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REVIEW

Modular organization of cardiac energy metabolism: energy conversion, transfer and feedback regulation

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Abstract

To meet high cellular demands, the energy metabolism of cardiac muscles is organized by precise and coordinated functioning of intracellular energetic units (ICEUs). ICEUs represent structural and functional modules integrating multiple fluxes at sites of ATP generation in mitochondria and ATP utilization by myofibrillar, sarcoplasmic reticulum and sarcolemma ion-pump ATPases. The role of ICEUs is to enhance the efficiency of vectorial intracellular energy transfer and fine tuning of oxidative ATP synthesis maintaining stable metabolite levels to adjust to intracellular energy needs through the dynamic system of compartmentalized phosphoryl transfer networks. One of the key elements in regulation of energy flux distribution and feedback communication is the selective permeability of mitochondrial outer membrane (MOM) which represents a bottleneck in adenine nucleotide and other energy metabolite transfer and microcompartmentalization. Based on the experimental and theoretical (mathematical modelling) arguments, we describe regulation of mitochondrial ATP synthesis within ICEUs allowing heart workload to be linearly correlated with oxygen consumption ensuring conditions of metabolic stability, signal communication and synchronization. Particular attention was paid to the structure–function relationship in the development of ICEU, and the role of mitochondria interaction with cytoskeletal proteins, like tubulin, in the regulation of MOM permeability in response to energy metabolic signals providing regulation of mitochondrial respiration. Emphasis was given to the importance of creatine metabolism for the cardiac energy homoeostasis.

Keywords cardiac metabolism, creatine kinase, mitochondria, respiration regulation.

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The mechanisms underlying the regulation of cardiac energy metabolism continues to be controversial (Saks *et al.* 2006a,b,c, Balaban 2009, Cortassa *et al.* 2009, Liu & O'Rourke 2009). On the one hand, the observation is that cardiac oxygen consumption depends linearly on the cardiac workload (Starling & Visscher 1927) outlining that under physiological conditions, there is a strict relationship between oxidative ATP synthesis and utilization. On the other hand, intracellular ATP concentration does not change regardless of the increase in cardiac workload (Balaban *et al.* 1986) with ATP synthesis per day exceeding many times the heart mass itself (Saks *et al.* 2012). An explanation of this remarkable heart energy homoeostasis can be found in the subtle mechanisms of cardiac energy metabolic regulation, including intracellular metabolite channelling through coupled reactions, Ca^{2+}/Mg^{2+} and AMP signalling and metabolic microcompartmentalization to match oxidative phosphorylation (OxPhosph) to intracellular energy demand under conditions of metabolic stability (Balaban *et al.* 1986, Dzeja & Terzic 2003, Saks *et al.* 2006a,b,c).

Biochemical reaction systems in living cells represent thermodynamically open systems functioning in a non-equilibrium steady state (Saks *et al.* 2007a, 2009, De la Fuente *et al.* 2010). The breakdown of compounds through catabolism and build-up through anabolism (i.e. metabolism) are coupled to energy conversion with subsequent ATP hydrolysis to perform cellular work. The role of mitochondrial OxPhosph in free energy transformation in catabolic reactions is to keep a high value of the phosphorylation potential displacing from equilibrium the mass action ratio of ATP synthesis (Saks *et al.* 2009, Nicholls & Ferguson 2013). The whole system includes metabolic fuel transport and degradation pathways, fatty acid β-oxidation, tricarboxylic acid cycle, electron transport chain, phosphoryl transfer networks, molecular motors (ATPases), as well as feedback signalling functioning in non-equilibrium steady state. Thus, the system perfectly adapts ATP synthesis to ATP hydrolysis (Dzeja & Terzic 2003, Jørgensen *et al.* 2005, Qian 2006, De la Fuente *et al.* 2010, Ge & Qian 2013). The non-equilibrium steady state maintains constant concentrations of metabolites at fluxes and chemical potential gradients different from zero (Qian 2006). Therefore, maintaining ATP, ADP and inorganic phosphate (Pi) by phosphoryl transfer reactions in close vicinity to ATPases prevents their inhibition by ADP (Dzeja & Terzic 2003, Qian 2006, Ge & Qian 2013). The non-linear reaction kinetics coupled with molecular diffusion through the non-equilibrium biochemical reaction systems leads to the formation of self-organized wave patterns facilitating metabolic communication (Mair & Müller 1996, Dzeja & Terzic 2003, Qian 2006). These structures called as dissipative metabolic systems maintain low level of internal entropy (high level of organization) by energy and matter dissipation (Prigogine & Nicolis 1977, Schneider & Sagan 2005, De la Fuente *et al.* 2010).

The structure and functional organization of cardiac energy metabolism into intracellular energetic units (ICEUs) embodies the theory of dissipative metabolic systems and principles of energetic efficiency (Saks *et al.* 2006a,b,c, 2012). At sites such as myofibrillar, sarcoplasmic reticulum (SR) and sarcolemma ion-pump, ATPases are linked to mitochondrial ATP synthesis through stoichiometric phosphoryl transfer in metabolic networks (Dzeja & Terzic 2003, Saks *et al.* 2006a,b,c), while ionic signalling activates a number of metabolic enzymes and primes energetic system for anticipated energy usage surge (Saks *et al.* 2006a,b,c, Glancy & Balaban 2012). Therefore, ATP synthesis is governed by energy-demanding processes through metabolic and ionic feedback regulation (Saks *et al.* 2001, 2007a, 2012).

The concept involves maintenance of energy metabolism homoeostasis and stable levels of intracellular concentrations of ATP, ADP and phosphocreatine (PCr) during cardiac cycles (Williamson 1979, Balaban *et al.* 1986, Saks *et al.* 2006a,b,c). Stability of intracellular PCr, ATP and ADP levels during wide range of heart workload and respiration rate changes (Balaban *et al.* 1986, Balaban 2009) suggests that efficient energetic signal communication systems operate within ICEUs between ATP consumption and ATP synthesis sites with minimal concentration changes and gradient (Dzeja & Terzic 2003, Saks *et al.* 2006a,b,c).

The review aims to describe advances in understanding of regulation of mitochondrial ATP synthesis within ICEUs permitting heart workload to be linearly correlated with oxygen consumption and ATP synthesis under conditions of metabolic stability.

Structural basis of modular organization of cardiac energy metabolism. Adenine nucleotides compartmentalization and restricted intracellular diffusion

Mitochondrial organization, dynamics and interactions with cytoskeleton govern regulation of energy metabolism (Hudder *et al.* 2003). In adult cardiomyocytes, intermyofibrillar mitochondria are localized at the A-band level of sarcomeres and are at Z-lines separated from each other by T-tubuls in close connection, to the SR (Katz 1992, Hayashi *et al.* 2009). Figure 1 shows an adult primary cardiomyocyte with

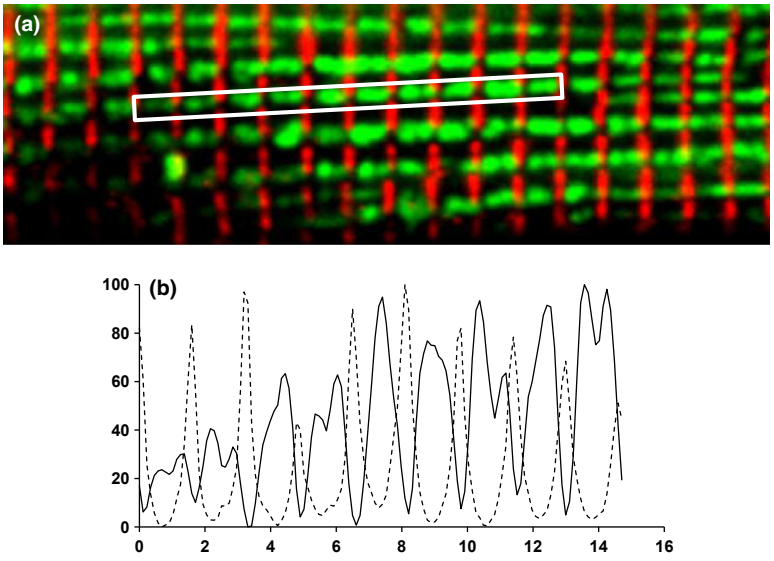

Figure 1 Mitochondria distribution in adult rat cardiomyocyte. (a) Individual mitochondria, visualized by green autofluorescence of flavoproteins, are localized at the A-band level of sarcomere. Immunofluorescent labelling of α-actinin (red colour) was used to mark sarcomeric Z-lines. (b) Fluorescence intensity plot shows peaks of mitochondrial flavoproteins intensities (dotted line) which correspond to regions of 'zero' intensity of α-actinin (solid line). Reproduced from Gonzalez-Granillo *et al.* (2012) with permission.

individual mitochondria localized at the A-band. The immunofluorescent labelling of α-actinin (red colour) was used to mark sarcomeric Z-lines (Gonzalez-Granillo *et al.* 2012). Estimation of mitochondrial centre distribution by probability density function demonstrated that in cardiomyocytes individual intermyofibrillar mitochondria are regularly arranged according to 'crystal-like' pattern (Vendelin *et al.* 2005). T-tubules, oriented longitudinally along myofibrils and transversely at the level of Z-line, potentially may supply each mitochondrion with oxygen (Soeller & Cannell 1999).

High temporal resolution of mitochondrial dynamics in adult cardiomyocytes revealed limited oscillations of mitochondrial fluorescence centres most probably due to conformational changes of the inner membrane and matrix volume rather than fusion-fission dynamics (Hackenbrock *et al.* 1986, Mannella 2006, Beraud *et al.* 2009). The changes of the mitochondrial matrix volume by about 10–15% can be associated with 40–50 mV changes in mitochondrial membrane potential ($\Delta\Psi_m$) and correspond to energetic/redox changes during State 4 – State 3 transitions (O'Reilly *et al.* 2003, Mannella 2006). Relatively fixed structural organization of intermyofibrillar mitochondria is a required condition for sarcomere contraction based on both, force generating displacement of myosin relative to actin and spatial rearrangement of other cytoskeleton proteins providing passive elasticity to cardiac muscle (Fukuda & Granzier 2005). Additionally, 'dyadic clefts' between T-tubules and SR shape calcium-induced calcium-release Units of compartmentalized Ca^{2+} 'sparks' (O'Rourke *et al.* 2005, Bers & Despa 2006, Hayashi *et al.* 2009, Liu & O'Rourke 2009, Soeller *et al.* 2009, Kembro *et al.* 2013).

Embedment of mitochondria into the cytoskeleton, their intimate localization relative to other membranous elements and enzymatic associations with structural proteins create mechanical barriers for diffusion of various metabolites, forming intracellular microcompartments (Saks *et al.* 1995). Kinetic analysis of respiration regulation by ADP revealed the dependence of the apparent affinity of mitochondrial respiration for ADP on cells structure. The apparent Michaelis constant for ADP (app. K_mADP), which inversely represents the apparent affinity of respiration for ADP, is of about 370.8 ± 30.6 μM in permeabilized adult cardiomyocytes and about 7.9 ± 1.6 μM in isolated heart mitochondria (the Michaelis–Menten representation of ADP-stimulated respiration can be seen in Fig. 8b, Saks *et al.* 2012). Trypsin proteolysis of cardiomyocytes decreased the apparent K_m for ADP up to the level characteristic for isolated heart mitochondria due to the disruption of orgelles interaction with cytoskeleton and mitochondria disorganization (Kuznetsov *et al.* 1996). The apparent affinity of mitochondrial respiration for ADP also strongly depends on its source. The respiration rate of permeabilized adult cardiomyocytes achieved maximum value (V_{max}) at <20 μM of endogenous ADP, while millimolar concentration of the exogenous ADP was needed to obtain the same effect (Saks *et al.* 2001). For this experiment, endogenous ADP was produced by intracellular ATPases during hydrolysis of exogenous ATP. Higher apparent affinity of respiration for endogenous ADP in permeabilized cardiomyocytes was explained by its direct channelling associated with the restricted diffusion and intracellular compartmentalization (Saks *et al.* 2001). This assumption was confirmed by the experiment using ADP-trapping system, consisting of pyruvate kinase (PK) and phosphoenolpyruvate (PEP),

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which competes with mitochondria for ADP (Gellerich & Saks 1982). The phosphoenolpyruvate–pyruvate kinase system completely suppressed the respiration stimulated by exogenous ADP and inhibited not more than by about 20% of respiration stimulated by endogenous ADP (Saks *et al.* 2001, 2003, 2008). Abraham *et al.* (2002) have been shown that the diffusion of ATP is also restricted in cardiac cells. Studying kinetics of phosphoryl exchange through the creatine kinase (CK) and adenylate kinase (AK) reactions by ^{31}P magnetic resonance spectroscopy (^{31}P MRS), Nabuurs *et al.* (2010) demonstrated that in muscle cells, ATP and even more ADP are bound to slowly rotating macromolecules or cytoskeletal proteins and are only transiently present as free ATP or ADP in the cytosol. In *in vivo* studies of ATP and PCr diffusion in rat skeletal muscles using ^{31}P MRS confirmed diffusional anisotropy induced by subcellular barriers (de Graaf *et al.* 2000). These and other studies have contributed to the formulation of hypothesis about modular organization of cardiac energy metabolism assuming that mitochondria, consuming ADP and re-sythesisyng ATP, are integrated with adjacent ADP-producing sites (ATPases of myofbrils and ion pumps) by means of proteins associated to cytoskeleton which contribute to mitochondrial organisation and intracellular energy flux transfer (Saks *et al.* 2001).

Compartmentalized energy transfer

Two theories were proposed to explain how metabolites overcome intracellular diffusion limitations: the theory of vectorial ligand conduction proposed by Mitchell (1979), and flux transfer in non-equilibrium steady state (Goldbeter & Nicolis 1976, Dzeja & Terzic 2003, Qian 2006, De la Fuente *et al.* 2010, Ge & Qian 2013). According to these theories, compartmentalized metabolic processes are integrated by metabolic channelling via enzymatic complexes which can associate physically with cytoskeleton creating metabolic pathways (Ovádi & Srere 2000, Huang *et al.* 2001). This kind of communication was named compartmentalized energy transfer flux (Saks & Ventura-Clapier 1994, Hudder *et al.* 2003, Ovádi & Saks 2004, Saks *et al.* 2008). High-energy phosphoryl flux between ATP-consuming and ATP-generating sites is transmitted by the driving force created by local disequilibrium in sequential rapidly equilibrating reactions catalysed by highly compartmentalized CK and AK iso-enzymes (Bessman & Carpenter 1985, Wallimann *et al.* 1992, Saks *et al.* 1994, Zeleznikar *et al.* 1995, Tuckerman *et al.* 2002, Dzeja & Terzic 2003, Aliev *et al.* 2011). It is known that flux wave propagation along coupled and rapid equilibrating chemical and biological reaction proceed much faster than diffusion of reactants and is capable of operating with minimal or no concentration gradients (Goldbeter & Nicolis 1976, Mair & Müller 1996, Dzeja *et al.* 1998, Dzeja & Terzic 2003). This explains why changes in cellular adenine nucleotide, which are intermediates in tightly coupled reactions, are not observed even with marked increases in metabolic flux (Balaban *et al.* 1986, Zeleznikar *et al.* 1995, Saks *et al.* 2006a,b,c).

Significant new insights regarding dynamics of muscle bioenergetics have been obtained from *in situ* measurements of intracellular energy fluxes by ^{18}O isotope-assisted ^{31}P MRS developed by Nelson Goldberg at University of Minnesota and Dzeja group at Mayo Clinic, Rochester, MN (Zeleznikar *et al.* 1995, Dzeja *et al.* 1998, Pucar *et al.* 2001, Dzeja & Terzic 2003). Cellular ATP hydrolysis in the presence of ^{18}O-labelled water results in the incorporation of ^{18}O into phosphoryl groups which movement through various phosphoryl transfer networks is detected by ^{31}P MRS. Using this technique, authors demonstrated that in normal heart about 80–88% of the intracellular energy flux is carried by PCr through the CK reaction, about 15% via AK reaction and remaining 5–7% via glycolysis (Pucar *et al.* 2001, Dzeja *et al.* 2011a, Nemutlu *et al.* 2012).

Creatine kinase catalyses the reversible reaction of adenine nucleotides transphosphorylation, the forward reaction of PCr and MgADP synthesis and the reverse reaction of creatine (Cr) and MgATP production (Wallimann *et al.* 1992, 2007, 2011, Schlattner & Wallimann 2004, Schlattner *et al.* 2006). Mitochondrial CK (MtCK, sarcomeric and ubiquitous isoforms) is an octameric protein situated in the contact sites of two mitochondrial boundary membranes, neighbouring voltage-depending anion channel (VDAC) at the outer membrane and adenine nucleotide translocase (ANT) at the inner membrane. In the mitochondrial inner membrane (MIM), positively charged MtCK is bound to negatively charged cardiolipin. MtCK shares the same cardiolipin patches with ANT (Schlattner & Wallimann 2004, Schlattner *et al.* 2006, Wallimann *et al.* 2011). Due to this close localization, MtCK is functionally coupled to ANT and catalyses the transfer of phosphoryl groups from ATP generated by OxPhosph with PCr production. PCr is used by cytoplasmic isoform of CK (MMCK) localized in myofibrils at M- and I-band of sarcomeres and in SR (Wallimann & Eppenberger 1985, Kraft *et al.* 2000). MMCK rephosphorylates ADP released from active centre of myosin ATPase to ATP used within contraction cycle. MMCK is associated also with sarcolemmal and SR membranes to regenerate continuously the local pools of ATP for membrane ATPases and for regulation of metabolic sensor – ATP-dependent K-channel in sarcolemma (Alekseev *et al.* 2012, Saks

et al. 2012). Thus, energetic communication between mitochondria and sites of ATP utilization proceeds through compartmentalized energy transfer mediated mostly by CK and also AK and glycolytic enzyme chains associated with mitochondria, myofibrils, nucleus and sarcolemma to provide energetic continuum throughout the cell. It also allows to avoid accumulation of MgADP close to ATPases. MgADP is an efficient competitive inhibitor of ATPases (myofibrillar, SERCA and sarcolemmal ion-pumps) and increased intracellular MgADP may reduce the rate of crossbridge detachment masking the length-dependent activation and Frank–Starling mechanism (Yamashita *et al.* 1994, Fukuda *et al.* 2000, Saks *et al.* 2006a,b, c). Due to the high affinity of MMCK for MgADP (app. K_m for ADP is about 10–35 μM), the ATP regeneration capacity of MMCK is very high preventing MgADP accumulation even when energy utilization exceeds energy production (Wallimann *et al.* 1992).

Modular organization of cardiac energy metabolism into ICEUs

Both, regularly arranged distinct mitochondria, integrated into a microdomain of energy producing/consuming cellular complexes, and compartmentalized energy phosphoryl transfer are met by the conditions of modular organisation of cardiac energy metabolism into ICEUs (Fig. 2). ICEU contains sites of ATP hydrolysis such as myofibrillar, SR, sarcolemmal, ion-pump ATPases which use mitochondrially synthesized ATP delivered through phosphotransfer networks (Fig. 2) (Saks *et al.* 1998, 2006a,b,c, 2007a,b, 2012, Kaasik *et al.* 2001). ICEUs are connected with each other; individual mitochondria or several adjacent mitochondria in cardiomyocytes can be taken to be in the centre of its own ICEU (Saks *et al.* 2009). Therefore, they may function as a coordinated network connected by messengers such as various metabolites or reactive oxygen species (Aon *et al.* 2003, 2007, O'Rourke *et al.* 2005, Liu & O'Rourke 2009, Aon & Cortassa 2012, Kembro *et al.* 2013). In spite of synchronized functioning, damage of one individual mitochondrion (e.g., depolarization by laser photoactivation) does not affect $\Delta\Psi_m$ of neighbouring mitochondria or the total energy state of the sum of cardiomyocytes (Aon *et al.* 2003, 2007, Saks *et al.* 2012). ICEUs are integrated with calcium-induced calcium-release units, forming a system of intracellular compartmentalized calcium and energy transfer to support electromechanical coupling of cardiac muscle contraction. (O'Rourke *et al.* 2005, Bers & Despa 2006, Rizzuto & Pozzan 2006, Maack & O'Rourke 2007, Gunter & Sheu 2009, Hayashi *et al.* 2009, Liu & O'Rourke 2009, Saks *et al.* 2012, Kembro *et al.* 2013). The disruption of ICEUs due to the fusion of individual mitochondrion into one reticulum or fragmentation with formation of mitochondria clusters will defeat the integrated organization of cardiac energy metabolism altering mitochondrial energy conversion, intracellular distribution of energy fluxes and controlling signals as well as Ca^{2+} transients (Scorrano 2013, Varikmaa *et al.* 2014). Alterations in mitochondrial morphology, frequently associated with modifications of sarcomer structures and energy metabolism, have been implicated in different heart pathologies such as ischaemia-reperfusion and heart failure (Chen *et al.* 2009, 2012, Ong *et al.* 2010, Dorn 2013). Active mitochondrial fusion–fission dynamic have been seen in embryonic cardiac development and in cardiac differentiation of stem cells (Chung *et al.* 2013). However, fusion–fission cannot be excluded in perinuclear mitochondrial clusters and may occur between two contiguous intermyofibrillar mitochondria in adult cardiomyocytes (Kuznetsov *et al.* 2009).

Mitochondria, as proposed by Aon *et al.* (2007), Aon & Cortassa (2012), function as metabolic 'hubs' for multiple catabolic and anabolic pathways. Figure 2 schematically represents ICEU in which free fatty acids and carbohydrates are taken up by the cell and oxidized via the Krebs cycle to CO_2, paralleled by the production of NADH and $FADH_2$. These substrates are then oxidized in the respiratory chain reactions producing electrons to reduce O_2 and pumping protons across the inner membrane to create the electrochemical potential. This potential is used by ATP synthase to regenerate ATP. Then, coupled mitochondrial CK catalyses the direct transphosphorylation of ATP to Cr, thus producing PCr. The ATPase reactions release the free energy of ATP hydrolysis to perform the cellular work. If workload increases, ATP production and respiration are increased due to the feedback regulation via the CK-system (Pucar *et al.* 2001, Ingwall & Weiss 2004, Ventura-Clapier *et al.* 2004, Ingwall 2006, Saks *et al.* 2006a,b,c, 2012). In the next part of this article, our interest will be focused in more detail on the mechanisms of this regulation.

Regulation of mitochondrial respiration in permeabilized adult cardiomyocytes

The role of MtCK-ANT functional coupling in respiration regulation in mitochondria in situ *in permeabilized* adult *cardiomyocytes*

The functional coupling of MtCK with ATP synthesis via ANT is a key element in the transfer of energy from mitochondrial ATP to PCr, which represents the basis for intracellular energy transport via CK/PCr shuttle (Wallimann *et al.* 1992, 2007, 2011,

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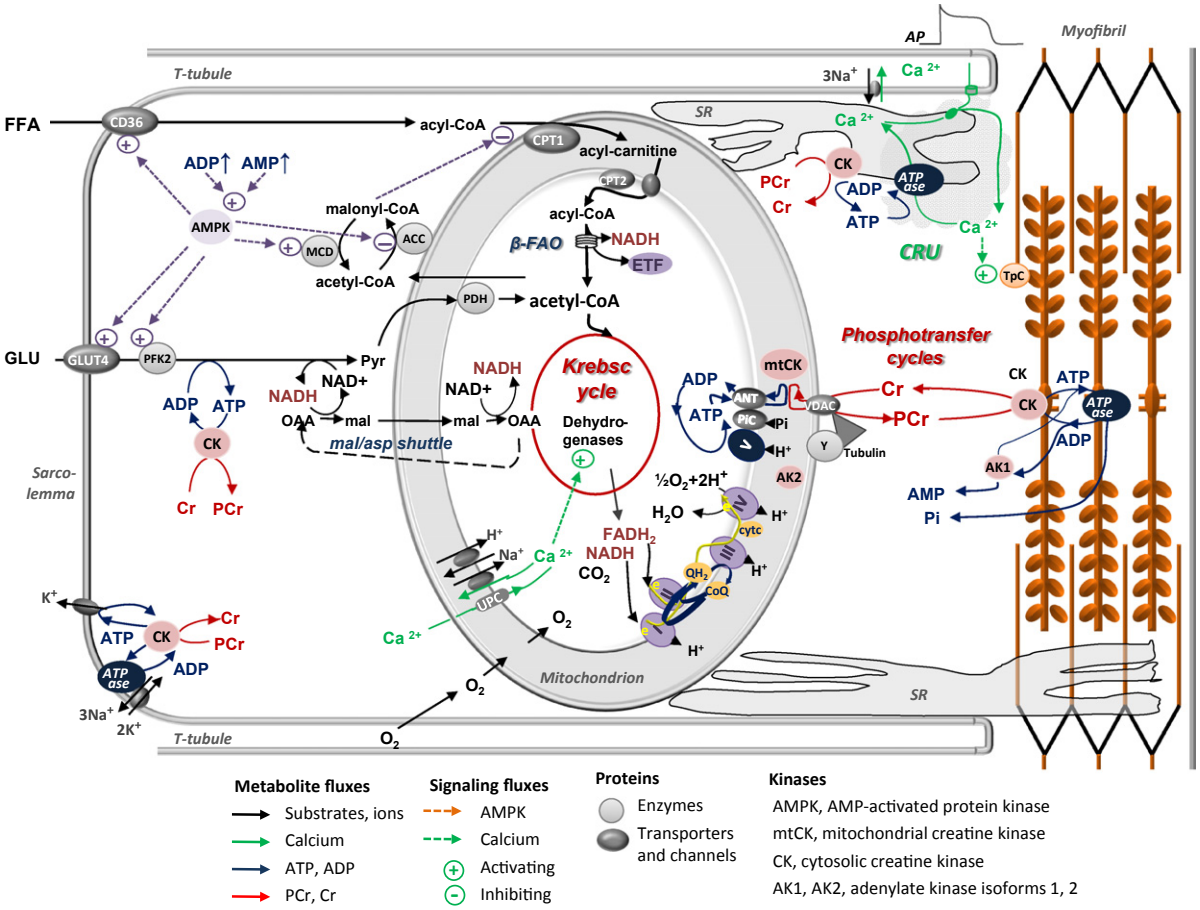

Figure 2 Intracellular energetic units (ICEUs). ICEUs include sites of ATP hydrolysis (myofibrillar ATPases, sarcoplasmic reticulum ATPase, ion-pump ATPase) which are relied to the mitochondrial ATP synthesis through the creatine kinase (CK)/PCr network. Metabolic or randle cycles include free fatty acids (FFA) and glucose (GLU) transport and degradation. FFA is taken up by a family of plasma membrane proteins (fatty acid transporter protein, FATP1, fatty acid translocase, CD36) and esterified to acyl-CoA via fatty acyl-CoA synthetase. The resulting acyl-CoA is then transported into mitochondria via carnitine palmitolyltransferase I (CPT and CPT II). Once inside, acyl-CoA becomes a substrate for the *β*-oxidation pathway (*β*-FAO), resulting in the production of one molecule of NADH, one molecule of $FADH_2$, and one molecule of Acetyl-CoA. Acetyl-CoA enters the TCA cycle, where it is further oxidized to CO_2 with the concomitant generation of three molecules of NADH, one molecule of $FADH_2$ and one molecule of ATP. GLU is taken up by glucose transporter-4 (GLUT4) and enters the glycolysis pathway, which converts glucose into two molecules of pyruvate (PYR), two net ATP and two NADH. NADH is transferred into mitochondria via the malate–aspartate shuttle (mal/asp shuttle). OAA, oxaloacetate. Pyruvate enters into the Krebs cycle and oxidative phosphorylation (OxPhosph) via Acetyl-CoA. NADH and $FADH_2$ issued from both metabolic pathways are oxidized in the respiratory chain. Mitochondrial creatine kinase (MtCK) catalyses the direct transphosphorylation of intramitochondrial ATP and cytosolic creatine (Cr) into ADP and phosphocreatine (PCr). ADP enters the matrix space to stimulate OxPhosph, while PCr is transferred via the cytosolic Cr/PCr shuttle to be used in the functional coupling between CK and ATPases (acto-myosin ATPase and ion pumps). Feedback regulation of mitochondrial ATP synthesis is performed by Cr/Pc, ADP, Pi ratios. Cell signaling via AMP kinase (AMPK) provides a parallel control of most of these processes, including substrate uptake via fatty acid and glucose transporters and flux via *β*-FAO and glycolysis. Reproduced from Saks *et al.* (2014) with permission.

Schlattner & Wallimann 2004, Schlattner *et al.* 2006). Comparative kinetic analysis of MtCK reaction in isolated heart mitochondria revealed that under conditions of activated OxPhosph, the MtCK reaction is strongly shifted to the direction of PCr synthesis using all mitochondrial ATP for PCr production (Saks *et al.* 1985, 2007a). The apparent constants of dissociation of MgATP from binary (MtCK.MgATP) and ternary (MtCK.Cr.MgATP) enzyme–substrate complexes (Kia and Ka, respectively, Fig. 3c) were decreased when OxPhosph of isolated heart mitochondria was activated (Table 1) (Jacobus & Saks 1982, Saks *et al.* 1985, 2007a). This decrease was explained by the direct transfer of ATP from ANT to MtCK due to their spatial proximity and functional coupling (Aliev & Saks 1993, Metelkin *et al.* 2006). A similar

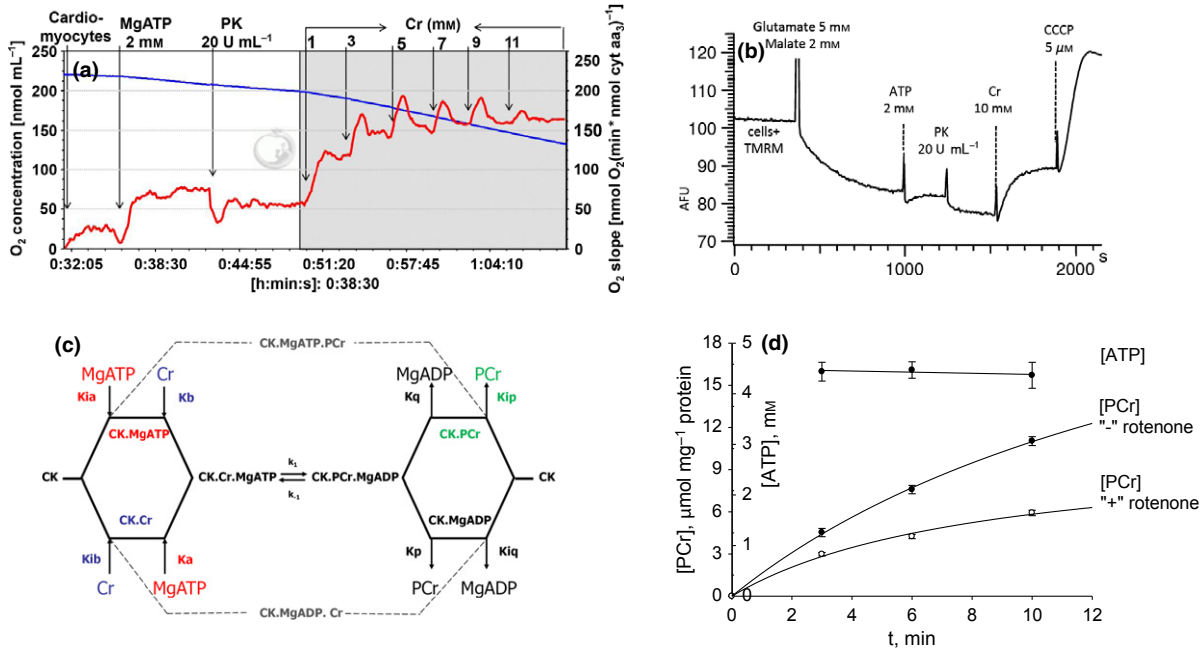

Figure 3 Kinetic properties of mitochondrial creatine kinase (MtCK) *in situ* in permeabilized adult primary cardiomyocytes. (a) The experimental procedure used for the complete kinetic analysis of MtCK in permeabilized adult primary cardiomyocyte. The left scale and the blue trace indicate the oxygen concentration (nmolO_2 mL^{-1}). The right scale and the red trace show the rate of oxygen uptake expressed in nmolO_2 min^{-1} $nmol^{-1}$ cyt. aa3. The experiment was carried out in solution containing 5 mM glutamate/2 mM malate as respiratory substrates. First, the respiration is activated by addition of MgATP-inducing production of endogenous ADP in MgATPase reaction. Then, phosphoenolpyruvate–pyruvate kinase (PEP–PK) system is added to trap all extramitochondrial free ADP. This decreases the respiration rate, but not to initial level, due to structural organization of intracellular energetic unit (ICEU). Mitochondria are in privileged position to trap some of endogenous ADP. Addition of creatine activates MtCK reaction. The oxidative phosphorylation (OxPhosph) is stimulated mostly by intramitochondrial ADP, produced by MtCK, which is not accessible for PEP–PK. (b) Measurement of mitochondrial membrane potential ($\Delta\Psi_m$) using the TMRM flurescence was applied to show the control that Cr exerts on the respiration. TMRM is a positively charged lipophyle fluorescent probe that enters into negatively charged matrix when the inner membrane is energized. Incubation of permeabilized adult cardiomyocytes with TMRM gives the detectable level of its fluorescence outside the mitochondria. Respiratory substrates induce membrane polarization corresponding to state 2 of respiration according to Chance. The addition of ATP induced small change in mitochondrial membrane potential. The remove of ADP by PEP–PK system induces state 4 of respiration and complete membrane polarisation. Cr strongly increases respiration corresponding to state 3 and rapid decrease in $\Delta\Psi_m$ due to the phosphorylation of ADP produced in activated MtCK reaction and transferred into the matrix due to MtCK/adenine nucleotide translocase (ANT) functional coupling. (c) Kinetic mechanism of MtCK reaction (bi-bi, random quasi-equilibrium type according to Cleland classification). Scheme shows the interconversion of productive ternary enzyme–substrate (CK.Cr.MgATP) and enzyme–product (CK.PCr.MgADP) complexes in the presence of $MgATP^{2-}$, $MgADP^{-}$, Cr and phosphocreatine (PCr). Every substrate and products is characterized by two constants of dissociation. (d) Measurement of ATP and PCr production during the stepwise addition of Cr (a) using ion pair HPLC/UPLC. Experiments were performed under conditions of activated (full circles) and inhibited (empty circles) complex I of the respiratory chain. The ATP level was stable during the experiment, while PCr production continuously increased. Adapted from Guzun *et al.* (2009) and Timohhina *et al.* (2009).

mechanism accounts for the reversed direct transfer of ADP produced by the MtCK reaction via ANT back to ATP synthase for rephosphorylation, as described later.

The apparent kinetics of MtCK in mitochondria *in situ* in permeabilized cardiomyocytes is totally different from that of in mitochondria *in vitro* (Table 1, Fig. 3a,c). The apparent constants of dissociation of MgATP from binary and, especially, ternary MtCK-substrate complexes are increased many times in mitochondria *in situ* in comparison to that *in vitro* (Table 1) (Guzun *et al.* 2009). This reflects the decrease in the apparent affinity of MtCK, situated behind mitochondrial outer membrane (MOM), for exogenous MgATP and indicates the enhanced restriction of exogenous MgATP diffusion at the level of MOM and within organized structures of ICEUs. The decrease in the apparent constants of dissociation of Cr from MtCK–substrate complexes suggests an increase in the apparent affinity of MtCK for Cr in

Table 1 Comparative kinetic analysis of mitochondrial creatine kinase in isolated mitochondria with and without activated oxidative phosphorylation (OxPhosph) and *in situ* in permeabilized cardiomyocytes

	K_{ia}MgATP (mM)	K_aMgATP (mM)	K_{ib}Cr (mM)	K_bCr (mM)	K_{ip}PCr (mM)	V_{max} ($nmolO_2$ min^{-1} nmol cyt aa_3^{-1})
Mitoch. *in vitro* − OxPhosph	0.92 ± 0.09	0.14 ± 0.02	29.4 ± 12	5.2 ± 2.3		187.9±40.7
Mitoch. *in vitro* + OxPhosph	0.44 ± 0.08	0.016 ± 0.01	28 ± 7	5 ± 12	0.84 ± 0.2	
Mitoch. *in situ, perm. cardiomyocytes*	1.94 ± 0.86	2.04 ± 0.14	2.12 ± 0.2	2.17 ± 0.4	0.89 ± 0.2	178.2 ± 33.9

PCr, phosphocreatine.
Values were summarized from Jacobus and Saks (1982), Saks *et al.* (1985, 2007a), and Guzun *et al.* (2009).

mitochondria *in situ* in comparison with *in vitro* (Table 1). The apparent constant of dissociation of PCr, however, did not change (Table 1). Increased affinity of MtCK for Cr and an unchanged affinity for PCr reveal their free diffusion through MOM in permeabilized cardiomyocytes (Guzun *et al.* 2009, Timohhina *et al.* 2009, Saks *et al.* 2012).

Measurement of phospho-metabolite concentrations (reflecting energy fluxes from mitochondria) in parallel with kinetic analysis of MtCK in permeabilized cardiomyocytes showed a continuous increase of PCr production in the presence of stable level of ATP (Fig. 3d) (Timohhina *et al.* 2009). The PCr production to oxygen consumption (V_{PCr}/V_{O2}) ratio was close to the theoretical ATP/O_2 ratio for cardiac cells *in vivo*. The V_{PCr}/V_{O2} of Cr-stimulated respiration was about 5.7, while the theoretical efficiency of OxPhosph (P/O_2) is 6 (Timohhina *et al.* 2009). Thus, Cr is an efficient feedback regulator of respiration in permeabilized cardiomyocytes, as well as in skeletal oxidative *m. soleus* and mixed human *m. vastus lateralis* (Guzun & Saks 2010). Low apparent affinity of MtCK for Cr in these muscles (the apparent K_mCr is about 1.0–1.5 mM) shows that low concentrations of Cr are capable to significantly increase respiration rates up to the maximal rate of ADP-stimulated respiration (Guzun *et al.* 2011, Saks *et al.* 2012, Varikmaa *et al.* 2014).

The ADP recycling in functionally coupled MtCK reaction was studied in permeabilized cardiomyocytes using tools of Metabolic Control Analysis (Tepp *et al.* 2011). Metabolic Control Analysis allows quantitative determination of the degree of control that a given enzyme exerts on specific metabolic flux (Moreno-Sánchez *et al.* 2008). Control coefficient of ANT and ATP synthase on the energy flux increased when respiration was activated with substrates of the CK (Cr and ATP in the absence of extramitochondrial ADP) in comparison with ADP-stimulated respiration (Tepp *et al.* 2011). The sum of flux control coefficients of the respiratory chain complexes, ATP synthase, inorganic phosphate carrier (PiC), MtCK and ANT larger than one can be explained by the local ADP recycling in coupled MtCK reaction and channelling of substrates through the macromolecular supercomplex (Kholodenko & Westerhoff 1993, Saks *et al.* 1996, Tepp *et al.* 2011).

Functional coupling of MtCK with ANT amplifies intramitochondrial adenine nucleotides turnover maintaining high rates of OxPhosph and coupled PCr production in the presence of Cr under conditions of limited MOM permeability enhancing adenine nucleotides microcompartmentalization (Saks *et al.* 2007a, 2012, 2014).

The role of mitochondria–cytoskeleton interactions in the regulation of mitochondrial respiration in situ *in permeabilized cardiomyocytes*

It is known that the selective permeability of VDAC depends on many factors, among them are the cell-specific pattern of VDAC isoforms; VDAC interaction with different proteins (tubulin, hexokinase II (HKII), macrotubule-associated proteins, plectin, desmin …); cell-specific patterns of intracellular proteins capable to interact with VDAC and their functional state (polymerization state or post-translational modifications); biophysical properties of the channel itself, the kind of molecules passing through the channel and MOM phospholipid composition.

Several cytoskeletal proteins were tested for their ability to connect to MOM and control mitochondrial metabolism via controlling MOM permeability. For example, such a role has been suggested for desmin or for the 1b isotype of plectin that may interact with VDAC at MOM in cardiomyocyte mitochondria (Capetanaki 2002, Carré *et al.* 2002, Schröder *et al.* 2002, Capetanaki *et al.* 2007). Our attention was focussed on the β-tubulin cytoskeletal protein with its known structural, transport and metabolic functions (Sackett 2010). Using immunogold labelling of total β-tubulin, the presence of this protein at the

mitochondrial surface in cardiomyocytes, between MOM and myofibrils, SR and sarcolemma membranes has been shown (Saetersdal *et al.* 1990), for example, a study of the distribution of β-tubulins by fluorescence confocal microscopy showed that βII-tubulin is codistributed with mitochondria in adult cardiomyocytes and permeabilized myocardial fibres (Fig. 4) (Guzun *et al.* 2011, Gonzalez-Granillo *et al.* 2012, Saks *et al.* 2012). Hetero-dimeric $\alpha\beta$-tubulin may induce *in vitro* a reversible closure of purified VDAC protein reconstituted into lipid monolayers (Rostovtseva *et al.* 2008). Notably, the addition of hetero-dimeric $\alpha\beta$-tubulin to isolated heart mitochondria significantly increased the apparent K_m(ADP) up to the value characteristic to that of mitochondria *in situ* in permeabilized cardiomyocytes (Monge *et al.* 2008).

Tubulin can create a heterodimeric complex formed by two globular and two C-terminal tails (CTT) of α- and β-proteins. Globular α- and β-proteins can be polymerized into microtubules, while α- and β-CTT can interact with other intracellular structures and proteins. Truncated tubulin without CTT did not induce reversible blockage of purified VDAC typical for tubulin (Rostovtseva 2010). The CTTs of different α- and β-tubulin isotypes have almost similar length (from 10 to 20 aminoacid residues) and electronegative charges (from about −7 to −9) suggesting the possibility of reciprocal interaction with VDAC (Sackett 2010). Taking into account that anti-βII-tubulin antibody oriented against CTT find its epitop and is visible in confocal fluorescent microscopy, we assumed that βII-tubulin binds to the VDAC protein binding site situated on the cytosolic face according to Colombini functional conformation of VDAC, rather than penetrate into the VDAC channel (Colombini 2009, Rostovtseva 2010). The CTT of α-tubulin itself, however, can slip into the channel as it is described by Rostovtseva (Rostovtseva *et al.* 2008, Rostovtseva 2010). The role of α-tubulin isotypes in the regulation of mitochondrial metabolism is still not fully understood. Therefore, future studies of the intracellular distribution of α-tubulin isotypes could help to understand their function relative to VDAC and βII-tubulin CTT binding. It is also conceivable that tubulin could increase VDAC affinity for other regulatory proteins that in our scheme are called 'linker protein'.

Importantly, both colchicin treatment inducing microtubules depolymerization, and treatment with 0.8 M KCl used to remove myosin from cardiac muscle fibres ('ghost' fibres'), did not dissociate tubulin from its colocalization with mitochondria and did not change significantly the apparent affinity of respiration

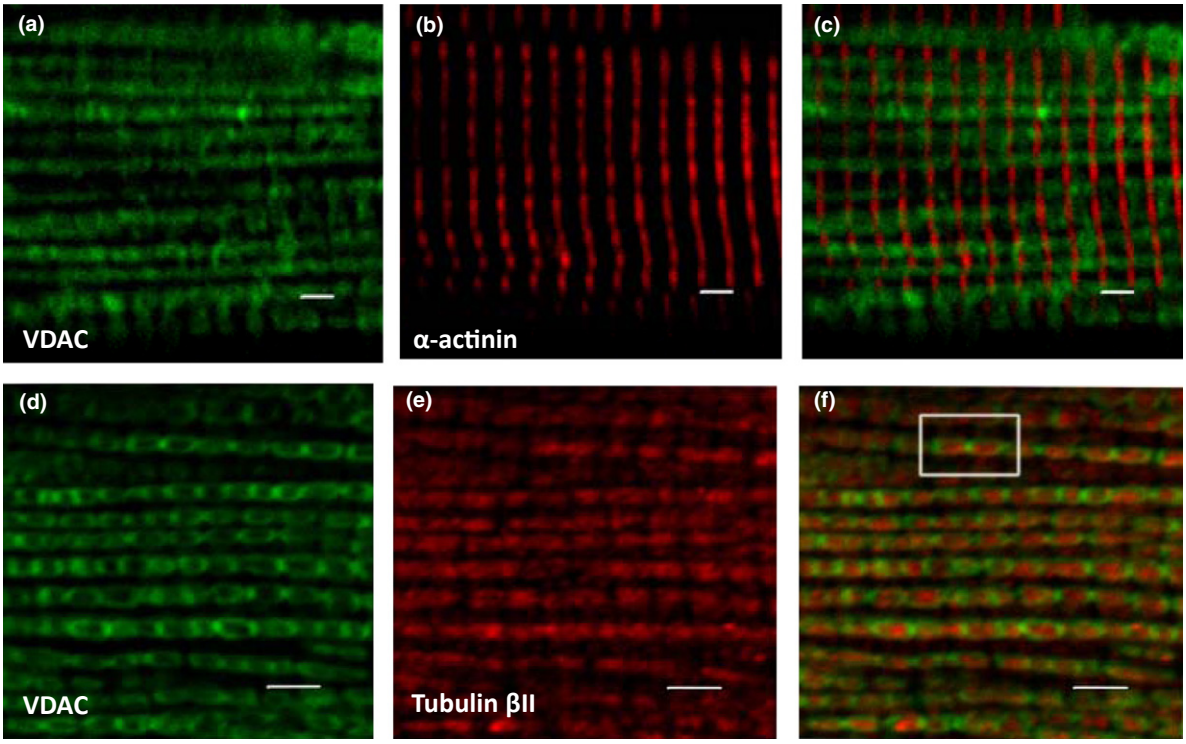

Figure 4 Confocal microscopy images of mitochondria [coimmunolabelled for voltage-depending anion channel (a, d)], α-actinin (b) and βII-tubulin (e) arrangement in adult cardiac muscle fibres. Images (c, f) show that both mitochondria and βII-tubulin are arranged regularly between Z-lines. Scale bar 2 μm. Reproduced from Varikmaa *et al.* (2014) with permission.

for ADP. The apparent K_m(ADP) of 'ghost' cardiac fibres was found to be close to intact permeabilized fibres and was about 280 μM (Guerrero *et al.* 2010). These findings indicate that the strong and intimate connection between MOM and tubulin is, most probably, due to the interaction with free dimeric tubulin. In adult cardiomyocytes, about 30% of tubulin is polymerized and 70% are in the heterodimeric state (Tagawa *et al.* 1998). These two conformational states of tubulin protein are in a dynamic balance driven by polymerization–depolymerization processes (Sackett 2010). Conversely, in cancer hepatoma cells, colchicin induces a decrease in the mitochondrial membrane potential (Maldonado *et al.* 2010). We assume that this essential difference with cardiac cells can be explained by the difference in regulation of energy metabolism. Intracellular energy transfer in hepatoma cells is directly carried out by ATP instead of PCr. This type of cells, as well as adult hepatocytes, does not contain MtCK (Fontaine *et al.* 1995). The restriction of ADP/ATP diffusion through VDAC, induced by depolymerized tubulin, is harmful for cells with mitochondrial OxPhosph stimulated notably by cytosolic ADP and missing (or insufficiently represented) alternative phosphoryl transfer networks (such as CK/PCr). Therefore, VDAC blockage, limiting ATP diffusion, results in the loss of mitochondrial membrane potential of hepatoma cells.

Differences in MOM permeability for ADP could also originate from the distinct expression patterns of VDAC isoforms. Striated muscles express three isoforms of VDAC (De Pinto *et al.* 2010). The decrease in the apparent affinity for ADP in permeabilized cardiac muscle fibres of VDAC1$^{-/-}$ mice and VDAC3$^{-/-}$ mice indicates the possible role of VDAC2 in the restriction of adenine nucleotides diffusion (Anflous *et al.* 2001, Anflous-Pharayra *et al.* 2007, 2011). VDAC2 isoform is mainly expressed in the heart of wild-type murins and its deletion is embryologically lethal (Anflous *et al.* 2001, Anflous-Pharayra *et al.* 2011). The interaction of microtubule-associated protein with VDAC2 (Lindén *et al.* 1989) reinforces our belief that βII-tubulin can bind to VDAC2 to regulate its permeability for adenine nucleotides. VDAC1 and VDAC3 are permeable to ATP/ADP, and this could be linked to the control by HKII (Anflous-Pharayra *et al.* 2007, Maldonado *et al.* 2010). VDAC1/3 null cells do not contain HKII bound to VDAC (Chiara *et al.* 2008). However, more studies are needed to address the specificity of VDAC interactions with proteins and to clarify mechanism of regulation of the VDAC-selective permeability using genetic manipulations by silencing βII-tubulin in adult cardiomyocytes or transfecting βII-tubulin into cells lacking this tubulin isotype.

Observations of adenine nucleotides microcompartmentalization and functional coupling of MtCK with ANT and limited MOM permeability for ADP demonstrate the important role of MtCK in the communication between mitochondria and intracellular energy-demanding processes (Dzeja & Terzic 2003, Saks *et al.* 2006b, Dzeja *et al.* 2011a).

To further investigate the role of MOM permeability in the regulation of respiration *in situ*, we analysed the effects of exogenous MgATP on respiration under different conditions (Fig. 5). Addition of ATP to permeabilized cardiomyocytes activates respiration due to production of endogenous ADP. The apparent K_m(ATP) was found to be about 160 μM due to restriction of ADP diffusion *in situ*. In case of MtCK activation by the addition of Cr, the respiration rate increases rapidly due to continuous regeneration of ADP in intermembrane space (local cycling by activated MtCK reaction), thus decreasing the apparent K_m(ATP) to 24 μM. Removal of extramitochondrial ADP by PEP-PK system (this system rephosphorylates ADP released from mitochondria to ATP) changes completely the kinetics of regulation of respiration controlled by MtCK by increasing the apparent K_m(ATP) to 2 mM. Figure 5 shows that *in situ* high

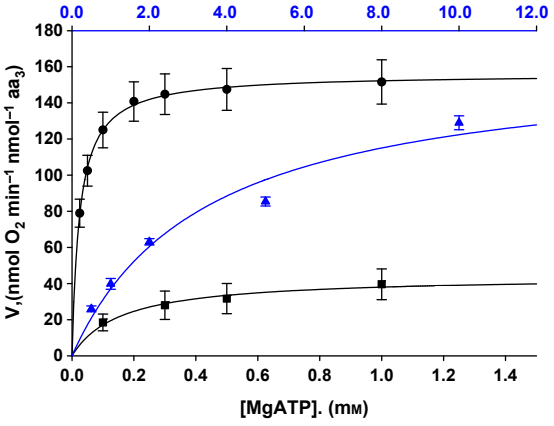

Figure 5 Role of mitochondrial outer membrane permeability and ADP signalling in the regulation of respiration of permeabilazed adult cardiomyocytes. (■) stepwise addition of MgATP activates respiration due to the production of endogenous extramitochondrial ADP. The apparent K_m(ATP) is equal to 158 ± 40.1 μM because of the restriction of ADP diffusion *in situ*. (●) stepwise addition of MgATP in the presence of 20 mM of creatine increases rapidly respiration rates due to the stimulation by endogenous extra- and intramitochondrial ADP decreasing the apparent K_m(ATP) to 24 ± 0.8 μM. (blue ▲) removal of extramitochondrial ADP by phosphoenolpyruvate –pyruvate kinase system mitochondrial creatine kinase increases significantly the apparent K_m(ATP) to 2 mM because of restricted diffusion. Reproduced from Guzun *et al.* (2009) with permission.

amounts of extramitochondrial ADP cannot effectively stimulate respiration because of restricted diffusion. At the same time, small amounts of extramitochondrial ADP, which can play the signalling role, are necessary for maximum activation of respiration, but exclusively in case of active MtCK in the presence of Cr (Guzun *et al.* 2009, Saks *et al.* 2012).

The results of these studies highlight the important role of the ANT – MtCK – VDAC - Tubulin system in the regulation of respiration and energy-transferring fluxes in cardiac cells. This system creates a supramacromolecular complex which we called 'Mitochondrial Interactosome' (MI) (Fig. 6) (Timohhina *et al.* 2009, Saks *et al.* 2012). Specifically, it represents the ATP synthasome proposed by Pedersen (2007) and formed by ATP synthase, ANT and PiC, as well as MtCK that is functionally coupled to OxPhosph via ANT and with VDAC, which, in turn, interacts with certain regulatory (cytoskeletal) proteins (Fig. 6). MI can include also the respiratory supercomplexes (Chen *et al.* 2004). Along the cristae membranes, the MI contains only MtCK and ATP Synthasome. ATP produced by ATP synthase is transferred to MtCK due to its functional coupling with ATP synthasome, and then, MtCK transfers the phosphoryl group from ATP to Cr. The final product, PCr, is then released from mitochondria as a main energy flux due to high selective permeability of VDAC. Recycled ADP is returned back to the matrix. However, VDAC is not completely impermeable for adenine nucleotides allowing small signalling amounts of ADP to reach ATP synthase. Regenerated ATP is transferred directly by ANT to MtCK which continuously recycles ADP and by doing so, maintains the production of the PCr energy flux. In this way, MtCK amplifies the cytosolic ADP signal and, due to its high selective permeability, VDAC separates mitochondrial energy PCr flux from cytosolic ADP signalling.

Formation of structure–function relationship in the development of ICEUs

According to our previous studies, the formation of regular arrangement of mitochondria and cytoskeletal components in rat cardiac cells in developmental terms takes place in the course of 3 month, in parallel with the maturation of energy transfer systems of mitochondrial metabolism (Table 2, Fig. 7) (Tiivel *et al.* 2000). During the first neonatal days, mitochondria of rat cardiac cells are randomly clustered in the cytosol and situated mostly in the perinuclear area. At this developmental state, functional interactions between mitochondrial and cytoskeletal proteins, characteristic for adult cardiomyocytes, have not been formed yet, and therefore, no ICEUs have yet been established. In this stage of development, cardiac cells already use OxPhosph, but are relatively more dependent on anaerobic glycolysis (Lopaschuk & Jaswal 2010). Accordingly, at birth, the apparent K_m(ADP) is relatively low (75.0 ± 4.5 μM, cardiomyocytes from 3-day-old rat) compared with the adult heart and increases steadily to the adult levels (317 ± 29.5 μM,

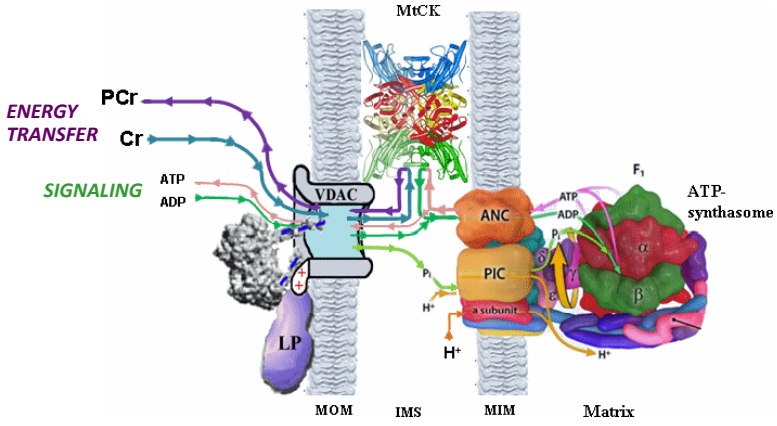

Figure 6 'Mitochondrial Interactosome' includes ATP synthasome formed by ATP synthase, adenine nucleotide translocase (ANT) and inorganic phosphate carrier (PiC), mitochondrial creatine kinase (MtCK) functionally coupled to ATP synthasome and voltage-dependent anion channel (VDAC) with regulatory proteins (βII-tubulin and other linker proteins). ATP regenerated by ATP synthase is transferred to MtCK due to its functional coupling with ATP syntasome. MtCK transfers the phosphate group from ATP to creatine. Produced PCr leaves mitochondria as a main energy flux due to high selective permeability of VDAC. Recycled ADP is returned to ATP syntasome via the functional coupling. Small signalling amounts of ADP can reach ATP synthase. Regenerated ATP is transferred directly to MtCK which recycles continuously the ADP maintaining production of the PCr energy flux. In this way, MtCK amplifies cytosolic ADP signal. Reproduced from Timohhina *et al.* (2009) with permission.

Table 2 Developmental changes in the values of maximal respiration rate (V_{max}) and the apparent K_m for exogenous ADP [K_m(ADP)] measured in isolated from rat

Days	App. K_m(ADP), μM	V_{max}, nmol min^{-1} mg^{-1} protein
3	75.0 ± 4.5***	44.9 ± 4.1***
14	81.9 ± 18.8***	70.5 ± 12.6*
21	138.4 ± 11.7**	110.3 ± 3.9
28	149.4 ± 8.1**	104.7 ± 4.7
60	185.3 ± 27.5*	109.8 ± 11.4
84	317.4 ± 29.5	104.6 ± 5.7

Calcium-tolerant cardiomyocytes were isolated by perfusion with collagenase as described previously (Tepp *et al.* 2011) Oxygen consumption was determined by a high-resolution respirometry (Oxygraph-2K; OROBOROS Instruments, Innsbruck, Austria) in Mitomed solution supplemented with respiratory substrates, 5 mM glutamate and 2 mM malate. Data are expressed as the mean ± standard error of the mean (SEM).
Statistical significance: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. $n \geq 5$.

84 days postnatal) as indicated in Table 2. One of the main factors, suggested to influence the diffusion restrictions for ADP, is the mitochondria–cytoskeleton interactions and binding of cytoskeletal elements as for example tubulin to VDAC (Monge *et al.* 2008, Rostovtseva *et al.* 2008). Moreover, at this early stage in development, MtCK, although present in the cell in small amount, probably is not yet properly localized and coupled to OxPhosph (Khuchua *et al.* 1998).

During the first postnatal week, βII-tubulin is localized mainly in the subsarcolemmal area, distant from mitochondria which are clustered predominantly to the perinuclear area (Fig 7a). After 2 weeks (14 postnatal days), βII-tubulin has expanded its localization throughout the cellular interior. After 21 days, colocalization of mitochondria with βII-tubulin is visible and βII-tubulin becomes more concentrated within the intermyofibrillar space. These studies show that the timeframe of the increase in apparent K_m(ADP) value (decrease of the affinity for ADP) is closely related to the formation of regular patterns of mitochondria and their colocalization with βII-tubulin. As a consequence of this structural organization, an increase in the diffusion restrictions for ADP can be measured that is an indicator for a more precise regulation of energy transfer.

The formation of regular arrangement of the mitochondria and cytoskeletal modifications occurs in parallel with the maturation of energy transfer kinase systems (Tiivel *et al.* 2000, Chung *et al.* 2008, Dzeja *et al.* 2011a,b) (Table 2, Fig. 7). An up-regulation of MtCK expression and increase in its activity at second postnatal week is observed (Fig. 7b, Table 2). Western blot analysis shows the presence of MtCK at low level already after three postnatal days; they reach 60% of the adult level during second week (Fig. 7b). The effect of MtCK/Cr system on the mitochondrial respiration at postnatal day 3 is low and its activation takes place progressively from 14 to 84 postnatal days, when MtCK expression approaches the adult levels (Fig. 7b). Thus, CK-system is becoming progressively more capacitive in mediating the feedback regulation between ATP consumption in cytosol and production in mitochondria.

Summarizing, existing data indicate clearly that functional alterations in energetic metabolism during development can be associated with parallel changes in intracellular structural organization of mitochondria relative to myofibrils and cytoskeletal proteins. The studies of postnatal formation of ICEUs showed that the increase in apparent K_m(ADP) value and activation of CK/PCr phosphoryl transpher system are interconnected and directly related to the time course of the visible appearance of mitochondria – βII-tubulin co-localization in the heart cells. These results indicate that functional interactions of mitochondria with cytoskeletal proteins could be important prerequisites for formation of highly regulated energy transfer network in adult cardiomyocytes. In conclusion, rat heart is structurally and metabolically mature at the age of 3 month, when ICEUs are fully formed and MtCK is active coupled to OxPhosph and forming microcompartmentalization for production of PCr and efficient intracellular energy transport facilitated by the PCr/Cr circuit shuttle.

Feedback signalling via near-equilibrium enzymatic phosphoryl transfer networks (regulation under working conditions)

Under physiological conditions, according to the Frank–Starling's law, the force of heart contraction is regulated by ventricular filling and sarcomere length-dependent mechanism at constant metabolite concentrations and constant amplitude of Ca^{2+} transients (Fukuda & Granzier 2005, Saks *et al.* 2006a,b,c). The high degree of metabolic stability displayed by cardiac muscle comes from the high coordination between ATP utilization and regeneration. The regulation of mitochondrial ATP synthesis is governed by metabolic feedback through communicating of changes in compartmentalized Pi, ADP, AMP and Cr/PCr ratios (Dzeja & Terzic 2003, Saks *et al.* 2006a,b,c, 2012, Aliev *et al.* 2011).

In several studies, the nature of the metabolic signalling within ICEU was studied using mathematical modelling of compartmentalized energy transfer based on experimental data (Aliev & Saks 1997, Dos Santos

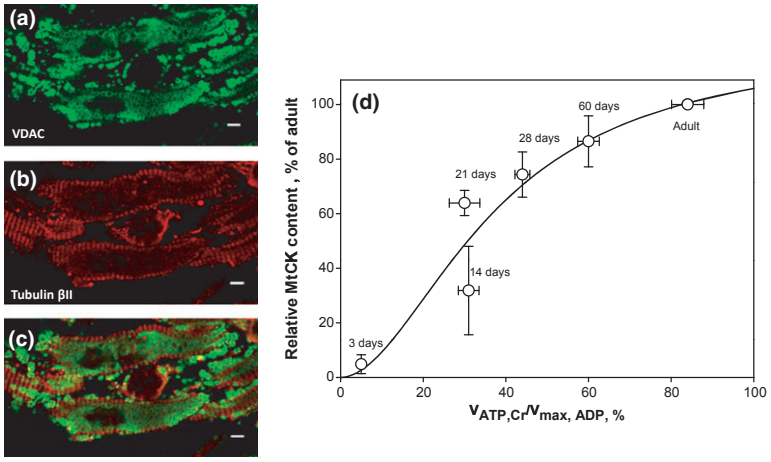

Figure 7 Development of intracellular energy units in cardiomyocytes isolated from rat hearts. (a–c) Immunofluorescent confocal micrograph of developmental distribution of mitochondria and βII-tubulin in 8-day-old cardiomyocytes coimmunolabelled for voltage-depending anion channel (VDAC) (a) and βII-tubulin (b). The lower panel (c) shows the colocalization of VDAC and βII-tubulin in the 8-day-old rat heart cells. Magnification bar: 2 μm. For immunolabelling mouse monoclonal antibody for βII-tubulin (Abcam, ab28036, Cambridge, UK) and the rabbit polyclonal serum for VDAC (kindly provided Dr. Catherine Brenner, Universite Paris-Sud, Paris, France) were used. Fluorescence images were acquired by Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany) equipped with a Plan-Apofluar 63×/1.30 glycerol objective. (d) Relationship between content of mitochondrial creatine kinase (MtCK) and activation of the MtCK – phosphoryl transfer system during rat heart postnatal development. Ordinate – percentage of MtCK protein expression relative to adult – densitometric quantification of Western blot. Abscissa – stimulatory effect of creatine on the respiration rate of cardiomyocytes in the presence of 2 mM ATP and the trapping system for exogenous ADP (10 IU mL^{-1} of the pyruvate kinase (PK) and 5 mM of the phosphoenolpyruvate (PEP). Under these conditions, changes in kinetics of respiration follow the kinetics of MtCK reaction. V_{max}(ADP) – theoretical maximal respiration rate in the presence of exogenous ADP (see Table 2).

et al. 2000, Aliev *et al.* 2011). The Aliev and Saks model considers the time-dependent diffusional exchange of ATP, ADP, PCr, Cr and Pi between myofibrils and intramyofibrillar mitochondria in cardiomyocytes. Mitochondrial OxPhosph is activated by ADP and Pi produced from ATP hydrolysis by myosin in the myofibril compartment. The model considers CK compartmentalization and the real non-equilibrium kinetics of the CK reactions in different cellular compartments (Aliev & Saks 1997, Dos Santos *et al.* 2000, Aliev *et al.* 2011). A detailed description of the last version of the model is given in the study described by Aliev *et al.* (2011).

According to this model, the cyclic ATP production in mitochondria during contractions is associated with cyclical oscillations of ADP and Pi concentrations in myofibrils. These data are confirmed by experiments *in vivo* using ^{31}P MRS of isolated perfused heart. Fluctuations of PCr and Cr were in the order of 8–15% during the different phases of the cardiac cycle (Honda *et al.* 2002). Inorganic phosphate, Pi, is not consumed by the myofibrillar MMCK reaction and therefore diffuses freely and enters mitochondrial matrix via its carrier (PiC). Part of ADP is used by the myofibrillar MMCK reaction due to its non-equilibrium steady-state, and part of the ADP forms a gradient of concentration transmitted towards the matrix. The rephosphorylation of ADP in myofibrillar MMCK reaction increases the Cr/PCr ratio which is transferred towards MtCK via CK/PCr shuttle. The ADP signal reaches mitochondria and increases ATP regeneration. Regenerated ATP, due to MtCK-ANT functional coupling, supplies the MtCK reaction (which is also in non-equilibrium steady state) to poduce PCr. As a result, the reactions catalysed by different isoforms of compartmentalized CK tend to maintain the intracellular metabolic stability (Saks *et al.* 1994, 2014, Dzeja & Terzic 2003, Schlattner & Wallimann 2004, Schlattner *et al.* 2006, Guzun *et al.* 2011).

Figure 8 shows the ADP dependence of rates of mitochondrial respiration and heart oxygen consumption under various conditions corresponding to different workloads (Fig. 8a) (Aliev & Saks 1997, Vendelin *et al.* 2000, Aliev *et al.* 2011). They are shown by coloured arrows and are confined to the area of physiological cytosolic ADP concentrations marked with a grey rectangle (Fig. 8b). When MOM is permeable, as in isolated mitochondria, the regulation of respiration by ADP is impossible because ADP concentrations are saturated even at the minimal workload and respiration therefore should be maximal. However, when ADP diffusion is restricted at the level of MOM, as in

mitochondria *in situ*, the respiration rates become linearly dependent on ADP concentrations (Fig. 8b), and in a fact, on heart workload, in accordance with the metabolic aspect of Frank–Starling law (Fig. 8c). The Frank–Starling law of the heart describes the ability of heart to change the force of contraction and stroke volume in response to changes in the end-diastolic volume (Opie 1998). The metabolic aspect of this law is expressed by linear dependence between the increase of left ventricular end-diastolic volume and the increase in respiration rates (Williamson *et al.* 1976) in the absence of dectable changes in the intracellular ATP and PCr content (Balaban *et al.* 1986). The linear dependence of oxygen consumption on [ADP], under physiological conditions, can be amplified by Cr in the presence of activated MtCK. So, this graph points to the importance of decreased MOM permeability in feedback regulation and restricted ADP diffusion *in vivo* (Fig. 8c).

It has been demonstrated that reduced function of CK-system and decreased energy flux is the most prominent energetic abnormality in human heart failure and myocardial infarction (Weiss *et al.* 2005, Neubauer 2007). Using technique of ^{31}P MRS by saturation transfer and synchronisation with cardiac cycle ATP flux via the CK reaction was analysed *in vivo* (Weiss *et al.* 2005). Authors' demonstred that the ATP flux through CK reaction is reduced by 50% in patients with heart failure even under conditions of relatively small changes of PCr and ATP. In a recent paper, the same group demonstrated that overexpression of MtCK improves energy reserves of heart and

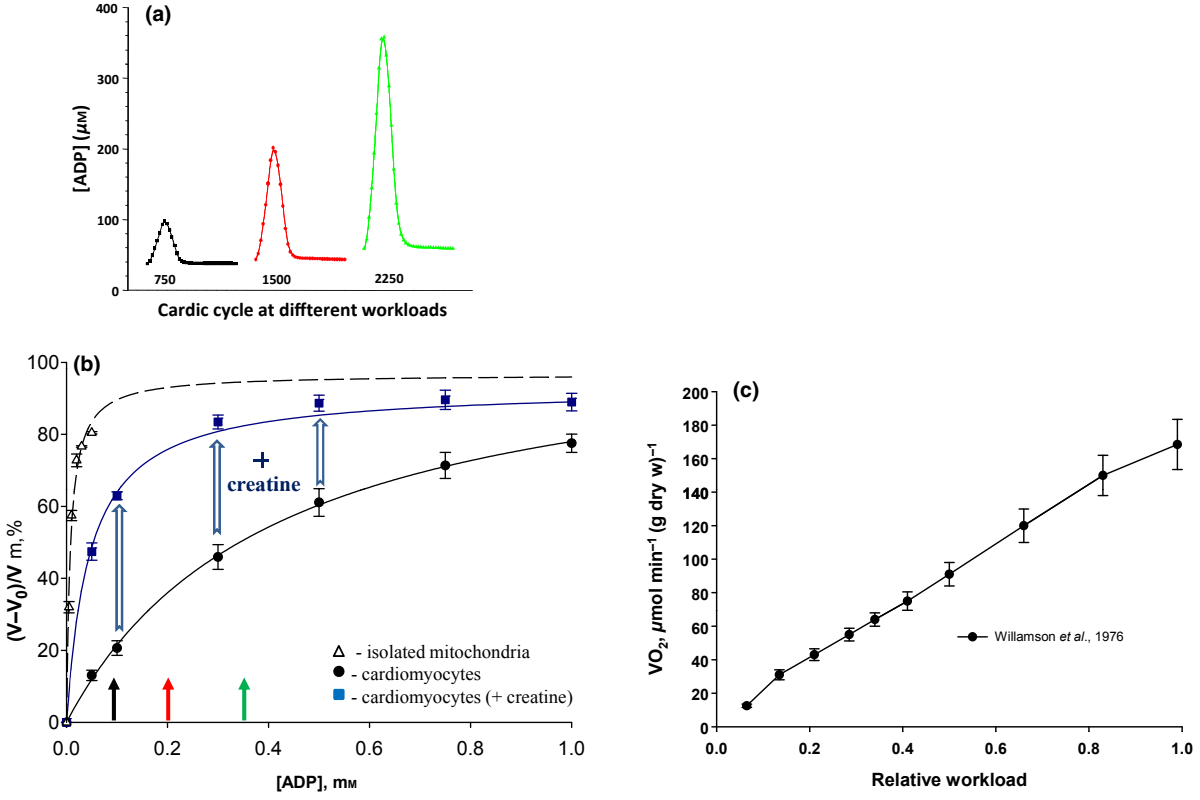

Figure 8 Mechanisms of regulation of respiration controlled by MtCK. (a) Mathematically modelled oscillations of ADP concentrations in the core of myofibrils over cardiac cycle at workloads equivalent to 750 (black), 1500 (red) and 2250 (green) μmol ATP s^{-1} kg^{-1}. (b) Graphical Michaelis–Menten representation of the dependence of mitochondrial respiration rate on the concentration of ADP. Coloured arrows on *X*-axes show ADP concentrations corresponding to increased workloads from panel (a). In isolated mitochondria (permeable mitochondrial outer membrane), ADP concentration corresponding to minimal workload falls in the region of maximal respiratory rate (saturated concentration) and does not allow any regulation. The apparent K_m for ADP in isolated mitochondria is 7.9 ± 1.6 μM. In permeabilized cardiomyocytes (restricted diffusion), the respiration rates become linearly dependent on ADP concentrations in agreement with heart workloads (Fig. 8a, c). The apparent K_m for ADP in permeabilized cardiomyocytes is 370.8 ± 30.6 μM. This linear dependence is amplified by creatine in the presence of activated MtCK. The apparent K_m for ADP in the presence of creatine decreases up to 50.2 ± 8.0 μM. (c) Linear increase in oxygen consumption rates as a function of increased relative workload. Reproduced from Saks *et al.* (2012) with permission.

protects from toxic insults, for example, by doxorubicin cardiotoxicity (Gupta *et al.* 2013). Augmentation of cardiac MMCK expression attenuated ischaemic acidosis, reduced injury and improved not only high-energy phosphate content and the rate of CK ATP synthesis in post-ischaemic myocardium, but also recovery of contractile function (Akki *et al.* 2012).

Recently, Phillips *et al.* (2012) hypothesized that the mitochondrial ATP synthesis matching in cardiac cells intracellular ATP hydrolysis under conditions of metabolic homoeostasis is regulated by posttranslational modifications (PTMs) of OxPhosph complexes, specifically of F1F0ATPase. Authors demonstrated that the activity of F1F0ATPase isolated from pig heart is lower than that from liver. This inhibition was due to the phosphorylation of several enzyme subunits. Modulation of isolated F1F0ATPase activity by dephosphorylation, on the one hand, and the increase in F1F0ATPase activity in response to the dobutamin stress, on the other hand, allowed authors to conclude that persistent reversible PTMs regulate variable F1F0ATPase activity in response to metabolic stress (Phillips *et al.* 2012). This conclusion reinforced authors' previous assumption. In Michaelis–Menten model of ATP production as a function of ADP concentrations, the rate of ATP production can be altered by changing the rate of the maximum ATP production at constant ADP concentration (Balaban 2012). Taking into account that the F1F0ATPase concentration is constant in the heart with workload, authors concluded that the change of maximum rate of ATP production can be acheived only by the modification of enzyme kinetics via PTMs (Balaban 2012, Phillips *et al.* 2012). However, this model of ADP-driven ATP production completed for isolated heart mitochondria (apparent K_m for ADP is about 30 μM) does not take into account intracellular phosphoryl transfer networks participating to the signal transduction and amplification in highly organized heterogeneous intracellular medium. Aditionally, the F1F0ATPase dephosphorylation resulted from the incubation of isolated heart mitochondria in calcium-containing buffer (Phillips *et al.* 2012) allowed authors to link the hypothesis on PTMs regulation of OxPhosph with the hypothesis of parallel activation of excitation–contraction coupling and OxPhosph by calcium (Balaban 2002, 2012). The last hypothesis is reviewed by authors taking into account the results of OxPhosph regulation in mice knockdown for mitochondrial calcium uniporter (Pan *et al.* 2013).

Among other hypothesis, it was also proposed that the OxPhosph complexes PTMs have cardioprotective role due to the inhibition of enzyme hydrolytic activity which reduces the reverse ATP breakdown during ischaemia (Sun *et al.* 2007, Wang *et al.* 2013) and the protection by reversible PTMs of F1FOATPase from permanent oxidative damage during ischaemia-reperfusion in spite at the expense of decreased ATP production (Sun *et al.* 2007, Sun & Murphy 2010, Murphy *et al.* 2012, Chung *et al.* 2013).

The role of creatine in cardiac energy metabolism

In spite of the long history based on the proof knowledge about the role of Cr in muscle cells respiration and important role of CK/PCr shuttle in energy transport in the heart and oxidative muscles, this topic continues to aliment scientific debates. Recently, it has been shown that mice, knockout for guanidine-acetic acid methyltransferase ($GAMT^{-/-}$), which was associated with Cr deficiency, may have unaltered maximal exercise capacity and response to chronic myocardial infarction due to the metabolic flexibility (Lygate *et al.* 2013). Quite opposite results were obtained in arginine/glycine amino-transferase (AGAT) knockout and Cr-deficient mice in the same year by Nabuurs *et al.* (2013). Cr depletion leads to several metabolic abnormalities in skeletalmuscle, including reduced ATP, increased inorganic phosphate levels and reduced activities of proton-pumping respiratory chain enzymes and an elevated glycolytic contribution in ischaemic circumstances (Nabuurs *et al.* 2013). These changes were reversed by oral Cr administration. Cr treatment is effective in humans with AGAT deficiency too, preventing many neurological and functional abnormalities (Leuzzi 2002). The reason for the differences between results of these papers may lay in the method used for attainment of Cr deficiency and metabolic adaptations.

The AGAT knockout mouse, in contrast to the GAMT knockout one, does not synthesize guanidino acetic acid (GAA). The latter in the GAMT knockout skeletal muscle was shown to be phosphorylated by CK to form an alternative energy-rich phosphagen, phospho-GAA (PGAA), which still can be utilized as high-energy phosphagen, albeit at lower efficiency (Heerschap *et al.* 2007). Therefore, only AGAT knockout model can be regarded as real Cr-deficient model, while in GAMT knockout model, the functions of Cr/PCr pair are partially delegated to GAA/PGAA couple.

The question of efficiency of GAA/PGAA couple was explored by Kan *et al.* (2004). According to their data, in skeletal muscle during ischaemia, PGAA 'was metabolically active in $GAMT^{-/-}$ mice and decreased at a rate comparable with the decrease of PCr in WT mice.' In fact, the recovery rate of PGAA in $GAMT^{-/-}$ mice after ischaemia was reduced compared with PCr in WT mice. According to their data, the initial

recovery rate of PGAA in GAMT$^{-/-}$ mice hindleg muscles is 0.034 ± 0.003 mM s^{-1} or about 3.44-fold lower than the rate of PCr restoration, 0.117 ± 0.01 mM s^{-1}. However, this PGAA turnover rate can still support almost 80% of ATP turnover rate (0.043 ± 0.006 mM s^{-1}) in GAMT$^{-/-}$ mice. These PCr and PGAA turnover rates are manifold times lower than the unidirectional exchange rates of phosphoryl transfer in the opposite direction, from PCr to ATP (9.7 ± 0.5 mM s^{-1}) and PGAA to ATP (<0.5 mM s^{-1}), detected in magnetization saturation transfer (MST) experiments. The reasons of these dramatic differences are not preciously settled (Kan *et al.* 2004) and ^{31}P NMR saturation transfer data are undergoing reinterpretation (Balaban & Koretsky 2011, From & Ugurbil 2011, Kemp & Brindle 2012). Of note is that MST experiments on mouse hearts knockout for GAMT still revealed about fivefold difference in PCr or PGAA signal intensity decrease upon 6 s saturation of [γ-P]-ATP peak (Lygate *et al.* 2013). Also *in vivo* in structurally organized and tightly coupled reactions the real kinetics may be very different from the classic kinetics of free enzymes in solution (Saks *et al.* 2007a,b).

Suppose, in accord with data of Kan *et al.* (2004), the real rate of PGAA restoration is 3.5-fold lower than that of PCr, and then, we can illustrate by modelling the main energetic consequences of PCr substitution in heart muscle by PGAA. The results of this modelling, presented in Appendix S1, indicate an impaired cardiac contractile function associated with possible slowing of CK reactions in PGAA substituted hearts. Such changes were really detected by Lygate *et al.* (2013).

There may be no differences in the response to chronic myocardial infarction observed between WT and GAMT knockout mice could be predicted. Since in acute ischaemic, insult phosphagens are rapidly depleted and hearts in that respect becoming almost equal. Interestingly, slowly metabolized phosphorylated Cr analogues cyclocreatine and β-guanidinopropionic acid (GP) can reduce ATP depletion and even prolong ischaemic survival (Turner & Walker 1985, Oudman *et al.* 2013). In this regard, reduction of basal PCr by GP feeding in myocardial infarction hearts did not further impair mechanical function (Horn *et al.* 2001). Moreover, in hearts, hardly survived after severe myocardial infarction, pathological relations between main cellular systems may be too far from regulatory events in normal hearts. In this regard, homoarginine, another metabolite of AGAT, which has a role in NO generation and perhaps in energy metabolism too, was absent in AGAT($^{-/-}$) mice and increased in GAMT($^{-/-}$) mice (Choe *et al.* 2013). Cerebral damage and neurological deficits in experimental stroke were increased in AGAT($^{-/-}$) mice and attenuated by homoarginine supplementation (Choe *et al.* 2013), which is unlikely to occur without energetic and metabolic rearrangements. Interestingly, homoarginine levels have strong association with cardiovascular risk and mortality (Pilz *et al.* 2013). In conclusion, highly probable operation of GAA/PGAA couple, which can support significant part of ATP turnover, in GAMT knockout mice distinguishes this model from AGAT knockout model in which Cr is really absent without any functional substitution. These data, as well as data on CK knockout mice (van Deursen *et al.* 1993, Saupe *et al.* 1998, Boehm *et al.* 2000, Dzeja *et al.* 2004, 2011a,b), provide strong evidences for the important role of CK in the energy homoeostasis in muscle cells with high and fluctuating energy demands.

Novel aspects and physiological relevance

It is beyond doubt that understanding of how well is organized intracellular energetic infrastructure in cardiac muscle cells, how it is functioning, and how this organization is disappearing in case of pathology is important for medical science. This may open new frontiers in future for modern metabolic medicine and therapy for cardiac infarction or metabolic syndrome diseases when energy metabolism is pathologically altered. The use of microtubules depolymerizing agent, colchicine, has been demonstrated to be efficient in the treatment of post-operative atrial fibrillation (Imazio *et al.* 2011), the atrial fibrilation ablation therapy (Deftereos *et al.* 2012) and prevention of secondary cardiovascular events in patients with stable coronary disease (Nidorf *et al.* 2013, 2014). In all these studies, colchicine was used due to its anti-inflammatory effects, and no attention was paid to its potential implication in cardiac energy metabolism through microtubules depolymerization. In cancer cells (for example hepatoma cells), microtubules depolymerization by colchicine-induced depletion of mitochondrial membrane potential and cells death (Maldonado *et al.* 2010, Lemasters *et al.* 2012). This effect of colchicine is opposite to that observed in adult cardiomyocytes because of the cardinal difference in the structure/functional organization of energy metabolism. A large number of cancer cell-types, also non-beating NB HL1 cells, do not express isotype of βII-tubulin (Hiser *et al.* 2006) and mitochondrial isoform of CK (Patra *et al.* 2008). Therefore, it will be interesting to see if transfection of both proteins in cancerous NB HL1 cells could restore restore restricted ADP diffusion, microcompartmentalization and remodel them into cells with like-primary cardomyocytes phenotype. One possibility for such

bioenergetic therapy is associated with reinforcement of the binding of tubulin to VDAC; in fact, this is may be capable to induce and maintain the regulation of mitochondrial respiration via microcompartmentalization of adenine nucleotides and the control of energy fluxes through external mitochondrial membrane.

Conflict of interest

None of the coauthors of this article have any conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Model simulation of cyclic bursts of ADP concentration in myoplasm.

Appendix S1. Modeling of creatine deficiency.

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