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REVIEW ARTICLE

Systems bioenergetics of creatine kinase networks: physiological roles of creatine and phosphocreatine in regulation of cardiac cell function

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Abstract Physiological role of creatine (Cr) became first evident in the experiments of Belitzer and Tsybakova in 1939, who showed that oxygen consumption in a well-washed skeletal muscle homogenate increases strongly in the presence of creatine and with this results in phosphocreatine (PCr) production with PCr/O_2 ratio of about 5–6. This was the beginning of quantitative analysis in bioenergetics. It was also observed in many physiological experiments that the contractile force changes in parallel with the alteration in the PCr content. On the other hand, it was shown that when heart function is governed by Frank–Starling law, work performance and oxygen consumption rate increase in parallel without any changes in PCr and ATP tissue contents (metabolic homeostasis). Studies of cellular mechanisms of all these important phenomena helped in shaping new approach to bioenergetics, Molecular System Bioenergetics, a part of Systems Biology. This approach takes into consideration intracellular interactions that lead to novel mechanisms of regulation of energy fluxes. In particular, interactions between mitochondria and cytoskeleton resulting in selective restriction of permeability of outer mitochondrial membrane anion channel (VDAC) for adenine nucleotides and thus their recycling in mitochondria coupled to effective synthesis of PCr by mitochondrial creatine kinase, MtCK. Therefore, Cr concentration and the PCr/Cr ratio became important kinetic parameters in the regulation of respiration and energy fluxes in muscle cells. Decrease in the intracellular contents of Cr and PCr results in a hypodynamic state of muscle and muscle pathology. Many experimental studies have revealed that PCr may play two important roles in the regulation of muscle energetics: first by maintaining local ATP pools via compartmentalized creatine kinase reactions, and secondly by stabilizing cellular membranes due to electrostatic interactions with phospholipids. The second mechanism decreases the production of lysophosphoglycerides in hypoxic heart, protects the cardiac cells sarcolemma against ischemic damage, decreases the frequency of arrhythmias and increases the post-ischemic recovery of contractile function. PCr is used as a pharmacological product Neoton in cardiac surgery as one of the components of cardioplegic solutions for protection of the heart against intraoperational injury and injected intravenously in acute myocardial ischemic conditions for improving the hemodynamic response and clinical conditions of patients with heart failure.

Keywords Creatine · Phosphotransfer networks · Mitochondria · Respiration · Regulation · Systems biology

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Introduction: historical note

Creatine was discovered 175 years ago by the French scientist Michel Eugene Chevreul. Almost one century later Otto Meyerhoff and Archibald Hill studied the energetics of carbohydrate catabolism in skeletal muscles, for which

they subsequently received the Nobel Prize (Hill et al. 1924; Kresge et al. 2005). Meyerhoff focused on the study of glycolytic pathways demonstrating that in the absence of oxygen glycolysis leads to production of lactic acid. Archibald Hill used these theoretical bases to explain mechanisms of transformation of energy liberated during metabolism into mechanical work of muscles. Hill and Meyerhoff hypothesized that muscle contraction is fuelled by the energy released from glycogen conversion into lactic acid. The role of oxygen in their theory was limited to the removal of lactic acid during the resting period. The worldwide recognized lactic acid theory of contraction (Mommaerts 1969; Ivanov et al. 1997) was ruled out by the very simple experiment performed by Lundsgaard, in 1930. Lundsgaard inhibited glycolysis in contracting frog muscle by iodoacetate and observed that despite inhibition of glycolysis, frog's muscle continued to contract during some time under hypoxic conditions (Lundsgaard 1930). By that time, a new compound in which phosphate is linked to creatine through a phosphoamide bond, the phosphagen (later named phosphocreatine, PCr), was already discovered by Grace and Philip Eggleton and by Fiske and Subbarow (Eggleton and Eggleton 1927; Fiske and Subbarao 1927). Lundsgaard observed that after a series of contractions under anaerobic conditions in the iodoacetate-injected muscle, the lactic acid content was similar to that of resting muscle and the phosphocreatine content was completely used up. In the control muscle contracting under the conditions of Hill's experiment (glycolysis and anoxia), the increase in lactic acid concentration was associated with only slight decrease in phosphocreatine content. In this way, Lundsgaard hypothesized that in muscle poisoned with iodoacetate the immediate energy source of muscle contraction was phosphocreatine. Poisoned cardiac muscle stopped its work in anoxic conditions after a few minutes of contraction and continued to contract for 1.5–2 h in the presence of oxygen even under very high concentrations of iodoacetate. Lohmann, in 1934, described the creatine kinase (CK) reaction which links PCr with ATP. In 1939, Belitzer and Tsybakova measured the phosphagene (PCr) production and oxygen consumption in pigeon's pectoral muscles homogenate stimulated by creatine without the addition of adenine nucleotides. These authors were the first to show that creatine stimulates respiration and that PCr production was associated with P/O_2 ratio between 5.7 and 7 (Belitzer and Tsybakova 1939). During the same time period, Frank and Starling discovered the basic law of the heart functioning—parallel increase in respiration and cardiac work with increase in left ventricle filling (Starling and Visscher 1926; Saks et al. 2006).

It took about 80 years to identify the cellular mechanisms of these important phenomena, which are all related to the physiological role of creatine and creatine kinase system in heart cells (Bessman and Geiger 1981; Bessman and Carpenter 1985; Wallimann et al. 1992, 2007; Saks 2007). The central role of Cr, PCr and CK (Cr/PCr/CK) system in cardiac cells is shown in Fig. 1. Their central role is based on the intracellular distribution of different isoforms of CK in muscle cells that has been previously described in great details elsewhere (Wallimann et al. 1992, 2007).

The aim of this review article is to describe the key mechanisms of the functioning of the Cr/PCr/CK system in the heart that has been revealed in functional and kinetic studies. Central among these mechanisms is the functioning of a supercomplex, mitochondrial interactosome (Timohhina et al. 2009). These mechanisms result from complex interactions in structurally highly organized cells and are therefore best understood using the approaches developed in Systems Biology (Saks 2007). The central role of the Cr/PCr/CK system in muscle and brain cells' energetics makes it an important target for understanding of the mechanisms of pathogenesis of many muscular and neuromuscular diseases and their possible treatment.

Mitochondrial interactosome

Evidence for functional coupling between MtCK and ANT in isolated heart mitochondria

The mitochondrial isoform of CK was discovered in 1964 in the Klingenberg's laboratory (Klingenberg 1970). Sarcomeric (sMtCK) is present in muscle cells and ubiquitous (uMtCK) is present in other tissues (Wallimann et al. 1992). Bessman and Fonyo (1966) and Jacobus and Lehninger (1973) showed that creatine stimulates respiration of isolated mitochondria in State 4 (when ADP is completely used up) up to State 3 (corresponding to maximal level of ADP-stimulated respiration) (Bessman and Fonyo 1966; Jacobus and Lehninger 1973). This and other similar experiments led to conclusion that MtCK controls the oxidative phosphorylation. Under respiring conditions, the MtCK reaction is influenced by its coupling with oxidative phosphorylation via adenine nucleotide translocase (ANT). Jacobus and Saks (1982) evaluated the kinetic properties of MtCK in isolated heart mitochondria under conditions of the ATP supply either by activated oxidative phosphorylation or by the exogenous addition (in the presence of inhibited oxidative phosphorylation (Jacobus and Saks 1982). In the first case, the MtCK reaction rate was evaluated by measuring oxygen consumption (process functionally coupled to the PCr production) while in the second case by measuring ADP production and subsequent NADH oxidation in phosphoenolpyruvate–pyruvate kinase–lactate dehydrogenase (PEP–PK–LDH) enzymes system (Fig. 2a). The results

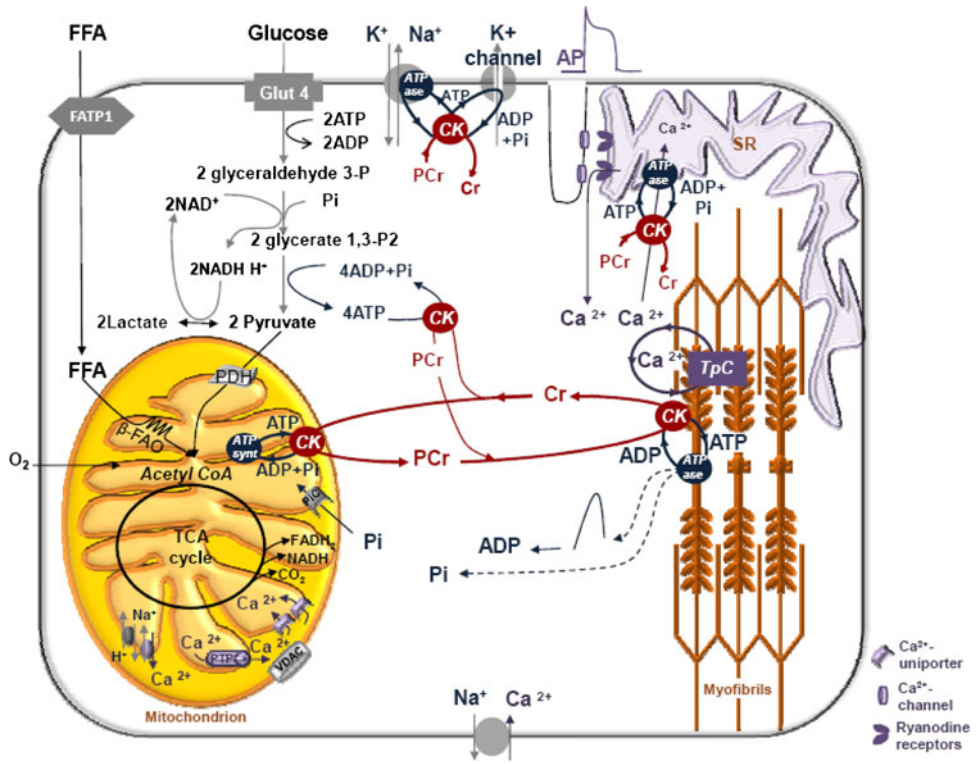
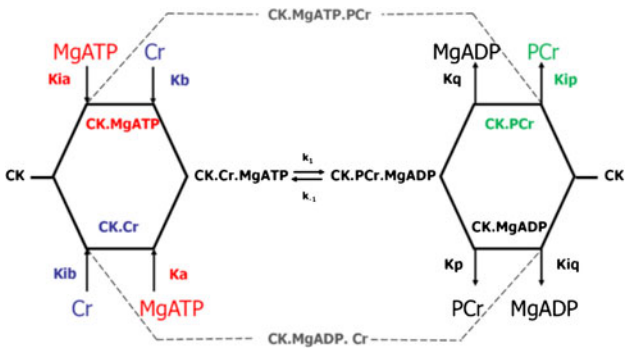


Fig. 1 Scheme of energy metabolism in heart and skeletal muscle cells. Two sources of ATP re-synthesis, mitochondrial oxidative phosphorylation and glycolysis, are interconnected with sites of ATP hydrolysis via the phosphocreatine/creatine kinase (PCr/CK) shuttle. *GLUT4* glucose transporter, *FATP1* fatty acid transport protein, *β-FAO* beta-oxidation of fatty acids, *PHD* pyruvate dehydrogenase, *PTP* permeability transition pore, *VDAC* voltage-dependent anion channel, *TpC* troponin C, *AP* action potential. The isoforms of the creatine kinase (CK) present in different subcellular compartments are coupled with both ATP producing (mitochondrial and glycolytic) and ATP consuming (contraction, ions pumping) processes. In muscle cells sarcomeric mitochondrial CK (MtCK) functionally coupled to ATP synthase via adenine nucleotide translocase (ANT) and cytosolic isoforms of CK (MMCK and MBCK) coupled to glycolytic enzymes (phosphoglycerate kinase (PGK) and pyruvate kinase (PK) catalyse forward reaction of phosphocreatine (PCr) synthesis from mitochondrial or glycolytic ATP and creatine). The MMCK functionally coupled to myosin ATPases, sarcoplasmic reticulum ATPases or ions-pumping-ATPases catalyse reverse reaction of ATP regeneration from PCr and locally produced ADP. The prevalence of one of the ways of PCr production is tissue specific. In cardiac and oxidative muscle cells PCr used for muscle contraction is produced mainly from mitochondrial ATP, while in fast twitch glycolytic muscle it is produced from ATP supplied by glycolysis

showed that the oxidative phosphorylation strongly increases the rate of PCr production (Fig. 2b). The explanation of this observation was given by a complete kinetic analysis of the creatine kinase reaction in isolated heart mitochondria which follows the Bi Bi random type quasi equilibrium mechanism (Jacobus and Saks 1982):



The apparent constant K_a of dissociation of MgATP from the ternary enzyme–substrates complex, CK.Cr.MgATP, decreased by an order of magnitude when ATP was supplied to MtCK from oxidative phosphorylation (Fig. 2c; Table 1). This was explained by the direct transfer of adenine nucleotides between MtCK and ANT. It was shown that MtCK and ANT are present in cardiac mitochondria in the 1:1 molar ratio (Kuznetsov and Saks 1986) and linked to each other by cardiolipin molecules (Schlattner et al. 2004). The results shown in Fig. 2b describe the increase in the rate of PCr production by oxidative phosphorylation that cannot be explained by the assumption of free ATP diffusion in the intermembrane space. Free diffusion of molecules by Brownian movement (Fig. 2d) is described by the Einstein–Smoluchowski equation: $D = \lambda^2/2t$, where D is diffusion coefficient, λ is the distance of displacement for a given time t (Saks et al. 2008). Employing this equation and knowing that the distance between two mitochondrial membranes is

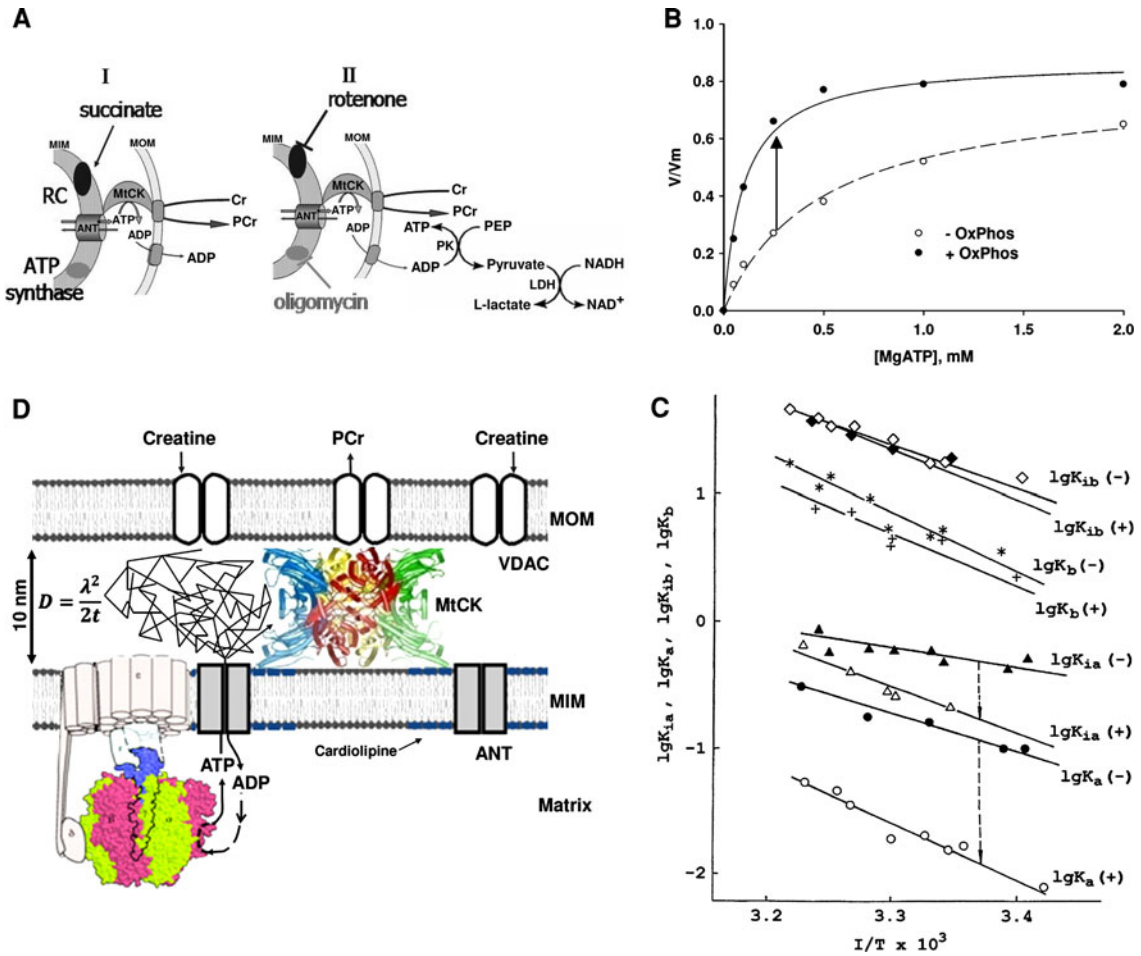


Fig. 2 **a** Scheme of experiments performed to study the kinetic properties of creatine kinase (CK) in isolated heart mitochondria. In the presence of oxidative phosphorylation (I), the measurement of reaction rates was carried out by recording the oxygen uptake in the presence of activated respiratory chain (by succinate). In the second experiment (II), the disappearance of NADH in pyruvate–lactate dehydrogenase reactions (LDH) was followed spectrophotometcally in the presence of inhibited respiratory chain by rotenone and oligomycin. *MIM* Mitochondrial inner membrane, *MOM* mitochondrial outer membrane, *RC* respiratory chain, *ANT* adenine nucleotide translocase, *MtCK* mitochondrial creatine kinase, *Cr* creatine, *PCr* phosphocreatine. **b** Michaelis–Menten representation of relative rates of MtCK reaction (V/V_m) in isolated heart mitochondria as a function of MgATP concentrations measured in the presence of 10 mM creatine and inhibited (*empty circles*) or activated oxidative phosphorylation (*filled circles*) (adapted from). **c** Complete kinetic analysis of the forward MtCK reaction in heart mitochondria. The temperature dependencies of the kinetic constants are shown at a semi-logarithmic scale in the presence (+) and absence (−) of oxidative phosphorylation. The dissociation constants were expressed in mM. Only K_a (dissociation constant of MgATP from ternary enzyme–substrate complex) is changed by oxidative phosphorylation by an order of magnitude; smaller changes are seen for Ki_a, but practically no changes are seen for the dissociation constant of creatine (reproduced from Saks et al. 1998] with permission). **d** 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). This complex allows the direct exchange-metabolite channelling, 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. The inconsistently of this hypothesis is argued in the text

about 0.01 μm, and admitting that the coefficient of ATP diffusion in water is 200 $\mu m^2/s$ (Saks et al. 2008), the time during which the molecule of ATP will cross this distance by free diffusion will be 0.25×10^{-6} s. Given that the turnover number (TN) of MtCK reaction in direction of PCr production is equal to 33–41 s^{-1} (Engelborghs et al. 1975), the time τ needed for MtCK to transfer the phosphoryl group from ATP to creatine, $\tau = 1/TN$ will be, respectively, 0.03–0.024 s, any theoretical attempt to explain the functional coupling of MtCK with ANT by the simple diffusion becomes futile: very rapid diffusion should equilibrate ATP concentration in intermembrane space and surrounding medium. The real apparent constant of ATP diffusion in (or from) the microcompartment between octameric MtCK and ANT, taking into consideration the above given values for λ, τ and Einstein–Smoluchowski diffusion equation, has to be

in the range of 10^{-3} $\mu m^2/s$ and not 200 $\mu m^2/s$ as it was supposed to be in a homogenous water bulk solution (Saks et al. 2008). Most probably, there is a vectorial movement of ATP molecules (channelling) directly from ANT to MtCK due to their close proximity without liberation into intermembrane space (Saks et al. 2007a, b). The molecular details of this channelling need to be revealed. After the utilisation of this ATP in the MtCK reaction for creatine phosphorylation, ADP may be channelled back to ANT, or liberated into intermembrane space and into the surrounding medium, if the voltage-dependent anion channel (VDAC) in the mitochondrial outer membrane is open. Thus, the functional coupling between ANT and MtCK is finally depending on the state of VDAC in the outer mitochondrial membrane, MOM.

Control of outer mitochondrial membrane permeability in cardiac cells in situ

Cells' permeabilisation technique revealed a remarkable decrease in affinity of oxidative phosphorylation for free ADP in permeabilized cardiac cells in situ (Fig. 3a) in comparison with isolated heart mitochondria (the apparent K_m for free ADP is respectively $\sim$350 and $\sim$10 μM) (Saks et al. 1995, 1998). The analysis of this data led to the conclusion of the restriction of movement of adenine nucleotides across the MOM in permeabilized cardiomyocytes, this resulting in increase in the functional coupling within MtCK-oxidative phosphorylation system, thereby increasing the role of the Cr/PCr/CK system in energy transfer (Aliev and Saks 1997). The restriction of ADP diffusion can be clearly seen in experiments in which the ADP-trapping system, pyruvate kinase-phosphoenolpyruvate (PK-PEP), proposed previously by F. Gellerich for isolated mitochondria (Gellerich and Saks 1982), is added to permeabilized cardiomyocytes in the presence of activated MtCK (by the addition of ATP and creatine). Under these conditions, the PEP–PK system allows us in detecting the possible leak of ADP produced by MtCK from the mitochondrial intermembrane space (Fig. 3b, c). As it is shown in Fig. 4a, the PK–PEP system cannot inhibit the respiration of permeabilized cardiomyocytes in contrast to that of isolated heart mitochondria where respiration rate is decreased by this system by about 50% (Gellerich and Saks 1982). The ADP produced locally in the MtCK reaction of permeabilized cardiomyocyte is returned entirely into the matrix without any leak of mitochondrial ADP towards intracellular milieu.

Recently, it was shown that the selective permeability of MOM for adenine nucleotides is due to the control of VDAC by certain cytoskeleton proteins, specifically by $\alpha\beta$-tubulin (Rostovtseva and Bezrukov 2008; Rostovtseva et al. 2008). Reconstructing purified VDAC into planar phospholipid membranes, author showed that dimeric tubulin (which in nanomolar concentration is insufficient for polymerization in the absence of GTP and Mg^{2+}) induced the reversible voltage-dependent partial block of the channel. The addition of dimeric tubulin to isolated heart mitochondria induced the decrease in the affinity of oxidative phosphorylation for free ADP (i.e. the increase in the apparent K_m for free ADP) (Fig. 4b). Rostovtseva et al. proposed the model for the control of the VDAC permeability by tubulin which is based on the interaction of positively charged domain of VDAC with negatively charged C-terminal tail of tubulin (Fig. 4c) (Rostovtseva and Bezrukov 2008; Rostovtseva et al. 2008). This model is in concord with the results of our recent immunolabelling studies on localization of tubulin isoforms in permeabilized heart cells which directly shows the connection of beta II tubulin with mitochondria (Fig. 4d).

By continuously removing extramitochondrial MgADP, the PEP–PK system allows us to avoid its stimulatory effect on respiration (Fig. 3c). Under these conditions, the MtCK reaction activated by MgATP and creatine controls oxidative phosphorylation via the MgADP produced inside the mitochondrial intermembrane space. We applied this protocol for investigation of the MtCK kinetic properties in permeabilized cardiac cells in situ. A series of experiments were performed in the presence of different fixed concentrations of MgATP and stepwise raised concentrations of creatine, to allow us to estimate kinetics parameters of the

Table 1 Complete kinetic analysis of the forward MtCK reaction in heart mitochondria

	K_{ia}^{app}MgATP (mM)	K_{a}^{app}MgATP (mM)	K_{ib}^{app}Cr (mM)	K_{b}^{app}Cr (mM)	K_{ip}^{app} PCr (mM)
Mitochondria in vitro					
−OxPhos	0.92 ± 0.09	0.15 ± 0.023	30 ± 4.5	5.2 ± 0.3	
+OxPhos	0.44 ± 0.08	0.016 ± 0.01	28 ± 7	5 ± 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

The decrease by about order of magnitude of the apparent constant of dissociation of MgATP from its ternary complex with MtCK under conditions when MgATP is regenerated by oxidative phosphorylation (OxPhos) in isolated mitochondria point on the presence of functional coupling of MtCK with OxPhos controlling MgATP direct transfer

In permeabilized cardiomyocytes in situ this constant is increased by two orders of magnitude, showing selective limitation of the permeability of the outer mitochondrial membrane for ATP

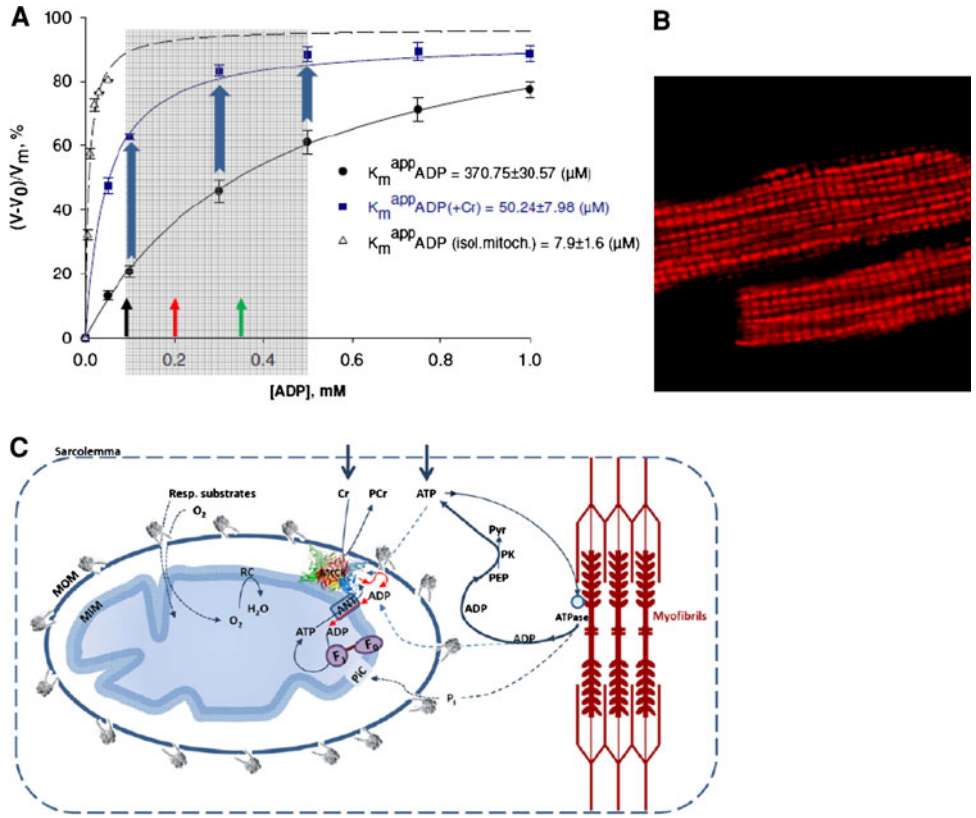


Fig. 3 **a** Michaelis–Menten representation of ADP-stimulated respiration of isolated heart mitochondria (*empty triangles*) and permeabilized cardiomyocytes measured under conditions of the absence (*full circles*) and presence of creatine (*full squares*). *Colour arrows* correspond to the fluctuations of ADP concentrations subsequent to different heart workloads. The *gray rectangle* delimits area of physiological cytosolic ADP concentration. Outer membrane of isolated mitochondria being highly permeable for adenine nucleotides limits the respiration regulation by free ADP. The maximal respiration rate is achieved by minimal ADP concentrations equivalent to the minimal workload. When the ADP diffusion is restricted at the level of MOM, as in mitochondria in situ, the respiration rates become linearly dependent on ADP concentrations. This linear dependence under physiological conditions can be amplified by creatine in the presence of activated MtCK. So, this *graph* explains the role of MOM permeability in feedback regulation. If there is no restriction of diffusion, there is no regulation (adapted from Guzun et al. 2009). **b** Confocal image of isolated rat cardiomyocyte. Mitochondrial labelling with MitoTracker Red. **c** Protocol of the method used to study interaction between mitochondrial and glycolytic systems in competition for endogenous ADP. Exogenous ATP is hydrolyzed by cellular ATPases into endogenous extramitochondrial ADP and inorganic phosphate (Pi). Mitochondrial (MtCK) and non-mitochondrial creatine kinases in myofibrils and at membrane of sarcoplasmic reticulum in the presence of creatine and ATP produce endogenous intra- and extramitochondrial ADP. Phosphoenolpyruvate (PEP) and pyruvate kinase (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 mitochondrial intermembrane space (IMS) and is re-imported into the matrix via adenine nucleotide translocase (ANT) due to its functional coupling with MtCK

MtCK reaction in the cells in situ (Guzun et al. 2009). These results are also shown in Fig. 5a and Table 1. Figure 5a shows rapid activation of respiration by creatine under these conditions. Figure 5b shows that this is directly related to the control of the mitochondrial membrane potential. On the other hand, the remarkable decrease in affinity for MgATP, seen as an increase in the apparent constant of dissociation for MgATP from ternary enzyme–substrates complex (MtCK.Cr.MgAT) (Table 1) is due to the restriction of MgATP diffusion at the level of mitochondrial outer membrane (MOM) in permeabilized cardiomyocytes in comparison with isolated mitochondria. Significant decrease in ATP diffusion through MOM results in the energy export out of the mitochondria almost exclusively by PCr. Concomitant measurements of PCr production and oxygen consumption through the experiment in which the respiration is stimulated by creatine in the presence of activated MtCK and removed extramitochondrial ADP, showed that the P/O ratio is close to theoretical value ~6 (Fig. 5c) allowing us to assume that PCr is the main energy flux exported from cardiac mitochondria (Timohhina et al. 2009). These experimental results fit and

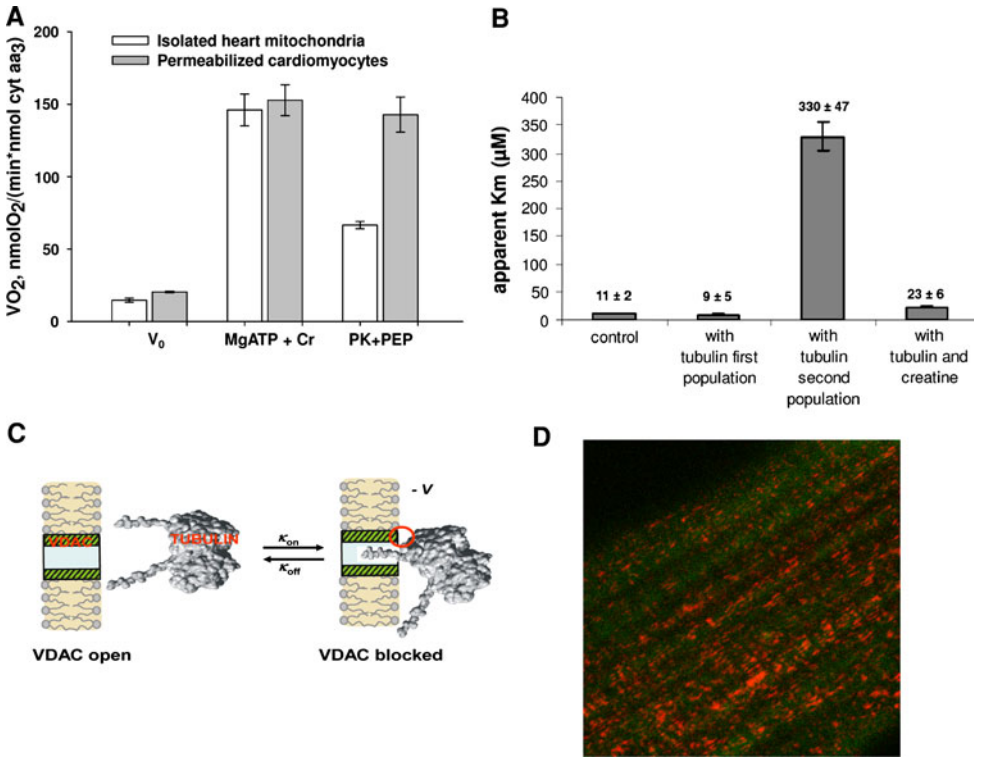


Fig. 4 **a** Full compartmentalization of intramitochondrial ADP produced by MtCK in the intermembrane space of mitochondria in permeabilized cardiomyocytes in situ. Respiration was activated by addition of MgATP (5 mM) and creatine (20 mM). Stable and maximal respiration rates are supported by MtCK activity in the presence of ATP and creatine. Under these conditions, approximately 50% of ADP, produced in the intermembrane space, can be trapped by the powerful PEP–PK system in the case of isolated heart mitochondria. The respiration is not completely inhibited because of the presence of direct ADP transfer from MtCK to ANT. In permeabilized cardiomyocytes in situ when mitochondrial 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. **b** Tubulin dramatically increases apparent K_m for ADP in regulation of respiration of isolated brain mitochondria. In the presence of creatine, the apparent K_m for ADP is again decreased. These effects reproduce the observations on permeabilized cardiomyocytes shown in Fig. 3a (adapted from Rostovtseva et al. 2008). **c** Model of tubulin–VDAC interaction. One tubulin C-terminal tail partially blocks channel conductance by entering VDAC pore. This process is voltage dependent and could be described by the first-order reaction of one-to-one binding of tubulin to VDAC. Some additional interaction between tubulin globular body and VDAC may be involved (adapted from). **d** Confocal image of immunofluorescent labeling of tubulin βII co-localisation with VDAC in fixed cardiomyocytes. The anti-tubulin βII antibody (Abcam, ab28036) with the Cy5 (Jackson Immunoresearch 115-175-146) antibody (*red color*) were used to label tubulin βII isoform, and the anti-VDAC1/2/3/ antibody IgG-FITC (*green color* Santa Cruz Biotechnology; sc-98708) were used to label VDAC. As we can see in a part tubulin βII co-localizes with VDAC

explain perfectly the earlier observation by Belitzer and Tsybakova (1939).

Thus, the apparent kinetic parameters of the MtCK reaction, its direction, velocity of reaction and the ability to function in non-equilibrium steady state are in a tight dependency upon the functional interaction of MtCK with ANT and VDAC due to the orchestrated functioning of oxidative phosphorylation/ANT/MtCK/VDAC selective permeability of which is governed by tubulin. All they may be grouped into structure–functional unit, a supercomplex that we called mitochondrial interactosome (MI) (Fig. 6a). The following complexes were integrated into the mitochondrial interactosome: ATP synthasome (Chen et al. 2004; Pedersen 2007, 2000), the functional complexes formed by MtCK, ANT, VDAC and tubulin (Saks 2007; Wallimann et al. 1992; Dolder et al. 2003; Timohhina et al. 2009) (Fig. 6a). This unit can also include the super complex formed by the respiratory chain (Lenaz and Genova 2007; Vonck 2009). The role of interactosome is to ensure the cycling of adenine nucleotides in mitochondria, coupled to PCr synthesis and facilitate the export of the free energy of mitochondrial ATP phosphorylation potential as energy of PCr fluxes. Functioning of MI is effectively controlled by creatine concentration and PC/Cr ratio. Interestingly, this supercomplex is significantly altered in cancerous HL-1 cells of cardiac phenotype (Fig. 6b). The analysis of mechanisms of energy metabolism regulation and MI

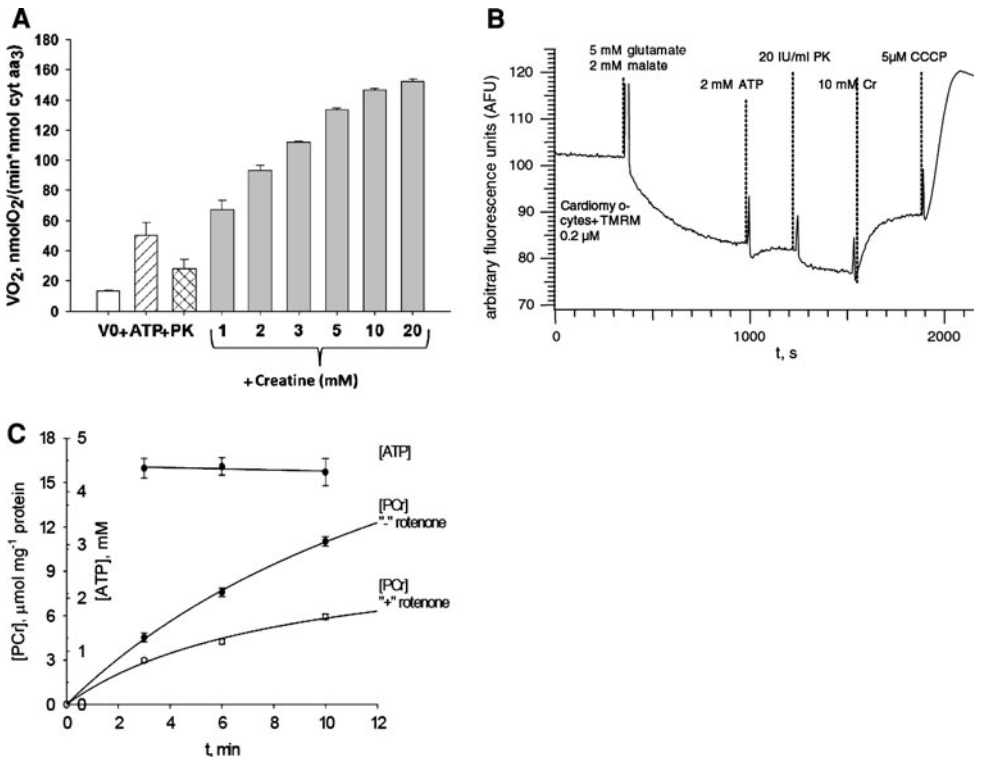


Fig. 5 **a** High efficiency of creatine in the control of respiration of permeabilized cardiomyocytes. The addition of 2 mM MgATP activates respiration due to production of endogenous MgADP in ATPase reaction. Pyruvate kinase (PK) and phosphoenolpyruvate (PEP) added to experimental milieu remove the extramitochondrial MgADP and thus inhibit respiration. Creatine under these conditions rapidly activates mitochondrial creatine kinase (MtCK) reaction and subsequently local MgADP recycling and oxygen consumption rate to the maximal values. **b** Measurement of mitochondrial membrane potential by using the fluorescence of TMRM (0.2 μM). The protocol described in Fig. 5a was applied in this experiment. The addition of 5 mM glutamate and 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, activation of MtCK and mitochondrial respiration by the addition of 10 mM creatine decreased membrane potential to a lower steady state level. The uncoupling agent CCCP 5 μM was used to dissipate the membrane potential. **c** The rate of phosphocreatine production by mitochondrial and cytoplasmic creatine kinases in permeabilized cardiomyocytes was measured using ion pair HPLC/UPLC during the experiment described in Fig. 5a. Experiments were performed under conditions of activated (*full circles*) and inhibited (*empty circles*) complex I of the respiratory chain and with addition of creatine in concentration of 20 mM. The ATP level, continuously regenerated by the PEP–PK system, was stable during the experiment. The between VO_2 (measured by oxygraphy represented in Fig. 5a) and VPCr was about 5.7. Adapted from Timohhina et al. (2009)

structure of tumour cardiac cells (NB HL-1 cells line) confirms this hypothesis. Mitochondria of NB HL-1, cells in comparison with adult cardiomyocytes, are filamentous and in a continuous fusion/fission (Pelloux et al. 2006; Beraud et al. 2009). The affinity of oxidative phosphorylation for free ADP is high (K_m^{app} ADP = 25 ± 4 μM) and identical to that of isolated heart mitochondria surrounded by a homogeneous medium (Anmann et al. 2006). The high affinity for free ADP indicates the important permeability of MOM for adenine nucleotides. Respiration of NB HL-1 cells cannot be stimulated by the addition of creatine. Eimre et al. (2008) and Monge et al. (2009) showed that NB HL-1 cells have a glycolytic metabolic profile characterized by a low activity of the total creatine kinase and a high activity of hexokinase II (Eimre et al. 2008; Monge et al. 2009). These properties are characteristic to energy metabolism of cancer cells, described by Otto Warburg in 1956, and more recently elucidated in Pedersen's work (Warburg 1956; Pedersen 2007).

Metabolic control analysis: increased flux control coefficients in mitochondrial interactosome

Both theoretically and experimentally, the most effective quantitative method for identification of the regulatory mechanisms in the complex metabolic systems is metabolic control analysis developed about 35 years ago by Heinrich and Rapoport and Kacser and Burns (Kholodenko et al. 1995). In this approach, the relative changes of total flux J through a metabolic system is studied in dependence of the fractional change in activity of the given enzyme *k*, and the role of the latter in flux regulation is quantitatively described by its flux control coefficient:

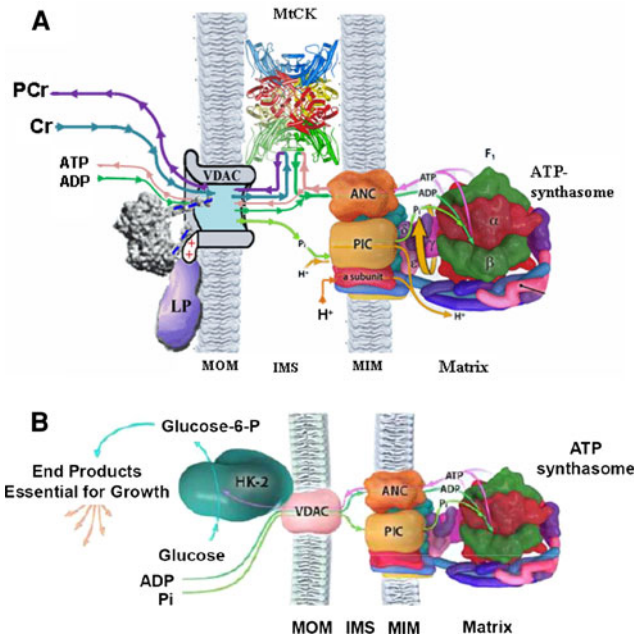


Fig. 6 **a** Mitochondrial interactosome of adult cardiomyocyte consists of functionally coupled supercomplex of ATP synthasome (formed by the ATP synthase, adenine nucleotides carrier, ANC, and phosphate carrier (PIC) Pedersen 2007, 2000), mitochondrial creatine kinase and VDAC with bound protein (tubulin and/or linker protein, LP) (Timohhina et al. 2009). MtCK transfer the phosphate group from mitochondrial ATP to creatine producing PCr and recycling ADP. Recycled ADP is returned to ATP synthasome due to its functional coupling with MtCK. PCr leaves mitochondria as a main energy flux due to high selective permeability of VDAC for this compound. **b** In HL-1 cancer cells of cardiac phenotype MtCK is absent and ATP synthesized by ATP synthasome leaves mitochondria. This ATP is used by HK-II, interacting with the VDAC in the outer mitochondrial membrane (effect Warburg) Pedersen (2007)

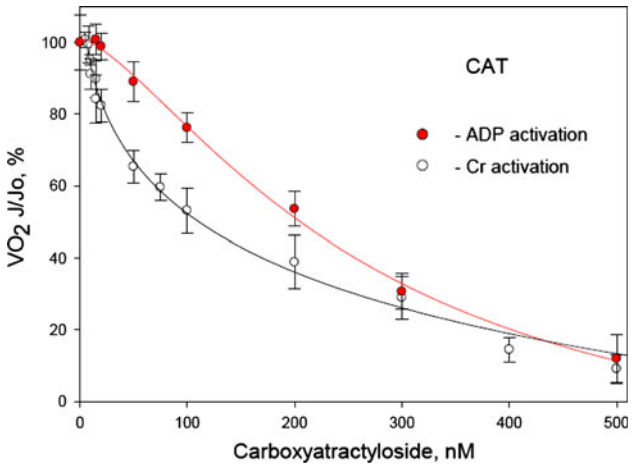


Fig. 7 The effect of functional coupling of mitochondrial CK and local ADP regeneration in the vicinity of adenine nucleotide translocase (ANT) on the flux control coefficient of the ANT. Inhibitor titration experiments were performed with a specific and irreversible inhibitor ANT carboxyatractyloside (CAT). Two titrations with carboxyatractyloside are shown, one under conditions of simple ADP activation of respiration by externally added ADP, at its saturating concentration (1 mM) (*filled circles*, *red curve*) and one under conditions of CK-ANT coupling and local ADP recycling, in the presence of activated CK (added creatine) and PK-PEP ADP trapping system (*open circle*, *black curve*). At similar rates of respiration, the same amount of carboxyatractyloside had a dramatically more pronounced inhibitory effect in coupled system, when compared with maximal respiration activated by externally added 1 mM ADP. This indicates that the flux control coefficient of ANT is significantly larger in the presence of functionally coupled CK. In both cases, values of the flux control coefficients for ANT were determined experimentally and calculated with the graphical method (Gellerich et al. 1990) from the initial slopes of the titration curves of respiration with CAT, estimated by linear regression of the first titration points

$$C_k^j = \frac{v_k}{J_j}\frac{\delta J_j}{\delta v_k}$$

For mitochondria in vivo, the total flux is the rate of respiration, VO_2, and the activities of different complexes of mitochondrial interactosome can be changed by using their irreversible inhibitors (Gellerich et al. 1990). Figure 7 shows the results of experimental determination of the flux control coefficients for ANT which was inhibited by increasing the concentration of carboxyatractyloside (CAT), for two different conditions: for direct activation of respiration by ADP in saturating concentration of 2 mM (ADP activation), and for conditions when respiration is controlled by the MtCK reaction in the presence of MgATP (2 mM), creatine (10 mM) and PK–PEP system. In the latter case, adenine nucleotides ADP and ATP are being continuously recycled in the mitochondria in the functionally coupled system within mitochondrial interactosome. Theoretical analysis control theory of metabolic channeling performed by Kholodenko, Cascante and Westerhoff some years ago revealed that in the system with metabolic channeling and functional coupling the control coefficients should be increased (Kholodenko et al. 1995). This is exactly what we see in Fig. 7: for direct activation of respiration by ADP the flux control coefficient for ANT is 0.21, and for the MtCK-activated respiration the flux control coefficient increases to 0.93 (unpublished results). Thus, the functional coupling between MtCK and ANT in MI strongly increases the efficiency of the control of mitochondrial oxidative phosphorylation by intracellular metabolic factors. Recycling of ADP in MI is controlled much more effectively than direct supply of ADP via ANT. The rate of this recycling of adenine nucleotides is controlled by creatine via MtCK reaction. It has also been shown that such a recycling of ADP and ATP in MI decreases the rate of reactive oxygen species (ROS) production (Meyer et al. 2006) and PTP opening (Dolder et al. 2003).

Systems biology of the creatine kinase pathway in the heart: feedback mechanism of regulation of energy fluxes

Localization of cytosolic MM creatine kinase in myofibrils and at the membranes of sarcoplasmic reticulum and sarcolemma has been described in details in previous studies (Wallimann et al. 1992, 2007; Saks et al. 2007a, b). These creatine kinases catalyze the reaction in the opposite direction, when compared with mitochondria: the use of PCr for rapid regeneration of local ATP (Guzun et al. 2009). Thus, in the creatine kinase pathway creatine kinases operate in mitochondria and at the sites of ATP utilization in the opposite direction in the non-equilibrium state (Guzun et al. 2009). In cardiac cells, the mitochondria and the whole phosphocreatine pathway are organized into regularly arranged intracellular energetic units (Saks et al. 2001) (Fig. 1). These units including phosphocreatine pathway are the results of specific interactions in the cells and best described by Systems Biology approaches in Molecular System Bioenergetics (Saks 2007). These specific interactions result in new, system level properties which do not exist if the system components are isolated (Saks 2007). Examples of these important new properties are compartmentalization of metabolites and enzymes, including creatine kinases in the cells, metabolic channeling and functional coupling—all characteristic for the PCr/CK pathway of energy transfer. One of the results of application of this approach is description of the cellular mechanisms of metabolic aspects of Frank–Starling law of the heart (Starling and Visscher 1926; Saks et al. 2006). Systems Biology approach relies on the use of mathematical models for description of complex phenomena (Saks 2007). Compartimentalized energy transfer by the creatine kinase system has been described mathematically by Vendelin–Aliev–Saks (VAS) model using enzyme localization data and their kinetics, metabolic channeling, functional coupling and limitation of MOM permeability (Aliev and Saks 1997; Vendelin et al. 2000, 2004; Dos Santos et al. 2000). The model describes well the Frank–Starling phenomenon under conditions of metabolic stability (Saks et al. 2006). According to the VAS model and the results of in vivo studies of heart energy metabolism using nuclear magnetic resonance spectroscopy by ^{31}P (Honda et al. 2002; Spindler et al. 2001), the ATP hydrolyzed in ATPase reactions during cyclic contractions is associated with cyclic oscillations of ADP, PCr/Cr ratio, Pi and proton concentrations in myofibrils (Fig. 8). The Pi is not consumed in the MMCK reaction, but diffuses freely and enters mitochondrial matrix via its carrier (PIC). The local impulse of ADP concentration is used up mainly in the MMCK reaction, due to its non-equilibrium state, and at the same time forms a gradient of ADP concentration transmitted towards the mitochondrial matrix. The rephosphorylation of ADP in the MMCK reaction using PCr changes the PCr/Cr ratio in the direction of the increase of creatine concentration. These signals are transferred towards MtCK via CK/PCr shuttle. ADP signal reaches mitochondria and increases ATP regeneration. Regenerated ATP, due to the MtCK-ANT functional coupling, supplies the MtCK reaction (which is also in non-equilibrium steady state). Thus, the cytosolic ADP signal is transformed into a PCr amplified energy flux which is maintained by the MtCK reaction due to its non-equilibrium steady-state, local turnover of ATP/ADP between ATPsynthase and MtCK within mitochondrial interactosome. The efficiency of PCr production by MtCK in cardiomyocytes is close to the maximum efficiency of oxidation phosphorylation ($PCr/O_2 = 5.7 \pm 0.7$) (Timohhina et al. 2009; Guzun et al. 2009). By applying the general principle of feedback regulation proposed by Norbert Wiener (1947), one can represent the mechanism of adjustment of intracellular ATP requirement to mitochondrial ATP regeneration through feedback signalling fluxes (cytosolic Cr/PCr, ADP, Pi) and energy fluxes (PCr) transformed in non-equilibrium steady-state compartmentalized CK reactions and ATPases reactions (Saks et al. 2007a, b; Guzun et al. 2009). The mechanism of feedback regulation of mitochondrial ATP regeneration tends to maintain the fundamental property of living system, intracellular metabolic stability (Dos Santos et al. 2000; Balaban et al. 1986; Williamson et al. 1976).

Mitochondrial interactosome in pathology, clinical effects of phosphocreatine (Neoton)

In myocardial infarction and in heart failure, rapid decrease in PCr content occurs due to lack of oxygen supply and pathological changes in the creatine kinase system (Neubauer 2007; Neubauer et al. 1997; Wyss and Kaddurah-Daouk 2000; Wallimann et al. 2007; Saks et al. 2007a, b). Total ATP content usually changes very slowly and its changes, as well as changes in the free energy of ATP hydrolysis calculated from total metabolites' contents, are dissociated from the rapid fall of the cardiac contractile force (Neely et al. 1973; Kammermeier et al. 1990), total depletion of ATP resulting in contracture of heart muscle (Koretsune and Marban 1990). Rapid decline in heart contractile function in hypoxia and ischemia are most likely to be related to changes in compartmentalized energy transfer systems, leading to decreased regeneration of ATP in functionally important cellular compartments, as shown in Fig. 1. First, rapid decline in ATP regeneration in subsarcolemmal area results in changes in ion currents across this membrane and thus in shortening of action potential (Dzeja et al. 2007; Saks et al. 2007a), and secondly, rapid

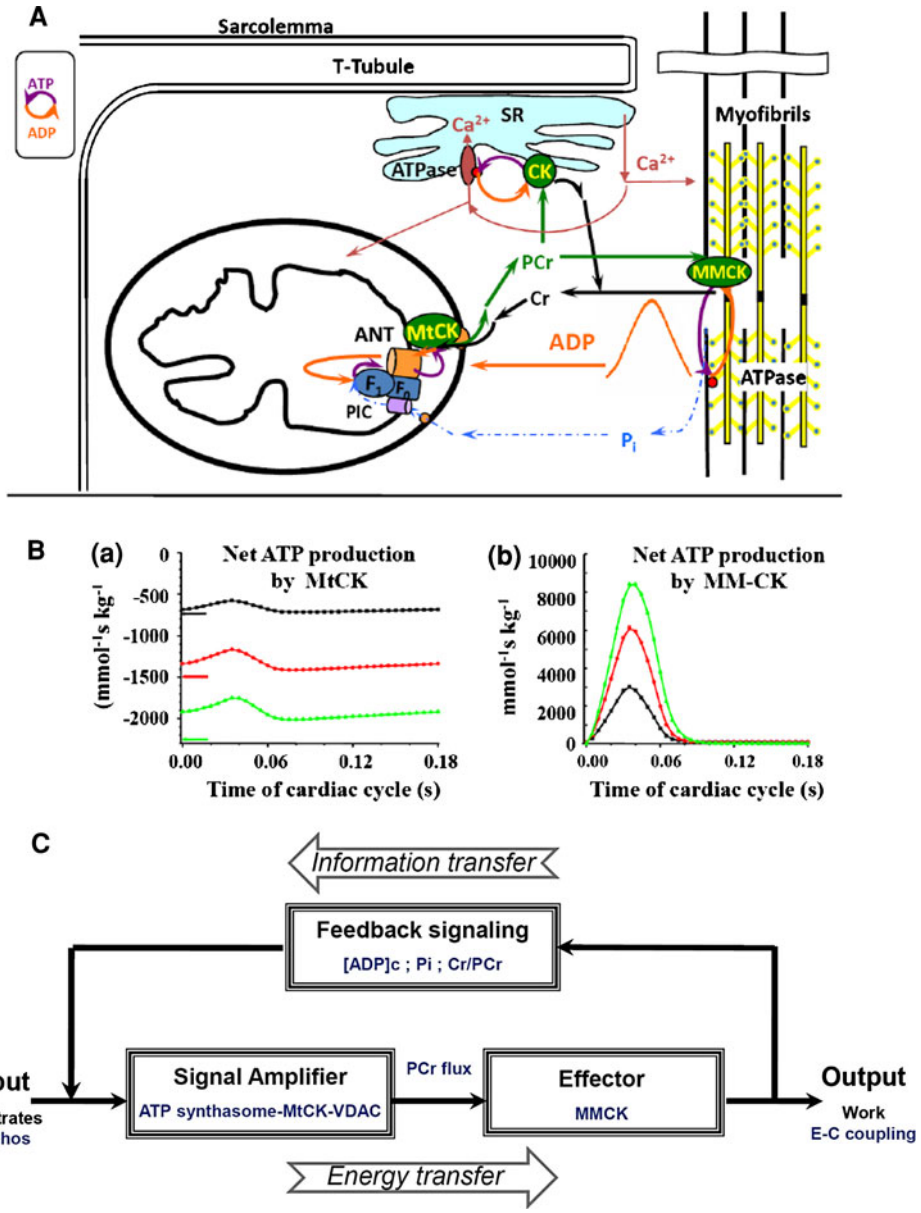


Fig. 8 **a** Intracellular energy unit of cardiomyocytes in which the sites of ATP utilization (ATPases of sarcoplasmic reticulum, of myosin, of Na^+/K^+ pumps) are connected to the site of ATP regeneration (F_0F_1 represents oxidative phosphorylation) by the phosphotransfer shuttle (CK/PCr). **b** The ATP hydrolysis during muscle contraction produces a fluctuation of ADP concentrations (Fig. 8 **b**-*b*) associated with the release of Pi. A part of released ADP is rephosphorylated by MMCK using PCr. Produced creatine serves as a feedback metabolic signal for mitochondrial respiration. Another part of ADP creates a concentration gradient from the core of myofibril toward mitochondrion, and is used to regenerate the mitochondrial ATP while entering the matrix via the ANT. Regenerated ATP supply MtCK reaction increasing local ATP/ADP turnover between ATP synthasome and MtCK with continuous PCr production (Fig. 8 **b**-*a*). **c** Feedback regulation of mitochondrial respiration within intracellular energy units in vivo. In this scheme, the ATP consumption to complete cellular work, marked as output, and ATP regeneration are interconnected via the feedback signalling through cytosolic ADP, Pi and Cr/PCr. PCr produced in MtCK reaction from mitochondrial ATP is exported towards intracellular milieu as energy flux and is used for the local ATP regeneration by myofibrillar MMCK [adapted from Guzun et al. (2009)]

decline in ATP regeneration in myofibrillar microcompartments due to lack of phosphocreatine slows down the contraction cycle (Saks et al. 2007a, b). Similar but slower changes are observed in chronic cardiac and skeletal muscle diseases (Nascimben et al. 1996; Ingwall 2004, 2006, 2009; Shen et al. 2005; Tian and Ingwall 1996; Weiss et al. 2005; Mettauer et al. 2006; Ventura-Clapier et al. 2002; De Sousa et al. 2000). In concord with this conclusion are the results published by Weiss et al. (2005) showing that cardiac ATP flux through CK is reduced by 50% in cases of human heart failure in the absence of reduction of ATP stores. Local phosphotransfer networks

in the subsarcolemmal area are an important part of the membrane sensors of the cellular energy state also in brain cells (Wallimann et al. 2007; Andres et al. 2008; Burklen et al. 2006; Adhihetty and Beal 2008), this explaining the dependence of functional state of these cells on phosphocreatine supply, and thus the central importance of the PCr/Cr system. In brain cells, ubiquitous mitochondrial creatine kinase is co-expressed with cytosolic BB isoenzyme localized both in cytoplasm and at the cell membrane. Alterations of these systems are observed in many neurodegenerative diseases (Andres et al. 2008; Burklen et al. 2006).

Both in experiments on frog hearts (Saks et al. 1976, 1978) and in patients with heart diseases (Nascimben et al. 1996) the significant decrease in intracellular creatine level resulting in decreased content of PCr has been shown to result in the hypodynamic state characterized by decreased contractility. In experiments, restorations of the intracellular Cr level by its inclusion into perfusate allowed completely normalize the PCr content and the contractile force (Saks et al. 1976).

All these results are in concord with the very early observation by Lundsgaard that intracellular PCr is one of the important factors of regulation of the force of muscle contraction (Lundsgaards 1930).

Intensive and numerous studies have been carried out on transgenic mice with knockout of different CK isoenzymes, or enzymes responsible for creatine metabolism and transport (Ingwall 2009; Ventura-Clapier et al. 2002; ten Hove et al. 2005). Despite multiple adaptive mechanisms—activation of alternative phosphotransfer pathways, such as adenylate kinase shuttle (Ventura-Clapier et al. 2002; Dzeja et al. 1996), structural changes in the cells and increase in oxidative capacity of skeletal muscle (Ingwall 2004; Ventura-Clapier et al. 2002), and many others (Ingwall and Weiss 2004), significant functional and metabolic changes especially related to calcium metabolism and contractile performance have been observed in these experiments (Ingwall and Weiss 2004; Momken et al. 2005; Spindler et al. 2004; Nahrendorf et al. 2006; ten Hove et al. 2005). Thus, Momken et al. (2005) have reported that double knockout of MtCK and MM CK very significantly impairs voluntary running capacity of mice. Knockout of enzymes of creatine biosynthesis in mice resulted in significantly reduced responses to inotropic stimulation (ten Hove et al. 2005). Similarly, hearts of rats treated with guanidinopropionic acid performed much less pressure–volume work (Kapelko et al. 1989). Most interestingly, recent works from Neubauer's laboratory have shown that overexpression of creatine transporter and supernormal myocardial creatine contents lead to heart failure (Phillips 2009; Wallis et al. 2005). In these hearts, creatine content is increased by more than a factor of 2 (Phillips 2009). Most interestingly, these experiments put into evidence the importance of the PCr shuttle: the heart failure may be due to the formation of dead-end complex CK.MgADP.Cr formation (Morrison and James 1965) and inhibition of PCr utilization for local ATP regeneration.

In summary, data obtained in experiments with CK knockout mice are in concord with our conclusion made in this work and before (Saks et al. 2006; Saks 2007; Wallimann et al. 1992; Saks et al. 2007a) that muscle (and other) cells are viable without MtCK and other CK isoenzymes, as HL-1 cells, but PCr-CK and other phosphotransfer pathways are necessary for effective energy transfer and metabolic regulation at higher energy demand, and thus for survival under stress conditions. An important observation is that exercise training results in cytoskeleton remodelling, including changes in Mitochondrial Interactosome and increased efficiency of energy transfer via PCr- CK pathway (Zoll et al. 2002; Walsh et al. 2001; Guerrero et al. 2005).

An important clinical study was performed by Neubauer's group in United Kingdom (Neubauer 2007; Neubauer et al. 1997). Using ^{31}P-NMR spectroscopy in combination with imaging for investigation of cardiac muscle energy metabolism in patients, the authors showed that in patients with cardiac disease—dilated cardiomyopathy (DCM) the decreased PCr/ATP ratio (lower than 1.6) is very clear and strong diagnostic index of increased mortality. In the heart of patients with DCM the ATP content remained the same as in healthy control patients, but PCr decreased by 70% as compared to control. This shows the vital importance of the phosphocreatine—creatine kinase energy transfer network for the cardiac muscle normal function and life (Neubauer 2007; Neubauer et al. 1997).

Thus, the PCr/ATP ratio is an important diagnostic parameter of heart disease, as is the total creatine content. Low PCr concentrations and low PCr/ATP ratios mean decreased regeneration of ATP by the PCr/CK system in microdomains (compartments) which are critically important for the function of the heart, skeletal muscle and brain. These microdomains are localised in myofibrils, near the sarcolemma and the membrane of sarcoplasmic reticulum in muscle cells and near cellular membrane in brain cells (see Fig. 1). There is a general consensus now among the researchers in muscle and brain energy metabolism that the further challenge and urgent need is to develop better bioprobes to image metabolic microdomains of ATP and functional proteomics to identify physical interactions between key proteins responsible for their formation (Neubauer 2007; Saks et al. 2007a, b; Weiss et al. 2005).

In addition to its important role in supporting regeneration of local ATP pools as a substrate for MM–CK reactions in myofibrils and cellular membranes, the PCr molecule appears to have another very useful property—membrane stabilizing action (Fig. 9c). This was revealed in

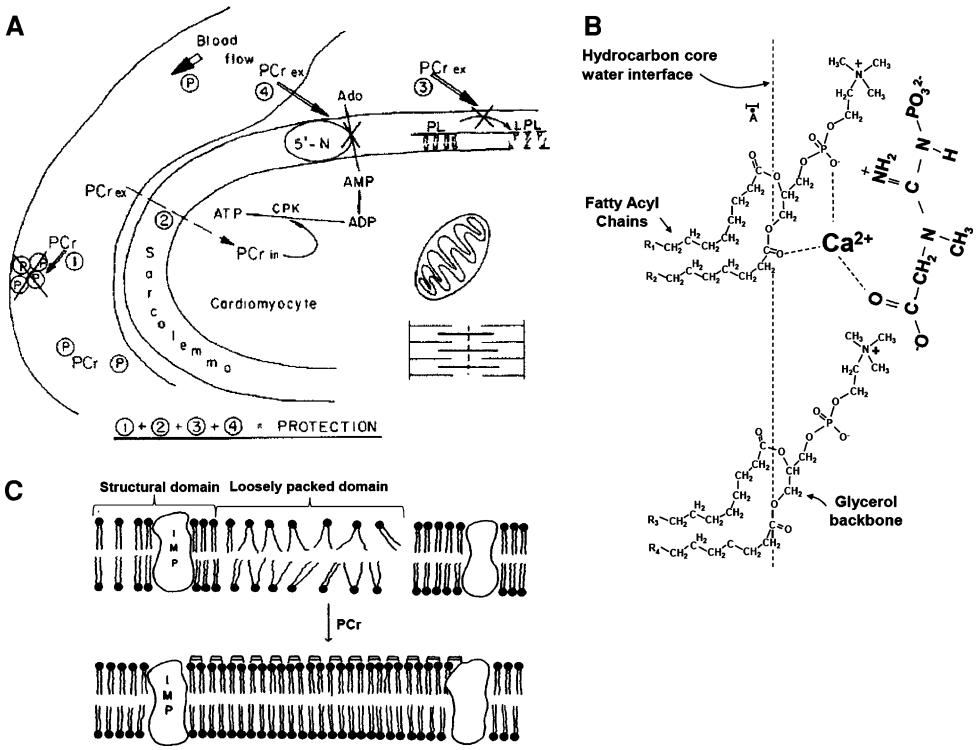


Fig. 9 **a** Scheme of the mechanisms of PCr protective action in cardiac ischemia. These mechanisms include *1* inhibition of platelet aggregation, extracellular mechanism, *2* possible penetration into the cells and participation in intracellular energy transfer (intracellular mechanism), *3* inhibition of accumulation of lysophosphoglycerides, *4* inhibition of 5′-nucleotidase of sarcolemma (adapted from). **b** Zwitterionic interaction of phosphocreatine with bipolar heads of phospholipid molecules in the membrane surface interphase. **c** Stabilization of the phospholipid bilayer by PCr molecules [adapted from Saks and Strumia (1993)]

long series of clinical use of extracellular phosphocreatine injection with clear protective effect on ischemic myocardium, and in detailed experimental studies (Semenovsky et al. 1987; Ruda et al. 1988; Robinson et al. 1984; Woo et al. 2005). In all these studies, extracellular phosphocreatine was used and shown to decrease the ischemic damage of heart muscle by multiple mechanisms (Fig. 9). Among others, there is clear membrane stabilizing effect of PCr (Saks and Strumia 1993) which may be explained by interaction of its zwitterionic molecule carrying positive and negative charges with opposite charges of phospholipids polar heads in the membrane surface interphase (Fig. 9b) that resulting in the transition of the mobile domain (fluid phase) of membranes into a structured domain (gel phase) as shown in Fig. 9c, leading to the decrease of the rate of phospholipids degradation into lysophospholipids and lipid peroxidation (Saks and Strumia 1993). Rapid fall of the intracellular PCr pool in hypoxia and ischemia may thus be a significant factor of destabilization of cellular membranes.

In conclusion, the PCr–CK pathway is as an efficient highway connecting ATP production and consumption sites. Without this highway, cells have to find other ways of ATP and energy transfer, but the efficiency of communication and regulation is lost and energy may be wasted (as in HL-1 cells). Under these conditions, muscle and brain cells degrade into a pathological state.

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