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

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

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

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Introduction

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

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

Materials and methods

Experimental protocols

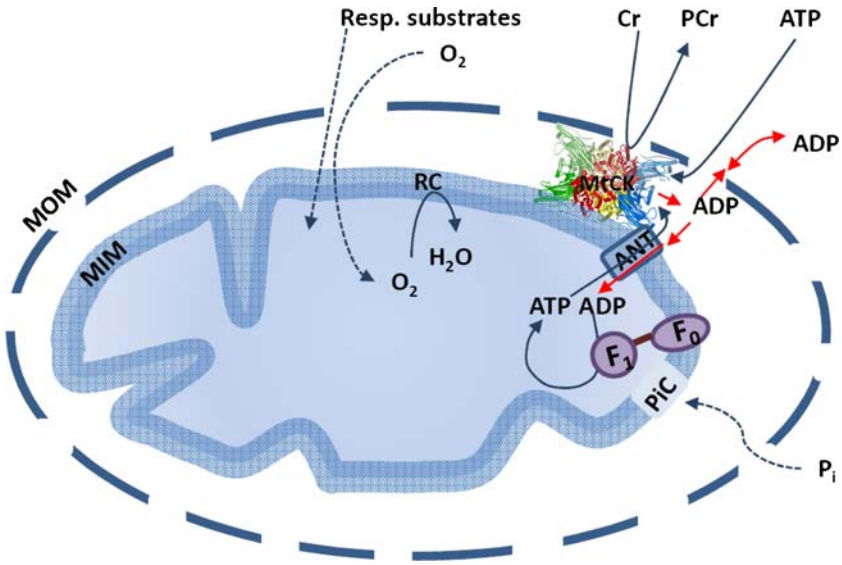
The principles of this study are illustrated by Schemes 1 and 2. Scheme 1 represents isolated mitochondria *in vitro* when mitochondrial creatine kinase is activated by addition of creatine, and Scheme 2 shows mitochondrion *in situ,* in permeabilized cardiac cells, surrounded by cytoskeleton proteins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

Isolation of mitochondria from cardiac muscle

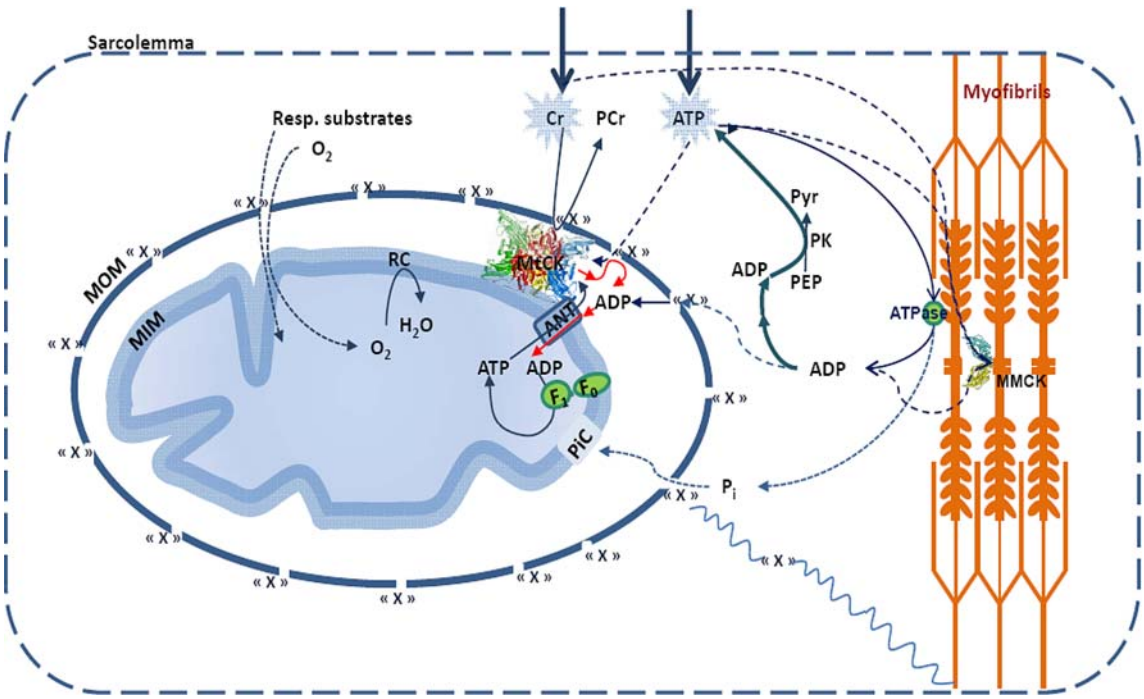
Mitochondria were isolated from adult rat hearts as described by Saks et al. 1975.

Isolation of adult cardiac myocytes

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



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



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

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

Permeabilization procedure

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

Measurements of oxygen consumption

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

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

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

Measurement of mitochondrial membrane potential

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

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

Measurements of mitochondrial cytochromes content

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

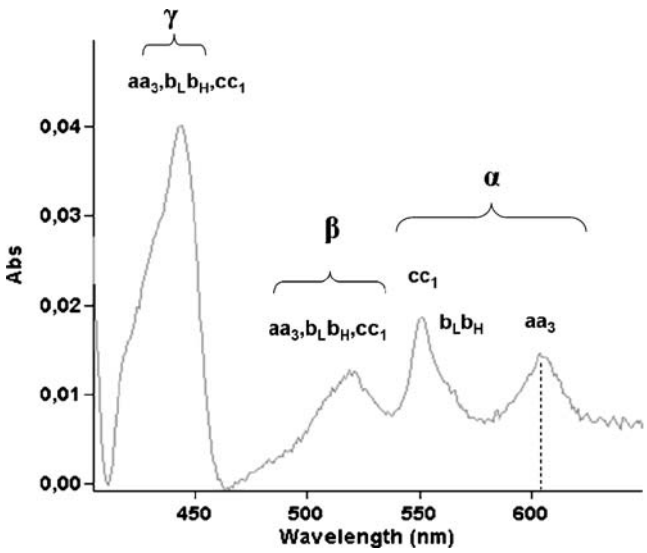


Fig. 1 The absorbance spectra of cytochromes (reduced versus oxidized), recorded by scanning the samples (isolated mitochondria or cardiomyocytes) from 530 to 650 nm in spectrophotometry

Isolation and purification of tubulin

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

Reconstitution studies

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

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

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

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

Results

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

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

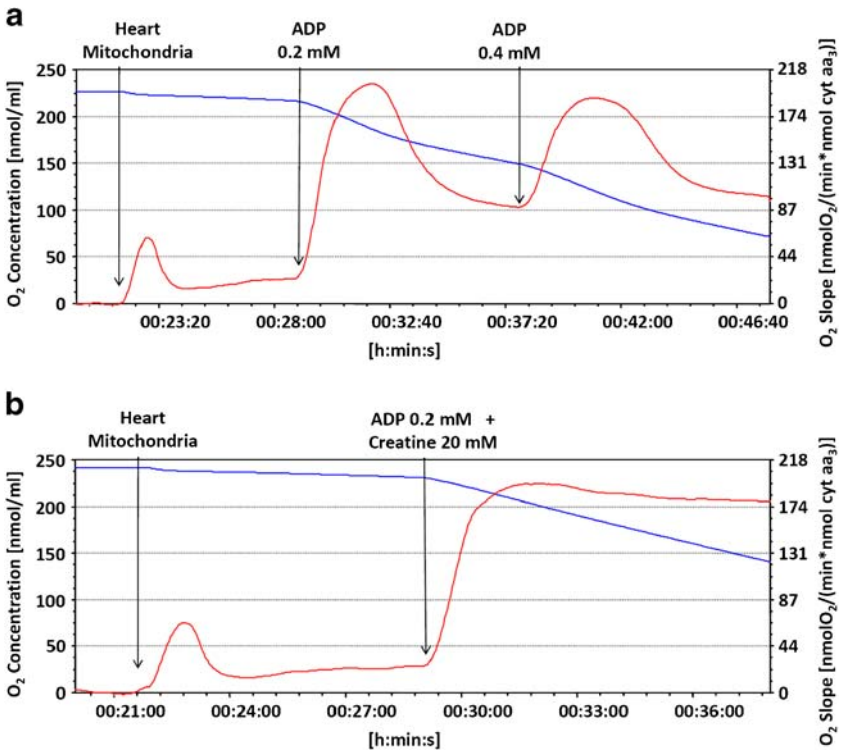


Fig. 2 **a** The classical respiratory control – transition between States 2, 3 and 4, according to Chance, in response to addition of limited amounts of ADP (0.2 mM; 0.4 mM) to isolated mitochondria, **b** Stable State 3 of respiration of isolated mitochondria stimulated by 0.2 mM ADP in the presence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

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

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

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

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

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

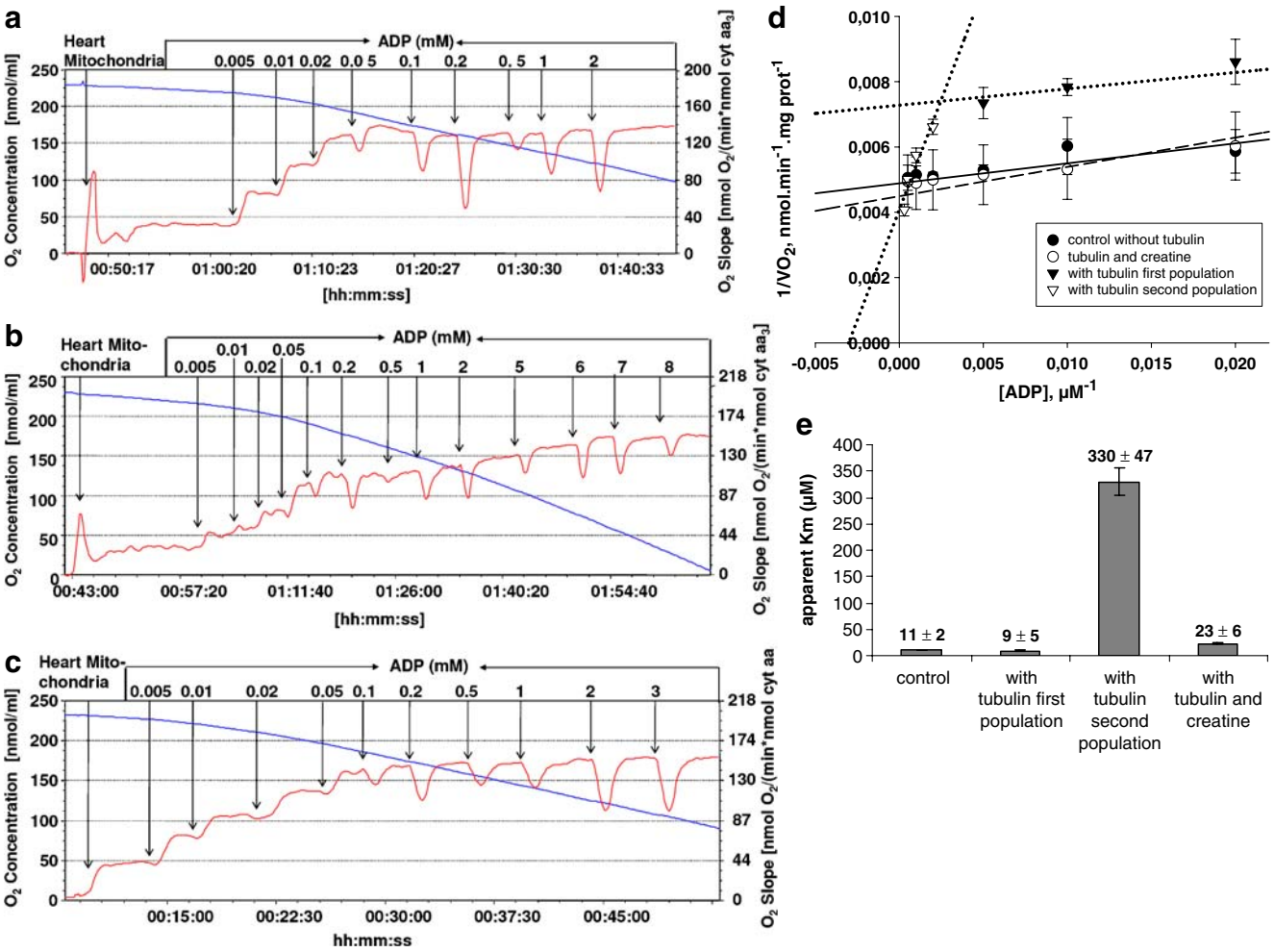


Fig. 3 **a** Oxygraph recording of the control kinetic of mitochondrial respiration activated by increasing concentrations of exogeneous ADP, **b** and **c** Oxygraph recording of the kinetics of mitochondrial respiration regulation in the presence of 1 μM tubulin (3B) and both 1 μM tubulin and 20 mM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

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

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

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

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

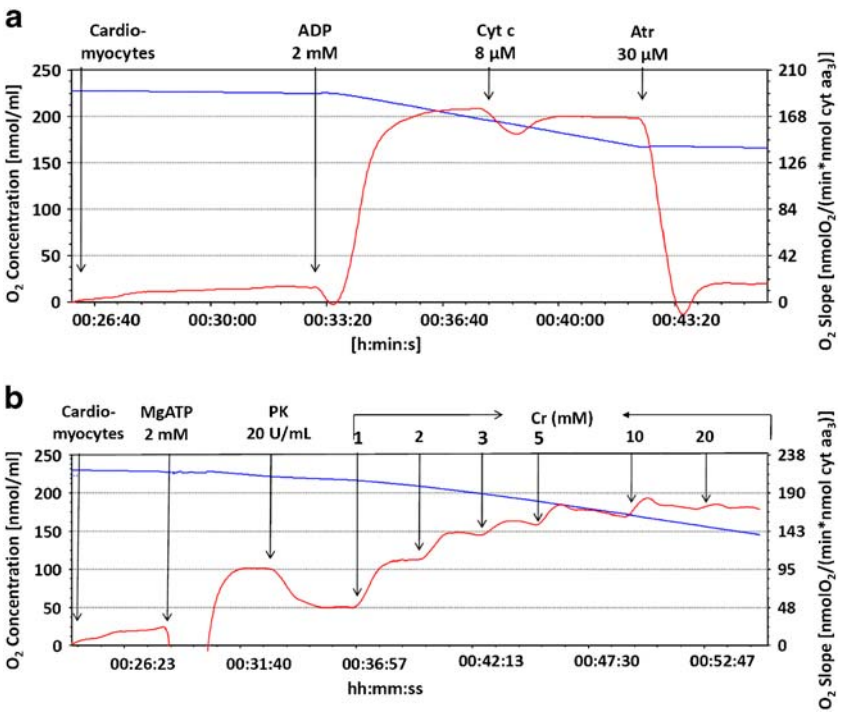


Fig. 4 **a** State 3 of mitochondrial respiration maintained in permeabilized cardiomyocytes. Cardiomyocytes were permeabilized with 25 μg/mL saponin in oxygraph cells during 10 min. Then respiration was activated in situ by addition of 2 mM exogenous ADP. Cytochrome c test shows intactness of MOM. Atractyloside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

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

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

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

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

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

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

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

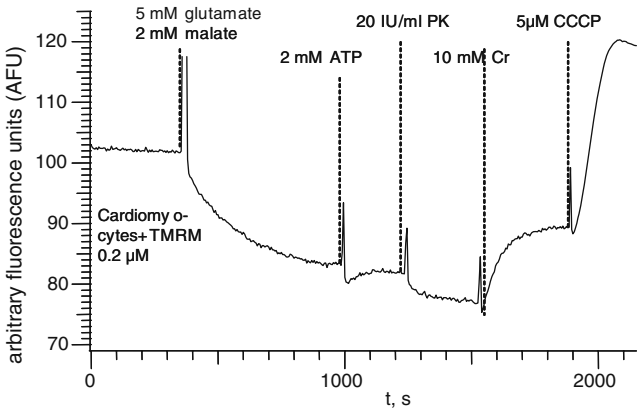


Fig. 5 Response of the TMRM fluorescence (excitation 548 nm, emission 574 nm) to mitochondrial respiration changes. Isolated cardiac cells were permeabilized with 25 μg/ml saponin and incubated in Mitomed solution supplied with 5 mM PEP and 0.2 μM TMRM in a thermostated fluorimeter cell. Addition of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

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

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

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

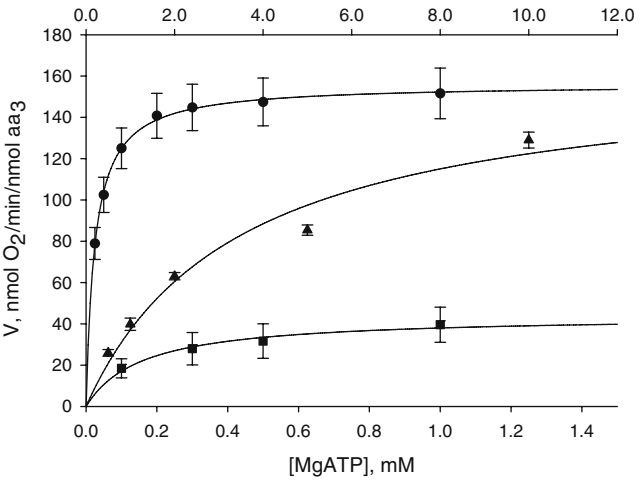


Fig. 6 Regulation of mitochondrial respiration as a function of the concentration of added exogenous MgATP in the absence (■) and the presence (●) of 20 mM Cr before addition of a PK-PEP system (bottom x-scale) and in the case of supplementation with PEP-PK system (▲, top x-scale). In the absence of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

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

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

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

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

Discussion

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

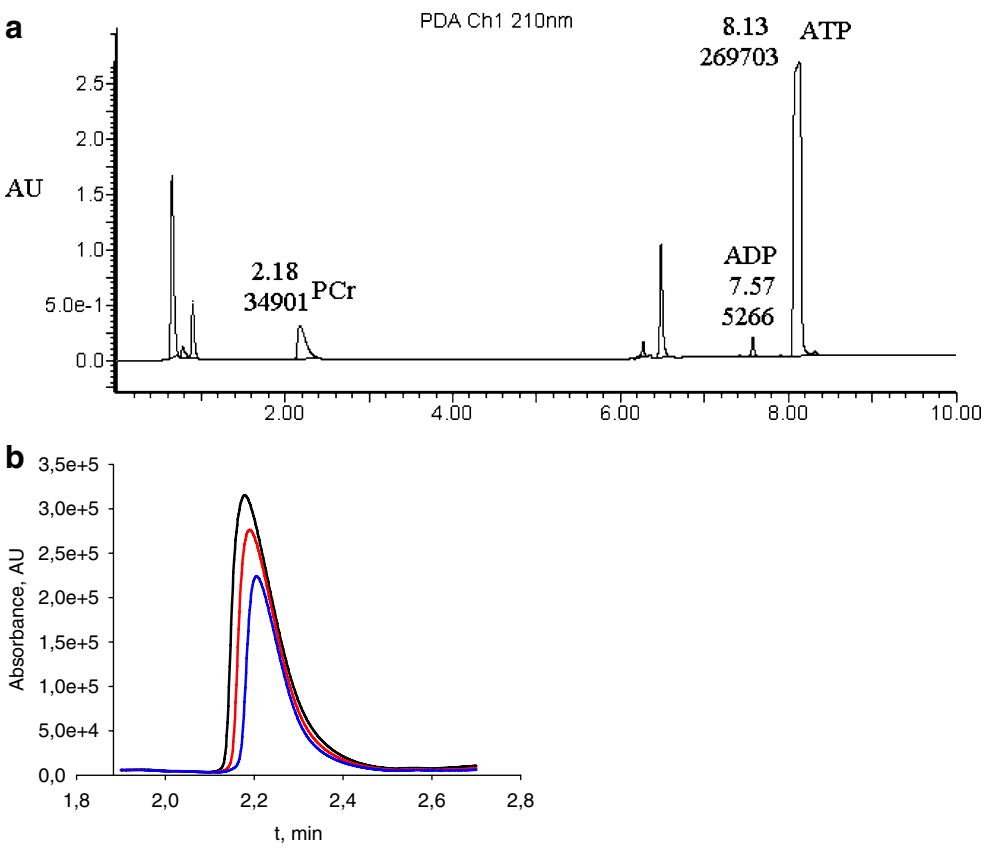


Fig. 7 **a** Chromatograms were obtained by and ACQUITY UPLC system from permeabilized cardiomyocyte incubations for 5 mM ATP. The samples of the reaction mixture were taken at specified time intervals after the initializing reaction. The positions of PCr and ATP traces on the retention time scale were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

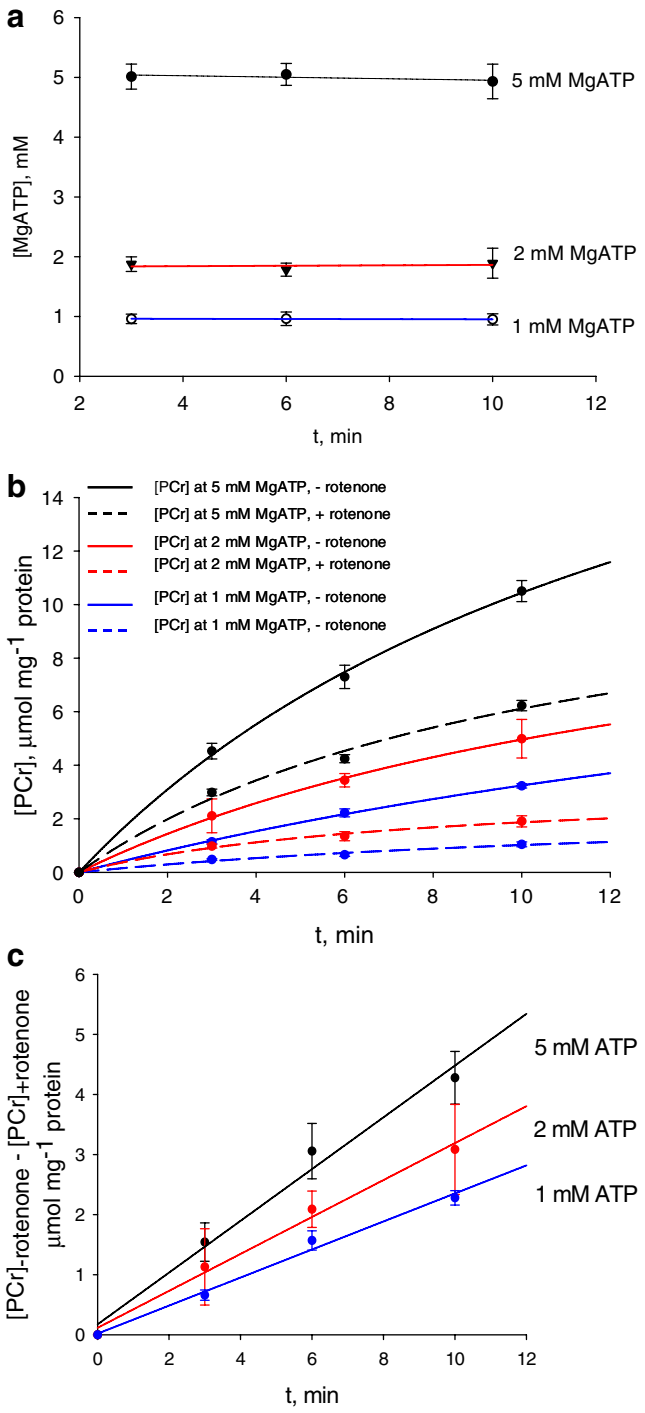


Fig. 8 **a** The ATP level, continuously regenerated by the PEP-PK system, was stable during the experimental procedure described in the Fig. 5, **b** The rate of phosphocreatine production by mitochondrial and cytoplasmic creatine kinases in permeabilized non-inhibited cardiomyocytes (solid lines).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

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

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

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

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

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

[b] V_{02} rate of oxygen consumption

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

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

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

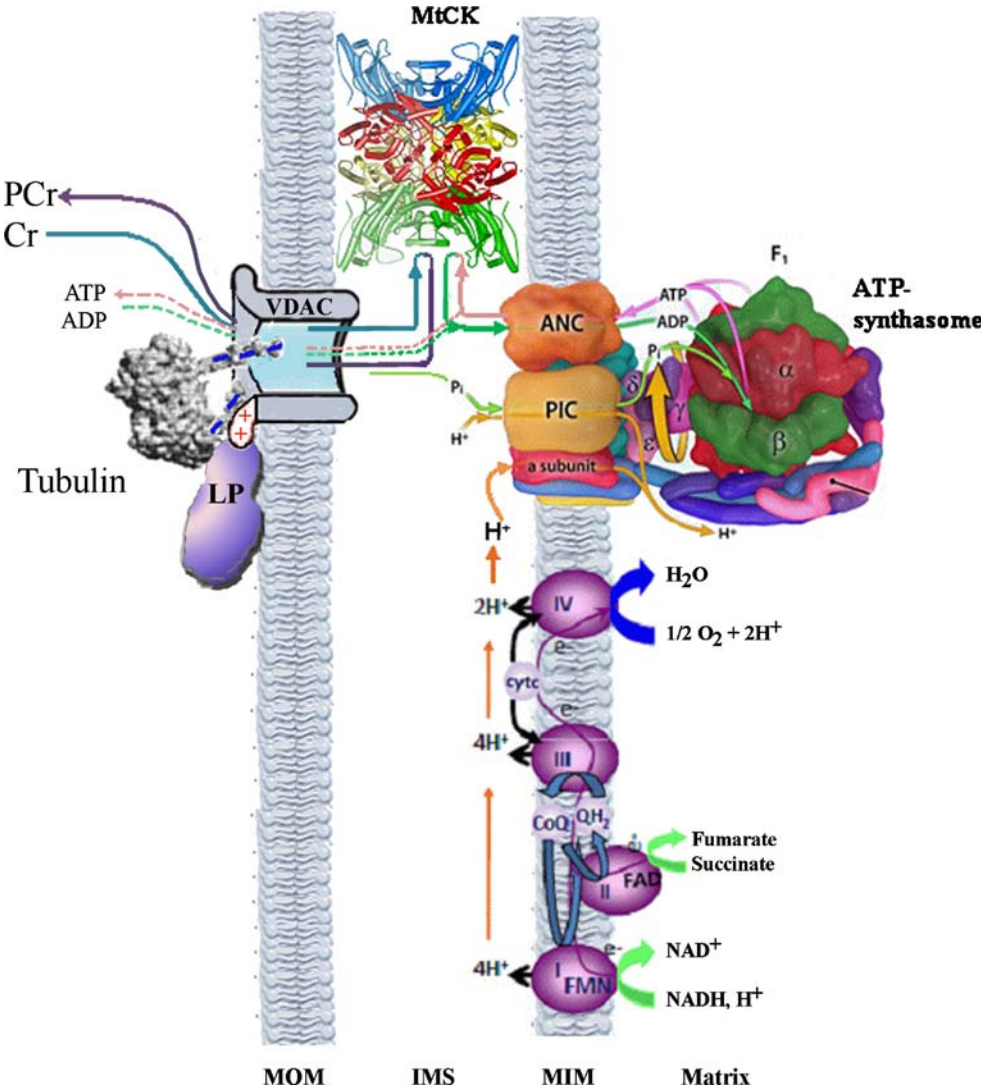


Fig. 9 Proposed model of regulation of respiration in a supercomplex named Mitochondrial Interactosome, consisting of ATP Synthasome - MtCK-VDAC- Tubulin. Macromolecular ATP synthase is represented as a part of the complex ATP Synthasome (*reprinted with kind permission from Peter L. Pedersen,* 2007a,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

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

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

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

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

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

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