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

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ABSTRACT

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

1. Introduction

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

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

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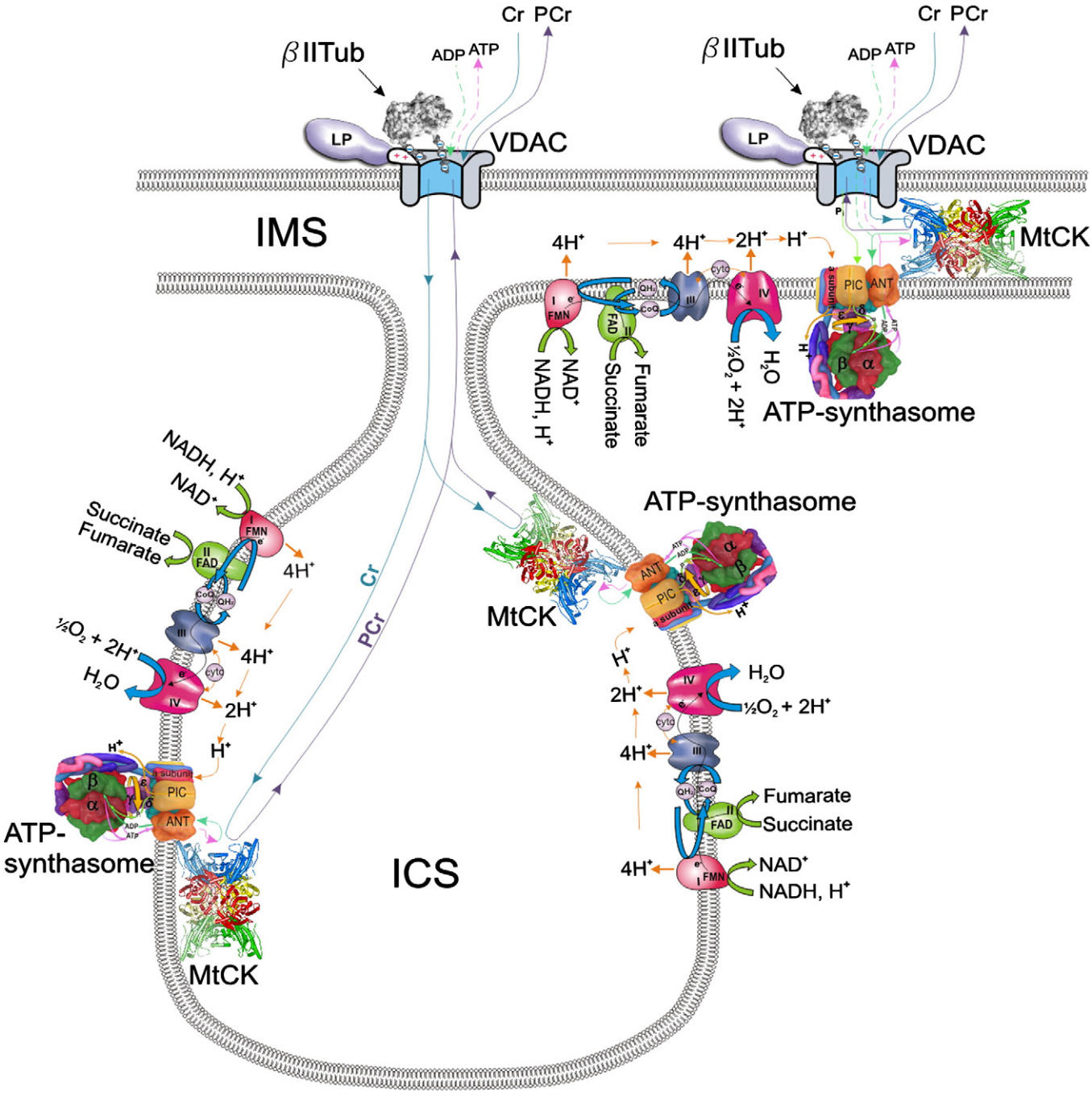


Fig. 1. Model of regulation of respiration in the Mitochondrial Interactosome (MI). MI is a supercomplex consisting of ATP Sythasome (reprinted with kind permission from Peter L. Pedersen [60,61]), respiratory chain complexes, mitochondrial creatine kinase (MtCK), voltage dependent anion channel (VDAC),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

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

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

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

2. Materials and methods

2.1. Laboratory animals and chemicals

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

2.2. Isolation of adult cardiac myocytes

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

2.3. Isolation of mitochondria from cardiac muscle

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

2.4. Permeabilization procedure

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

2.5. Measurements of oxygen consumption

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

2.7. Data analysis

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

3. Results

3.1. Quality control tests for preparations used

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

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

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

3.2. Equality of maximal rates of respiration in all preparations

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

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

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

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

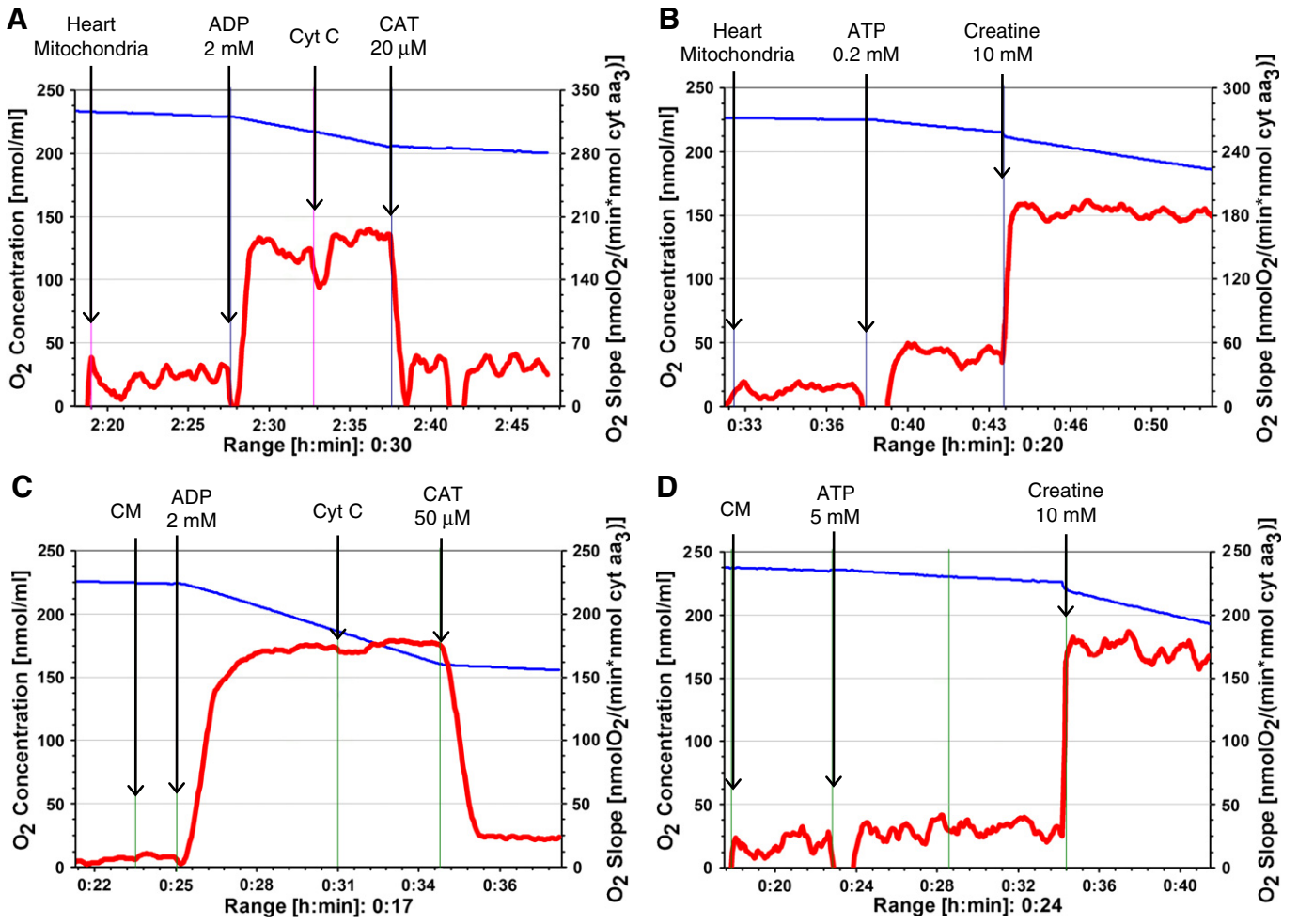


Fig. 2. The quality tests. A. Quality test of intactness of mitochondrial membranes in isolated mitochondria. Representative respiration traces of isolated mitochondria recorded using a two-channel high-resolution respirometer (Oroboros oxygraph 2 k). Left-hand scale and blue trace indicate oxygen concentration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

produced by MtCK in microcompartments within the IMS (Fig. 1), may be re-imported into the matrix *via* adenine nucleotide translocase (ANT) due to its functional coupling with MtCK [2,3,43] or leave IMS *via* VDAC in dependence upon permeability of this channel. If ADP can leave IMS *via* VDAC, it will be trapped by PEP-PK system and the respiration rate decreases. Fig. 3B and C show the respiration

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

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

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

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

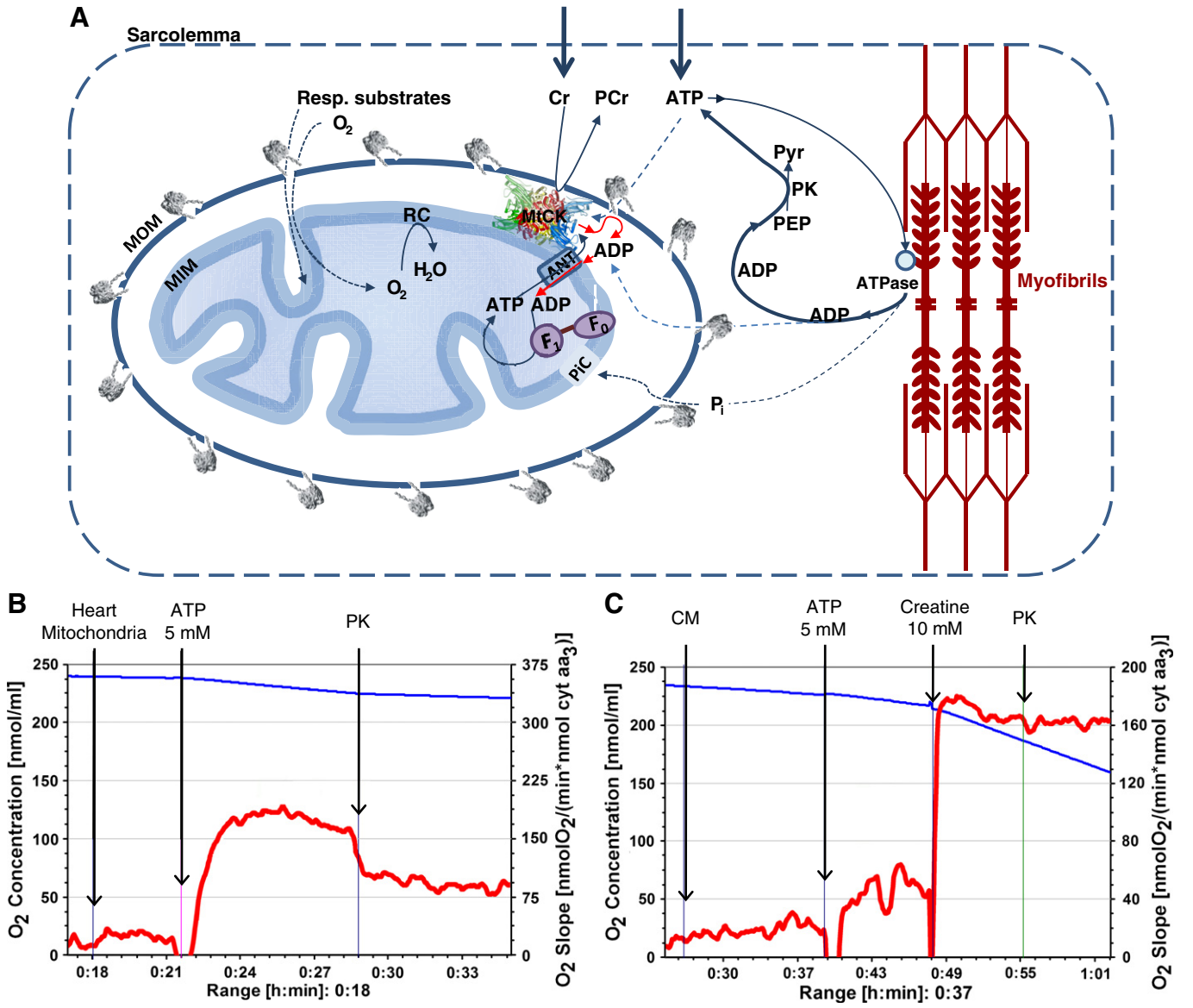


Fig. 3. Influence of PK-PEP system on the mitochondrial respiration. A. Scheme of the experimental protocols. The method is called Gellerich–Guzun protocol [2,29]. It represents mitochondrion *in situ*, in permeabilized cardiac cell, surrounded by cytoskeletal proteins and myofibrils. The mitochondrial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

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

3.4. Specific inhibition of MtCK by DNFB

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

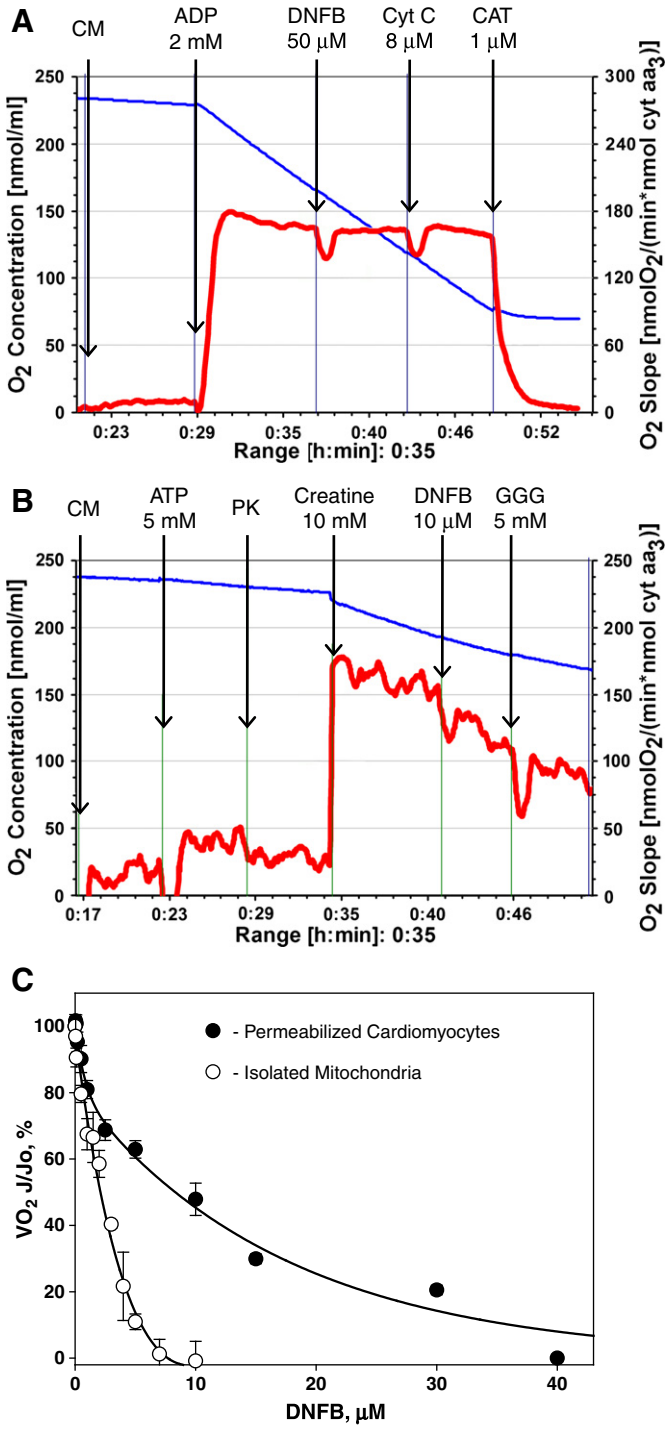


Fig. 4. Specificity of dinitrofluorobenzene (DNFB) for inhibition of MtCK. A. ADP activation. Respiration of cardiomyocytes (CM) activated with 2 mM ADP. Without activation of MI complex by creatine (Cr), the respiration rate was not affected by addition of 50 μM DNFB, ATP Synthasome was not affected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

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

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

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

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

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

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

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

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

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

All coefficients were determined by measurements according two protocols: 1) direct activation with 2 mM ADP when MtCK and Mitochondrial Interactosome (MI) complex are not activated (ADP activation) and: 2) under conditions of Cr/MtCK activation when after addition of ATP and PK-PEP system, the addition of Cr activates MtCK and all the MI supercomplex coupling system. The FCC of the complexes is several times higher with Cr for Complex I of the respiratory chain, ANT and ATP synthase. FCC for MtCK is remarkably high. MtCK together with ANT can be considered as most important regulatory parts of the MI. The sum of the measured coefficients is 3 times higher upon Cr activation, suggesting the direct metabolic channelling in MI.
Results are presented as average values ± SEM for 10–15 experiments.

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

3.6. Flux control by ATP Synthasome complexes

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

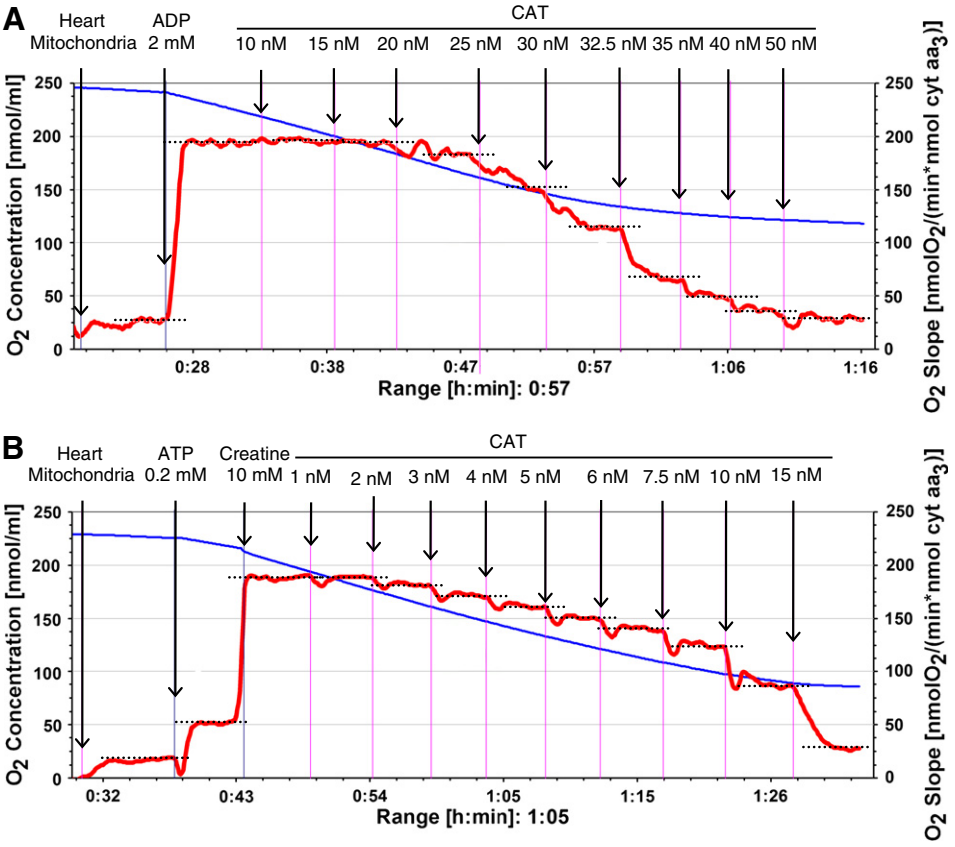
Fig. 7 shows that similar effect was also seen for the case of inhibition of Complex I by its specific inhibitor rotenone. The initial slope of inhibition curve of rotenone (Complex I) was similar in isolated mitochondria and CM with ADP activation (C_{vi}^{ATP} = 0.19 and 0.2; Table 2 and 3), decline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

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

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

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

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


A
Heart Mitochondria
ADP 2 mM
CAT
10 nM 15 nM 20 nM 25 nM 30 nM 32.5 nM 35 nM 40 nM 50 nM
O2 Concentration [nmol/ml]
O2 Slope [nmolO2/(min*nmol cyt aa3)]
0:28 0:38 0:47 0:57 1:06 1:16
Range [h:min]: 0:57
B
Heart Mitochondria
ATP 0.2 mM
Creatine 10 mM
CAT
1 nM 2 nM 3 nM 4 nM 5 nM 6 nM 7.5 nM 10 nM 15 nM
O2 Concentration [nmol/ml]
O2 Slope [nmolO2/(min*nmol cyt aa3)]
0:32 0:43 0:54 1:05 1:15 1:26
Range [h:min]: 1:05
C
- Cr activation (Mito)
- ADP activation (Mito)
- Cr activation (CM)
- ADP activation (CM)
VO2 J/Jo, %
Carboxyatractyloside, nM
D
- ADP activation
- Creatine activation
FCC value
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CM

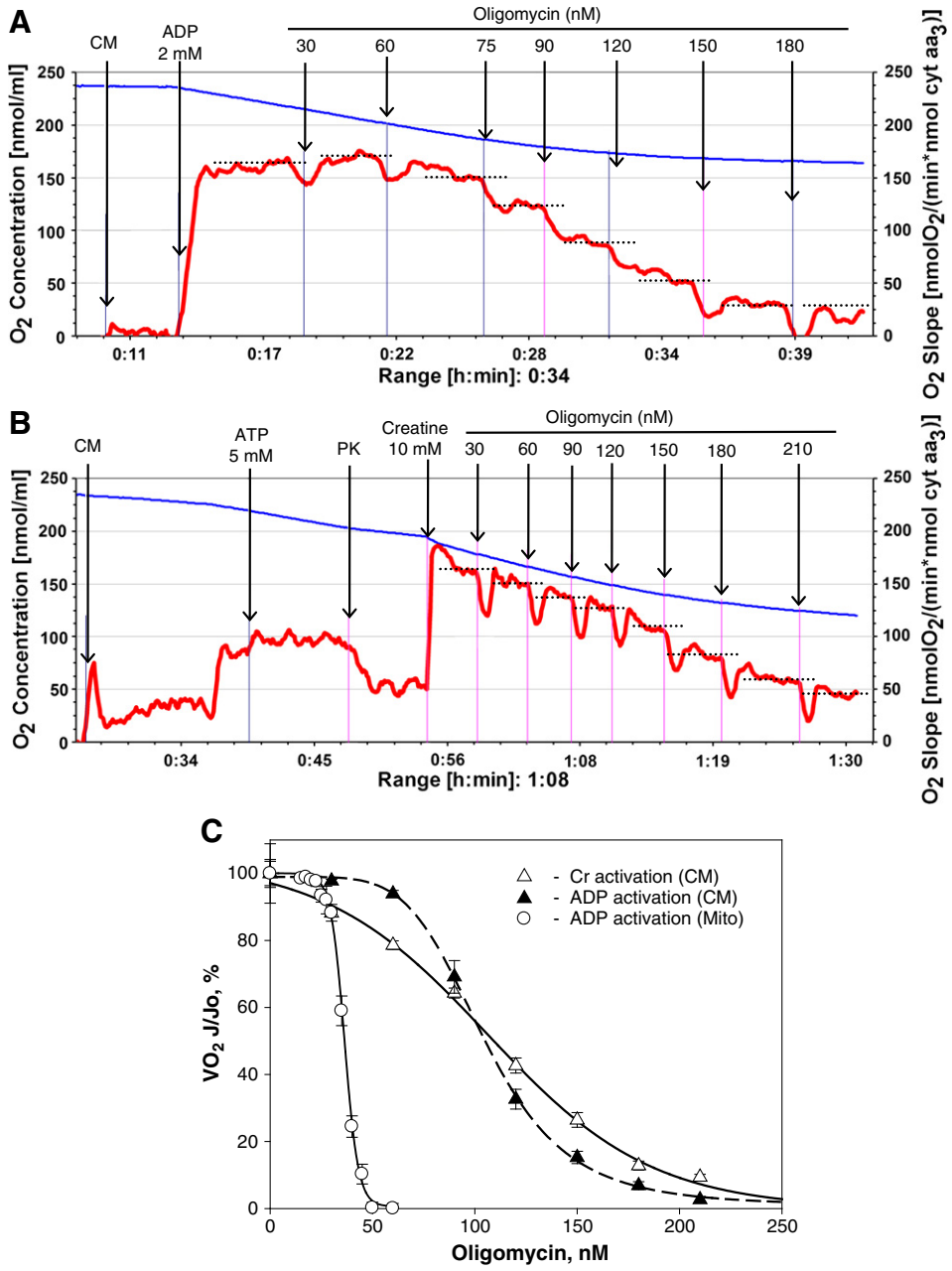

Fig. 6. Respiration traces of inhibition of cardiomyocytes with oligomycin. A. Direct ADP activation; B. Cr activation. Respiration was activated with 2 mM ADP (A) or with addition of 10 mM Cr in the presence of 5 mM ATP and PK-PEP system (B). Dotted lines mark steady states in each inhibitor concentration.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

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

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

3.7. Application of mathematical model in flux control coefficient calculation

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

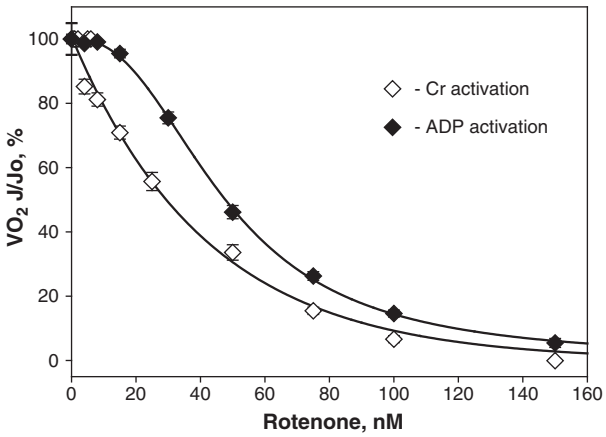

Fig. 7. Titration curves for Complex I by rotenone in permeabilized cardiomyocytes. The respiration inhibition curves are presented for two experimental conditions: with external ADP activation and with Cr activation (physiological conditions).

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

4. Discussion

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

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

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

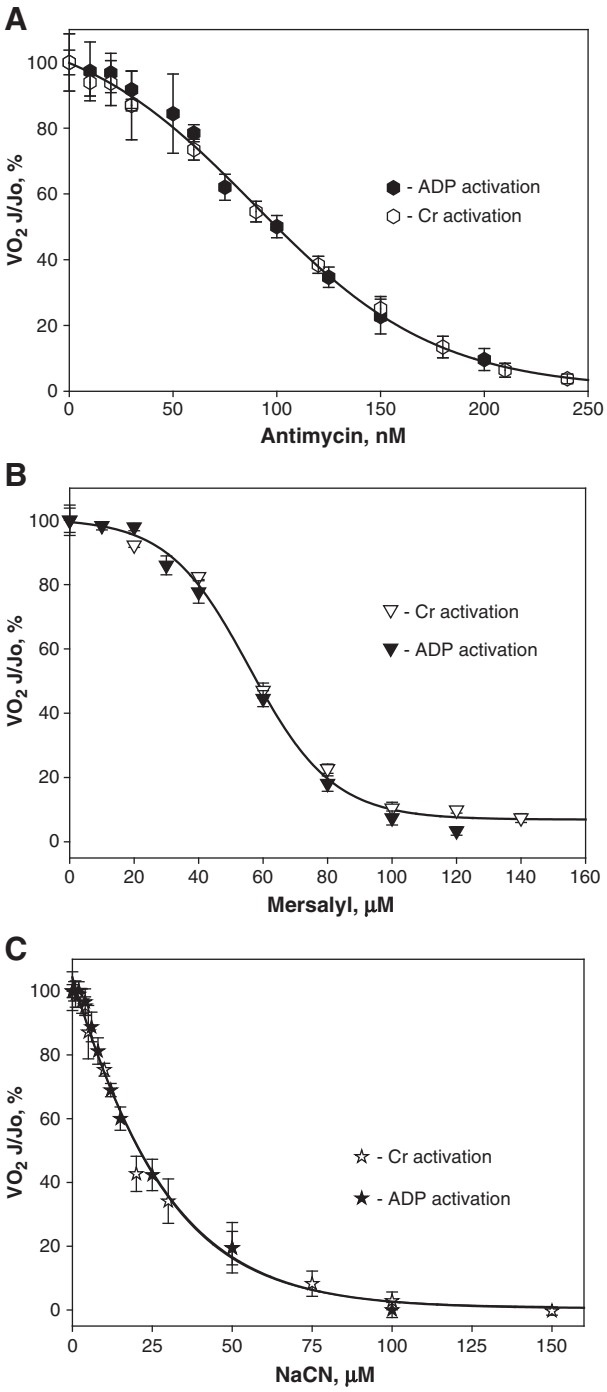

Fig. 8. Titration curves for respiratory chain complexes and phosphate carrier by their specific inhibitors in permeabilized cardiomyocytes. The respiration inhibition curves are presented for two experimental conditions: with external ADP activation and with Cr activation (physiological conditions): A. CoQ cytochrome-c oxidoreductase (Antimycin). B. Pi carrier (Mersalyl); C. Complex IV (sodium cyanide). Inhibition curves with Antimycin, Mersalyl and Cyanide have no significant difference in ADP activation versus Cr activation; although complex III and IV are important regulators in CM with direct ADP activation, it is relatively decreased in physiological conditions with Cr activation (Table 3).

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

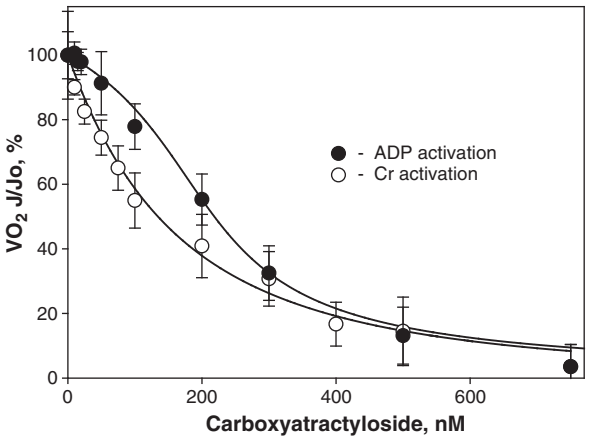

Fig. 9. The experimental points of CAT titration for the ADP (filled cycles) and Cr (open cycles) activated systems, fitted to mathematical model by Gellerich [11]. Rather good fitting was seen in both cases between the computed curve and experimental data (analogous comparison was done for the all components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

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

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

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

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

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