Mitochondrial creatine kinase isoform expression does not correlate with its mode of action

Keltoum ANFLOUS*, Vladimir VEKSLER*, Philippe MATEO*, Françoise SAMSON†, Valdour SAKS‡ and Renée VENTURA-CLAPIER*§

*U-446 INSERM, Cardiologie Cellulaire et Moléculaire, Faculté de Pharmacie, Université Paris-Sud, 5 rue Jean-Baptiste Clément, 92296 Châtenay-Malabry, France,

†CNRS URA 1159, Hôpital Marie-Lannelongue, 133 Ave. de la Résistance, 92350 le Plessis-Robinson, France, and ‡Bioenergetic Laboratory, Institute of Chemical

Physics and Biophysics, Estonian Academy of Sciences, Rävala puistee 10, Tallin EE01100, Estonia

In adult mammalian ventricular tissue, mitochondrial creatine kinase (mi-CK), which is bound to the outer surface of the mitochondrial inner membrane, is functionally coupled to oxidative phosphorylation. This is shown, in saponin-permeabilized rat ventricular fibres, by a shift in the apparent K_m of mitochondrial respiration for ADP from $300 \pm 56 \mu\text{M}$ to $111 \pm 40 \mu\text{M}$ ($P < 0.001$) on the addition of 25 mM creatine, due to a local accumulation of ADP close to the ATP/ADP translocator. We have found that, in atrial fibres, the apparent K_m for ADP is high, but is not decreased by creatine, suggesting an absence of coupling in this tissue, as has previously been observed in smooth muscle. mi-CK is encoded by two different genes, which are expressed in a tissue-specific manner: the sarcomeric isoform is expressed in ventricular and skeletal muscles, while the ubiquitous isoform is expressed in smooth muscle, brain and other tissues. In order to determine whether a specific function can be attributed to the expression of a specific isoform, we investigated mi-CK

mRNA expression by Northern blot analysis. Hybridization with synthetic oligonucleotides specific for each mi-CK isoform showed the expression of only the sarcomeric isoform in rat atria. This result was confirmed by PCR using primers specific for each isoform. In addition, electrophoretic analysis of CK isoforms showed no difference in the octamer/dimer ratio of mi-CK in the atria and ventricles. In atria, unlike the soleus or ventricles, the maximum potential rate of mitochondrial phosphocreatine synthesis was lower than the maximal rate of ATP production by the mitochondria. The total CK/adenylate kinase ratio was also lower in atria than in the other tissues, suggesting a greater contribution of adenylate kinase to adenine nucleotide compartmentation in this tissue. The functional differences between mi-CK in the two cardiac tissues seem to imply a specific arrangement of the proteins in the intermembrane space rather than the expression of specific isoforms.

INTRODUCTION

In mammals, creatine kinase (CK) exists as mitochondrial and cytosolic isoforms. In tissues with high and fluctuating energy requirements, such as the ventricles and slow-twitch skeletal muscle (soleus), the CK/phosphocreatine (PCr) system connects sites of ATP production (mitochondria or glycolysis) with those of its utilization (ATPases), thereby playing a major role in energy transfer [1,2]. The structural basis for this concept is the compartmentation of CK isoforms. CK is encoded by four different genes, which express M-CK, B-CK and two mitochondrial (mi-CK) isoforms in a developmental and tissue-specific manner [3,4]. Mi-CK_s (the sarcomeric isoform) is expressed in the ventricles and slow-twitch skeletal muscle in co-ordination with M-CK, and mi-CK_u (the ubiquitous isoform) is mainly expressed in brain and smooth muscle, in co-ordination with B-CK [3]. In contrast with cytosolic CK isoenzymes, which exist as dimers, mi-CK forms both octameric and dimeric structures [5]. The significance and role of the two mitochondrial isoforms is not known. In striated muscles, which express mi-CK_s, this isoenzyme is located at the outer surface of the mitochondrial inner membrane, and is functionally coupled to respiration, thus supporting the shuttle model for the CK/PCr system (for reviews, see [1,2,6]). In ventricular and slow-twitch skeletal muscle, this coupling results in local augmentation of the ADP concentration in close proximity to adenine nucleotide translocase (ANT). In skinned fibres, this leads to a significant decrease by creatine of the apparent mitochondrial K_m for ADP, the diffusion of which is thought to be restricted *in vivo*, probably

by the mitochondrial outer membrane [7,8]. This restriction of diffusion or functional coupling is absent from fast-twitch skeletal muscle, where the CK/PCr system is thought to play a buffer role. Fast-twitch skeletal muscle almost exclusively expresses muscle cytosolic CK (MM-CK), with a low apparent K_m for ADP [9].

It was shown recently that, in rat skinned atrial fibres, creatine does not stimulate respiration in the presence of submaximal concentrations of ADP, in spite of the presence of active mi-CK [10]. Similarly, in uterine smooth muscle, which expresses mi-CK_u, mi-CK activity is present and even increased during uterus growth, but no coupling between CK activity and oxidative phosphorylation could be observed [11,12]. From these observations it seems that mi-CK could operate in mitochondria according to two functional modes. One of these modes would be an 'ADP amplification mode' whose paradigm is adult ventricular tissue, and the second an 'ADP regeneration mode', which is encountered in smooth muscle.

The aim of the present study was to investigate the reasons for the absence of functional coupling between mi-CK and respiration in rat atrial mitochondria, and to determine whether mi-CK_u and mi-CK_s could form the structural basis of the two functional modes of mi-CK. The results show that neither the expression of a specific isoform nor the presence of the oligomeric form is correlated with the functional state of mi-CK. These observations prompt us to consider that the spatial arrangement and organization of the mitochondrial intermembrane compartment play a crucial role in the regulation of energy fluxes in muscle cells.

Abbreviations used: AK, adenylate kinase; ANT, adenine nucleotide translocase; CK, creatine kinase; DTT, dithiothreitol; mi-CK, mitochondrial CK; mi-CK_u, ubiquitous mi-CK isoform; mi-CK_s, sarcomeric mi-CK isoform; PCr, phosphocreatine.

§ To whom correspondence should be addressed.

EXPERIMENTAL

Tissue respiration experiments

Male Wistar rats weighing 250–350 g were used. Bundles of fibres, 200–300 μm in diameter, were isolated from the endocardial surface of the left ventricle, slow-twitch skeletal muscle and atria, and permeabilized as described by Veksler et al. [13]. Fibre bundles of 5–10 mg were incubated for 30 min in solution S (skinning) containing 2.77 mM CaK_2EGTA , 7.23 mM K_2EGTA (100 nM free Ca^{2+}), 6.56 mM MgCl_2 (1 mM free Mg^{2+}), 5.7 mM Na_2ATP , 15 mM PCr , 20 mM taurine, 0.5 mM dithiothreitol, 50 mM potassium methanesulphonate (160 mM ionic strength), 20 mM imidazole (pH 7.1) and 50 $\mu\text{g}/\text{ml}$ saponin. Bundles were washed for 10 min in solution R (respiration), whose composition was the same as that of solution S except that 5 mM glutamic acid, 2 mM malic acid and 3 mM K_2HPO_4 were present instead of the high-energy phosphates. All steps were carried out at 4 °C with vigorous stirring.

The respiration of skinned fibres was determined at 22 °C (except where otherwise stated) in 3 ml of solution R containing 2 mg/ml BSA, using a Yellow Spring Instruments Oxygraph. The stock solution of ADP was titrated to determine the precise ADP concentration, and the range of ADP concentrations used was 0.1–3.0 mM and 0.025–1.0 mM for measurements without and with 25 mM creatine respectively. After measurements, fibres were removed, dried and weighed. Calculations were made assuming that the solubility of oxygen at 22 °C is 230 nmol of oxygen/ml when equilibrated with ambient air. Rates of respiration are given in μmol of oxygen/min per g dry wt.

Biochemical determinations and data evaluation

Total CK and adenylate kinase (AK) activities were determined after homogenization of tissues (ventricles and soleus at 50 mg/ml; atria and brain at 100 mg/ml) using an Ultra-Turrax Polytron, in a solution containing 5 mM Hepes (pH 8.7), 1 mM EDTA, 1 mM DTT and 0.1 % Triton X-100. Homogenates were incubated on ice for 1 h, with shaking every 15 min.

Whole homogenates were used for enzymic activity measurements and isoenzyme pattern determination. CK activity was determined using the coupled enzyme assay of glucose-6-phosphate dehydrogenase and hexokinase, producing NADPH. NADPH production was measured spectrophotometrically at 340 nm (Gilford Spectrophotometer) and 30 °C. The solution used for the CK activity assay contained 20 mM Hepes (pH 7.4), 5 mM MgCl_2 , 20 mM glucose, 0.5 mM DTT, 1 mM ADP, 0.6 mM NADP^+ , 20 mM PCr , 100 μM diadenosine pentaphosphate (for inhibition of AK), 2 units/ml hexokinase and 2.5 units/ml glucose-6-phosphate dehydrogenase. AK activity was determined as described above, but the medium did not contain diadenosine pentaphosphate or PCr .

Samples of 1 μl of homogenate from each tissue were applied to a 1 % agarose gel and subjected to electrophoresis for 2 h at 200 V in Tris/barbital buffer (50 mM, pH 8.9). CK isoenzyme activities were revealed after incubating the gel with a staining-solution-soaked paper for 20 min at room temperature, and the fluorescence of the NADPH produced was visualized under UV light. The staining solution contained 22 mM Mes (pH 7.4), 50 mM magnesium acetate, 70 mM glucose, 120 mM DTT, 100 mM PCr , 9 mM ADP, 9 mM NADP^+ , 4 units/ml hexokinase and 7 units/ml glucose-6-phosphate dehydrogenase. The octamer/dimer ratio of mi-CK was calculated by scanning on a densitometer (Desaga CD60, Sarstedt, Germany), and the area under the peak of each oligomer was integrated.

Differences between tissues were compared using Student's *t*

test for unpaired data. Results are expressed as means $\pm$ S.D., and are considered significantly different at $P < 0.05$.

Molecular study

Total RNAs from rat ventricles, atria, brain and soleus were prepared according to the Chomczynski and Sacchi method [14]. The integrity of all RNA samples was checked using ethidium bromide. Quantification was carried out by absorption at 260 nm. Northern blotting was performed using 15 μg of total RNA from each tissue, which was electrophoresed on a 0.8 % agarose/formaldehyde gel and transferred to nylon membranes (Hybond- N^+ ; Amersham).

Northern blot hybridization was performed using two 36-mer oligonucleotide synthetic probes (GENOSYS Products). The first probe (41R) was specific for the rat mi-CK_u mRNA and corresponded to the 3' untranslated region between nucleotides 1348 and 1383 [4]. The second probe (42R) was specific for the rat mi-CK_s mRNA and corresponded to the 3' untranslated region between nucleotides 1290 and 1325 [4]. The two probes were labelled using [γ - ^{32}P]ATP, which was incorporated into the 5' region by incubation with T4 polynucleotide kinase for 1 h at 37 °C. Hybridization was performed at 42 °C for 2 h in Rapid-hybridization buffer (Amersham). The membranes were washed in $5 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl, 0.015 M sodium citrate) at room temperature for 2×15 min, and in $1 \times \text{SSC}$ at 40 °C for 5 and then 3 min. They were then autoradiographed with intensifying screens for up to 3 weeks at -80 °C.

The two synthetic oligonucleotides (41R and 42R) were used for reverse transcription using 1 μg of total RNA from each tissue surveyed. Annealing was performed at 45 °C. A 29-mer synthetic oligonucleotide (43F), which is common to the 5' coding regions of rat mi-CK_u and mi-CK_s (nucleotides 898–926 [4]), was used as an upstream primer. Thirty PCR cycles were carried out. Samples of 20 μl of each amplified product were analysed by electrophoresis on a 1 % agarose gel, and the size of the products was determined by comparison with fragment sizes of a lambda DNA *Hind*III/ Φ X-174 *Hae*III Digest (Stratagene).

RESULTS

Respiratory parameters of permeabilized preparations of ventricles, atria and soleus muscle

In order to study the role of mi-CK in mitochondria, oxygen consumption and respiratory parameters were measured in saponin-permeabilized preparations of the different muscles in the presence of creatine (Table 1). The basal respiration rate (V_0), measured in the absence of ADP, was significantly higher in

Table 1 Respiratory parameters of mitochondria *in situ*

Experiments were carried out using saponin-permeabilized skinned fibres. Values (means $\pm$ S.D. of results from five animals) are expressed in μmol of O_2 /min per g dry weight. Significant differences compared with ventricular values are indicated by ** $P < 0.01$. V_0 , basal respiration rate; V_{max} , maximal respiration in the presence of 1 mM ADP and 25 mM creatine; V_{max}/V_0 , respiratory control index. Also given is the percentage inhibition of respiration by atractyloside, an inhibitor of the ADP/ATP carrier.

	V_0 ($\mu\text{mol}/\text{min}$ per g)	V_{max} ($\mu\text{mol}/\text{min}$ per g)	V_{max}/V_0	Inhibition by atractyloside (%)
Ventricles	6.1 $\pm$ 2.0	27.8 $\pm$ 5.9	4.90 $\pm$ 1.60	84.0 $\pm$ 2.1
Atria	3.84 $\pm$ 0.20**	18.6 $\pm$ 2.3**	4.83 $\pm$ 0.45	82.1 $\pm$ 1.4
Soleus	3.46 $\pm$ 0.86**	12.3 $\pm$ 3.2**	3.58 $\pm$ 0.50	81.6 $\pm$ 6.3

Figure 1 Dependence of respiration rate on external ADP concentration

The respiration rate was measured in the absence (■) or in the presence (●) of 25 mM creatine in ventricular (left) and atrial (right) saponin-permeabilized fibres. The K_m for ADP was 335 μ M without and 85 μ M with creatine in ventricles, and 315 μ M without and 470 μ M with creatine in atria.

ventricles than in the other tissues. The maximal respiration rate (V_{max}), expressed per g of tissue dry wt., is an index of the oxidative capacity of the tissues. This parameter was significantly higher in ventricles than in atria, and decreased in order ventricles > atria > soleus. The V_{max}/V_0 ratio, which reflects the acceptor control ratio, did not differ significantly between the different tissues. In all cases, the integrity of the mitochondrial inner membrane was maintained, since respiration was greatly inhibited by atractyloside (Table 1). Since addition of cytochrome *c* did not stimulate respiration, it could also be assumed that the mitochondrial outer membrane was intact (results not shown).

Apparent K_m of respiration for ADP

In ventricular tissue, it has been shown that mi-CK is coupled to oxidative phosphorylation and that creatine is able to stimulate mitochondrial respiration in the presence of submaximal ADP concentrations [13] at 22 °C. In order to eliminate a possible influence of temperature, we checked the effect of creatine on respiration at higher temperatures. At 30 °C, creatine was still able to stimulate respiration at a submaximal ADP concentration in ventricular fibres, but not in atria (results not shown). We also checked that the increase in respiration induced by 25 mM creatine was not due to osmotic effects. The addition of 25 mM mannitol instead of creatine was without effect on the respiration rate at submaximal ADP concentrations in ventricular fibres (results not shown).

Stimulation of respiration by creatine was previously shown to be due to compartmentation of adenine nucleotides by the functional interaction between ANT and mi-CK, and could be demonstrated by a creatine-induced shift in the apparent K_m for ADP of mitochondria *in situ* [8]. The K_m values for ADP were thus determined in the presence and in the absence of creatine in the different tissues. These measurements were carried out at 22 °C, to ensure the stability of the preparations. Figure 1 shows the respiration rate as a function of ADP concentration in the presence and in the absence of 25 mM creatine in representative ventricular and atrial fibres. Although no difference was found between atria and ventricles in the absence of creatine, in the presence of creatine the relative respiration rates were shifted towards lower ADP concentrations in ventricles but not in atria.

Figure 2 Northern blot analysis of mi-CK_s

Samples of 15 μ g of total RNA from rat brain (B), soleus (S), ventricles and atria were loaded in the indicated lanes. RNA blot hybridization with a synthesized oligonucleotide probe specific for mi-CK_s was performed at 42 °C. The migration positions of 28 S and 18 S rRNAs are shown by arrows. The arrowhead indicates the mRNA species corresponding to mi-CK_s.

The mean value of the apparent K_m for ADP in the absence of creatine was higher in soleus muscle ($625 \pm 203 \mu$ M, $n = 5$) than in ventricles ($299 \pm 56 \mu$ M, $n = 5$, $P < 0.05$) or atria ($350 \pm 90 \mu$ M, $n = 5$, $P < 0.05$). Addition of 25 mM creatine induced a 3–4-fold decrease in the apparent K_m for ADP in both soleus ($172 \pm 66 \mu$ M, $n = 4$, $P < 0.01$) and ventricle ($111 \pm 40 \mu$ M, $n = 5$, $P < 0.001$), but not in atria ($347 \pm 132 \mu$ M, $n = 3$).

Northern hybridization and PCR amplification

Since the absence of coupling has also been observed previously in smooth muscles that express mi-CK_u [11,12], the expression of both mi-CK isoforms was studied in atria, ventricles and soleus, and compared with that in brain. Northern blot analysis using the synthetic oligonucleotide probe specific for rat mi-CK_s showed a single RNA, of about 1500 nucleotides, which corresponded to the size of rat mi-CK_s mRNA [4] in ventricles, soleus and atria; no signal was detected in the brain after the same period of exposure (Figure 2). Northern blot analysis using the

Figure 3 Reverse transcription PCR of mi-CK cDNA

mi-CK_s and mi-CK_u cDNAs from brain (B), slow-twitch skeletal muscle (S), ventricle (V) and atrial (A) mRNAs were reverse-transcribed using two synthetic oligonucleotides specific for each isoform in the 3' untranslated region and one common oligonucleotide in the coding region (see the Experimental section). The amplified products, visualized by ethidium bromide staining, are localized between the size markers of 603 and 310 bp (lane M; arrows).

Table 2 Total tissue activities of CK and AK

Values are means $\pm$ S.D. of the numbers of animals given in parentheses. Significance of differences compared with ventricles: ** $P < 0.01$; *** $P < 0.001$.

	Activity (units/g wet weight)		
	AK	CK	CK/AK
Ventricles (8)	226 $\pm$ 35	908 $\pm$ 150	4.08 $\pm$ 0.73
Atria (8)	139 $\pm$ 40***	332 $\pm$ 74***	2.46 $\pm$ 0.47***
Brain (8)	96 $\pm$ 21***	399 $\pm$ 94***	5.8 $\pm$ 2.2
Soleus (5)	354 $\pm$ 230	1813 $\pm$ 615**	4.15 $\pm$ 0.76

synthetic oligonucleotide probe specific for rat mi-CK_u did not give any signal, even in brain, which was taken as a reference for this isoform. We thus performed RT-PCR, which is more sensitive for detecting mRNA species with low levels of expression, using the same oligonucleotides as reverse primers (41R and 42R) and an additional common oligonucleotide from the coding region as forward primer (43F). PCR products, visualized by ethidium bromide, showed the presence of one amplified product of the expected size for mi-CK_s (427 bp) in ventricles, soleus and atria, but not in brain, whereas amplification using the mi-CK_u-specific primer revealed the presence of a single band of the expected length (485 bp) in brain only (Figure 3).

CK and AK activities

Total CK and AK activities were measured in crude extracts of all tissues. Atria had the lowest total CK activity (Table 2), whereas the total AK activity of this tissue was in the same range as that in brain, but significantly lower than that in ventricles and soleus. The CK/AK ratio showed clear differences between atria and other tissues, the relative AK activity being 66% higher in atria.

To determine whether the level of the oligomeric form of mi-CK might be different between ventricles and atria, CK isoenzymes were separated by agarose-gel electrophoresis. Table 3

Table 3 Distribution of CK isoenzymes

Values (means $\pm$ S.D. of numbers of animals given in parentheses) are expressed as a percentage of total CK activity. Significance of differences compared with ventricular values: ** $P < 0.01$; *** $P < 0.001$.

	Content (%) mi-CK	MM-CK	MB-CK	BB-CK
Ventricle (8)	22.0 $\pm$ 4.5	53.3 $\pm$ 6.7	20.2 $\pm$ 2.7	4.5 $\pm$ 2.0
Atria (7)	6.1 $\pm$ 3.1***	52.9 $\pm$ 5.8	29.9 $\pm$ 1.5***	11.2 $\pm$ 4.5**
Brain (5)	10.4 $\pm$ 4.6***	—	—	91.2 $\pm$ 2.6***
Soleus (7)	6.8 $\pm$ 2.8***	89.8 $\pm$ 7.5***	1.1 $\pm$ 1.5***	2.2 $\pm$ 3.7

shows the CK isoenzyme distribution among the different tissues. Ventricular tissue showed the highest relative amount of mi-CK. Soleus had a strong signal for MM-CK and a weaker one for BB-CK. No detectable MM-CK or MB-CK isoforms could be seen in brain. Atria showed a lower amount of mi-CK, but a higher amount of BB-CK and MB-CK, which is characteristic of foetal muscle tissues. The mi-CK signal could be resolved in two sub-bands, corresponding to octameric and dimeric oligomers, for both mi-CK_u and mi-CK_s [15]. The octamer/dimer ratio was measured in the different tissues. This ratio was similar in atria and ventricles (4.51 $\pm$ 1.7 and 5.1 $\pm$ 1.9 respectively), whereas soleus and brain had a lower proportion of octamers (2.7 $\pm$ 1.4 and 1.7 $\pm$ 0.6 respectively).

From the above data, the maximal rates of ATP and PCr production by mitochondria could be estimated (Table 4). The maximal rate of ATP production was calculated for each tissue from ADP-stimulated respiration ($V_{\max} - V_0$, given in Table 1) multiplied by a factor of 6, assuming that 6 ATP molecules are formed per O₂ molecule. This value can reasonably be corrected by a factor of 2 for 30 °C. The maximal rate of PCr production can be estimated from the total CK activity (given in Table 2) and the percentage of mi-CK (given in Table 3), assuming a 4-fold lower $V_{\max}$ for the CK reaction in the direction of PCr production [16] and a wet weight/dry weight ratio of 5. These estimations show that, for ventricles as well as soleus, the rate of PCr production matches the rate of ATP production in mitochondria. This is clearly not the case for atria, where V_{PCr} is 7-fold less than V_{ATP} .

DISCUSSION

It has been proposed that the functional coupling of mi-CK to ANT increases the turnover of adenine nucleotides and controls their local concentration in the mitochondrial intermembrane space. This coupling behaves as a powerful amplifier for the regulatory action of ADP on oxidative phosphorylation (for a review, see [2]). Indeed, *in situ*, the mitochondrial matrix is poorly accessible to ADP [8]. Limitation of diffusion by the mitochondrial outer membrane may account in part for the dynamic adenine nucleotide compartmentation in the mitochondrial intermembrane space, and is dependent on the colloid osmotic pressure [17,18]. This restricted ADP diffusion has been demonstrated in skinned cardiac fibres and in permeabilized cardiomyocytes, which exhibit a high apparent K_m for ADP, as opposed to isolated mitochondria which have a low K_m for ADP [7]. Later it was shown that such restricted diffusion occurs in slow but not in fast skeletal muscle [9]. In addition, coupling of mi-CK to respiration and the restricted diffusion of ADP represent adaptable mechanisms for mitochondrial regulation. Using rats fed on an analogue of creatine, β -guanidinopropionic

Table 4 Maximal rates of ATP and PCr production by mitochondria

V_{ATP} was calculated from Table 1 as $6 \times (V_{max} - I_0)$. A factor of 2 was used to correct for the temperature difference (second column). V_{PCr} was calculated from the relative amount of mi-CK (Table 3) and total CK activity (Table 2), taking into account the fact that the maximal rate of the CK reaction for PCr production is four times lower than the maximal rate of CK reaction for ATP production [16], and using a wet weight/dry weight ratio of 5.

	Rate ($\mu\text{mol/min per g dry wt.}$)		
	V_{ATP}		V_{PCr} (30 °C)
	22 °C	30 °C	
Ventricle	130	~ 260	250
Atria	89	177	25
Soleus	53	106	154

acid, which competitively inhibits creatine transport across the plasmalemma, Clark et al. [19] have shown a 3-fold decrease in the apparent K_m for ADP in skinned ventricular fibres, to a value close to that observed in isolated mitochondria. Similarly, Veksler et al. [9] found a decrease in the K_m for ADP in skinned ventricular and slow-twitch skeletal muscle fibres in M-CK knock-out mice. This kind of adaptation probably occurs to overcome the lack of a high-energy transfer mechanism between sites of energy production (mitochondria) and those of energy consumption (myofibrils and ion pumps).

The results presented in this study show that rat atria lack the property of coupled mi-CK, in spite of a high apparent K_m for ADP. This raises two questions: (1) what determines the coupling of mi-CK to respiration?, and (2) if mi-CK is not involved in adenine nucleotide compartmentation, how is the low accessibility of the mitochondrial inner compartment to ADP overcome in atria?

It was not clear whether both atrial and ventricular tissues, which exhibit different structural and metabolic properties, express the same mi-CK isoform [3,4,20]. Northern blot analysis demonstrated the expression of mi-CK_s in ventricle, soleus and atria, whereas no signal was observed in brain. However, no signal was obtained, even in brain, using the specific oligonucleotide for mi-CK_u. This could be due to insufficient sensitivity of our Northern blot analysis, since the oligonucleotide probe labelled using T4 polynucleotide kinase does not have a high specific activity. We therefore performed PCR amplification analysis using two reverse primers specific for each form of rat mi-CK and a common forward primer in the coding region. These primers were designed from the known cDNA sequences of rat mi-CK_s and mi-CK_u, and were chosen so that they were separated by at least one intron according to the known human gene sequences, in order to rule out an eventual amplification of DNA. The results clearly established that atria, like ventricles and slow-twitch skeletal muscle, express mi-CK_s only. Thus the functional differences between atrial and ventricular mi-CKs could not be explained by the expression of a specific isoform.

One interesting question regarding adenine nucleotide compartmentation is that of its structural basis. One model for the functional coupling of mi-CK to respiration is the theory of microcompartmentation and metabolic channelling within mitochondrial contact sites. In this model, ANT (located in the mitochondrial inner membrane), mi-CK (located in the intermembrane space between the mitochondrial inner and outer membranes) and porin (located in the mitochondrial outer membrane) form a highly organized multienzyme complex at contact sites,

thereby creating a microcompartment that allows efficient substrate channelling between the three enzymes [21–23]. In this model ATP, newly synthesized by respiration, is transported by ANT and converted *in situ* by mi-CK into ADP and PCr. PCr is exported via porin, which is considered as the main channel for nucleotides and small substrates in the mitochondrial outer membrane, whereas ADP is transported back by ANT into the matrix to be rephosphorylated. It has been shown that purified octameric mi-CK from chicken heart is preferentially suited to lipid binding, and interacts simultaneously with purified mitochondrial inner and outer membranes, thereby creating an intermembrane contact [24]. Complex-formation and functional interaction between the octameric oligomer of mi-CK and porin has been demonstrated *in vitro* [25]. In addition, kinetic differences between chicken heart dimeric and octameric oligomers of mi-CK have been observed, pointing to a possible physiological role for the oligomerization of mi-CK [26]. Therefore the oligomeric state of mi-CK might play an important role in adenine nucleotide compartmentation. However, different oligomerization patterns did not appear to account for atrio-ventricular differences, since no difference in the mi-CK octameric/dimeric ratio was observed between atria and ventricles.

Additional sites for mi-CK binding exist, however, on the mitochondrial inner membrane that are not in contact with the mitochondrial outer membrane, and the stoichiometry of ANT to mi-CK (one CK monomer for two ANT monomers) is not in complete accordance with such a strict molecular model [2]. Other molecular arrangements may be involved in the functional coupling. Mi-CK is bound to cardiolipins of the mitochondrial inner membrane in which ANT is embedded [27]. In ventricular tissue, ANT and CK are structurally close to each other; inhibition of respiration by antibodies against CK suggests that the distance between ANT and mi-CK is less than the molecular size of the antibodies (about 16 nm) [28]. The best way to accommodate these data with the stoichiometry of 1:2 between mi-CK and ANT is to suppose that the dimeric molecule of CK is structurally and functionally related to the tetramer of ANT [2]. Clearly, the precise structure of mi-CK–ANT interactions awaits further studies. However, multienzyme complex organization seems to be the main determinant of the functional coupling between mi-CK and oxidative phosphorylation. This may also involve tissue-specific isoforms of ANT, the amounts of cardiolipins, isoforms of porin and the spatial arrangement of the complexes. Our data suggest that atria may lack this type of organization, leading to a lack of coupling between mi-CK and respiration.

The lack of coupling of mi-CK to respiration in atria, in spite of a high K_m for ADP, raises the question of the accessibility of cytosolic ADP to mitochondria as an acceptor for respiration, and of the importance of oxidative phosphorylation for this tissue. The present results suggest that atria are less dependent on the shuttle function of the CK/PCr system. In this tissue, mi-CK would function as in smooth muscle in the 'ADP regeneration mode' only. Quantitative analysis of reaction rates in mitochondria shows that, whereas in ventricles and soleus mi-CK is expressed in sufficient amounts to precisely match the maximal rate of ATP production and its conversion into PCr, in atria the maximal PCr production rate by mi-CK is only 15% of that of ATP production. Clearly, other mitochondrial kinases may supply ADP for oxidative phosphorylation [29], and more work is needed to identify the mitochondrial kinases that fulfil this function in rat atrial fibres.

Atria express more BB-CK and MB-CK isoforms than ventricles, which is in accordance with the data of Savabi and

Kirsch [30]; this tissue has a lower oxidative capacity and depends more on the energy produced from glycolysis [10,30]. Since the CK/AK ratio is lower in atria than in the other tissues studied, it is likely that AK plays a more important role in energy transfer than in the ventricles. AK is present at sites of energy production (mitochondria) and utilization (myofibrils). It has been suggested that AKs, by close interaction with CKs, may also play an important role in cellular high-energy phosphate metabolism and transport (for a review, see [31]) and may participate in adenine nucleotide compartmentation within the mitochondrial intermembrane space [29]. It is not clear whether both mitochondrial and cytosolic AK activities are increased in atria. It has been shown that AK may take over a substantial proportion of energy-rich phosphoryl transfer when CK activity is reduced [32]. From studies of the kinetic behaviour of AK in intact diaphragm muscle, it was suggested that AK-catalysed phosphotransfer functionally couples ATP-consuming processes to anaerobic glycolytic ATP generation [32]. Atria contract against a lower pressure and for a shorter duration than ventricles, and cellular energy turnover is lower. Ventricular function would thus be more critically dependent on a well developed and effective energy producing and transport system (mi-CK coupled to mitochondrial respiration), tightly coupling respiration to energy consumption. In contrast, atria, which possess higher relative glycolytic capacities and lower energy requirements, would be less critically dependent on such a specialized mitochondrial transport system, but rather on a more efficient local ATP regenerating system through cytosolic or myofibrillar AK, CK and glycolytic enzymes.

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