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

Kersti Tepp[1]✉, Natalja Timohhina[1], Vladimir Chekulayev[1], Igor Shevchuk[1], Tuuli Kaambre[1] and Valdur Saks[1,2]

[1]Laboratory of Bioenergetics, National Institute of Chemical Physics and Biophysics, Tallinn, Estonia; [2]Laboratory of Fundamental and Applied Bioenergetics, INSERM U884, Joseph Fourier University, Grenoble, France

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

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

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INTRODUCTION

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

✉e-mail: Kersti.Tepp@kbfi.ee

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

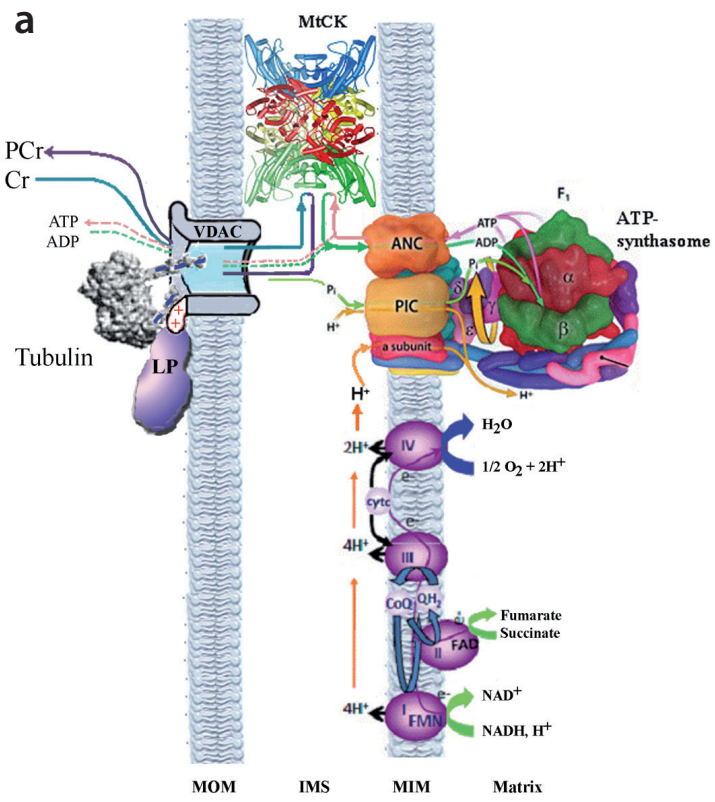

Figure 1a. Model of regulation of respiration in the Mitochondrial Interactosome (MI)
MI is a supercomplex consisting of ATP Sythasome (reprinted with kind permission from Peter L. Pedersen (2007; 2008), mitochondrial creatine kinase (MtCK), voltage dependent anion channel (VDAC), and tubulin. Octameric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

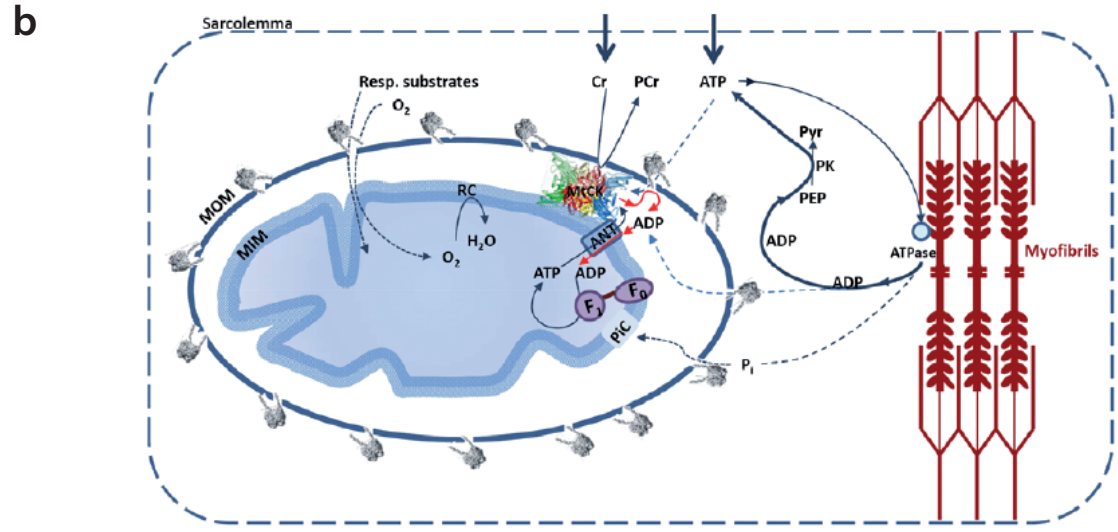

Figure 1b. Scheme of experimental protocols
This mode of representation is called Gellerich–Guzun protocol (Gellerich 1982; Guzun 2009). It represents mitochondrion *in situ* in a permeabilized cardiac cell, surrounded by cytoskeletal proteins and myofibrils. The mitochondrial outer membrane (MOM) is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

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

MATERIALS AND METHODS

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

RESULTS

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

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

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

Table 1. Inhibitors used for Mitochondrial Interactosome complexes

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

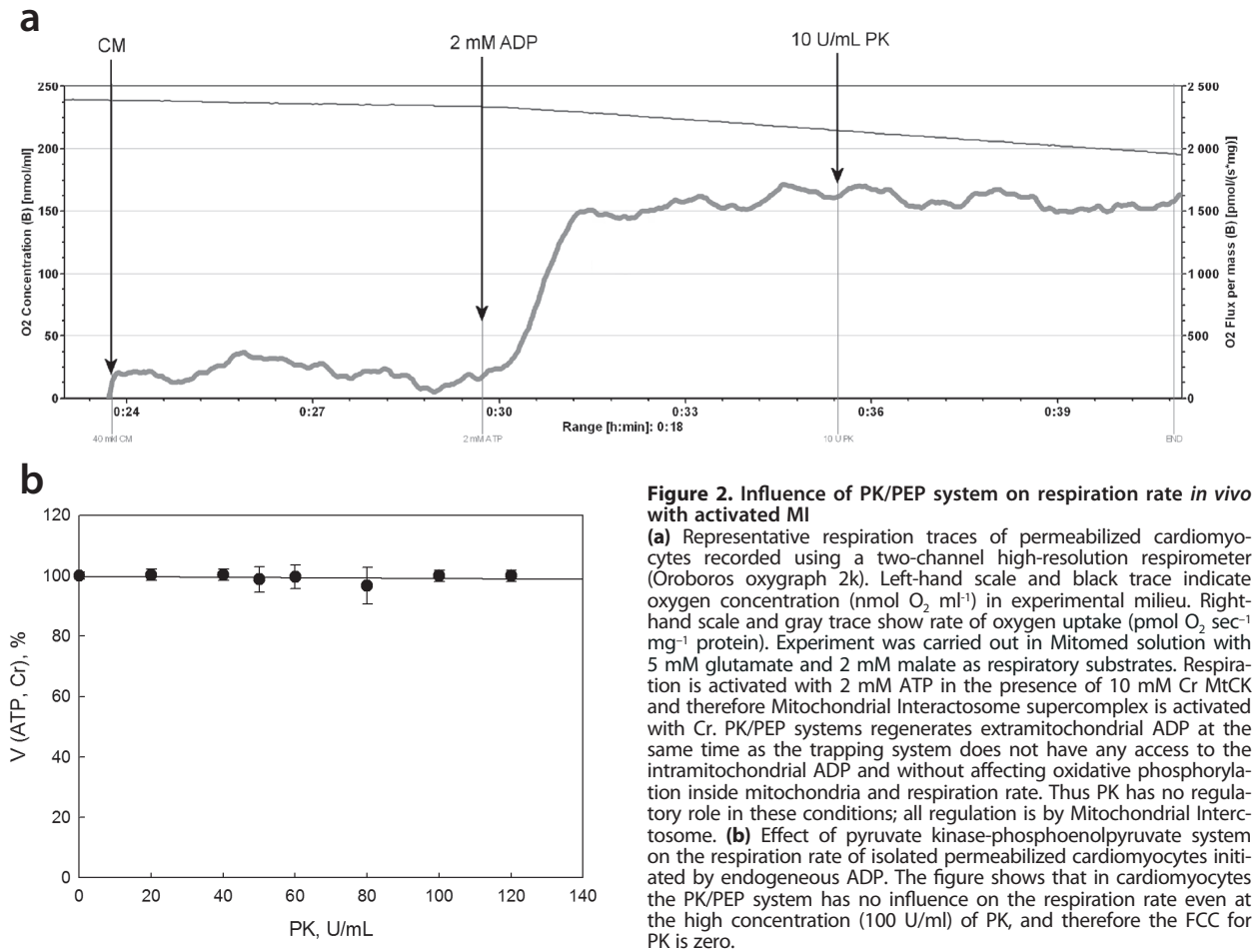

Figure 2. Influence of PK/PEP system on respiration rate *in vivo* with activated MI
(a) Representative respiration traces of permeabilized cardiomyocytes recorded using a two-channel high-resolution respirometer (Oroboros oxygraph 2k). Left-hand scale and black trace indicate oxygen concentration (nmol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

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

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

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

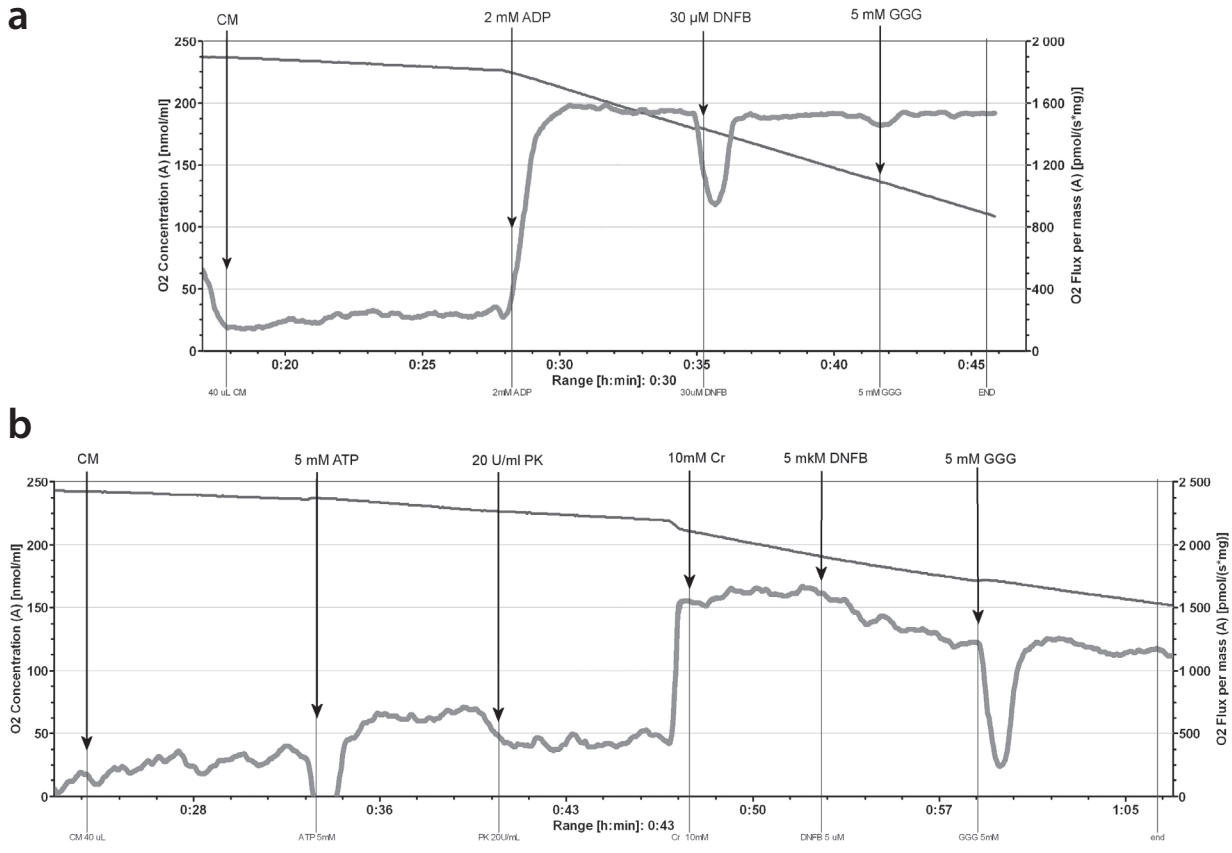

Figure 3. Specificity of dinitrofluorobenzene (DNFB) for inhibition of MtCK
(a) Respiration activated with ADP. Without activation of MI complex by creatine (Cr), the respiration rate was not affected by addition of 30 µM DNFB. **(b)** Respiration activated by Cr. In the second protocol, the respiration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

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

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

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

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

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

Table 2. Respiration rates

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

DISCUSSION

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

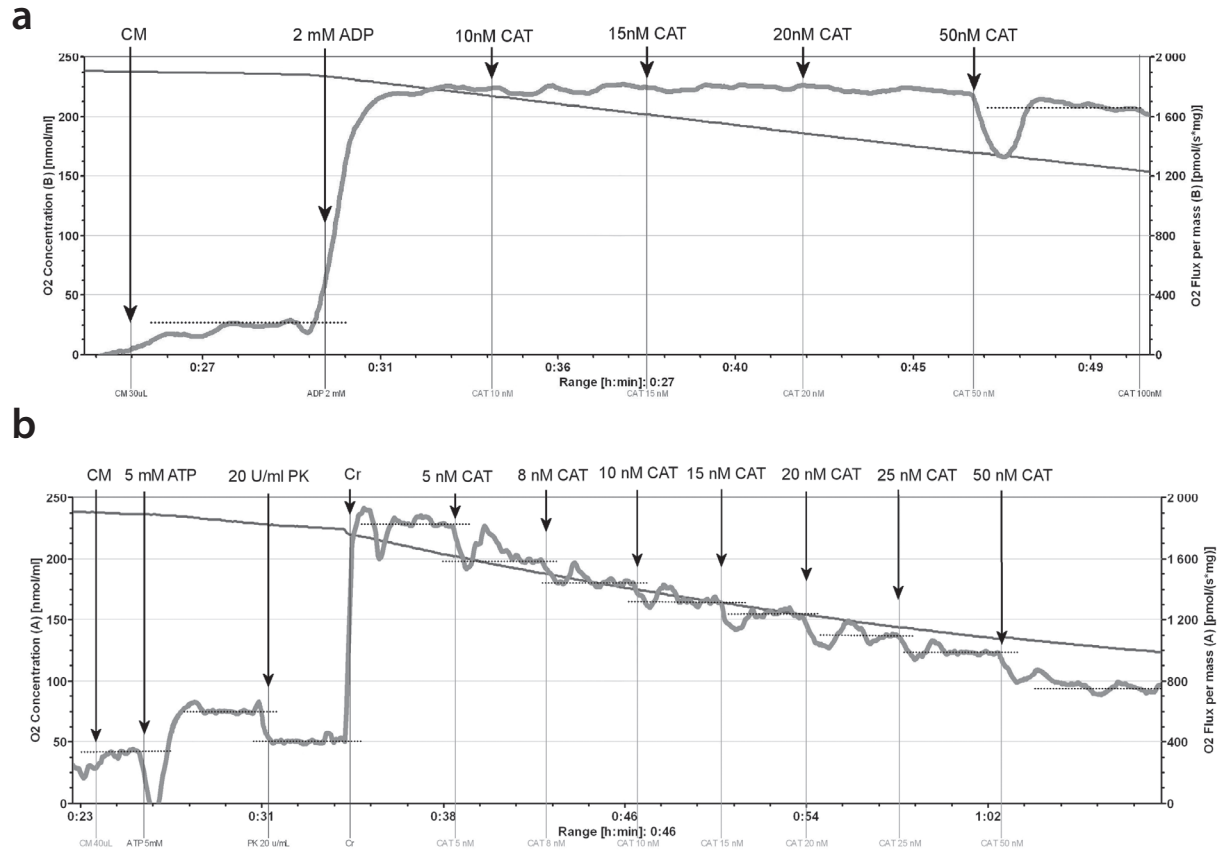

Figure 4. Respiration inhibition with carboxyatractyloside (CAT)
(a) Direct ADP activation and **(b)** the MI complex is activated with addition of 10 mM Cr in the presence of ATP and PK/PEP system. Dotted lines mark steady states. In the case of activated MI complex the respiration rate decreases significantly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

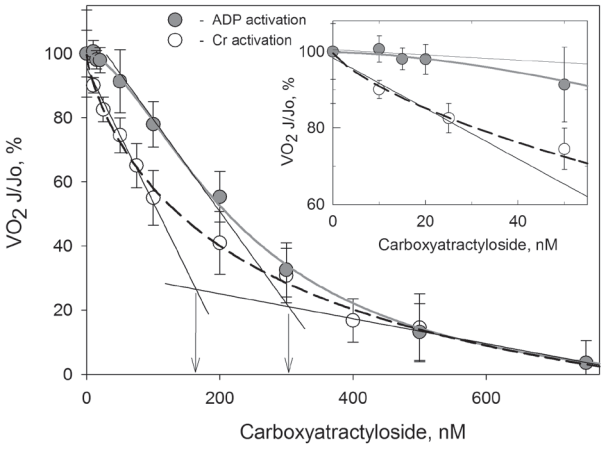

Figure 5. Titration curves of CAT
Respiration inhibition curves of two experimental conditions: with external ADP activation and Cr activation. In the Fig. 5 and in the insert of the figure the respiration rates are presented. From these data initial slope and I_{max} was calculated, and the FCC for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

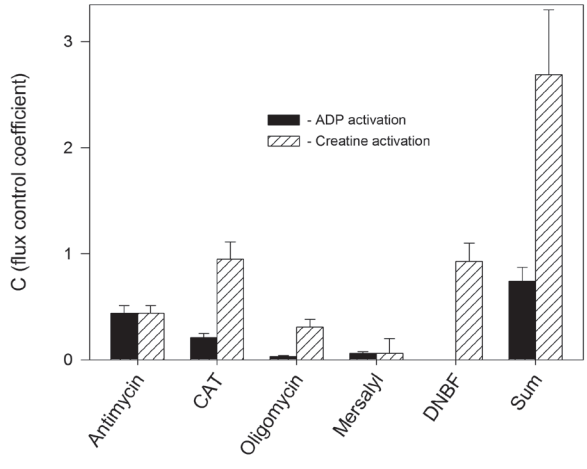

Figure 6. Flux control coefficients (FCC)
The coefficients were determined by two different protocols: the respiration activated with ADP (MtCK and Mitochondrial Interactosome (MI) complex are not activated) and activation with Cr, in the presence of ATP and PK-PEP system. The FCC was measured for ATP/ADP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

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

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

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

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

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

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

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

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

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

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