioning by rapid turnover of ADP and ATP in mitochondria coupled to PCr synthesis. (Articles II, IV, VII and VIII).

3. Direct measurement of energy fluxes from mitochondria into cytoplasm in permeabilized cardiac cells *in situ*: further evidence for existence of mitochondrial interactosomes (Article III-IV)

In order to verify the effectiveness of control of respiration by MtCK we measured simultaneously the content of PCr and ATP in cytosol and oxygen consumption rate in the conditions according the Gellerich-Guzun protocol used in the study of kinetics of MtCK (Figure 26). This test was performed in the presence of different fixed concentrations of MgATP (1, 2, 5 mM) in the presence of Cr. The primary purpose of this part of our study was to evaluate the production of PCr by mitochondrial creatine kinase and the efficiency of oxidative phosphorylation stimulated by endogenous ADP produced locally by MtCK.

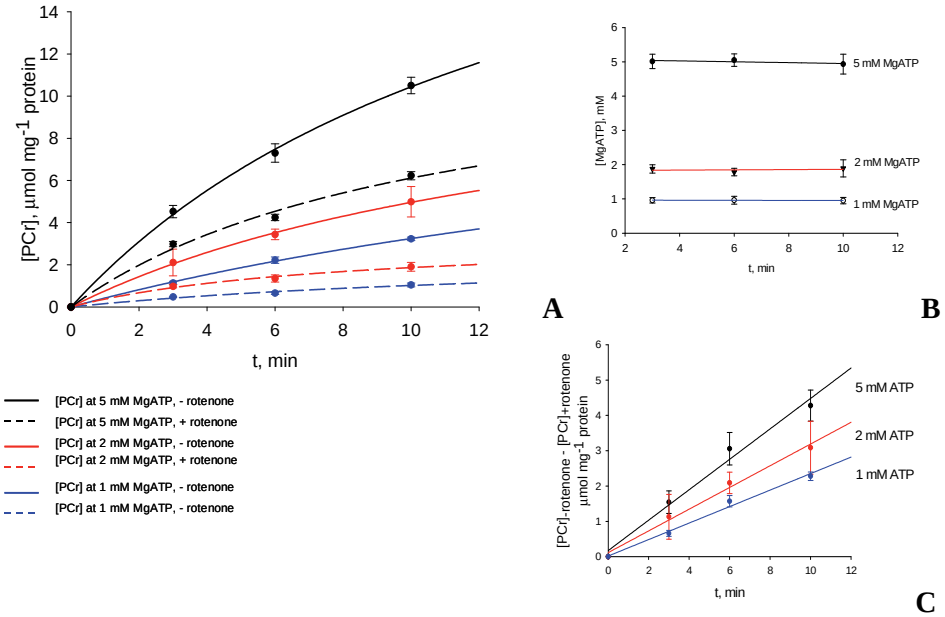


Figure 28. **The rate of phosphocreatine (PCr) and ATP production**

Fig. 28A The rate of phosphocreatine production by mitochondrial and cytoplasmic creatine kinases in permeabilized non-inhibited cardiomyocytes (solid lines) and the rate of PCr production only by cytoplasmic creatine kinases MMCK (dashed lines). Oxidative phosphorylation is inhibited by 10µM rotenone.

Fig. 28B The ATP level continuously regenerated by the PEP-PK system was stable during the experimental procedure described in the Fig.24

Fig. 28C Difference in phosphocreatine production rates under conditions of activated and inhibited (by rotenone) respiratory chain calculated from Fig. 26A.

The reaction catalyzed by CK is reversible; PCr can be produced by two sources: intra-mitochondrial creatine kinase (MtCK) and extra-mitochondrial creatine kinase (MMCK). The total production of PCr was measured in the

presence of oxidative phosphorylation activated by glutamate and malate. Extra-mitochondrial production of PCr was assayed in the presence of inhibited mitochondrial respiration achieved via inhibiting the Complex I of respiratory chain by rotenone. The mitochondrial production of PCr by MtCK was calculated by subtracting the PCr flux produced by MMCK from the total flow of PCr (Figure 28C).

Samples were collected after 3, 6 and 10 min stimulation of respiration by creatine, analyzed by HPLC/UPLC [210]. Oxygen consumption rates were measured in the same conditions in oxygraph. The results allowed us to estimate the efficiency of oxidative phosphorylation when the energy transfer is regulated by MtCK. Results of the study showed that the PCr/O_2 ratio was equal to 5.7 (Table 5). This value is close to the theoretical value of $ATP/O_2 = 6$ which means that 3 molecules of ATP are synthesized as mitochondria reduce one oxygen atom. In our case the mitochondrial ATP is transphosphorylated by MtCK with the production of PCr that leaves mitochondria freely into cytosol. The concentration of MgATP, measured by HPLC remained stable over time (Fig. 28B) confirming a strong restriction of ATP diffusion at the level of outer mitochondrial membrane.

Table 5. Measured rates of PCr production, corresponding oxygen consumption and their calculated ratios for fixed ATP concentration in mitochondria *in situ*.

	V_{PCr}	V_{O2}	V_{PCr}/V_{O2}
1 mM ATP	0.23±0.02	0.041±0.001	5.80±0.45
1 mM ATP	0.31±0.02	0.056±0.02	5.44±0.44
1 mM ATP	0.43±0.04	0.074±0.003	5.81±0.48
Average			5.68±0.14

V_{PCr} rate of PCr production measured with the use of HPLC/UPLC $\mu molmg^{-1}protein\ min^{-1}$
V_{O2} rate of oxygen consumption $\mu molmg^{-1}protein\ min^{-1}$
V_{PCr}/V_{O2} calculated ratio of V_{PCr} / V_{O2}

These results confirm the hypothesis of functional coupling between MtCK and ANT whereby the direct channeling of ATP from the matrix to MtCK is possible, at the same time as there are strong restrictions for diffusion for ATP in the IMS. The mitochondrial outer membrane channels are selectively permeable for PCr at the same time as ADP is channeled through ANT back to matrix.

The regulatory effect of mitochondrial outer membrane in diffusion of metabolites was observed also before and explained by binding of some cytoskeletal elements (called factor X) to mitochondrial outer membrane [157, 221]. Rostovtseva et al. identified this factor X by showing the direct interaction of heterodimeric tubulin with the VDAC inserted into phopholipid membrane [166-167]. It is shown in this work that the kinetics of regulation of respiration of isolated mitochondria with added tubulin is similar to that in permeabilized cardiomyocytes. The addition of heterodimeric tubulin to isolated mitochondria was found to increase apparent Km for exogenous ADP from 11±2μM to

330±47 μM but creatine decreased it to 23±6 μM [179]. The same phenomenon, the decrease of apparent Km in the presence of Cr was observed also in cardiomyocytes in the previous stage of the work (Figure 25). The decrease of Km in the presence of activated MtCK shows formation of selective permeability of mitochondrial outer membrane VDAC. In these conditions ATP and ADP are circulating inside mitochondria and amplifying the signal entering from the cytosol. These results prove that tubulin is at least one of the proteins forming the regulatory complex with VDAC to regulate selectively permeability. It is found that other cytoskeletal proteins also form complexes with VDAC in the outer mitochondrial membrane, particularly desmin [222-223] and plectin [224]. Recent studies have show that in cardiomyocytes the prevalent isoform of tubulin is βII [152], missing in the cancer cells [183] . Immunolabelling with anti-βII tubulin antibody (Figure 29) shows that arrangement of βII tubulin resembles regular arrangement of mitochondria in cardiac cells (Figure 21A). Therefore we could conclude colocalization of βII isoform with mitochondria. These results give clear evidence that βII tubulin is responsible for selective permeability of VDAC.

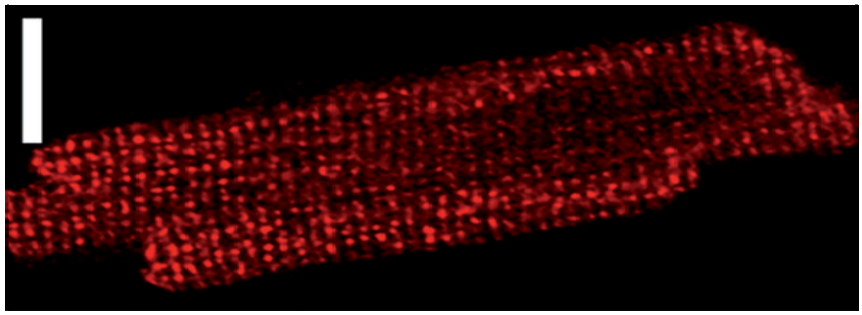


Figure 29. Confocal image of regularly arranged proteins labelled with anti-βII-tubulin antibody and Cy5. Separate fluorescent spots are organized in distinct longitudinally oriented parallel lines repeating mitochondrial arrangement in cardiomyocytes. Scale bar 14μm

The results show directly that under physiological conditions the permeability of the VDAC is selective; the major energy carrier from mitochondria into cytoplasm is PCr, produced by mitochondrial creatine kinase, which functional coupling to adenine nucleotide translocase is enhanced by selective limitation of permeability of mitochondrial outer membrane. As the conclusion of the results of the study concept of supercomplex of Mitochondrial Interactosome (MI) was proposed including ATP Synthasome, MtCK and VDAC associated with heteromeric tubulin and probably other complexes regulating permeability of the channel (Figure 30).

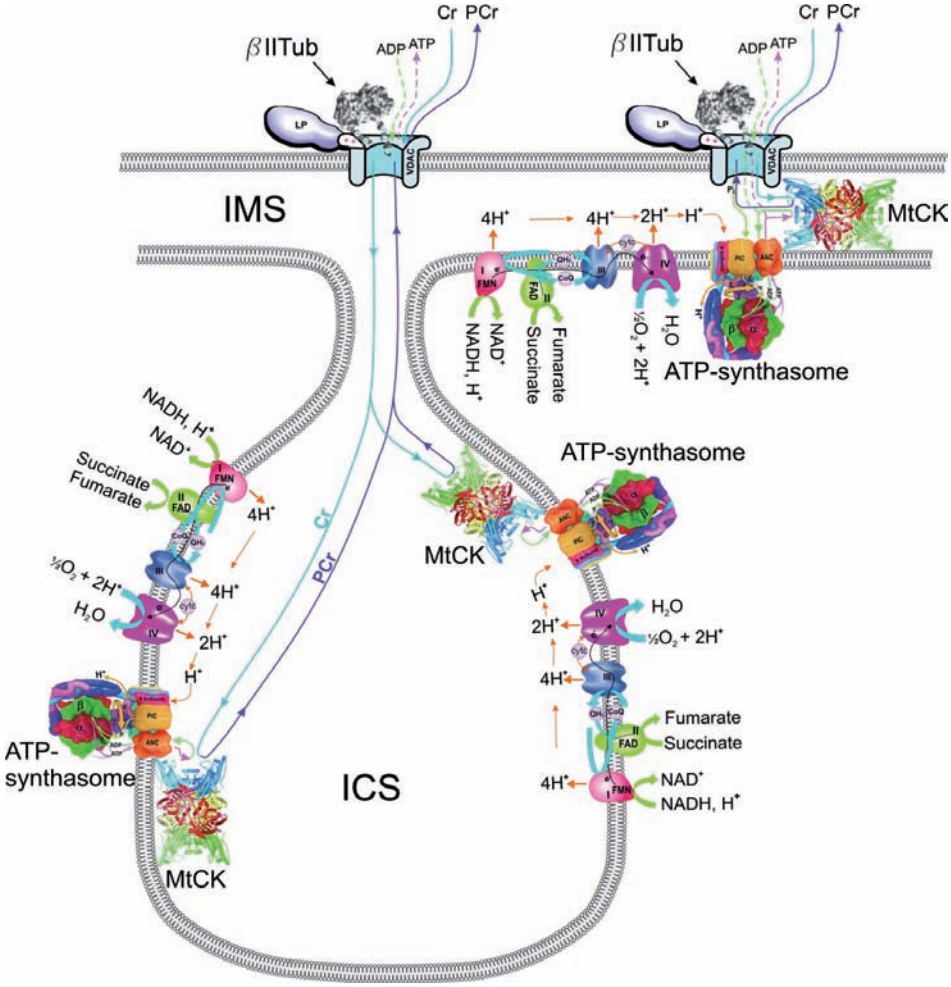
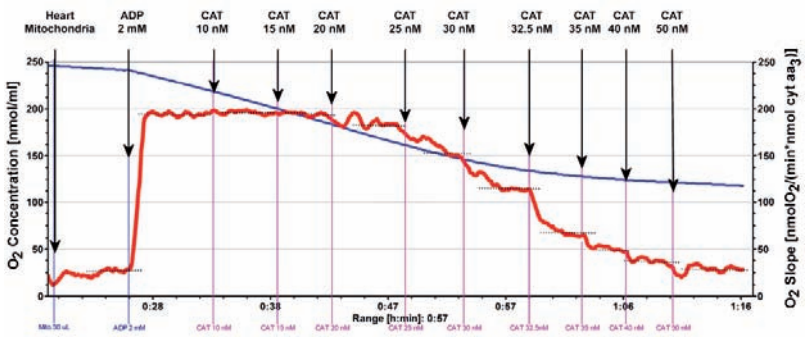


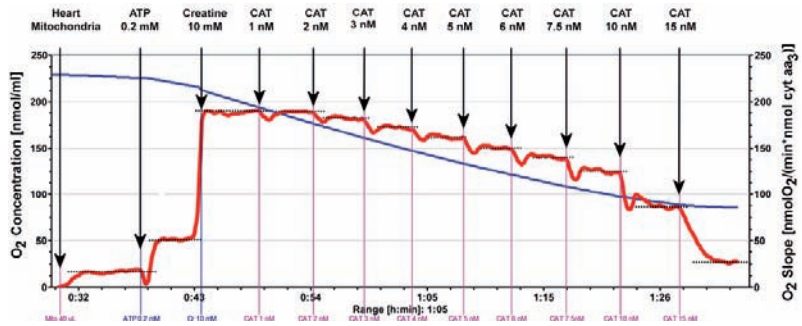
Figure 30. Mitochondrial Interactosome - supercomplex consisting of ATP Sythasome (reprinted from [225-226] with permission), mitochondrial creatine kinase (MtCK), voltage dependent anion channel (VDAC), and tubulin. Octameric MtCK (from [71] with permission) located in the mitochondrial intermembrane space (IMS) is attached to mitochondrial inner membrane [14] and in the contact sites to the outer membranes [71]. VDAC permeability is selectively regulated by heterodimeric tubulin, whose binding to VDAC in intact mitochondrial membrane may be either direct or by some linker proteins (LP). This complex of VDAC with other proteins controls fluxes of adenine nucleotides and phosphocreatine (PCr) into surrounding medium, and phosphorylation by the ATP Synthasome system is effectively regulated by creatine (Cr) *via* MtCK. Here: ANC - adenine nucleotide carrier; and PIC-phosphate carrier. Assumed direct metabolite channeling in intermebrane space is depicted by arrows. Reprinted from [138] Article III.

4. Metabolic Control Analysis of energy fluxes in heart cells (Article V-VI)

In the previous parts of the study the mechanism of the energy transfer regulation was elucidated. It was shown that kinetic parameters of the regulation of mitochondrial respiration measured in cells *in situ* are very different from those *in vitro* [137]. In adult cardiac cells mitochondria are regularly arranged into ICEUs due to their interaction with tubulin, microtubular system and probably other cytoskeletal structures. Functional coupling to adenine nucleotide translocase with MtCK is enhanced by selective limitation of permeability of mitochondrial outer membrane within supercomplex ATP Synthasome-MtCK-VDAC-tubulin, Mitochondrial Interactosome (Figure 30) [138, 227]. Within this supercomplex its different components may have various contributions in the overall control of the mitochondrial respiration rate and energy fluxes in the heart. The contribution of different components to the control is quantitatively elucidated by the application of the Metabolic Control Analysis (MCA). In previous experimental studies the method of MCA has been very intensively applied to the analysis of the control of respiration in isolated mitochondria [196-201] and also to some experiments with fibers [160, 203]. In this study we completed the Metabolic Control Analysis for measuring the flux control coefficients for respiratory chain complexes, ATP Synthasome, ANT, Pi carrier and MtCK (Table 6) in permeabilized cardiomyocytes under two conditions: with direct exogenous ADP activation and with endogenous ADP activation (ATP in the presence of Cr and PK/PEP system). The second protocol corresponds more closely to the physiological conditions in healthy heart cells *in vivo.* In these conditions both MgATP and creatine are always present and mitochondria compete with glycolytic system for cytoplasmic ADP, glycolysis being represented by PK-PEP system in our protocol. As explained before (see Figure 26), the PK-PEP trapping system removes extra-mitochondrial ADP, however, in the case of cardiomyocytes with activated MtCK, intramitochondrial ATP/ADP is circulating inside mitochondria due to the diffusion restriction for ADP in the IMS and selective permaeability of VDAC and addition of PK have no influence on respiration rate (Figure 24A). Therefore, there in no need to measure FCC for PK, in these conditions the FCC for PK is zero.



A



B

Figure 31. Respiration traces of inhibition of isolated mitochondria with CAT.
A Respiration was activated with 2 mM ADP, preceded by the stepwise addition of inhibitor. Subsequent steady states are marked by dotted lines. **B** Influence of the inhibitor on the respiration rate if the respiration was activated by Cr.

To calculate the flux control coefficients from experimental data two calculation methods were used in parallel: calculation from initial slope developed by Groen [200] and calculation using equation developed by Gellerich [198]. The computed flux control coefficients are comparable with estimations obtained using the graph method in the linear system in the case with ADP activation (C_{vi}^{JATP} computed 0.25, and determined by graph method 0.20). The estimated dissociation constant K_d = 15 nM and E_0=225 nM (ADP activation) was close to that reported earlier [228-229]. However, when the calculations were made for the system with activated MtCK, the Gellerich model overestimates the value of flux control coefficient (C_{vi}^{JATP} computed 1.53, determined by graph method 0.92). Therefore we concluded that the Gellerich model has to be developed to use it in non-linear pathway as is the case with activated MtCK. The results presented in Table 6 and figures are calculated from initial slope of inhibition curve.

Previous works of MCA with isolated mitochondria were performed using different conditions, which can explain also differences of the results. In order to

compare the influence of diffusion restrictions at the level of mitochondrial outer membrane on the FCC, we also measured the flux control coefficients in isolated mitochondria in the same conditions used for cardiomyocytes,

It was shown by Gellerich et. al. and Groen et al. [198, 200] already in studies of isolated mitochondria that the flux control coefficient value might change depending upon the presence of the ADP regenerating system. Since under physiological conditions creatine is always present in cardiac cells and MtCK coupled with ATP-Synthasome within MI is always activated, mitochondrial respiration in these cells is largely controlled by the MtCK reaction.

As could be seen from the results presented in Table 6 the FCC of the several complexes increases significantly on physiological conditions (activated MI).

Table 6. Flux control coefficients of MI complexes in CM.

	Inhibitor	Flux control coefficient	
		ADP activation	Cr activation
NADH-CoQoxidoreductase (Complex I)	Rotenone	0.20 ± 0.04	0.64 ±0.03
CoQ cytochrome-c oxidoreductase (Complex III)	Antimycin	0.41± 0.08	0.40 ±0.01
Cytochrome c oxidase Complex IV	Na cyanide	0.39 ± 0.09	0.49 ± 0.08
ATP/ADP carrier	CAT	0.20 ± 0.05	0.92 ± 0.05
ATP synthase	Oligomycin	0.065 ± 0.01	0.38 ± 0.05
Pi carrier	Mersalyl	0.064 ± 0.04	0.06 ± 0.05
MtCK	DNFB		0.95 ± 0.02
Sum		1.33± 0.31	3.84 ± 0.29

The values of FCC were determined by measurements according to two protocols: a) direct activation with 2 mM ADP when MtCK and Mitochondrial Interactosome (MI) complex were not activated (ADP activation) and b) Cr activation: after addition of ATP and PK-PEP system, the addition of Cr activates MtCK and all the MI supercomplex coupling system. Results are presented as average values ± SEM for 10-15 experiments.

The data obtained in this work show that the flux control is much more efficient in the system with ADP-ATP recycling within mitochondria with activated MtCK than in the mitochondria with direct supply of ADP (Table 6). Significant increase of flux control coefficients was observed for ANT (increases five times), ATP Synthase complex and also for Complex I of respiratory chain. Complexes III and IV of the respiratory chain are important regulators in the MI with direct ADP supply, but with activated MtCK their relative regulatory role decreased.

In order to to evaluate the coupling of the main regulatory complexes in the MI MtCK and ANT measurements were made to compare the FCC of these complexes in isolated mitochondria versus mitochondria in situ with and without activated MtCK. As seen from the results (Figure 33) the FCC of ANT in isolated mitochondria is four times higher with Cr activation, thus supporting the

concept of the direct transfer of adenine nucleotides between these complexes, indicated by other authors [29, 34]. It shows that in the presence of Cr MtCK is always an important regulator of oxidative phosphorylation even in isolated mitochondria without respiration regulation by selective permeability of VDAC (Figure 34). FCC of ANT and MtCK have very high and the same time close values; therefore there is also a possibility that under these circumstances these complexes could be counted as one functional unit.

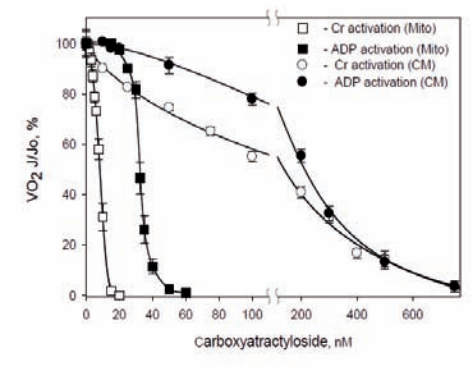


Figure 32. Titration curves for ANT with carboxyatractiloside in isolated mitochondria (Mito) and in permeabilized cardiomyocytes (CM). The respiration inhibition curves are presented for two experimental conditions: with external ADP activation and *in vivo* conditions with Cr activation.

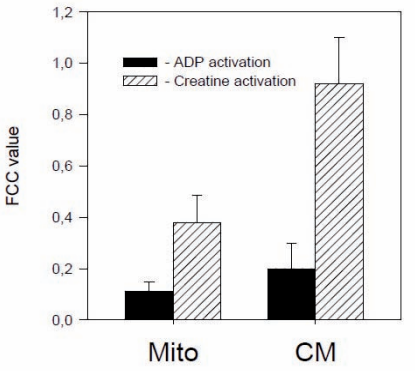


Figure 33. FCC with CAT in isolated mitochondria and CM. Flux control coefficients were determined with ADP activation and with activated MI – *in vivo* conditions. The FCC for ANT is higher almost four times in isolated mitochondria if MtCK is activated with Cr. In physiological conditions, in CM in the presence of Cr, the increase of FCC values is five times compared with CM with ADP activation and the value is almost tenfold higher than in isolated mitochondria.

In comparison with the isolated mitochondria the maximal concentrations of inhibitors needed for the complete inhibition of respiration in permeabilized cardiomyocytes increased 4-10 times (Fig. 32 and 34). These results emphasize the influence of diffusion restrictions in cells due to such physical factors as macromolecular crowding, heterogeneity of diffusion due to the cell structure and selective permeability of membranes, in particular due to the interaction with cytoskeleton [116, 138]. Any quantitative model describing mitochondrial metabolism should be based on the extensive experimental data taking into account not only the data of individual isolated enzymes present in the cell but also the regulation caused by the intracellular structural interactions such as physical barriers, compartmentalization phenomenon and possible direct

interaction between metabolic complexes, including the phenomenon of metabolic channeling etc.

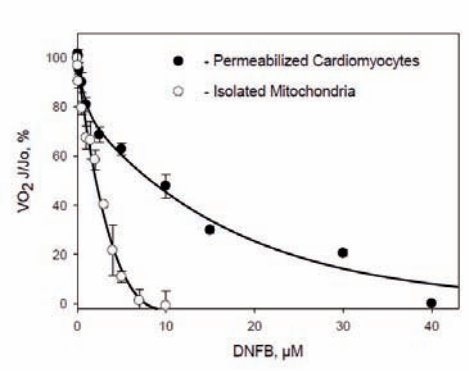


Figure 34. Inhibition titration curve of isolated mitochondria and CM with DNFB Isolated mitochondria or CM were inhibited with DNFB in the presence of 10 mM Cr and ATP. Inhibition was terminated with triglycine and the respiration rate was registered from the following steady state. Maximal inhibiting concentration of DNFB is four times higher than in CM due to the diffusion restrictions.

One aim of the study was also to investigate the possibility of direct channeling in the energy transfer regulation in MI. It was found in the experimental studies of the control of respiration in isolated mitochondria that the sum of flux control coefficients of respiratory chain complexes, ATP synthase and metabolite carriers is close to 1 which corresponds to the behavior of a linear metabolic system [188-189, 200]. Analogous works with similar results were also carried out incase of permeabilized cardiac fibers if respiration was activated by direct addition of ADP [160, 203]. Kholodenko, Westerhoff and their coworkers showed investigating theorethical aspects of MCA that the increased sum of the FCC is an indication of direct channeling in the pathway [190]. The results of our study showed that in mitochondria *in situ* with activated MI the sum of the calculated FCC is more than three times higher than in the conditions with exogenous ADP activation. It could be concluded that the sensitivity of the complexes to the metabolic signals is significantly higher in these conditions and that there is an indication of the possibility of direct channeling of intermediates in the physiological conditions as presented in [190].

The results of this study show high efficiency of regulation of energy fluxes in the coupled to MtCK-ANT reaction within Mitochondrial Interactosome in comparison with the linear sequence of reactions in the ATP Synthasome activated by direct addition of ADP in mitochondria *in situ* as well as in isolated mitochondria. These results are consistent with the proposal of the central role of MtCK in the regulation of mitochondrial respiration and energy fluxes in normal adult cardiac cells [9, 68]. High efficiency of energy flux control in MI makes

this supercomplex a key site for the feedback of metabolic regulation of mitochondrial respiration in cardiac cells. These results demonstrate directly that under physiological conditions the major energy carrier from mitochondria into cytoplasm is PCr, produced by mitochondrial creatine kinase (MtCK), and the part of the direct ATP transfer under physiological conditions could remain approximately 10%.

There are also several other authors who emphasize the importance of MtCK as central complex not only in energy transfer regulation but in the regulation of cell lifecycle. Max Dolder [76] has shown that substrates of MtCK inhibit mitochondrial permeability transition. The effect was visible only when CK was located between mitochondrial membranes in functional coupling with ANT. Externally added CK did not have the protecting effect. There is also evidence for MtCK activity having a key role as a preventive antioxidant against oxidative stress, reducing mitochondrial ROS generation through an ADP recycling mechanism [230].

5. Feedback metabolic regulation within ICEUs (Article VII)

The results obtained in this work allow us to draw principle conclusions that the kinetics *in vivo* is different from that of *in vitro* the regulation of energy fluxes is a system level property which could be investigated only in mitochondria *in situ*, studing the regulation of energy transfer by mitochondrial outer membrane VDAC associated with βII-tubulin and other proteins. It is shown in our study that in CM with activated MtCK (physiological conditions), the complexes of energy transfer have increased sensitivity to metabolic signals in comparision to CM with direct ADP activation or in the case of isolated mitochondria.

Many authors have recorded cyclic changes of the concentrations of main metabolites within cardiac cycle [231-233], showing the slight (8-12%) oscillations of PCr and Cr concentrations during one cardiac cycle on the background of the remarkable stability of the average values of the concentrations of ATP and PCr (metabolic homeostasis). The amplitude of displacement of MMCK from equilibrium, as well as cyclic changes of ADP concentration are proportionally increased with workload [74, 152-153, 234-236]. The rephosphorylation of ADP in MMCK reaction increases locally the Cr/PCr ratio, which is transferred towards MtCK via CK/PCr shuttle [237]. The amplitudes of ADP concentration changes within contraction cycle are to our knowledge the only parameters, which meet the requirements for metabolic signal formulated by Liu and O'Rourke [131], see section 3.3.

When mitochondrial outer membrane is permeable, as in isolated mitochondria, the regulation of respiration is impossible because of a saturating concentration of intracellular ADP which exceeds manifold even in diastolic phase (about 40 μM) the apparent affinity of oxidative phosphorylation for free ADP (Km^{app} ADP = 7.9±1.6 μM). On the contrary, when the ADP diffusion is

restricted at the level of MOM, as in mitochondria *in situ*, the apparent Km for free ADP increases to about 370.75±30.57 μM. As is shown in the study, the change in the kinetic of oxidative phosphorylation is due to the selective permeability of VDAC caused by the assotsiation of βII-tubulin and other regulatory protein complexes. It is quantitatively shown by using the method of MCA that metabolic sensitivity of complexes of MI is significantly increased, the sum of flux control coefficients, close to four. According to theories developed by Kholodenko, Westerhoff and others (see section 4) this result prove that the diffusion restrictions for adenine nucleotides in the level of MOM are surpassed by direct channeling between the complexes of MI and the ADP/ATP are recycling inside mitochondria is amplifying the entering signal. High FCC of MtCK and ANT also prove the recycling process of adenine nucleotides in the presence of creatine. These results quantitatively show that creatine, by activating the coupled MtCK within Mitochondrial Interactosome, induces ADP/ATP recycling and thus amplifying the effect of cytoplasmic ADP.

Thus, the regulation of respiration by local changes in ADP concentration under condition of restriction of adenine nucleotides diffusion across the mitochondrial membrane is possible exclusively due to the specific structure of Mitochondrial Interactosome where MtCK reaction amplifies this signal due to its functional coupling with ATP Synthas.ome, increasing the steady state rate of recycling adenine nucleotides in mitochondria and the rate of respiration. Thus, as is presented in the study in physiological conditions, cyclic changes in local ADP concentrations in myofibrillar space of ICEUs due to nonequilibrium state of CK reactions become an effective regulatory signal, but only if the VDAC permeability is restricted by association of βII-tubulin and if creatine is present. The coupled MM-CK reaction in myofibrils and coupled MtCK reaction in mitochondria run in non-equilibrium state in opposite directions, resulting in the separation of energy fluxes (mass and energy transfer by PCr) and signaling (information transfer by oscillations of cytosolic ADP concentrations, Pi and PCr/Cr ratio) amplified within Mitochondrial Interactosome. As a result, the reactions catalysed by different isoforms of compartmentalized CK tend to maintain the intracellular metabolic stability. It has been also shown by mathematical modeling that not more than 10% of free energy is transported out of mitochondria by ATP flux needed to equilibrate the information-carrying flux of ADP into mitochondria [111].

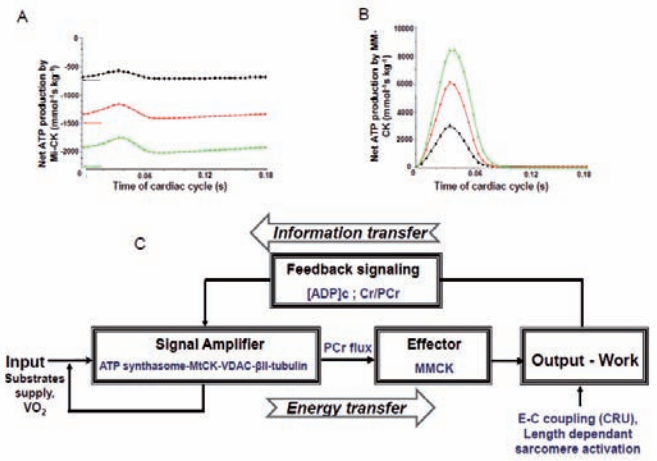


Figure 35. General presentation of the feedback metabolic signaling in regulation of energy metabolism within Intracellular Energetic Units in cardiac cells. Due to the non-equilibrium steady-state MtCK and non-equilibrium cyclic MMCK reactions intracellular ATP utilization (marked as output) and mitochondrial ATP regeneration (marked as input) are interconnected via the cyclic fluctuations of cytosolic ADP and Cr/PCr.Reprinted from [111], Article VII.

The separation of energy and information transfer illustrated by the general scheme in Figure 35 shows the feedback regulation of respiration *in vivo* corresponding to the Norbert Wiener's cybernetic principles [11, 153, 227]: the usage of ATP (or release of free energy of ATP hydrolysis (ΔG_{ATP}) to perform work, marked as output) and the ATP regeneration (or extraction of ΔG_{ATP} from substrates by oxidative phosphorylation corresponding to input) are interconnected via the feedback signaling through oscillations of cytosolic concentrations in ADP, Pi and Cr/PCr amplified within MI.

Therefore, as the result of the study it was proved that the mystery of cardiac metabolism can be explained only within the framework of Molecular System Biology, using studies of mitochondria *in situ* which make it possible to investigate the regulation by strucuture-function relationship in mitochondria. It was shown that regulation by VDAC, associated with βII–tubulin in MOM in cardiomyocytes induces the linear response of mitochondrial respiration to workload-dependent metabolic signals. This feedback mechanism of the regulation of respiration on beat-to-beat basis ensures the metabolic stability necessary for normal heart functioning and explains well the metabolic aspect of the Frank-Starling's law of the heart – linear dependence of the respiration rate upon workload [94, 112, 235-236]. In physiological conditions when diffusion restrictions for adenine nucleotides have increased the apparent Km value more than tenfold and the signal is amplified inside mitochondria, the oscillations of ADP and Cr concentrations are sufficient to regulate energy fluxes in cardiac cell.

CONCLUSIONS

1. There is direct relationship between the cell structure and the regulation of energy fluxes. In cardiomyocytes mitochondrial arrangement is very regular, they are organized into intracellular energy structures by tubulin system. There is supercomplex Mitochondrial Interactosome (MI) formed by ATP synthasome, mitochondrial creatine kinase (MtCK), voltage dependent anion channel (VDAC) in mitochondrial outer membrane and associated to VDAC regulative protein complexes including tubulin βII. MI is a central structure for energy transfer and respiration regulation. Oxidative phosphorylation is a predominant provider of ATP and the CK system is the main carrier of energy from mitochondria to ATPases.

2. In comparision to cardiomyocytes, in cardiac phenotype cancerous HL-1 cells mitochondria are chaotically organized within the cell interior. Their shape is dynamically changing from granular to filamentous and the energy metabolism is based mostly on the glycolytic reactions coupled to oxidative phosphorylation through hexokinase (HK).

3. Complete kinetic analysis of the regulation of respiration by mitochondrial creatine kinase in the presence of pyruvate kinase and phosphoenolpyruvate system in permeabilized cardiomyocytes was carried out. It revealed striking differences in the kinetic behavior of the MtCK-activated mitochondrial respiration i*n situ* and *in vitro* (in isolated mitochondria). Apparent dissociation constants of MgATP from its binary and ternary complexes with MtCK, K_{ia} and K_a were increased by several orders of magnitude *in situ* in comparison with the same constants *in vitro* (0.44 and 1.94 for K_{ia}MgATP and 0.016 and 2.04 K_aMgATP). At the same time apparent dissociation constants of creatine, K_{ib} and K_b were significantly decreased *in situ* in comparison with isolated mitochondria (28.0 and 2.12 for K_{ib}, 5.0 and 2.17 for K_b).

4. Direct measurements of energy fluxes from mitochondria in isolated permeabilized cardiomyocytes into cytosol by use of HPLC method showed that while ATP concentration did not change in time, mitochondria effectively produced phosphocreatine (PCr). Calculated PCr/O_2 ratio equal to 5.68±0.14 proving that energy is carried out of mitochondria by 90% in the form of flux of PCr under physiological conditions. Adding heterodimeric tubulin to isolated mitochondria was found to increase the apparent Km for an exogenous ADP more than tenfold, indicating directly that the permeability of mitochondrial outer membrane VDAC under physiological conditions is selective.

5. By using the method of MCA, it was shown quantitatively that the key regulatory complexes of the MI are MtCK and ANT and the sum of flux control coefficients is close to four, which according to the theoretical analysis prove direct channeling of substrates in MI.

6. The results obtained in the work allow to conclude that due to the selective permeability of VDAC in association of tubulin βII and other protein complexes, ADP/ATP is circulating inside mitochondria, amplifying the metabolic signal, which comes to mitochondria in the form of cyclic changes in local ADP, Pi and PCr concentrations from the sites of energy consumption through PCr/Cr shuttle. The coupled creatine kinase (MMCK) reaction in myofibrils and coupled MtCK reaction in mitochondria run in non-equilibrium state in opposite directions, resulting in the separation of energy fluxes (mass and energy transfer by PCr) and signaling (information transfer by oscillations of cytosolic ADP concentrations, Pi and PCr/Cr ratio) amplified within Mitochondrial Interactosome. This mechanism enables to induce the linear response of mitochondrial respiration to workload-dependent metabolic signals. Therefore the results of the study explain the metabolic aspect of the Frank-Starling law, the regulation of energy fluxes at different workload.

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ABSTRACT

In this work the mechanism of regulation of mitochondrial respiration and energy fluxes in cardiomyocytes was investigated in the framework of Molecular System Bioenergetics, part of Systems Biology. It has been shown by Starling 80 years ago that cardiac respiration linearly depends on workload, but the mechanism of that dependence remains unknown.

In the first part of the work the complete kinetic analysis of mitochondrial creatine kinase (MtCK) reaction in permeabilized cardiomyocytes was performed and energy fluxes from mitochondria to cytosol were directly measured. On the basis of these results a concept of super-complex of Mitochondrial Interactosome (MI) was developed. According to the theory, MI consists of complexes of respiratory chain with ATP synthase and Pi transporter, adenine nucleotide translocase, MtCK and voltage-dependent anion channel (VDAC) with protein complexes regulating its permeability. In the second part of the study Metabolic Control Analysis was used to determine which complexes of MI are main regulators of oxidative phosphorylation in close to physiological conditions.

We show that there are strong diffusion restrictions for ATP at the level of mitochondrial outer membrane, while there is no restriction for creatine (Cr) and phosphocreatine (PCr). ADP, synthesized by MtCK in mitochonrial intermembrane space, is directly channeled back into mitochondrial matrix through adenine nucleotide translocator (ANT). Direct measurements showed that energy is carried out of mitochondria by 90% in the form of flux of PCr under physiological conditions (with activated MtCK in the structure of MI). The metabolic sensitivity of complexes of MI is significantly increased under physiological conditions in comparison with respiration activated by direct addition of exogenous ADP to mitochondria. The sum of flux control coefficients in MI is close to four, which according to the theoretical analysis prove the existence of ADP/ATP recycling in the MI. The most important regulatory complexes in MI in cardiac cell are MtCK and ANT.

It can be concluded from these results that due to selective permeability of VDAC the ADP/ATP are circulating inside mitochondria, amplifying the metabolic signal, which comes to mitochondria in the form of cyclic changes in local ADP, Pi and PCr concentrations from the sites of energy consumption through PCr/Cr shuttle. These results explain the metabolic mechanisms of Frank-Starling law in the heart – linear dependence of respiration rate upon workload.

KOKKUVÕTE

Antud dissertatsiooni eesmärgiks oli uurida mitokondriaalse oksüdeeriva fosforüleerimise regulatsiooni südamerakus süsteemibioenergeetika vaatepunktist, lähtudes eeldusest, et raku struktuur ja seda moodustavad komponendid (põhiliselt tubuliin) osalevad aktiivselt energiavoo regulatsioonis. Töö esimeses etapis määrati mitokondriaalse kreatiinkinaasi (MtCK) reaktsiooni dissotsiatsioonikonstandid ATP, kreatiini ja fosfokreatiini suhtes ning mõõdeti mitokondrist tsütosooli väljuvate oksüdeeriva fosforüleerimisega seotud metaboliitide vooge. Selle tulemusena pakuti välja Mitokondriaalse Interaktosoomi (MI) mudel, mis sisaldab hingamisahela kompleksid koos ATP süntaasi ja fosfori transporteriga, adeniin nukleotiid translokaasi, MtCK ning mitokondri välismembraani VDAC kanali koos seda reguleerivate valgukompleksidega. Töö teises etapis määrati Metaboolse Kontrolli Analüüsi meetodit kasutades energiavoo kontrollkoefitsendid Mitokondriaalset Interaktosoomi modustavatele kompleksidele.

Töö tulemused võimaldavad järeldada, et mitokondri välismembraanil on tugevad difusioonitakistused ATP suhtes, samal ajal kui Cr ja PCr liikumisele takistusi ei ole. Mitokondri membraanidevahelises ruumis MtCK reaktsioonis ATP-st sünteesitud ADP suunatakse koheselt ANT kanali kaudu tagasi maatriksisse, mitokondrist väljub energiavoog 90 % ulatuses PCr kujul. Mitokondri välismembraani asuva VDAC kanali läbitavus on selektiivne, seega osaleb ta väga olulise komponendina oksüdeeriva fosforüleerimise regulatsioonis. Kanali läbitavuse regulatsioon on määratud sellega seotud valgukomplekside poolt, millest südamerakkudes üks olulisemaid on tubuliini βII isovorm. Mitokondriaalses Interaktosoomis on paljude komplekside puhul metaboolne tundlikkus tunduvalt kõrgem füsioloogilistel tingimustel (aktiveeritud MtCK puhul) võrreldes tingimustega, kus hingamine on aktiveeritud otseselt ADP lisamisega. Voo kontrollkoefitsientide summa läheneb MIs neljale, millest võib järeldada, et tegemist on substraatide otsese ülekandega antud kompleksi piires. Olulisemad regulatiivsed kompleksid oksüdeeriva fosforüleerimise regulatsioonis südamerakkudes on mitokondriaalne kreatiinkinaas ja adeniin nukleotiid translokaas.

Lähtudes antud tulemustest saab väita, et VDAC selektiivsest läbitavusest tulenevalt mitokondris ringlev ADP/ATP võimendab signaali, mis saabub mitokondrisse energia tarbimise punktidest CK/PCr võrgustiku kaudu. Seoses sellega, et difusioonitakistustest tulenevalt näiline Km (ADP) MtCK suhtes kasvab ja saabuva signaali suhtes on tundlikkus väga kõrge, on võimalik oksüdeeriva fosforüleerimise paindlik regulatsioon vastavalt energia ülekandega seotud metaboliitide kontsentratsioonide hetkelistele muutustele lähtudes südameraku kiirelt muutuvast energiavajadusest. Antud töö tulemused seletavad Frank- Starlingi seaduse metaboolset aspekti: lineaarset sõltuvust hapniku tarbimise ja südame töö vahel.

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Comparative analysis of the bioenergetics of adult cardiomyocytes and nonbeating HL-1 cells: respiratory chain activities, glycolytic enzyme profiles, and metabolic fluxes[1]

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Abstract: Comparative analysis of the bioenergetic parameters of adult rat cardiomyocytes (CM) and HL-1 cells with very different structure but similar cardiac phenotype was carried out with the aim of revealing the importance of the cell structure for regulation of its energy fluxes. Confocal microscopic analysis showed very different mitochondrial arrangement in these cells. The cytochrome content per milligram of cell protein was decreased in HL-1 cells by a factor of 7 compared with CM. In parallel, the respiratory chain complex activities were decreased by 4–8 times in the HL-1 cells. On the contrary, the activities of glycolytic enzymes, hexokinase (HK), and pyruvate kinase (PK) were increased in HL-1 cells, and these cells effectively transformed glucose into lactate. At the same time, the creatine kinase (CK) activity was significantly decreased in HL-1 cells. In conclusion, the results of this study comply with the assumption that in contrast to CM in which oxidative phosphorylation is a predominant provider of ATP and the CK system is a main carrier of energy from mitochondria to ATPases, in HL-1 cells the energy metabolism is based mostly on the glycolytic reactions coupled to oxidative phosphorylation through HK.

Key words: cardiomyocytes, HL-1 cells, respiration, cytochromes, glycolysis, energy metabolism.

Résumé : On a effectué chez des rats adultes une analyse comparée des paramètres bioénergétique des cardiomyocytes (CM) et des cellules HL-1, qui ont une structure très différente mais un phénotype cardiaque similaire, pour montrer l'importance de la structure cellulaire pour la régulation de ses flux énergétiques. L'analyse par microscopie confocale a montré une répartition mitochondriale différente dans ces cellules. La teneur en cytochromes par mg de protéine cellulaire a diminué d'un facteur 7 dans les cellules HL-1 par comparaison à celles des CM. Parallèlement, les activités complexes de la chaîne respiratoire ont diminué de 4 à 8 fois dans les cellules HL-1. En revanche, les activités des enzymes glycolytiques, hexokinase (HK) et pyruvate kinase (PK), ont augmenté dans les cellules HL-1, lesquelles ont transformé efficacement le glucose en lactate. L'activité de la créatine kinase (CK) des cellules HL-1 a aussi diminué significativement. En conclusion, les résultats de cette étude viennent étayer à l'hypothèse que, contrairement aux CM où la phosphorylation oxydative est un fournisseur important d'ATP et le système CK, le principal transporteur d'énergie des mitochondries vers les ATPases, dans les cellules HL-1, le métabolisme énergétique repose principalement sur les réactions glycolytiques couplées à la phosphorylation oxydative par l'HK.

Mots-clés : cardiomyocytes, cellules HL-1, respiration, cytochromes, glycolyse, métabolisme énergétique.

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Introduction

Studies of energy metabolism in muscle cells have led to a conclusion that the metabolic compartmentalization and mechanisms of regulation of oxidative phosphorylation are system-level properties giving rise to the unitary organization of metabolic processes in the heart and in oxidative skeletal muscle cells (Seppet et al. 2001; Weiss et al. 2006; Saks et al. 2006; Saks 2007; Saks et al. 2008). The basis of this type of organization is the specific structure–function relationship characteristic of these cells. For example, in heart cells mitochondria are arranged regularly in a longitudinal lattice between the myofibrils and are located within the limits of the sarcomeres (Vendelin et al. 2005). Such a high level of structural organization results in the formation of complexes between ATPases and mitochondria that are termed intracellular energy units (ICEUs) (Saks et al. 2001; Seppet et al. 2001). Energy transfer and feedback metabolic regulation between mitochondria and ATPases occur via specialized pathways in the ICEUs that are mediated by creatine kinase (CK) and adenylate kinase (AK), and to some extent by direct adenine nucleotide channeling (Wallimann et al. 1992; Dzeja et al. 2007; Saks 2007). This organization is achieved by interaction of mitochondria with cytoskeletal structures (Seppet et al. 2001; Saks et al. 2001; Capetanaki 2002). Direct structural contacts between mitochondria and sarcoplasmic (and endoplasmic) reticulum (SR), mediated by cytoskeletal proteins and enabling the delivery of calcium from SR into mitochondrial intermembrane space and establishing local microdomains of high calcium concentration, have also been documented in many studies and are also consistent with the point of view of unitary organization of the cell metabolism (Sharma et al. 2000; Anmann et al. 2005; Csordás et al. 2006; Wilding et al. 2006). A useful reference system in these studies of the role of structure–function relationships in establishing the control over mitochondrial function has been the immortalized HL-1 mouse cardiomyocyte cell line (Seppet et al. 2006; Anmann et al. 2006). This cell line, first developed by Claycomb and colleagues, can continuously divide and spontaneously contract (beating, B HL-1 cells) while maintaining a differentiated cardiac phenotype through indefinite passages in culture (Claycomb et al. 1998; White et al. 2004). Recently, a novel subtype of HL-1 cells, the nonbeating (NB) cells, was developed by culturing B HL-1 cells in the medium with specific serum that led the cells to lose their beating properties without stopping their proliferation in normal culture medium (Pelloux et al. 2006). The NB HL-1 cells do not present the pacemaker current, spontaneous depolarization, or calcium oscillations of the original B HL-1 cells (Pelloux et al. 2006). Confocal imaging of mitochondria revealed very different structural organization of the HL-1 cells compared with adult cardiomyocytes (CM). Whereas the B HL-1 cells possess some residual sarcomeres, the NB HL-1 cells are devoid of these structures (Pelloux et al. 2006). Our previous studies have shown that these structural peculiarities are associated with striking differences in the kinetics of respiration regulation by exogenous ADP in the HL-1 cells compared with that of normal cardiomyocytes. We found that both the B and NB HL-1 cells exhibited much lower K_m for ADP in stimulating respiration than the cardiomyocytes did (Anmann et al. 2006). It was also observed that the HL-1 cells differ from normal cardiomyocytes by their gene expression profile of the AK and CK isoforms and by the absence of functional coupling of mitochondrial and cytosolic CK isoforms to oxidative phosphorylation and Ca^{2+}-Mg^{2+}-ATPases, respectively (Eimre et al. 2008). From these studies, it was concluded that contrary to CM in which mitochondria and Ca^{2+}-Mg^{2+}-ATPases are organized into the ICEUs, these complexes do not exist in HL-1 cells as a result of a less organized energy metabolism (Eimre et al. 2008).

However, the quantitative comparative analysis of different pathways of ATP production in the HL-1 cells and adult CM is still lacking. Therefore, in this study we investigated the differences in the activities of key glycolytic enzymes and respiratory chain complexes in adult rat CM, HL-1 cells, and m. gastrocnemius from rat (as a reference system for glycolytic muscles). The results show that in contrast to the adult CM, the HL-1 cells exhibit very low capacity of oxidative phosphorylation but high capacity of glycolytic system, which is the predominate source of ATP for energy-consuming reactions. The CK system that mediates energy transfer in CM cannot effectively operate in HL-1 cells owing to much lower activity compared with CM.

Materials and methods

Animals

Adult Wistar rats weighing 300–350 g were used in the experiments. The animals were maintained and studied in accordance with the US National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Preparation of isolated CM, HL-1 cell culture, and homogenates of m. gastrocnemius

The CM were isolated from adult rat ventricular myocardium as described previously (Anmann et al. 2006). The cells were washed and kept in Mitomed solution (Anmann et al. 2006) of the following composition (in mmol/L): EGTA 0.5, sucrose 110, potassium lactobionate 60, $MgCl_2$ 3, dithiothreitol 0.5, taurine 20, KH_2PO_4 3, and K-HEPES 20, pH 7.1, at 4 °C before the experiments.

The NB HL-1 cells were cultured as described previously (Pelloux et al. 2006). The cells were detached by trypsinization and the cell suspension was washed 3 times and centrifuged for 5 min at 1000*g* with Mitomed medium at 4 °C and stocked in this medium at 4 °C. The cells were cultured and kept in an atmosphere of 95% oxygen and 5% of air to avoid hypoxia.

The homogenates of glycolytic skeletal muscle, m. gastrocnemius as a reference tissue, were prepared in phosphate buffer (100 mmol/L KH_2PO_4, pH 8) from the muscle of adult rat by using a Polytron homogenizer. The suspensions were centrifuged at 6000*g* for 10 min and the supernatants were kept in this solution at 4 °C until the enzyme assays were started.

Laser confocal microscopy

For confocal microscopy, the HL-1 cells were detached by trypsin incubation and washed 5 times with Mitomed solution. These cells or CM were then incubated with a mitochondria-specific dye, MitoTracker Green FM (excitation

488 nm, emission 516 nm), which becomes fluorescent while accumulating in the lipid environment of mitochondria and is insensitive to membrane potential, and with a nucleus dye, Hoechst 342 (excitation 461 nm, emission 350 nm), which is a cell-permeable nucleic acid stain (DNA bound). The digital images of MitoTracker and Hoechst 342 fluorescence were acquired with a Leica DM IRE2 inverted confocal microscope with a 63× water immersion lens. The MitoTracker Green fluorescence was excited with the 488 nm line of argon laser, using 510–550 nm for emission.

Measurement of cytochrome content in different muscle cells

The HL-1 cardiac cells were thawed at 4 °C and rapidly disrupted in ice-cold PBS by vigorous vortex and Ultra-Turrax T25 homogenizator (Janke and Kunkel, Germany) at medium speed for 30 s. Insoluble material was removed by centrifugation at 2500*g* for 10 min. The supernatant was centrifuged at 15 000*g* for 30 min to remove myoglobin. The supernatant was discarded and the sediment was rehomogenized in 1.5 mL PBS. To obtain the cytochrome difference spectrum (reduced minus oxidized spectra), the mitochondria were solubilized by addition of 5% sodium deoxycholate (Sigma) into the cuvette to a final concentration of 0.3% (*v*/*v*), thereafter oxidized with potassium ferricyanide (reference spectrum), and then reduced with sodium dithionite in the same cuvette, as described previously (Fuller et al. 1985). Absorbance spectra of cytochromes were recorded by scanning the samples at 535–630 nm with a dual-beam spectrophotometer Lambda 900 (Perkin-Elmer) or with a Cary 50 Bio UV-Vis spectrophotometer (Varian, Palo Alto, USA). The cytochrome aa_3 content was calculated from the difference spectrum (reduced minus oxidized spectra) at the maximum absorption value in the range of 603–605 nm normalized for the absorbance of the isosbestic point at 630 nm; the content of cytochrome *c* was determined at the maximum absorption value of 550 nm normalized for the absorbance of the isosbestic point at 540 nm; the content of cytochrome *b* was calculated at the maximum absorption value of 562 nm normalized for the absorbance of the isosbestic point at 577 nm using the relevant extinction coefficient ε values according to Maguire et al. (1982). The same technique was applied to measure the cytochrome aa_3 content in CM and m. gastrocnemius. The data obtained were normalized for whole-cell or tissue protein content measured in the primary homogenates according to Bradford (1976) with bovine serum albumin (BSA) as a standard.

Determination of activity of the respiratory chain complexes

The activity of mitochondrial respiratory segments was measured in CM and B and NB HL-1 cells by using a high-resolution Oroboros oxygraph (Innsbruck, Austria). The cells were permeabilized in an oxygraph chamber containing the Mitomed solution with 25 µg/mL saponin and 2 mg/mL essential fatty acid-free BSA at 25 °C. Mitochondrial respiration was first activated by 5 mmol/L glutamate and 2 mmol/L malate via complex I in the presence of ADP in saturating concentration (2 mmol/L). Thereafter complex I was inhibited by rotenone (5 µmol/L) and complex II was activated by succinate (10 mmol/L), followed by inhibition of complex III by antimycin A (10 µmol/L). Finally the artificial substrates of complex IV, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) and ascorbate, were added (at 1 mmol/L and 5 mmol/L, respectively) to determine the activity of that complex.

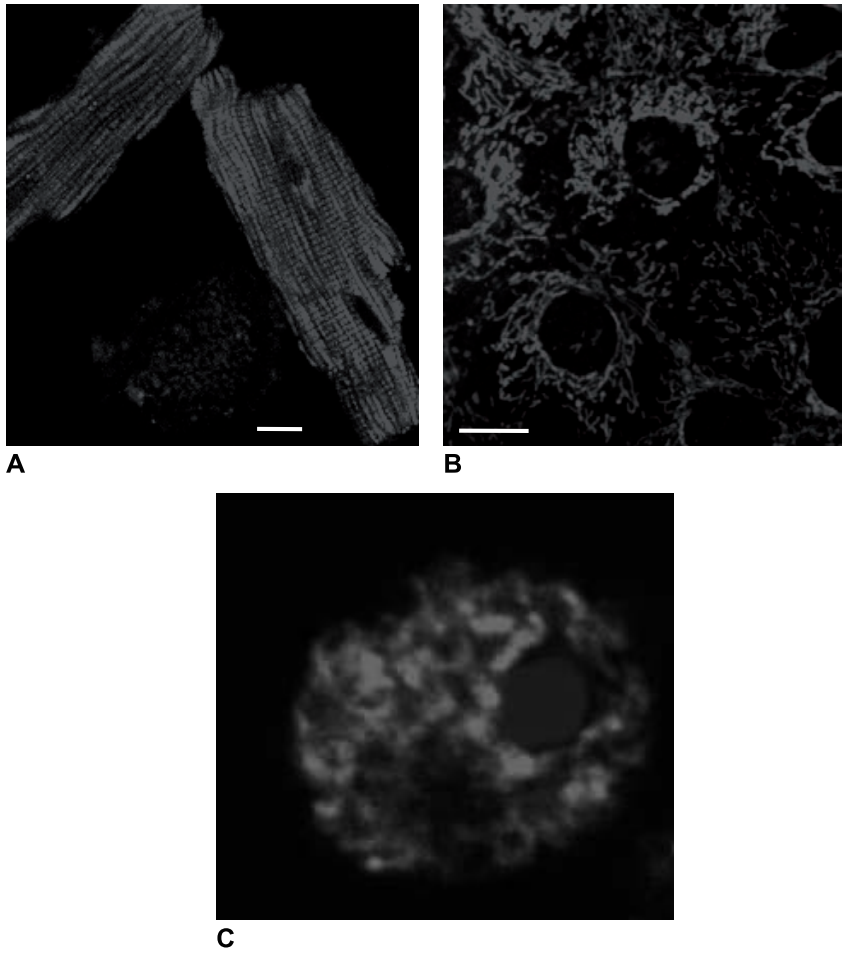
Assessment of enzyme activity

The enzyme activity in adult rat CM, NB HL-1 cells, and m. gastrocnemius homogenates was measured by the Cary 50 Bio UV-Vis spectrophotometer at 340 nm at 30 °C in the following basic buffer (BB) (in mmol/L): HEPES 50, magnesium acetate 5, postassium acetate 100, and EGTA 0.5; the pH was varied according to the optimum for the enzyme or coupled enzymes assessed, and 1% Triton X-100 was added to destroy the cellular membranes. The hexokinase (HK) activity was measured at pH 7.1 in BB supplemented with the following: 2 IU/mL glucose-6-phosphate dehydrogenase (G6PDH) and (in mmol/L) glucose 20, ATP 1.1, NADP 0.6, and P^1,P^5-di(adenosine-5′) pentaphosphate (Ap_5A) 0.01. Fructose-6-phosphokinase (FPK) activity was measured at pH 8.5 in BB supplemented with the following: 9.6 IU/mL lactate dehydrogenase (LDH), 5 IU/mL pyruvate kinase (PK), and (in mmol/L) phosphoenolpyruvate 0.4, fructose-1,6-diphosphate 0.64, fructose-6-phosphate 1.8, ATP 1.1, and NADH 0.2. The glyceraldehyde-3-phosphate dehydrogenase activity was assessed at pH 7.6 in BB additionally containing 15 IU/mL 3-phosphoglycerate kinase and (in mmol/L) 3-phosphoglycerate 6, ATP 1.1, and NADH 0.2. The PK activity was measured at pH 7.6 in BB containing 9.2 IU/mL LDH and (in mmol/L) phosphoenolpyruvate 6, ADP 1.2, and NADH 0.2. The LDH activity was assessed by registering the rate of NADH oxidation at pH 7.4 in BB containing (in mmol/L) pyruvate 10 and NADH 0.2. The CK activity was measured in the direction of ATP formation at pH 7.1 in BB supplemented with (in mmol/L) glucose 20, ADP 1.2, NADP 0.6, Ap_5A 0.01, and phosphocreatine 20, and with 2 IU/mL G6PDH and 40 IU/mL HK as the coupled enzymes.

Measurement of ATP and lactate/glucose ratio

For ATP measurement in cell lysates, the NB HL-1 cells were first incubated for 10 min in Tyrode's solution containing (in mmol/L) NaCl 150, KCl 5.4, NaH_2PO_4 0.9, $CaCl_2$ 1.8, $MgCl_2$ 1.2, HEPES 10, pH 7.2, adjusted with NaOH. This solution also contained 10 mmol/L glucose for control condition, or no glucose and 2 mmol/L 2-deoxyglucose to inhibit glycolysis. Then the cell plates were plunged into boiling water for 1.5 min, detergent solution was added (25 mmol/L Tris, 2 mmol/L EDTA, 0.5 mmol/L dithiothreitol (DTT), 0.1% Tween, pH 7.75 with acetic acid), and the plates were set to freeze at –80 °C. Thereafter the cells were thawed, sonicated, and scraped off on ice. The cell extracts were pipetted in precooled tubes, spun at 10 000*g* for 15 min at +4 °C. The ATP concentration was measured in supernatants using Prolux luciferase–luciferin kit (Euralam, France) in an Optocomp luminometer (MGM Instruments, Hamden, USA). For measurement of the lactate/glucose ratio, supernatants from 25 cm^2 culture flasks of NB HL-1 cells (10^7

Fig. 1. Confocal fluorescent imaging of mitochondria in normal CM in respiration medium (A) and in NB HL-1 cells in the culture medium (B). The mitochondria were visualized by cell incubation (20–30 min) with mitochondrial membrane potential-sensitive probe TMRM (100 nmol/L). Each fluorescent spot represents a mitochondrion. Scale bars, 10 μm. (C) NB HL-1 cells after detachment from the cell plates. Lighter grey shading is mitochondrial labelling with MitoTracker green (in the Web version, mitochondrial labelling appears as green fluorescence), and the darker grey shading is nuclear labelling with Hoechst 342 (in the Web version, this appears as blue fluorescence). CM, adult rat cardiomyocytes; NB HL-1, nonbeating mouse cardiomyocyte cell line; TMRM, tetramethylrhodamine methyl ester.



cells) kept for 24, 48, and 72 h of culturing in normal culture medium (supplemented Claycomb medium) were taken and incubated in the same medium containing 21 mmol/L glucose and 2 mmol/L lactate. The glucose and lactate concentrations were determined by using glucose-sensitive and lactate-sensitive CCX analyzer electrodes (Nova Biomedical, Waltham, USA). Glucose consumption and lactate production were calculated from the differences of their concentrations in samples and control medium.

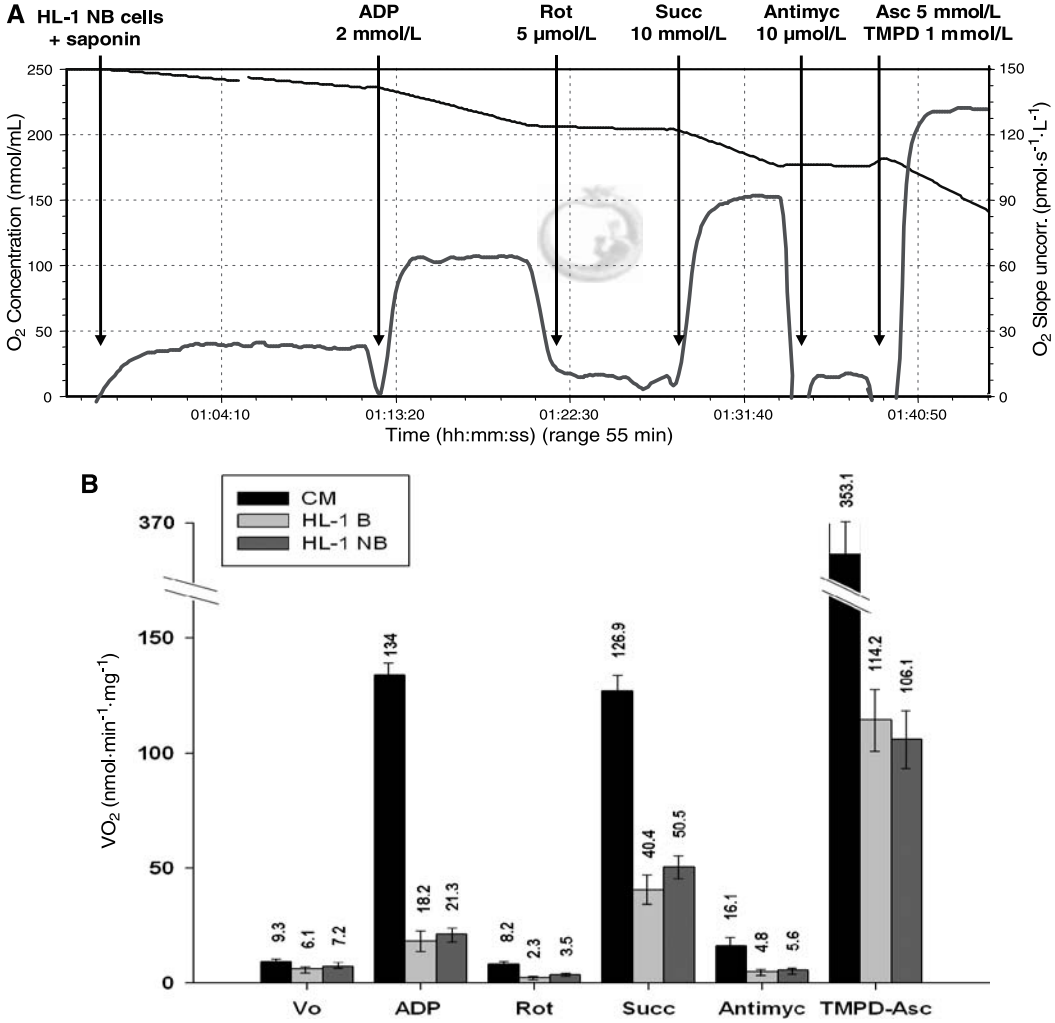
Statistical analysis

Means ± SE are presented. Statistical analysis of data was performed by one-way ANOVA with Bonferroni or Dunnett's post test.

Results and discussion

Figure 1 demonstrates that in contrast to CM, in which the mitochondria are regularly positioned between the myofibrils in a crystal-like manner, each at the level of the adjacent sarcomere (Fig. 1A), the mitochondria in NB HL-1 cells are chaotically organized within the cell interior, presenting the dynamically changing (from granular to filamentous) mitochondrial pattern (Fig. 1B). Figure 1C shows that after detachment of these cells by trypsinization they acquired a rounded shape. Previously we have shown that after saponin treatment to permeabilize the HL-1 cells, the mitochondria still remain within the cell interior despite marked deterioration of the cell membrane. From these experiments it was

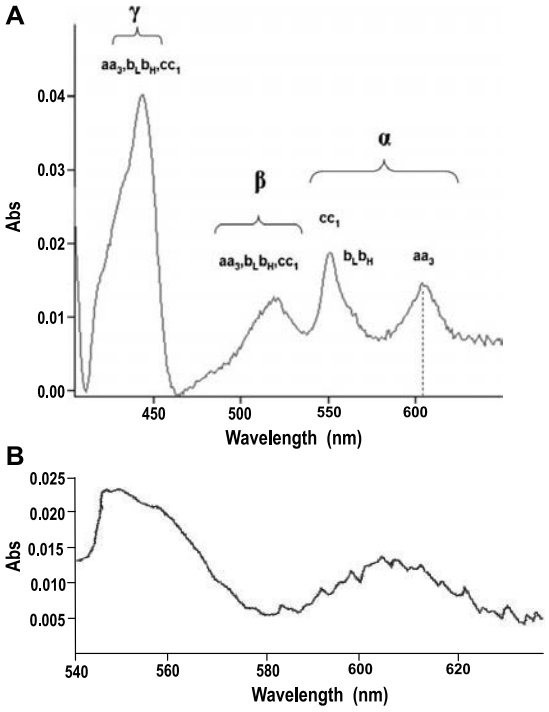
Fig. 2. (A) Original recording of the respiratory chain function in NB HL-1 cells permeabilized in Mitomed solution at 25 °C in the presence of 25 µg/mL saponin (plus 5 mmol/L glutamate and 2 mmol/L malate). Then 2 mmol/L ADP, 5 µmol/L rotenone (Rot), 10 mmol/L succinate (Succ), 10 µmol/L antimycin A (Antimyc), and 1 mmol/L TMPD with 5 mmol/L ascorbate (Asc+TMPD) were added to the oxygraph chamber. (B) The activity of the respiratory chain complexes in CM, B HL-1, and NB HL-1 cells, n = 5–7. B HL-1, beating HL-1 cell line.



also found that 15 min treatment of cells with saponin was optimal for selective permeabilization of the sarcolemma (Eimre et al. 2008).

Figure 2A shows the original recording of respirometric assessment of the activity of different complexes of the respiratory chain in NB HL-1 cells. Similar determinations for muscle and cardiac cells have been reported elsewhere (Eimre et al. 2008; Kuznetsov et al. 2008). Figure 2B demonstrates the mean levels of the respiratory data for CM and B and NB HL-1 cells. First, it can be seen that addition of 2 mmol/L ADP strongly activated respiration, thus showing effective control of oxidative phosphorylation by ADP in permeabilized HL-1 cells. Second, the respiration rate with complex I-dependent substrates was lower than with succinate, a complex II-dependent substrate, in NB HL-1 cells, whereas in CM the complex I-dependent respiration was close to that of the complex II-dependent one. These results, being in agreement with previous data (Eimre et al. 2008), suggest a relative deficiency in complex I in HL-1 cells, because in normal adult permeabilized cardiac cells the respiration rate with either glutamate or malate usually exceeds that with succinate (Seppet et al. 2005). Remarkably, the respiratory activities of the B and NB HL-1 cells were close to each other but about 4–8 times lower than in CM (Fig. 2B). Figure 3 demonstrates the cytochrome difference spectrum (reduced minus oxidized spectra) of isolated CM

Fig. 3. Original recordings of cytochrome spectra in the CM (A) and NB HL-1 cells (B).



(Fig. 3A) and HL-1 cells (Fig. 3B). Figure 3A shows the whole spectrum of cytochromes, while Fig. 3B shows only the α-peak area. From these spectra, the cellular content of cytochromes was calculated (Table 1). It can be seen that the content of cytochromes aa_3 is about 7 times less in HL-1 cells than in CM. Similarly, the cytochrome *c* content was much less in NB HL-1 cells than in adult CM (Table 1). Taken together these data allow us to conclude that the lower content of mitochondrial respiratory chains in HL-1 cells is responsible for their diminished respiratory capacity compared with CM (Fig. 2).

Determination of enzyme profiles (Table 2) revealed 5-fold elevated levels of the HK activity in the NB HL-1 cells. Also, the activity of PK was higher than in CM (Table 2). In general, the activities of glycolytic enzymes in HL-1 cells remained far less than in m. gastrocnemius cells. The activity of FPK was close to the activity of HK in the HL-1 cells, but lower than its activity in CM. However, FPK activity is known to follow sigmoid kinetics and is regulated allosterically by many intracellular factors (Drozdov-Tikhomirov et al. 1999), which is apparently favourable to keep the enzyme in HL-1 cells sufficiently active to maintain high glycolytic flux with glucose/lactate stoichiometry about 2, as shown in Fig. 4. Considering the effective transformation of glucose into lactate, the increased activity of several glycolytic enzymes, and the very low activity of oxidative phosphorylation, it appears that glycolysis may serve as the main energy-providing system in HL-1 cells. At the same time, the capacity of the glycolytic system to produce ATP is probably much lower in HL-1 cells than in glycolytic skeletal muscle (m. gastrocnemius), as indicated by comparison of the activity of glycolytic enzymes (Table 2). This difference may be explained by low energy requirements of HL-1 cells, since they do not contract because of the absence of sarcomeric structures (Pelloux et al. 2006).

Interestingly, our previous data have shown that unlike the case in adult CM, the HK in HL-1 cells effectively stimulates mitochondrial respiration, for it is bound to mitochondrial outer membrane that enables its coupling to oxidative phosphorylation (Eimre et al. 2008). The current study shows that this type of interaction between mitochondria and HK is ensured by very high activity of that enzyme. It is known that several cancer cell types overexpress HK (Mathupala et al. 2006; Gwak et al. 2005) and that in these cells HK is also coupled to oxidative phosphorylation so that mitochondrially produced ATP is preferably used for glucose phosphorylation by HK, whereas ADP liberated in that process is returned to mitochondria to stimulate ATP synthesis (Shinohara et al. 1997). It has been suggested that this mechanism allows the cancer cells to overcome potential inhibition of glycolysis due to lactate accumulation, particularly in conditions of hypoxia. Moreover, interaction of HK with mitochondria protects cells from apoptosis and excess reactive oxygen species production (Pastorino et al. 2002; da Silva et al. 2004) and provides cells with glycolytic intermediates as important components for different biosynthetic pathways, thus favouring proliferation of the cells and cancer growth (Mathupala et al. 2006, also reviewed by Seppet et al. 2007). In light of these data, our current results suggest that HL-1 are similar to cancer cells not only in their capacity for unlimited proliferation but also in their underlying mechanism: coupling glycolysis to oxidative phosphorylation, which helps to facilitate the glycolytic flux at the expense of mitochondrially produced ATP. These results are all consistent with and help to explain Warburg's original observation made in the 1920s that cancer cells are capable of increased lactate production under aerobic conditions (see Mazurek 2007 for a review).

Our earlier studies have revealed that permeabilized HL-1 cells exhibit much lower apparent K_m for ADP in regulation of oxidative phosphorylation (25 ± 4 μmol/L for NB and 47 ± 15 μmol/L for B) than that observed in adult permeabilized CM (360 ± 51 μmol/L) (Anmann et al. 2006). High K_m for ADP in CM stems from local restrictions of the ADP diffusion in the cells, including limited diffusion across the mitochondrial outer membrane (Saks et al. 2001; Seppet et al. 2001). This conclusion is substantiated by the observation that treatment of CM with trypsin leads to a 3-fold decrease in K_m value, along with dramatic changes in intracellular structure caused by this protease (Anmann et al. 2005, 2006). It is noticeable by their high apparent affinity to exogenous ADP that the HL-1 cells are similar not only to trypsin-treated adult CM but also to neonatal rat CM, which after permeabilization with saponin also exhibit very low K_m for ADP in stimulation of respiration (Tiivel et al. 2000). In addition, the neonatal CM are characterized by underdeveloped cellular structure, low capacity

Table 1. Cytochrome content in CM and in NB HL-1 cells.

Preparation	Cytochrome aa_3	Cytochrome b	Cytochrome cc_1
NB HL-1 cells	0.075±0.026*	0.096±0.036	0.051±0.01**
CM	0.5±0.16	0.45±0.17	0.49±0.1

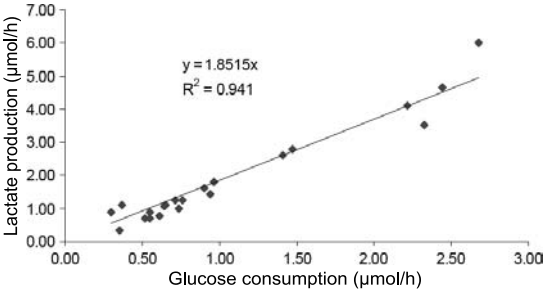
Note: Cytochrome content is given in nanomoles per milligram of cell protein (nmol/mg), n = 3–7. *, Significant at $p < 0.05$ and **, $p < 0.02$ compared with CM group.

Table 2. Enzyme activity of CM, NB HL-1 cells, and m. gastrocnemius homogenate (GH).

	CM	NB HL-1 cells	GH
Hexokinase	33±3*	148±12††	31±4
Fructose 6-phosphate kinase	698±23*	124±5††	1339±124
Creatine kinase	6327±724*	395±43††	83556±3866
Lactate dehydrogenase	4414±265	4069±449††	13985±800
Pyruvate kinase	637±69*	1375±187†	11571±722
Glyceraldehyde 3-phosphate dehydrogenase	2331±391	2693±490††	7511±701

Note: Activity is given in nanomoles per minute (nmol/min) per milligram of protein, n = 5–7. *, Significant at $p < 0.001$ vs. NB HL-1 cells. †, $p < 0.01$ and ††, $p < 0.001$ vs. GH.

Fig. 4. Lactate/glucose ratio represented by the slope of the correlation between the rates of lactate production and glucose consumption in NB HL-1 cells.



of oxidative phosphorylation, high rates of glycolysis, and absence of CK-mediated system of energy transfer compared with adult CM (Tiivel et al. 2000). Our recent data suggest that in the HL-1 cells, the CK-mediated energy transfer system is also not functional. Indeed, although creatine significantly activated the respiration and decreased the apparent K_m for ADP in normal adult CM, in HL-1 cells it exerted little, if any, influence because of the downregulation of mitochondrial CK and the absence of its coupling to oxidative phosphorylation (Anmann et al. 2006; Eimre et al. 2008). Likewise, the cytosolic CK isoforms were found not to be coupled to ATPases in HL-1 cells (Eimre et al. 2008). The current study explains these observations by extremely low activity of CK in these cells compared with CM (16-fold difference, as shown in Table 2), corresponding well to the marked downregulation of genes encoding mitochondrial and M and B isoforms of the CK at mRNA and protein levels in HL-1 cells (Eimre et al. 2008). On the basis of these data, it is evident that in contrast to CM in which the CK system represents the main mechanism of energy transfer, CK cannot play such a role in HL-1 cells.

Striking structural dissimilarities between the CM and HL-1 cells (Fig. 1) lead to the assumption that the mechanisms of interaction between the systems of producing and consuming ATP may be entirely different in these cells. Indeed, in contrast to normal CM, in which the intermyofibrillar mitochondria are strictly positioned at the level of sarcomeres, thus allowing formation of ICEUs, in HL-1 cells these complexes cannot be established because of the lack of sarcomeres (particularly in the NB subtype) and because of the continuous fusion and fission of mitochondria in HL-1 cells (Pelloux et al. 2006; Anmann et al. 2006). As a result of the less organized structure (loosely packed and even slowly moving mitochondria (Pelloux et al. 2006)), which leads to diminished restrictions for adenine nucleotides compared with those in CM, in HL-1 cells the mitochondria and ATPases may communicate via simple diffusion of adenine nucleotides, which is suggested by the low apparent K_m for ADP in regulation of respiration (Anmann et al. 2006). Another possibility is that the intracellular transfer of energy-rich phosphoryls is mediated by AK and HK, since the isoforms of these enzymes are strongly upregulated (this study and Eimre et al. 2008). The AK-mediated system of energy transfer is based on functional coupling of the mitochondrial isoform AK2 to adenine nucleotide translocase and the cytosolic isoform AK1 to ATPases (Dzeja and Terzic 2003). Regarding the role of HK, it is possible that because of its coupling to mitochondrial ATP synthesis, a situation is established in which glycolysis serves not only as a source of ATP, but also as a system of linear transfer of ATP from mitochondria to ATPases, with PK functioning as a terminal effector in local provision of ATP for ATPases (Dzeja and Terzic 2003). These modes of energy transfer may be sufficient to support the relatively low metabolic needs of HL-1 cells compared with those of CM.

In conclusion, the results of this study comply with the assumption that in contrast to CM in which oxidative phosphorylation is a predominant provider of ATP and the CK system is a main carrier of energy from mitochondria to ATPases, in HL-1 cells the energy metabolism is based primarily on the glycolytic reactions coupled to oxidative phosphorylation through HK.

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PUBLICATION II

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Regulation of respiration controlled by mitochondrial creatine kinase in permeabilized cardiac cells in situ
Importance of system level properties

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ABSTRACT

The main focus of this investigation is steady state kinetics of regulation of mitochondrial respiration in permeabilized cardiomyocytes in situ. Complete kinetic analysis of the regulation of respiration by mitochondrial creatine kinase was performed in the presence of pyruvate kinase and phosphoenolpyruvate to simulate interaction of mitochondria with glycolytic enzymes. Such a system analysis revealed striking differences in kinetic behaviour of the MtCK-activated mitochondrial respiration in situ and in vitro. Apparent dissociation constants of MgATP from its binary and ternary complexes with MtCK, K_{ia} and K_a (1.94 ± 0.86 mM and 2.04 ± 0.14 mM, correspondingly) were increased by several orders of magnitude in situ in comparison with same constants in vitro (0.44 ± 0.08 mM and 0.016 ± 0.01 mM, respectively). Apparent dissociation constants of creatine, K_{ib} and K_b (2.12 ± 0.21 mM 2.17 ± 0.40 Mm, correspondingly) were significantly decreased in situ in comparison with in vitro mitochondria (28 ± 7 mM and 5 ± 1.2 mM, respectively). Dissociation constant for phosphocreatine was not changed. These data may indicate selective restriction of metabolites' diffusion at the level of mitochondrial outer membrane. It is concluded that mechanisms of the regulation of respiration and energy fluxes in vivo are system level properties which depend on intracellular interactions of mitochondria with cytoskeleton, intracellular MgATPases and cytoplasmic glycolytic system.

1. Introduction

In the beginning, the stimulating effect of creatine on respiration in muscle homogenates was recognised when studies in bioenergetics begun in 1930 with Engelhardt discovering ATP synthesis related to oxygen consumption [1–3], followed in 1939 by Belitzer and Tsybakova's discovery of the effects of creatine on oxygen consumption [4]. Belitzer and Tsybakova showed that creatine added to well washed homogenate of pigeon pectorals muscle strongly increased oxygen uptake and production of phosphagen (as phosphocreatine, PCr, was called at that time) without any added adenine nucleotides, which were present only in trace amounts. The efficiency the coefficient of aerobic synthesis of phosphagen, the PCr/O_2 ratio was between 5.2 and 7 [4]. Now we know that the high efficiency of the control of oxidative phosphorylation by creatine is due to the functional coupling of mitochondrial creatine kinase (MtCK) with adenine nucleotide translocase (ANT) [5–16]. However, the role of this coupling in the regulation of cardiac respiration and energetics is still not widely recognised [17–23].

Mitochondrial respiration and fine regulation of energy fluxes are of vital importance for normal cell life, especially for cells with high energy demand, such as cells of the heart, brain and skeletal muscle. In spite of the fundamental progress of knowledge of mitochondrial bioenergetics [24], the nature of respiratory control and in more general sense, the mechanisms of regulation of energy fluxes in the cardiac and other cells in vivo are still highly debated [11,12,18–20,25–30]. For further progress in this area and for elucidation of these complex mechanisms, application of newly developed Systems Biology approaches for analysis of complex integrated systems may be very helpful [31–34]. Systems Biology as a new paradigm provides novel concepts and approaches for the analysis of complex biological systems [35,36], including integrated energy metabolism of muscle and brain cells [12]. For these studies, the most useful and constructive concept of Systems Biology is that of system-level properties which are direct consequences of interactions between cellular components and not known for isolated components [28,32,33]. In the intact heart, the respiration rate is linearly dependent on the workload which itself is governed by the Frank–Starling law [11,37,38]. Remarkably, this

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occurs under conditions of metabolic stability (homeostasis), implying stable intracellular levels of metabolites such as phosphocreatine and ATP during workload and respiration rate changes [39,40]. Furthermore, under physiological conditions when workload is changed by alteration of left ventricular filling, the calcium transients in cytoplasm stay unchanged [41]. These fundamental observations evidently contradict the popular creatine kinase equilibrium theory in establishing cytoplasmic ADP concentration for respiration regulation (the latter does not change under conditions of metabolic stability), and exclude the explanation of respiration regulation by calcium ions only [25,42]. Calcium may help to activate respiration only under conditions of adrenergic stimulation which increases its entry into cells [43,44]. Further, in explaining respiration regulation the cell is often considered as a homogeneous reaction medium [18,21–23,45–48], thus ignoring the impact of the high degree of structural organization of the cell, in particular cardiomyocytes, macromolecular crowding phenomena, etc. (for a critical review see ref. [14]). However, multiple experimental studies of the kinetics of respiration regulation performed on permeabilized cells indicate the heterogeneity of ADP diffusion [49,50–69]. Local restrictions of intracellular diffusion of ADP and ATP are the basis for modular organization of cardiac energy metabolism [70–74]. Of major importance in bypassing these restrictions is the creatine kinase–phosphocreatine circuit (or shuttle) which includes both MtCK functionally coupled to the oxidative phosphorylation via ANT [74] and MM isoform of creatine kinase coupled to MgATPase reactions in myofibrils and in cellular membranes [5,6,11,12,28]. Under in vivo conditions oxidative phosphorylation is also influenced by the presence of other ADP utilizing systems within the cells such as glycolysis. System analysis of the regulation of mitochondrial respiration and oxidative phosphorylation taking into account all these complex interactions in organized intracellular medium in the cells is still absent. Our aim was to perform such a system analysis of the regulation of respiration experimentally in permeabilized cardiomyocytes in situ for further realistic developments of modelling of energy metabolism in the future. To achieve this aim, we investigated the kinetics of the regulation of MtCK-activated respiration both in isolated mitochondria and in permeabilized cardiomyocytes in the absence and presence of the glycolytic system (represented by pyruvate kinase and phosphoenolpyruvate) of trapping free ADP produced by MgATPases. The use of this protocol allowed us to study the kinetics of MtCK reaction in situ in permeabilized cardiomyocytes by excluding the influence of other ATP-consuming reactions. This molecular system analysis of a complex situation close to that in cardiac cells in vivo revealed novel aspects of the respiration regulation in cardiac cells not present in isolated mitochondria, showing that mechanisms of the regulation of respiration and energy fluxes in the cells are system-level properties dependent on the multiple interactions of mitochondria with cellular structures.

2. Materials and methods

2.1. Experimental protocols

The principles of this study are illustrated by four schemes of increasing complexity: Schemes 1 and 2 represent isolated mitochondrion as a reference system, and Schemes 3 and 4 illustrate permeabilized cardiomyocyte chosen as experimental study model. Experiments were performed first without or with activated MtCK reaction and then in the presence of ADP trapping system. This system, consisting of phosphoenolpyruvate (PEP) and pyruvate kinase (PK), traps extramitochondrial ADP produced by cytoplasmic isoforms of creatine kinases (MMCK) and MgATPase reactions and subsequently regenerates extramitochondrial ATP. Endogenous intramitochondrial ADP produced by MtCK forms a micro-domain within the intermembrane space (IMS) and is re-imported into the matrix via ANT due to its functional coupling with MtCK. A series of experiments were performed to check the properties of this model in order to make it useful for complete MtCK kinetic analysis.

2.2. Isolation of mitochondria from cardiac muscle

Heart mitochondria were isolated from adult white Wistar rats 300 g body weight, as described by Saks et al. [75]. The final pellet containing mitochondria was re-suspended in 1 ml of isolation medium containing 300 mM sucrose, 10 mM HEPES, pH 7.2, and 0.2 mM EDTA and kept in ice for no longer than 3 h.

2.3. Isolation of adult cardiac myocytes

Adult cardiomyocytes were isolated after perfusion of the rat heart with collagenase using the adaptation of the technique described previously [52]. Isolated cells were re-suspended in 1–2 ml of Mitomed solution [76] described below for respiration measurements and stored on ice during measurements. Isolated cardiomyocytes contained 70–90% of rod-like cells when observed under a light microscope.

2.4. Permeabilization procedure

In order to study the kinetics of the regulation of mitochondrial respiration in cardiomyocytes using different metabolites, the cell sarcolemma was permeabilized by saponin keeping the mitochondrial membranes intact [76,77]. The tests for intactness of the outer and inner mitochondrial membranes are described in the Results section. The permeabilization procedure was carried out directly in an oxygraph chamber with 25 μg/ml saponin during 10 min before starting the measurements of respiration rates at 25 °C and continuous stirring. To study the role of cytoskeleton in the regulation

Schemes 1–4.

Schemes 1 and 2 represent a system related to isolated heart mitochondrion. The respiratory chain (RC) complexes, ATPsynthase (F_1F_0) and Pi carrier PIC are integrated within the mitochondrial inner membrane (MIM). Mitochondrial creatine kinase (MtCK) is depicted as an octamer [15,16], located in the mitochondrial inter-membrane space (IMS) and attached to the inner membrane surface. In our experiments MtCK is activated by creatine (Cr) in the presence of ATP. The final products of MtCK-forward reaction are phosphocreatine (PCr) and endogenous ADP. The ADP phosphorylation is visualized by recording the oxygen consumption. In Scheme 1 endogenous intramitochondrial ADP produced by MtCK reaction forms a micro-domain within intermembrane space. The micro-compartmentalized ADP can either enter into mitochondrial matrix for phosphorylation or escape into the surrounding medium via voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane (MOM).

In Scheme 2 the model is supplemented with ADP-trapping system consisting of pyruvate kinase (PK) and phosphoenolpyruvate (PEP). This system utilizes all ADP leaving mitochondria to regenerate extramitochondrial ATP.

Scheme 3 represents mitochondrion in situ, in permeabilized cardiac cell, surrounded by cytoskeleton proteins (depicted as "x" factor) and myofibrils. The MOM is less permeable than in isolated mitochondrion, due to the interactions of VDAC with cytoskeleton proteins. Exogenous ATP is hydrolyzed by cellular ATPases into endogenous extramitochondrial ADP and inorganic phosphate (Pi). Mitochondrial (MtCK) and non-mitochondrial MM creatine kinases (cytosolic, myofibrillar, SERCA, sarcolemmal), activated by creatine in the presence of ATP, produce endogenous intra- and extramitochondrial ADP. Thus the oxidative phosphorylation is controlled by endogenous ADP produced by the MtCK, MMCK and ATPase reactions.

Scheme 4 represents system III supplemented with phosphoenolpyruvate (PEP) and pyruvate kinase (PK). PEP–PK system removes extramitochondrial ADP produced by intracellular ATP-consuming reactions and continuously regenerate extramitochondrial ATP. Endogenous intramitochondrial ADP produced by MtCK forms microcompartments within the IMS and is re-imported into the matrix via adenine nucleotide translocase (ANT) due to its functional coupling with MtCK. A series of experiments were performed to check the properties of this model in order to use it for complete MtCK kinetic analysis.

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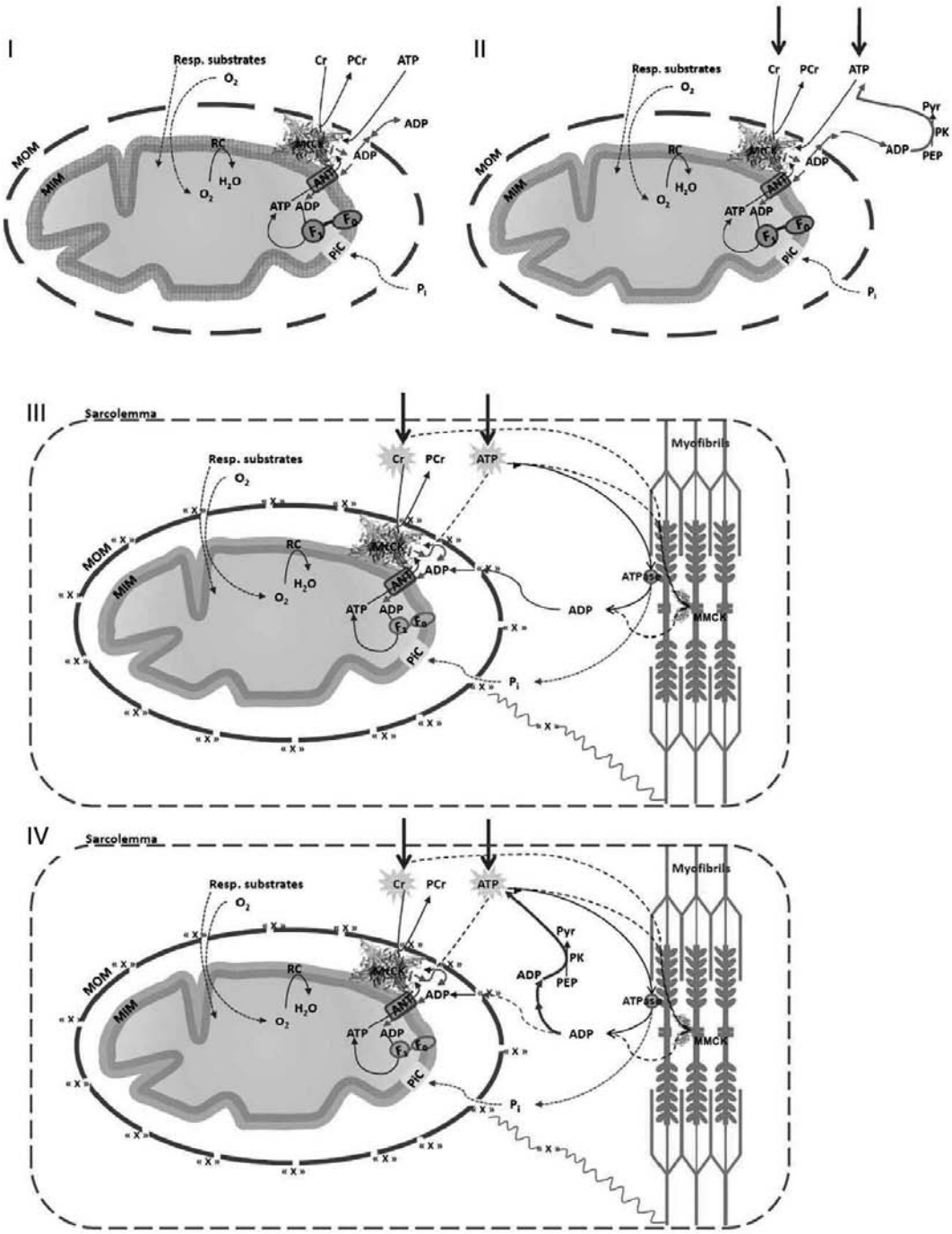
of mitochondrial respiration, permeabilized cardiomyocytes were treated with 0.3 μM trypsin, added into the oxygraph chambers. After 6 min of incubation, trypsin activity was inhibited by 2 μM Soybean trypsin inhibitor (STI) and 5 mg/ml fatty acid free BSA. Then the experiment was continued using these disorganized cells.

2.5. Measurements of oxygen consumption

The rates of oxygen uptake were determined with a high-resolution respirometer Oxygraph-2K (OROBOROS Instruments, Austria) in Mitomed solution [76] containing 0.5 mM EGTA, 3 mM $MgCl_2$,



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60 mM K-lactobionate, 3 mM KH_2PO_4, 20 mM taurine, 20 mM HEPES, 110 mM sucrose, 0.5 mM dithiothreitol (DTT), pH 7.1, 2 mg/ml fatty acid free BSA, complemented with 5 mM glutamate and 2 mM malate as respiratory substrates.

Measurements were carried out at 25 °C; solubility of oxygen was taken as 240 nmol/ml [78].

In kinetic experiments with different fixed MgATP concentrations, stock solution of 100 mM MgATP was prepared by mixing equimolar amounts of $MgCl_2$ and ATP; pH was adjusted to 7.2.

2.6. Measurements of mitochondrial cytochrome content

For comparative quantitative analysis of the kinetics of the regulation of respiration in isolated mitochondria and permeabilized cardiomyocytes, the respiration rates were expressed in nmol of oxygen consumed per minute per nmol of cytochrome aa_3, but not per mg of protein (if not indicated differently). Cytochrome aa_3 content in both cases is representative of respiratory chain, while proteins contained in cardiomyocytes are not all present in mitochondria. The contents of mitochondrial cytochrome aa_3 in the isolated mitochondria and cardiomyocytes were measured spectrophotometrically according to the method described before [79,80]. The cells or mitochondria were solubilized with 1% of sodium deoxycholate in phosphate buffer (100 mM KH_2PO_4, pH 8). The differential spectrum (reduced by dithionite versus oxidized cytochromes) was obtained by scanning from 400 to 650 nm using a Cary 50 Bio spectrophotometer (Varian, Palo Alto, USA) or Evolution 600 spectrophotometer (Thermo Electron Scientific Instruments, UK). Cytochromes of the respiratory chain were reduced by addition of several crystals of sodium dithionite to 1 ml of suspension of mitochondria (4 mg/ml) or cardiomyocytes (2 mg/ml). The value of peak at 605 nm was used for quantification of respiratory chain cytochrome aa_3 contents (cytochrome *c* oxidase) both in isolated mitochondria and cardiomyocytes, using the extinction coefficient ε value equal to 24 mM^{-1} cm^{-1} [80,81]. Protein concentrations were determined using a BCA protein assay kit (Pierce, USA) as a standard.

2.7. Determination of the rate of PCr production in cardiomyocytes in situ by ion pair HPLC/UPLC

Determination of the rates of PCr synthesis in permeabilized cardiomyocytes in situ under conditions used in respirometry experiments was carried out using ion pair HPLC/UPLC by stopping the reaction typically at 3, 6 and 10 min. 100 µl aliquots of the reaction mixture were withdrawn and added to a 200 µl ice-cold 1 M $HClO_4$ solution, immediately supplemented with 5 µl of 100 mM EDTA and neutralized with 210 µl of 0.952 M KOH in 0.5–1 min. The samples were held on ice for additional 10–15 min for proper precipitate formation and centrifuged at 16,000 *g* and 4 °C for 2–3 min. The supernatants were immediately frozen (−40 °C) and analyzed within 5–6 h. Addition of EDTA (final 1 mM) proved to be useful in order to bind traces of Mg^{2+} to suppress any residual enzyme (particularly adenylate kinase, unpublished observations) activity and stabilize the preparations. Separations of Cr, PCr and adenine nucleotides were performed by ultraperformance ion-pair chromatography (UPLC) on a 2.1×100 mm ACQUITY UPLC HSS T3

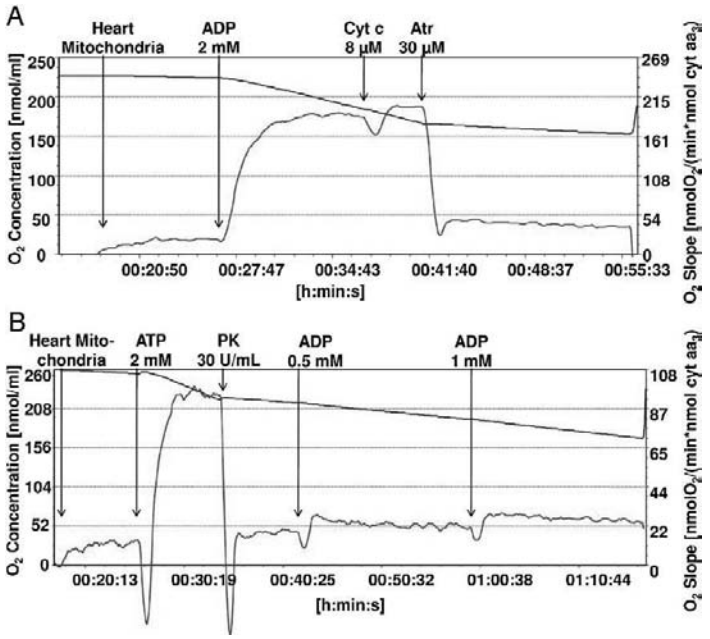

Fig. 1. (A) Representative respiration traces of isolated mitochondria recorded using a two-channel high resolution respirometer (Oroboros oxygraph 2k, Oroboros, Innsbruck, Austria). The left scale and the blue trace indicate the oxygen concentration ($nmolO_2$ ml^{-1}) in the experimental milieu. The right scale and the red trace show the rate of oxygen uptake expressed in $nmolO_2$ min^{-1} $nmol^{-1}$cyt. aa_3. The experiment was carried out in Mitomed solution with 5 mM glutamate/2 mM malate as respiratory substrates. State 3 of respiration rate (according to Chance) is achieved by adding 2 mM ADP. The integrity of the outer and inner mitochondrial membranes (MOM and MIM) was tested by addition of 8 µM cytochrome *c* and 30 µM atractyloside (Atr) respectively. Only samples with the respiratory control index ($RCI = V^3/V^2$) exceeding 7 and activation of respiration by exogenous cytochrome c less than 7% were used for experiments. (B) Effect of ADP-trapping system on respiration of isolated mitochondria stimulated by ATP. When the stable level of respiration is achieved 30 U/ml PK and 5 mM PEP are added. The PEP–PK system inhibits respiration using all free ADP. Subsequent addition of ADP in increasing amounts did not stimulate respiration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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C_{18} column packed with 1.7 μm particles (Waters) by recording optical density simultaneously at 210 nm and 254 nm for creatine and PCr, and adenine nucleotides, respectively. Sample volumes of 10 μl were injected by autosampler. The mobile phase consisted of buffer A (20 mM KH_2PO_4, 3 mM tetrabutylammonium bisulfate (TBAS)) and buffer B (200 mM KH_2PO_4, 10% (v/v) acetonitrile, 0.3 mM TBAS), both adjusted to pH 5.0 with 2 M phosphoric acid and filtered through a 0.2 μm membrane filter. The elution was performed at a flow rate 0.4 ml/min in buffer A for 2 min followed by 1:1 gradient elution with buffers A and B up to 8.5 min and additionally with buffer B up to 10 min. After the analysis, the column was re-equilibriated by washing for 1 min with water and buffer A for 9 min thus resulting in total time for an analysis of 20 min. The column was periodically cleaned by washing with 80% methanol. The retention time for the reagents were, in minutes, 0.63 (Cr), 1.70 (PCr), 6.33 (AMP, traces), 6.95 (ADP) and 7.29 (ATP), all within ±0.01 min. Stock solutions for calibration (0.1 M) were prepared in 0.2 M KH_2PO_4 at pH 7.0 and stored at −40 °C for not more than 2–3 days in order to minimize PCr and ATP degradation. Calibration solutions were prepared in supernatant solutions obtained after addition and precipitation of cardiomyocytes as described above.

2.8. Analysis of the steady state kinetics of the MtCK reaction coupled to respiration

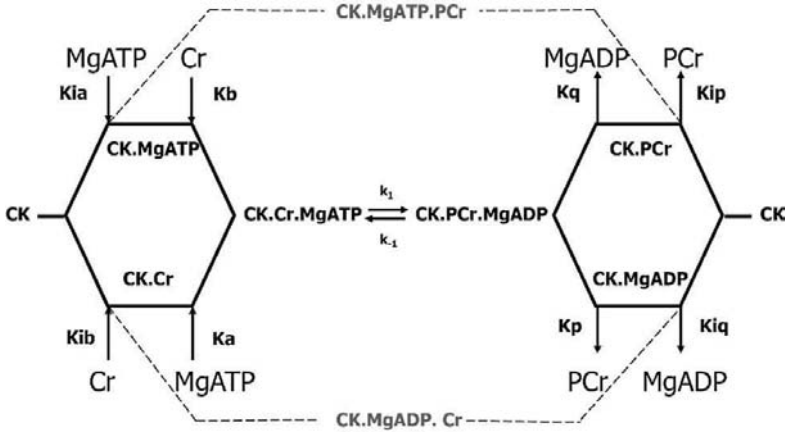
The steady state kinetics of MtCK reaction coupled to oxidative phosphorylation via ANT in permeabilized cardiomyocytes in situ was studied using the protocol shown in Scheme 4. Cardiac cells were injected into an oxygraph chamber and permeabilized with 25 μg/ml saponin by incubation for 10 min. Then respiration was activated by addition of MgATP at different fixed concentrations to initiate the endogenous MgADP production by MgATPases. Then 20 U/ml PK and 5 mM PEP were added for the trapping of MgADP and ATP regeneration. This significantly reduced the respiration rate. Then the MtCK reaction was activated by adding creatine in increasing concentrations. Under these conditions steady state kinetics of respiration follows the kinetics of MtCK reaction. For kinetic analyses only the respiration rates dependent on creatine were used; these rates were found by subtraction of the respiration rates after PK–PEP addition in the absence of creatine from the total respiration rates measured in the presence of ATP and creatine. Because of the constant PCr/O_2 ratio found in the experiments (see Results section), the steady state reaction rates were expressed as VO_2, since the main aim was to study the kinetics of the regulation of respiration dependent on MtCK. These rates can be easily converted into the MtCK reaction rates by using this PCr/O ratio according to the equation $V_{CK} = 5.7\ VO_2$. Experimental data were analyzed by applying the method of complete kinetic analysis of MtCK reaction described earlier for isolated mitochondria by W. Jacobus and V. Saks [82]. The methods of complete kinetic analysis of the creatine kinase reaction [75,83] are following. According to Cleland classification, the creatine kinase reaction mechanism is Bi-Bi random quasi-equilibrium type [83] (see Scheme 5).

Scheme 5 shows the interconversion of productive ternary enzyme–substrate (CK·Cr·MgATP) and enzyme–product (CK·PCr·MgADP) complexes in the presence of $MgATP^{2-}$, MgADP-, creatine and phosphocreatine. K_{ia}, K_{ib}, K_{iq}, and K_{ip} are the constants of dissociation from the binary and K_a, K_b, K_q, and K_p from the ternary complexes with creatine kinase. k_1 and $k_{-1}$are the rate constants of the forward and reverse reactions. The dead-end ternary complexes CK·MgATP·PCr and CK·MgADP·Cr are abortive complexes limiting enzyme activity [75].

Dissociation constants for MgATP K_{ia}, K_a and for Cr K_{ib}, K_b from their binary and ternary complexes with MtCK are:

$$K_{ia} = \frac{[CK] \cdot [MgATP]}{[CK \cdot MgATP]};\ K_b = \frac{[CK \cdot MgATP] \cdot [Cr]}{[CK \cdot Cr \cdot MgATP]};\ K_{ib} = \frac{[CK] \cdot [Cr]}{[CK \cdot Cr]};$$
$$K_a = \frac{[CK \cdot Cr] \cdot [MgATP]}{[CK \cdot Cr \cdot MgATP]}. \quad (1)$$

The limiting effect of the final product MgADP accumulation was avoided in experiments by using ATP regeneration system: oxidative phosphorylation or/and PEP–PK system. To study the kinetic properties of MtCK in situ, in the permeabilized cardiomyocytes, several conditions have to be fulfilled: firstly, MtCK must totally control oxidative phosphorylation, secondly, MgADP produced by MtCK should be compartmentalized within the intermembrane space without leaking into extramitochondrial medium but taken back by ANT into mitochondrial matrix. This has been shown to be the case in well-prepared cardiomyocytes with the content of rod-like Ca-tolerant cells higher than 70–90% and stable after permeabilization procedure. The last condition is to exclude maximally the stimulatory effect of extramitochondrial ADP on oxidative phosphorylation, and that was achieved by adding at least 20 U/ml of PK with 5 mM PEP.



Scheme 5. The kinetic mechanism of MtCK reaction.

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2.8.1. Kinetics of the forward MtCK reaction

The rate of forward CK reaction in the presence of MgATP and creatine is defined by Eq. (2) if the formation of dead-end complexes is ignored:

$$v = \frac{V_m \cdot [\mathrm{MgATP}] \cdot [\mathrm{Cr}]}{K_{ia}K_b + K_b[\mathrm{MgATP}] + K_a[\mathrm{Cr}] + [\mathrm{MgATP}] \cdot [\mathrm{Cr}]}. \quad (2)$$

Primary analysis of data derived from Eq. (2) in double-reciprocal coordinates of 1/v versus 1/[S] is:

$$\frac{1}{v} = \left[\frac{K_b}{V_m}\left(\frac{K_{ia}}{[\mathrm{MgATP}]} + 1\right)\right]\frac{1}{[\mathrm{Cr}]} + \frac{1}{V_m}\left(\frac{K_a}{[\mathrm{MgATP}]} + 1\right) \quad (3)$$

for fixed [MgATP] and varying [Cr],and:

$$\frac{1}{v} = \left[\frac{K_a}{V_m}\left(\frac{K_{ib}}{[\mathrm{Cr}]} + 1\right)\right]\frac{1}{[\mathrm{MgATP}]} + \frac{1}{V_m}\left(\frac{K_b}{[\mathrm{Cr}]} + 1\right) \quad (4)$$

for fixed [Cr] and varying [MgATP].

This primary analysis provides the values of ordinate intercepts (i_1, i_2) and slopes (s_1, s_2) for secondary analysis (Eqs. (5) and (6)).

$$i_1 = \frac{1}{V_m}\left(\frac{K_a}{[\mathrm{MgATP}]} + 1\right);\ s_1 = \frac{K_b}{V_m}\left(\frac{K_{ia}}{[\mathrm{MgATP}]} + 1\right) \quad (5)$$

$$i_2 = \frac{1}{V_m}\left(\frac{K_b}{[\mathrm{Cr}]} + 1\right);\ s_2 = \frac{K_a}{V_m}\left[\frac{K_{ib}}{[\mathrm{Cr}]} + 1\right]. \quad (6)$$

By replotting the estimated regression parameters as functions of secondary substrates, one can obtain the values of dissociation constants for MgATP (K_{ia} and $K_{a,}$) and creatine (K_{ib} and K_b) from their binary and ternary complexes with MtCK.

At $i_1 = 0$ and $i_2 = 0$ the values for K_b and K_a, are acquired. Constants K_{ib} and K_{ia} are obtained in the case of $s_1 = 0$ and $s_2 = 0$.

2.8.2. Product inhibition of MtCK by PCr

For the reaction in the presence of PCr the total enzyme exists in five forms.

$$[\mathrm{CK}]_t = [\mathrm{CK}] + [\mathrm{CK} \cdot \mathrm{MgATP}] + [\mathrm{CK} \cdot \mathrm{Cr}] + [\mathrm{CK} \cdot \mathrm{MgATP} \cdot \mathrm{Cr}] + [\mathrm{CK} \cdot \mathrm{PCr}]. \quad (7)$$

Again, the formation of the dead-end complex CK·MgATP·PCr is ignored because of its very high dissociation constant [75]. The rate of the forward reaction in this case is defined by Eq. (8), where K_{ip} is the dissociation constant (Eq. (9)) from its binary complex with MtCK.

$$v = \frac{V_m \cdot [\mathrm{MgATP}] \cdot [\mathrm{Cr}]}{K_{ia}K_b + K_b[\mathrm{MgATP}] + K_a[\mathrm{Cr}] + \frac{[\mathrm{PCr}]K_{ia}K_b}{K_{ip}} + [\mathrm{MgATP}] \cdot [\mathrm{Cr}]} \quad (8)$$

$$K_{ip} = \frac{[\mathrm{PCr}] \cdot [\mathrm{CK}]}{[\mathrm{CK} \cdot \mathrm{PCr}]}. \quad (9)$$

The slopes s_1 of the series of straight lines, obtained from double reciprocal coordinates 1/v versus 1/[Cr] at varying [PCr] and [MgATP] concentrations are analyzed by Eq. (10).

$$s_3 = \frac{K_{ia}K_b}{V_m}\left(1 + \frac{[\mathrm{PCr}]}{K_{ip}}\right)\frac{1}{[\mathrm{MgATP}]} + \frac{K_b}{V_m}. \quad (10)$$

By plotting the resulting slopes $s(s_3)$ versus [PCr], according to Eq. (11), abscissa intercept directly provides the value of $-K_{ip}$.

$$s(s_3) = \frac{K_{ia}K_b}{V_m} + \frac{K_{ia}K_b}{V_m}\frac{[\mathrm{PCr}]}{K_{ip}}. \quad (11)$$

2.9. Data analysis

All data are presented as mean ± SEM. Statistical analysis were performed using Student's *t*-test and $p < 0.05$ was taken as the level of significance.

2.10. Reagents

Ethylene glycol-bis(2-aminoethyl)-N,N,N′,N′ether-tetraacetic acid (EGTA), lactobionic acid, 1,4-dithio-DL-threitol (DDT), imidazole, potassium dihydrogen phosphate (KH_2PO_4), 2-aminoethanesulfonic acid (taurine), 2-morpholinoethanesulfonic acid monohydrate (MES), L(−)-malic acid, L-glutamic acid and creatine monohydrate, tetrabutylammonium bisulfate (TBAS)—Fluka; magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), sodium bicarbonate ($NaHCO_3$), potassium chloride (KCl), sodium chloride (NaCl), glucose, sucrose (cell culture tested) HEPES, BES pyruvate, BES, Mg acetate—Sigma; Na_2-ATP, leupeptine collagenase A, blendzyme 1, albumin, from bovine serum, essentially fatty acid free (BSA), STI, phosphocreatine disodium salt (PCr)—Roche.

3. Results

3.1. Reference systems I and II: regulation of respiration by MtCK in isolated mitochondria in the absence and presence of PEP–PK

Fig. 1A shows a recording of oxygen consumption by isolated heart mitochondria used in this work as a reference system for comparison with permeabilized cardiomyocytes. Addition of ADP in a saturating concentration of 2 mM to isolated mitochondria induced State 3 high respiration rate. The respiratory control index (RCI) usually exceeded 7 (Fig. 1A). Addition of exogenous cytochrome *c* only slightly increased the respiration rate showing the intactness of the outer mitochondrial membrane, and addition of atractyloside decreased the respiration rate close to the State 2 value showing the intactness of the inner mitochondrial membrane [76,77]. These characteristics show the quality of isolated mitochondrial preparation needed for kinetic analysis. Fig. 1B shows the experimental test of the ADP trapping power of the PEP–PK system. In the presence of such a system, exogenous ADP has no effect on respiration, since all added ADP is rapidly consumed by PEP–PK. However, when respiration of isolated heart mitochondria was stimulated by creatine in the presence of ATP (i.e. MtCK was activated), addition of PK and PEP decreases respiration rate only about 50% of its maximal value (Fig. 2A). The remnant rate of respiration (up to 50% of VO_2max) was due to the functional coupling between MtCK and ANT with the direct transfer of ADP into the matrix. Gellerich et al. [84–87] have proposed the hypothesis of the dynamic compartmentation of ADP in the mitochondrial intermembrane space, according to which ADP concentration gradients across the outer mitochondrial membrane may explain the respiration stimulated by creatine in the presence of the PEP–PK system. However, Vendelin et al. [8] showed by applying mathematical modelling that the direct ATP transfer between ANT and MtCK is more important for respiratory control by creatine in isolated mitochondria than the dynamic ADP compartmentation proposed by Gellerich [66]. Fig. 2B shows the kinetics of activation of respiration in isolated heart mitochondria by addition of increasing amounts of creatine up to 20 mM in the presence of ATP and ADP-trapping PEP–PK system. Again not more than 50% of V_{max} can be reached in this experiment due to the functional coupling of MtCK with ANT. That means that at least 50% of ADP produced locally by MtCK is accessible for the extramitochondrial PEP–PK system due to high permeability of VDAC for adenine nucleotides in isolated mitochondria, in accordance with classical data from Klingenberg's [88] and Colombini's laboratories [89]. At the same time, stimulation of respiration up to 50% of V_{max} by creatine in the presence of PEP–

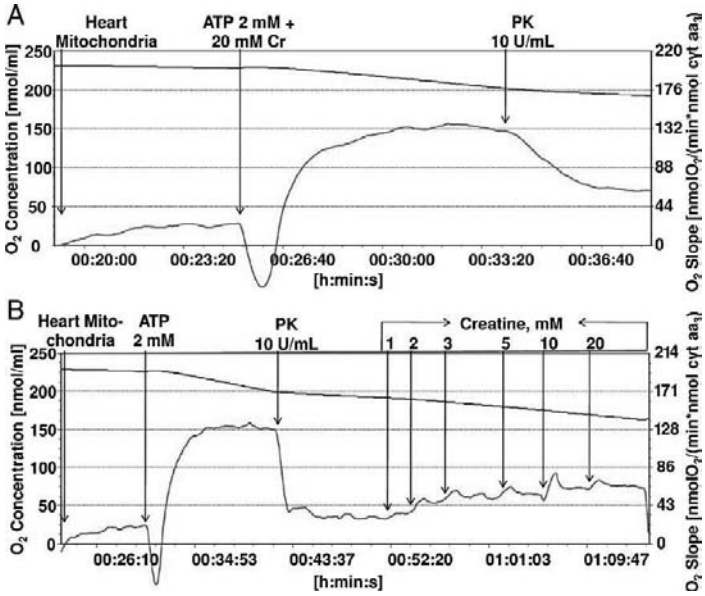

Fig. 2. (A) Stable respiration rate supported by MtCK activity in the presence of ATP and creatine. Under these conditions, approximately 50% of ADP, produced in the intermembrane space, can be trapped by powerful the PEP–PK system. The respiration is not completely inhibited because of the presence of direct ADP transfer from MtCK to ANT. (B) The kinetics of activation of respiration in isolated mitochondria stimulated by increasing amounts of creatine in the presence of ATP (i.e. activated MtCK reaction) and the absence of extramitochondrial ADP, which is efficiently consumed by the PEP–PK reaction.

PK system shows the existence of the functional coupling between MtCK and ANT.

3.2. Regulation of respiration in experimental system III—permeabilized cardiac cells without PEP–PK

Regular quality tests for isolated cardiomyocytes used in this work were similar to those reported for isolated mitochondria in Fig. 1A. The effect of the addition of exogenous cytochrome *c* on State 3 respiration was absent showing the intactness of MOM. Only preparations with these characteristics were used in the experiments reported in this work.

Table 1 summarizes the respiratory parameters of the isolated mitochondria and isolated cardiomyocytes. As it can be seen from Table 1, maximal respiration rates are equal to both isolated mitochondria and cardiomyocytes if calculated per nmol of cytochrome aa_3. Expressed in this way, the kinetic data reported in this work may be easily used for the quantitative analysis by mathematical modelling in the future.

Table 1
Basic respiration parameters of isolated rat heart mitochondria and of mitochondria in situ in permeabilized cardiomyocytes.

Parameter	Mitochondria in vitro	Mitochondria in situ (permeabilized cardiomyocytes)
V_0, nmolO_2 min^{-1} mg prot^{-1}	26.37 ± 7.93	7.53 ± 1.61
V_3 (2 mM ADP), nmolO_2 min^{-1} mg prot^{-1}	187.94 ± 40.68	84.45 ± 13.85
[Cyt aa_3], nmol mg prot^{-1}	1.00 ± 0.012	0.46 ± 0.09
V_3 (2 mM ADP), nmolO_2 min^{-1} nmol cyt aa_3^{-1}	187.94 ± 40.68	178.23 ± 33.96
$V_{Cr,ATP}$, nmolO_2 min^{-1} nmol cyt aa_3^{-1}	197.90 ± 31.86	162.63 ± 26.87

V_0—respiration rate in State 2 in the presence of substrates before addition of ADP or ATP.
V_3—respiration rate in the presence of 2 mM ADP.
$V_{Cr,ATP}$—respiration rate in the presence of activated MtCK by 2 mM ATP and 20 mM creatine.

In cells in vivo, endogenous ADP may be produced in MgATPase and in creatine kinase (MtCK and MMCK) reactions from ATP (see Scheme 3). In this scheme exogenous ATP added to permeabilized cardiomyocytes is hydrolyzed by cellular ATPases with the formation of endogenous extramitochondrial ADP which subsequently stimulates mitochondrial respiration. The apparent kinetics of activation of respiration by exogenous MgATP evidently follows the kinetics of activation of cellular ATPases by this substrate and is shown in Fig. 3A. In the absence of creatine, the apparent K_m (K_m^{app}) for exogenous MgATP is 157.75 ± 40.06 μM. In the presence of creatine the K_m^{app} for MgATP decreases to 24.89 ± 0.81 μM (Fig. 3A). Almost maximum activation of respiration in the presence of creatine and significant decrease of apparent K_m for MgATP under these conditions are related to the control of respiration by both intramitochondrial ADP locally produced in the MtCK reaction and extramitochondrial ADP produced in MMCK in MgATPases reactions. Functional coupling of MtCK with ANT is a powerful amplification mechanism of a regulatory ADP metabolic signal from cytoplasm, due to a manifold increase of ADP and ATP recycling rate in mitochondria. In the presence of both MgATP and creatine one can see the overall effects of all these mechanisms.

3.3. Regulation of respiration in experimental system IV—permeabilized cardiac cells supplemented with PEP–PK

To study the role of the coupled MtCK alone in the regulation of respiration in situ in the cells, the stimulatory effect of extramitochondrial ADP produced by MgATPases and MMCK can be extinguished by the PEP–PK system (Scheme 4). Remarkably, addition of the PEP–PK system helps to simulate the in vivo conditions in the cells, where the glycolytic system always is present and by consuming ADP competes with mitochondrial respiration. An interesting and important finding in these experiments was the fact that in the presence of fully activated MtCK the addition of competitive ADP-trapping PEP–PK system could not inhibit the mitochondrial respiration in permeabilized cardiac cells in situ (Fig. 3B), in contrast with the isolated

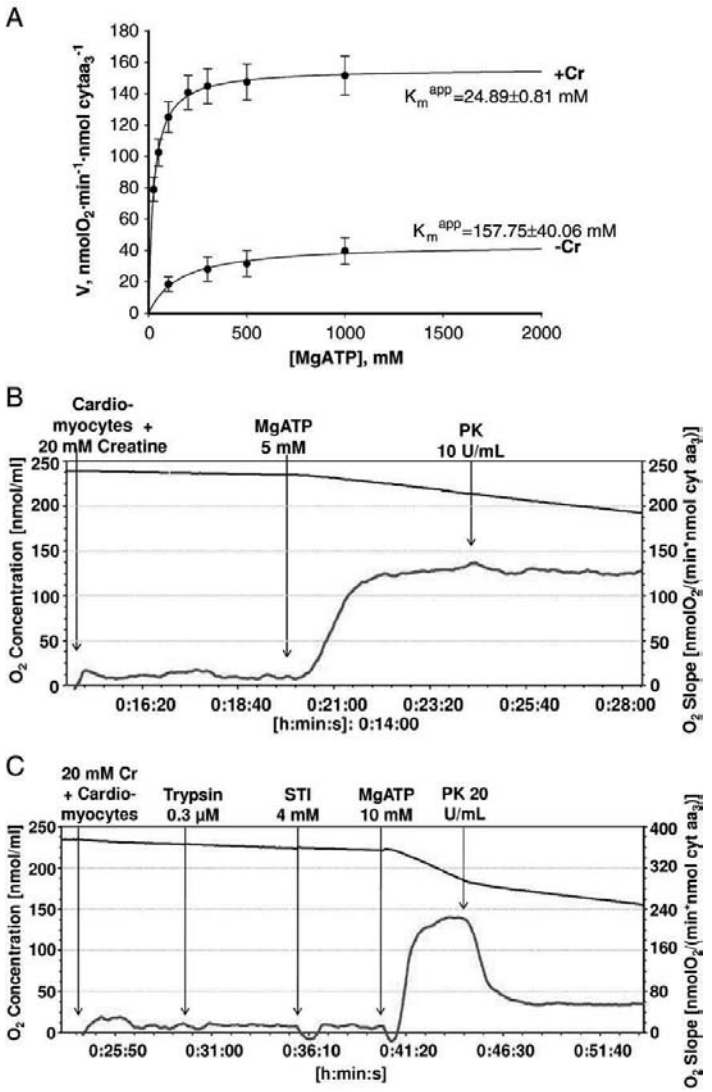

Fig. 3. (A) Rates of mitochondrial respiration in permeabilized cardiomyocytes as function of added exogenous MgATP in absence and in presence of 20 mM of creatine. After permeabilization, respiration rates were recorded after addition of different amounts of MgATP in absence and presence of creatine. Manifold increase in V_{max} and decrease in the apparent K_m for ATP in the presence of 20 mM creatine (i.e. under conditions of activated MtCK) is seen. (B) Full compartmentalization of intramitochondrial ADP produced by MtCK in the intermembrane space of mitochondria in permeabilized cardiomyocytes in situ. Instead of 2 mM ADP, respiration was activated by addition of MgATP (5 mM) and creatine (20 mM). Addition of PK in the presence of PEP (5 mM added into medium before) did not change significantly the respiration rate. In mitochondria in situ when respiration is controlled by the MtCK reaction, the powerful PEP–PK system is not able to inhibit respiration. The permeability of VDAC for ADP seems strongly decreased. (C) Proteolytic treatment of permeabilized cardiomyocytes eliminates the diffusion restrictions for endogenously produced ADP. Note, that after the treatment of isolated cardiomyocytes with trypsin in low concentration (0.3 μM) ADP becomes accessible to the PEP–PK trapping system. Rate of respiration in the presence of activated MtCK (by creatine and ATP) is due to the channelling of some part of ADP from MtCK to ANT within intermembrane space (see Fig. 2A).

mitochondria (Fig. 2A). Fig. 3B shows that the respiration rate of mitochondria in situ stimulated by 20 mM creatine and 5 mM MgATP did not decrease after addition of 10 U/ml PK in spite of the fact that the extramitochondrial ADP must have been significantly reduced. Under these conditions respiration is fully maintained by intramitochondrial ADP produced in MtCK reaction, which is not accessible for the PEP–PK system. In vivo and in situ the mitochondrial outer membrane in cardiomyocyte is most probably not easily permeable for ADP which is mainly transported by ANT back into the matrix for subsequent rephosphorylation (see Scheme 4). However, after treatment of permeabilized cardiomyocytes with trypsin, compartmentalized in the intermembrane space ADP becomes accessible for the PEP–PK system (Fig. 3C). In these experiments, cardiomyocytes were incubated in an oxygraph chamber in solution supplemented with trypsin (0.3 μM). After 6 min trypsin was inhibited by STI+ BSA, and mitochondrial respiration was stimulated by addition of 2 mM ATP and 20 mM creatine. The PK and PEP added under these conditions inhibit respiration by trapping almost all extramitochondrial ADP and a significant part of ADP produced by MtCK. A similar effect was observed in isolated mitochondria (Fig. 2A). These results

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show that in the permeabilized cardiomyocytes ADP produced by MtCK in mitochondrial intermembrane space is not easily accessible for PEP–PK, most probably due to decreased permeability of VDAC in the mitochondrial outer membrane. The decrease of VDAC permeability for adenine nucleotides in mitochondria in situ as compared with mitochondria in vitro is caused probably by its interaction with cytoskeleton proteins [90–93], sensitive to trypsin treatment. Activation of respiration under these conditions directly follows the activation of MtCK by its substrates and the respiration rates can be used to study the steady state kinetics of MtCK activation by its substrates (MgATP and creatine). The micro-compartmentation of all intramitochondrial ADP produced by the MtCK reaction in the absence of its leak toward the extramitochondrial space is one of the most important properties of our experimental model—permeabilized cardiomyocyte (Scheme 4).

3.4. Regulation of respiration in experimental system IV—MtCK kinetic analysis

Such characteristics of permeabilized intact cardiomyocytes as complete micro-compartmentation of ADP produced in MtCK reaction in situ, the functional coupling of MtCK with ANT, and significant elimination of the stimulatory effect of extramitochondrial ADP by the PEP–PK system, has led us to elaborate the experimental protocol for studying in situ the role of MtCK in the regulation of mitochondrial respiration described in Materials and methods. The experimental protocol is shown in Fig. 4 and its principles are illustrated by Scheme 4. First, after permeabilization, mitochondrial respiration is stimulated by the addition of 2 mM ATP which is hydrolyzed by cellular ATPases with production of endogenous ADP. Secondly, when the stable level of respiration is achieved, the adequate quantity of PK and PEP are added to maximally uptake all free ADP and regenerate extramitochondrial ATP. Thus, the stable level of extramitochondrial ATP is maintained. *Finally*, the MtCK reaction is activated by increasing the amounts of creatine. As a result, oxidative phosphorylation is stimulated only by intramitochondrial ADP produced by the MtCK reaction (Fig. 4). To study the role of this ADP in the regulation of oxidative phosphorylation, we subtracted the oxygen consumption rate under PEP–PK (VO_{2PK}) before creatine addition from creatine-stimulated respiration rate (VO_{2Cr}).

This is the complete and the most precise protocol for studies of the MtCK functions in situ in permeabilized cells. Fig. 4 shows that the respiration rate determined by this protocol is very sensitive to changes in creatine concentration. It rapidly increases in response to the addition of creatine in rather low concentrations, 1–3 mM. To understand the reason for this high sensitivity to creatine, we performed the complete kinetic analysis of MtCK reaction under these conditions using different fixed concentrations of MgATP (0.5 to 10 mM) and varying creatine concentrations (1 to 10 mM). Graphical presentation of creatine activated respiration rates (VO_2) in function of varying concentrations of creatine and fixed MgATP and *vice versa*: in function of varying [MgATP] and fixed [Cr] are shown in Figs. 5A and 6A, respectively. The primary analysis of data in double reciprocal plots (1/V as a function of 1/[S]) according to Eqs. (3) and (4) are represented in Figs. 5B and 6B. This linearization gives the family of straight lines with one common point of interception, corresponding to the kinetic behaviour of the Bi-Bi random type quasi equilibrium reaction mechanism of creatine kinase (Figs. 5B and 6B). This primary analysis of kinetic data gives us values of ordinate intercepts and slopes for secondary linearization according to Eqs. (5) and (6) (Figs. 5C and 6C), which provides the dissociation constants for MgATP and creatine from their binary and ternary complexes with MtCK (Table 2).

This complete kinetic analysis of the MtCK reaction in cardiomyocytes in situ provides results remarkably different from MtCK kinetic properties of isolated mitochondria reported before. The kinetic analysis of the MtCK reaction of isolated mitochondria was performed applying the protocol described by Jacobus and Saks in 1982 and have been described in several previous publications [12,80,82,94]. The apparent constant of dissociation for MgATP from its binary complex with MtCK (K_{ia}) is 4 times higher (from 0.44 ± 0.08 to 1.94 ± 0.86 mM) and from ternary complex (K_a) is 100 times higher (from 0.016 ± 0.01 to 2.04 ± 0.14 mM) in mitochondria in situ than one in vitro. These data evidence strong decrease of mitochondrial affinity for free MgATP added into the medium, suggesting significant restriction of ATP diffusion in some local areas in cardiomyocytes, most probably at the level of MOM. On the other hand, we observed a strong decrease of the apparent constant of dissociation for creatine in situ. The K_{ib} for creatine (binary MtCK–substrate complex) was 15 times lower (from 28 ± 7 to 2.12 ± 0.21 mM) and K_bCr −2.5 times lower (from 5.0 ± 1.2 to 2.17 ± 0.40 mM) than in isolated mitochondria. These results show increased apparent affinity of MtCK in mitochondria in situ for creatine. Table 2 shows also the maximal steady state rates expressed as the maximal creatine-stimulated respiration rates. The rates of PCr production can be calculated from these respiration rates by using the PCr/O_2 ratio (see below). The maximal steady state rate of PCr production in isolated mitochondria calculated in this way is 1.1 $\mu mol \cdot min^{-1} \cdot nmol$ cyt. aa_3^{-1} that is practically equal to the activity of MtCK measured in the direction of PCr production at saturating substrates' concentrations, and to the maximal activity of ATP synthesis reaction [75,95,96]. For cardiomyocytes, this rate

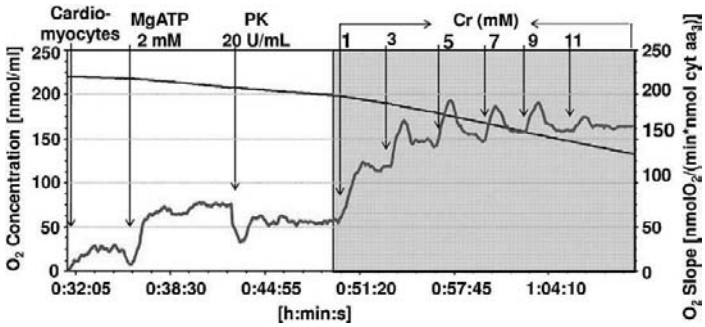

Fig. 4. The experimental procedure used for complete kinetic analysis of MtCK in mitochondria in situ (permeabilized cardiomyocyte). First, the respiration is activated by addition of MgATP after permeabilization of cardiomyocytes by saponin inducing production of endogenous ADP in MgATPase reaction (Schemes 3 and 4). Then PEP–PK is added to trap all extramitochondrial free ADP. This decreases the respiration rate, but not to initial level, due to structural organization of ICEU (see Schemes 3 and 4). Mitochondria are in privileged position to trap some of endogenous ADP. Under this conditions addition of creatine in different amounts activates MtCK reaction. The oxidative phosphorylation is stimulated mostly by intramitochondrial ADP, produced by MtCK reaction, which is not accessible for PEP–PK.

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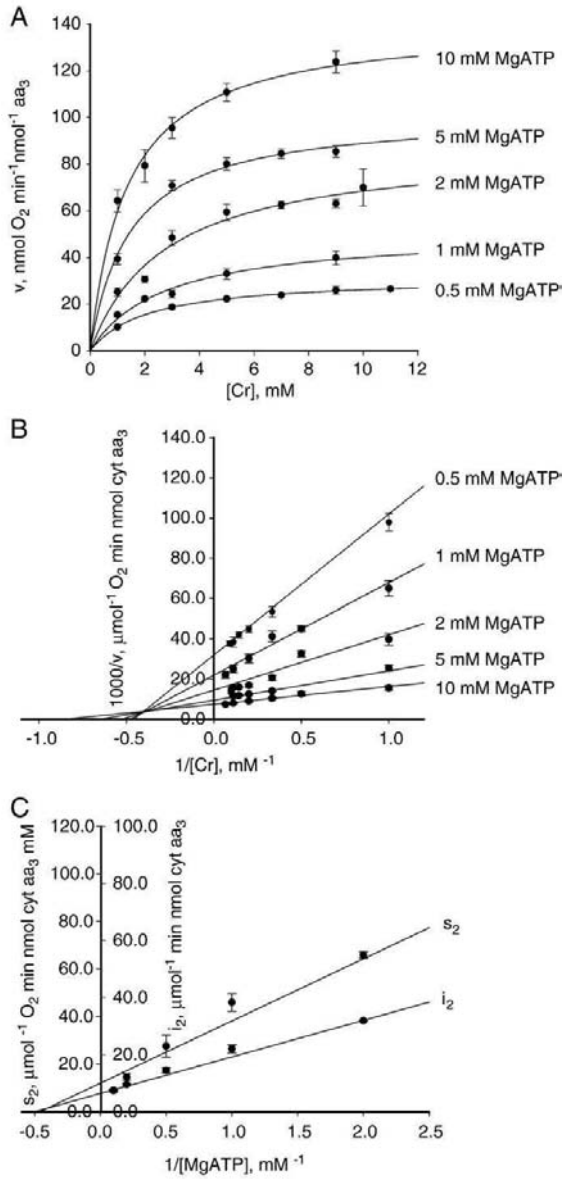

Fig. 5. (A) The rates of creatine activated respiration of permeabilized cardiomyocytes as functions of varying creatine concentrations at fixed MgATP concentrations. The respiration rates (VO_2) were calculated by subtraction of oxygen consumption rates in the presence of PEP–PK (VO_{2PK}) from the creatine activated respiration rates (VO_{2Cr}) ($VO_2 = VO_{2Cr} - VO_{2PK}$), (see Fig. 4 right panel). (B) The primary analysis of data (A) in double reciprocal coordinates of 1/v (1/respiration rate) versus 1/[Cr] (substrate with varying concentration) for different fixed MgATP concentration. (C) The secondary analysis of the primary plots from panel B. Slopes (s) and intercepts of y-axis (i) are plotted as a function of reciprocal coordinate of secondary substrate MgATP. The intercepts of x-axis provide directly the reciprocal values of dissociation constants of MgATP (K_{ia} and K_a) from the binary and ternary complexes of MtCK.

measured directly as described below (Kinetics of respiration in experimental system IV—MtCK kinetic analysis) is 0.51 ± 0.04 μmol min^{-1} mg^{-1}protein or again 1.1 μmol min^{-1} nmol cyt. aa_3^{-1}. That means that MtCK reaction coupled to ANT functions in steady state far from equilibrium, in good agreement with the results of mathematical modelling of this coupling [97] and confirmed by direct measurements with ^{31}P NMR inversion transfer technique [98].

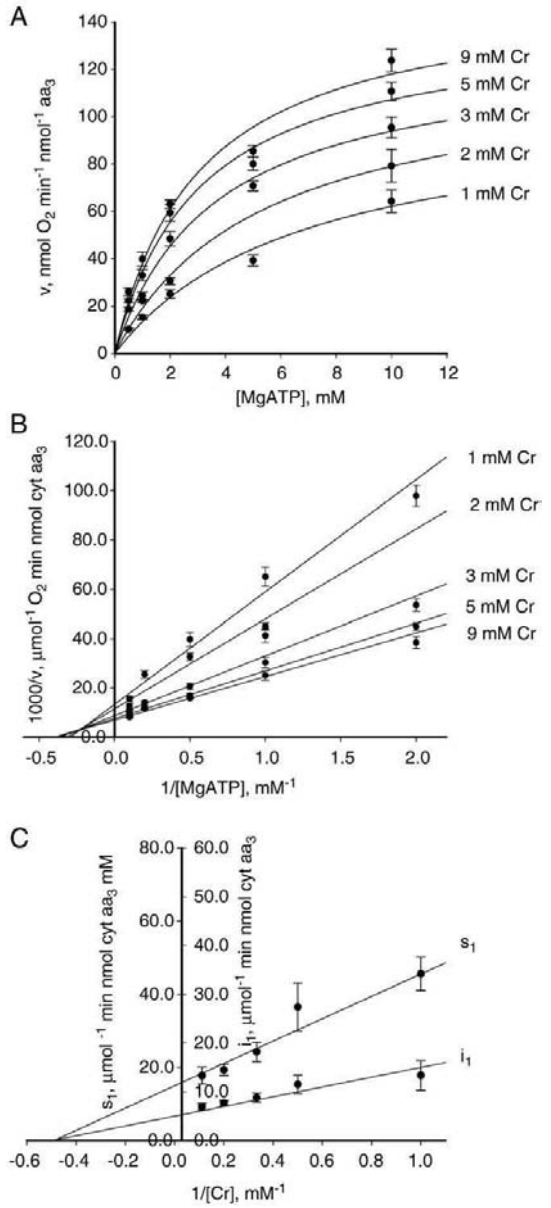

Fig. 6. (A) The rates of creatine activated respiration of permeabilized cardiomyocytes as functions of varying MgATP concentrations at fixed creatine concentrations. The respiration rates (VO_2) were calculated by subtraction of oxygen consumption rates in the presence of PEP–PK (VO_{2PK}) from the creatine activated respiration rates (VO_{2Cr}) ($VO_2 = VO_{2Cr} - VO_{2PK}$), (see Fig. 4 right panel). (B) The primary analysis of data (A) in double reciprocal coordinates of 1/v (1/respiration rate) versus 1/[MgATP] (substrate with varying concentration) for different fixed creatines concentration. (C) The secondary analysis of the primary plots from panel B. Slopes (s) and intercepts of y-axis (i) are plotted as a function of reciprocal coordinate of secondary substrate creatine. The intercepts of x-axis provide directly the reciprocal values of dissociation constants of creatine (K_{ib} and K_b) from the binary and ternary complexes of MtCK.

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Table 2
Apparent kinetic constants of rat heart mitochondrial creatine kinase (MtCK) in mitochondria in vitro and in situ in permeabilized cardiomyocytes.

	K_{ia}MgATP (mM)	K_aMgATP (mM)	K_{ib} Cr (mM)	K_b Cr (mM)	V_{max}	K_{ip} (mM)
Mitoch. in vitro*	0.44 ± 0.08	0.016 ± 0.01	28 ± 7	5 ± 1.2	187.9 ± 40	0.84 ± 0.22
Mitoch. in situ (with PEP–PK)	1.94 ± 0.86	2.04 ± 0.14	2.12 ± 0.21	2.17 ± 0.40	161.65 ± 11.38	0.89 ± 0.17

K_{ia}MgATP and K_aMgATP—constants of dissociation of MgATP from its binary (K_{ia}) and ternary (K_a) complexes with mitochondrial creatine kinase (MtCK). K_{ib}Cr and K_bCr—constants of dissociation of creatine from its binary (K_{ib}) and ternary (K_b) complexes with MtCK. K_{ip}—constant of dissociation of phosphocreatine from its binary (K_{ip}) complex with MtCK. V_{max}—maximal oxygen consumption rates in nmol O_2/min/nmol cyt. aa_3 corresponding to the State 3 of respiration.
* Kinetic constants for isolated mitochondria are taken from references [12], [80] and [82].

It is most interesting to compare the kinetic behaviour of systems III and IV—the respiration rate in permeabilized cardiomyocytes in the presence of 10 mM creatine as a function of exogenous MgATP without and with added PEP–PK system. The data for these conditions were taken from Figs. 3A and 6A and their comparison is shown in Fig. 7. The PEP–PK system very strongly changes the kinetics from high apparent affinity to low apparent affinity for MgATP. In the case of system III there is the production of endogenous MgADP by MgATPases and extramitochondrial CK reactions; in the case of system IV the MgADP is trapped and respiration maintained only by mitochondrial MtCK reaction in the intermembrane space of mitochondria. An interesting observation was that pre-incubation with PK, before the addition of creatine, (see Fig. 3B) was needed to see the difference between systems III and IV—most probably this time was needed to allow penetration of PK molecules into the permeabilized cell interior and into the ICEUs. The presence of the PEP–PK system did not change the kinetic parameters of soluble MM creatine kinase (unpublished data). Therefore, the data in Fig. 7 show the importance of small endogenous MgADP fluxes from MgATPases to mitochondria where their effect on respiration is manifoldly amplified by the functionally coupled MtCK–ANT system. This is consistent with earlier findings from our and Theo Wallimann's laboratories showing high sensitivity of respiration of permeabilized cells in the presence of creatine to endogenous MgADP fluxes induced by addition of exogenous MgATP [71,72,99]. Under these conditions high respiration rates are observed when MgADP concentration in the medium is very low [99].

3.5. Kinetics of respiration regulation in system IV in the presence of PCr

To study the apparent affinity of MtCK in situ for PCr, we applied the protocol illustrated in Fig. 4. Experiments described in Fig. 5A were repeated in the presence of different fixed concentrations of PCr. Phosphocreatine induced the decrease of respiration rate in a dose-dependent way in both models: isolated mitochondria [82] and permeabilized cardiomyocytes (Fig. 8). The primary and secondary kinetic analyses according to Eqs. (10) and (11), presented in Fig. 8A and B for cardiomyocytes, provide the value of dissociation constant for PCr from the binary complex MtCK·PCr, K_{ip}. Interestingly, the K_{ip} in isolated mitochondria and in mitochondria in situ are identical and thus independent of the change in mitochondrial outer membrane permeability; in both cases it was close to 0.9 mM (Table 2).

Decreased affinity of MtCK for exogenous MgATP in situ may be explained by the existence of strong restrictions of MgATP diffusion at the level of mitochondrial outer membrane, or within ICEU structures

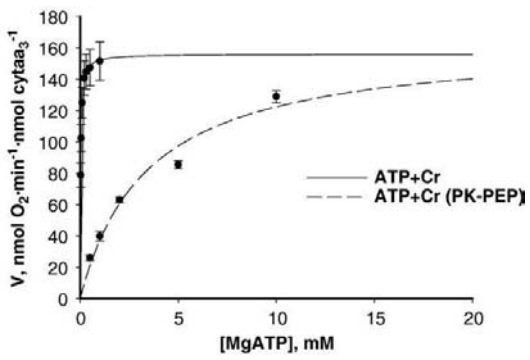

Fig. 7. Summary of the kinetics of regulation of mitochondrial respiration in situ in permeabilized cardiomyocytes by creatine in the presence of ATP without or with the PEP–PK system. The curve solid lines represents rate of respiration stimulated by endogenous ADP produced by ATPases from exogenous ATP and by creatine kinases via in the presence of 20 mM creatine (from Fig. 3A). The curve with dotted line represents rate of respiration stimulated by creatine via activated MtCK, while the endogenous ADP fluxes are excluded by the PEP–PK system (from Fig. 6A).

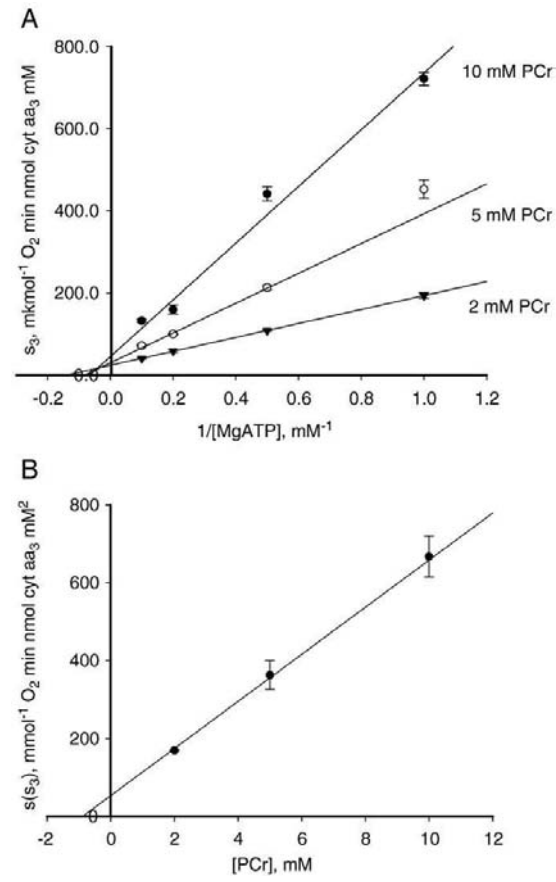

Fig. 8. (A) Competitive inhibition of MtCK in permeabilized cardiomyocytes by phosphocreatine. The slopes of straight lines obtained in double reciprocal plots of 1/v versus 1/[MgATP] for 9 mM of creatine (see Fig. 6B) for three different pfosphocreatine concentrations. (B) Secondary linearization of slopes of straight lines from panel A as a function of concentration of PCr provides value of K_{ip} (Eqs. 10 and 11 in Materials and methods).

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near mitochondria. The unchanged constant of dissociation for PCr (K_{ip}) and decreased constant of dissociation for creatine (K_{ib} and K_b) evidence on the other hand the lack of these restrictions for creatine and PCr. Therefore it was interesting and important to verify that PCr produced by MtCK easily diffuses into cytoplasm. Therefore, we measured directly the rate of PCr production in mitochondria and its transfer into the cytoplasm (surrounding medium) under these experimental conditions in the presence of creatine and the PEP–PK system described in Fig. 4. Measurements were realised in the same conditions as kinetic studies on respiration: i.e. activation of mitochondrial respiration by the addition of creatine (20 mM) in the presence of fixed amounts of MgATP (5 mM) and the PEP–PK system. The samples were taken 3, 6 and 10 min after activation of the MtCK reaction by creatine. After the treatment described above (see Materials and methods) the concentrations of ATP, PCr and creatine in the medium were measured with UPLC. Concentrations of PCr increase with time, while ATP concentrations stay always constant (Fig. 9A). The PCr in cardiac cells can be produced by different isoforms of creatine kinase. To ascertain which amount of PCr content in the samples is of mitochondrial origin, the oxidative phosphorylation was inhibited by 10 mM rotenone (Fig. 9A). The difference of the rates of PCr synthesis in the absence and presence of rotenone is due exclusively to the activation of mitochondrial synthesis of PCr in MtCK reaction (Fig. 9B). From this rate and from V_{O2} measured in the parallel experiments, the PCr/O_2 ratio can be calculated. The PCr/O_2 ratio allows to quantitatively evaluate the efficiency of free energy conversion in the coupled MtCK–ANT-oxidative phosphorylation reactions. The high value of PCr/O_2 equal to 5.7 ± 0.7 is remarkably close to that found by Belitser and Tsybakova in 1939 and shows that all mitochondrial ATP is rapidly used up for PCr synthesis which does not accumulate in the intermembrane space but easily leaves mitochondria. This means that the VDAC permeability is high for PCr and changes selectively by mitochondrial–cytoskeleton interactions for adenine nucleotides.

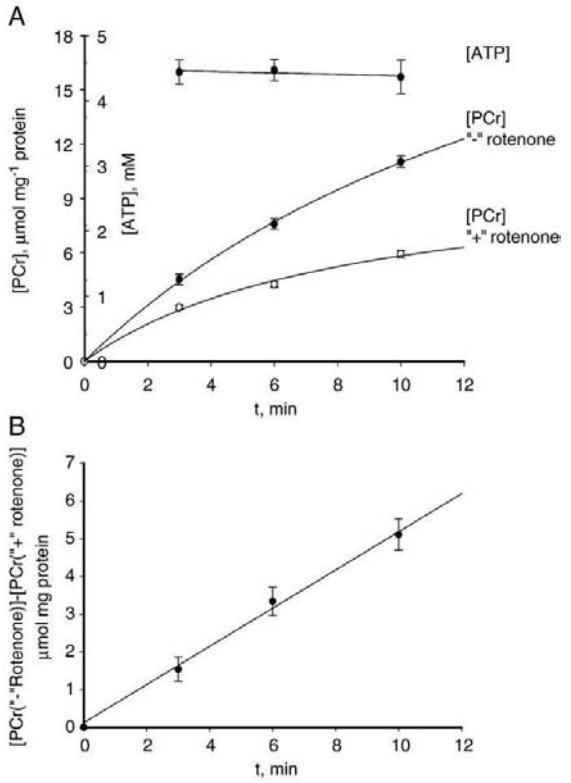

Fig. 9. (A) The rate of phosphocreatine production by mitochondrial and cytoplasmic creatine kinases in permeabilized non-inhibited cardiomyocytes (●). Analysis of samples of reaction mixture taken at 3, 6 and 10 min after activation of MtCK by creatine (20 mM) in permeabilized cardiomyocytes in the presence of MgATP (initially added to about 5 mM) and PEP (5 mM) and PK (20 U/ml) (see Fig. 4) were performed by using ion pair HPLC/UPLC as described in Materials and methods. The ATP level, continuously regenerated by the PEP–PK system, was stable during the experiment. When oxidative phosphorylation is inhibited by rotenone (○), the PCr can be produced only by cytoplasmic creatine kinases, MMCK. (B) The difference in phosphocreatine production rates under conditions of activated and inhibited (by rotenone) respiratory chain calculated from panel A. In separate experiments the oxygen consumption rates were measured, the creatine activated respiration rate in these experiments was 0.088 ± 0.007 $\mu molO_2\ min^{-1}\ mg^{-1}$protein. These data give the ratio of $PCr/O_2 = 5.7 \pm 0.7$.

4. Discussion

The results of this study show very clearly that the mechanisms of the regulation of mitochondrial respiration and energy fluxes in the cardiac cells are system-level properties dependent on the interaction of mitochondria with intracellular structures and functional interactions with metabolic systems including glycolysis, which are not predictable on the basis of properties of isolated mitochondria only. Cytoskeletal components like tubulin, and probably also desmin, plectin and others which are responsible for regular arrangement of mitochondria in cardiac cells and are sensitive to proteolysis most probably control also the permeability of VDAC in MOM [91–93]. The results of our study show that these proteins selectively limit the VDAC permeability, decreasing it mostly for ATP and ADP but not for creatine or phosphocreatine. Strongly decreased permeability of MOM for adenine nucleotides significantly enhances the functional coupling between MtCK and ANT increasing the rate of recycling of ADP and ATP in mitochondrial matrix-inner membrane space. Especially interesting and important is the significantly enhanced apparent affinity of MtCK for creatine in the cells in situ.

For some unknown reasons, numerous groups of investigators still consistently insist on the validity of the concept of the creatine kinase equilibrium in muscle cells which is taken as homogenous medium [18–20,22,23,26,27,45–47,100,101] and support the point of view that simple calculations of cytoplasmic ADP concentration and related parameters such as free energy of ATP hydrolysis in homogeneous intracellular medium are sufficient to explain the mechanisms of regulation of energy fluxes in the cells [23,47]. However, there are surprising and very obvious controversies in this point of view. It was already mentioned above that under conditions of metabolic stability the ADP concentration calculated from CK equilibrium is also constant, while the rate of oxygen consumption changes [37]. The calculated ADP concentration in resting heart cells is in the range of 50–100 μM [102]. If mitochondria in the cells behave as they do in vitro and ADP freely diffuses between cytoplasm and mitochondrial intermembrane space, ANT with its apparent K_m(ADP) around 10 μM [82] should be always almost saturated and respiration rate in the resting state almost maximal with glucose as substrate when Pi is also elevated [103]. However this is not true; heart respiration rate in the resting state is only 5% of V_{max} [37]. Low respiration rate of the heart in the resting state is well explained by decreased affinity (increased apparent K_m) for cytoplasmic ADP measured in experiments with permeabilized cells. This means that ADP diffusion across outer mitochondrial membrane is rather limited. The results of current study directly confirm this conclusion: while in isolated mitochondria the PEP–PK system decreases creatine-activated respiration rate by about 50% (Fig. 2A), in permeabilized cardiomyocytes the effect of PEP–PK is practically absent (Fig. 3B) but observed again after selective proteolysis (Fig. 3C).

Thus, the results of this work and many others show that the assumption of the creatine kinase equilibrium is an unnecessary

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limitation [104]. Our data show that effective phosphocreatine production may occur in mitochondria with the rate close to maximal activity of MtCK and ATP synthesis, and it has been shown in direct measurements with ^{31}P NMR inversion transfer that in different cellular compartments in hearts the creatine kinase isoenzymes function in steady state in the direction dependent on their location and functional coupling either with oxidative phosphorylation via ANT in mitochondria (as MtCK), or with MgATPases in myofibrils and cellular membranes (as MMCK bound to these structures) [98]. Only in cytoplasmic compartment creatine kinase may approach quasi-equilibrium in the resting state, especially in resting glycolytic muscles with very high cytoplasmic CK activity. This conclusion is confirmed by the results of a mathematical model of compartmentalized energy transfer for analysis of experimental data (see below).

4.1. Peculiarities of the kinetics of regulation of respiration coupled to the MtCK reaction in mitochondria in situ

Classical works by Lardy and Wellman [105], Britton Chance [106,107] and many others [24] have established that the rate of mitochondrial respiration in isolated mitochondria in vitro is strictly regulated by availability of ADP for ANT in the mitochondrial inner membrane (MIM). In isolated mitochondria in vitro mitochondrial outer membrane is permeable for metabolites with molecular mass lower than 7 kDa due to the open state of the VDAC in MOM [88], and the efficiency of the regulation of mitochondrial functions in vitro by extramitochondrial ADP depending only upon the affinity of ANT for ADP, which is very high (the apparent K_m for ADP is in the range of 10–20 μM) [108].

However, in the cardiac and many other cells in vivo, mitochondria are involved in multiple structural interactions with other cellular structures, and functional interactions with other metabolic systems, such as the glycolytic system and cellular ATPases. All these interactions play important roles in the regulation of mitochondrial activities and energy fluxes in the cells, resulting in appearance of new, system-level properties. The interesting result is the strengthening of the role of functional coupling between MtCK and ANT in the regulation of energy fluxes and respiration.

Both the structure of MtCK and its interaction with ANT have been studied by using isolated and purified enzymes or isolated mitochondria. Classical studies by Theo Wallimann's group have given detailed structure of CK including MtCK and direct localisation of octameric MtCK close to ANT due to C-terminal ligand to cardiolipin negative charges [15,109–112]. The respiratory control by creatine was first discovered by Bessman and Fonio [113], Jacobus and Lehninger [114] and Vial [115]. All of these groups showed that in isolated heart mitochondria in the presence of ATP creatine exerts acceptor control of respiration by activating MtCK and supplying locally ADP for ANT. Quantitative analysis of this phenomenon showed that oxidative phosphorylation in mitochondria specifically accelerates the forward creatine kinase reaction of phosphocreatine production leading to an assumption of functional coupling between MtCK and ANT [75]. Jacobus and Saks have studied functional coupling between MtCK and ANT in isolated heart mitochondria by kinetic methods [82]. Then, the functional coupling has been shown repeatedly in kinetic [94] and thermodynamic experiments [96], directly confirmed by tracer and other methods by many independent groups [116–118]. One of the most important works was done by Barbour et al. who directly showed, using radioactive ADP, the recycling of ADP and ATP due to functional coupling of MtCK and ANT [116]. Bessman's laboratory showed the interaction of mitochondrially bound creatine kinase with oxidative phosphorylation by measuring the isotope incorporation into newly synthesized ATP and PCr [117,118] and thus also confirmed the functional coupling of MtCK with ANT. Detachment of MtCK from its binding site on mitochondrial membranes into intermembrane space results in loss of the effects of oxidative phosphorylation on the kinetics of MtCK reaction even in the presence of intact MOM [94]. Monoclonal inhibitory antibodies against MtCK have been shown to inhibit also the ADP/ATP exchange in mitoplasts [119]. Kim and Lee [120] showed that isolated pig heart mitochondria can form phosphocreatine continuously in the respiration medium without externally added adenine nucleotides, due to rapid recycling of their trace amounts in mitochondrial creatine kinase-oxidative phosphorylating system functionally coupled via the action of the adenine nucleotide translocase. Similar data were reported by Dolder et al. [10]. The functional coupling of MtCK and ANT has been found to be vital for protection of mitochondria from permeability transition pore (PTP) opening and from production of reactive oxygen species, ROS. Dolder et al. showed in experiments with the transgenic liver-MtCK mice that mitochondria are protected from PTP opening via functional coupling of the MtCK reaction to oxidative phosphorylation [10]. Meyer et al. demonstrated that in the presence of creatine, MtCK coupled to ANT increases recycling of adenine nucleotides in mitochondria, accelerates respiration, thus diminishing the reduced state of electron carriers and production of oxygen radicals, ROS, that are further converted to H_2O_2 by superoxide dismutase [121]. All these multiple works from different laboratories show the tight functional coupling of MtCK and ANT in heart and skeletal mitochondria. Similar coupling exists in brain mitochondria [80]. Vendelin et al. applying mathematical modelling, analyzed in details the mechanism of this functional coupling, showing that the direct ATP transfer from ANT to MtCK is more important for accelerated PCr production and respiratory control than dynamic ADP compartmentation in the intermembrane space proposed by Gellerich [8,85,86]. However, in cells in vivo this dynamic compartmentation of ADP may become an important additional factor of functional coupling between MtCK and ANT due to interaction of MOM with cytoskeletal elements which selectively limit VDAC permeability in cells in situ [91]. Numerous laboratories have recorded manifold differences in mitochondrial apparent affinity for free exogenous ADP in vitro (K_m^{app} for free ADP ~10 μM), in permeabilized cardiomyocytes in situ (K_m^{app} ~350 μM) and in cardiomyocytes pre-treated with trypsin (K_m^{app} ~70–100 μM) [50–53,55–65,77]. In the reconstituted complete system of mitochondria in situ in cardiac permeabilized cells complemented with glycolytic ADP-trapping system (Scheme 4) the apparent kinetics of the MtCK dependent respiration regulation is totally different from that seen in mitochondria in vitro. In fact, there are two remarkable differences. The first is the decrease in apparent affinity of MtCK for exogenous MgATP (apparent K_a increased more than 100 times, Table 2) in mitochondria in situ as compared to in vitro, most probably due the enhanced restriction of MgATP diffusion within organized structures of intracellular energetic units, ICEUs, most probably locally at the level of MOM. Due to the control of VDAC permeability by cytoskeleton, apparent affinity of MtCK to exogenous (cytoplasmic) MgATP is strongly decreased. In vivo the elements of cytoskeleton, most probably tubulin and some other proteins of this network, limit VDAC permeability. Rostovtseva et al., showed very recently that dimeric tubulin in nanomolar concentrations induce highly voltage sensible reversible closure of VDAC reconstituted into planar phospholipid membrane [92,93]. Added to isolated brain or heart mitochondria tubulin induces decrease of apparent affinity of ANT for free ADP (increase of the K_m^{app} ADP from ~10 μM to ~169 μM for isolated brain mitochondria and for sample supplemented with tubulin respectively) [80,92,93]. Strong diffusion restriction at the level of MOM in vivo increases the effective adenine nucleotides micro-compartmentation within intermembrane space and influences the respiratory control of oxidative phosphorylation.

Diffusion restrictions for ATP in permeabilized cardiac fibers have been registered also by Ventura-Clapier's group in studies of calcium uptake by sarcoplasmic reticulum (SR): they found that this uptake was much more effective when supported by mitochondrial oxidative phosphorylation or by PCr than in the presence of exogenous ATP

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[122]. These data show the restrictions of diffusion of adenine nucleotides into some important areas of ICEUs. In their experimental protocol SR were preloaded by calcium during 5 min period of preincubations and caffeine-induced release of calcium seen as contraction transients were measured [122]. Since both parameters depend on local ATP/ADP ratios, both different rates of ADP removal and ATP supply in these local areas explain these results. The authors concluded by comparing the results of separate experiments with oxidative phosphorylation and exogenous ATP that ATP fluxes from mitochondria are equal to fluxes of PCr in energy supply to cytoplasm [122]. However, the kinetic studies of ATP fluxes were not performed in these works. These fluxes were measured in the present study as described in Fig. 9, and the measured PCr/O_2 ratio equal to 5.7 leaves little room for ATP fluxes in energy supply into cytoplasm in the presence of creatine when MtCK coupled to ANT is activated.

The second remarkable difference observed in this work is that apparent constant of dissociation of creatine from the binary complex with MtCK (K_{ib}) decreases about 10 times in mitochondria in situ, in permeabilized cardiomyocytes, as compared with isolated mitochondria (Table 2). At the same time, the apparent affinity of MtCK for phosphocreatine is similar in vitro and in situ (K_{ip} is about 2 mM, Table 2). Thus, there is no diffusion restriction for diffusion of these guanidino substrates across MOM into intermembrane space where MtCK is located. The remarkably high affinity of MtCK in mitochondria in situ for creatine may be a result of specific conformational state of the enzyme dependent of both configuration of cristae surface where MtCK is localized, and ATP supply by ANT. Recently Hornikova et al. have shown the importance of the binding of MM–CK to myofibrils for maintaining active conformation the presence of substrates [123]. The substrate-induced conformational changes are required in order to bring the substrates closer to each other for spontaneous catalysis [123]. We may assume that similar conformation changes may occur also in the MtCK in situ resulting in increased affinity for creatine when MtCK is continuously supplied by mitochondrially produced ATP. Vendelin et al. found that functional coupling of MtCK to ANT changes significantly the free energy profile of the MtCK reaction [8]. The precise mechanism of this important phenomenon needs, however, further detailed study by FRET and other available new methods.

As a result, creatine becomes one of the main regulators of the rate of coupled MtCK–oxidative phosphorylation reactions and of ADP/ATP turnover in mitochondria in cardiac cells in vivo, resulting in effective production of PCr. High $PCr/O_2 =$ 5.7 ratio found in experiments with permeabilized cells shows that all mitochondrially produced ATP is rapidly used up for PCr synthesis which easily leaves mitochondria (Fig. 9B). These results conform to the now commonly accepted theory of the role of PCr/CK phosphotransfer system [5–9,11,12,14–16,70,124,125] as the main pathway of energy transfer and feedback metabolic communication between mitochondria and cytoplasm in heart cells and further confirm its validity. All these are consistent with first classical observations by Belitzer and Tsybakova [4] and many other observations.

4.2. ADP flux amplified by coupled MtCK–ANT is a necessary component of metabolic feedback signaling

Our results allow us to make several important conclusions about the nature of metabolic feedback signaling between ATPases and mitochondria in the heart and probably skeletal muscle and the brain in vivo. This signaling seems to include three different components: Pi fluxes, changes in Cr/PCr ratios and fluxes of endogenous ADP strongly amplified by the functional coupling between MtCK and ANT [11,18,28,126,127].

The role of extramitochondrial MgADP fluxes in the regulation of mitochondrial respiration in the whole system (cardiac cell) can be seen from comparison of data from the difference of apparent kinetics of mitochondrial respiration controlled by activated MtCK in the presence and absence of free MgADP fluxes (Fig. 7, Schemes 3 and 4). In both situations endogenous extramitochondrial MgADP is produced by hydrolysis of exogenous MgATP in ATPase-reactions and by MM creatine kinases bound to myofibrils and sarcoplasmic reticulum. This MgADP is generated within ICEUs near mitochondria and diffuses into the intermembrane space even if VDAC permeability is restricted, and free ADP enters into the matrix for rephosphorylation. Creatine added under these conditions changes strongly the apparent affinity of mitochondrial respiration switching it from the control by endogenous extramitochondrial MgADP toward that produced in MtCK reaction (K_m^{app} for exogenous MgATP in the presence of creatine decreases from ~160 μM to ~24 μM), (Fig. 3A, Fig. 7). A similar tendency was observed when mitochondrial respiration was stimulated by exogenous ADP without and with creatine [49,71,91]. The apparent K_m for free ADP in the presence of creatine is typically ten times smaller than the K_m^{app} for ADP alone [49,71,91]. Kinetics becomes different when we eliminate extramitochondrial MgADP by trapping it in the PEP–PK reaction (Fig. 7, experimental model is represented in Scheme 4). One explanation could be that this difference is due to the inhibition of the MtCK reaction by its final product phosphocreatine, which as we can see in HPLC measurements (Fig. 9A) is accumulating in the experimental medium. The PCr production can be carried out by the forward MtCK and MMCK reactions. In our experiment, due to the PEP–PK system, the free MgADP is efficiently removed from the medium (Fig. 1B) and the extramitochondrial pool of free MgATP is maintained at a high stable level (Fig. 9A). Under these conditions and the stepwise increase of creatine concentrations, the PCr production by cellular creatine kinases increases (see Fig. 9B), showing that MOM is highly permeable to PCr. The constant of dissociation of PCr from MtCK·PCr complex (K_{ip}) is similar (~0.9 mM) for mitochondria in vitro and in situ in permeabilized cardiomyocyte, and thus does not depend on changes in MOM permeability (Table 2). At the same time, as we can see from Table 2 the apparent affinity of MtCK in situ for creatine increases manifoldly even in the presence of freely available PCr. Therefore the observed differences are not explained by inhibitory effects of PCr, which should result first in the increase of apparent dissociation constants for creatine, since they both have the same binding site in the active centre of MtCK. However, for future mathematical modelling we have to take into account the PCr production in the steady state by cellular creatine kinases, induced by our experimental conditions (Scheme 4).

Thus, the observed differences between systems III and IV (see Fig. 7) are most probably due to the presence of ADP fluxes in the absence of the PEP–PK system. The high respiration rate is maintained in situ in the absence of the PEP–PK system by endogenous MgADP but only if creatine is present to activate MtCK (Fig. 3A). Even under conditions of limited MOM permeability in vivo, in the presence of creatine the respiration is maximally activated due to amplification of stimulatory effect of MgADP from cytoplasm by the mechanism of metabolic channeling within coupled MtCK/ANT reactions [128]. Thus, the functional coupling is a powerful amplifier of the regulatory action of cytoplasmic MgADP. This conclusion is fully consistent with the results of the mathematical modelling of compartmentalized energy transfer [97,104,126,129]. This model quantitatively reproduced the linear dependence of oxygen uptake on workload (metabolic aspect of Frank–Starling mechanism) and showed firstly the remarkable stability of average values of ATP, phosphocreatine and creatine, (PCr/ATP ratio) when workload (MgATPase activity) was changed and secondly the significant workload (and VO_2) dependent variations of cytoplasmic ADP concentrations within the cardiac cycle. In the systolic phase the ADP level may be increased by a factor of 10 when VO_2 changed 10 times, induced by non-equilibrium behaviour of myofibrillar MMCK and MtCK [130]. These remarkable changes in MgADP concentrations are related to small cyclic changes (within the range of 5–10%) of creatine and PCr levels around their stable steady state levels [97,126]. These cyclic small scale changes in PCr and

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creatine have been recorded in several careful experiments with the use of gated ^{31}PNMR [131,132]. Application of the Metabolic Control Analysis to this model showed that respiration rate may be controlled by Pi and by changes in creatine and ADP concentration, their flux control coefficients depending on workload and corresponding steady state values of respiration rates [133].

Our experimental results described in this work are in concord with the predictions of the mathematical model of compartmentalized energy transfer and may be used for further corrections of parameters of this model [126] when it will be included into future general mathematical model of heart energy metabolism which is still absent. The complete mathematical model of mitochondrial processes of energy conversion is also needed for further quantitative analysis of experimental data reported in this work and calculation of metabolic fluxes across the mitochondrial outer membrane in vivo. Further, in our experiments the ATPase reactions were limited by the lack of Ca^{2+} in solution. Further studies with different concentrations of Ca^{2+} to selectively activate MgATPases can better clarify the role of endogenous extramitochondrial ADP fluxes in regulation of mitochondrial respiration in situ. The oscillations of ADP concentration may be synchronized with localized changes of Ca^{2+}in cytoplasm and in mitochondria, modifying the activity of the enzymes of mitochondrial systems to increase the rate of ADP rephosphorylation and consecutively that of PCr production [126].

Our results are also consistent with important clinical observations described by Neubauer and others, who showed that in patients with heart failure myocardial ATP level remain normal (10 mM) until the advanced stage of disease, but the creatine and PCr levels decrease at earlier stages by 30–70% [125,134,135]. Thus the PCr/ATP ratio becomes a stronger predictor of mortality as a result of cardiovascular diseases than functional or clinical indexes [125]. All these changes can be explained by the important role of PCr in maintaining ATP in vitally important local cellular compartments, while the intracellular steady state concentration depend both on mitochondrial activities and intactness of functional coupling between MtCK and ANT.

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PUBLICATION III

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Direct measurement of energy fluxes from mitochondria into cytoplasm in permeabilized cardiac cells *in situ:* some evidence for mitochondrial interactosome

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Abstract The aim of this study was to measure energy fluxes from mitochondria in isolated permeabilized cardiomyocytes. Respiration of permeabilized cardiomyocytes and mitochondrial membrane potential were measured in presence of MgATP, pyruvate kinase – phosphoenolpyruvate and creatine. ATP and phosphocreatine concentrations in medium surrounding cardiomyocytes were determined. While ATP concentration did not change in time, mitochondria effectively produced phosphocreatine (PCr) with PCr/O_2 ratio equal to 5.68±0.14. Addition of heterodimeric tubulin to isolated mitochondria was found to increase apparent Km for exogenous ADP from 11±2 μM to 330±47 μM, but creatine again decreased it to 23±6 μM. These results show directly that under physiological conditions the major energy carrier from mitochondria into cytoplasm is PCr, produced by mitochondrial creatine kinase (MtCK), which functional coupling to adenine nucleotide translocase is enhanced by selective limitation of permeability of mitochondrial outer membrane within supercomplex ATP Synthasome-MtCK-VDAC-tubulin, Mitochondrial Interactosome.

Keywords Respiration · Cardiomyocytes · Mitochondria · Creatine kinase · Creatine · Phosphocreatine · Tubulin

Introduction

Mitochondrial respiration, coupled to production of ATP and fine regulation of energy fluxes to the sites of ATP utilization are vital for normal cell life. In spite of the fundamental progress of knowledge of mitochondrial bioenergetics (Nicholls and Ferguson 2002), the nature of respiratory control and in more general sense, the mechanisms of regulation of energy fluxes during workload changes in the cardiac and other cells *in vivo* are still highly debated (Balaban 2009; Beard 2005, 2006; Guzun et al. 2009; Saks et al. 2007a, 2006, 2007c; Van Beek 2007, 2008; Vendelin et al. 2000; Wu et al. 2007, 2008). Intensive studies during several decades have accumulated an abundance of data showing compartmentation of adenine nucleotides and the role of phosphotransfer networks in energy transfer (Dzeja et al. 2007; Dzeja and Terzic 2003; Saks et al. 2008, 2007a, 2006, 2007c, 2004, Schlattner and Wallimann 2004; Schlattner et al. 2006; Vendelin et al. 2004a; Wallimann et al. 1992, 2007; Wyss et al. 1992). Of major importance are the creatine kinase – phosphocreatine circuit (or shuttle) which includes both mitochondrial creatine kinase (MtCK) functionally coupled to the oxidative phosphorylation via adenine nucleotide translocase (ANT) and MM isoform of creatine kinase coupled to MgATPase reactions in myofibrils and at cellular membranes, and the adenylate kinase shuttle (Dzeja et al. 2007; Dzeja and Terzic 2003; Saks et al. 2008, 2007a, 2006,

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2007c, 2004, Schlattner and Wallimann 2004; Schlattner et al. 2006; Vendelin et al. 2004a; Wallimann et al. 1992, 2007; Wyss et al. 1992). However, in very many other works, while explaining respiration regulation the cell is often considered as a homogeneous reaction medium, thus ignoring the impact of the high degree of structural organization of the cell, in particular cardiomyocytes, macromolecular crowding phenomena etc. (for critical review see Saks et al. 2008). Usually, in these works the creatine kinase system is either totally ignored (Hom and Sheu 2009) or taken to be a cytoplasmic reaction in equilibrium providing researchers a simple method of calculation of ADP concentration and then free energy of ATP hydrolysis (Beard 2005; Wu et al. 2008). In many of these works, ATP utilization is taken to be matched to its production by changes in cytoplasmic calcium concentration (Balaban 2009; Hom and Sheu 2009). There are other works to propose the compromise that both pathways of energy transfer – by phosphotransfer networks and direct diffusion of ATP – play equal roles, both carrying 50 % of energy fluxes out of mitochondria (Joubert et al. 2008; Kaasik et al. 2001). To solve these controversies, in this work we measured directly the energy fluxes from mitochondria in permeabilized cardiomyocytes *in situ* under conditions close to those *in vivo* – in the presence of ATP, creatine and the glycolytic system (represented by pyruvate kinase and phosphoenolpyruvate) for trapping free ADP produced by MgATPases. Changes in ATP and phosphocreatine contents in the surrounding medium were measured by HPLC/UPLC technique and respiration rates were measured by oxygraphy. The rates of PCr production and respiration were used to determine the PCr/O_2 ratios to evaluate quantitatively the energy fluxes carried out in mitochondria *in situ* by PCr. The results show that at any initial ATP concentration high rates of respiration were maintained by MtCK reaction, the high PCr/O_2 ratios being close to the theoretically maximal value of P/O_2 equal to 6 (Nicholls and Ferguson 2002) showing directly that under physiological conditions the main carriers of energy into cytoplasm are phosphocreatine molecules.

Materials and methods

Experimental protocols

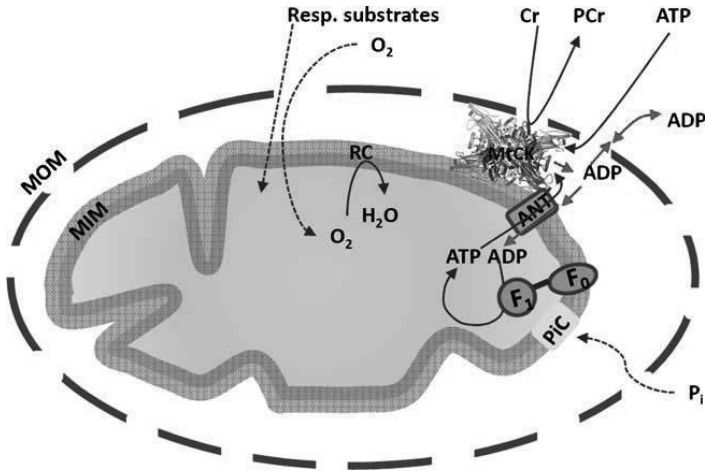
The principles of this study are illustrated by Schemes 1 and 2. Scheme 1 represents isolated mitochondria *in vitro* when mitochondrial creatine kinase is activated by addition of creatine, and Scheme 2 shows mitochondrion *in situ,* in permeabilized cardiac cells, surrounded by cytoskeleton proteins (depicted as "X" factor) and myofibrils. The respiratory chain (RC) complexes, ATP synthase (F_1F_0) and Pi carrier PIC are integrated within the mitochondrial inner membrane (MIM). Mitochondrial creatine kinase (MtCK) is depicted as an octamer, located in the mitochondrial intermembrane space (IMS) and attached to the inner membrane surface. In our experiments MtCK is activated by creatine (Cr) in the presence of ATP. The final products of MtCK-forward reaction are phosphocreatine (PCr) and endogenous ADP. The MOM is less permeable than in isolated mitochondrion, due to the interactions of VDAC with cytoskeleton proteins. Exogenous ATP is hydrolyzed by cellular ATPases into endogenous extramitochondrial ADP and inorganic phosphate (Pi). Mitochondrial (MtCK) and non-mitochondrial MM creatine kinases (cytosolic, myofibrillar, SERCA, sarcolemmal) activated by creatine in the presence of ATP, produce endogenous intra- and extramitochondrial ADP. Thus the oxidative phosphorylation is controlled by endogenous ADP produced by the MtCK, MMCK and ATPase reactions. The permeabilized cardiomyocytes were supplemented with phosphoenolpyruvate (PEP) and pyruvate kinase (PK). PEP-PK system removes extramitochondrial ADP produced by intracellular ATP consuming reactions and continuously regenerates extramitochondrial ATP. Endogenous intramitochondrial ADP produced by MtCK forms microcompartments within the IMS and is re-imported into the matrix via adenine nucleotide translocase (ANT) due to its functional coupling with MtCK.

Isolation of mitochondria from cardiac muscle

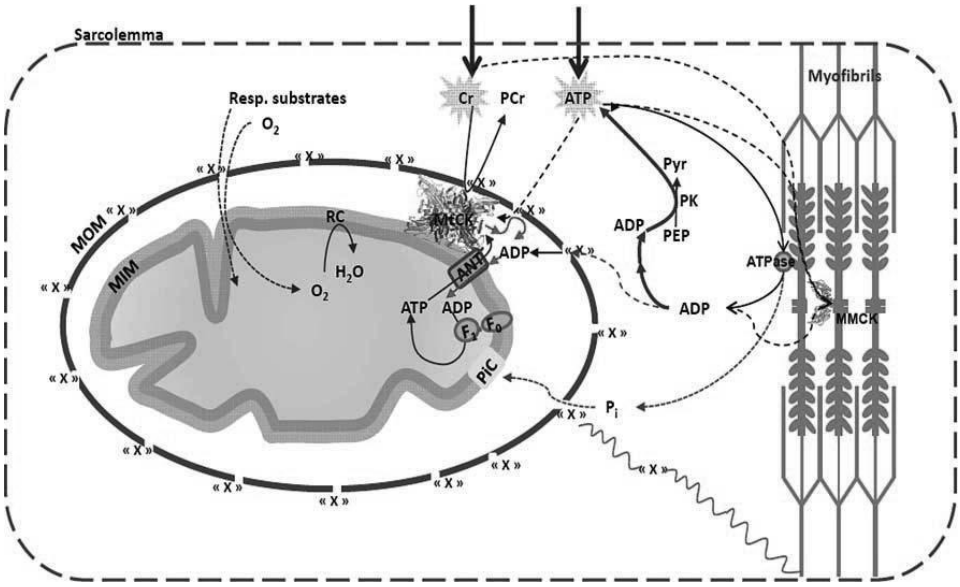
Mitochondria were isolated from adult rat hearts as described by Saks et al. 1975.

Isolation of adult cardiac myocytes

Adult cardiomyocytes were isolated after perfusion of the rat heart with collagenase using the adaptation of the technique described previously Saks et al. 1991. Wistar male rats (300–350 g) were anaesthetized with pentobarbital and de-coagulated using 500 U of heparin. The heart was quickly excised preserving a part of aorta and placed into isolation medium (IM) of the following composition: 117 mM NaCl, 5.7 mM KCl, 4.4 mM $NaHCO_3$, 1.5 mM KH_2PO_4, 1.7 mM $MgCl_2$, 11.7 mM glucose, 10 mM creatine, 20 mM taurine, 10 mM PCr, 2 mM pyruvate and 21 mM HEPES, pH 7.1. The excised rat heart was cannulated by aorta and suspended in Langendorf system for perfusion and washed for 5 min with a flow rate of 15–20 mL/min. The collagenase treatment was performed by switching the perfusion to circulating isolation medium supplemented with 0.03 mg/ml liberase Blendzyme I (Roche) and BSA 2 mg/ml at the flow rate of 5 ml/min for 20–30 min. The end of the digestion was determined



Scheme 1 represents a system related to isolated heart mitochondrion, used as a reference system in this work. The respiratory chain (RC) complexes, ATPsynthase (F_1F_0) and Pi carrier PIC are integrated within the mitochondrial inner membrane (MIM). Mitochondrial creatine kinase (MtCK) is depicted as an octamer, located in the mitochondrial inter-membrane space (IMS) and attached to the inner membrane surface. In the experiments MtCK is activated by creatine (Cr) in the presence of ATP. The final products of MtCK-forward reaction are phosphocreatine (PCr) and endogenous ADP. The ADP phosphorylation is visualized by recording the oxygen consumption. In scheme I endogenous intramitochondrial ADP produced by MtCK reaction forms a micro-domain within the intermembrane space. The micro-compartmentalized ADP can either enter into the mitochondrial matrix for phosphorylation or escape into the surrounding medium via voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane (MOM)



Scheme 2 represents a mitochondrion *in situ*, in a permeabilized cardiac cell, surrounded by cytoskeleton proteins (depicted as "X" factor) and myofibrils. The MOM is less permeable than in isolated mitochondrion, due to the interactions of VDAC with cytoskeleton proteins. Exogenous ATP is hydrolyzed by cellular ATPases into endogenous extramitochondrial ADP and inorganic phosphate (Pi). Mitochondrial (MtCK) and non-mitochondrial MM creatine kinases (cytosolic, myofibrillar, SERCA, sarcolemmal) activated by creatine in the presence of ATP, produce endogenous intra- and extramitochondrial ADP. The system is supplemented with phosphoenolpyruvate (PEP) and pyruvate kinase (PK). PEP-PK system removes extramitochondrial ADP produced by intracellular ATP consuming reactions and continuously regenerates extramitochondrial ATP. Endogenous intramitochondrial ADP produced by MtCK forms microcompartments within the IMS and is re-imported into the matrix via adenine nucleotide translocase (ANT) due to its functional coupling with MtCK

following the decrease in perfusion pressure measured by a manometer. After the digestion the heart was washed with IM for 2–3 min and transferred into IM containing 20 μM $CaCl_2$, 10 μM leupeptin, 2 μM STI and 2 mg/ml fatty acid free BSA. The cardiomyocytes were then gently dissociated using forceps and pipette suction. Cell suspension was filtered through a crude net to remove tissue remnants and let to settle for 3–4 min at room temperature. After 3–4 min the initial supernatant was discarded and the pellet of cardiomyocytes resuspended in 10 ml of IM containing 20 μM $CaCl_2$ and the protease inhibitors. This resuspension-sedimentation cycle with calcium-tolerant cells was performed twice. After that cardiomyocytes were gradually transferred from 20 μM Ca^{2+} IM into free calcium Mitomed (supplemented with protease inhibitors and BSA) and washed 5 times. Each time, slightly turbid supernatant was removed after 4–5 min of the cells' sedimentation. Isolated cells were re-suspended in 1 – 2 ml of Mitomed solution (Kuznetsov et al. 2008) described below for respiration measurements and stored on ice during measurements. Isolated cardiomyocytes contained 70–90% of rod-like cells when observed under the light microscope.

Permeabilization procedure

In order to study the kinetics of regulation of mitochondrial respiration in cardiomycytes using different metabolites, the cells sarcolemma was permeabilized by saponin keeping the mitochondrial membranes intact (Kuznetsov et al. 2008; Saks et al. 1998b). The tests for intactness of the outer and inner mitochondrial membranes are described in "Results" section. The permeabilization procedure was carried out directly in an oxygraph chamber with 25 μg/mL saponin during 10 min before starting measurements of respiration rates at 25°C and continuous stirring.

Measurements of oxygen consumption

The rates of oxygen uptake were determined with a high-resolution respirometer Oxygraph-2K (OROBOROS Instruments, Austria) in Mitomed solution (Kuznetsov et al. 2008) containing 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 3 mM KH_2PO_4, 20 mM taurine, 20 mM HEPES, 110 mM sucrose, 0.5 mM dithiothreitol (DTT), pH 7.1, 2 mg/mL fatty acid free BSA, complemented with 5 mM glutamate and 2 mM malate as respiratory substrates.

Measurements were carried out at 25°C; solubility of oxygen was taken as 240 nmol/ml (Gnaiger 2001).

In kinetic experiments with different fixed MgATP concentrations, a stock solution of 100 mM MgATP was prepared by mixing equimolar amounts of $MgCl_2$ and ATP, pH was adjusted to 7.2.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi$m) was measured by a spectrofluorimeter (F 2500 DIGILAB, HITACHI, Tokyo, Japan) with a fluorescent cationic dye tetramethylrodamine methyl ester (TMRM) according to the protocol decribed earlier (Freedman and Novak 1989). This indicator dye is a lipophilic fluorescent cation which passes cellular and mitochondrial membranes and accumulates within the mitochondrial matrix in a $\Delta\Psi m$-dependent manner, its fluorescence intensity decreases when the dye is accumulated by mitochondria (Nicholls and Ferguson 2002). The decrease of fluorescence intensity shows mitochondrial energization and is proportional to $\Delta\Psi$m. This property has been used to dynamically monitor $\Delta\Psi m$ in mitochondria *in situ* in permeabilized cardiomyocytes. The excitation wavelength was 548 nm and emission wavelength 574 nm. Data are reported as arbitrary fluorescence units (AFUs). TMRM was obtained from FluoProbes®, Interchim, France, dissolved in DMSO to a concentration of 1 mM. Aliquots of this stock solution were diluted in Mitomed solution described above and used in a final concentration of 0.2 μM.

The measurements of changes in $\Delta\Psi$ in isolated mitochondria and in mitochondria *in situ* in permeabilized cardiomyocytes induced by substrates, MgATP, PK-PEP system and creatine were performed in the same medium as described above for measurements of oxygen consumption (see above). Shortly, permeabilized cardiomyocytes (or isolated mitochondria) were incubated with 0.2 μM TMRM. This stage corresponds to zero polarization of mitochondrial inner membrane or to State 1 of respiration according to Chance (Chance and Williams 1956). Addition of respiratory substrates (5 mM Glutamate and 2 mM Malate) induces polarization of the mitochondrial inner membrane (decrease of AFU due to the accumulation of TMRM inside the matrix). This energy state corresponds to State 2 of respiration according to Chance. The subsequent addition of 2 mM ATP followed by 20 U/mL PK and 5 mM PEP (ADP-trapping system) should induce maximal energization of mitochondria, which corresponds to state 4 respiration. Creatine (10 mM) added in the presence of MgATP and the trapping system for free ADP activates MtCK reaction. Intramitochondrial ADP produced by activated MtCK is expected to decrease $\Delta\Psi$m due to its use for ATP synthesis respiration (State 3 according to Chance) and ADP/ATP translocation, both dependent on MtCK-ANT functional coupling. The experiment is terminated by addition of 5 μM uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) which provokes the $\Delta\Psi$m collapse and thus allows zero level of membrane potential.

Measurements of mitochondrial cytochromes content

For comparative quantitative analysis of the kinetics of regulation of respiration in isolated mitochondria and permeabilized cardiomyocytes, the respiration rates were expressed in nmoles of oxygen consumed per minute per nmoles of cytochrome aa_3, but not per mg of protein (if not indicated differently). Cytochrome aa_3 content in both cases is representative of the respiratory chain, while proteins contained in cardiomyocytes are not all present in mitochondria. The contents of mitochondrial cytochrome aa_3 in the isolated mitochondria and cardiomyocytes were measured spectrophotometrically according to the method described before (Fuller et al. 1985; Monge et al. 2008). The cells or mitochondria were solubilized with 1 % of sodium deoxycholate in phosphate buffer (100 mM KH_2PO_4, pH 8). The differential spectrum (reduced by dithionite *versus* oxidized cytochromes) was obtained by scanning from 400 to 650 nm using a Cary 50 Bio spectrophotometer (Varian, Palo Alto, USA) or Evolution 600 spectrophotometer (Thermo Electron Scientific Instruments, UK). Figure 1 shows the difference spectrum of cytochromes for isolated mitochondria. The value of peak at 605 nm was used for quantification of respiratory chain cytochrome aa_3 contents (cytochrome c oxidase) both in isolated mitochondria and cardiomyocytes using the extinction coefficient ε value equal to 24 $mM^{-1}.cm^{-1}$ (Monge et al. 2008; Van Gelder 1966). Protein concentrations were determined using a BCA protein assay kit (Pierce, USA) as a standard.

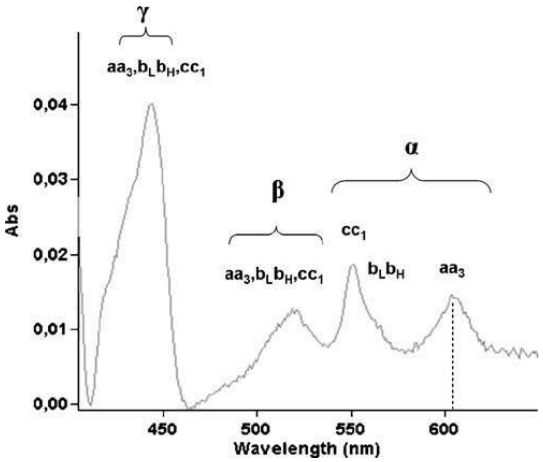


Fig. 1 The absorbance spectra of cytochromes (reduced versus oxidized), recorded by scanning the samples (isolated mitochondria or cardiomyocytes) from 530 to 650 nm in spectrophotometry

Isolation and purification of tubulin

Tubulin from rat brain and bovine brain was used with equivalent results. The bovine tubulin was obtained from Cytoskeleton (Boulter, CO, USA). The rat brain tubulin was purified as previously described (Sackett et al. 1991; Wolff et al. 1996). Frozen rat brains were thawed, homogenized in Assembly Buffer (0.1 M MES, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.9), and centrifuged at 100,000 g. Microtubule protein (tubulin plus microtubule associated proteins) was purified by several rounds of GTP-driven, temperature-dependent polymerization and depolymerization (Sackett et al. 1991). Tubulin was then purified from this material by selective polymerization in high buffer concentration, pelleted by centrifugation, redissolved in Assembly Buffer at 25 mg/ml, and drop frozen in liquid nitrogen (Wolff et al. 1996). In its final form the tubulin used was the $\alpha\beta$-heterodimer (Sackett et al. 1991; Wolff et al. 1996).

Reconstitution studies

Isolated and purified rat heart mitochondria (8 mg/ml) were incubated in Mitomed solution (see above) with 1 µM tubulin for 30 min at room temperature (22°C). After that, the samples were injected into an oxygraph chamber in presence or in absence of 20 mM creatine. Kinetics of activation of respiration were analyzed by successive addition of ADP (0.005–0.01–0.02–0.05–0.1–0.2–0.5–1–2–3 mM). Assay medium additionally contained 0.2% of serum bovine albumin and 1 IU/ml apyrase from potato (Sigma-Aldrich) as an ADP regeneration system. This isoenzyme of apyrase has an exceptionally high ATPase/ADPase ratio (10:1) and can be used for effective regeneration of ADP to maintain steady-state of respiration in the presence of limited amounts of ADP in kinetic studies.

Determination of the rate of PCr production in cardiomyocytes *in situ* by ion pair HPLC/UPLC

Determination of the rates of PCr synthesis in permeabilized cardiomyocytes *in situ* under conditions used in respirometry experiments was carried out using ion pair HPLC/UPLC by stopping the reaction typically at 3, 6 and 10 min. 100 µl aliquots of the reaction mixture were withdrawn and added to 200 µl ice-cold 1 M $HClO_4$ solution, immediately supplemented with 5 µl of 100 mM EDTA and neutralized with 210 µl of 0.952 M KOH in 0.5 – 1 min. The samples were held on ice for additional 10 – 15 min for proper precipitate formation and centrifuged at 16 000 g and 4ºC for 2 – 3 min. The supernatants were immediately frozen (−40˚C) and analyzed within 5 – 6 h.

Addition of EDTA (final 1 mM) proved to be useful in order to bind traces of Mg^{2+} to suppress any residual enzyme (particularly adenylate kinase, unpublished observations) activity and stabilize the preparations. Separations of Cr, PCr and adenine nucleotides were performed by ultra-performance ion-pair chromatography (UPLC) on a 2.1× 100 mm ACQUITY UPLC HSS T3 C_{18} column packed with 1.7 μm particles (Waters) by recording optical density simultaneously at 210 nm and 254 nm for creatine and PCr, and adenine nucleotides, respectively. Sample volumes of 10 μl were injected by autosampler. The mobile phase consisted of buffer A (20 mM KH_2PO_4, 3 mM tetrabutylammonium bisulfate (TBAS)) and buffer B (200 mM KH_2PO_4, 10% (v/v) acetonitrile, 0.3 mM TBAS), both adjusted to pH 5.0 with 2 M phosphoric acid and filtered through a 0.2 μm membrane filter. The elution was performed at a flow rate 0.4 ml/min in buffer A for 2 min followed by 1:1 gradient elution with buffers A and B up to 8.5 min and additionally with buffer B up to 10 min. After the analysis the column was re-equilibriated by washing for 1 min with water and buffer A for 9 min thus resulting in total time for an analysis 20 min. The column was periodically cleaned by washing with 80% methanol. The retention time for the reagents were defined/checked by measurements with the standard solutions prior to every test series Stock solutions for calibration (0.1 M) were prepared in 0.2 M KH_2PO_4 at pH 7.0 and stored at −40°C for not more than 2 – 3 days in order to minimize PCr and ATP degradation. Calibration solutions were prepared in supernatant solutions obtained after addition and precipitation of cardiomyocytes as described above.

Results

Figure 2a shows the classical respiratory control analysis. ADP in limited concentrations (0.2 – 0.4 mM) activates respiration but does not maintain the stable value of the rate of oxygen consumption by isolated mitochondria due to its rapid phosphorylation into ATP. As a result the fast transition of respiration from State 3 to the State 4 is observed (Fig. 2a). Addition of 20 mM creatine leads to stabilization of respiratory rate at the level close to the State 3 value (Fig. 2b). In this case a stable level of respiration is maintained by phosphorylation of endogenous ADP produced locally by activated MtCK. In accordance with many earlier data (Jacobus and Lehninger 1973; Meyer et al. 2006; Monge et al. 2008, Saks et al. 2007c, 1975, 2004), these results show that MtCK is able to maintain a maximal rate of respiration by supplying endogenous, locally produced ADP to ANT. Phosphocreatine produced in these coupled reactions leaves mitochondria via VDAC in the outer mitochondrial membrane. It was shown by Gellerich and Saks 1982 that part of ADP locally produced by MtCK in isolated mitochondria is equilibrated between intermembrane space and surrounding medium due to high permeability of the VDAC, but an equal amount of ADP is taken by ANT back to the mitochondrial matrix. This phenomenon can be easily revealed by addition of the ADP trapping system consisting of PK and PEP (Gellerich and Saks 1982; Gellerich et al. 1987, 2002, 2000). When respiration of isolated heart mitochondria was stimulated by creatine in the presence of ATP, addition of PK and PEP decreases the respiration rate to about 50 % of its maximal value. The remnant rate of respiration (up to 50% of VO_2max) was due to the functional coupling between MtCK and ANT with the direct transfer of ADP into the matrix (Vendelin et al. 2004a).

Movement of ADP across the outer membrane of isolated mitochondria can be limited by association of heterodimeric tubulin to VDAC (Monge et al. 2008; Rostovtseva and Bezrukov 2008; Rostovtseva et al. 2008). Under these conditions creatine effectively regulates respiration by increasing the rate of ADP-ATP recycling in the coupled MtCK-ANT system. The fact that tubulin in its dimeric form is able to interact directly with heart mitochondria was confirmed in experiments with isolated heart mitochondria (Monge et al. 2008), by a partial reconstruction of the cytoskeleton surrounding mitochondria. The results shown in Fig. 3 demonstrate that addition of tubulin (1 μM) to isolated heart mitochondria induce an increase in apparent Km for ADP from 11±2 μM to 330±47 μM. The Fig. 3a/b/c show oxygraph recordings of the activation of the mitochondrial respiration by exogenous ADP. Figure 3a is control kinetics of respiration regulation of isolated mitochondria. The maximal rate of respiration is observed in the presence of ADP at a concentration of 20 – 50 μM and the apparent Km is very low (11±2 μM), demonstrating the absence of diffusion barriers for ADP into the intermembrane space. Figure 3b shows the increase in diffusion constraints caused by tubulin obviously by direct interaction with VDAC in the outer mitochondrial membrane (Monge et al. 2008; Rostovtseva et al. 2008): respiration rate continues to increase even after addition of ADP at a concentration of 2 mM. In this case, kinetic analysis showed that in the presence of tubulin two populations of mitochondria with different apparent Km appear, one with very high apparent Km for exogenous ADP equal to 330±47 μM (Fig. 3d and e). In the presence of creatine (Fig. 3c) ADP again rapidly activated the respiration and only one population with an apparent Km equal to 23±6 μM was seen due to activation MtCK and increasing the recycling of ADP and ATP in mitochondrial matrix and inner membrane (Kim and Lee 1987; Meyer et al. 2006; Saks et al. 1998a, 1991, 1993). Evidently, creatine easily diffuses into the intermembrane

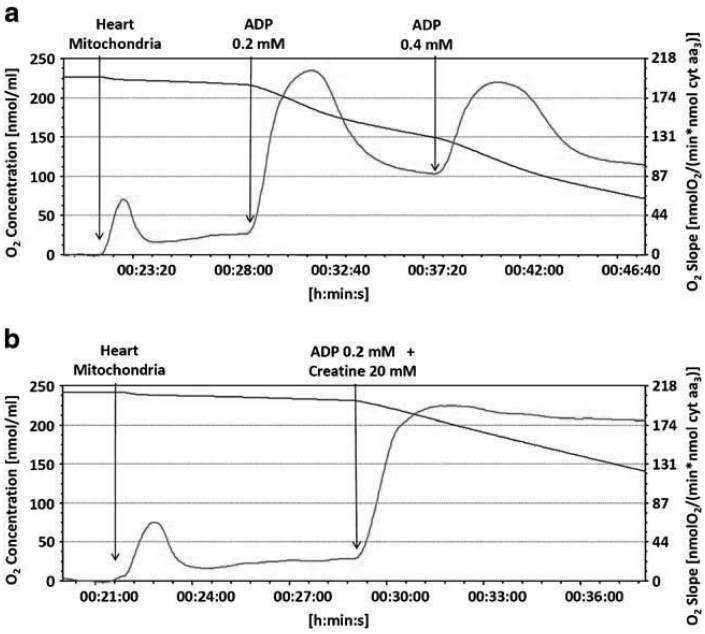


Fig. 2 **a** The classical respiratory control – transition between States 2, 3 and 4, according to Chance, in response to addition of limited amounts of ADP (0.2 mM; 0.4 mM) to isolated mitochondria, **b** Stable State 3 of respiration of isolated mitochondria stimulated by 0.2 mM ADP in the presence of 20 mM creatine. This stability is explained by the continuous production of endogenous ADP by mitochondrial creatine kinase (MtCK) reaction

space via the tubulin-VDAC complex (Rostovtseva et al. 2008) which in the mitochondrial membrane may include other cytoskeletal proteins (see "Discussion"). Thus, activation of the mitochondrial creatine kinase (MtCK) allowed overcoming the diffusion restriction for ADP provoked by the presence of tubulin in the vicinity of VDAC. The apparent Km for ADP in the control and in the presence of both tubulin and creatine are very close (Fig. 3d and e).

The experiments with isolated mitochondria and added tubulin shown in Fig. 3 reproduce well the kinetics of respiration regulation in permeabilized heart cells where the apparent Km for exogenous ADP is very high but decreased significantly by creatine (Appaix et al. 2003; Saks et al. 1998a, 1991, 1993, 1995).

To evaluate quantitatively the relative role of the phosphocreatine flux in energy transfer from mitochondria into cytoplasm in the cardiac cells *in vivo*, we used the permeabilized cardiac cells in combination with the added, exogenous PK-PEP system to simulate the interaction between mitochondria and glycolytic systems and their competition for extramitochondrial ADP. In permeabilized cardiac cells *in situ* in the presence of creatine and MgATP (see Scheme 2), MgADP is produced in the MgATPase reactions in myofibrils and sarcoplasmic reticulum (SR), in the MtCK reaction in the mitochondrial intermembrane space and in the MM creatine kinase reaction both in myofibrils and at SR membranes. If there is direct crosstalk between mitochondria and MgATPases by MgATP supply from mitochondria to ATPases and MgADP back as supposed in several studies (Joubert et al. 2008; Kaasik et al. 2001; Kuum et al. 2009), the PCr/O_2 ratio should be significantly less than the theoretically maximal P/O_2 ratio, which is equal to 6 (Nicholls and Ferguson 2002). Thus determination of PCr/O_2 ratio in a system described in Scheme 2 allows us to measure directly the energy fluxes between mitochondria and cytoplasm. Exogenous PK-PEP helps to keep extramitochondrial ADP concentration low and avoid rapid consumption of PCr in the coupled MMCK- MgATPase reactions.

Figure 4a shows a regular quality test for isolated cardiomyocytes used in this work. Addition of ADP in saturating concentration of 2 mM to permeabilized cardiomyocytes induced a State 3 high respiration rate. The respiratory control index usually exceeded 7 (Fig. 4a). Addition of exogenous cytochrome c did not increase the respiration rate, this showing the intactness of the outer mitochondrial membrane, and addition of atractyloside decreased the respiration rate close to the State 2 value, this showing the intactness of the inner mitochondrial membrane (Kuznetsov et al. 2008; Saks et al. 1998b). Only preparations with these characteristics were used in the experiments reported in this work.

Figure 4b shows the behaviour of the study system described by Scheme 2. Cardiomyocytes were permeabilized by saponin and State 2 respiration recorded. MgATP was added to a 2 mM final concentration to stimulate MgATPases, this increasing the respiration rate. This rate was decreased by addition of PK (20 IU/mL) in the

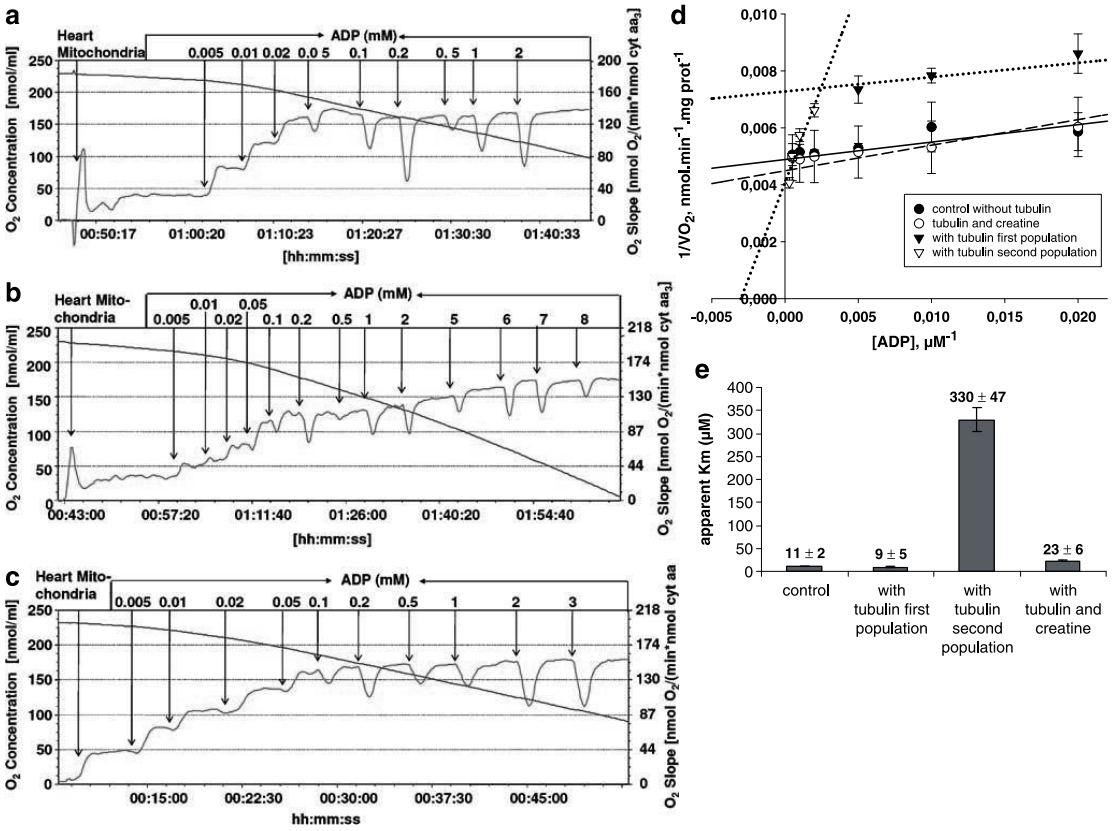


Fig. 3 **a** Oxygraph recording of the control kinetic of mitochondrial respiration activated by increasing concentrations of exogeneous ADP, **b** and **c** Oxygraph recording of the kinetics of mitochondrial respiration regulation in the presence of 1 μM tubulin (3B) and both 1 μM tubulin and 20 mM creatine (3C), **d** Double reciprocal representation (Lineweaver-Burk) of the kinetic of respiration regulation for isolated mitochondria (control, solid circles and straight line), with 1 μM tubulin (triangles and dotted lines) and with either 1 μM tubulin or either 20 mM creatine (empty circles and dashed line), **e** Comparison of the apparent Kms for exogenous ADP in the presence of tubulin and/or creatine. The values of the Kms indicated above the bars are in μM

presence of PEP (5 mM) due to trapping of a significant part of extramitochondrial MgADP. The respiration rate did not return to the State 2 level, this showing that some part of MgADP was channelled back to mitochondria. Subsequent addition of creatine rapidly increased the respiration rate. At a creatine concentration of 10 mM the maximal respiration rate was achieved; therefore, this concentration was used in further experiments. It was shown before that when respiration of mitochondria in permeabilized cardiomyocytes *in situ* is activated by creatine and MgATP (Guzun et al. 2009) and a high respiration rate is achieved, addition of PK – PEP does not result in a decrease of rate of oxygen consumption. That shows that ADP locally produced by MtCK in the intermembrane space is not accessible for exogenous ADP – trapping system, obviously, due to decreased permeability of VDAC as a result of binding of some cytoskeletal protein(s) to this channel. Selective treatment of permeabilized cardiomyocytes with trypsin to digest these proteins PK-PEP system again decreased the respiration rate, exactly as in isolated mitochondria in vitro (Guzun et al. 2009).

Table 1 summarizes the respiratory parameters of the isolated mitochondria and isolated cardiomyocytes. As it can be seen from Table 1, maximal respiration rates are equal both in isolated mitochondria and cardiomyocytes if calculated per nmol of cytochrome aa_3.

Since the mechanism of functioning of ANT is dependent upon and governed by the mitochondrial membrane potential $\Delta\Psi$ (Klingenberg 2008), it was important to record the changes in $\Delta\Psi$ under conditions described in Fig. 4b. The results of these measurements are shown in Fig. 5.

Figure 5 shows the recordings of changes in membrane potential in mitochondria *in situ* in permeabilized cardio-

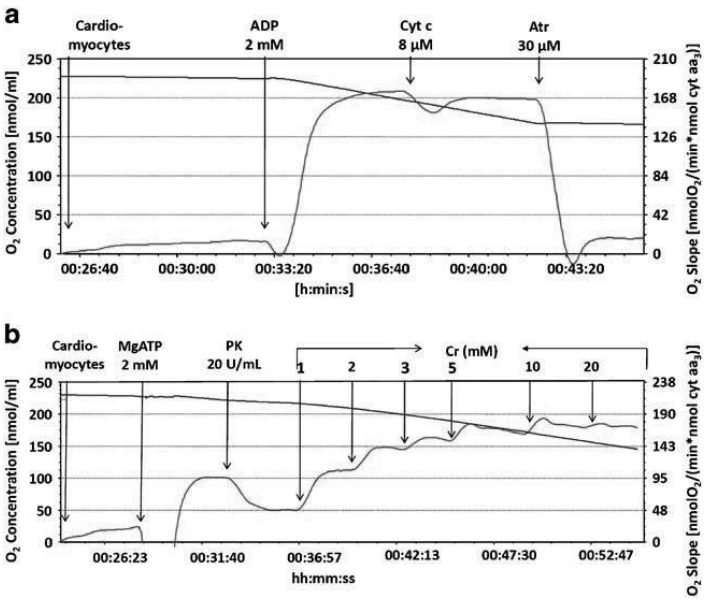


Fig. 4 **a** State 3 of mitochondrial respiration maintained in permeabilized cardiomyocytes. Cardiomyocytes were permeabilized with 25 µg/mL saponin in oxygraph cells during 10 min. Then respiration was activated in situ by addition of 2 mM exogenous ADP. Cytochrome c test shows intactness of MOM. Atractyloside test shows that respiration is totally controlled by ANT, **b** The respiration recording of the study system described by Scheme 2. Cardiomyocytes were permeabilized by saponin and State 2 respiration recorded. MgATP was added to 2 mM final concentration to stimulate MgATPases, this increasing the respiration rate. This rate was decreased by addition of PK (20 IU/mL) in the presence of PEP (5 mM) due to trapping of a significant part of extramitochondrial MgADP. Subsequent addition of creatine rapidly increased the respiration rate

myocytes. First, cardiomyocytes were permeabilized in fluorimeter cells into which TMRM and PEP were also added. Because of the presence of some endogenous substrates in cardiomyocytes, already some energization of the membrane was observed compared to the zero level at the end of experiments (Fig. 5). This energization was increased (fluorescence decreased) further after addition of glutamate and malate to induce the State 2 respiration. Subsequent addition of ATP did not lead to additional changes in membrane potential: energization of membrane due to the presence of ATP was equilibrated by ADP production in MgATPase reactions. Addition of PK induced transition into a true State 4 respiration and maximal energization of mitochondria due to effective removal of this extramitochondrial ADP. Addition of creatine in a final concentration of 10 mM induced a remarkable decrease in membrane potential and its transition to a new lower steady state level was observed. Addition of an uncoupler CCCP decreased the membrane potential to zero. These experiments show that MtCK in the presence of creatine effectively supplies local ADP to ANT which operates a in so called "productive" exchange mode (ADPin-ATPout) at a high value of $\Delta\Psi$ (as compared with the "unproductive" exchange ATPin-ADPout at low membrane potential) (Klingenberg 2008). These data directly show the effective functional coupling between ANT and MtCK.

Very interestingly, measurements of the respiration rates as a function of MgATP concentration at different steps of the experimental protocol described by Scheme 2 and in Figs. 4b and 5 gave remarkable and important results

Table 1 Basic respiration parameters of isolated rat heart mitochondria and of mitochondria in situ in permeabilized cardiomyocytes

Parameter	Mitochondria in vitro	Mitochondria in situ (permeabilized cardiomyocytes)
V_0,[a] $nmolO_2 \cdot min^{-1} \cdot mg\ prot^{-1}$	26.37±7.93	7.53±1.61
V_3[b], $nmolO_2 \cdot min^{-1} \cdot mg\ prot^{-1}$	187.94±40.68	84.45±13.85
[Cyt aa_3], $nmol \cdot mg\ prot^{-1}$	1.00±0.012	0.46±0.09
V_3, $nmolO_2 \cdot min^{-1} \cdot nmol\ cyt\ aa_3^{-1}$	187.94±40.68	178.23±33.96
$V_{Cr,ATP}$[c], $nmolO_2 \cdot min^{-1} \cdot nmol\ cyt\ aa_3^{-1}$	197.90±31.86	162.63±26.87

[a] V_0 respiration rate in State 2 in the presence of substrates before addition of ADP or ATP

[b] V_3 respiration rate in the presence of 2 mM ADP

[c] $V_{Cr,ATP}$ respiration rate in the presence of activated MtCK by 2 mM ATP and 20 mM creatine

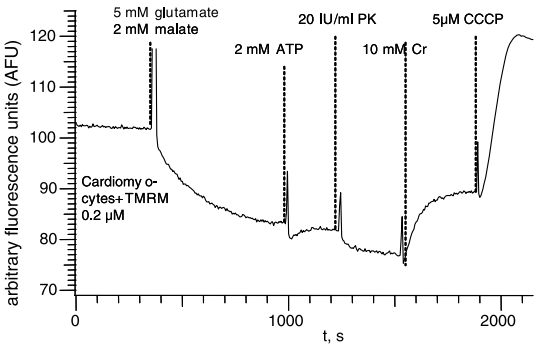


Fig. 5 Response of the TMRM fluorescence (excitation 548 nm, emission 574 nm) to mitochondrial respiration changes. Isolated cardiac cells were permeabilized with 25 μg/ml saponin and incubated in Mitomed solution supplied with 5 mM PEP and 0.2 μM TMRM in a thermostated fluorimeter cell. Addition of the substrates 5 mM glutamate, 2 mM malate caused a decrease in fluorescence indicating the accumulation of TMRM in mitochondrial matrix. 2 mM ATP produced a small change in mitochondrial membrane potential, $\Delta\Psi$. Activation of MtCK and mitochondrial respiration by addition of 10 mM creatine decreased $\Delta\Psi$ to a lower steady state level. The uncoupling agent CCCP 5 μM was used to dissipate the membrane potential

concerning the role of ATP, ADP creatine and also selective restriction of diffusion of adenine nucleotides in cardiomyocytes and particularly across the mitochondrial outer membrane in regulation of mitochondrial respiration *in vivo*. First, these measurements were made in the absence and presence of creatine before addition of PEP-PK system. The MgATP concentrations added in these experiments are shown at the lower abscissa axis in Fig. 6. In the absence of creatine, the respiration rate was increased in response to addition of MgATP due to activation of extramitochondrial MgATPases with the apparent Km equal to 158±40 μM in accordance with many earlier determinations (Saks et al. 2001; Seppet et al. 2001), but the Vmax value was low due to absence of calcium ions in these experiments. In the presence of creatine, the addition of MgATP very rapidly increased the respiration rate to its maximal value and the apparent Km for MgATP decreased to 24±0.8 μM. Under these conditions, MgADP is produced both extramitochondrially in the MgATPase and MM-CK reactions and in the MtCK reaction coupled to ANT (see Scheme 2). To differentiate between these two sources of MgADP, PEP and PK were added. This completely changed the kinetics of respiration regulation: for activation of respiration, addition of much higher concentrations of MgATP was needed; these concentrations are shown on the upper abscissa axis in Fig. 6. In these experiments, the apparent Km for MgATP was increased to 2 mM, in accordance with our recent observations (Guzun et al. 2009). Under these conditions, almost all extramitochondrial ADP is trapped and mitochondrial respiration is exclusively dependent upon ADP supply by MtCK (as seen in Fig. 5b), which is only slowly activated by exogenous MgATP most possibly by limited permeability of VDAC in permeabilized cells *in situ* (Guzun et al. 2009). Thus, for maximal activation of respiration, some extramitochondrial MgADP is needed, and in the presence of creatine rapid recycling of this ADP in the coupled MtCK- ATP Synthasome system maintains a high respiration rate (Guzun et al. 2009).

Based on the results of experiments shown in Fig. 6, in the next experiments MgATP was used in concentrations of 1, 2 and 5 mM in the presence of creatine (10 mM) and the PEP-PK system for determination of the PCr/O_2 ratio in permeabilized cardiomyocytes.

Determination of PCr/O_2 ratio was performed with the use of HPLC/UPLC technique for detection and quantification of the compounds of interest, PCr and ATP, in the reaction mixture and with separate measurements of corresponding oxygen uptake with a high-resolution ORO-BOROS respirometer. Under experimental conditions described above mitochondrial respiration was activated by addition of 10 mM Cr in the presence of fixed MgATP (1, 2, 5 mM) concentration, and extramitochondrial ADP produced by MMCK and ATPase was trapped by the PEP (5 mM) – PK (20 IU/mL) system (Fig. 4b and 5). The product mixture samples were collected at 3, 6 and 10 min

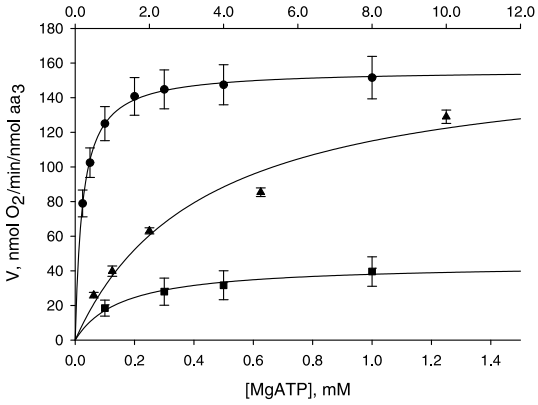


Fig. 6 Regulation of mitochondrial respiration as a function of the concentration of added exogenous MgATP in the absence (■) and the presence (●) of 20 mM Cr before addition of a PK-PEP system (bottom x-scale) and in the case of supplementation with PEP-PK system (▲, top x-scale). In the absence of PK-PEP system, the apparent affinity for exogenous MgATP without Cr (K_m^{app}=157,8± 40,1 μM), produced by hydrolysis in MgATPase reactions, is diminished due to addition of 20 mM Cr (K_m^{app}=24,9±0.8 μM). In the presence of PK-PEP system, apparent affinity for MgATP is significantly decreased (see the text). Maximum rate of respiration in all cases was similar. Addition of ADP-trapping PEP-PK system drastically changes the kinetics of regulation

after initializing MtCK reaction. Separation and analysis of the mixture of components were performed according to a standard HPLC/UPLC operating procedure (described in Materials and Methods section). The output presents a series of peaks located on the time axis, each corresponding to a compound in the test solution, which passed through UV detectors (Fig. 7a). Estimation of these substances was identified by the authentic samples. The concentration of the components was calculated from the area under corresponding peaks. By plotting peaks matching PCr it could be seen how its amount is increasing in time in the test medium (Fig. 7b).

ATP levels continuously regenerated by the PEP-PK system exhibited no significant change in the trial mixtures (Fig. 8a). However, PCr concentration in the surrounding medium increased rapidly in dependence on MgATP concentration (Fig. 8b).

Since PCr in permeabilized cardiac cells can be produced by different isoforms of creatine kinase (MtCK and non-mitochondrial creatine kinases (myofibrillar, SR and sarcolemmal), to ascertain which amount of PCr content in the samples is of mitochondrial origin, the oxidative phosphorylation was inhibited by 10 μM rotenone (Fig. 8b). The differences of the rates of PCr synthesis in the absence and presence of rotenone at defined MgATP concentrations estimate MtCK contribution in each case (Fig. 8c). Oxygen consumption rises with an increase of MgATP concentration (Table 2) in accordance with kinetic data shown in Fig. 6. The same tendency is observed for the rate of PCr production.

Table 2 summarizes the rates of PCr production and corresponding respiration rates at fixed ATP concentrations. From these data the PCr/O_2 ratio is calculated, the average value is equal to 5.68±0.14, which is close to the theoretical maximal value of 6 (Nicholls and Ferguson 2002).

Discussion

The results of this study show clearly the important role of the ANT - MtCK-VDAC- Tubulin system in regulation of respiration and energy fluxes in the cardiac cells (Fig. 9). ANT in the mitochondrial inner membrane is an integral part of the ATP Synthasome (Chen et al. 2004; Pedersen 2007a, b). Therefore, there seems to be a supercomplex of ATP Synthasome - MtCK – VDAC – Tubulin in contact sites (Brdizka 2007) in heart mitochondria which controls the regulation of respiration. This whole complex may be shortly named “Mitochondrial Interactosome,” MI (Fig. 9). This Mitochondrial Interactosome may in some cases

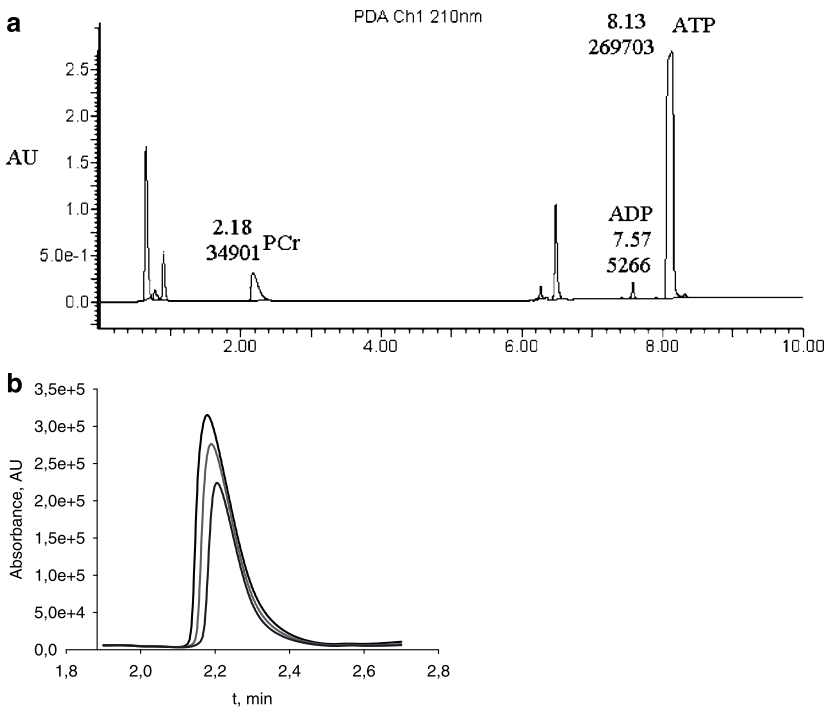


Fig. 7 **a** Chromatograms were obtained by and ACQUITY UPLC system from permeabilized cardiomyocyte incubations for 5 mM ATP. The samples of the reaction mixture were taken at specified time intervals after the initializing reaction. The positions of PCr and ATP traces on the retention time scale were detected at 2.2 and 8.1 min, respectively. Quantitative assessment of the concentrations of the mixture components was obtained from the peak area, **b** Replotted from original chromatograms graph with the peaks correponding to PCr appereance after 3, 6 and 10 min after activation MtCK reaction by adding 10 mM Cr into the medium

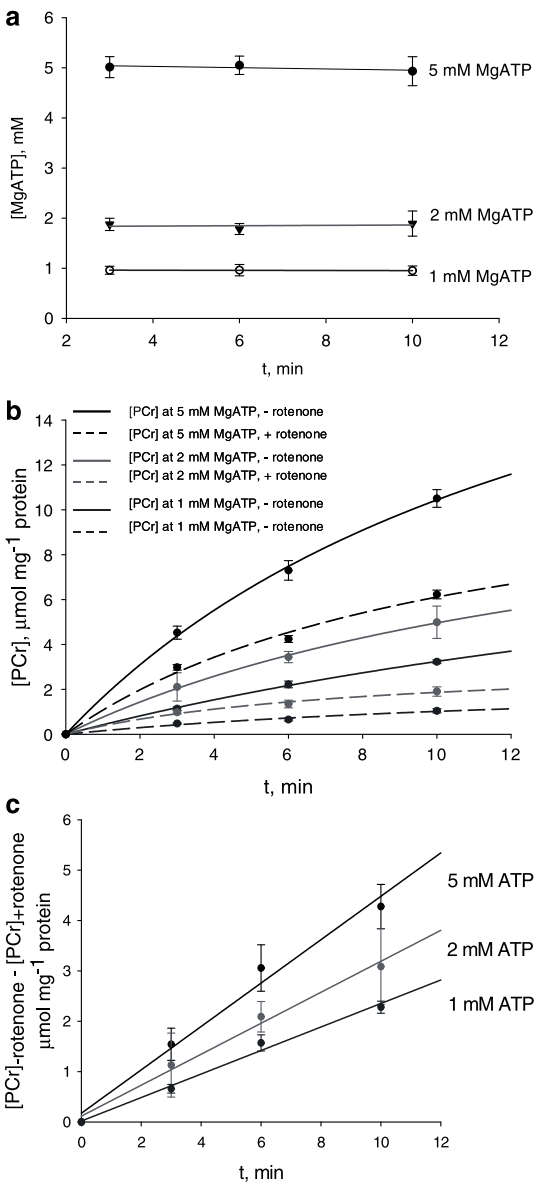


Fig. 8 **a** The ATP level, continuously regenerated by the PEP-PK system, was stable during the experimental procedure described in the Fig. 5, **b** The rate of phosphocreatine production by mitochondrial and cytoplasmic creatine kinases in permeabilized non-inhibited cardiomyocytes (solid lines). After activation of MtCK by creatine (10 mM) in permeabilized cardiomyocytes in the presence of MgATP (1, 2, 5 mM) and PEP (5 mM) and PK (20 U/ml) reaction was stopped after 3, 6 and 10 min. Analyses of the collected mixture were performed by using ion pair HPLC/UPLC as described in Materials and Methods. When oxidative phosphorylation is inhibited by rotenone, 10μM (dashed lines), the PCr can be produced only by cytoplasmic creatine kinases, MMCK, **c** The difference in phosphocreatine production rates under conditions of activated and inhibited (by rotenone) respiratory chain calculated from Fig. 6b. In parallel experiments corresponding oxygen consumption rates were measured. The creatine (10 mM) activated respiration rates rises with the increase of MgATP concentrations. For any MgATP concentrations PCr/O_2 is equal to 5.68±0.14

include also supercomplexes of the respiratory chain (Lenaz and Genova 2007; Vonck and Schafer 2009). Along the cristae membranes the MI contain only MtCK and ATP Synthasome. Direct measurements of energy fluxes from mitochondria into cytoplasm (surrounding medium in experiments with permeabilized cardiac cells) show PCr/O_2 ratios close to the theoretical maximal P/O_2 ratio under conditions similar to those *in vivo*. These high PCr/O_2 ratios are seen for all three MgATP concentrations up to 5 mM, which is close to physiological concentrations of ATP in cells. Thus, our data clearly show the effectiveness of the transmission of high energy bond from ATP to PCr within the MI. These data leave also little room for direct crosstalk between mitochondria and MgATPases by MgATP and MgADP channeling (Joubert et al. 2008; Kaasik et al. 2001; Kuum et al. 2009) this shows that energy is carried out of mitochondria by PCr fluxes and creatine effectively regulates the MtCK – ATP Synthasome complex due to selective restriction by heterodimeric tubulin of VDAC permeability only for adenine nucleotides but not for creatine or PCr (Guzun et al. 2009). Under these conditions, the amount of ATP and ADP diffusing through MOM is minimal but not zero. Kaasik et al. 2001 and Kuum et al. 2009 made their conclusion of the crosstalk between mitochondria and MgATPases by direct transfer of MgATP and MgADP on the basis of recordings in separate experiments of the amount of calcium in sarcoplasmic reticulum after a rather long period of incubation of permeabilized cells either with ATP, phosphocreatine and ATP, or ATP and respiratory substrates. No reaction rates or energy fluxes were recorded under physiological conditions – activation of MtCK and interaction of mitochondria with other cellular systems, including ATPases, cytoskeleton and the glycolytic system. The absence of such a system analysis does not allow conclusions to be made of the distribution of energy fluxes between mitochondria and cytoplasm *in vivo*.

In our experiments, both MtCK-controlled respiration and PCr production rates were dependent on MgATP concentration and increased with the elevation of the latter in the interval of 1 – 5 mM (Fig. 6 and 8c). This conforms to our recent kinetic data showing that in permeabilized cardiomyocytes *in situ* the diffusion of ATP into the intermembrane space is restricted (Guzun et al. 2009). At the same time PCr evidently rather easily diffuses through VDAC into the surrounding medium. Kinetic determina-

Table 2 Measured rates of PCr production, corresponding oxygen consumption and their calculated ratios for fixed ATP concentration in mitochondria in situ

	V_{PCr}[a] μmolmg−1 protein min−1	V_{O2}[b] μmolmg−1 protein min−1	V_{PCr}/V_{O2}[c]
1 mM ATP	0.23±0.02	0.041±0.001	5.80±0.45
2 mM ATP	0.31±0.02	0.056±0.02	5.44±0.44
5 mM ATP	0.43±0.04	0.074±0.003	5.81±0.48
		Average	5.68±0.14

[a] V_{PCr} rate of PCr production measured with the use of HPLC/UPLC

[b] V_{02} rate of oxygen consumption

[c] V_{PCr}/V_{O2} calculated ratio of PCr/O_2

tions showed that the affinity of MtCK for exogenous creatine in the permeabilized cardiomyocytes was even increased in comparison with isolated mitochondria, and not changed for phosphocreatine. Because of this rather selective control of VDAC permeability (Rostovtseva et al. 2008, Monge et al. 2008) and functional coupling between MtCK and ANT (Saks et al. 2004; Vendelin et al. 2004b), all ATP produced in oxidative phosphorylation is practically completely used for PCr production and ADP is rapidly channeled back through ANT to the mitochondrial matrix.

Our data are in line with an increasing understanding of the importance of the contacts of outer mitochondrial

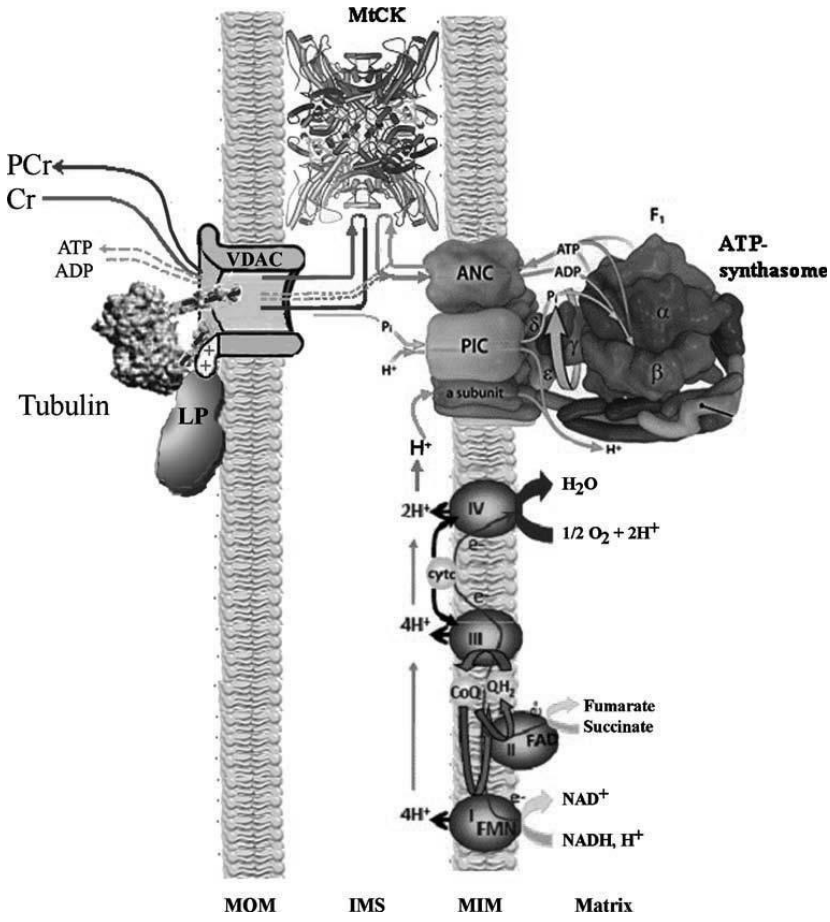


Fig. 9 Proposed model of regulation of respiration in a supercomplex named Mitochondrial Interactosome, consisting of ATP Synthasome - MtCK-VDAC- Tubulin. Macromolecular ATP synthase is represented as a part of the complex ATP Synthasome (*reprinted with kind permission from Peter L. Pedersen,* 2007a, b, 2008), with adenine nucleotides carriers (ANC) and phosphate carriers (PIC). Octameric mitochondrial creatine kinase (MtCK) (*the structure was kindly supplied by U.Schlattner*), located in the mitochondrial intermembrane space (IMS) and attached to mitochondrial inner (MIM) and in the contact sites to outer membranes (MOM). VDAC permeability is selectively regulated by heterodimeric tubulin, which binding to VDAC in intact mitochondrial membrane may be either direct or by some linker proteins (LP). This complex of VDAC with other proteins controls the outcome of adenine nucleotides and PCr fluxes into surrounding medium, and phosporylation by the ATP Synthasome system is effectivily regulated by creatine via MtCK

membrane VDAC with the cytoskeleton for regulation of energy fluxes and mitochondrial respiration in cardiac cells (Aliev and Saks 1997; Anflous et al. 2001; Boudina et al. 2002; Burelle and Hochachka 2002; Capetenaki 2002; Colombini 2004; Guerrero et al. 2005; Kummel 1988; Kuznetsov et al. 1989, 1996; Linden et al. 1989; Liobikas et al. 2001; Rostovtseva and Bezrukov 2008; Rostovtseva et al. 2008; Saks et al. 1998a, 2003, 1991, 1974, 1985, 1993, 1989; Veksler et al. 1995; Vendelin et al. 2004b; Zoll et al. 2003a, 2005, 2003b, 2002). Studies of permeabilized cells in many laboratories have shown an increased apparent Km for ADP for exogenous ADP in the regulation of respiration in comparison with isolated mitochondria (Anflous et al. 2001; Boudina et al. 2002; Burelle and Hochachka 2002; Guerrero et al. 2005; Kummel 1988; Kuznetsov et al. 1996; Liobikas et al. 2001; Saks et al. 1998a, 2007d, 2003, 1991, 1993, 1989; Veksler et al. 1995; Zoll et al. 2003a, 2005, 2003b, 2002). This was explained by local restriction of ADP diffusion in the cells due to binding of some cytoskeletal elements (called factor X) to the mitochondrial outer membrane (Appaix et al. 2003; Saks et al. 1995). Very recently, Rostovtseva et al. identified this factor X by showing direct interaction of heterodimeric tubulin with VDAC (Rostovtseva and Bezrukov 2008; Rostovtseva et al. 2008). In this work we show that kinetics of regulation of respiration of isolated mitochondria with added tubulin is similar to that in permeabilized cardiomyocytes. In both cases a high apparent Km for exogenous ADP is decreased when MtCK is activated by creatine (Fig. 3). In intact cells, other cytoskeletal proteins are also shown to form contacts with VDAC in the outer mitochondrial membrane, particularly desmin (Capetenaki 2002; Linden et al. 2001) and plectin (Reipert et al. 1999). Rostovtseva et al. 2008 have directly shown a strong interaction of purified tubulin with VDAC inserted into phospholipid membranes. In the cardiac cells *in vivo*, usually only about 30% of tubulin exists in the polymerized state within the microtubular system, the remaining part being in the free heterodimeric form (Tagawa et al. 1998). Therefore, the effects observed by Rostovtseva et al. 2008 and shown in Fig. 3 may well be valid for *in vivo* conditions. Interestingly, however, high apparent Km values are also characteristic for permeabilized cells (see above) from which dimeric tubulin may be thought to leak out. However, that does not happen: in experiments with use of colchicine (Guerrero 2005) to depolymerized tubulin in permeabilized cardiomyocytes, immunolabelling of tubulin by antibodies against β subunits and studies of its localization by confocal microscopy showed disappearance of the microtubular network but intensive labeling and diffused intracellular localization of tubulin, which diffusion may be limited due to its binding to other cytoskeletal elements and particularly to the outer mitochondrial membrane (Guerrero 2005). Correspondingly, only a minor decrease of apparent Km for endogenous ADP was seen (Guerrero 2005).

In the intact mitochondrial outer membrane some other proteins may also be associated with VDAC. These interactions may result in specific restriction of VDAC permeability only for adenine nucleotides, but not creatine or PCr. It has been shown that one of these proteins may be microtubule-associated protein 2 (MAP2) (Linden and Karlsson 1996; Linden et al. 1989) and cyclic nucleotide phosphodiesterase (Bifulco et al. 2002). Interestingly, similar association of tubulin via binding to linker proteins has been shown for the plasma membrane (Wolff 2009). These data allow supposing that in the contact sites between inner and outer mitochondrial membranes there is the supercomplex MI in the cells *in situ* where tubulin is associated either directly or via linker proteins to VDAC, which is associated with MtCK - ATP Synthasome complex (Fig. 9). Earlier, Pedersen et al. have shown the existence of a similar "supercomplex", i.e., the ATP Synthasome-VDAC-Hexokinase 2 in cancer cells that helps in explaining the Warburg effect (Chen et al. 2004; Pedersen 2007a, b, 2008). Our earlier studies of cancer cells of the cardiac phenotype - continuously dividing HL-1 cells are consistent with the explanation proposed by Pedersen. These studies have shown that in HL-1 cells apparent Km for exogenous ADP is very low, creatine kinase is downregulated and creatine has no effect on respiration (Anmann et al. 2006). On the contrary, in these cells hexokinase activity is increased manifold and glucose activates respiration via activation of membrane-bound hexokinase (not seen in normal cardiomyocytes) (Eimre et al. 2008). These results show that in the HL-1 cells tubulin is replaced by hexokinase 2 and creatine kinase is absent in the Mitochondrial Interactosome. Thus, alterations in the structure of MI may contribute in cancerogenesis. Another way to change the MI structure is knock-out MtCK by genetic manipulations resulting in increasing the energy transfer in the cells via adenylate kinase pathway (Dzeja et al. 2007).

The hypothesis of Mitochondrial Interactosome conforms to the fundamental theory of Peter Mitchell about vectorial metabolism (Mitchell 1979, 2004). According to this theory, an important consequence of the organization of the enzymes into multienzyme complexes is vectorial metabolism and ligand conduction which brings together "transport and metabolism into one and the same chemiosmotic molecular level - biochemical process catalyzed by group-conducting or a conformationally mobile group-translocating enzyme system" (Mitchell 1979). For enzymes and catalytic carriers that have spatially separated binding sites for donor and acceptor (as MgATP and creatine depicted for MtCK in Fig. 9), group transfer can be considered as vectorial group translo-

cation (Mitchell 1979). This is true also for movement of substrates and products from carrier to enzyme and via VDAC with selective permeability (see Fig. 9). In his latest reviews Peter Mitchell encouraged a wider use of the chemiosmotic principle and the biochemical concept of specific ligand conduction in explaining organization and operation of metabolic and transport processes within the cell (1979). Today this idea receives increased attention and is certainly another important insight of Peter Mitchell to the understanding of cellular energy conversion processes (Dzeja et al. 2007).

All data reported in this work and recently (Guzun et al. 2009) strongly support the theories of intracellular energy transport by phosphotransfer networks (Dzeja et al. 2007, Saks et al. 2008, 2007a, 2006, 2007c, 2004, Schlattner and Wallimann 2004; Schlattner et al. 2006; Vendelin et al. 2004a; Wallimann et al. 1992, 2007; Wyss et al. 1992). They also show that the popular theories of cells as homogenous medium (Barros and Martinez 2007; Meyer et al. 1984; Wu and Beard 2009) are not compatible with experimental data. An extreme case of these theories is an explanation, which from time to time appears in literature, perfect in its naïve simplicity and obviously based on observation of electron micrographs of cardiac cells saying that "mitochondria are "wrapped" partially around the myofilaments with certain degrees of variations. This close apposition of mitochondria to the contractile machinery strategically allows mitochondria to deliver ATP more efficiently to the sites where energy demands are high." (Hom and Sheu 2009). Others support this view, saying that "since the myofibrils generally have small diameters and are surrounded by tense mitochondria, it is possible that CK-facilitated transport does not play a significant role *in vivo*" (Wu and Beard 2009). This is some kind of "mechanical bioenergetics" when just looking at electron microscopic images is taken to replace careful biochemical research. Electron micrographs give useful information, but not enough. Our present study presents more clear evidence that the regulation of the cells' metabolism is a system level property dependent on the interactions of many intracellular structures and systems in the cell (Guzun et al. 2009) These interactions within MI, which lead to new, system level properties, occur within micro- and nanometer scales, much smaller than the diameter of myofibrils. From the point of view of Molecular System Bioenergetics (Guzun et al. 2009; Saks et al. 2007b), an important task is to clarify and describe quantitatively the regulatory mechanisms of the tubulin-VDAC-MtCK-ATP Synthasome supercomplex (Fig. 9), in the interaction with all the other metabolic systems in the cell. In this complex, the behaviour of MtCK cannot be described either by simple solution kinetics nor by even a more simple equilibrium equation (Vendelin et al. 2004b).

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PUBLICATION IV

Tepp, K.**,** Chekulayev, V., Shevchuk I.**,** Timohhina, N., Kaambre, T., Saks, V. (2010) Metabolic control analysis of integrated energy metabolism in permeabilized cardiomyocytes - experimental study

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Regular paper

Metabolic control analysis of integrated energy metabolism in permeabilized cardiomyocytes — experimental study★❋

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The main focus of this research was to apply Metabolic Control Analysis to quantitative investigation of the regulation of respiration by components of the Mitochondrial Interactosome (MI, a supercomplex consisting of ATP Synthasome, mitochondrial creatine kinase (MtCK), voltage dependent anion channel (VDAC), and tubulin) in permeabilized cardiomyocytes. Flux control coefficients (FCC) were measured using two protocols: 1) with direct ADP activation, and 2) with MtCK activation by creatine (Cr) in the presence of ATP and pyruvate kinase-phosphoenolpyruvate system. The results show that the metabolic control is much stronger in the latter case: the sum of the measured FCC is 2.7 *versus* 0.74 (ADP activation). This is consistent with previous data showing recycling of ADP and ATP inside the MI due to the functional coupling between MtCK and ANT and limited permeability of VDAC for these compounds, PCr being the major energy carrier between the mitochondria and ATPases. In physiological conditions, when the MI is activated, the key sites of regulation of respiration in mitochondria are MtCK (FCC = 0.93), adenine nucleotide translocase ANT (FCC = 0.95) and CoQ cytochrome *c* oxidoreductase (FCC = 0.4). These results show clearly that under the physiological conditions the energy transfer from mitochondria to the cytoplasm is regulated by the MI supercomplex and is very sensitive to metabolic signals.

Keywords: mitochondria, respiration, cardiomyocytes, metabolic control analysis, creatine kinase

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INTRODUCTION

Mitochondrial respiration, coupled to production of ATP and fine regulation of energy fluxes to the sites of ATP utilization, is vital for normal cell life. The regulation at different workloads is of main importance in the high energy demanding brain and heart cells. In spite of the fundamental progress in the understanding of mitochondrial bioenergetics (Nicholls & Ferguson, 2002), the nature of respiratory control and, in a more general sense, the mechanisms of regulation of energy fluxes during workload changes in the cardiac and other cells *in vivo* are still highly debated (Vendelin *et al.*, 2000; Beard, 2005; 2006; Saks *et al.*, 2006; 2007a; 2007b; Saks, 2007; Van Beek, 2007; 2008; Wu *et al.*, 2008; Balaban, 2009; Guzun *et al.*, 2009; Wu & Beard, 2009). In explaining respiration regulation, the cell is often described as a homogenous reaction medium, thus ignoring the impact of the high degree of structural organization of the cell, in particular of cardiomyocytes. In this context, it is especially important to use Systems Biology approaches to analyze the complex biological systems *in situ* to discover system-level properties (Saks *et al.*, 2008), which are direct consequences of interactions between cellular components and are absent for isolated components. Recent research of steady state kinetics of respiration regulation in permeabilized cardiomyocytes has revealed striking differences in the behavior of mitochondria *in vitro* and *in situ*: the apparent K_m for ADP is more than ten times higher *in situ* than *in vitro* (Guzun *et al.*, 2009; Saks *et al.*, 2007c). The apparent dissociation constants of Mg-ATP from complexes with mitochondrial creatine kinase (MtCK) were several orders of magnitude higher *in situ* than *in vitro*, while the apparent dissociation constants of creatine (Cr) were significantly lower *in situ* than *in vitro* (Guzun *et al.*, 2009). These results show clearly that the mechanisms of regulation of mitochondrial respiration and energy fluxes in the cardiac cells are system-level properties dependent on the interaction of mitochondria with intracellular structures, which are not predictable on the basis of the properties of isolated mitochondria only (Saks, 2007). It has also been shown that the cytoskeletal component tubulin, which is responsible for the regular arrangement of mitochondria in cardiac cells, also controls the permeability of voltage dependent anion channel (VDAC) in mitochondrial outer membrane (MOM) (Rostovtseva & Bezrukov, 2008). Recently, we have demonstrated that the selective permeability of the MOM–VDAC–tubulin complex is crucial for the regulation of energy transfer in cardiac cells (Timohhina *et al.*, 2009): a supercomplex consisting of ATP Synthasome, MtCK,

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Abbreviations: ANT, adenine nucleotide translocase; BSA, bovine serum albumin; CAT, carboxyatractyloside; CK, creatine kinase; CM, cardiomyocytes; Cr, creatine; DNFB, 2,4-dinitrofluorobenzene; FCC, flux control coefficient; GGG, triglycine; IMS, mitochondrial intermembrane space; IM, isolation medium; MI, Mitochondrial Interactosome; MtCK, mitochondrial creatine kinase; MCA, Metabolic Control Analysis; MCC, Metabolic Control Coefficient; MOM, mitochondrial outer membrane; PIC, inorganic phosphate carrier; PCr, phosphocreatine; PEP, phosphoenolpyruvate; PK, pyruvate kinase; VDAC, voltage-dependent anion channel

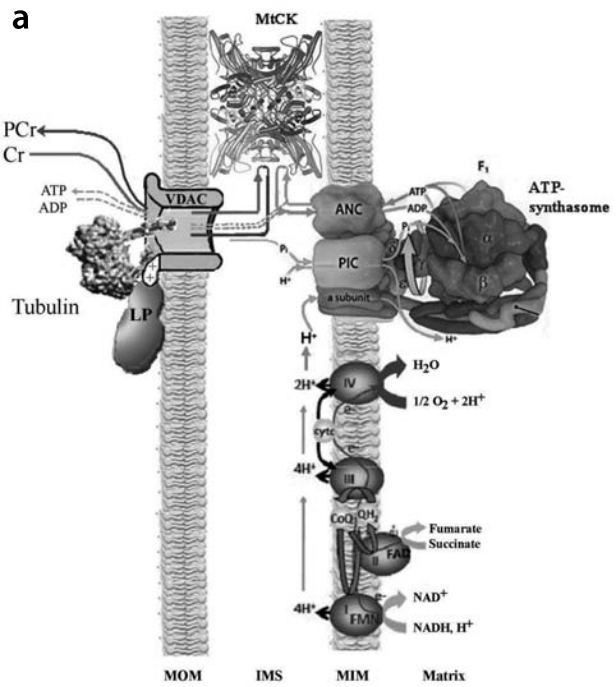

Figure 1a. Model of regulation of respiration in the Mitochondrial Interactosome (MI)
MI is a supercomplex consisting of ATP Sythasome (reprinted with kind permission from Peter L. Pedersen (2007; 2008), mitochondrial creatine kinase (MtCK), voltage dependent anion channel (VDAC), and tubulin. Octameric MtCK (structure kindly supplied by U. Schlattner (2006)) located in the mitochondrial intermembrane space (IMS) is attached to mitochondrial inner membrane (MIM) and in the contact sites to the outer membrane (MOM). VDAC permeability is selectively regulated by heterodimeric tubulin, whose binding to VDAC in intact mitochondrial membrane may be either direct or through linker proteins (LP). This complex of VDAC with other proteins controls fluxes of adenine nucleotides and phosphocreatine (PCr) into surrounding medium, and phosphorylation by the ATP Synthasome system is effectively regulated by creatine (Cr) *via* MtCK. Here: ANC, adenine nucleotide carrier; PIC, phosphate carrier. Reproduced from Saks *et al*. (2010) with permission.

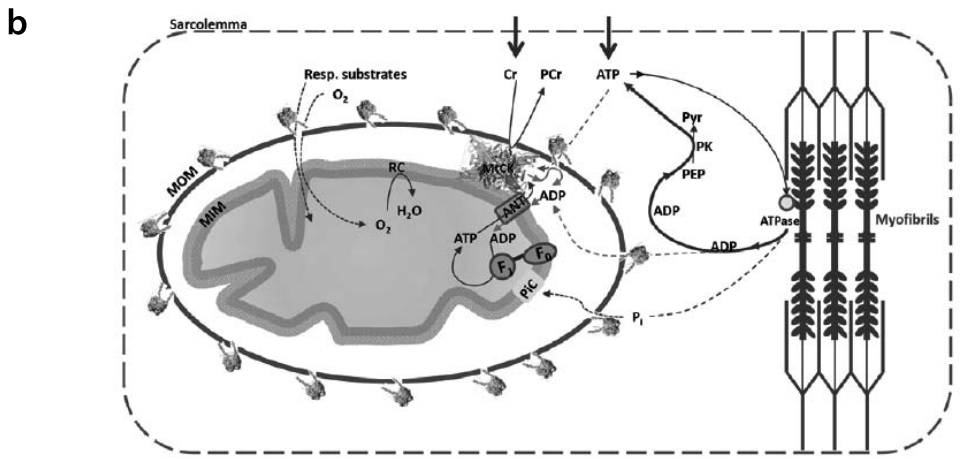

Figure 1b. Scheme of experimental protocols
This mode of representation is called Gellerich–Guzun protocol (Gellerich 1982; Guzun 2009). It represents mitochondrion *in situ* in a permeabilized cardiac cell, surrounded by cytoskeletal proteins and myofibrils. The mitochondrial outer membrane (MOM) is less permeable than in isolated mitochondria, due to the interactions of VDAC with cytoskeletal proteins. The system is supplemented with phosphoenolpyruvate (PEP) and pyruvate kinase (PK). This PK/PEP system removes extramitochondrial ADP produced by intracellular ATP-consuming reactions and continuously regenerates extramitochondrial ATP. Endogenous ADP produced by MtCK is re-imported back to the matrix *via* adenine nucleotide translocase (ANT) due to the functional coupling with MtCK. Reproduced from Timohhina *et al*. (2009) with permission.

VDAC and tubulin, or Mitochondrial Interactosome (MI) (Fig. 1a) in heart mitochondria has been shown to control the regulation of respiration (Saks *et al*., 2010). It is especially interesting to use the method of Metabolic Control Analysis (MCA) to describe quantitatively the distribution of control between the complexes of energy transfer in MI. In the present work we use permeabilized cardiomyocytes (Fig. 1b), which means that the complex structural and functional organization of cardiomyocytes and its importance for metabolic regulation are taken into account. To determine the flux control coefficient (FCC), the flux was measured as the rate of oxygen consumption in permeabilized cardiomyocytes when the MI complexes were titrated with their specific inhibitors to stepwise decrease their activity.

MATERIALS AND METHODS

Experimental protocols. The principles of our study are illustrated by Figs. 1a and 1b. Figure 1a represents a model of the MI supercomplex (consisting of ATP Synthasome, MtCK, VDAC and tubulin in contact sites) in heart mitochondria, which controls the regulation of respiration. This MI in some cases includes supercomplexes of the respiratory chain (Lenaz & Genova, 2007; Vonck & Schafer, 2009). Along the cristae membranes the MI contains only MtCK and ATP Synthasome.

Figure 1b represents the setup of our experiments; the mitochondrion *in situ,* in a permeabilized cardiac cell, surrounded by cytoskeleton proteins (tubulin) and myofibrils. The respiratory chain complexes, ATP Synthasome (F_1F_O) and inorganic phosphate (Pi) carrier (PIC) are integrated within the mitochondrial inner membrane (MIM). MtCK is depicted as an octamer, located in the mitochondrial intermembrane space (IMS) and attached to the inner membrane surface.

In the first part of our experiments (ADP activation) respiration is activated by extramitochondrial ADP. The Cr/phosphocreatine (PCr) transfer network and the MI supercomplex of energy transfer regulation are not activated. In this case, ATP produced in the mitochondria is leaving it through the MOM (Guzun *et al.*, 2009; Timohhina *et al.*, 2009).

In the second part of our experiments, MtCK is activated by Cr in the presence of ATP (see Fig. 1b). The permeabilized cardiomyocytes were supplemented with phosphoenolpyruvate (PEP) and pyruvate kinase (PK). This PK/PEP system removes extramitochondrial ADP produced by intracellular ATP-consuming reactions and continuously regenerates extramitochondrial ATP. Endogenous intramitochondrial ADP produced by MtCK forms microcompartments within the IMS and is re-imported into the matrix *via* adenine nucleotide translocase (ANT) due to its functional coupling with MtCK. The final products of MtCK-forward reaction are PCr and endogenous ADP. Due to the activation of the MI, direct interactions of ANT, MtCK, VDAC and cytoskeleton proteins endogenous ADP and ATP are circulating inside the mitochondria and oxidative phosphorylation is controlled by endogenous ADP.

Animals. Male Wistar rats weighing 300–350 g were used. The animals were housed five per cage at constant temperature (22 °C) in environmental facilities with a 12:12 h light-dark cycle and were given standard laboratory chow *ad libitum.* Animal procedures were approved by the Estonian National Committee for Ethics in Animal Experimentation (Estonian Ministry of Agriculture).

Isolation of adult cardiac myocytes. Adult cardiomyocytes were isolated after perfusion of the rat heart with collagenase A (Roche) using an adaptation of the technique described previously (Saks *et al.*, 1991). Rats were anaesthetized with medetomidine and ketamine, decapitated and the heart was quickly excised preserving a part of the aorta and placed into isolation medium (IM) of the following composition: 117 mM NaCl, 5.7 mM KCl, 4.4 mM $NaHCO_3$, 1.5 mM KH_2PO_4, 1.7 mM $MgCl_2$, 11.7 mM glucose, 10 mM Cr, 20 mM taurine, 10 mM PCr, 2 mM pyruvate and 21 mM Hepes, pH 7.1. The excised heart was cannulated by the aorta, suspended in the Langendorf system for perfusion and washed for 5 min with a flow rate of 15–20 ml/min. The collagenase treatment was performed at 37 °C by switching the perfusion to circulating O_2-saturated IM supplemented with 1 mg/ml collagenase A and 2 mg/ml BSA at flow rate of 5 ml/min for 20–30 min. The end of the digestion was determined following the decrease in perfusion pressure measured by a manometer. After the digestion the heart was washed with IM for 2–3 min and transferred into IM containing 20 μM $CaCl_2$, 10 μM leupeptin, 2 μM soybean trypsin inhibitor and 2 mg/ml fatty acid free BSA. Cardiomyocytes were then gently dissociated using forceps and pipette suction. Cell suspension was filtered through a crude net to remove tissue remnants and let to settle for 3–4 min at room temperature. After 3–4 min the initial supernatant was discarded, pellet of cardiomyocytes resuspended in 10 ml of IM containing 20 μM $CaCl_2$ and the protease inhibitors. This resuspension-sedimentation cycle with calcium-tolerant cells was performed twice, after that the cardiomyocytes were gradually transferred from IM with 20 μM Ca^{2+} into calcium-free Mitomed (supplemented with protease inhibitors and BSA) and washed 5 times. Each time, slightly turbid supernatant was removed after 4–5 min of the cells' sedimentation. Isolated cells were re-suspended in 1–2 ml of Mitomed solution described below for respiration measurements and stored on melting ice. Isolated cardiomyocytes contained 70–90 % of rod-like cells when observed under a light microscope.

Isolation of mitochondria from cardiac muscle. Mitochondria were isolated from adult rat hearts as described in Saks *et al.* (1975).

Permeabilization procedure. In order to study the regulation of mitochondrial respiration in cardiomyocytes, the sarcolemma was permeabilized by saponin treatment keeping the mitochondrial membranes intact (Saks *et al.*, 1998; Kuznetsov *et al.*, 2008). The permeabilization procedure was carried out at 25 °C with 20 μg/ml saponin for 10 min and then resuspension-sedimentation cycle with Mitomed solution was performed twice to remove saponine from solution and Ca^{2+} from cells.

Measurements of oxygen consumption. The rates of oxygen uptake were determined with a high-resolution respirometer (Oxygraph-2K, from OROBOROS Instruments, Austria) in Mitomed solution (Kuznetsov *et al.*, 2008) containing 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 3 mM KH_2PO_4, 20 mM taurine, 20 mM Hepes (pH 7.1), 110 mM sucrose, 0.5 mM dithiothreitol, 5 mg/ml fatty acid free BSA, supplemented with 5 mM glutamate and 2 mM malate as respiratory substrates. These measurements were carried out at 25 °C; solubility of oxygen was taken as 240 nmol/ml (Gnaiger, 2001). In kinetic experiments with Mg-ATP, stock solution of 100 mM Mg-ATP was prepared by mixing equimolar amounts of $MgCl_2$ and ATP, pH was adjusted to 7.2. The respiration rates were expressed in pmol of oxygen consumed per second per mg of protein or in nmol of oxygen consumed per minute per nmol cytochrome *aa3*. The content of mitochondrial cytochrome *aa3* in the cardiomyocytes was measured spectrophotometrically according to the method described before (Monge *et al.*, 2008). Protein concentration was determined using a BCA protein assay kit (Pierce, USA) with BSA as a standard.

Measurements of MtCK activity. For calculation of inhibition curve of MtCK, the creatine kinase (CK) activity was measured at different concentrations of its inhibitor (2,4-dinitrofluorobenzene, DNFB) in a Cary 100 Bio spectrophotometer according to the method described before (Monge *et al.*, 2009). The MtCK activity was measured at the same conditions as was the oxygen consumption change in the Oxygraph. Isolated mitochondria were used to measure selectively the activity of MtCK. These measurements were performed at 25 °C.

After the addition of DNFB, a 5-min inhibition period of CK was allowed and then the triglycine (GGG) was added to stop the inhibition.

Metabolic Control Coefficient (MCC) determination. MCA helps to understand the mechanisms by which a given enzyme exerts high or low control of metabolic flux and how the control of the pathway is shared by several pathway enzymes and transporters. By applying MCA it is possible to identify the steps that could be modified to achieve a successful alteration of flux or metabolite concentration in pathways.

The control coefficients are defined as the ratios of the fractional changes in the system variables to the fractional change in the biochemical activity that caused the system change. It allows the identification of system components that are crucial in the control of pathway flux or metabolite concentration and thus also in the regulation of energy transfer and regulatory networks.

FCC quantifies the control that a certain reaction exerts on the steady-state flux.

$$S_1 + E_1 \rightarrow S_2 + E_2 \rightarrow S_3 + E_3 \rightarrow P$$

$$J = dP/dt = -dS_1/dt$$

Groen *et al.* (1982) derived a method to determine experimentally the FCC using titration with specific enzyme inhibitors. As the amount of inhibitor tends to zero the response of the flux to the inhibitor can be expressed in MCA terms.

The FCC is defined according to the equation (Fell, 1997):

$$C_{vi}^{J} = \left(\frac{dJ}{dv_i}\right) \Big/ \left(\frac{J}{v_i}\right) = \frac{d\ln J}{d\ln v_i}$$

in which the expression dJ/dv_i describes the variation in flux (J) when an infinitesimal change takes place in the enzyme i concentration or activity. In practice, the infinitesimal changes in v_i are undetectable, and hence measurable noninfinitesimal changes are undertaken. If a small change in v_i promotes a significant variation in J, then this enzyme exerts a high flux control. In contrast, if a rather small or negligible change in the flux is observed when v_i is greatly varied, then the enzyme does not exert a significant flux control.

For the case of an irreversible specific inhibitor, an estimation of the value of the FCC coefficient is given by Groen *et al.* (1982) and Moreno-Sanches *et al.* (2008):

$$C_E^J = (\Delta J/\Delta I) \times (I_{max}/J_0)$$

where $(\Delta J/\Delta I)$ is initial slope of the flux/inhibition graph.

Until now the method of MCA has been used to measure FCC in mitochondria (Moreno-Sanchez *et al.*, 2008). In our work we use the tool to measure the coefficient of the complexes in the MI, the model proposed in our previous article (Timohhina *et al.*, 2009).

The inhibitors used were: carboxyatractyloside (CAT) for ATP/ADP transporter, oligomycin for complex V (ATP Synthase), mersalyl for Pi transporter, antimycin for complex III, and DNFB for MtCK (see Table 1). Of the inhibitors used CAT, oligomycin, mersalyl, and antimycin are considered irreversible and noncompetitive in these conditions. For mitochondrial CK was measured also the enzyme activity decrease during inhibition. The impact of each complex is calculated according the methods described by Fell (1997) and Westerhoff (2008).

Enzymes and other chemicals were obtained from Sigma, Fluka and Roche.

Data analysis. To reduce the possibility of random error the experiments were repeated seven to twenty times and fitting was used to calculate the FCC. All data are presented as mean ±S.E.M. Statistical analysis was performed using Student's *t*-test, and $P<0.05$ was taken as the level of significance.

RESULTS

To study the role of the Mitochondrial Interactosome complex in the regulation of oxidative phosphorylation we used the protocol described in Fig. 1b. This protocol has been developed and used in our recent studies of respiration regulation *in situ* (Guzun *et al.*, 2009; Timohhina *et al.*, 2009). Permeabilized cells contain extramitochondrial ATPases and creatine kinases which produce ADP after addition of ATP and Cr. To trap all the extramitochondrial ADP we used the pyruvate kinase–phosphoenolpyruvate system that keeps ATP concentration at the initial constant level. As it is shown in Fig. 2a, when mitochondrial respiration is controlled by MtCK, activated with 2 mM ATP and 10 mM Cr, PK/PEP addition does not influence the respiration rate and thus has no role in the control of respiration. This is explained by the closure of VDAC by tubulin (Rostovtseva & Bezrukov, 2008) which makes VDAC selectively permeable towards Cr and PCr (Guzun *et al.*, 2009; Timohhina *et al.*, 2009; Saks *et al.*, 2010). Therefore, ADP produced by MtCK is not accessible for the extramitochondrial PK/PEP and the respiration rate is regulated by reactions in the MI. Figure 2b shows that the PK/PEP system does not have any influence on the respiration rate even at the PK concentration of 100 U/ml. These results show that for mitochondria *in vivo* the FCC for PK is zero.

Table 2 shows the maximal oxygen consumption rates measured according to protocols 1 and 2. It is clear from these data that the V_3 rates are the same in both systems within the statistical error. This equality of respiration rates makes it possible to compare the flux control coefficients determined by the two protocols used.

Figure 3 shows the recordings of oxygen consumption by permeabilized cardiomyocytes to demonstrate the specificity of inhibition of MtCK by DNFB. It has been shown by Infante and Davies (1965) that DNFB is an effective and specific inhibitor of the CK reaction. In Fig. 3a the respiration was activated with 2 mM ADP. In this case, the MI is not activated and the inhibition of MtCK with 30 μM DNFB does not have any influ-

Table 1. Inhibitors used for Mitochondrial Interactosome complexes

MI complex	Inhibitor	Concentration range
ATP/ADP carrier	CAT	10–750 nM
ATP Synthase	Oligomycin	30–210 nM
Pi carrier	Mersalyl	10–120 μM
MtCK	DNFB	0.05–40 μM
CoQ cytochrome *c* oxidoreductase (Complex III)	Antimycin	10–240 nM

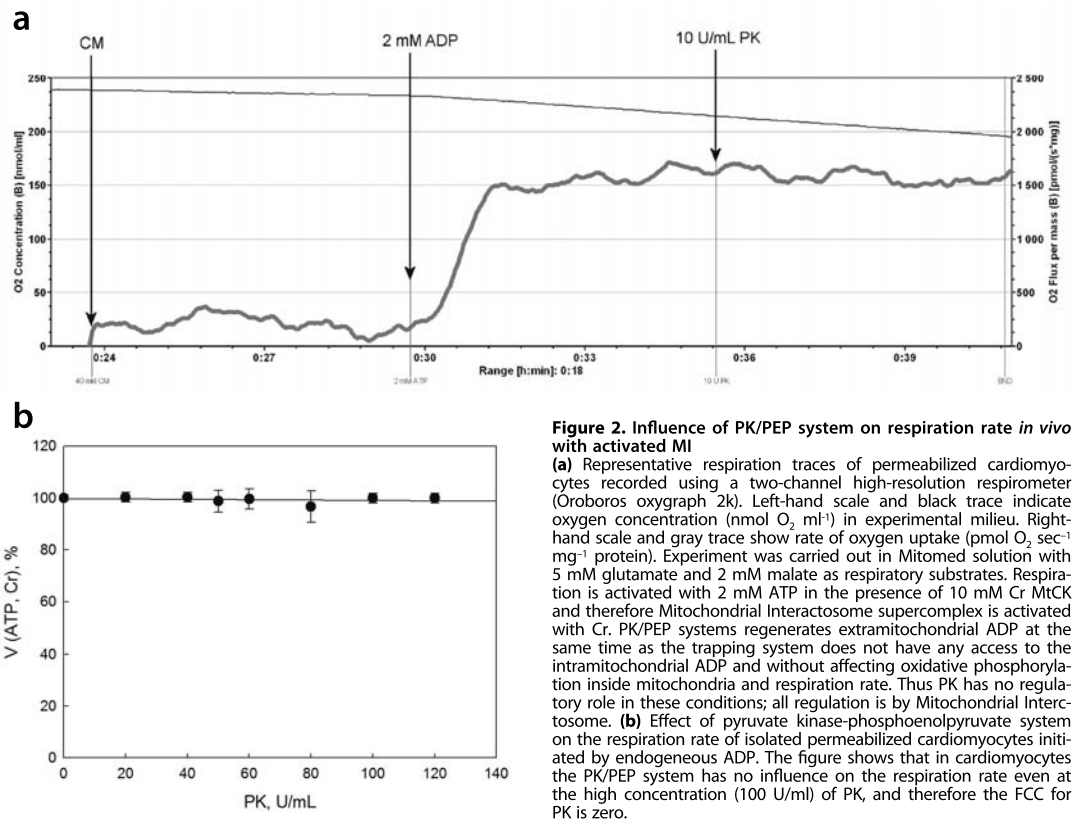

Figure 2. Influence of PK/PEP system on respiration rate *in vivo* with activated MI

(a) Representative respiration traces of permeabilized cardiomyocytes recorded using a two-channel high-resolution respirometer (Oroboros oxygraph 2k). Left-hand scale and black trace indicate oxygen concentration (nmol O_2 ml^{-1}) in experimental milieu. Right-hand scale and gray trace show rate of oxygen uptake (pmol O_2 sec^{-1} mg^{-1} protein). Experiment was carried out in Mitomed solution with 5 mM glutamate and 2 mM malate as respiratory substrates. Respiration is activated with 2 mM ATP in the presence of 10 mM Cr MtCK and therefore Mitochondrial Interactosome supercomplex is activated with Cr. PK/PEP systems regenerates extramitochondrial ADP at the same time as the trapping system does not have any access to the intramitochondrial ADP and without affecting oxidative phosphorylation inside mitochondria and respiration rate. Thus PK has no regulatory role in these conditions; all regulation is by Mitochondrial Interctosome. **(b)** Effect of pyruvate kinase-phosphoenolpyruvate system on the respiration rate of isolated permeabilized cardiomyocytes initiated by endogeneous ADP. The figure shows that in cardiomyocytes the PK/PEP system has no influence on the respiration rate even at the high concentration (100 U/ml) of PK, and therefore the FCC for PK is zero.

ence on the respiration rate of mitochondria. This shows that the ATP Synthasome was not inhibited by DNFB. Figure 3b shows recordings of oxygen consumption when the respiration was activated with 5 mM ATP and Cr (up to 10 mM): the maximal oxygen consumption rate is equal to that in the presence of ADP. In this case, the MI is activated, and the ATP produced in the ATP Synthasome is channelled *via* the ANT to the intermembrane space where in the MtCK reaction PCr is formed. The PCr is leaving the mitochondria *via* the selectively regulated VDAC complex at the same time as ADP is channeled back through the ANT due to the functional coupling between MtCK and ANT with a direct transfer of ADP into the matrix. The recycling ATP and ADP do not leave the mitochondria. In this case DNFB, even at a concentration as low as 15 μM, has a significant influence on the respiration rate due to the inhibition of MtCK. This inhibitory action develops in time, as can be seen from the recording. After the five-minute inhibition period the addition of GGG stops further inhibition and the respiration rate remains unchanged. This method was used to measure the oxygen consumption rate at different concentrations of DNFB and to measure the inhibition of activation of MtCK. From these data the FCC for MtCK can be precisely determined.

Figure 4 represents respiration traces of cardiomyocytes titrated by CAT according to two different protocols; namely, Fig. 4a represents direct ADP activation whereas Fig. 4b shows Cr activation when the respiration is initially activated with ATP, and then in the presence of the PK/PEP system 10 mM Cr activates the MtCK. Figure 5 shows the respiration rates for all CAT concentrations, as measured according to both protocols as described in Fig. 4. In Fig. 5 one can also see the method used for I_{max} calculation. In the inset of Fig. 5 initial slopes are presented. The I_{max} values were calculated from the points of interception of the straight line at high concentrations of the inhibitor and the straight lines at the beginning of inhibition curves, the slopes of which were used to calculate the FCC values. The respiration rate values at the higher concentrations of the inhibitor are very close in the case of the two protocols used; therefore the change in the FCC is caused by the change of initial slopes of the inhibition curves, indicating change of the mechanism of energy transfer regulation due to the activation of MI.

It is clear from a comparison of these results that in the second case, at the same concentrations of CAT, the effect of this inhibitor is much stronger. At 20 nM CAT (see inset in Fig. 5) the respiration rate with external ADP activation is almost the same as without the inhibitor. At the same concentration of CAT with the Cr protocol, the respiration rate is reduced by about 25%. A slight inhibition of respiration by CAT in the direct ADP activation protocol is visible at 50 nM CAT; at the same concentration of this inhibitor but with an activated MI system the respiration rate is reduced almost two-fold. Similar analyses of titration with specific inhibitors (Table 1) and recording the respiration rate were made with oligomycin for complex V (ATP Synthase), mersalyl for Pi transporter, and antimycin for complex III. Results presented in Supplementary Material show different pattern of

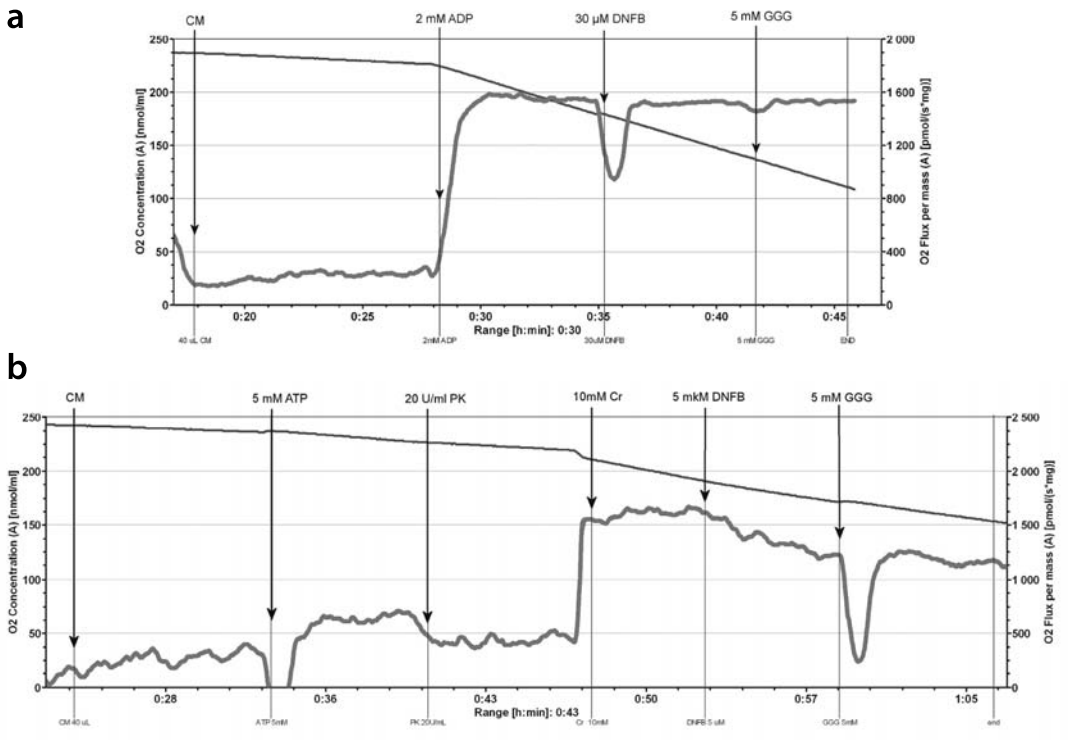

Figure 3. Specificity of dinitrofluorobenzene (DNFB) for inhibition of MtCK
(a) Respiration activated with ADP. Without activation of MI complex by creatine (Cr), the respiration rate was not affected by addition of 30 μM DNFB. **(b)** Respiration activated by Cr. In the second protocol, the respiration of cardiomyocytes (CM) was activated with ATP then PK/PEP system was added to trap extramitochondrial ADP and then 10 mM Cr to activate MtCK. Addition of 15 μM DNFB gives significant decrease at respiration rate. The inhibitory effect was terminated with triglycine (GGG), resulting in stable level of respiration showing steady state of the system. PEP was added to 5 mM and PK to 20 units/ml.

inhibition as compared with the data shown in Fig. 5. The inhibition of the Pi transporter by mersalyl is shown where no differences between two protocols used can be observed. Thus, the differences between FCC of the same complex according the protocols used are specific with respect to the transporters investigated.

From these data flux control coefficients were calculated and are shown in Fig. 6. It is apparent that the metabolic control is much stronger in the MI when the MI supercomplex is activated with Cr than in the case of respiration activated by exogenous ADP. The sum of the FCC for external ADP activation is less than 1 because not all the complexes of MI are accounted for. The sum of the FCC in the second protocol is more than three times higher: it shows a direct channeling in physiological conditions (Kholodenko *et al.*, 1993).

The FCC of the ANT increases from 0.21 to 0.95 with Cr activation. The same is seen with oligomycin: the FCC with activated MI complex is ten times higher (0.3 *versus* 0.03) The FCC for antimycin is the same in both conditions, which shows that the complex has an important regulatory role in energy transfer, but the regulatory impact of the complex III of the respiratory chain is the same in both protocols. The FCC for Pi carrier (mersalyl) is minor (0.06) with direct ADP activation as well as with Cr activation. From these results it is clear that the sensitivity increase (with activated MI) is specific for the complexes involved in the ADP-ATP turnover.

Though some researches presume that the Pi carrier has an important regulatory role in mitochondrial respiration (Beard 2006; Balaban 2009) our results show that the impact of the complex in the regulation is minimal in both protocols.

The results suggest that the key sites of the regulation of respiration in MI are MtCK (FCC = 0.93) and ANT (FCC = 0.95).

Table 2. Respiration rates

	V_3, nmol O_2 min^{-1}(mg prot)$^{-1}$	V_3, nmol O_2 min^{-1}(cyt aa3)$^{-1}$
ADP (protocol 1)	77.08 ± 6.62	160.60 ± 13.79
Cr (protocol 2)	80.40 ± 7.34	167.50 ± 15.30

DISCUSSION

In previous work with mitochondria *in vitro* Metabolic Control Analysis was applied to study the regulation of the respiration rate (Groen *et al.*, 1982) in the presence of different extramitochondrial ATP-consuming systems (Gellerich *et al.*, 1983; Wanders *et al.*, 1984) and in the presence

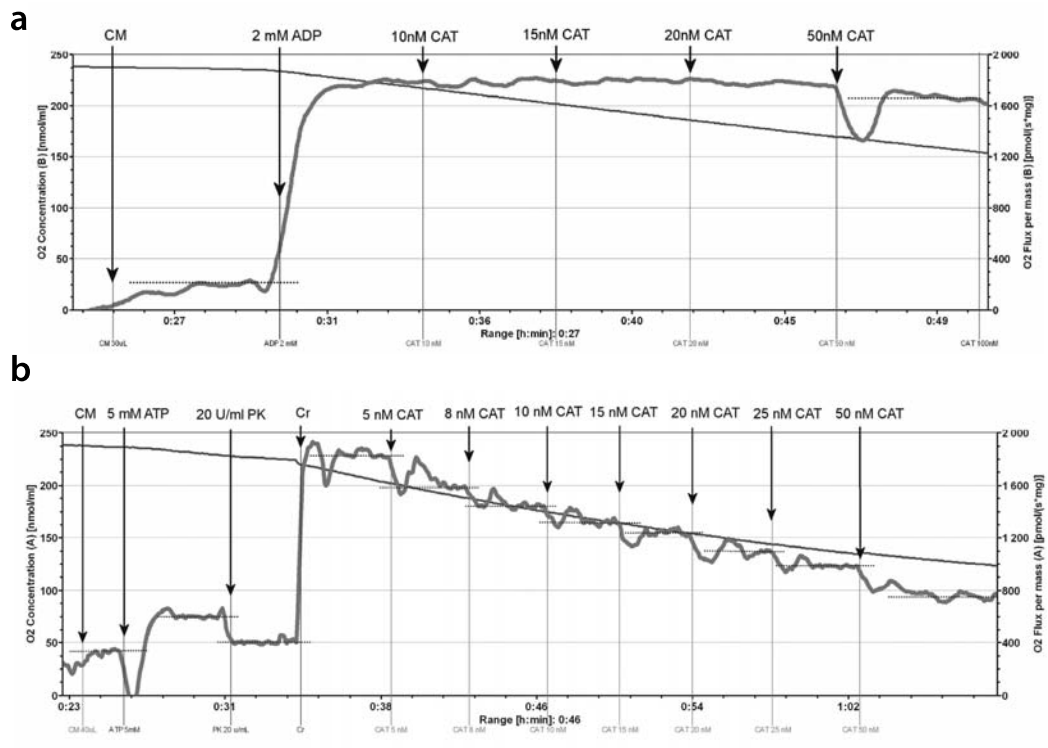

Figure 4. Respiration inhibition with carboxyatractyloside (CAT)
(a) Direct ADP activation and **(b)** the MI complex is activated with addition of 10 mM Cr in the presence of ATP and PK/PEP system. Dotted lines mark steady states. In the case of activated MI complex the respiration rate decreases significantly even at lower concentrations of inhibitor.

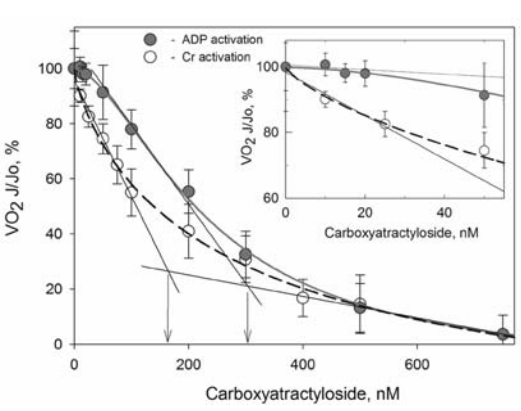

Figure 5. Titration curves of CAT
Respiration inhibition curves of two experimental conditions: with external ADP activation and Cr activation. In the Fig. 5 and in the insert of the figure the respiration rates are presented. From these data initial slope and I_{max} was calculated, and the FCC for ATP/ADP carrier estimated. The I_{max} values were calculated from the interception point of the straight line at the high concentrations of inhibitor and the straight line at the beginning of inhibition curve. The inhibition titration points on the higher concentrations are very close; therefore the initial slope of the curve causes the relative change in the FCC.

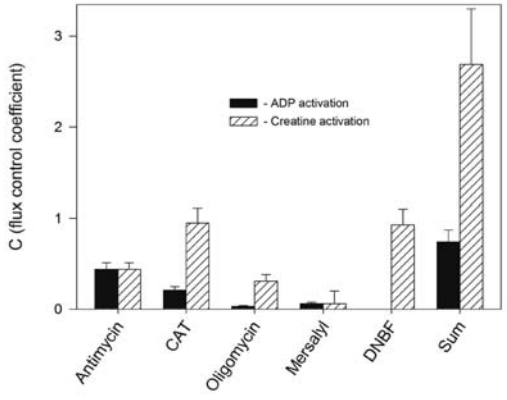

Figure 6. Flux control coefficients (FCC)
The coefficients were determined by two different protocols: the respiration activated with ADP (MtCK and Mitochondrial Interactosome (MI) complex are not activated) and activation with Cr, in the presence of ATP and PK-PEP system. The FCC was measured for ATP/ADP carrier (inhibitor, CAT), ATP Synthase (Oligomycin), Pi carrier (Mersalyl), MtCK (DNFB) and CoQ cytochrome *c* oxidoreductase (Antimycin). Also the sum of the measured coefficients is presented. As could be seen from the figure, the sum of the FCC as well as most of the FCC of complexes are multiple times higher upon the Cr activation (respiration is regulated by MI) The results show clearly that direct channeling is taking place in the regulation of respiration in MI.

of the PK/PEPsystem which competes with mitochondria for ADP (Gellerich & Saks, 1982; Gellerich *et al.*, 1982; 1983). However, these studies did not take into account the mitochondrial behavior *in vivo*. In isolated mitochondria the VDAC in the outer mitochondrial membrane is completely open and the PK/PEP system decreases the MtCK controlled respiration rate by approx. 50% (Gellerich & Saks, 1982). As is shown in Fig. 2, in permeabilized cardiomyocytes even at the high concentration of 100 U/ml PK does not have any influence on the respiration rate of mitochondria *in situ* and therefore the FCC of PK is zero.

According to our best knowledge the MCA has not been used as yet for analysis of the regulation *in vivo* when the respiration is controlled by the MtCK reaction. Our aim was to investigate this important question using two protocols. In the first protocol, we activated the respiration with extramitochondrial ADP; in this scheme the coupled MtCK-ANT-VDAC system is not activated and ATP leaves the mitochondria (Saks *et al.*, 2010). In the second protocol, the respiration was activated with ATP (endogenous ADP activation), then the PK/PEP system was added to trap the extramitochondrial ADP and MtCK and the MI supercomplex were activated by addition of Cr. This protocol represents real physiological conditions when respiration is regulated by coupled reactions at the ATP Synthasome–ANT–MtCK–VDAC–tubulin complex as was shown in our previous works (Timohhina *et al.*, 2009; Saks *et al.*, 2010). In the second experimental conditions, representing *in vivo* conditions, when the PK/PEP system is present and MtCK and therefore the MI supercomplex is activated by Cr, the mitochondrial outer membrane has very low permeability for ADP and ATP (Timohhina *et al.*, 2009). The ADP produced by MtCK is directly channeled back into the mitochondrial matrix through the ANT; ATP and ADP do not leave mitochondria and therefore are not accessible for the PK/PEP system. The PK/PEP system traps all the extramitochondrial ADP, but it does not have any access to the intramitochondrial ADP and has no influence on the oxidative phosphorylation inside mitochondria and the respiration rate as shown in Fig. 2. This means that the PK does not have any regulative role in these conditions; all the regulation is by the MI.

The results of this study show very clearly that the metabolic control is much stronger in the MI when the MtCK–ANT–VDAC complex is activated by creatine and the ADP-ATP recycling in the coupled reactions of MtCK–ANT–ATP Synthasome: the sum of measured FCC is 2.7 *versus* 0.74 (ADP activation). This indicates that the responses of mitochondria to metabolic changes in the cell are more extensive with an activated MI.

Our studies confirm the theory of Kholodenko, Westerhoff and their coworkers, who investigated theoretically the problem of "simple" metabolic pathways *versus* channeled pathways and showed that in channeled pathways the responsiveness to an external signal is enhanced and corresponding coefficients are larger than in non-channeled pathways (Kholodenko & Westerhoff, 1993; Peletier *et al.*, 2003). From our results it can be seen that the sum of FCC is increased several-fold when the MI is activated. The exact value of FCC is rather difficult to determine and therefore we used a comparison of two protocols to investigate the relative changes of FCC in the CM with activated and non-activated MI. Even if the absolute values of the coefficients are burdened with an error, the differences of FCC calculated here are very significant and the sum of the coefficients shows good evidence of ADP recycling between MtCK and ATP Sythasome in the MI.

Our results show that the relative role of most complexes in respiration regulation is also significantly changed when the MI is activated (activation with Cr). Complex III of the respiratory chain has a relatively high FCC in the case of ADP activation, but under physiological conditions when the MI is activated its relative regulatory power is much lower. With Cr activation the key sites of energy transfer regulation are ANT and MtCK. Also for ATP Synthase the FCC is significantly different for ADP and Cr activation, while the Pi carrier has no significant regulatory role in either case. As can be seen from the results, the FCC of MtCK and ANT are very high and of similar value. These results show that MtCK and ANT are the key sites of the regulation of energy fluxes from mitochondria into the cytoplasm and the results suggest the possibility of a direct channelling between these complexes. These results also completely exclude any role of a direct transfer of adenine nucleotides between mitochondria and ATPases as recently proposed by other (Kaasik *et al.*, 2001; Piquereau *et al.*, 2010).

We can conclude that under physiological conditions the responsiveness of the energy transfer pathway to metabolic signals is very high and the oxidative phosphorylation in mitochondria is very effectively regulated by the coupled MI supercomplex.

These results confirm our previous conclusions (Guzun *et al.*, 2009; Timohhina *et al.*, 2009) that the mechanisms of the regulation of mitochondrial respiration and energy fluxes in cardiac cells are system-level properties dependent on the interaction of mitochondria with intracellular structures and functional interactions with metabolic systems, which are not predictable on the basis of the properties of isolated mitochondria only. Similar conclusions have been made by several other authors (Kadenbach *et al.*, 2010; Guzun *et al.*, 2010). The strongly decreased permeability of MOM for adenine nucleotides in the IM significantly enhances the functional coupling between MtCK and ANT and the rate of recycling of ADP and ATP inside the mitochondria is very high. However, there is no restriction of diffusion for Cr and PCr, the latter being the major energy carrier between the mitochondria and ATPases (Saks, 2007). These data and the results of this work show clearly that under physiological conditions regulation of energy transfer by the supercomplex of MI takes place in mitochondria and is very sensitive to metabolic signals. The novel concept of the MI should be taken into account by any intended mathematical modeling.

An interesting task of further studies is to show the closeness of ANT and MtCK by FRET and other biophysical methods. Gel electrophoresis shows the physical association between different proteins and has been useful in several cases, such as determination of the structure of ATP Synthase (Chen *et al.*, 2004), but it is not suitable for experiments in *in vivo* systems where the intracellular structure has an important regulatory role. It is shown by experiments with mitochondria *in vitro versus in vivo* that the MOM and its VDAC complex with cytoskeleton proteins (one of which is tubulin) have an important regulatory role in energy transfer regulation in cardiomyocytes (Rostovt-

seva & Bezrukov, 2008; Guzun *et al.*, 2009) and there is direct channeling between MtCK and ANT in mitochondria *in vivo*. In mitochondria *in vitro* the regulatory part of MOM is lost and therefore the energy transfer regulation is different from the one *in vivo*, when the energy transfer is regulated by the MI.

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High efficiency of energy flux controls within mitochondrial interactosome in cardiac intracellular energetic units

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ABSTRACT

The aim of our study was to analyze a distribution of metabolic flux controls of all mitochondrial complexes of ATP-Synthasome and mitochondrial creatine kinase (MtCK) in situ in permeabilized cardiac cells. For this we used their specific inhibitors to measure flux control coefficients (C_{vi}^{JATP}) in two different systems: A) direct stimulation of respiration by ADP and B) activation of respiration by coupled MtCK reaction in the presence of MgATP and creatine. In isolated mitochondria the C_{vi}^{JATP} were for system A: Complex I - 0.19, Complex III – 0.06, Complex IV 0.18, adenine nucleotide translocase (ANT) – 0.11, ATP synthase – 0.01, Pi carrier - 0.20, and the sum of C_{vi}^{JATP} was 0.75. In the presence of 10 mM creatine (system B) the C_{vi}^{JATP} were 0.38 for ANT and 0.80 for MtCK. In the permeabilized cardiomyocytes inhibitors had to be added in much higher final concentration, and the following values of C_{vi}^{JATP} were determined for condition A and B, respectively: Complex I - 0.20 and 0.64, Complex III - 0.41 and 0.40, Complex IV - 0.40 and 0.49, ANT - 0.20 and 0.92, ATP synthase - 0.065 and 0.38, Pi carrier - 0.06 and 0.06, MtCK 0.95. The sum of C_{vi}^{JATP} was 1.33 and 3.84, respectively. Thus, C_{vi}^{JATP} were specifically increased under conditions B only for steps involved in ADP turnover and for Complex I in permeabilized cardiomyocytes within Mitochondrial Interactosome, a supercomplex consisting of MtCK, ATP-Synthasome, voltage dependent anion channel associated with tubulin βII which restricts permeability of the mitochondrial outer membrane.

1. Introduction

Studies with permeabilized cardiomyocytes have shown that in the cells *in situ* kinetic parameters of regulation of mitochondrial respiration are very different from those *in vitro* [1–4]. These data show that mechanisms of fine regulation of integrated metabolism and energy fluxes *in vivo* cannot be completely understood by studying separately isolated cellular components – mitochondria, sarcoplasmic reticulum, myofibrils etc. [4]. Specific structural organization of the cell and interactions between cellular components, including cytoskeleton, result in formation of dissipative metabolic structures [5], such as Intracellular Energetic Units (ICEUs)[1] in cardiac and oxidative skeletal muscle cells [1,6], glycolytic metabolons etc. [5]. In adult cardiac cells mitochondria are very regularly arranged into ICEUs due to their interaction with tubulin, microtubular system and probably other cytoskeletal structures [7,8]. Immunolabelling studies with the use of confocal microscopy have shown association of beta II tubulin with the mitochondrial outer membrane [9]. This interaction results in formation of a supercomplex Mitochondrial Interactosome (MI) in contact sites of outer and inner mitochondrial membranes, consisting of ATP Synthasome (including respiratory chain complexes), mitochondrial creatine kinase (MtCK), voltage-dependent anion channel (VDAC), tubulin αβII heterodimers and probably some other „linker proteins“ [8]. Within the cristae membranes, MI contains only ATP Synthasome connected to MtCK by adenine nucleotide translocase (ANT). The localization of these supercomplexes in heart mitochondria is shown in Fig. 1. Tubulin binding to VDAC very significantly decreases the permeability of mitochondrial outer membrane for adenine nucleotides, but not for creatine (Cr) and phosphocreatine (PCr), and recycling of ADP and ATP within mitochondria is coupled to effective synthesis of PCr with PCr/O_2 ratio 5.7 [10]. Within this supercomplex, its different components may have various contributions in the overall control of the mitochondrial respiration rate and energy fluxes in the heart. This question can be quantitatively solved by application of the Metabolic Control Analysis [11],

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[1] ***Abbreviations:*** ANT, adenine nucleotide translocase; BSA, bovine serum albumin; CAT, carboxyatractyloside; CK, creatine kinase; CM, cardiomyocytes; Cr, creatine; DNFB, 2,4 dinitrofluorobenzene; C_{Ji}^{j}, flux control coefficient; GGG, triglycine; ICEU, intracellular energetic unit; IM, isolation medium; IMS, mitochondrial intermembrane space; MCA, metabolic control analysis; MI, mitochondrial Interactosome; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane; MtCK, mitochondrial creatine kinase; PCr, phosphocreatine; PEP, phosphoenolpyruvate; PIC, phosphate carrier; PK, pyruvate kinase; VDAC, voltage-dependent anion channel

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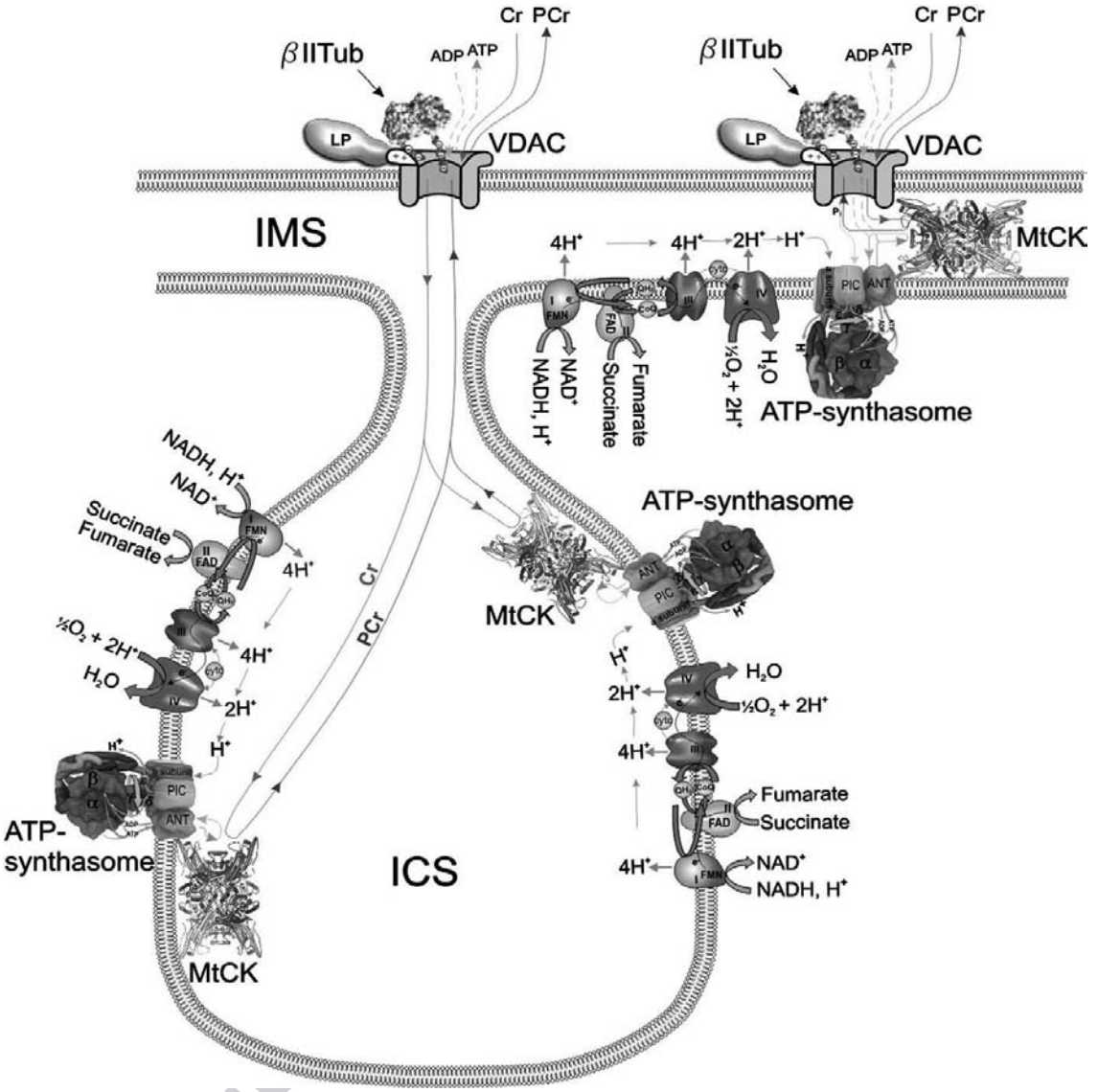

Fig. 1. Model of regulation of respiration in the Mitochondrial Interactosome (MI). MI is a supercomplex consisting of ATP Sythasome (reprinted with kind permission from Peter L. Pedersen [60,61]), respiratory chain complexes, mitochondrial creatine kinase (MtCK), voltage dependent anion channel (VDAC), and tubulin. Octameric MtCK (the structure was kindly supplied by U.Schlattner [33]) located in the mitochondrial intermembrane space (IMS) is attached to mitochondrial inner membrane and in the contact sites to the outer membranes [33]. VDAC permeability is selectively regulated by heterodimeric tubulin [42], whose binding to VDAC in intact mitochondrial membrane may be either direct or by some linker proteins (LP). This complex of VDAC with other proteins controls fluxes of adenine nucleotides and phosphocreatine (PCr) into surrounding medium, and phosphorylation by the ATP Synthasome system is effectively regulated by creatine (Cr) *via* MtCK. Here: ANT - adenine nucleotide carrier; and PIC-phosphate carrier.

developed in 1974 in the works by Kacser and Bruns [12,13] and Heinrich and Rapoport [14,15]. Theoretical aspects of MCA have been subsequently analyzed in many works by Kholodenko, Westerhoff, Cascante and others [16–20]. In experimental studies this method has been very intensively applied for analysis of the control of respiration in isolated mitochondria [21–24]. It was found in these works that the sum of the flux control coefficients of respiratory chain complexes, ATP synthase and metabolite carriers is close to 1 that corresponding to the behaviour of a linear metabolic system [19–22]. Analogous works with similar results were carried out also in permeabilized cardiac fibers when respiration was activated by direct addition of ADP [25,26]. Interestingly, already in studies of isolated mitochondria it was shown by Gellerich et al. and Groen et al. [27,28] that the flux control coefficient value may change in dependence upon the presence of ADP regenerating system. Since under physiological conditions creatine is always present in cardiac cells and MtCK coupled to ATP-Synthasome within MI is always activated, mitochondrial respiration in these cells is largely controlled by the MtCK reaction. In our recent preliminary study with application of MCA we measured C_{VI}^{IATP} for two key enzymes and complexes of MI

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in permeabilized cardiomyocytes under two conditions: 1) direct activation of respiration by addition of ADP in concentration of 2 mM in the presence of respiratory substrates and inorganic phosphate (Pi); 2) MI was activated by addition of creatine and MgATP in the presence of a system consisting of pyruvate kinase (PK) and phosphoenolpyruvate (PEP) for trapping extramitochondrial ADP [29]. The results showed that the flux control coefficients for ANT and ATP Synthase were significantly higher in the latter case [30]. The second protocol corresponds more closely to the physiological conditions in healthy heart cells *in vivo*. In these conditions both MgATP and creatine are always present and mitochondria compete with glycolytic system for cytosolic ADP. Therefore, to complete this study we performed the MCA with measuring the flux control coefficients for all respiratory chain complexes in permeabilized cardiomyocytes under two conditions described above. For comparison, the flux control coefficients were measured also for MtCK and ANT *in vitro* in isolated cardiac mitochondria.

The data obtained in the present work show that the flux control of several steps is much more efficient in a system with ADP-ATP recycling (Mitochondrial Interactosome with activated MtCK, our system 2) than in mitochondria with direct supply of ADP. These results are consistent with the proposal of the central role of Mitochondrial Interactosome with activated MtCK in regulation of mitochondrial respiration and energy fluxes in adult normal cardiac cells [1,31–34]. High efficiency of energy flux control in MI makes this supercomplex a key site for feedback metabolic regulation of mitochondrial respiration in cardiac cells.

2. Materials and methods

2.1. Laboratory animals and chemicals

Male Wistar rats weighing 300–350 g were used in the experiments. The animals were housed at constant temperature (22 °C) in environmental facilities with a 12:12 h light-dark cycle and were given standard laboratory chow *ad libitum*. Animal procedures were approved by the Estonian National Committee for Ethics in Animal Experimentation (Estonian Ministry of Agriculture).

2.2. Isolation of adult cardiac myocytes

Adult cardiomyocytes were isolated after perfusion of the rat heart with collagenase A (Roche), using an adaptation of the technique described previously [35]. Rats were anaesthetized with medetomidine and ketamine, decapitated and, the heart was quickly excised preserving a part of the aorta and placed into isolation medium (IM) of the following composition: 117 mM NaCl, 5.7 mM KCl, 4.4 mM $NaHCO_3$, 1.5 mM KH_2PO_4, 1.7 mM $MgCl_2$, 11.7 mM glucose, 10 mM Cr, 20 mM taurine, 10 mM PCr, 2 mM pyruvate and 21 mM HEPES, pH 7.1. The excised heart was cannulated by the aorta and suspended in Langendorf system for perfusion and washed for 5 min with a flow rate of 15–20 ml/min. The collagenase treatment was performed at 37 °C by switching the perfusion to circulating O_2-saturated IM supplemented with 1 mg/ml collagenase A and 2 mg/ml BSA at flow rate of 5 ml/min for 20–30 min. After the digestion the heart was washed with IM for 2–3 min and transferred into IM containing 20 μM $CaCl_2$, 10 μM leupeptin, 2 μM soybean trypsin inhibitor and 2 mg/ml fatty acid free BSA. The cardiomyocytes were then gently dissociated using forceps and pipette suction. Cell suspension was filtered through a crude net to remove tissue remnants and let to settle for 3–4 min at room temperature. After 3–4 min the initial supernatant was discarded, pellet of cardiomyocytes resuspended in 10 ml of IM containing 20 μM $CaCl_2$ and the protease inhibitors. After the resuspension-sedimentation cycles the cardiomyocytes were gradually transferred from IM with 20 μM Ca^{2+} into calcium free Mitomed (supplemented with protease inhibitors and BSA) and washed. Isolated cells were re-suspended in 1–2 ml of Mitomed solution [36] described below for respiration measurements and stored on melting ice before measurements. Isolated cardiomyocytes contained 70–90% of rod-like cells.

2.3. Isolation of mitochondria from cardiac muscle

Mitochondria were isolated from adult rat hearts as described in [37].

2.4. Permeabilization procedure

In order to study the regulation of mitochondrial respiration in cardiomyocytes, the cells sarcolemma was permeabilized by saponine treatment keeping the mitochondrial membranes intact [36,38]. The permeabilization procedure was carried out at 25 °C with 20 μg/ml saponine for 10 min and then resuspension-sedimentation cycle with Mitomed solution.

2.5. Measurements of oxygen consumption

The rates of oxygen uptake were determined with a high-resolution respirometer (Oxygraph-2 K, from OROBOROS Instruments, Austria) in Mitomed solution [36] containing 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 3 mM KH_2PO_4, 20 mM taurine, 20 mM HEPES (pH 7.1), 110 mM sucrose, 0.5 mM dithiothreitol, 2 mg/ml fatty acid free BSA, supplemented with 5 mM glutamate and 2 mM malate as respiratory substrates. These measurements were carried out at 25 °C; solubility of oxygen was taken as 240 nmol/ml [39]. The decision to make measurements at 25 °C and not at physiological 37 °C was made after several experiments and careful consideration. It is crucial, when using the method of MCA that the decrease of the respiration rate during the measurement is only due to the inhibition. The experiments of stepwise inhibition of oxygen consumption were taking a long time. At 37 °C due to the activation of lysosomal enzymes, the respiration rate decreases during the time because of instability of preparation. Also the solubility of oxygen is decreasing significantly from 25 °C to 37 °C, the oxygen concentration in the cell of the oxygraph at the end of the experiment could not be in the area of anoxia.

In kinetic experiments with MgATP, stock solution of 100 mM MgATP was prepared by mixing equimolar amounts of $MgCl_2$ and ATP, pH was adjusted to 7.2. The respiration rates were expressed in pmol of oxygen consumed per second per mg of protein or in nmol of oxygen consumed per nmol cytochrome aa_3. Protein concentrations were determined using a BCA protein assay kit (Pierce, USA) with BSA as a standard.

2.6. Determination of flux control coefficients (C^J_{vi})

Control coefficient is defined as the ratio of the fractional change in the system variable to the fractional change in the biochemical activity that caused the system change [40]. It allows the identification of system components that are crucial in the control of pathway flux or metabolite concentration and thus also in the regulation of energy transfer and regulatory networks. The C^J_{vi} is defined according to the equation [40,41]:

$$C^J_{vi} = \left(\frac{dJ}{dv_i}\right) / \left(\frac{J}{v_i}\right) = \frac{d\ln J}{d\ln v_i}$$

in which the expression dJ/dv_i describes the variation in flux (J) when an infinitesimal change takes place in the enzyme i concentration or activity. In practice, the infinitesimal changes in v_i are undetectable, and hence measurable noninfinitesimal changes are undertaken. If a small change in v_i promotes a significant variation in J, then this

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enzyme exerts an elevated flux control. In contrast, if a rather small or negligible change in flux is observed when v_i is greatly varied, then the enzyme does not exert significant flux control [30,40].

Groen et al. [27] derived a method to determine experimentally the $C_{vi}^{[ATP}$ using titration with specific enzyme inhibitors. As the amount of inhibitor tends to zero the response of the flux to the inhibitor can be expressed in MCA terms.

For the case of irreversible specific inhibitor, an estimation of the value of the C_{vi}^{J} coefficient is given by Groen [27] and Moreno-Sanches [41]:

$$C_E^J = (\Delta J/\Delta I)\ (I_{\max}/J_0),$$

where $(\Delta J/\Delta I)$ is initial slope of the flux/inhibition graph, I_{max} is the inhibitor concentration giving complete inhibition, and J_0 is the initial steady-state flux value.

The flux control coefficients in permeabilized cardiomyocytes were determined by using graphical method described by Fell [40]. Additionally, obtained results were compared with the computer estimated coefficients. In this case non-linear regression analysis was used by fitting experimental data to the mathematical model, developed by Gellerich [11]. The fitting was performed with the use of the MathCad Professional 2001 (MathSoft, PTC) by providing best-fit values of three parameters: K_d (dissociation constant of the enzyme-inhibitor complex), E_0 (concentration of inhibitor binding sites) and C_0 ($C_0 = (\mathrm{dln}J/\mathrm{dln}E)_{E=E0}$ in the absence of the inhibitor).

The inhibitors used were: rotenone for Complex I of respiratory chain (concentration range 2–150 nM), antimycin for Complex III (10–240 nM), sodium cyanide for Complex IV (1–150 μM), oligomycin for Complex V (ATP synthase, 30–210 nM), carboxyatractyloside (CAT) for ATP/ADP transporter (10–750 nM), mersalyl for PIC (10–120 μM), and DNFB for MtCK .(0,05-40 μM).

From the inhibitors used rotenone, antimycin, cyanide, CAT, oligomycin and mersalyl were considered as pseudo-irreversible and non-competitive in these conditions.

Enzymes and other chemicals were obtained from Sigma, Fluka and Roche.

2.7. Data analysis

To reduce the possibility of random error the experiments were repeated seven to twenty times and the fitting technique was used to calculate the $C_{vi}^{[ATP}$. All data are presented as mean ± SEM. Statistical analysis was performed using Student's t-test, and $p<0.05$ was taken as the level of significance.

3. Results

3.1. Quality control tests for preparations used

There are several important quality tests of intactness of membrane structures that should be used in kinetic studies both with isolated mitochondria and especially with permeabilized cardiomyocytes [36]. When isolated mitochondria were used, prior to study experiments, respiratory control ratio was checked and only mitochondria, having state 3/4 ratio 6 and higher was used in experiments. The first of the quality tests used both for isolated mitochondria and mitochondria *in situ*, is the cytochrome c test shown in Fig. 2. Cytochrome c, a highly soluble hemoprotein of the respiratory chain is loosely associated with the outer side of the inner membrane of the mitochondria. If the outer membrane is disrupted, cytochrome c leaves mitochondria and consequently, in this situation its addition increases respiration rate. After activation of respiration of isolated mitochondria with ADP, addition of cytochrome c gives only insignificant (not more than 10%) increase in oxygen consumption rate in mitochondria (Fig. 2A). Addition of CAT is quality test for mitochondrial inner membrane. CAT entirely blocks ANT and therefore, if the inner membrane is intact, addition of CAT stops ATP/ADP exchange between mitochondrial matrix and intermembrane space; oxygen consumption rate decreases back to initial v_0 level (Fig. 2A). Analogous quality test for cardiomyocytes (CM) is shown in Fig. 2C. In mitochondria *in situ*, in permeabilized CM, the addition of cytochrome c does not increase oxygen consumption rate (Fig. 2C), demonstrating that the mitochondrial outer membrane is intact. Also, an addition of CAT decreases respiration rate back to the initial level, showing intactness of the mitochondria inner membrane. All together these results show the high quality and selectivity of the permeabilization procedure. In our experiments preparations of mitochondria and cardiomyocytes meeting the requirements of this quality test were always used.

Another important quality indicator, especially in the case of isolated mitochondria, is low MgATPase activity (showing low amount of damaged/destroyed mitochondria) and strong activation of respiration up to maximal values by creatine in the presence of ATP (Fig. 2B). Similar results were obtained for permeabilized cardiomyocytes in the absence of calcium (Fig. 2D).

Third important and most sensitive quality marker is the pyruvate kinase (PK) –PEP [29] test described below.

3.2. Equality of maximal rates of respiration in all preparations

The flux control coefficients are dependent on the steady state rates of the fluxes [27,28,40]. Therefore, the proper measurements always require intact preparations with high initial (non-inhibited) maximal respiration rates per nmol of cytochromes aa_3. The values of these maximal steady state respiration rates are shown in separate recordings in Fig. 2 and summarized in Table 1. The steady states, reached according to each protocol, differ by energy flux transfer regulation. However, it can be seen from Table 1 that the maximal rates are equal in the case of direct activation by ADP (Fig. 2A and B) and activation by MgATP in the presence of Cr (Fig. 2C - isolated mitochondria and 2D - mitochondria *in situ*). In all cases, the maximal respiration rates calculated per nmol of cytochrome aa_3 were in the range of 160–172 nmol O_2 min^{-1} nmol *cyt* aa_3^{-1}. Therefore these systems are comparable by the values of maximal steady state rates of respiration and could be used to study changes in energy flux regulation by MCA.

3.3. Experimental protocols for analysis of flux control coefficients in isolated mitochondria and permeabilized cardiomyocytes

Fig. 3A represents a scheme of protocol of our experiments with permeabilized cardiomyocytes. Mitochondrion *in situ,* in a permeabilized cardiac cell, is associated with cytoskeleton proteins (tubulin) and surrounded by myofibrils. The respiratory chain complexes, ATP Synthasome with ATP synthase, ANT and PIC are integrated within the mitochondrial inner membrane [42]. MtCK is depicted as an octamer, located in the mitochondrial intermembrane space (IMS) and attached to the inner membrane surface [33].

In the first part of our experiments respiration was activated by the addition of exogenous ADP to the final concentration of 2 mM, and thus Cr/phosphocreatine (PCr) transfer network and the MI supercomplex of energy transfer were not activated. In this case, ATP produced in mitochondria is transported out of the mitochondria through MOM channels [2,10]. In the second part of experiments, MtCK was activated by Cr in the presence of MgATP. Under these conditions, the cellular MgATPases are also activated. Therefore, during the experiment permeabilized cardiomyocytes were supplemented with PEP and PK to eliminate the influence of extramitochondrial MgADP: the PEP-PK system removes exogenous ADP, produced by intracellular ATP consuming reactions and continuously regenerates extramitochondrial ATP [29]. Endogenous intramitochondrial ADP,

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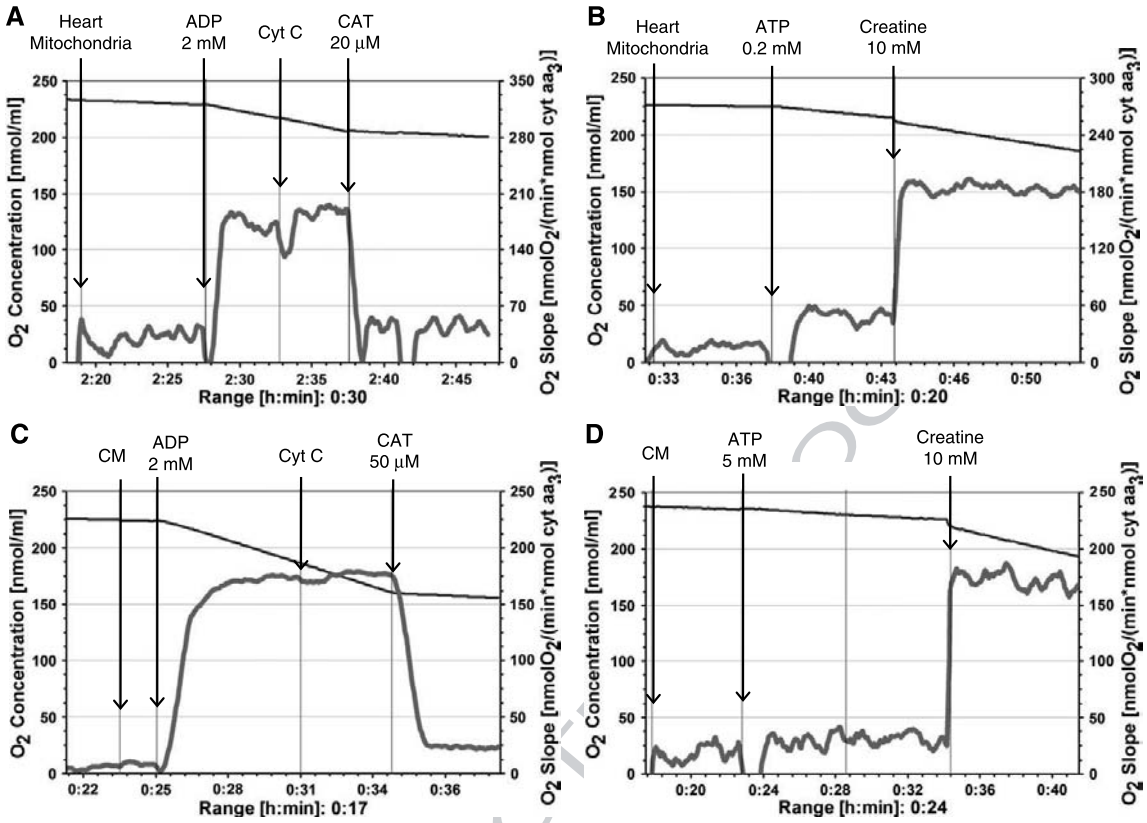

Fig. 2. The quality tests. A. Quality test of intactness of mitochondrial membranes in isolated mitochondria. Representative respiration traces of isolated mitochondria recorded using a two-channel high-resolution respirometer (Oroboros oxygraph 2 k), Left-hand scale and blue trace indicate oxygen concentration (nmol O_2 ml^{-1}) in the experimental milieu. Right-hand scale and the red trace show rate of oxygen uptake (nmol O_2 min^{-1} nmol *cyt* aa_3^{-1}). Experiment was carried out in Mitomed solution with 5 mM glutamate and 2 mM malate as respiratory substrates. Respiration is activated with 2 mM ADP; addition of cytochrome c gives only insignificant increase in oxygen consumption, – the outer membrane of mitochondria is only slightly affected by isolation: after addition of CAT respiration decreases back to v_0 level thus showing that the inner membrane of the mitochondria is intact. The test shows quality of isolation of mitochondria. B. Maximal respiration rate with Cr activation in isolated mitochondria. Respiration was activated with 0.2 mM ATP, addition of 10 mM Cr increases respiration rate due to the activation of MtCK. The maximal respiration rate is equal of the V_{max} (ADP) (Fig. 2A) and consequently these protocols could be used as comparative systems to measure the influence of MtCK and MI activation on C_{vi}^{ATP} of complexes of oxidative phosphorylation. C. Quality test of intactness of mitochondrial membranes in permeabilized cardiomyocytes. Respiration was activated with 2 mM ADP. In CM outer membrane of the mitochondria is intact, addition of cytochrome c does not increase oxygen consumption rate, showing the intactness of the outer membrane, Addition of CAT decreases respiration rate back to the v_0 level due to the closure of ANT (showing intactness of mitochondrial inner membrane). D. Maximal respiration rate with Cr activation in CM. Respiration was activated with 5 mM ATP, after addition of 10 mM Cr maximal respiration rate was achieved; equal to that with direct ADP activation (Fig. 2A and C) as well as with ATP activation in the presence of Cr (Fig. 2B and D). Consequently we could use these four protocols for MCA to investigate an influence of in vivo conditions (activated MI) on C_{vi}^{ATP} of the MI complexes.

produced by MtCK in microcompartments within the IMS (Fig. 1), may be re-imported into the matrix *via* adenine nucleotide translocase (ANT) due to its functional coupling with MtCK [2,3,43] or leave IMS *via* VDAC in dependence upon permeability of this channel. If ADP can leave IMS *via* VDAC, it will be trapped by PEP-PK system and the respiration rate decreases. Fig. 3B and C show the respiration recordings of isolated mitochondria and cardiomyocytes according to this protocol for the experimental conditions 2 (Cr activation in the presence of PK-PEP system). In intact cardiomyocytes with rod-like shape PK-PEP addition does not influence the respiration rate, as it is seen in Fig. 3C. This is explained by closure of VDAC by tubulin βII [44] which makes VDAC selectively permeable only for Cr and PCr [2,3,10]. ADP produced by MtCK is channelled *via* ANT into mitochondrial matrix and is not accessible to extramitochondrial PK-PEP trapping system. In this case MI complexes completely control the oxidative phosphorylation and respiration that makes it possible to measure C_{vi}^{ATP} for these complexes, since the C_{vi}^{ATP} for PK and MgATPases are zero under these experimental conditions. Cytoskeletal proteins associated with the outer mitochondrial membrane and controlling VDAC permeability are separated during isolation of mitochondria. Therefore, in isolated mitochondria the selective permeability of mitochondrial outer membrane VDAC is lost and part of ADP is accessible for the PK-PEP system [2,43,45]: Fig. 3B shows that in this case, addition of PK in the presence of 10 mM Cr and 5 mM ATP

Table 1
Maximal respiration rates of isolated mitochondria and of mitochondria in situ in permeabilized cardiomyocytes from rat heart.

Conditions	V_{max}, nmol O_2 min^{-1} nmol *cyt* aa_3^{-1}	
	Mitochondria	CM
ADP activation	172 ± 8	161 ± 14
Cr activation	173 ± 17	168 ± 10

ADP activation - respiration rate in the presence of 2 mM ADP.
Cr activation - respiration rate in the presence of activated MtCK by 2 mM ATP and 20 mM creatine. CM – permeabilized cardiomyocytes.
Average values and SD are based on 14–17 experiments.

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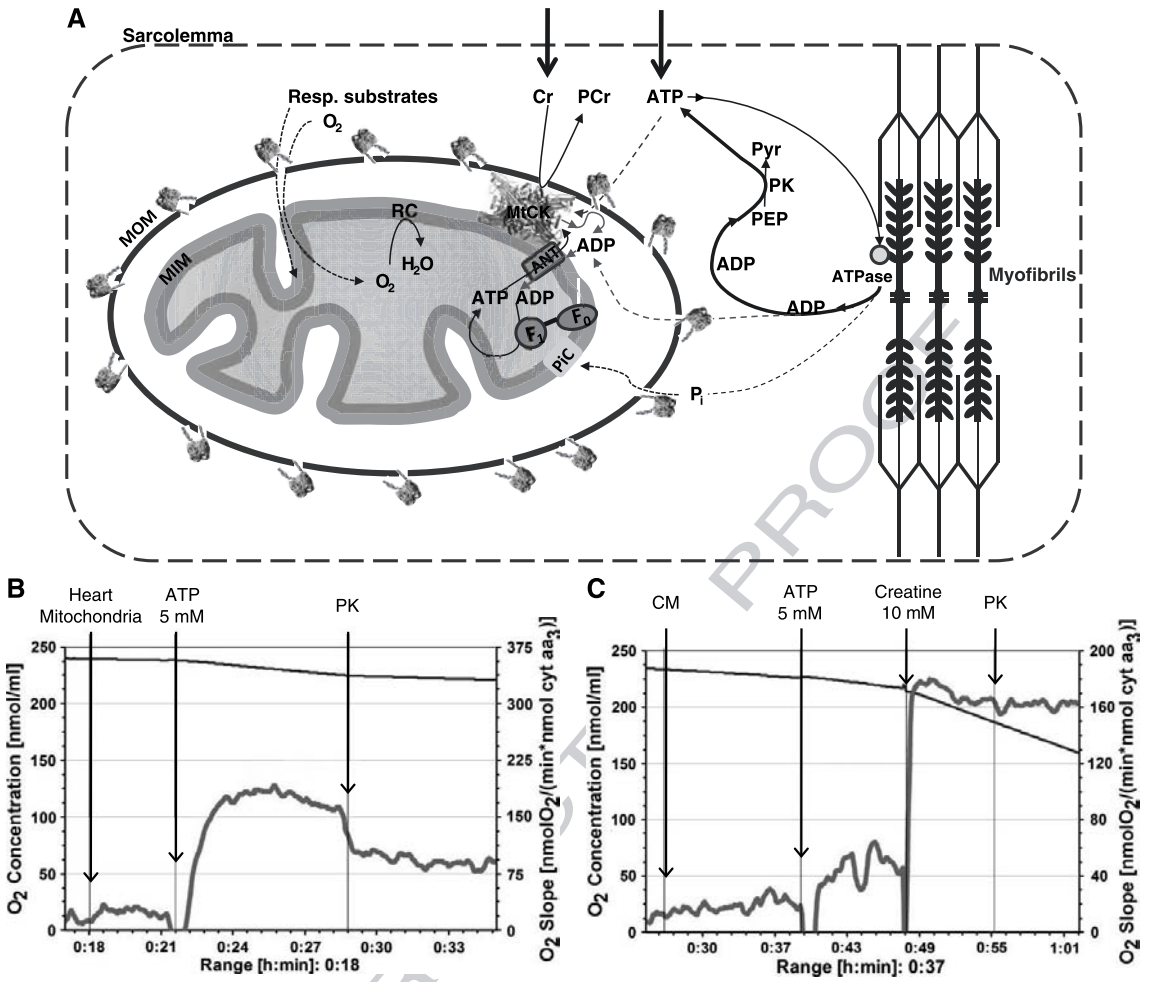

Fig. 3. Influence of PK-PEP system on the mitochondrial respiration. A. Scheme of the experimental protocols. The method is called Gellerich–Guzun protocol [2,29]. It represents mitochondrion *in situ*, in permeabilized cardiac cell, surrounded by cytoskeletal proteins and myofibrils. The mitochondrial outer membrane is less permeable than in isolated mitochondria, due to the interactions of VDAC with cytoskeletal proteins [2,9]. The system is supplemented with phosphoenolpyruvate (PEP) and pyruvate kinase (PK). This PEP-PK system removes exogenous ADP produced by intracellular ATP-consuming reactions and continuously regenerates extramitochondrial ATP. Endogenous ADP produced by MtCK is re-imported back to the matrix *via* adenine nucleotide translocase (ANT) due to the functional coupling with MtCK. Reproduced from Timohhina et al. with permission [10]. B. Influence of PK-PEP system on the respiration on isolated heart mitochondria. Respiration is activated with 5 mM ATP, in the presence of 10 mM Cr in the solution. Addition of 10 U/ml PK (with 5 mM PEP in the solution) decreases oxygen consumption rate about 50% due to detachment of the regulatory proteins from VDAC on the mitochondrial outer membrane during isolation of mitochondria; the result show that PK-PEP system have an influence on oxidative phosphorylation in isolated mitochondria and therefore the protocol with PK trapping system could not be used in isolated mitochondria MCA measurements. C. Influence of PK-PEP system on respiration rate *in situ* with activated MI. Respiration is activated with 5 mM ATP, in the presence of 10 mM Cr in the solution. The MtCK and therefore Mitochondrial Interactosome supercomplex is activated with Cr. The addition of PK does affect oxidative phosphorylation and respiration rate in CM. The PK-PEP systems regenerates all the exogenous ATP, at the same time as the trapping system does not have any access to the intramitochondrial ADP due to the selective permeability of VDAC. PK does not have any regulatory role and control strength in these conditions; all the regulation is by Mitochondrial Interactosome. The maximal respiration rate in these conditions is equal with those with ADP activation; therefore we could use these systems as comparative in MCA measurements.

decreases respiration rate to the half of maximum. The remnant rate of respiration was due to the functional coupling between MtCK and ANT with the direct transfer of ADP into the matrix [2]. Therefore, in isolated mitochondria the PK-PEP protocol cannot be used for $C_{vi}^{[ATP]}$ determination. They can be measured only in the case of direct ADP activation of respiration.

3.4. Specific inhibition of MtCK by DNFB

$C_{vi}^{[ATP]}$ for the MtCK reaction was determined by stepwise inhibition of the reaction by 2,4 dinitrofluorobenzene (DNFB), which is an effective and specific inhibitor of the CK reaction [46]. Recordings of oxygen consumption by permeabilized cardiomyocytes shown in Fig. 4 confirm the selectivity of MtCK inhibition by DNFB. Fig. 4A shows the results of experiments in which the respiration was activated with 2 mM ADP (protocol 1). MtCK and therefore MI were not activated; addition of 50 μM DNFB did not have any influence on the respiration rate. The experiment shows that ATP Synthasome was not inhibited by DNFB. When respiration was activated with 5 mM ATP and 10 mM Cr (Fig. 4B), DNFB, even in a concentration of 10 μM, had a significant influence on the respiration rate, showing the specific inhibition of MtCK by DNFB. As the inhibition develops

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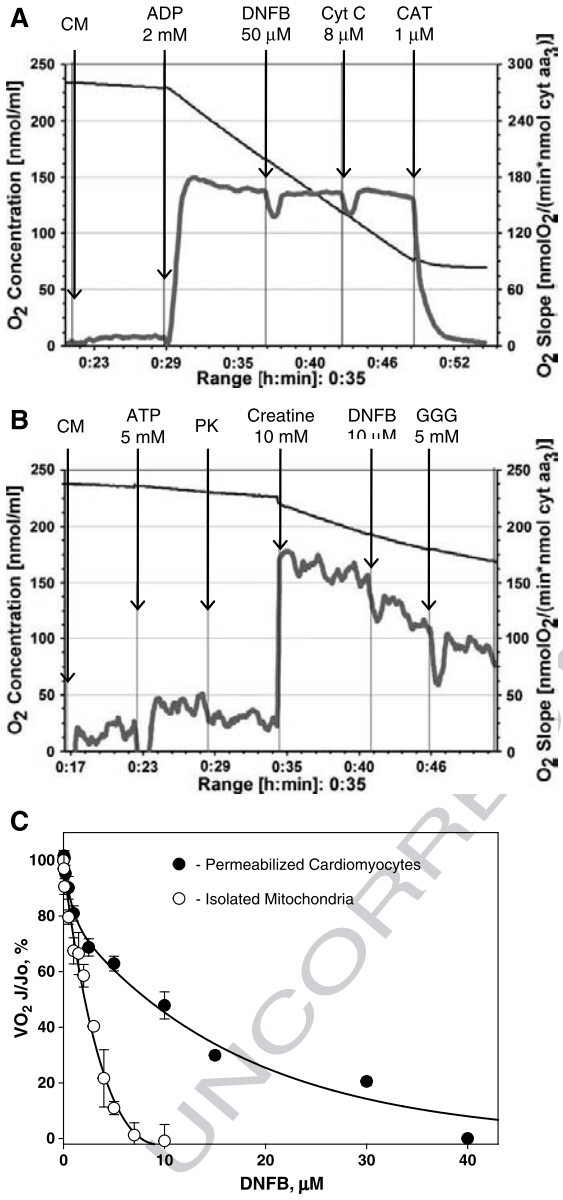

Fig. 4. Specificity of dinitrofluorobenzene (DNFB) for inhibition of MtCK. A. ADP activation. Respiration of cardiomyocytes (CM) activated with 2 mM ADP. Without activation of MI complex by creatine (Cr), the respiration rate was not affected by addition of 50 μM DNFB, ATP Synthasome was not affected by DNFB. B. Cr activation. Respiration was activated with 5 mM ATP; the PK-PEP system was added to trap exogenous ADP and then 10 mM Cr to activate MtCK. Addition of 10 μM of DNFB gives significant decrease at respiration rate. The inhibitory effect was terminated with 5 mM triglycine (GGG) resulting in stable level of respiration, showing steady state of the system. PEP was added to 5 mM and PK to 10 units/ml. C. Inhibition titration curve of isolated mitochondria and CM with DNFB. Isolated mitochondria were inhibited with DNFB in the presence of 10 mM Cr and 0.2 mM ATP. Inhibition was terminated with triglycine and the respiration rate was registered from the following steady state. Maximal inhibition concentration is four times higher in CM due to the diffusion restrictions.

in time, in all the measurements with DNFB after exact 5 minutes period further inhibition was stopped by triglycine (GGG) and the respiration rate remains unchanged (steady state) (Fig. 4B). This method was used to measure the oxygen consumption rate at different concentrations of DNFB both in permeabilized cardiomyocytes and isolated heart mitochondria. Fig. 4C shows the titration curves of MtCK activity in both these preparations. Remarkably, the DNFB concentration needed for maximal inhibition of MtCK activity was much higher in permeabilized cardiomyocytes than in isolated mitochondria (Fig. 4C). This may be explained at least partially by the diffusion restriction across the MOM, since similar observation was made also for other inhibitors. Nevertheless, in both cases the $C_{vi}^{[ATP]}$ for MtCK was very close: 0.95 and 0.8, respectively (Table 2 and 3).

3.5. Flux control by ANT and MtCK in isolated mitochondria and permeabilized cardiomyocytes

In many previous studies $C_{vi}^{[ATP]}$ of respiratory chain complexes in isolated mitochondria have been measured [21,22,27,28,41,47,48], but these measurements were performed using different conditions, explaining a divergence of the results (see [41] for review). Several authors have also estimated the $C_{vi}^{[ATP]}$ in permeabilized muscle fibers [26,49]. To evaluate the influence of restriction of mitochondrial outer membrane permeability on the metabolic sensitivity of MI complexes, we measured the $C_{vi}^{[ATP]}$ values of MI complexes in isolated mitochondria as well as mitochondria *in situ* according the protocols, described in Methods. Our preliminary results have shown the major role of complexes of MtCK and ANT in flux control in the MI [30]. In this context it was interesting to perform MCA analysis of the complexes comparing direct ADP and Cr/MtCK activated respiration also in isolated mitochondria.

Fig. 5A shows representative traces of oxygen consumption by isolated heart mitochondria, when respiration was activated by ADP. ANT was stepwise inhibited by carboxyatractyloside (to the concentrations indicated in Fig. 5A). Fig. 5B shows similar traces for the system where respiration was maintained by activated MtCK. As it was observed in the case of permeabilized cardiomyocytes before [11], the effect of the inhibitor on respiration in the same concentration range on the respiration is much stronger under the conditions of activated MtCK (Fig. 5A and B). Significant increase of sensitivity of ANT with Cr/MtCK activation to CAT is consistent with direct functional coupling of MtCK-ANT and ADP recycling, as it was shown before [3,31,32,50,51]. The titration curves for respiration inhibition by CAT both for isolated mitochondria and permeabilized cardiomyocytes under these two conditions are shown in Fig. 5C. Remarkably, as it was seen in the case of inhibition of MtCK with DNFB (Fig. 4C), the concentrations of CAT needed for complete inhibition of ADP -

Table 2
Flux control coefficients of respiratory chain complexes in isolated mitochondria.

Complex	Inhibitor	1	2	3
NADH-CoQ oxidoreductase Complex I	Rotenone	0.19 ± 0.07	0.15	0.26
CoQ cytochrome-c oxidoreductase (Complex III)	Antimycin	0.06 ± 0.03	0.01	0.19
Cytochrome c oxidase (Complex IV)	Na cyanide	0.18 ± 0.07	0.11	0.13
ATP/ADP carrier	CAT	0.11 ± 0.02	0.24	0.04
ATP synthase	Oligomycin	0.01 ± 0.01	0.34	0.12
Pi carrier	Mersalyl	0.20 ± 0.06	0.15	0.14
Sum		***0.75 ± 0.26***	*1.00*	*0.88*
MtCK*	DNFB	0.80 ± 0.04	-	-

Flux control coefficients of various complexes measured by different authors in isolated mitochondria.
1 – Heart mitochondria: results of our laboratory. Respiration rate measurements were made in the presence of 2 mM ADP, except * when measurements were made in the presence of 10 mM Cr and 0.2 mM ATP. Results were presented as average values ± SEM for 8–10 experiments.
2 - Heart mitochondria; 0.5 mM pyruvate and 0.2 μM Ca^{2+} [21].
3 - Heart mitochondria; 10 mM pyruvate and 10 mM malate [22].

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Table 3
Flux control coefficients (FCC) for various complexes of Mitochondrial Interactosome in permeabilized cardiomyocytes from rat heart.

Complex	Inhibitor	FCC (flux control coefficient) ± SEM	
		ADP activation	Cr activation
NADH-CoQ oxidoreductase Complex I	Rotenone	0.20 ± 0.04	0.64 ± 0.03
CoQ cytochrome-c oxidoreductase (Complex III)	Antimycin	0.41 ± 0.08	0.4 ± 0.01
Cytochrome c oxidase (Complex IV)	Na cyanide	0.39 ± 0.09	0.49 ± 0.08
ATP/ADP carrier	CAT (carboxyatractyloside)	0.20 ± 0.05	0.92 ± 0.05
ATP synthase	Oligomycin	0.065 ± 0.01	0.38 ± 0.05
Pi carrier	Mersalyl	0.064 ± 0.04	0.06 ± 0.05
MtCK	DNFB		0.95 ± 0.02
Sum		**1.33 ± 0.31**	**3.84 ± 0.29**

All coefficients were determined by measurements according two protocols: 1) direct activation with 2 mM ADP when MtCK and Mitochondrial Interactosome (MI) complex are not activated (ADP activation) and: 2) under conditions of Cr/MtCK activation when after addition of ATP and PK-PEP system, the addition of Cr activates MtCK and all the MI supercomplex coupling system. The FCC of the complexes is several times higher with Cr for Complex I of the respiratory chain, ANT and ATP synthase. FCC for MtCK is remarkably high. MtCK together with ANT can be considered as most important regulatory parts of the MI. The sum of the measured coefficients is 3 times higher upon Cr activation, suggesting the direct metabolic channelling in MI.

Results are presented as average values ± SEM for 10–15 experiments.

dependent respiration are by order of magnitude higher in the case of permeabilized cardiomyocytes than in isolated mitochondria. Similar high concentrations of CAT were also used by Wisniewski et al. for inhibition of mitochondrial respiration in permeabilized rat soleus skeletal muscle [26]. Evidently, this shows that there are diffusion restrictions across mitochondrial outer membrane also for CAT, similarly with ADP. In both cases the inhibition is more effective for MtCK - activated respiration than for ADP – activated respiration (Fig. 5C). Fig. 5D shows calculated C_{vi}^{IATP} for ANT for isolated mitochondria and mitochondria *in situ* in cardiomyocytes. In both cases the $C_{vi}^{IATP}$with Cr activation (activated MI) is by factor higher than with external ADP activation, and both parameters are higher in cardiomyocytes. In permeabilized cardiomyocytes ANT is not the most important regulator of the oxidative phosphorylation, when measured with external ADP activation (see Table 3): C_{vi}^{IATP} for CAT is 0.20 versus 0.41 for antimycin and 0.40 for cyanide. However, the value of the coefficient and therefore sensitivity of the complex to a metabolic signal increases in isolated mitochondria almost four times when MtCK is activated with Cr (Fig. 5D): under the physiological conditions (with activated MI) the C_{vi}^{IATP} is almost tenfold higher than in isolated mitochondria and five times higher than in permeabilized cardiomyocytes with ADP activation.

3.6. Flux control by ATP Synthasome complexes

Fig. 6 shows the oxygraph traces for permeabilized cardiomyocytes, titrated by oligomycin, according the two different protocols: direct ADP activation (Fig. 6A) and Cr activation (Fig. 6B), when the respiration is initially activated with ATP, and then in the presence of PK-PEP system the 10 mM Cr activates the MtCK. Again, in the second case, at the same concentrations of oligomycin, the effect of this inhibitor is stronger. This indicates that the complex is more sensitive to the metabolic regulation with activated MI. In Fig. 6C the inhibition titration curves for permeabilized cardiomyocytes in both cases are represented, and compared with titration curve for isolated mitochondria. Again, isolated mitochondria are inhibited much more rapidly, and the slope of the titration curve for permeabilized cardiomyocytes is steeper, when the MI supercomplex is activated with Cr *versus* respiration activated by exogenous ADP.

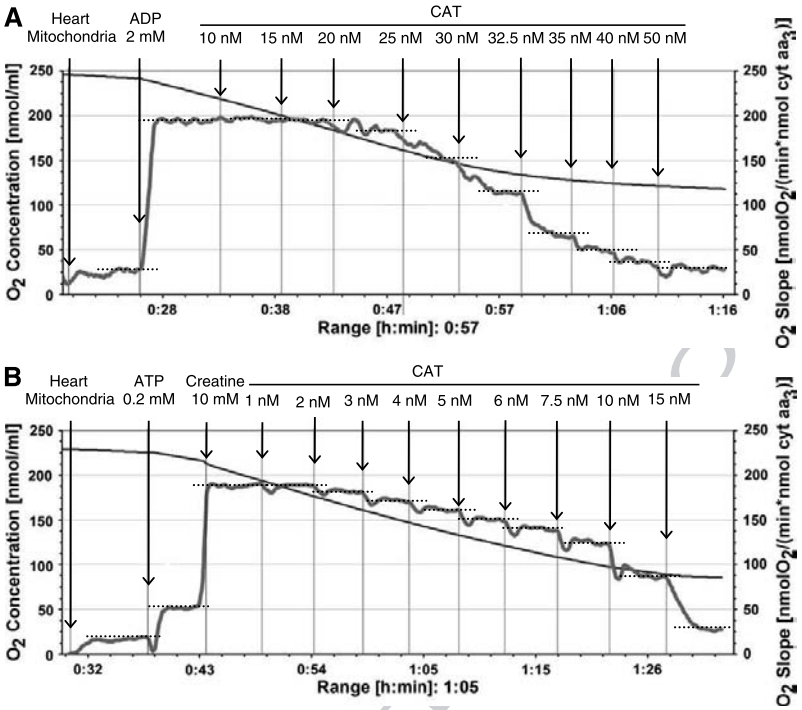
Fig. 7 shows that similar effect was also seen for the case of inhibition of Complex I by its specific inhibitor rotenone. The initial slope of inhibition curve of rotenone (Complex I) was similar in isolated mitochondria and CM with ADP activation (C_{vi}^{IATP} = 0.19 and 0.2; Table 2 and 3), decline of the inhibition curve with Cr activation is significantly stronger, the value of coefficient has increased three times (C_{vi}^{IATP} = 0.64).

Fig. 8 shows that no differences were observed in titration curves between two protocols used for inhibition of Complex III by antimycin (Fig. 8A), PIC by mersalyl (Fig. 8B) and Complex IV by NaCN (Fig. 8C).

Table 2 shows all C_{vi}^{IATP} for MI complexes in isolated heart mitochondria measured with direct ADP activation. Besides our results, data of some other authors have presented [21,22]. C_{vi}^{IATP}s of several complexes are quite close; differences in results could be caused by dissimilarity in measurement conditions. It could be also concluded from the results of our work that the metabolic regulation is very sensitive of minor biochemical as well as structural changes in cell. Our results show that in isolated mitochondria the main regulatory complexes are Complex I and Complex IV in the respiratory chain, ANT and PIC (Table 3). In mitochondria *in vitro* the impact of Complex III (antimycin) is insignificant (C_{vi}^{IATP} 0.06), that is in agreement with the results of previous studies. Table 3 shows the calculated C_{vi}^{IATP} for permeabilized cardiomyocytes. Calculated coefficients of Complex III in mitochondria *in situ* are the same in both protocols: with Cr activation and ADP activation and the C_{vi}^{IATP} is significantly important (C_{vi}^{IATP} 0.41). It shows that the complex has important regulatory role in the regulation of electron transfer in these conditions. The value of C_{vi}^{IATP} of Complex IV is higher in mitochondria *in situ* (C_{vi}^{IATP} 0.40 and 0.49 in CM versus 0.18 in mitochondria). It is clear from these results that the metabolic control of the MI complexes is higher in physiological conditions than could be concluded from the *in vitro* studies. The sum of C_{vi}^{IATP} in isolated mitochondria for the case of ADP activation is 0.75 and in the permeabilized cells it is 1.33 (Table 3 and 4).

The influence of inhibition of ATP Synthase in energy flux control is similar in mitochondria *in vitro* (C_{vi}^{IATP} = 0.01) and *in situ* with ADP activation (C_{vi}^{IATP} = 0.06). In cardiomyocytes under physiological conditions (Cr activation) the regulatory weight of ATP Synthase complex has increased six fold (C_{vi}^{IATP} 0.38). The most important regulatory sites in fluxes energy in MI are functionally coupled ANT and MtCK. The C_{vi}^{IATP} for ANT increases from 0.11 (isolated mitochondria, ADP activation) to 0.92 in cardiomyocytes with Cr activation. For MtCK the value of C_{vi}^{IATP} is high in both conditions: 0.80 in isolated mitochondria to 0.95 in cardiomyocytes. This result shows that in the presence of Cr the MtCK is always important regulator of the

Fig. 5. Respiration traces of inhibition of isolated mitochondria with CAT. A. Respiration was activated with 2 mM ADP, preceded by inhibitor addition according protocol. With dotted lines subsequent steady states are marked. B. Influence of the inhibitor on the respiration rate when the respiration was activated with 10 mM Cr and 0.2 mM ATP. C. Titration curves of respiration of isolated mitochondria and permeabilized cardiomyocytes with CAT in the case of ADP or Cr activation. D. Flux control coefficients for ANT in isolated mitochondria and permeabilized cardiomyocytes for respiration activation by ADP or creatine.



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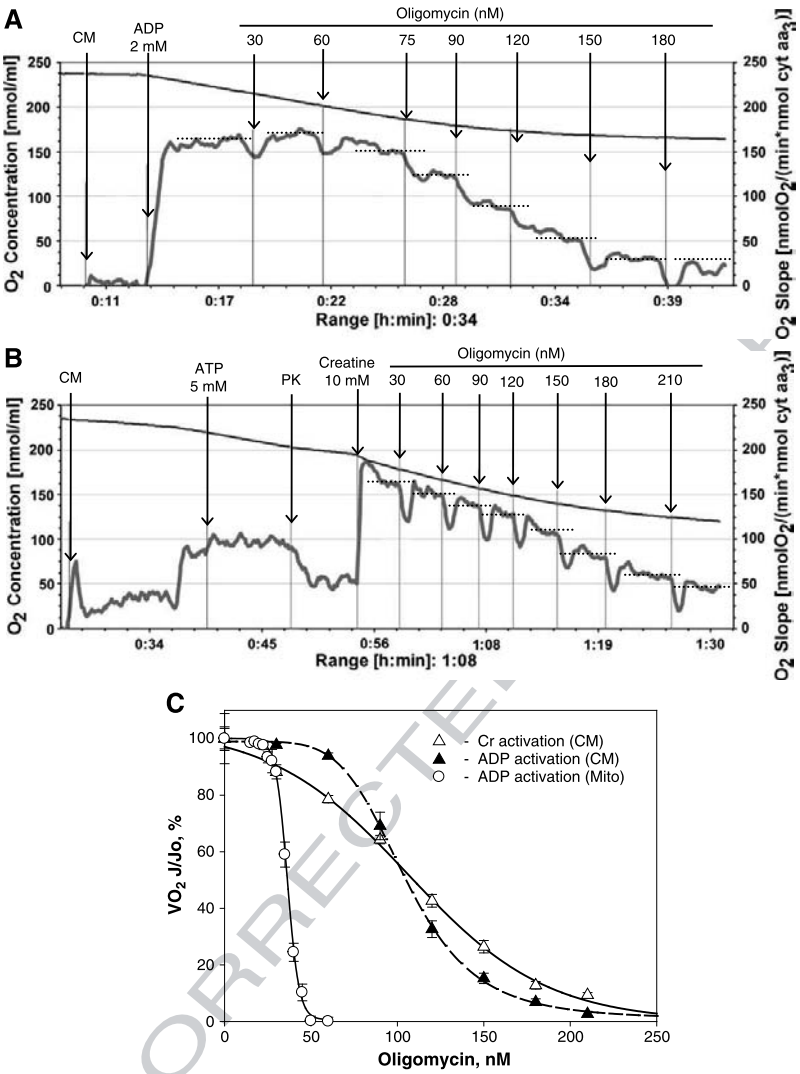

Fig. 6. Respiration traces of inhibition of cardiomyocytes with oligomycin. A. Direct ADP activation; B. Cr activation. Respiration was activated with 2 mM ADP (A) or with addition of 10 mM Cr in the presence of 5 mM ATP and PK-PEP system (B). Dotted lines mark steady states in each inhibitor concentration. In the case of activated MI complex the respiration rate decreases is stronger: with 30 nM there is no decrease in respiration rate in ADP activation, with activated MI decrease is significant. C. Inhibition titration curves of isolated mitochondria (circles) and CM with Oligomycin. The titration curves are represented with ADP activation (filled triangles) and with Cr activation (activated MI). The concentration needed for maximal inhibition was increased five times in CM versus isolated mitochondria.

energy transfer. The regulatory role of PIC is insignificant in the case of mitochondria *in situ* (C_{vi}^{ATP} 0.06) but increases to 0.20 in isolated mitochondria.

Table 4 shows that due to increases in C_{vi}^{ATP} for Complex I, ANT and ATP Synthase in the case of MtCK activation by creatine the sum of C_{vi}^{ATP} increases to 3.84 (Table 4).

3.7. Application of mathematical model in flux control coefficient calculation

The flux control coefficients in permeabilized cardiomyocytes were determined by using graphical method. Additionally, obtained results were compared with the computer estimated coefficients. In this case non-linear regression analysis was used by fitting experimental data to the mathematical model, developed by Gellerich [11]. The fitting was performed by providing best-fit values of three parameters: K_d (dissociation constant of the enzyme-inhibitor complex), E_0 (concentration of inhibitor binding sites) and C_0 ($C_0 = (dlnJ/dlnE)_{E=E0}$ in the absence of the inhibitor). As it seen from the Fig. 9, rather good fitting was seen in both cases between the computed curve and experimental data. The computed flux control coefficients are comparable with estimations done using the graph method in the linear system for the case with ADP activation (C_{vi}^{ATP} computed 0.25, graph 0.20). Activation of mitochondrial respiration by addition of creatine to MI system presents more complex, non-linear system, with the restrictive regulation of energy fluxes on the level of MOM through VDAC by cytoskeletal proteins and the mechanisms of metabolic channeling, functional coupling between ANT

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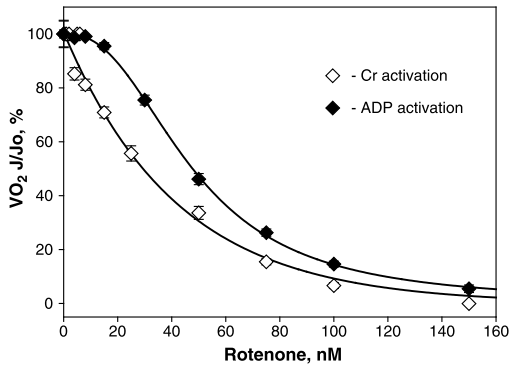

Fig. 7. Titration curves for Complex I by rotenone in permeabilized cardiomyocytes. The respiration inhibition curves are presented for two experimental conditions: with external ADP activation and with Cr activation (physiological conditions).

and MtCK. Under these conditions the Gellerich's model overestimates flux control coefficient (C_{vi}^{JATP}computed 1.53, graph 0.92 - Fig. 9). It may be concluded at present that the graphical method is better adapted for comparative studies, since it is not dependent on the model. A method proposed by Gellerich needs evidently to be revised in order to be applied to the calculations of FCC for the MI system with the activated by creatine MtCK - for that more complete model of the processes of metabolic channeling, functional coupling and ADP recycling in MI is needed. Since the Gellerich's method gave even bigger differences between the results of the use of two protocols, it even more strengthens our conclusions that the activated MI is very effective amplifier of the metabolic signals.

4. Discussion

Metabolic Control Analysis, MCA is a precise, objective and effective method of quantitative analysis of regulation of metabolic systems and has been applied intensively in the experimental research in bioenergetics [22,28,40,41]. MCA helps to understand the mechanisms by which a given enzyme exerts high or low control of metabolic flux and how the control of the pathway is shared by several pathway enzymes and transporters. By applying MCA it is possible to identify the steps that could be modified to achieve a successful alteration of flux or metabolite concentration in pathways. Till now the method of MCA has been used to measure C_{vi}^{JATP} in mitochondria [41] and in permeabilized muscle fibers when respiration was activated by addition of ADP [25,26]. Practically all studies with isolated mitochondria and permeabilized muscle fibers [21,22,25,27,41,49,52]have been carried out by using titration with specific irreversible or pseudo-irreversible inhibitors (see Materials and Methods section). This method can be used experimentally to study directly the distribution of flux control within a metabolic system. In our work we use this tool to measure the coefficient of the complexes in MI, the model proposed in our previous article [10].

The results of our present study confirm our previous conclusion that the in case of several complexes their metabolic flux control of mitochondrial respiration in cardiomyocytes is much more efficient under conditions of ADP-ATP recycling in mitochondria within MI coupled to creatine phosphorylation and PCr production (MtCK reaction) than in the case of direct exchange of ADP and ATP between mitochondria and cytoplasm. These results are consistent with our earlier conclusion that the coupled reactions in MI are very effective amplifiers of the feedback metabolic signals, connecting heart work with mitochondrial respiration in the heart [1–3,43].

In previous studies we have shown that the steady-state kinetics of the regulation of mitochondrial respiration is different in

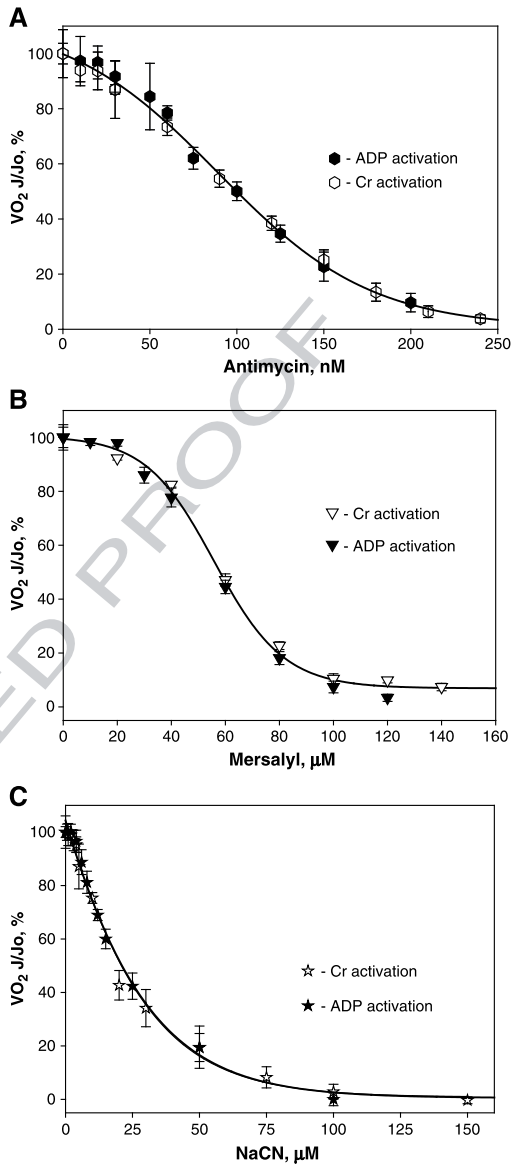

Fig. 8. Titration curves for respiratory chain complexes and phosphate carrier by their specific inhibitors in permeabilized cardiomyocytes. The respiration inhibition curves are presented for two experimental conditions: with external ADP activation and with Cr activation (physiological conditions): A. CoQ cytochrome-c oxidoreductase (Antimycin). B. Pi carrier (Mersalyl); C. Complex IV (sodium cyanide). Inhibition curves with Antimycin, Mersalyl and Cyanide have no significant difference in ADP activation versus Cr activation; although complex III and IV are important regulators in CM with direct ADP activation, it is relatively decreased in physiological conditions with Cr activation (Table 4).

permeabilized cardiomyocytes *in situ* versus in isolated mitochondria *in vitro* [1,3,6,7,36,38]. The apparent dissociation constants of MgATP from binary and ternary complexes with MtCK have increased by several orders in permeabilized cardiomyocytes, at the same time as the dissociation constant of creatine decreases about 10 times and the constant for PCr is similar in mitochondria *in situ*, as compared with isolated mitochondria [2]. Thus, there is a diffusion restriction for

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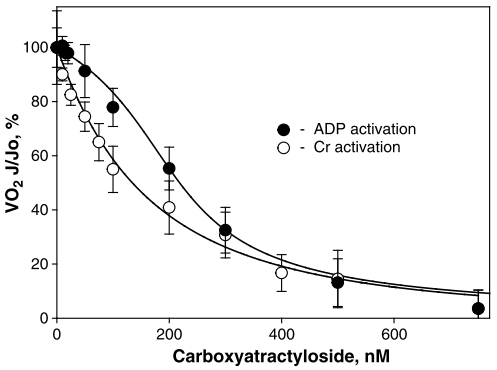

Fig. 9. The experimental points of CAT titration for the ADP (filled cycles) and Cr (open cycles) activated systems, fitted to mathematical model by Gellerich [11]. Rather good fitting was seen in both cases between the computed curve and experimental data (analogous comparison was done for the all components pathway). The computed flux control coefficients are comparable with estimations done using the graph method in the linear system for the case with ADP activation ($C_{vi}^{[ATP}$ computed 0.25, graph 0.20). The estimated dissociation constant $K_d = 15$nM and $E_0 = 225$nM (ADP activation) was close to that reported earlier [62,63]. Activation of mitochondrial respiration by addition of creatine to MI system presents more complex, non-linear system, with the restrictive regulation of energy fluxes on the level of MOM through VDAC by cytoskeletal proteins and the mechanisms of metabolic channeling, functional coupling between ANT and MtCK. Under these conditions the Gellerich's model overestimates flux control coefficient ($C_{vi}^{[ATP}$computed 1.53, graph 0.92 - Fig. 9). The computed FCCs were 0.25 and 1.53 respectively.

MgATP and not for PCr/Cr across MOM into intermembrane space, where MtCK is located. Direct measurements of energy fluxes from mitochondria in isolated permeabilized cardiomyocytes confirmed this conclusion [10]. Respiration rate of permeabilized cardiomyocytes was determined in the presence of MgATP, PK–PEP and Cr, at the same time as ATP and PCr concentrations in the medium surrounding cardiomyocytes were measured. While ATP concentration did not change in time, mitochondria effectively produced phosphocreatine with PCr/O_2 ratio equal to 5.68 ± 0.14 [10].These results showed that under physiological conditions the major energy carrier from mitochondria into cytoplasm is PCr, produced by mitochondrial creatine kinase (MtCK), and the part of the direct ATP transfer under physiological conditions could remain approximately 10%. Functional coupling to ANT is enhanced by selective limitation of permeability of MOM within supercomplex ATP Synthasome-MtCK-VDAC-tubulin, Mitochondrial Interactosome [1–3,10,44,53].

As could be seen from the results of our analysis $C_{vi}^{[ATP}$ of the several complexes increase significantly when MI functional coupling is activated and the sensitivity of the complexes to the metabolic signals is becoming significantly higher in these conditions. The main regulatory complexes in the MI are MtCK and ANT, which emphasize again the MtCK and the PCr/Cr as a main energy flux passway in cardiomyocytes. It has been shown in the works of Kholodenko et al. that the sum of $C_{vi}^{[ATP}$ could exceed 1 in the case of direct channelling (and branching or internal cycling), taking place in the investigated system [16,54]. Our results show that in mitochondria *in situ* with activated MI, the sum of the calculated $C_{vi}^{[ATP}$ is more than three times higher than in the conditions with exogenous ADP activation. This indicates the possibility of direct channelling in the physiological conditions in energy transfer regulation in MI, according to theories presented by [16]. Also $C_{vi}^{[ATP}$ of ANT and of MtCK have very high and at the same time close values, which supports the concept of direct transfer of adenine nucleotides between these complexes, shown by other authors [3,31,32,43,51,55]. There is also a possibility that under these circumstances these complexes could be counted as a one functional unit. In activated MI, the flux control coefficient is increased also for Complex I.

In comparison with isolated mitochondria the maximal concentrations of inhibitors needed for complete inhibition of respiration in permeabilized cardiomyocytes increased 4–10 times (Figs. 5 and 6). These results indicate again influence of the diffusion restrictions in cells due to the physical factors as macromolecular crowding, heterogeneity of diffusion due to the cell structure and selective permeability of membranes, in particular due to interaction with cytoskeleton [10,56]. Results of our study emphasize once again that the cell could not considered as a system where metabolites diffuse in an homogenous isotropic medium [57]. Any quantitative model describing mitochondrial metabolism should be based on the extensive experimental data, taking into account not only separated the enzymes present in the cell but also the regulation caused by the intracellular structural interactions such as physical barriers, compartmentalization phenomenon and possible direct interaction between metabolic complexes, including the phenomenon of metabolic channelling etc.

Also, there are also several other authors who emphasize the importance of MtCK as central complex not only in energy transfer regulation but also in the regulation of cell lifecycle. For example, Max Dolder [58] has shown that the substrates of MtCK (by activating ADP recycling) can inhibit mitochondrial permeability transition. This effect was seen only when MtCK was located between mitochondrial membranes and functionally coupled with ANT, whereas externally added CK did not produce any protecting effect. There is also an evidence that active MtCK may play a key role as protective antioxidant enzyme against oxidative stress, reducing mitochondrial ROS generation through functional coupling and ADP recycling mechanism [59].

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Review article

Intracellular Energetic Units regulate metabolism in cardiac cells

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ABSTRACT

This review describes developments in historical perspective as well as recent results of investigations of cellular mechanisms of regulation of energy fluxes and mitochondrial respiration by cardiac work — the metabolic aspect of the Frank–Starling law of the heart. A Systems Biology solution to this problem needs the integration of physiological and biochemical mechanisms that take into account intracellular interactions of mitochondria with other cellular systems, in particular with cytoskeleton components. Recent data show that different tubulin isotypes are involved in the regular arrangement exhibited by mitochondria and ATP-consuming systems into Intracellular Energetic Units (ICEUs). Beta II tubulin association with the mitochondrial outer membrane, when co-expressed with mitochondrial creatine kinase (MtCK) specifically limits the permeability of voltage-dependent anion channel for adenine nucleotides. In the MtCK reaction this interaction changes the regulatory kinetics of respiration through a decrease in the affinity for adenine nucleotides and an increase in the affinity for creatine. Metabolic Control Analysis of the coupled MtCK–ATP Synthasome in permeabilized cardiomyocytes showed a significant increase in flux control by steps involved in ADP recycling. Mathematical modeling of compartmentalized energy transfer represented by ICEUs shows that cyclic changes in local ADP, Pi, phosphocreatine and creatine concentrations during contraction cycle represent effective metabolic feedback signals when amplified in the coupled non-equilibrium MtCK–ATP Synthasome reactions in mitochondria. This mechanism explains the regulation of respiration on beat to beat basis during workload changes under conditions of metabolic stability.
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Contents

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"The essential thing in integrated metabolism is that the organism succeeds in freeing itself from all entropy it cannot help producing while alive". E. Schrödinger "What is life?" Cambridge, 1944

1. Introduction

In this work we describe application of Systems Biology and Molecular System Bioenergetics for solution of fundamental problems of cardiac energetics and metabolism. A Systems Biology solution needs the integration of physiological and biochemical mechanisms that take into account intracellular interactions of mitochondria with other cellular systems [1–10], in particular with cytoskeleton components [5]. This approach needs both experimental studies and computer analyses [6–10], and it takes into account the wealth of knowledge acquired during earlier periods of studies of these important problems [3,8,10], thus avoiding the limitations of narrowly focused reductionist studies [10]. The history of research is an important part of Systems Biology [8,10], and accordingly, we begin this review with short description of the history of studies of cardiac energy metabolism.

1.1. Historical background: oxygen consumption and substrate selection by the heart

Research in the area of energy metabolism of mammalian cells goes back to 1783 when Lavoisier and Laplace discovered biological oxidation. They showed that oxygen is consumed during respiration and CO_2 produced in animals, and concluded that "respiration is a process analogous to burning of coal" that gives energy to live [11]. About 150 years later, Ernest Starling applied this knowledge in his classical studies of cardiac function by using isolated heart and lung preparations, accepting that "rate of oxygen consumption is taken as a measure of the total energy set free in the heart during its activity" [12]. In these studies Starling discovered the capacity of the intact ventricle to vary its contraction force on a beat-to-beat basis as a function of its preload, generally referred to as the Frank–Starling law of the heart [13]. This law states that cardiac function, quantitatively characterized as work performance, is a function of ventricular filling, which in turn depends on pre- or afterload [13–15]. We know now that an increase in the length of sarcomeres of striated cardiac muscle results in changes in the overlapping between thin and thick filaments, alteration in myofilament lattice spacing, increased thin filament cooperativity and, consequently, in the number of force-generating cross-bridges [16]. Length-dependent activation of sarcomeres includes also changes of calcium sensitivity by cardiac myofilaments, which is of major importance particularly for low sarcomere length values [16]. A diminished functional response of the heart to changes in ventricle filling is observed in heart failure [14,15]. Furthermore, Starling et al. discovered the linear dependence of the rate of oxygen consumption upon cardiac work — this is the metabolic aspect of Frank–Starling law of the heart [12].Coupling of ATP synthesis to oxygen consumption was shown by Engelhard and Kalckar [17]. In 1939 Belitzer and Tsybakova found that oxygen uptake in the homogenate of the pectoral muscle of a pigeon was activated by creatine without addition of exogenous ADP (trace amounts of endogenous adenine nucleotides present were sufficient to catalyze coupling of the mitochondrial creatine kinase reaction and oxidative phosphorylation), and resulted in phosphocreatine (PCr) production with PCr/O_2 ratio equal to 5.2–7 [18]. These pioneering studies opened the way for a rapid development of bioenergetics and detailed description of the mechanism of ATP synthesis coupled to respiration in mitochondria [11,19,20], which in cardiac cell occupy about 30% of volume [21]. Mitochondrial respiration and ATP synthesis are coupled to phosphorylation of creatine and synthesis of phosphocreatine (PCr) by the mitochondrial creatine kinase (MtCK), a key component of the cellular system of creatine kinases [22–34].

Cardiac work determines not only the rate of oxygen consumption, as discovered by Starling, but also substrate uptake and fuel selection [15,35–37]. Major substrates used by the heart are carbohydrates (glucose, lactate, glycogen) and fatty acids [5,35–37]. Oxidation of carbohydrates accounts for not more than 20% of the oxygen consumed (or ATP produced) by the heart [15,35–37]. During the 1950s, Richard Bing initiated intensive research on metabolism of the human heart, and observed that the preferred substrates are fatty acids [38,39]. This phenomenon and the mechanisms of regulation of substrate uptake by the heart and skeletal muscle were studied and described in detail by Randle, Newsholm and their colleagues in Cambridge, UK [40–43]. They discovered the glucose–fatty acid cycle, now known as Randle cycle, which describes the cellular mechanisms by which fatty acid oxidation inhibits glycolysis [40,41,44,45]. The reason for natural selection of fatty acids by oxidative muscle as preferred substrates for energy metabolism was well explained by Newsholme [43]. The content of free energy per gram of mass that can be released during oxidation and converted into chemical energy in the form of ATP, is much higher for fatty acids than for carbohydrates due to the much higher content of nonoxidized-C—C— and —C—H chemical bonds; depending on the amount of bound water the difference in carbohydrates can range from 3- to 9-fold. Neely, Morgan and Williamson et al. investigated the regulation of the Randle cycle, *via* changes in Krebs cycle intermediates as a function of workload in isolated-perfused working heart [46–51]. This technique allows a precise examination of the Frank–Starling law by achieving high workloads and respiration rates [37,46–51]. Both carbohydrates and fatty acids are oxidized in mitochondria *via* Krebs cycle after formation of acetyl-CoA (see Fig. 1), the main difference being the amount of reducing equivalents produced by both fuels [36,44]. Formation of pyruvate from carbohydrates occurs in the cytoplasm through glycolysis. It is associated with NADH production, which enters rather slowly the mitochondria only *via* malate–aspartate shuttle (Fig. 1), and may limit the rate of glycolysis, particularly under high energy demand [46,52]. On the contrary, β-oxidation of fatty acids occurs in the mitochondrial matrix within an enzymatic supercomplex (metabolon) that appear to operate without rate limitations [53–55]. Therefore, fatty acid oxidation is free from kinetic limitation by the malate–aspartate shuttle, especially at high workloads when malonyl-CoA is decreased due to diminished acetyl-CoA concentration arising from Krebs cycle activation [56]. Malonyl-CoA is an effective inhibitor of the carnitine palmitoyltransferase I (CPT I), and thus of the transfer of acyl-groups into mitochondria for beta-oxidation [35,36,44,57]. It is produced from acetyl-CoA through the acetyl-CoA carboxylase (ACC) reaction, and in the malonyl-CoA decarboxylase (MCD) reaction converted back into acetyl-CoA (reviewed in references [36,44,56,57]). Among the metabolic changes in the heart at high workloads is a decrease of the content of acetyl-CoA, that as a consequence is leaving ACC with much less substrate and thus resulting in a significant decrease of malonyl-CoA in the presence of active MCD. Alterations in malonyl-CoA are the consequence of the increased fatty acid oxidation at elevated workloads, when the level of

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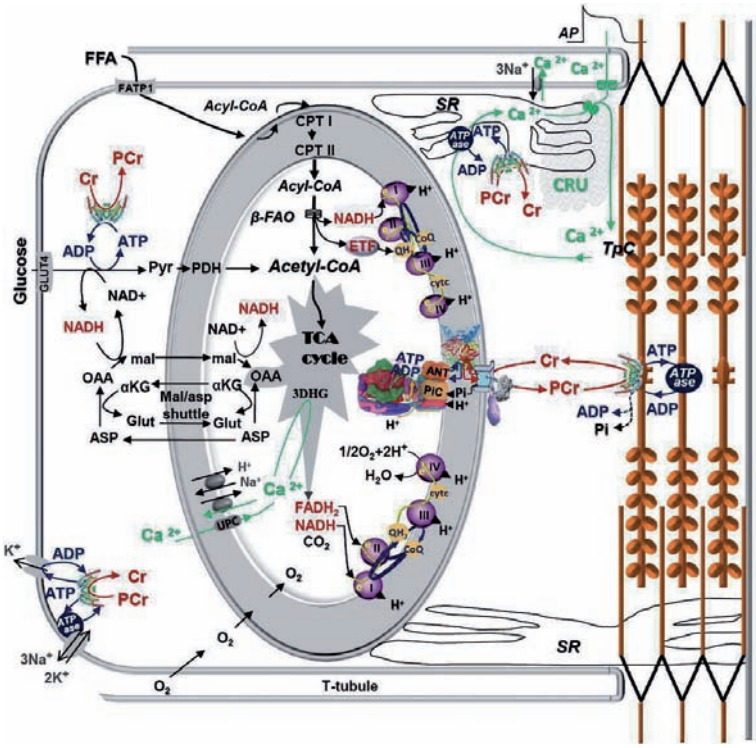

Fig. 1. Functional scheme of the Intracellular Energetic Units of adult cardiac muscle cell. Free fatty acids (FFA) taken up by a family of plasma membrane proteins (FATP1), are esterified to acyl-CoA which entering further the β-fatty acids oxidation (β-FAO) pathway which results in acetyl-CoA production. CPT I and CPT II — carnitine palmitoyltransferases I and II, respectively. Electron-transferring flavoprotein (ETF)-ubiquinone oxidoreductase delivers electrons from β-FAO *directly* to complex III of the respiratory chain (RC). NADH produced by β-FAO is oxidized in the complex I of the RC passing along two electrons and two protons which contribute to the polarization of mitochondrial inner membrane (MIM). Glucose (GLU) is taken up by glucose transporter-4 (GLUT-4) and oxidized via Embden–Meyerhof pathway. Pyruvate produced from glucose oxidation is transformed by the pyruvate dehydrogenise complex (PDH) into acetyl-CoA. The NADH redox potential resulted from glycolysis enters mitochondrial matrix via malate–aspartate shuttle. Malate generated in the cytosol enters the matrix in exchange for α-ketoglutarate (αKG) and can be used to produce matrix NADH. Matrix oxaloacetate (OAA) is returned to the cytosol by conversion to aspartate (ASP) and exchange with glutamate (Glut). Acetyl-CoA is oxidized to CO_2 in the tricarboxylic acids (TCA) cycle generating NADH and $FADH_2$ which are further oxidized in the RC (complexes I, II) with final ATP synthesis. G6P inhibits HK decreasing the rate of glucolysis. The key system in energy transfer from mitochondria to cytoplasm is Mitochondrial Interactosome (MI). MI is a supercomplex, formed by ATP synthase, adenine nucleotides translocase (ANT), phosphate carriers (PIC), mitochondrial creatine kinase (MtCK), voltage-dependent anion channel (VDAC) with bound cytoskeleton proteins (specifically βII-tubulin). MI is responsible for the narrow coupling of ATP/ADP intramitochondria turnover with phosphorylation of creatine (Cr) into phosphocreatine (PCr). PCr is then used to regenerate ATP locally by CK with ATPases (actomyosin ATPase, sarcoplasmic reticulum SERCA and ion pumps ATPases). The rephosphorylation of ADP in MMCK reaction increases the Cr/PCr ratio which is transferred towards MtCK via CK/PCr shuttle. A small part of ADP issued from ATP hydrolysis creates gradient of concentration transmitted towards the matrix. The shaded area in the upper right corner shows the Calcium Release Unit [87–89]. Calcium liberated from local intracellular stores during excitation–contraction coupling through calcium-induced calcium release mechanism, (1) activates contraction cycle by binding to troponin C in the troponin–tropomyosin complex of thin filaments and (2) enters the mitochondria mainly via the mitochondrial Ca^{2+} uniporter (UPC) to activate 3 Krebs cycle dehydrogenases: PDH, αKG, isocitrate dehydrogenase.

acetyl-CoA decreases due to shifts in the kinetics of the Krebs cycle [56]. This makes malonyl-CoA a negative metabolic feedback regulator that allows acyl-CoA entry into mitochondrial matrix space only when it is needed [56]. While all electrons from NADH, and thus from glycolysis, enter the respiratory chain *via* complex I, those obtained from β-oxidation are transferred *via* electron transferring flavoprotein (ETF) and complex III (Fig. 1), resulting in lower ATP/O ratio [36,44,57]. Under aerobic conditions, however, oxygen supply is not a limiting factor for ATP production and differences in ATP/O ratios are only of minor importance. Therefore, the increase in workload is more adequately supported by increase in fatty acid oxidation due to much more favorable kinetics of mass transfer and substrate supply, allowing maximal respiration rates of 160 μmol O_2/min/gdw in working hearts perfused with octanoate as a substrate [47] (Fig. 2A). Only under hypoxic or ischemic conditions, reduction or cessation of mitochondrial respiration results in the decrease or cessation of fatty acid oxidation, while that of carbohydrates is increased [37]. Under these conditions, glucose oxidation provides a potential advantage compared to that of fatty acids.

These classical mechanisms explain well the failure of recent attempts to metabolically modulate heart contractility by reducing fatty acid oxidation and increasing carbohydrate utilization (see [36,58]).

1.2. Cellular bioenergetics in the framework of Systems Biology: Molecular System Bioenergetics

In spite of the brilliant achievements of membrane bioenergetics in explaining the mechanism of ATP synthesis in mitochondrial oxidative phosphorylation [20], the central question of cardiac metabolism – how

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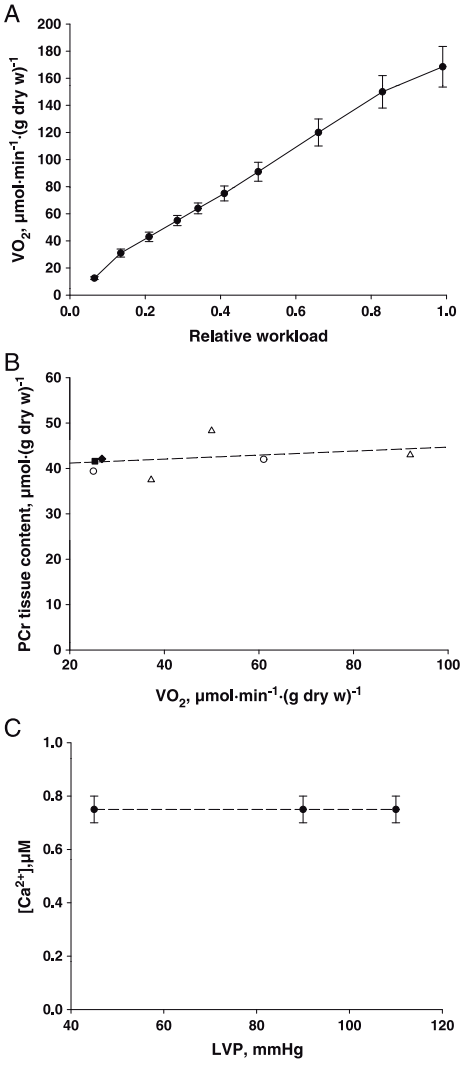

Fig. 2. Metabolic aspect of the Frank–Starling's low of the heart and the mystery of cardiac metabolism. A. Linear increase of oxygen consumption rates as a function of increased relative workload (which is a fraction of maximal workload). Experimental data are taken from [47]. B. Cardiac intracellular PCr homeostasis for different increasing respiration rates. Experimental data were summarized from different studies [48,64,65]. C. Intracellular calcium homeostasis: stable calcium transients for different heart workloads (different left ventricular pressure, LVP) of canine's heart. Experimental data are adapted from [72].

and by which mechanism cardiac work regulates energy fluxes, mitochondrial respiration and substrate uptake – remains to be elucidated despite 80 years of intense research effort. Fig. 2 shows the apparent puzzle of cardiac energy metabolism. For isolated mitochondria, control of respiration by ADP and Pi is indisputable [20,59,60]. Also, activation of Krebs cycle dehydrogenases by Ca^{2+} ions has been well established [61,62]. Although the experimental data obtained with isolated mitochondria and other cellular structures are valuable, they are not sufficient to explain the central problem of cardiac energetics. Two main observations lead us to this conclusion.

1. In aerobic hearts, increase of cardiac work and mitochondrial respiration rate occur at unchanged cellular amount of both ATP and PCr. This phenomenon, discovered by Neely et al. [48] and later confirmed by Balaban and others [63–66], is called metabolic stability or homeostasis (Fig. 2B). This initial set of key observations was interpreted to exclude any explanation of workload dependence of cardiac oxygen consumption upon a mechanism involving the control of mitochondrial respiration by ADP or Pi [67–70]. The basis of that interpretation is the assumption that these metabolites are related through equilibrium relationships [67–70]. The popular assumption of the creatine kinase equilibrium is, however, unnecessary limitation in contradiction with very many experimental data [27,29,31], including recent high resolution ^{31}P NMR experiments [71], showing that the major part of adenine nucleotides, notably ATP in muscle cells exists in the state of association with macromolecules and free ADP may be only transiently be present is cytosol [71].
2. Multiple and detailed physiological experiments using optical methods for monitoring intracellular Ca^{2+} concentrations showed that workload and oxygen consumption changes induced by alteration of left ventricle filling – referred to as the Frank–Starling phenomenon – are observed at unchanged calcium transients [16,72] (Fig. 2C). This crucial observation excludes any explanation involving a mechanism of control of mitochondrial respiration by intracellular calcium, as it has been proposed by several authors ([73], reviewed in [74]). A calcium-mediated mechanism may be important only in the case of adrenergic activation of the heart [75]).

O'Rourke proposed the two main conditions that need to be fulfilled by a metabolic signal regulating respiration *in vivo*: 1) its change must correlate with workload; and 2) it must be able to regulate ATP synthesis [76]. Obviously, the two popular hypotheses described above do not meet these criteria and leave the mechanism underlying the metabolic aspect of the Starling law unexplained.

Manifestly, the problem of regulation of oxygen consumption and substrate uptake by cardiac work demands the use of methods developed in the framework of Systems Biology for its solution. The aim is to explain the physiological phenomena observed by investigating intracellular interactions and resulting regulatory mechanisms, using both experimental and computer modeling methods [1–10,27,29–31]. This approach is rooted in the work of Claude Bernard [8–10] and has been intensively developed during the last decade [1–10,29–31,33]. Revealing the mechanisms of regulation of integrated energy metabolism *in vivo* in the framework of Systems Biology is the objective of Molecular System Bioenergetics [5,10,27]. This research area aims at studying system level properties arising from intracellular interactions, such as metabolic compartmentation, channeling and functional coupling [5,10,26–33]. In this review, we show that the mechanism of feedback regulation in cellular metabolism, linking the cardiac work with mitochondrial respiration under conditions of metabolic stability, can be analyzed quantitatively from experimental data describing intracellular interactions, structure–function relationships, and steady state kinetics of non-equilibrium reactions in cardiac cells.

2. Intracellular Energetic Units (ICEUs): organization of mitochondria by the cytoskeleton and control of mitochondrial morphodynamics

Cells are open systems that operate far from thermodynamic equilibrium while exchanging energy and matter with the external environment [77–84]. Therefore, the intracellular processes are best

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described by using the methods of non-equilibrium thermodynamics [79–84] and non-equilibrium steady state kinetics [78].A part of the energy inflow is used to lower entropy as reflected by the emergence of highly ordered intracellular structures which exhibit functionally complex dynamic behavior as a result of collective spatio-temporal organization [79–84]. These structurally and functionally organized metabolic systems may be described as cellular metabolic dissipative structures, representing functional enzymatic associations that form a catalytic entity as a whole and carry out their activities relatively independently [80–84].

In the heart cell, Intracellular Energetic Units, ICEUs [5,10,26–34,85,86] can be interpreted as metabolic dissipative structures. The concept of ICEUs (Fig. 1) was developed on the basis of information of cardiac cell structure and experimental data obtained in the studies of permeabilized cardiac cells and fibers. These studies revealed the major importance of structure–function relationships in the regulation of cardiac cell metabolism [26–34]. As shown in Fig. 1, an ICEU is a structural and functional unit of striated muscle cells consisting of distinct mitochondria localized at the level of sarcomeres between Z-lines and interacting with surrounding myofibrils, sarcoplasmic reticulum, cytoskeleton and cytoplasmic enzymes [5,10,26–34]. In adult cardiomyocytes, ICEUs interact with other dissipative metabolic structures such as calcium release units, CRUs (Fig. 1, shaded area at the upper right corner)[87–89]. Electron microscopic studies have always shown very regular arrangement of mitochondria in cardiomyocytes at the level of A-bands of sarcomeres in myofibrils in turn surrounded by the sarcoplasmic reticulum, a network on its own [90–92] . These results were later confirmed in studies with confocal microscopy [93,94] which also revealed crystal-like arrangement of mitochondria in cardic cells, as illustrated in Fig. 3A. The green fluorescence of antibodies against α-actinin at Z-lines does not colocalize with mitochondria (Figs. 3B and C). Thus, mitochondria are very regularly localized in the space between Z-lines as separate entities in close connection to sarcomeres and sarcoplasmic reticulum to form ICEUs. This conclusion was further confirmed by results of rapid scanning confocal microscopy shown in Fig. 3D, and by direct evidence of the absence of electrical connectivity between adjacent mitochondria (Fig. 3E). High speed scanning (1 frame per 400 ms) revealed very rapid position fluctuations of fluorescence centers of mitochondria labeled with MitoTracker® Green. No mitochondrial fusion or fission was observed in adult cardiomyocytes in contrast to cancerous HL-1 cells of cardiac phenotype [94]. Electrical discontinuity of mitochondria in adult cardiomyocytes has been reported by many authors [95–101]. It has been shown that laser irradiation resulted in the collapse of the membrane potential of individual mitochondria due to local ROS production and permeability transition (Fig. 3E; see also refs. [97,99–105]. These results show the presence of closely located but differentially energized mitochondria. This may have important physiological consequences. Depolarization and functional damage of distinct mitochondria under various pathological conditions like apoptosis will not translate to other mitochondria thus collapsing the entire cell energetics. In addition, mitochondrial discontinuity can prevent propagation of calcium or ROS signals. These results do not support the increasingly popular point of view that mitochondrial fusion is necessary for their normal functional activity [105–110]. Fusion becomes characteristic of mitochondria under pathophysiological states of cardiac cells, and is usually observed in continuously dividing tumor-like cells in culture [94,97,99,111–115].

2.1. Role of the microtubular system and β-tubulin isotypes in mitochondrial arrangement into ICEUs

Regular arrangement of mitochondria in cardiac cells is mediated by their association with three major cytoskeletal structures: microfilaments, microtubules and intermediate filaments [116–122]. It is well known that the cytoskeleton is most important for mitochondria, cell morphology and motility, intracellular traffic and mitosis [121,122]. By its nature, the contraction process needs very precise structural organization of sarcomeres and also mitochondria [123] which in muscle cells is maintained by cytoskeletal proteins. Among them, tubulin [124–126] is one of the most prominent components of the cytoskeleton that, among other functions, plays a role in energy metabolism. By establishing the boundaries of intracellular micro-compartments and of dissipative metabolic structures [127,128] it may intervene in the regulation of metabolic fluxes. In cardiac cells, the microtubular network together with intermediate filaments (plectin, desmin) and microfilaments (actin), form specific structures that represent a vital organization during the contraction cycle, and for regulation of energy supply [129–134]. About 30% of total tubulin is present in the form of αβ heterodimer, and 70% in polymerized form [135]. Remodeling of cytoskeleton, in particular of the microtubular system, is observed in cardiac hypertrophy and cardiomyopathies [132–134].

Most interestingly, already 20 years ago Saetersdal et al. discovered the association of tubulin with the mitochondrial outer membrane (MOM) by immunogold labeling and electron microscopy in cardiac cells [136]. Some of these results are reproduced in Fig. 4B. More recent investigations using immunofluorescence confocal microscopy allowed identifying the beta II isoform of tubulin interacting with MOM (Fig. 4C). This tubulin isotype exhibits a different distribution as compared with other β isotypes in cardiac cells [34]. Similarly to βII-tubulin, we have found that βIII- and βIV-tubulin were present in the left ventricular muscle and adult cardiac cells (Figs. 4D and E). Immunofluorescent labeling allowed us to show that βIV-tubulin is polymerized forming a characteristic microtubular network, and that βIII-tubulin colocalizes with Z-lines [34]. Thus, it appears that the main role of βIV-tubulin is to support the whole cellular morphology. The arrangement of βIV-tubulin in cardiac cancer cells (HL-1) is very different from that in adult cardiomyocytes. This observation may explain their different morphology together with the fact that HL-1 cells completely lack βII-tubulin [34].

The intracellular distribution of β-tubulin isotypes and the dense and complementary distributed system of polymerized and depolymerized tubulin isotypes, contribute to the very regular arrangement of distinct mitochondria into ICEUs in cardiomyocytes. Thus, βII tubulin fixes mitochondria concomitantly controlling their functioning and anchoring them to clusters exhibiting 3D structuring of βIII and βIV-tubulins. Under pathological conditions, such as in cancerous cardiac HL-1 cells, when the β-tubulin isotypes IV and II are absent, and βIII tubulin is diffusely distributed, mitochondria form a dynamic filamentous reticulum characterized by continuous fusion/fission movements (speed of mitochondrial movement ~90 nm/s) [34,94].

In these works described above the immunostaining by antibodies against C-terminal tail of the tubulin βII has been used to study their localization in permeabilized cardiomyocytes [34]. However, the tubulin exists in forms of non-polymerized or polymerized αβ-heterodimers [124–126], and there are several isotypes of both subunits which differ mostly by the structure of C-terminal tail [124,126]. Further intensive studies by immunocoprecipitation, mutagenesis and use of chemical inhibitors are needed to find out why only βII-tubulin is associated with mitochondria, what is the possible role of the α isotype and how they both interact with VDAC in the mitochondrial outer membrane. Also, the role of different VDAC isoforms in the interaction with cytoskeletal proteins in muscle cells is of interest, and how these interactions are influenced by other membrane-bound proteins [124–126].

The proper cyto-architecture and positioning of other cellular structures in adult cardiomyocytes are also dependent on the cytoskeletal proteins plectin and desmin [137–141]. Importantly, mutations of the human plectin gene showed that pathological disorganization of the intermediate filament cytoskeleton can be associated with severe mitochondrial dysfunction. Moreover, plectin deficiency in mice results in significantly decreased mitochondrial number and function [139–142]. Among many known isoforms of plectin, skeletal and cardiac muscle are characterized by high expression

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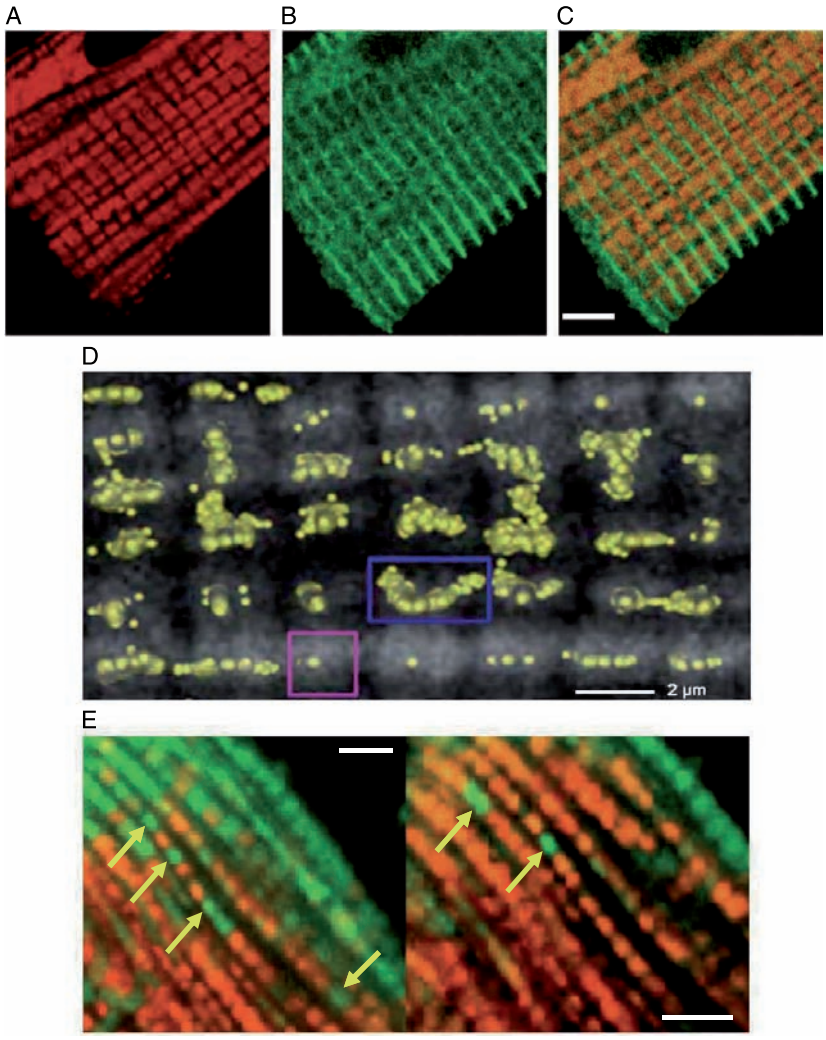

Fig. 3. A. Confocal image of regularly arranged mitochondria in fixed cardiomyocytes labeled by Mito-ID. B. Immunofluorescence labeling of α-actinin showing its specific localization at Z-lines (second antibody with FITC). C. Superposition of A and B shows clearly that mitochondria are localized between Z-lines (green fluorescence) without any fusion processes. Scale bar 5 μm. Reproduced from [34] with permission from Elsevier. D. 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. These fluorescence centers (which are assimilated to the center of mitochondria in cardiomyocytes) are shown as small yellow spheres. The position of fluorescent centers were superimposed with a reference confocal image of MitoTracker® Green fluorescence (in gray) 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 [94] with permission from Springer. E. Imaging of double-labeled cardiomyocytes mitochondria with ΔΨ-sensitive probe TMRM (red) and MitoTracker™ Green. Representative merge images of MitoTracker Green and TMRM fluorescence show very closely located normally energized and fully depolarized mitochondria (indicated by arrows), demonstrating their electrical disconnectivity. Scale bar 5 μm.

of plectins 1, 1b, 1d, and 1f isoforms [140,141]. Plectin 1d is specifically associated with Z-disks, whereas plectin 1b is shown to colocalize with mitochondria [139–142]. Plectin 1b is inserted into the MOM with the exon 1b-encoded N-terminal sequence serving as a target to mitochondria and anchoring signal that may also directly interact with the voltage-dependent anion channel (VDAC) [142]. Notably, in addition to its anchoring function, plectin 1b may participate also in promoting proper mitochondrial shape, since plectin 1b-deficient fibroblasts and myoblasts exhibit remarkably elongated mitochondria.

Likely, association of βII-tubulin and plectin with MOM may prevent binding of proteins both pro-apoptotic and those inducing mitochondrial fission or fusion.

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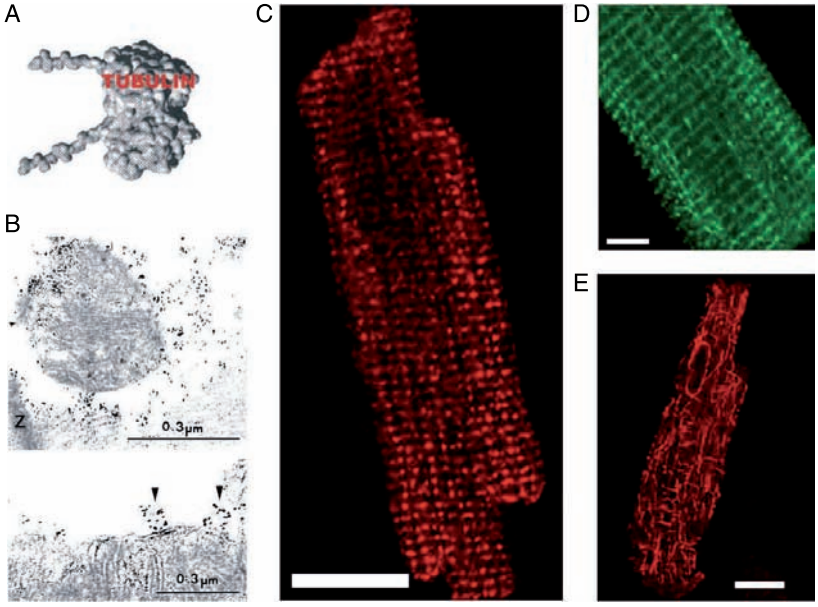

Fig. 4. A. Tubulin αβ-dimer with C-terminal tails. Adapted from [153]. B. Immunogold electron micrography showing the presence of β-tubulin in close proximity to mitochondria in freshly isolated cardiomyocytes. Figure reproduced from [136] with permission from Springer. C. Regularly arranged proteins labeled with anti-βII-tubulin antibody and Cy5 Separate fluorescent spots are organized in distinct longitudinally oriented parallel lines repeating mitochondrial arrangement in cardiomyocytes. Scale bar 14 μm. D. Tubulin labeled with anti-βIII-tubulin antibody and FITC demonstrates clearly distinguishable prevalent arrangement in transversal lines co-localized with sarcomeric Z-lines. Scale bar 6 μm. E. Tubulin labeled with anti-βIV-tubulin antibody and Cy5. βIV-tubulins form polymerised longitudinally and obliquely oriented microtubules. Scale bar 21 μm. Reproduced from [34] with permission from Elsevier.

2.2. Mitochondrial functional properties in ICEUs — selective control of outer membrane permeability by tubulin, Mitochondrial Interactosome

2.2.1. Role of cytoskeleton

Comparative studies of permeabilized adult cardiomyocytes with cancerous, cardiac-like non-beating HL-1 cells [26,27] showed that tubulin βII isotype co-expresses with MtCK in adult cardiomyocytes, while they are both absent in NB HL-1 cells (Fig. 5A). Non-beating (NB) HL-1 cells are derived from tumoral atrial cardiac myocytes (HL-1 cell line) grown up in Clycomb's laboratory [143]. These cells express cardiac proteins connexin 43, desmin, developmental myosin, cardiac isoforms of dihydropyridin receptors and are devoid of sarcomere structures [144]. NB HL-1e cells are characterized by the presence of sodium–calcium exchanger, the rapid delayed potassium current, but not pacemaker current. The spontaneous depolarization also was not observed. They possess randomly organized filamentous dynamic mitochondria. [34,94,144]. Fusion or fission was seen only in cancerous NB HL-1 cells but not in adult cardiomyocytes [94]. The differences observed in mitochondrial dynamics are related to distinct specific structural organization and mitochondria–cytoskeleton interactions in these cells [29] In contrast to adult cardiomyocytes, the NB HL-1 cells lack MtCK and βII-tubulin [34]. Thus, co-expression of MtCK and βII-tubulin correlate with contractile function in cardiac cells. The initial observation by Saertesdal et al. showing the association of tubulin with MOM in cardiac cells [136] remained unnoticed for a long time. A possible explanation of the functional role of βII-tubulin, however, became evident from studies of regulation of mitochondrial respiration in permeabilized cardiac cells and muscle fibers in many laboratories during the last two decades [145–151] (reviewed in [5,29]). In these studies, the apparent affinity of oxidative phosphorylation for exogenous ADP was characterized by an apparent Km of an order of magnitude lower in permeabilized cardiomyocytes than in isolated mitochondria. The apparent Km decreases after proteolytic treatment with trypsin [148] or addition of creatine (Fig. 5B, see ref. [29] for review). This effect of trypsin on mitochondrial respiration regulation *in situ* suggested that cytoskeletal proteins, then called Factor X, could be involved in the control of MOM permeability [149,150]. Appaix et al. showed in permeabilized cardiomyocytes that tubulin and plectin are very sensitive to proteolytic digestion [152]. Finally, Rostovtseva et al. showed direct interaction of tubulin with VDAC while reconstitution experiments by Monge et al. indicated that association with tubulin increased the apparent Km for ADP in isolated mitochondria [153–155]. F. Bernier-Valentin, B. Rousset and Carre et al. have shown association of tubulin with mitochondria from liver and several other type of cells [156,157]. Association of βII-tubulin with mitochondria in adult cardiomyocytes (Fig. 4) and its absence in HL-1 cells with very low apparent Km for ADP (Fig. 5B) further supported the idea that this tubulin isotype likely is Factor X, which controls VDAC permeability in the MOM [158–160].

Association of βII-tubulin with VDAC and its coexpression with MtCK has fundamental consequences for the regulation of metabolite and energy fluxes between mitochondria and cytoplasm in cardiac cells. One of them is that it strongly increases the functional compartmentation of adenine nucleotides in mitochondria while shifting the energy transfer via the phosphocreatine pathway [26–34]. These proteins were supposed to form a supercomplex, dubbed the Mitochondrial Interactosome (MI) in contact sites of the inner and outer mitochondrial membranes [29,33,34,161]. MI represents a key structure of regulation

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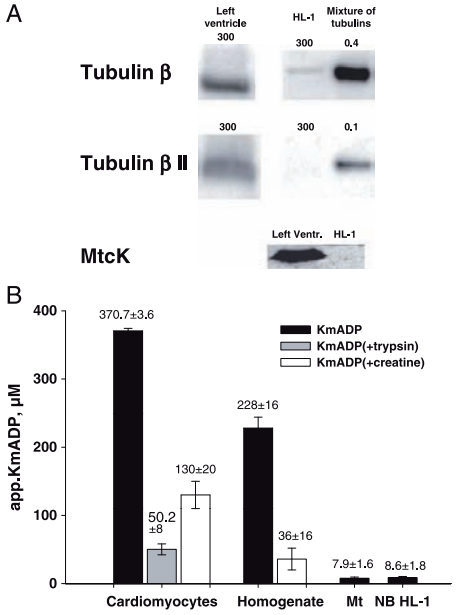

Fig. 5. A. Western Blot analysis of β and βII-tubulin isotypes, and MtCK in rat heart left ventricle and non beatting HL-1 cancer cells of cardiac phenotype. Numbers above the gel images show the amount of added total protein (in μg). Mixture of purified tubulins from brain was used as a reference [34]. Studied proteins are absent in HL-1 cells. B. Regulation of mitochondrial respiration by exogenous ADP in permeabilized muscle cells and fibers. High apparent Km for ADP in permeabilized cardiomyocytes and cardiac muscle homogenate decreases after trypsin proteolysis showing the increase of mitochondrial outer membrane (MOM) permeability for ADP. Increase of MOM permeability is caused by the proteolytic remove of βII-tubulin and other linker proteins from VDAC's protein-binding site. Isolated mitochondria and non beating HL-1 cells are characterized by very low KmADP and very high MOM permeability for ADP, respectively. This high permeability is caused by the absence of βII-tubulin (see Western blot). Creatine regulates respiration of permeabilized cardiomyocytes (decrease of the app. KmADP in the presence of creatine) and has no effect on the respiration of non-beating HL-1 cells because of the absence of MtCK (see Western blot). Data are taken from references [29,32,158,159].

of energy fluxes in ICEUs that strongly increases the efficiency of functional coupling between MtCK and the ATPSynthasome (Fig. 6). The MI supercomplex includes βII tubulin, VDAC, MtCK and ATPsynthasome, consisting of structurally bound ATPsynthase, ANT and PIC [29,33,34,161]; in the cristae membranes, there are only functionally coupled MtCK and ATP synthasome (Fig. 6B). The latter system is also present in isolated mitochondria (Fig. 6A) which have lost tubulin and therefore VDAC permeability is high (low apparent KmADP).

2.2.2. Kinetics of respiration regulation within MI

MtCK is present in mitochondria in octameric form [24,25] and bound to the outer surface of the inner membrane through electrostatic interactions involving positive charges of lysine residues and negative charges from cardiolipin which is also associated with the ANT [162,163]. The ANT carries ATP out of the mitochondrial matrix in exchange for ADP [164]. Due to its close proximity with MtCK, ATP is directly channeled to the active cite of MtCK and effectively used for PCr production if creatine is present (Fig. 6A). Such functional coupling of ANT and MtCK by direct channeling of ATP is well documented in many experimental studies with isolated mitochondria [165–171]. ADP formed at the active site of MtCK is released into intermembraine space (IMS) and may either return to the matrix via ANT or leave mitochondria via VDAC [172,173]. The flux distribution between these two routes depends upon VDAC permeability for adenine nucleotides. Binding of βII-tubulin to the MOM in cardiomyocytes limits this permeability further enhancing functional coupling between ANT and MtCK while increasing ADP transfer to the matrix *via* ANT. The overall effect of this structural–functional coupling is to increase the functional compartmentation of adenine nucleotides in the cell (Fig. 6B). Most remarkably, interaction of βII tubulin with VDAC significantly changes the kinetics of respiration by the MtCK reaction within MI [32,33].

It is important to notice that in permeabilized cardiomyocytes, mitochondria in all ICEUs have similar functional characteristics, as revealed by the kinetic analysis of respiration regulation by exogenous ADP (Figs. 6D–F). First, as it is shown in Table 1 and in Fig. 6D, the maximal ADP-dependent respiration rates when calculated per nmol of cytochromes aa3, are equal in isolated mitochondria (isolated with the use of trypsin) and in permeabilized cardiomyocytes, that meaning that in the permeabilized cells the activities of the whole mitochondrial population were measured. Second, the kinetic curves of the dependence of the respiration rates on ADP concentration are always represented by one hyperbolic curve (both in the absence and presence of creatine) (Fig. 6E), which are linearized by one straight line in double-reciprocal plots (Fig. 6F). Thus, there is no difference in the kinetic behavior of subsarcolemmal and intermyofibrillar mitochondria in permeabilized cardiomyocytes. In the intact non-permeabilized cells, subsarcolemmal ICEUs include both myofibrillar and sarcolemmal ATP consuming systems (Fig. 1), while in the cell interior mitochondria interact with ATP consuming systems of adjacent sarcomeres and sarcoplasmic reticulum (calcium release units, CRU). In all cases the permeability of MOM seems to be controlled by tubulin and possibly other cytoskeletal proteins.

The experimental protocol utilized to study MtCK properties *in situ* in permeabilized cardiomyocytes is schematically represented in Fig. 6C, and illustrated by oxygraph recordings shown in Fig. 6G. In this experimental setting, the kinetics of regulation of respiration is stimulated by endogenous ADP, in turn produced by MtCK in mitochondrial intermembrane space (MIM). The influence of extra-mitochondrial ADP is ruled out by adding exogenous pyruvate kinase (PK)–phosphoenolpyruvate (PEP) which are used to trap ADP outside the mitochondria [26,29,32,174]. Addition of MgATP activated ADP synthesis by MgATPases and stimulated respiration. Subsequent addition of PK + PEP decreased the respiration rate, which was rapidly increased again by stepwise addition of creatine. The maximal respiration rate achieved by activation of MtCK is equal to the State 3 rate [29,32] (Fig. 6G and Table 1). These results are in agreement with the idea that all ADP produced by the MtCK in the intermembrane space is not accessible for external PK–PEP system but is continuously recycled within MI, maintaining high rates of respiration and coupled PCr production [26,28–30,32]. In isolated mitochondria in the absence of bound βII tubulin, PK–PEP decreased the respiration rate by 50% as a result of the open state of VDAC (Table 1) [32,174]. The efficiency of intramitochondrial ATP/ADP turnover in the ATP synthasome [175] coupled to the MtCK reaction is confirmed by the high ratio of PCr production over oxygen consumption ($P/O_2 \sim 5.98$) [29,32,161]. This ratio is close to the theoretical value of P/O for mitochondrial oxidative phosphorylation [20], thus indicating the high efficiency of MI function within ICEUs in cardiac muscle cells [161]. As a result, almost all mitochondrial ATP is dephosphorylated by MtCK within MI and the free energy of phosphoryl bonds transferred to the cytoplasm through the PCr/Cr pathway [24–34].

This conclusion is further strengthening by the results of experiments shown in Fig. 6H. Since one of the enzymes which may be associated with the mitochondrial outer membrane may be hexokinase [176], we compared the effects of glucose and creatine on the respiration rate of permeabilized cardiomyocytes in the presence of MgATP and PK–PEP system. Glucose addition had only

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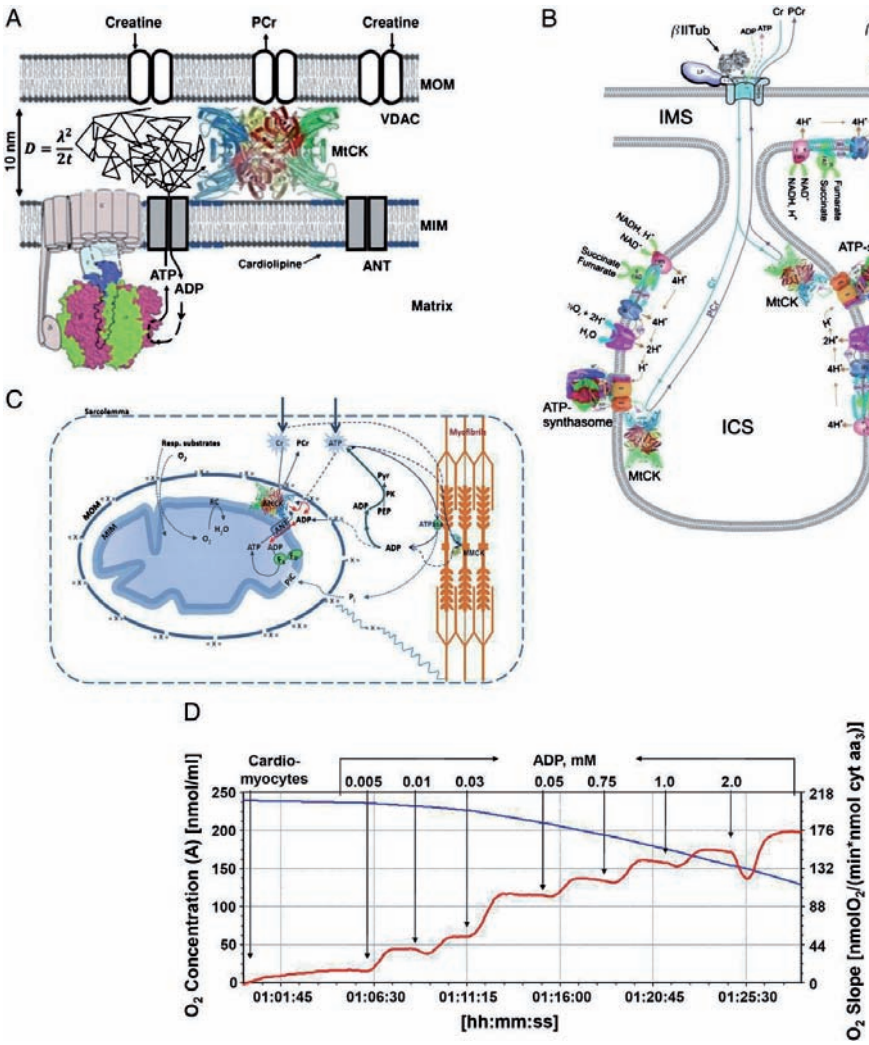

Fig. 6. A and B: Scheme of the microcompartment of adenine nucleotides in mitochondrial intermembrane space created by proteolipid complexes of MtCK with VDAC and ANT, and limited by mitochondrial outer membrane (MOM). LP – still unknown cytoskeletal linker protein which may help tubuli binding to VDAC. This complex allows the direct exchange — metabolite channeling, depicted by arrows. This figure represents also schematically hypothesis of free diffusion of adenine nucleotides molecules within mitochondrial intermembrane space, which can be described by Einstein–Smoluchowski diffusion equation. C shows the protocol of studies of the kinetics of the respiration rate regulation by ADP produced by MtCK in permeabilized cardiomyocytes. D. Oxygraph recording of oxygen consumption by permeabilized cardiomyocytes during titration with increased concentrations of ADP (from 0.05 to 2 mM) in the presence of 5 mM glutamate, 2 mM malate. The blue slope and left y-axes correspond to O_2 concentration; the red slope and right y-axes correspond to O_2 flux. E. Graphical presentation of experimental data of respiration rates as a function of ADP concentrations. This dependence is described by Henri–Michaelis–Menten equation. Empty bullets correspond to experiments performed in the presence of 20 mM creatine. F. Analyses of experimental data by linearization of dependences from panel E in double reciprocal plot of $1/VO_2$ as a function of 1/[ADP]. This plot gives value of the apparent Km for ADP (the abscise intercept corresponds to −1/Km). G. Experimental oxygraph recording of the respiration rate regulated by ADP produced by MtCK in permeabilized cardiomyocytes. First, the basal rate of respiration (V_0) of permeabilized cardiomyocytes (25 μg/mL saponin, 10 min, 25 °C) was recorded in the presence of respiratory substrates for complex I (5 mM glutamate and 2 mM malate). After that, respiration was activated by the addition of 2 mM MgATP which is hydrolyzed by myofibrillar ATPases releasing endogenous MgADP, inorganic phosphate (Pi) and proton. On the oxygraph trace this step is seen as an increase in oxygen consumption stimulated by endogenous MgADP. Next, extramitochondrial MgADP was removed by the addition of phosphoenolpyruvate (PEP, 5 mM) and pyruvate kinase (PK, 20 IU/mL) [32]. The PEP dephosphorylation catalyzed by PK uses ADP to regenerat extramitochondrial pool of ATP. This step is seen on oxygraph trace as an inhibition of respiration. Finally, stepwise creatine in the presence of MgATP activates MtCK and in thus MgADP and PCr production from MgATP and creatine. Respiration rate rapidly increases to its maximal value (Table 1), this showing that MgADP produced by MtCK behind the MOM is not accessible for PK + PEP system [29,32]. H. Comparison of the regulation of mitochondrial respiration by glucose and creatine in presence of MgATP and PK–PEP system in permeabilized cardiomyocytes (CM). Cardiomyocytes were permeabilized by saponin and State 2 respiration recorded. MgATP was added to a 2 mM final concentration to stimulate MgATPases, increasing the respiration rate. This rate was decreased by the addition of PK (20 U/mL) in the presence of PEP (5 mM). Thereafter, no effect on respiration rate was seen after the addition of glucose (10 mM). Subsequent addition of creatine (20 mM) rapidly increased the respiration rate up to maximal value. ADP produced by MtCK is not accessible for the PK–PEP system and is rapidly taken up by ANT into mitochondrial matrix.

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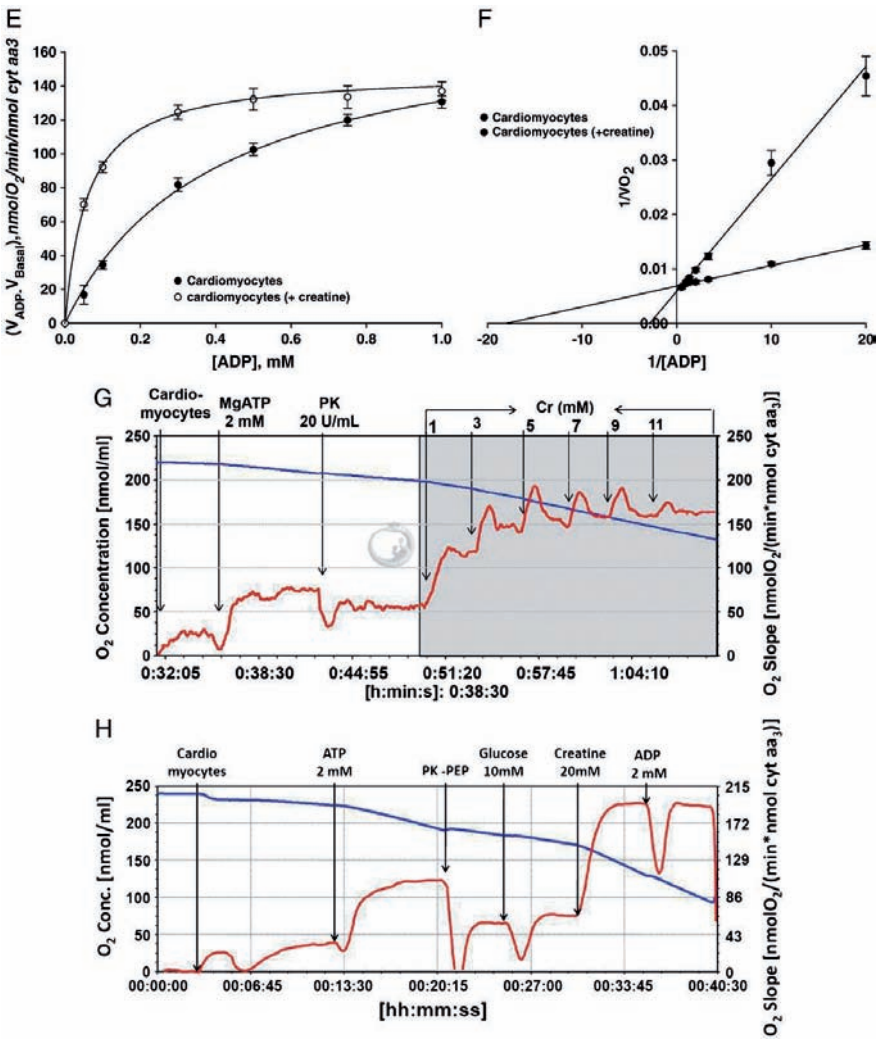

Fig. 6 (*continued*).

minor effect on the respiration rate, while creatine maximally activated the respiration. This shows that MgADP produced by hexokinase is accessible for PK–PEP system, while MgADP produced by MtCK is not accessible and is only recycling in coupled reactions in mitochondria resulting in PCr production.

As compared to isolated mitochondria, complete kinetic analyses of MtCK-controlled respiration performed in permeabilized cardiomyocytes using the protocol shown in Fig. 6C revealed significant changes in the kinetics of MtCK as a result of molecular interactions within MI [32]. The MtCK reaction follows a BiBi quasi-equilibrium random type mechanism, according to Cleland's classification [165]. The constants Kia, Ka, Kib and Kb are dissociation constants, showing the affinity of the free enzyme or its binary complexes for MgATP and creatine (see Table 2). When the MtCK reaction is coupled to the ATP synthasome, these constants reflect apparent affinities of the whole system to these substrates. Table 2 shows that while in isolated mitochondria coupling of MtCK to oxidative phosphorylation decreases both Kia and Ka for MgATP due to recycling of adenine nucleotides [165–171], in permeabilized cardiomyocytes these constants are much higher; the apparent Ka is increased by a factor of 100. At the same time, dissociation constants for creatine are decreased, resulting in the increased apparent affinity for this substrate by MtCK within MI [32]. These results show that βII-tubulin association with MOM decreases VDAC permeability selectively, *i.e.* limiting for MgATP and MgADP but not for creatine and PCr. To explain the observed specific increase of the apparent dissociation constants for MgATP in

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Table 1
Basic respiration parameters of isolated rat heart mitochondria and of mitochondria *in situ* in permeabilized cardiomyocytes. 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; $V_{Cr,ATP+PK,PEP}$ — respiration rate in the presence of activated MtCK by 2 mM ATP and 20 mM Creatine in the presence of 20 IU/mL PK and 5 mM PEP. Data from [29,32].

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 ± 0.04	0.46 ± 0.09
V_3 (2 mM ADP), nmolO_2 min^{-1}cyt aa_3^{-1}	187.94 ± 40.68	178.23 ± 33.96
$V_{Cr,ATP}$, nmol O_2 min^{-1} cyt aa_3^{-1}	197.90 ± 31.86	162.63 ± 26.87
$V_{Cr,ATP+PK,PEP}$, nmol O_2 min^{-1} cyt aa_3^{-1}	82.1 ± 10.5	160.45 ± 26.87

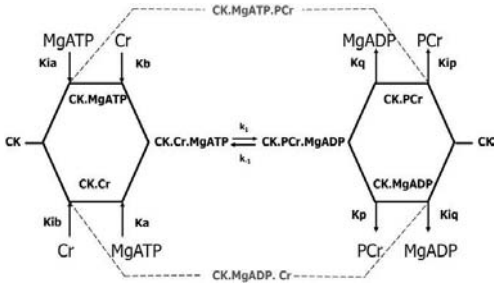
the MtCK reaction in permeabilized cardiomyocytes (Table 2) and apparent Km for exogenous ADP in respiration rate regulation (Fig. 6D–F) we may refer to the specific voltage gating mechanism of the VDAC channel. VDAC has a mobile and positively charged domain that forms part of the wall of the channel, according to Colombini's functional model [172,173,177]. It was proposed that the channel undergoes significant structural rearrangements upon voltage-gating when a positively charged voltage sensor domain translocates across the membrane towards one of the membrane interfaces depending on the sign of the applied voltage under conditions when purified VDAC is integrated into the phospholipid membrane [177]. Its conformational change results in a channel with reduced pore size. The metabolic anions (such as ATP) that moved easily through the open state face a barrier to flow through the closed state. Under *in vivo* conditions the conformational state and permeability of VDAC can be modified by its interaction with proteins from the intracellular environment, such as tubulin. At the same time, the absence of significant change in the apparent affinity of MtCK for creatine and PCr is in accordance with findings that VDAC in so called "closed" states are still permeable to small ions including [177].

Thus, 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 [178]. 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 [178]. This theory corresponds to the increasing amount of experimental data showing that in the living systems proteins function in concentrated and complicated environments [5,10,27,29,179] within organized metabolic dissipative structures [79–84] and metabolic networks [4]. Vectorial metabolism by ligand conduction within multienzyme complexes allows overcoming the diffusion problems for metabolites including ATP, the major part of which has been found to be associated with proteins in muscle cells [71].

2.2.3. MI as an amplifier of metabolic signals from cytoplasm

Fig. 7A shows that as a result of these kinetic features MI is a powerful amplifier of the effects of ADP in regulating mitochondrial respiration [29–34,161]. The role of extra- and intra-mitochondrial ADP in respiration regulation was studied by MgATP titration in the presence of creatine, *i.e.*, activated MtCK [32,161]. The influence of intramitochondrial ADP alone on respiration was estimated by removing extramitochondrial ADP according to the method described above (PEP–PK system mimicking glycolytic ADP consumption). We can see from Fig. 7A that the extra-mitochondrial ADP producing system alone cannot effectively activate respiration. The high apparent Km for exogenous MgATP (157.8 ± 40.1 μM) corresponds to the apparent Km of myofibrillar ATPase reaction for MgATP. When oxidative phosphorylation is stimulated by both extra- and intra-mitochondrial ADP (in the presence of creatine to activate MtCK), the respiration rate increases rapidly up to maximal values and the apparent Km for ATP decreases from 157.8 ± 40.1 μM to 24.9 ± 0.8 μM. Removal of extra-mitochondrial ADP by PEP–

Table 2
Kinetic properties of MtCK *in situ* in cardiomyocytes.



		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

Values of constants for isolated mitochondria are taken from [165]. In isolated mitochondria the oxidative phosphorylation decreases dissociation constants of MgATP from MtCK–substrates complexes suggesting the privileged up-take of all ATP by MtCK [32,33,165–171]. 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–substrates 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 [29,32] with permission from Elsevier.

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PK provokes the increase of Km for MgATP up to 2.04 ± 0.10 mM. These results show that endogenous ADP is an important regulator of respiration but only in the presence of creatine and activated MtCK. The stimulatory effect of respiration by endogenous ADP is strongly amplified by functional coupling of MtCK with ANT that increases adenine nucleotides recycling within the MI [29–34,161].

2.2.4. Metabolic control analysis of mitochondrial interactosome

In order to characterize quantitatively the role of each MI component in the control regulation of respiration within ICEUs, Metabolic Control Analysis was utilized [180]. Permeabilized cardiomyocytes were utilized under two conditions: i) direct activation of respiration by ADP (without MtCK), ii) and activation of respiration via MtCK, using the protocol described in Fig. 7B. Specific inhibitors of the MI components were used to titrate the respiratory flux in order to calculate flux control coefficients under both conditions [180]. The results obtained show high flux control by ANT and the ATP synthasome, both involved in ADP/ATP cycling. Interestingly, the flux control coefficient is increased in the presence of creatine also for the Complex I, probably due to the possible differences in red-ox state of its components. In both cases the Pi carrier PIC has been found to play only a minor role in the control of energy fluxes (Fig. 7B). The sum of flux control coefficients in the case of MtCK activation is close to 4, while in the case of ADP activation is close to one, as previously described [180]. Earlier theoretical work by Kholodenko et al. [181,182] showed that the sum of flux control coefficients may exceed unity in metabolic pathways exhibiting channeling as in the case of the ADP/ATP recycling exerted by MtCK. These results are in agreement with the notion that the MI is rate-controlling of respiration, conveying local metabolic signals in the ICEUs [180–182].

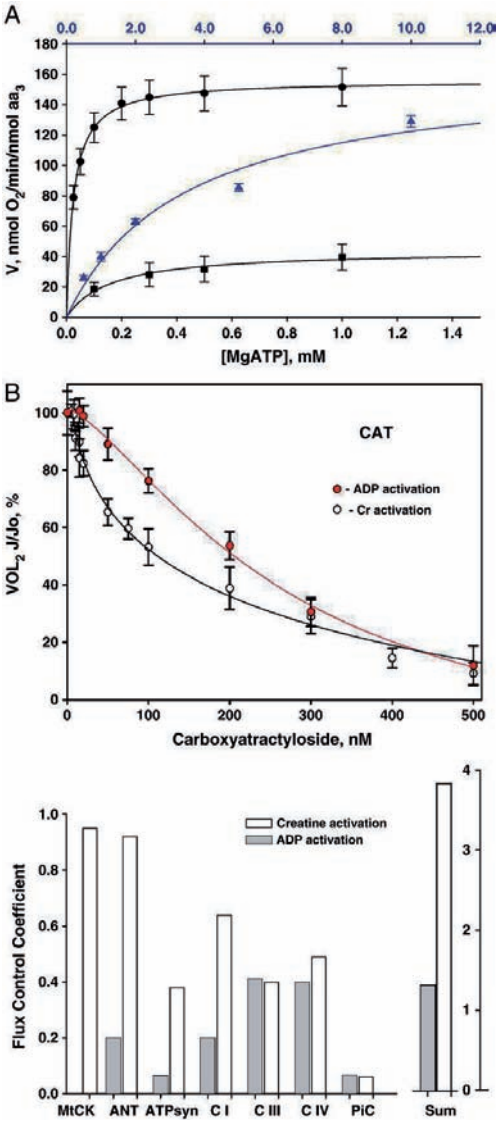

Fig. 7. Amplification of the metabolic signals by Mitochondrial Interactosome. A. Dependence of the rate of respiration in permeabilized cardiomyocytes on the concentration of MgATP added into the medium. This graphical representation reveals the role of endogenous ADP produced by MtCK within mitochondrial intermembrane space for the respiration regulation. (■) — respiration regulation by extramitochondrial MgADP issued from MgATP hydrolysis in MgATP reactions (experiment performed without ADP trapping system (PEP-PK) and in the absence of creatine); (●) — respiration regulation by extramitochondrial and intramitochondrial ADP produced by activated MtCK reaction (experiment performed without PEP–PK system but in the presence of 20 mM creatine); (▲) — respiration regulation by intramitochondrial ADP produced by activated MtCK only (experiment performed in the presence of both trapping system for free ADP and 20 mM creatine). Reproduced with permission from the reference 162. B. Metabolic Control Analysis of the MI. Flux control coefficients were measured as described in [169] under conditions of direct activation of respiration by ADP (2 mM), or by activation of MtCK by ATP (2 mM) and creatine (20 mM) in the presence of PK (20 IU/mL) and PEP (5 mM). A. Changes in the respiration rates in permeabilized cardiomyocytes during inhibition by carboxyatractyloside. B. Flux control coefficients for MtCK, adenine nucleotide translocase (ANT), ATP synthasome (ATPsyn), respiratory complexes I (C I), III (C III), IV (C IV), and inorganic phosphate carrier (PiC). The right panel shows the sum of flux control coefficients.

3. Feedback metabolic regulation within ICEUs

Fig. 2 depicts the riddle of cardiac metabolism: the manifold increase in the rate of myocardial oxygen consumption in response to elevated workload, which is induced by increased left ventricle filling (Starling's law) at apparently constant level of energy metabolites and calcium transients. Setting aside trivial explanations of this puzzle, what we need is to analyze experimental data obtained in studies of intracellular interactions in permeabilized cardiomyocytes described above with an adequate mathematic model, in combination with more detailed analyses of metabolic changes during the cardiac contraction cycle. This analysis will also help to understand the physiological meaning of VDAC interaction with βII-tubulin that restricts permeability of the outer mitochondrial membrane to adenine nucleotides.

Several authors have recorded cyclic changes of main metabolite concentrations during the cardiac cycle [183–185]. Fig. 8A reproduces experimental results of Wikman-Coffelt et al. in 1983 [183], Honda et al. in 2002 [184] and Spindler et al. in 2001 [185] showing the slight (8–12%) oscillations of PCr and Cr concentrations during 1 cycle. These cyclic changes were reproduced by the mathematical model of compartmentalized energy transfer corresponding to the concept of ICEUs, including the restriction of VDAC permeability [186–189]. This model was initially proposed by Aliev and Saks [186,187] and further developed by Vendelin et al. [188,189]. Mathematical modeling shows that not more than 10% of free energy is transported out of mitochondria by ATP flux needed to equilibrate the information-carrying flux of ADP into mitochondria [186]. According to this model,

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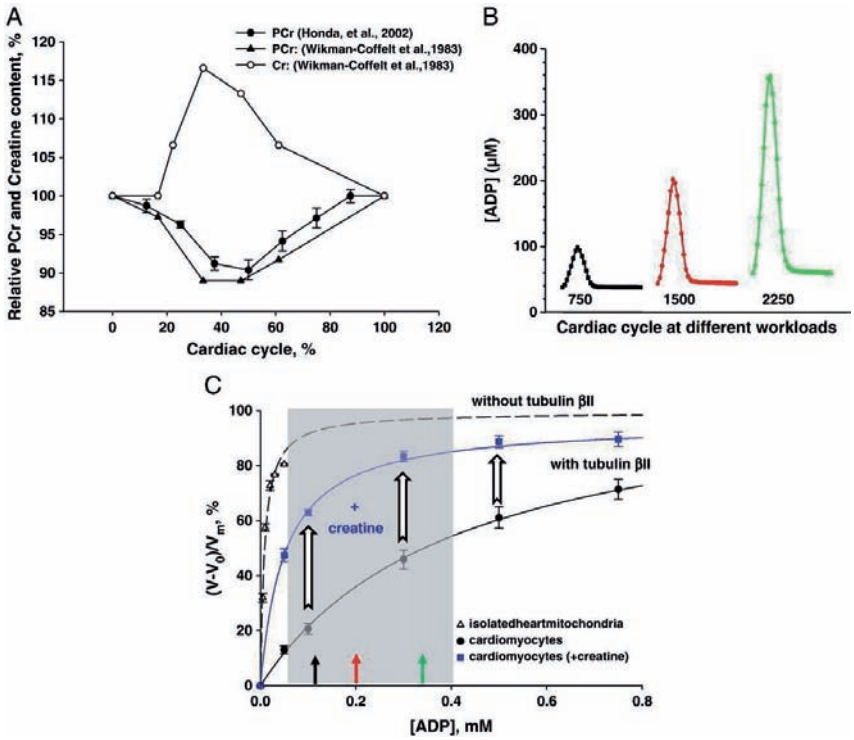

Fig. 8. Oscillations of PCr and creatine concentrations during cardiac cycle in perfused rat heart under conditions of metabolic stability. Experimental data adapted from Honda et al., 2002 [184] from gate-pacing ^{31}PMRS measurements and Wikman-Coffelt et al., 1983 [183] from rapid freeze clamp experiments. B. Mathematically modeled oscillations of ADP concentrations in the core of myofibrils over cardiac cycle at workloads equivalent to 750 (black), 1500 (red) and 2250 (green) μmol ATP s^{-1} kg^{-1}. Reproduced from [28] with permission. C. Graphical Michaelis–Menten representation of the dependence of mitochondrial respiration rate on the concentration of ADP. Colored arrows on X-axes show ADP concentrations corresponding to increased workloads from panel B. For explanation see the text.

ADP released from actomyosin crossbridges stimulates the local MMCK reaction in myofibrillar space of ICEUs and at the same time forms a gradient of concentration transmitted towards the mitochondria [186–188], Figs. 8B and C). The amplitude of displacement of MMCK from equilibrium, as well as cyclic changes of ADP are proportionally increased with workload (Fig. 8B) [28,29,31,186–188]. The rephosphorylation of ADP in MMCK reaction increases locally the Cr/PCr ratio which is transferred towards MtCK via CK/PCr shuttle [186]. The amplitude of ADP concentration changes during contraction are to our knowledge the only variables that meet the requirements for a metabolic signal formulated by O'Rourke (see above). Fig. 8C shows that the regulation of VDAC permeability by βII-tubulin is needed to induce linear responses of mitochondrial respiration to these local signals within ICEUs. When the mitochondrial outer membrane is permeable, as in isolated mitochondria, the regulation of respiration is impossible because of saturating concentrations in intracellular ADP. The latter exceeds manifold the apparent affinity of oxidative phosphorylation for free ADP ($K_m^{app}ADP = 7.9 \pm 1.6$ μM), even in diastolic phase (about 40 μM). On the contrary, when ADP diffusion is restricted at the level of MOM, as in mitochondria *in situ*, the apparent Km for free ADP increases to about 370.75 ± 30.57 μM and the respiration rate becomes almost linearly dependent on local ADP concentrations. Under these conditions, the initial rate of the hyberbolic curve can be approximated by linear dependence within the range of values corresponding to the increase in workload (Fig. 8B). Thus, cyclic changes in local ADP concentrations within the myofibrillar space of ICEUs become an effective regulatory signal due to: *i*) the non-equilibrium state of CK reactions, *ii*) the restricted VDAC permeability to metabolites elicited by association with βII-tubulin, and *iii)* the presence of creatine. When these conditions are fulfilled, activation of the coupled MtCK with MI by creatine, induces ADP/ATP recycling, increases respiration rate and displaces this linear dependence upward and to the left as shown in Fig. 8C, thus amplifying the effect of cytoplasmic ADP; and the apparent Km for ADP becomes equal to 50.24 ± 7.98 μM. These data suggest that regulation of respiration by local changes in ADP concentration, under condition of restriction of adenine nucleotides diffusion across mitochondrial membrane, is mediated by the specific structure of the MI. According to this view, the MtCK reaction amplifies the ADP signal due to its functional coupling with ATP Synthasome, thus increasing the steady state rate of adenine nucleotides recycling in mitochondria and the rate of respiration. The coupled reactions of muscle type MM CK in myofibrils and MtCK in mitochondria perform under non-equilibrium conditions and proceed in opposite directions (Fig. 9A and B) [24–34]. This mode of function results in separation of energy fluxes (mass and energy transfer by PCr) and signaling (information transfer by oscillations of cytosolic ADP concentrations, Pi and PCr/Cr ratio) which is amplified

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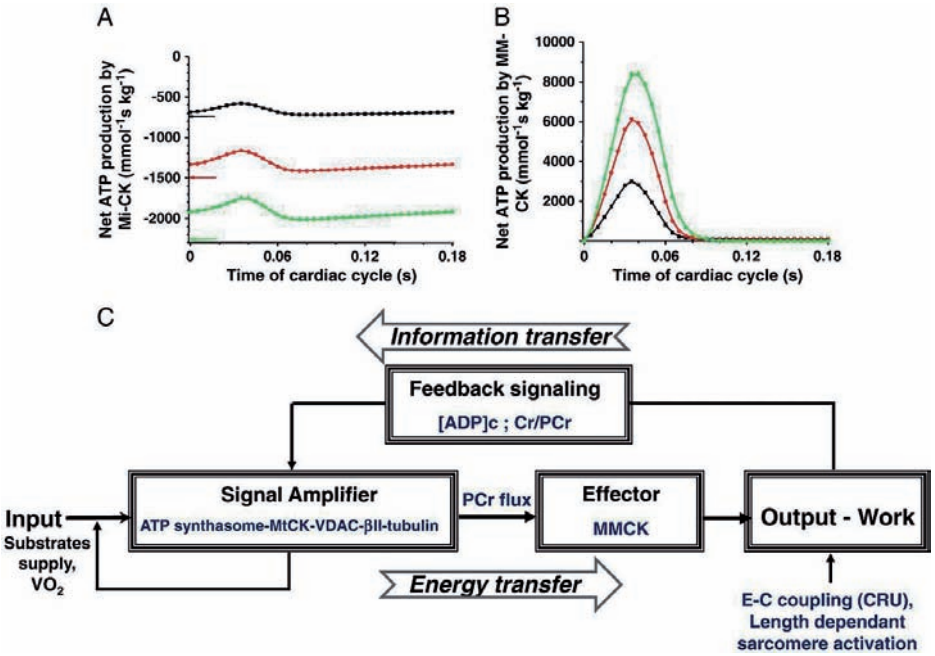

Fig. 9. A and B. Calculated net PCr production rates in non-equilibrium steady state MtCK reaction and cyclic changes in rates of ATP regeneration in non-equilibrium myofibrillar MMCK reaction during contraction cycles at different workloads correspond to oscillations of [ADP]c described in Fig. 8B (Reproduced from [28] with permission from Wiley & Sons). C. General presentation of the feedback metabolic signaling in regulation of energy metabolism within Intracellular Energetic Units in cardiac cells. Due to the non-equilibrium steady-state MtCK and non-equilibrium cyclic MMCK reactions intracellular ATP utilization (marked as output) and mitochondrial ATP regeneration (marked as input) are interconnected via the cyclic fluctuations of cytosolic ADP and Cr/PCr. For explanation see the text.

within the MI. As a result, reactions catalyzed by different isoforms of compartmentalized CK tend to maintain the intracellular metabolic stability.

The separation of energy and information transfer is illustrated by the scheme depicted in Fig. 9C. This Scheme shows feedback regulation of respiration *in vivo* corresponding to the Norbert Wiener's cybernetic principles [29,31,190]: the usage of ATP (or release of free energy of ATP hydrolysis, ΔG_{ATP}, to perform work (marked as output) and the ATP regeneration (or extraction of ΔG_{ATP} from substrates by oxidative phosphorylation; corresponding to input) are interconnected via the feedback signaling through oscillations of cytosolic concentrations in ADP, Pi and Cr/PCr amplified within MI. In this framework, the role of βII-tubulin association with MOM in cardiomyocytes would be to induce the linear response of mitochondrial respiration to workload-dependent metabolic signals. This elegant feedback mechanism of regulation of respiration on a beat-to beat basis ensures metabolic stability necessary for normal heart function, and explains well the metabolic aspect of the Frank–Starling's law of the heart, *i.e.* the linear dependence of respiration rate upon workload [12,13,28–30,186,188]. Importantly, recycling of adenine nucleotides within MI when coupled to PCr production significantly decreases also ROS levels ensuring maximal efficiency of free energy transduction in mitochondria and inhibits permeability transition pore opening [191–193], thus protecting the heart under stress conditions.

Interaction of βII-tubulin with VDAC within MI may be modified by phosphorylation [194], cytoskeletal remodeling or by structural rearrangements during the contraction cycle [195]. Most interestingly, if tubulin connects MOM to some structural elements in sarcomere, length-dependent activation of the latter may also change mitochondrial sensitivity to metabolic signaling.

4. Conclusions, perspectives and clinical significance

It is clear from the data presented in this review that the tubulin system plays important role in organizing mitochondria into Intracellular Energetic Units in cardiac cells and in the control of their functional properties. However, molecular mechanisms of the interaction of tubulin β II isotype with VDAC needs detailed further studies. In order to further verify *in vivo* some of the findings described throughout this work, we need to develop fluorescent or bioluminescent probes sensitive to ATP and ADP to monitor ICEUs function and the molecular interactions involved in the MI using FRET, fluorescence correlation spectroscopy and other techniques. Similarly to the discoveries of localized calcium domains (sparks) within CRUs [87–89], this will be most important for a quantitative description of localized mechanisms of regulation of energy fluxes in the heart. Very significant information about metabolic compartmentation of adenine nucleotides, their turnover and interaction with cellular structures can be obtained also by analysis of ^{31}P relaxation properties by NMR saturation transfer spectroscopy [71]. Numerous studies have shown that knock-out of creatine kinase isozymes or replacement of creatine by its analogs results in very significant structural and functional changes in muscle cell, a significant loss of contractile force, and perturbation of calcium metabolism (reviewed in [29,33]). In these cases, regulatory properties and the role of MI in information transfer are lost. The increase in energy transfer by other phosphotransfer pathways is another potential compensatory mechanism [196].

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The results described in this review are in agreement with important clinical observations performed by Neubauer's group [197], showing that in patients with dilated cardiomyopathy a decreased PCr/ATP ratio is a strong predictor of increased mortality. In myocardial infarction and in heart failure, rapid decrease of PCr content and heart function occur, possibly due to lack of oxygen supply and pathological changes in the creatine kinase system. A significant decrease of Cr and PCr content is observed in chronic cardiac and skeletal muscle diseases [198]. Data published by Weiss et al. [199] showing that cardiac ATP flux through CK is reduced by 50% in human heart failure in the absence of reduction of ATP stores, is also in agreement with the key role played by the CK/PCr system. The links between alterations in the CK/PCr system and cardiac pathology have been extensively reviewed elsewhere [200–204]. Finally, lack of βII-tubulin and MtCK in HL-1 cells may be helpful for explaining the Warburg mechanism in cancer cells [29,33,34].

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Original article

Studies of the role of tubulin beta II isotype in regulation of mitochondrial respiration in intracellular energetic units in cardiac cells

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ABSTRACT

The aim of this study was to investigate the possible role of tubulin βII, a cytoskeletal protein, in regulation of mitochondrial oxidative phosphorylation and energy fluxes in heart cells. This isotype of tubulin is closely associated with mitochondria and co-expressed with mitochondrial creatine kinase (MtCK). It can be rapidly removed by mild proteolytic treatment of permeabilized cardiomyocytes in the absence of stimulatory effect of cytochrome c, that demonstrating the intactness of the outer mitochondrial membrane. Contrary to isolated mitochondria, in permeabilized cardiomyocytes (*in situ* mitochondria) the addition of pyruvate kinase (PK) and phosphoenolpyruvate (PEP) in the presence of creatine had no effect on the rate of respiration controlled by activated MtCK, showing limited permeability of voltage-dependent anion channel (VDAC) in mitochondrial outer membrane (MOM) for ADP regenerated by MtCK. Under normal conditions, this effect can be considered as one of the most sensitive tests of the intactness of cardiomyocytes and controlled permeability of MOM for adenine nucleotides. However, proteolytic treatment of permeabilized cardiomyocytes with trypsin, by removing mitochondrial βII tubulin, induces high sensitivity of MtCK-regulated respiration to PK–PEP, significantly changes its kinetics and the affinity to exogenous ADP. MtCK coupled to ATP synthasome and to VDAC controlled by tubulin βII provides functional compartmentation of ATP in mitochondria and energy channeling into cytoplasm via phosphotransfer network. Therefore, direct transfer of mitochondrially produced ATP to sites of its utilization is largely avoided under physiological conditions, but may occur in pathology when mitochondria are damaged. This article is part of a Special Issue entitled 'Local Signaling in Myocytes'.

1. Introduction

Experimental studies of the mechanisms of regulation of mitochondrial function by feedback metabolic signaling *in vivo*[1–15] need the use of the permeabilized cells or fibers technique [16–24] and methods of *in vivo* kinetic studies [4–7]. Intensive investigations during the last two decades with use of these techniques have shown that the regulation of mitochondrial function *in vivo* is very different from that *in vitro*: the apparent Km for exogenous ADP in regulation of respiration is 20–30 times higher in the permeabilized cells than in isolated mitochondria *in vitro*[8–24]. This high apparent Km for ADP can be decreased by addition of creatine that activates mitochondrial creatine kinase, MtCK [8,13,19,20], or by the controlled proteolytic treatment [21–24]. The apparent Km for exogenous ADP shows the availability of ADP for the adenine nucleotide translocase (ANT) in mitochondrial inner membrane (MIM) and was proposed to be dependent on the permeability of the mitochondrial outer membrane's (MOM) voltage-dependent anion channel (VDAC) [22,23]. A strong decrease of the apparent Km for exogenous ADP produced by trypsin treatment pointed to the possible involvement of some cytoskeleton-related protein(s) in the control of the VDAC permeability originally referred to as "factor X" [22,23]. Appaix et al. [24] have shown that among cytoskeletal proteins sensitive to short

Abbreviations: ANT, adenine nucleotide translocase; BSA, bovine serum albumin; CAT, carboxyatractyloside; CK, creatine kinase; Cr, creatine; DTT, dithiothreitol; IM, isolation medium; IMS, mitochondrial intermembrane space; MI, Mitochondrial Interactosome; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane; MtCK, mitochondrial creatine kinase; PCr, phosphocreatine; PBS, phosphate buffer solution; PEP, phosphoenolpyruvate; PK, pyruvate kinase; STI, soybean trypsin inhibitor; VDAC, voltage-dependent anion channel; WS, washing solution.

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proteolytic treatment are tubulin and plectin. Rostovtseva et al. [25,26] established that the first candidate for the role of "factor X" is αβ heterodimeric tubulin, which upon binding to VDAC reconstructed into a planar lipid membrane strongly modulated the channel's conductance. Reconstitution experiments indicated that the addition of the heterodimeric tubulin to isolated mitochondria strongly increased the apparent Km for ADP [27]. Recent immunofluorescence confocal microscopic studies allowed to identify the tubulin associated with mitochondrial outer membrane in cardiomyocytes as its βII isotype [14]. The aim of this study was to investigate further the role of this tubulin-βII isotype in the regulation of respiration in cardiac cells. We show by immunofluorescence confocal microscopy and respirometry that short proteolytic treatment of permeabilized cardiomyocytes removes tubulin-βII from MOM. This significantly increases the MOM permeability for ADP as measured by activation of the MtCK located in the outer surface of inner mitochondrial membrane with trapping of extramitochondrial ADP by the pyruvate kinase (PK) – phosphoenolpyruvate (PEP) system. In accurately prepared permeabilized cardiomyocytes PK–PEP system has no effect on respiration, while in damaged cardiomyocytes and after proteolytic treatment MOM permeability is increased and respiration rate decreased due to ADP tapping by PK–PEP. This permeability test of MOM controlled by tubulin-βII can be used as the most sensitive quality control for intactness of mitochondria in permeabilized cardiomyocytes. Removal of tubulin-βII by proteolytic treatment does not damage the outer mitochondrial membrane itself (as shown by cytochrome c test), but significantly decreases the apparent Km for ADP via an increase of the permeability of VDAC.

2. Materials and methods

2.1. Isolation of cardiac myocytes with perfect rod-like shape, description of various troubleshooting

Adult cardiomyocytes were isolated by adaptation of the technique described previously [19]. Male Wistar rats (300–350 g) were anesthetized and the heart was quickly excised preserving a part of aorta and placed into washing solution (WS) (117 mM NaCl, 5.7 mM KCl, 4.4 mM $NaHCO_3$, 1.5 mM KH_2PO_4, 1.7 mM $MgCl_2$, 11.7 mM glucose, 120 mM sucrose, 10 mM Cr, 20 mM taurine, and 21 mM BES, pH 7.1). All solutions used during the procedure of isolation were saturated with oxygen. The heart was cannulated and washed with WS at a flow rate of 15–20 mL/min for 5 min. At that, the coronary flow should exceed ca. 20 mL/min; otherwise the heart has to be discarded. The collagenase treatment was performed by switching the perfusion to recirculation isolation medium (IM), (117 mM NaCl, 5.7 mM KCl, 4.4 mM $NaHCO_3$, 1.5 mM KH_2PO_4, 1.7 mM $MgCl_2$, 11.7 mM glucose, 10 mM creatine (Cr), 20 mM taurine, 10 mM PCr, 2 mM pyruvate, and 21 mM HEPES, pH 7.1), supplemented by collagenase (0.75 mg/mL) at a flow rate of 5 mL/min for 50 min at 37 °C. After the collagenase treatment the system was switched to the initial solution WS for 1–2 min and then the heart was transferred into the IM supplemented with 20 μM $CaCl_2$, 10 μM leupeptin, 2 μM soybean trypsin inhibitor (STI), and 2 mg/mL bovine serum albumin (BSA). The cardiomyocytes were then gently dissociated by pipette suction. The cell suspension was filtered and transferred into a test tube for sedimentation where the calcium-tolerant cells were allowed to freely sediment. After 3–4 min the initial supernatant was discarded and the pellet of cardiomyocytes resuspended in IM containing 20 μM $CaCl_2$, STI and leupeptine. The rod shaped intact cells sedimentated within 2–3 min and the supernatant with damaged cells was discarded. This resuspension–sedimentation cycle with calcium-tolerant cells was performed twice and after that cardiomyocytes were gradually transferred from calcium containing solution into calcium-free Mitomed [17]. Then, the cardiomyocytes were washed 5 times with the Mitomed containing 5 mg/mL BSA, 10 μM leupeptin, and 2 μM STI. Isolated cells were stocked in 1–2 mL volume and stored on ice during further experiments. Isolated cardiomyocytes contained 85–100% of rod-like cells when observed under a light microscope. Final quality of isolated rat cardiomyocytes was found to depend on a number of minor variations in different isolation steps beginning from the severing of the aorta, removal of the heart from the thorax and initial heart perfusion in order to remove Ca^{2+} and the remainder of blood before the collagenase treatment. It is also advisable to perform this operation in ≤1 min to avoid oxygen deficiency and hypoxia. The choice of the collagenase type is the next crucial step; to our experience, collagenase A (Roche) or Liberase Blendzyme 1 (Roche, similar to the new product Liberase DL Research Grade), an artificial mixture of purified enzymes with carefully controlled specific activities (Roche), results in satisfactory results. Caution should be taken in an attempt to reduce duration of the collagenase perfusion time at the expense of the increase in the enzymes activity. For every lot of collagenase the time of dissociation, enzyme ratios, and enzyme concentration affect tissue dissociation outcomes. The perfusion should be performed at controlled rate by pumping and, advisably, under manometric control in order to follow a decrease in the developed pressure from 55 to 60 mm Hg (which corresponds to ≈80 cm H_2O) to that less than 10 mm Hg. Collagenase solution should be washed out in the presence of the mixture of strong inhibitors of serine and thiol proteases and further operations also performed in the presence of these inhibitors. STI is capable of binding to different serine proteases, and leupeptin is the best choice for thiol proteases.

Usually, the obtained preparation is stable enough during 4–5 h needed for measurements. Used saponin concentration and permeabilization time should also be carefully adjusted by studies of the extent of permeabilization by respirometry.

An alternative to isolation of cardiomyocytes is the use of skinned cardiac fibers isolated according to the method described by Kuznetsov et al. and Saks et al. [17,18]. When correctly used, both methods allow obtaining identical results in studies of respiration regulation after cell or fiber permeabilization [8,9,16–24]. In both cases, it is important to avoid artifacts of cell or fiber isolation resulting in misleading and incorrect experimental data, sometimes reported in the literature, when permeabilized cells and fibers have very different properties [28]. The method of preparation of skinned fibers was in details described by Kuznetsov et al.[17]. To isolate high quality cardiomyocytes needed for functional studies it is equally important to avoid multiple errors, which are listed below in the Table 1.

2.2. Cell preparation for confocal microscopy Q7

Freshly isolated cardiomyocytes and cultured cells were fixed in 4% paraformaldehyde at 37 °C for 15 min. After rinsing with phosphate buffer solution (PBS, containing 2% BSA) cells were permeabilized with 1% Triton X-100 at 25 °C for 30 min. Finally, cells were rinsed repeatedly and incubated with primary antibody as described above for immunoblotting using concentrations indicated in the Table 1 (in 2% BSA containing PBS solution). The next day samples were rinsed and stained for 30 min at room temperature with secondary antibody. Secondary antibodies: Cy™ 5-conjugated Affini-Pure goat anti-mouse IgG (Jackson Immunoresearch 115-175-146), goat polyclonal secondary antibody to mouse IgG-FITC (Abcam ab6785), were used respecting concentrations recommended by the providers (Table 2). Q3

The same proceeding was done during trypsinization of cells but before being fixed, cells were trypsinized by 0.05 or 0.3 μM (0.1–4 mg

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Table 1
Useful advises for high quality cardiomyocyte isolation.

Steps	Problems	Possible reasons	Solutions
Heart dissection and hanging to start perfusion	Improper or too high flow rate, see below	Damage of the wall of aorta or aortic valve	Discard this heart. For dissection of the heart holding this between fingers, gently stretch the aorta and cut it to get long aorta to preserve aortic valve from damage
Initial perfusion (80 cm H_2O)	Perfusion pressure too high (>69 mm Hg), coronary flow rate too low (≤15 mL/min)	Aorta partially clogged up	i. Wait for some minutes, small embolus might flow out ii. Remove heart and hang up once again, otherwise discard the heart
	Coronary flow>25 mL/min, abnormally low perfusion pressure	Leak of perfusate due to improper hanging, see above	Hang up the heart once again, otherwise discard the heart
Collagenase perfusion	Perfusion pressure >10–15 mm Hg after 50 min perfusion	Protease concentration too low	Increase the concentration of the collagenase preparation
		Enzyme inactivation	Check storage conditions and the enzyme activity
		Perfusion temperature is too low	Verify temperature
	Too rapid drop perfusion pressure down to zero (in 10–15 min)	Protease concentration too high	Decrease the concentration of the collagenase preparation
	Stained heart surface	Uneven perfusate flow in the heart body due to clogging in some capillaries. Ischemic regions in the heart	Discard the heart, otherwise the yield will be low and quality doubtful
Preparing and washing of the cells	Too low cell sedimentation rate	Substantial amounts of damaged cells present	Normal intact cells sediment in 2–4 min. Elongation of sedimentation time in an attempt to improve the yield could exclusively result in collecting damaged cells
	Low cell viability and yield	Mechanical force for heart dissection is too excessive	Reduce shear force and use the pipette more gently
Saponin treatment	Too low activation of respiration by exogenous ADP	Incomplete permeabilization of sarcolemma	Cell permeabilization has to be checked in the oxygraph cells by addition of the saponin stock solution, the activation of respiration should be complete in ca. 10 min. and the final oxygen consumption rate remain unaltered at least for 20 min, otherwise the saponin concentration should be adjusted
Stirring	Gradual decay in the oxygen consumption rate	Cell damaging due to too vigorous stirring	Decrease in the stirring rate to sufficiently low value

of TR/mg cardiomyocytes protein) for 10 min at 25 °C and then STI, up to a final concentration of 0.02 mM, was added.

2.3. Confocal imaging

The fluorescence images were acquired with a Leica TCS SP2 AOBS inverted laser scanning confocal microscope (Leika, Heidleberg, Germany) equipped with a 63× water immersion objective (HCX PL APO 63.0×1.20 W Corr). Laser excitation was 488 nm for FITC (green fluorescence) and 633 nm for Cy 5 (red fluorescence). Images were then analyzed using Volocity softwere (Improvision, France).

2.4. Colocalization studies

α-actinin and βII-tubulin were immunostained with Cy5-labeled antibody according to the protocol described elsewhere [14]. They were imaged using the 63×/1.4 oil immersion Plan Apo objective, 633 nm HeNe laser and 638–747 nm detection of LSM710NLO confocal microscope (Carl Zeiss). The pinhole value was set to 1 Airy unit. Optical slices closest to the glass surface were analyzed in order to minimize the optical distortions in cardiomyocytes. Mitochondria distribution in fixed cardiomyocytes was visualized using flavoprotein autofluorescence signal excited with the two-photon laser at 720 nm and integrated between 408 and 546 nm. For increasing the autofluorecence of flavoproteins and improving the imaging of mitochondria, the permeabilized cells were treated before fixation with 10 μM rotenone for 10 min under aerobic conditions and washed twice in Mitomed solution described in the next section. The choice of this label-free imaging of mitochondria allowed one to avoid any possible spectral bleed-through to the near-infrared detection channel for α-actinin or βII tubulin immunofluorescence. Indeed, no cell specific background was detected in this channel in unlabelled cardiomyocytes. The very low background signal was detected in case of nonspecific control with the Cy5-labeled secondary antibody. The signal to noise was improved using 16 line scan repetitions and 6 μs pixel dwell time. Overall photobleaching with the used laser intensities did not exceed 1%. The red channel images were not treated for the sake of intensity comparison; the green channel images were processed with a Top-hat square shape filter to improve the contrast of rectangular mitochondria pattern (MetaMorph, Universal Imaging).

Table 2
List of antibodies used.

Commercial name	Dilution for immunofluorescence	Immunogen
Primary antibodies:		
mouse anti-tubulin βII(β2),(Abcam ab28036)	1/1000	Amino acids CEEEEGEDEA at the C terminus
alpha-actinin rabbit (Abcam, ab82247)	1/100	
Secondary antibodies:		
a) Cy™ 5-conjugated AffiniPure goat anti-mouse IgG (Jackson Immunoresearch 115-175-146)	1/100	
b) goat anti- mouse polyclonal secondary antibody IgG-FITC (Abcam ab6785)	1/800	

2.5. Measurements of oxygen consumption

The rates of oxygen uptake were determined with high-resolution respirometer Oxygraph-2K (OROBOROS Instruments, Austria) in Mitomed solution [17] containing 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 3 mM KH_2PO_4, 20 mM taurine, 20 mM HEPES (pH 7.1), 110 mM sucrose, 0.5 mM dithiothreitol (DTT), 2 mg/mL fatty

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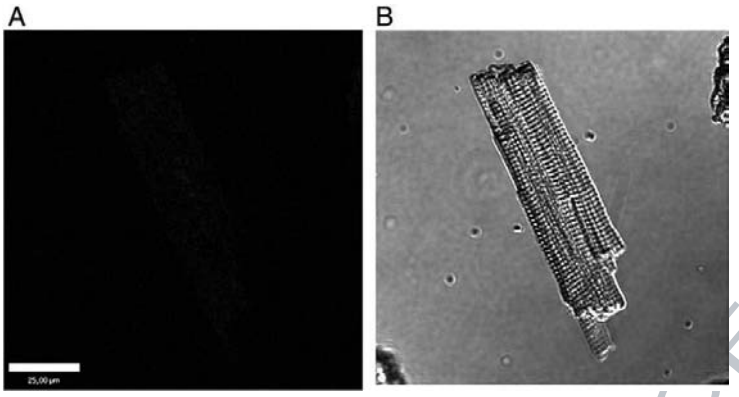

Fig. 1. Specificity test for immunofluorescence labeling of tubulin in cardiomyocytes. A. Confocal image of isolated cardiomyocytes after labeling with secondary antibodies Cy™ 5-conjugated AffiniPure goat anti-mouse IgG (Jackson Immunoresearch) without primary antibodies. B. Transmission image of the same cardiomyocyte.

acids free BSA, complemented with 5 mM glutamate and 2 mM malate as respiratory substrates. Respiration was activated by addition of creatine to a final concentration of 20 mM in the presence of 2 mM ATP. Maximal respiration rate was measured in the presence of 2 mM ADP. The measurements were carried out at 25 °C; solubility of oxygen was taken as 240 nmoL/mL [17].

2.6. Data analysis

The experiments were carried out independently in two different laboratories. The apparent Km for ADP or ATP was estimated from a linear regression of double-reciprocal plots or by non-linear least-squares.

3. Results

3.1. Confocal immunofluorescence imaging of tubulin-βII in permeabilized cardiomyocytes

Our recent study showed regular localization of βII-tubulin in cardiac cells [14], similar to the "crystal-like" arrangement of mitochondria [29–33]. Therefore, in this work we further investigated the correlation between localization of tubulin-βII close to the outer mitochondrial membrane in adult cardiomyocytes with several important parameters of regulation of mitochondrial respiration in permeabilized cardiac cells *in situ*. The second aim of this study was to describe the necessary quality tests of the intactness of mitochondria in permeabilized cardiomyocytes, required for the proper studies of the interaction of mitochondria with cytoskeleton *in situ*. A short-time proteolysis of permeabilized cells was optimized and used to remove tubulin-βII from the cells, since our earlier studies have shown that tubulin is one of the most sensitive proteins to this kind of treatment [24]. Trypsin treatment is also routinely used for isolation of intact mitochondria from heart muscle [12,24]. The localization of tubulin--βII in fixed cardiac cells was visualized by immunofluorescence confocal microscopy (Figs. 1–4). Fig. 1 shows the high selectivity of this method, demonstrating that incubation of cells with only secondary antibodies does not result in any labeling of intracellular structures. Only after incubation of the fixed and permeabilized cells with primary antibodies against C-terminal tail of tubulin-βII and subsequent incubation with secondary fluorescent antibodies intensive immunofluorescence labeling of tubulin-βII associated with mitochondria can be seen (Figs. 2 and 3). Fig. 2A shows the very regular immunofluorescence labeling of tubulin-βII by using secondary antibodies with green fluorescence before trypsin treatment. Fig. 2B shows that after short proteolysis the fluorescence intensity decreases significantly and regular arrangement of tubulin disappears. Since green fluorescence seen in Fig. 2A and B may be influenced by the autofluorescence of oxidized mitochondrial flavoproteins [18], localization of tubulin-βII was studied also by using secondary antibodies with red fluorescence (Fig. 3). Again, very regular labeling of mitochondria was seen. Similar to the results

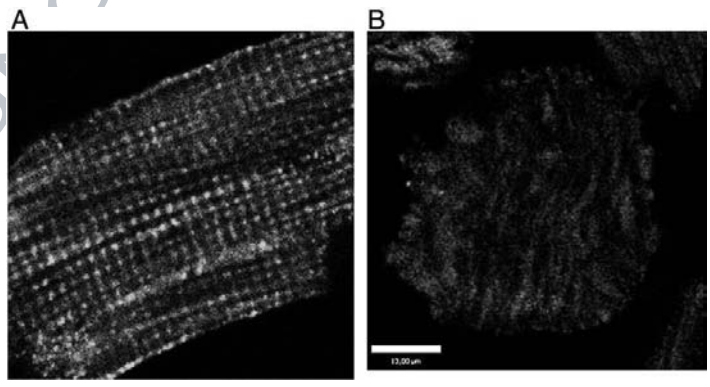

Fig. 2. Immunofluorescence labeling of βII-tubulin before and after trypsin treatment. Labeling with primary antibody and goat anti-mouse polyclonal secondary antibody IgG-FITC (Abcam ab6785). A. Before trypsin treatment. B. After trypsin (0.05 μM) treatment.

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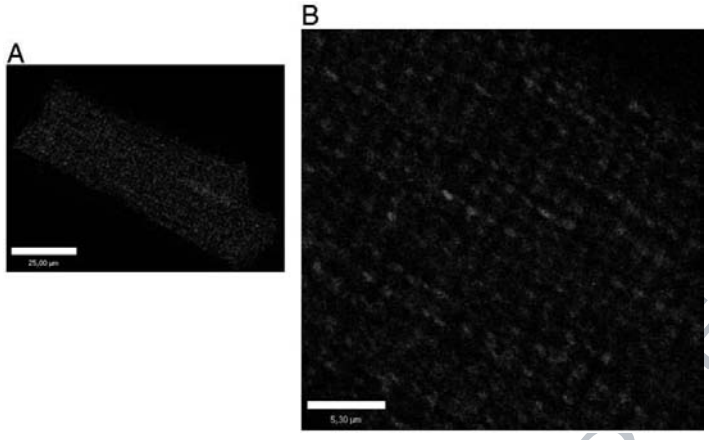

Fig. 3. Immunofluorescence labeling of βII-tubulin in isolated cardiomyocytes with primary and Cy™ 5-conjugated AffiniPure goat secondary anti-mouse IgG (Jackson Immunoresearch). Labeling of mitochondria in parallel rows parallel to long axis of the cell is seen. For further details see Ref. [14].

presented on Figs. 2B, Fig. 4 shows again that short treatment of permeabilized cardiomyocytes with trypsin completely removes the tubulin-βII, also changing the cell shape due to destruction of tubulin and other cytoskeletal systems, and changes intracellular arrangement of mitochondria from regular into irregular clustered one, in accordance with our earlier observations [24,32].

3.2. Colocalization of mitochondria and tubulin βII

To answer the question whether and how tubulin-βII is colocalized with mitochondria in cardiac cells, α-actinin in the Z-lines (Figs. 5A, C) and tubulin-βII (Figs. 5E, G) were immunostained with Cy5-labeled secondary antibody and mitochondrial localization was detected by imaging of the autofluorescence of mitochondrial flavoproteins in oxidized state. Figs. 5A and E show merged images, Figs. 5B, C and F, G show images recorded by separate channels. Figs. 5D and H show the results of quantitative analysis of these images — the fluorescence intensity plots along white lines drawn through representative sequences of 4 mitochondria (see panels B, C and F, G). The very low background signal (dashed lines in Figs. 5D, H) was detected in case of nonspecific control with only the Cy5-labeled secondary antibody. The amplitude of Cy5 fluorescence signal of α-actinin is strongly modulated along a mitochondrial chaplet with the period equal to that of mitochondria but with the inversed phase, indicating essential localisation of α-actinin on Z-lines (Figs. 5A–D). Remarkably, mitochondrial green autofluorescence was not detected in the Z-line area (Fig. 5D), showing the absence of mitochondrial fusion in cardiomyocytes, confirming our previous observation [8]. Contrary to the α-actinin staining, the tubulin-βII fluorescence amplitude modulation is very weak along the line of mitochondrial localization, showing the overall staining of the mitochondria (Figs. 5E–H). Since tubulin βII was detected also in the Z-line area, it seems to form a network-like structures connecting mitochondria to the other cytoskeletal structures. Thus, Figs. 3 and 5 confirm with higher resolution our earlier observations of colocalization of tubulin-βII with mitochondria. However, the resolution limit of confocal microscope (about 0.2 μm) does not allow more detailed analysis of protein localisation on the submitochondrial level (which can be done in the future by using FRET approach).

3.3. Alteration of parameters of respiratory regulation after removal of tubulin βII

The common tests of mitochondrial intactness, which include activation of mitochondrial respiration by ADP, are the cytochrome c test of intactness of the outer membrane of mitochondria and

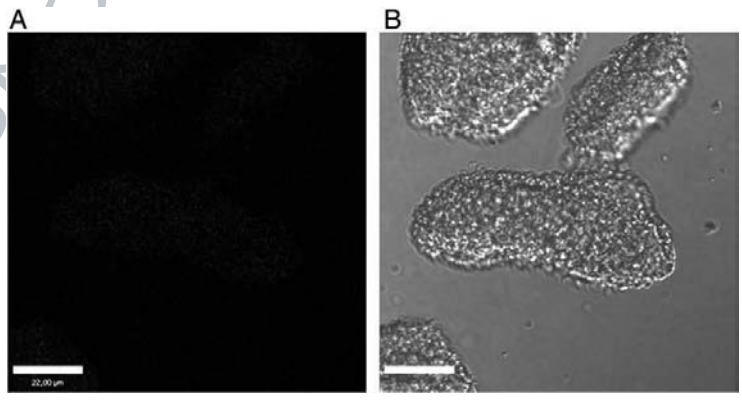

Fig. 4. Immunofluorescence labeling of βII-tubulin after short proteolysis of permeabilized cardiomyocytes with 0.05 μM trypsin before fixation (see Materials and methods). Cy™ 5-conjugated AffiniPure goat anti-mouse IgG (Jackson Immunoresearch 115-175-146) was used. A. Confocal image. B. Transmission image of the same cells.

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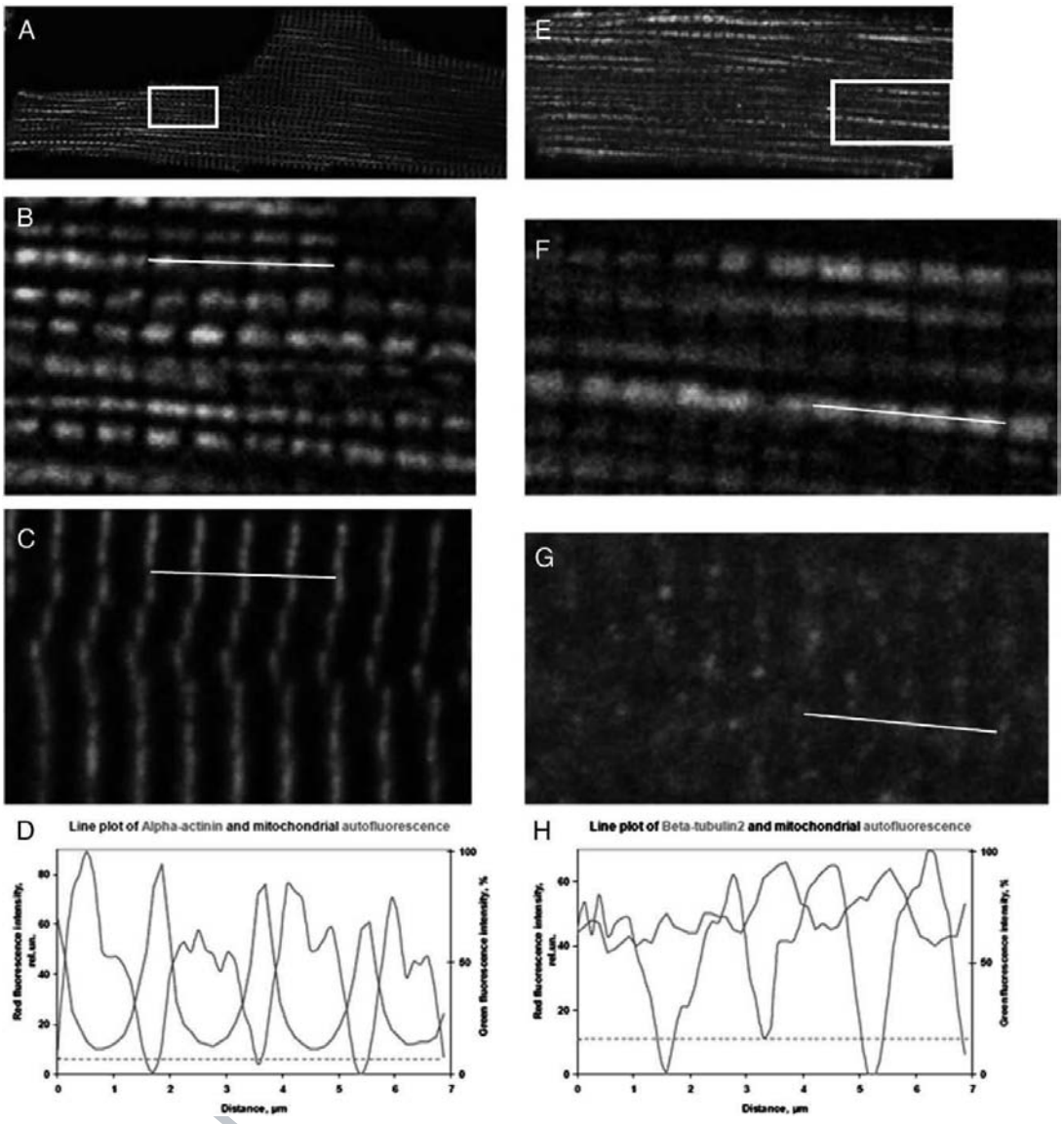

Q6 **Fig. 5.** Comparison of the intracellular distribution of βII-tubulin, α-actinine, a « Z-line label », and mitochondria. Confocal merged images of immunofluorescence labeling of α-actinin (A) or βII-tubulin (E), red color and mitochondrial autofluorescence, green color. B, C ,F, G — zoom in regions of interest highlighted by the white rectangles in the panels A and E, separated channels. D, H — intensity plots along white lines drawn through representative sequences of 4 mitochondria (panels B, C, F, G).Dashed red lines indicate the background level of unspecific fluorescence staining measured in control experiments. Red plots are presented in relative units using the same scale for βII-tubulin and α-actinin. Green plots were normalized to the 100% of the maximal intensity of autofluorescence after the background subtraction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

inhibition of ADP-stimulated respiration by carboxyatractyloside (CAT); they are shown in Fig. 6. In permeabilized cardiomyocytes ADP (2 mM) increases respiration rate more than 10 times and this rate is not changed by addition of cytochrome c (Fig. 6A). Cytochrome c, a highly soluble hemoprotein of the respiratory chain is loosely associated with the outer side of the inner membrane of the mitochondria. If the outer membrane is disrupted, cytochrome c leaves mitochondria, and in this situation addition of the protein increases respiration rate [34]. Thus, the cytochrome c test (Fig. 6A) shows that in permeabilized cardiomyocytes mitochondrial outer membrane is entirely intact. CAT completely inhibits ADP-activated respiration, showing that all ADP is imported into mitochondrial matrix *via* ANT [35]. Remarkably, all these parameters are not changed after treatment of permeabilized cardiomyocytes by trypsin (Fig. 6B) that showing that short proteolytic treatment leaves mitochondrial membranes completely intact, in accordance with all earlier data of studies of isolated heart mitochondria [24,34].

More sensitive test which shows clear changes in parameters of regulation of mitochondrial respiration after removal of βII-tubulin by short proteolysis is shown in Fig. 7. This Figure shows the parameters

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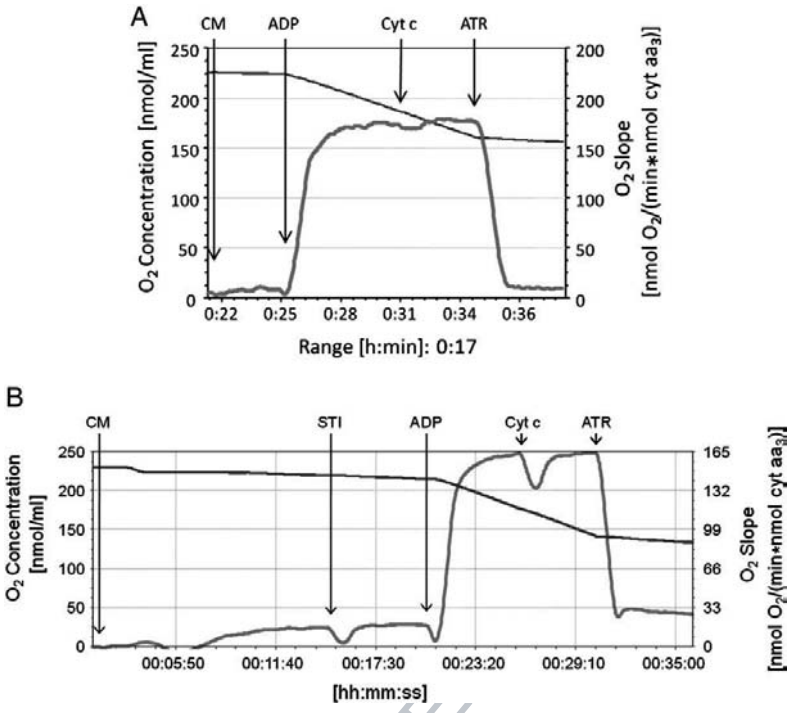

Fig. 6. Representative respiration traces before trypsin treatment of permeabilized cardiomyocytes (CM). A. Respiration is activated with 2 mM exogenous ADP. Cytochrome c (Cyt c) test (10 μM cytochrome) shows the intactness of MOM. Atractyloside (ATR, 0.1 mM) test shows that respiration is totally controlled by ANT. B. The same as A, but after treatment with trypsin at 0.05 μM for 10 min, then soybean trypsin inhibitor (STI) was added.

of regulation of mitochondrial respiration by MtCK activated by addition of creatine and MgATP. MtCK is located at the outer surface of mitochondrial inner membrane in close vicinity of ANT [2,3,36,37] and produces MgADP behind the outer mitochondrial membrane (Fig. 7A). This ADP formed in the active site of MtCK is released into intermembrane space of mitochondria and may either return to matrix *via* ANT or leave mitochondria through VDAC [38,39], the flux distribution between these two routes depending on the permeability of this channel for adenine nucleotides. The ADP flux distribution can be easily revealed by addition of exogenous ADP trapping system consisting of PK (20 U/mL) and PEP (5 mM) (Fig. 7B). Fig. 7C shows that in intact permeabilized cardiomyocytes (more than 90% of rod-like cells) addition of PK–PEP system does not change the rate of respiration, which is maintained at the maximal value by activated MtCK within mitochondrial interactosome. However after short proteolysis, removing βII-tubulin from MOM, addition of PK–PEP system decreases the respiration rate to half of its maximal value (Fig. 7D), as observed for isolated mitochondria before [8,12,40]. That means that about 50% of MgADP produced by MtCK can leave now mitochondria *via* VDAC which permeability for MgADP is increased. Remarkably, the effect of PK–PEP system on the respiration was also seen when the preparation of isolated cardiomyocytes contained, without use of trypsin, about 50% of rod-like intact cardiomyocytes and 50% of round-shape cells, probably due to some damaging factors listed in Table 1 (Fig. 7E). Thus, the PK–PEP test is an important quality control which has to be used in such studies to demonstrate intactness of isolated cardiomyocytes (see in details in Materials and methods section).

Fig. 8 shows that removal of βII-tubulin from mitochondrial membrane decreases the apparent Km for exogenous ADP in regulation of mitochondrial of mitochondrial respiration. This is in good agreement with earlier observation of Kuznetsov et al. and Appaix [21,24]. The results shown in Figs. 7 and 8 support the assumption that βII-tubulin bound to MOM in intact permeabilized cardiomyocytes *in vivo* limits the permeability of VDAC channel and increases ADP transfer to matrix *via* ANT, further enhancing the functional coupling between ANT and MtCK [5,7] and thus increases the functional compartmentation of adenine nucleotides within mitochondria in the cells (Fig. 7B). Under these conditions, the MtCK reaction completely controls the respiration rate even in the presence of cytoplasmic ADP trapping system: increase in creatine concentration rapidly increases the respiration rate to its maximal value (Figs. 9A, C). Under these conditions oxidative phosphorylation is maintained by ADP regeneration and recycling within mitochondrial interactosome coupled to permanent creatine phosphorylation and phosphocreatine production with high PCr/O_2 ratio equal to about 6 [41]. When the βII-tubulin is removed from MOM by proteolytic treatment and the VDAC permeability increased, exchange of adenine nucleotides between mitochondria and medium is increased and MtCK only partially controls the respiration (Figs. 9B, D).

4. Discussion

The results of this work are consistent with an assumption that βII-tubulin is one of the cytoskeletal proteins in heart cells which are able to control selectively the VDAC permeability in mitochondrial outer membrane for adenine nucleotides [14]. This restricted permeability for ADP and ATP favors their recycling in the coupled MtCK–ATP synthasome reactions in mitochondria connecting oxidative phosphorylation to PCr synthesis within a supercomplex, which we called "Mitochondrial Interactosome" [8,41], a key structure of phosphocreatine pathway of intracellular energy transfer [1–15]. Also, limited permeability of VDAC for ADP

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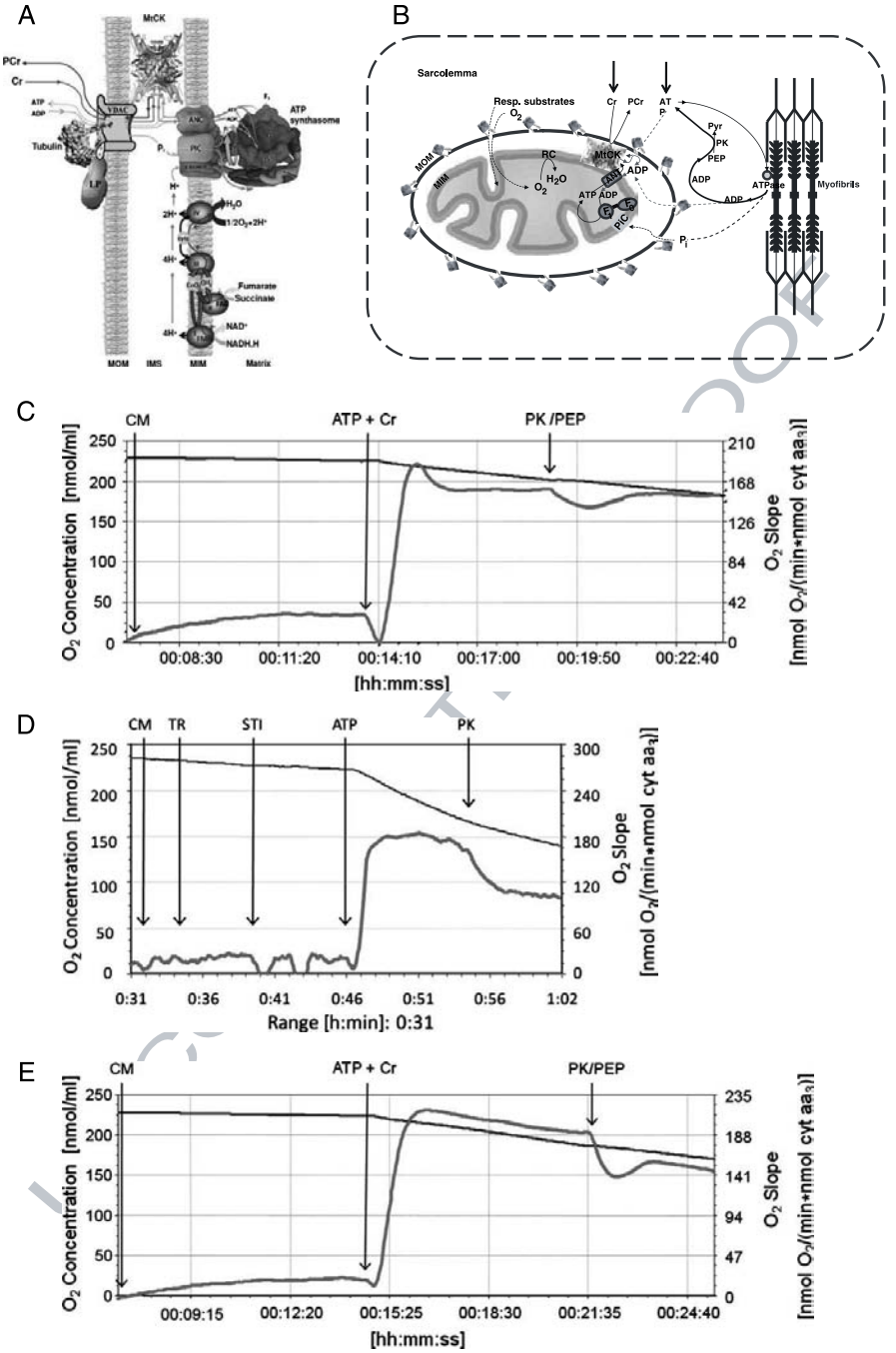

Fig. 7. A. The structure of mitochondrial interactosome showing the localization of MtCK coupled to ATP synthasome in cardiac cells. Adapted from [8,41]. B. The principle of the protocol of the study of ADP fluxes in permeabilized cells by PK–PEP system. C. Respiration of permeabilized cardiomyocytes (CM) was activated by MgATP (2 mM) and creatine (Cr, 20 mM). No effect of the addition of PK–PEP system on the respiration is seen, showing that the MgADP produced by MtCK is not accessible for this system. D. Respiration of trypsin (TR) treated cardiomyocytes in the presence of 20 mM Cr and 2 mM ATP. The oxygen uptake expressed in nmoL O_2/(min nmol cyt aa_3). Proteolytic treatment inhibited by addition of soybean trypsin inhibitor (STI, 0.02 mM) and BSA (5 mg/mL). Even after treatment of isolated cardiomyocytes with very low trypsin concentration (0.05 μM) ADP becomes accessible to the PEP–PK trapping system (PK 20 U/mL, PEP 5 mM). E. The effect of PK–PEP system on respiration of permeabilized cardiomyocytes which contained only about 50% of rod-like intact cells.

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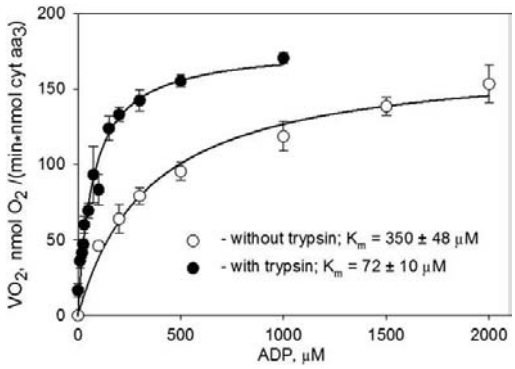

Fig. 8. Effect of trypsin on apparent Km for ADP of mitochondrial respiration in isolated permeabilized cardiomyocytes: respiration rates without (○) and with (●) trypsin treatment. Proteolytic treatment with trypsin resulted in an increase in the affinity of respiration for free ADP due to the proteolytic removal of βII-tubulin. Mean values and standard deviations for 9 experiments are shown.

has an important physiological function preventing from rapid saturation of ANT by this substrate and thus making possible the feedback metabolic regulation of mitochondrial respiration during workload changes [4,7,8,11,13]. Revealing the nature of interaction of tubulins with VDAC needs however further studies by using more selective methods than proteolysis.

Two decades ago two important observations were made almost simultaneously in the studies of cardiac cell bioenergetics. Using electron microscopy, Saetersdal et al. [42] have demonstrated in 1990 the presence of the immunogold anti-β-tubulin labeling at the outer mitochondrial membrane in cardiomyocytes, as well as in myofibers in close opposition to this membrane. This observation rested almost unnoticed and its importance unexplained for these two decades. In parallel, first Kummel in 1988 [16] and then many other investigators in different laboratories ([17–24], reviewed in Ref. [8,9]) discovered the differences in mitochondrial behavior *in vitro* and in permeabilized cardiomyocytes *in situ*: apparent Km for exogenous ADP in regulation of mitochondrial respiration was shown to be 20–30 times higher in the latter case than in isolated mitochondria [8,9]. Detailed investigation of this phenomenon in our laboratories led to conclusion that this phenomenon is related to the tight interactions between mitochondria and cytoskeleton in cardiac cells [22,23]. It was proposed that some components of cytoskeleton may control the permeability of the VDAC channel in the outer mitochondrial membrane in cardiac cells *in vivo*[22,23]. The results of the present and several other recent investigations confirm this suggestion and demonstrate directly that there is a specific isotype of tubulin-βII which is attached to the outer mitochondrial membrane and controls its permeability [14]. Mitochondrial βII-tubulin is co-expressed with MtCK and together with ATP Synthasome they were assumed to form a Mitochondrial Interactosome (MI), a key structure of the phosphotransfer pathway of energy transport into cytoplasm [14]. Evidently, this shows the important role of mitochondrial tubulin, discovered by Saetersdal et al. [42] in 1990.

Nevertheless, many questions still remain unanswered. Tubulin in non-polymerized form exists as αβ-heterodimer [43–45], and there are several isotypes of both subunits which differ mostly by the structure of C-terminal tail [43]. The questions that remain unanswered are: 1) why only βII-tubulin is associated with mitochondria; 2) which is the α isotype; 3) how they both interact with VDAC; and 4) what kind of other cytoskeletal proteins may be involved.

In this work we describe also the very simple and effective tests for investigation of the intactness of MI structure and function, energy fluxes from mitochondria into cytoplasm and functioning of MI which can be used as important quality controls for preparations of cardiac cells or myocardial fibers. Among other methods the cytochrome c test (Fig. 6) is first of them to be used for the detection of intactness of MOM in isolated mitochondria as well as in skinned fibers and permeabilized cardiomyocytes [17,18,34]. The loss of relatively weekly bound cytochrome c from MIM (as an important component of respiratory chain), especially at elevated ionic strength is accompanied by a significant decrease of the oxygen consumption and ATP synthesis [34]. Addition of saturating amount of exogenous cytochrome c to cytochrome c depleted mitochondria in cells or fibers in respiration medium results in restoration of the oxygen consumption and ATP synthesis from exogenous ADP, thus enabling to estimate the degree of damage and an amount of mitochondria with disrupted MOM. However, this effect does not allow estimating the state and quality of MI intactness, functioning and regulation of ATP/PCr synthesis. Inhibition of ANT by CAT [17,35] is another useful tool to check intactness of MIM, since increased rate in the residual oxygen consumption after inhibition by CAT is indicative for bypass of ADP–ATP and thus damage of MIM.

Some indication of functionally coupled MtCK could be observed from the creatine effect on the cellular respiration under conditions of externally added ADP, where creatine added to the experimental medium switches on the MtCK activity, resulting in a substantial decrease in Km (ADP) from values >300 μM down to 80–100 μM due to recycling of ADP in intermembrane space [19,24].

The use of the PK/PEP system is the most sensitive and comprehensive test for intactness of the whole MI system including the regulations at MOM. This simple and effective competitive enzyme method for studying the functional coupling phenomenon, namely the pathway of ADP movement from MtCK back to mitochondria or into the medium, was developed by Gellerich et al. and Guzun et al. [12,13,40,41]. These authors used an external PEP–PK system to trap ADP and thus to compete with ANT for this substrate. This competitive enzyme system was able to suppress 50% of Cr-stimulated respiration in isolated heart mitochondria, thereby showing the rather effective channeling of ADP from MtCK to the medium [12,40]. However, in permeabilized cardiomyocytes when MI is activated with 20 mMCr, PK/PEP system does not have any access to the intramitochondrial ADP and it is not affecting oxidative phosphorylation inside mitochondria and respiration rate. This protocol is excellent to elucidate the role of the mitochondrial outer membrane in the control of MI function, and foresaw many important functional aspects of the control of mitochondrial function *in vivo*. All these tests show that there is practically no measurable direct flux of ATP from mitochondria when MI is actively functioning. Direct transfer of ATP is observed under pathological conditions when the MOM is broken or tubulin lost from MI.

Any disturbances in MOM permeability regulation, including mild protease treatment, result in leakage of ADP and its competing trapping by the excessive PK/PEP and, finally, in a remarkable decrease of respiration. Only in the case of high cell quality (more than 95% intact rod like cells) cell respiration shows absence of the PK/PEP system effect.

The normal shape of the cardiac cells and mitochondrial arrangement are maintained by cytoskeletal structures, including tubulins, plectin, desmin and others [46–58]. In normal adult saponin-skinned fibers intermyofibrillar mitochondria retain their crystal-like pattern along with a relatively slow fluctuations around their position [32,33]. It has been supposed [33] that these fluctuations reflect the configurational changes of mitochondrial matrix between two classical condensed and orthodox

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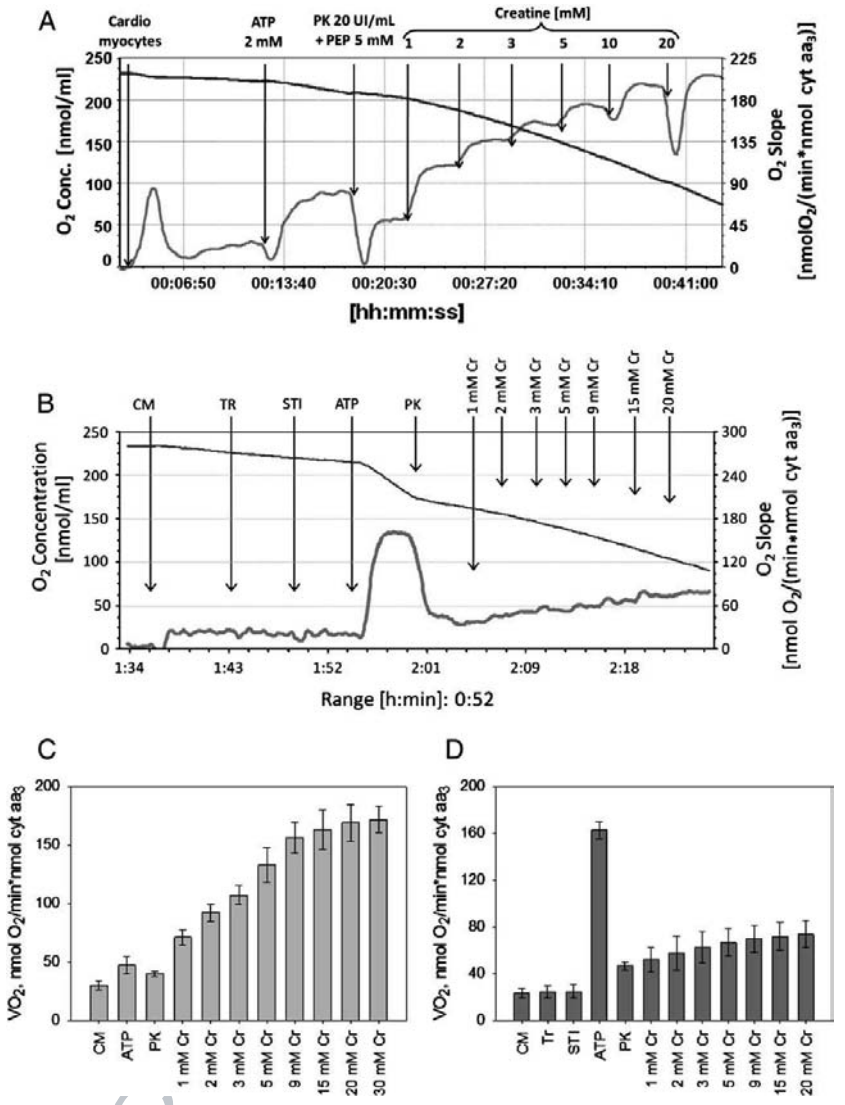

Fig. 9. Regulation of mitochondrial respiration by creatine (Cr) in the presence of activated MtCK, in cardiomyocytes (CM) non-treated and treated with trypsin. A. Cardiomyocytes were permeabilized by saponin and State 2 respiration recorded. MgATP was added to 5 mM final concentration to stimulate MgATPases, this increasing the respiration rate. This rate was decreased by addition of PK (20 U/mL) in the presence of PEP (5 mM). Subsequent addition of creatine rapidly increased the respiration rate up to maximal value. ADP produced by MtCK is not accessible for the PK–PEP system and is rapidly taken up by ANT into mitochondrial matrix. B. The same protocol after trypsin treatment: extramitochondrial ADP is more accessible to the PEP–PK reaction due to the proteolytic treatment, which destroys cytoskeletal proteins involved to the regulation of MOM. C. Mean values and standard errors for 7 experiments described in panel A. D. Mean values and standard errors for 7 experiments described in panel B.

states. Mitochondrial fusion and fission were not seen in adult intact cardiomyocytes [33]. This conclusion is confirmed by the results shown in Fig. 5D in this study.

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Teadustöö põhisuunad

- Integreeritud energeetilise metabolismi regulatsioonimehhanismid lihasrakkudes.
- Raku energiaühikute moodustumise dünaamika tüvirakkude diferentseerumise protsessis lihasrakkudeks ja nende degradatsioon vananemise käigus.

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