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The role of creatine kinase in inhibition of mitochondrial permeability transition

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Abstract Cyclosporin A sensitive swelling of mitochondria isolated from control mouse livers and from the livers of transgenic mice expressing human ubiquitous mitochondrial creatine kinase occurred in the presence of both 40 μM calcium and 5 μM atractyloside which was accompanied by a 2.5-fold increase over state 4 respiration rates. Creatine and cyclocreatine inhibited the latter only in transgenic liver mitochondria. Protein complexes isolated from detergent solubilised rat brain extracts, containing octameric mitochondrial creatine kinase, porin and the adenine nucleotide translocator, were reconstituted into malate loaded lipid vesicles. Dimerisation of creatine kinase in the complexes and exposure of the reconstituted complexes to >200 μM calcium induced a cyclosporin A sensitive malate release. No malate release occurred with complexes containing octameric creatine kinase under the same conditions.
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Key words: Adenine nucleotide translocator; Apoptosis; Creatine kinase; Cyclocreatine; Mitochondria; Permeability transition

1. Introduction

Mitochondrial permeability transition is a phenomenon that occurs during calcium loading of isolated mitochondria due to opening of an unspecific pore in the mitochondrial inner membrane, i.e. the permeability transition pore (PTP) (for reviews see [1,2]). PTP formation is enhanced by increased production of reactive oxygen species [3–6] and allows the flow of solutes < 1.5 kDa out from the mitochondrial matrix [3,7]. Opening of the pore may be involved in cellular apoptosis as a 50 kDa apoptosis inducing factor protein (AIF) is released from the mitochondrial intermembrane space (IMS), due to destruction of the mitochondrial outer membrane after excessive mitochondrial matrix swelling [8]. PTP manifestation is inhibited by substrates for the mitochondrial adenine nucleotide translocator (ANT), e.g. ADP and bonkregic acid, or the immuno-suppressant drug cyclosporin A (CspA) [9–11]. Recent publications have indicated that the PTP consists of either ANT alone [12,13], or a multi-protein complex consisting, in part, of hexokinase, porin and ANT [14].

An enzyme believed to have a structural and functional interaction with ANT is the mitochondrial isoform of creatine (Cr) kinase (CK; EC 2.7.3.2) (see [15] for review). CK is an enzyme involved in ATP homeostasis in cells of high and fluctuating energy demand and catalyses the following reaction: $MgATP+Cr \leftrightarrow PCr+MgADP+H^+$. The mitochondrial isoforms of CK are the muscle specific sarcomeric Mi_b-CK, and the ubiquitous Mi_a-CK which is mainly found in brain tissue mitochondria. Mi-CK resides in the IMS, is believed to occur as an octamer in vivo [16] and has preferential access to ATP exported from the mitochondrial matrix by ANT [17,18]. The forward Mi-CK reaction (see above) keeps ANT saturated with ADP, which in turn has the effect of bringing mitochondria close to maximal state 3 respiration at relatively low ADP concentrations [17,18]. However, a structural interaction between Mi-CK and ANT has not been demonstrated experimentally, so far. The dimeric cytosolic enzyme isoforms are also expressed in a tissue specific fashion and are cytoplasmically compartmentalised [15], i.e. brain specific BB-CK, cardiac muscle specific heterodimeric MB-CK and the skeletal muscle cytosolic isoform MM-CK [15,17].

No CK isoform is expressed in mammalian liver, however new transgenic mice have been produced which express the BB-CK isoform specifically in their livers which has facilitated calculation of free ADP levels by ^{31}P-NMR [19]. These mice have acquired resistance to induced hepatic insults, e.g. fructose loading, major hepatectomy, ischaemia, hypoxia, as well as to endotoxins [20–23]. Another transgenic mouse has been developed in which the human Mi_a-CK gene is specifically expressed in liver cells and imported successfully into the mitochondria of these cells [24]. Interestingly these Mi_a-CK containing liver mitochondria were shown to contain IMS electron densities, similar to those seen in Cr deficient rat solei [17] and Cr deficient adult rat cardiomyocytes [25]. Considering that Mi-CK is proposed to interact with ANT, and ANT may be a major component of the PTP [3,12–14], we induced PTP opening in control and transgenic liver mitochondria and quantitated the effects of the CK substrates PCr, Cr and the Cr analogues cyclocreatine (cycloCr) and guanidinopropionic acid (GPA) on PTP manifestation in control and transgenic liver mitochondria containing Mi-CK. In addition, we determined the functionality of Mi_a-CK in relation to its coupling to ANT and oxidative phosphorylation (OXPHOS) in these mitochondria [17]. Finally, previously characterised isolated complexes from detergent solubilised rat brain extracts, known to contain porin, octameric Mi_a-CK and ANT [14], were reconstituted into malate loaded vesicles. CspA sensitive malate release from these vesicles was used to analyse the role

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of Mi_a-CK in PTP opening or closing within the reconstituted complexes, in the presence of increasing concentrations of Ca^{2+}.

2. Materials and methods

2.1. Transgenic mice and creatine analogue production

Transgenic mice produced according to [24] were kindly provided on request by A.P. Koretsky. Guanidinopropionic acid and 1-carboxymethyl-3-iminoimidazolidine (cyclocreatine) were produced according to [26,27].

2.2. Mitochondrial isolation

Mice were killed and livers were immediately removed and placed into ice cold MSH buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EGTA, pH 7.4). The tissue was minced and washed with fresh MSH buffer until most of the blood was removed. Mitochondria were isolated and further purified on a 20% Percoll gradient as described in [17]. Protein determinations were carried out according to [28].

2.3. Creatine kinase activity measurements

Mitochondria of transgenic and control liver were analysed for creatine kinase activity in the pH-stat in 75 mM KCl, 10 mM $MgCl_2$, 0.1 mM EGTA, 1 mM β-mercaptoethanol and 4 mM ADP. The CK reaction was started upon addition of 10 mM PCr [29].

2.4. Spectrophotometry and oxygraph respirometry measurements of isolated liver mitochondria

CspA sensitive swelling of control and transgenic liver mitochondria (0.5–1 mg/ml in a buffer containing 40 μM $CaCl_2$, 5 mM succinate, 20 mM Na_2HPO_4, 75 mM mannitol, 250 mM sucrose, 10 mM HEPES, pH 7.4) was induced by addition of 5 μM atractyloside (Atr). Absorption was monitored spectrophotometrically at 540 nm. Decreased absorption at 540 nm, due to mitochondrial swelling, is taken as an indicator of PTP opening [1].

Respiration measurements were carried out with isolated mitochondria from control and transgenic liver at 25°C, using a Cyclobios oxygraph (Anton Paar, Innsbruck, Austria) in respiration buffer (75 mM mannitol, 250 mM sucrose, 10 mM HEPES, pH 7.4) [17]. Stimulation of state 4 respiration was carried out with 5 mM succinate and in the presence of 5 mM $MgCl_2$, 20 mM Na_2HPO_4. Low and maximum state 3 respiration were stimulated with 25 μM ADP followed by 50 μM ADP, respectively. This was repeated in the presence of 10 mM Cr to analyse Mi_a-CK mediated stimulation of state 3 respiration [17,18]. Furthermore, after stimulating state 4 respiration (with 5 mM succinate), oxygen consumption caused by PTP formation was measured first by introduction of 40 μM $CaCl_2$ (final concentration) to the electrode chamber, followed by 5 μM Atr. Additionally, measurements were also performed in the presence of CK substrates, i.e. 10 mM Cr, PCr, cycloCr or GPA. To verify PTP opening 2 μM CspA (Calbiochem) was added prior to the additions of 40 μM $CaCl_2$ and 5 μM Atr.

2.5. Reconstitution of complexes isolated from rat brains

Rat brains, stored frozen, were homogenised with a Teflon potter in a medium composed of 10 mM glucose, 10 mM monothioglycerol, at pH 8. The membranes were washed 3 times by centrifugation for 15 min at $12000 \times g$. The complex was extracted from the final pellet by resuspension in isolation medium containing 0.5% Triton X-100 and incubation for 45 min at room temperature. The undissolved membrane material was removed by centrifugation for 45 min at $40000 \times g$ in a Beckman Ti 50 rotor. The supernate was stirred for 30 min at room temperature with diethylaminoethyl (DEAE) cellulose (DE52 Whatman) that had been equilibrated with 1.5 mM Na_2HPO_4, 1.5 mM K_2HPO_4, 1.0 mM dithioerythritol, 0.1 M glucose pH 8.0 (buffer A). The amount of DEAE cellulose used was 3 g per U of creatine activity. Mitochondrial creatine kinase was eluted from the column by a KCl gradient between 40 and 400 mM [14]. As shown recently [14] this fraction is also composed of porin and ANT.

2.6. Reconstitution of the complexes into vesicles

The complex fraction containing Mi-CK, ANT and porin was reconstituted in turkey egg yolk lecithin+2% cholesterol. The phospholipid complex mixture was dialysed overnight against 10 mM HEPES pH 7.4. The vesicles were loaded with 5 mM malate by sonication, purified and freed of excess malate by subsequent chromatography on Sephadex G50 in 10 mM HEPES buffer. After chromatography the permeability of the loaded vesicles for malate was tested. Vesicles containing the protein complexes, and no protein as control, were centrifuged for 30 min at $400000 \times g$ and the supernate was tested for malate enzymatically [14].

2.7. Dissociation of the creatine kinase in the complex

Dissociation of octameric Mi_a-CK was performed according to [30] in the reconstituted liposomes and in aliquots of the DEAE cellulose column fractions by incubation of the samples for 20 min at room temperature with 5 mM $MgCl_2$, 20 mM creatine, 50 mM KNO_3 and 4 mM ADP (a mixture mimicking the transition state analogue complex or TSAC and known to dissociate octameric Mi-CK into dimers) [30]. The dissociation of the creatine kinase octamer was verified by Superdex 200 chromatography of the treated and untreated DEAE fractions. Malate loaded liposomes containing reconstituted octameric creatine kinase complex or dissociated dimeric creatine kinase complex were suspended in a buffer containing 125 mM sucrose, 10 mM HEPES (pH 7.4). The vesicles were analysed for malate release by addition of Ca^{2+} between 0 and 400 μM in the presence or absence of 0.5 μM *N*-methylVal-4-cyclosporin (a gift from P. Bernardi to D. Brdiczka).

3. Results and discussion

3.1. Mitochondrial respiration analyses and spectrophotometry

Transgenic liver mitochondria displayed 5.6 ± 1.6 units of specific Mi_a-CK activity per mg of mitochondrial protein ($n = 4$), determined by the pH-stat assay [29]. No CK activity was measured in control liver mitochondria. We compared the rates of oxygen consumption due to coupled OXPHOS of control and transgenic liver mitochondria. As shown in Table 1, under conditions of 25 and 50 μM ADP the transgenic mice showed slightly lower levels of oxygen consumption than controls. Consequently the transgenic liver mitochondria had a lower respiratory control ratio (RCR) than control, i.e. 3 and

Table 1
Rates of oxygen consumption (nmol O/mg mitochondrial protein/min) by control and transgenic mouse mitochondria ($n = 3$)

Substrate	Control mitos	Trans. mitos
5 mM succ.	13 ± 3	14 ± 1.5
25 μM ADP	33 ± 1	28 ± 1*
50 μM ADP	65 ± 3	55 ± 2
RCR	4	3
+10 mM creatine		
5 mM succ.	15 ± 2	15.5 ± 2
25 μM ADP	34 ± 1	45.5 ± 3*
50 μM ADP	62 ± 3	56 ± 3

Rates of oxygen consumption of control and transgenic (Trans.) mouse liver mitochondria (mitos) were carried out at 25°C with a Cyclobios oxygraph (Anton Paar, Innsbruck, Austria) in respiration buffer (75 mM mannitol, 250 mM sucrose, 10 mM HEPES, pH 7.4). Stimulation of OXPHOS was carried out in the presence of 5 mM $MgCl_2$, 20 mM Na_2HPO_4. State 4 respiration was the rate seen in the presence of 5 mM succinate only (succ.). Low state 3 was the rate of oxygen consumption seen after the addition of 25 μM ADP, and maximal state 3 that observed in the presence of 50 μM ADP, with 5 mM succinate already present. These experiments were repeated in the presence of 10 mM Cr to analyse Mi_a-CK mediated stimulation of state 3 respiration. Note that in mitochondria of transgenic mice expressing Mi_a-CK in their livers (Trans. mitos), creatine stimulated respiration at 25 μM ADP (*) relative to the values reached with the same ADP concentration. No comparable effect was observed in control mitochondria.

4, respectively (Table 1). In the presence of 10 mM Cr, 25 μM ADP brought state 3 from 50% of maximum (as seen without Cr) to 80% of maximal state 3 (Table 1), only in transgenic liver mitochondria. This stimulation of respiration by Cr via Mi-CK is normally found with isolated brain, skeletal and cardiac muscle mitochondria [17]. This clearly indicates that the Mi_a-CK in the transgenic liver mitochondria is active and conveys Cr stimulated respiration to mitochondria. No such effect was seen in control liver mitochondria, as expected (Table 1). Gel permeation chromatography of transgenic liver mitochondria extracts confirmed the prevalence (>95%) of octameric Mi-CK (data not shown).

In control and transgenic liver mitochondria, swelling (indicative of PTP opening) was observed only after the addition of 5 μM Atr to the mitochondrial suspensions containing 40 μM Ca^{2+} (data not shown). No swelling was observed in the presence of 0.5 μM CspA. In the next set of mitochondrial experiments we measured the rates of oxygen consumption above state 4 (with 5 mM succinate) after the addition of 40 μM Ca^{2+} followed by 5 μM Atr. 40 μM Ca^{2+} alone induced a 50–60% increase of state 4 oxygen consumption (Fig. 1), further addition of 5 μM Atr led to a final 2.5-fold increase of state 4 oxygen consumption in both control and transgenic liver mitochondria (Fig. 1). 10 mM Cr or cycloCr inhibited this stimulation of OXPHOS observed with either 40 μM Ca^{2+} or 5 μM Atr, only with transgenic mouse mitochondria (Fig. 1). Neither PCr nor GPA had any observable effect on oxygen consumption rates under these conditions of control and transgenic liver mitochondria, under the same conditions.

3.2. PTP-like properties in vesicles containing protein complexes isolated from rat brain

Experiments were carried out with previously characterised protein complexes isolated from rat brain [14], consisting of ANT, porin and octameric Mi_a-CK. These complexes were reconstituted into malate loaded phospholipid vesicles and exposed to 0–400 μM Ca^{2+} in the absence or presence of TSAC (to dimerise Mi-CK) (Fig. 2A). CspA sensitive malate release from the vesicles served as an indicator of PTP-like behaviour. Malate release was not observed upon loading of the vesicles with Ca^{2+} ranging from 0 to 400 μM (Fig. 2A). In contrast, treatment of the vesicles with TSAC caused a 6-fold increase of malate release in the presence of 400 μM Ca^{2+}, over that seen without Ca^{2+}. In the presence of CspA plus TSAC, malate release was efficiently blocked (Fig. 2A). These results indicate that octameric Mi_a-CK within the reconstituted complexes is necessary for inhibition of permeability transition, without the addition of any CK substrate. TSAC induced dimerisation of Mi-CK was confirmed by gel permeation chromatography, revealing that all measurable CK activity eluted in a peak at a MW of 80 kDa, representing dimeric Mi_a-CK (Fig. 2B).

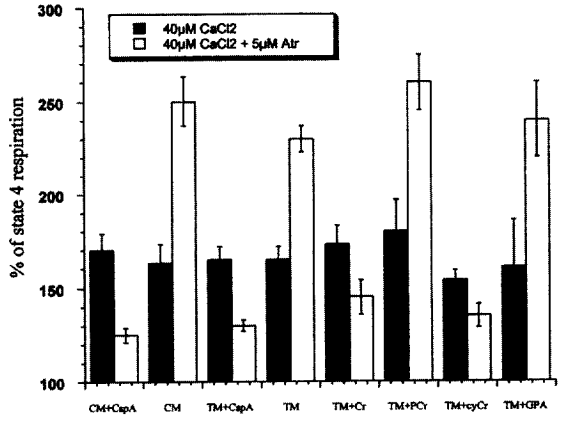

Fig. 1. Histogram representing the rates of oxygen consumption as a percentage of state 4 (100% = state 4 with 5 mM succinate) in the presence of 40 μM $CaCl_2$ followed by 5 μM atractyloside. CM = control liver mitochondria, TM = transgenic liver mitochondria, error bars indicate the standard deviation. All experiments were carried out at 25°C, with a Cyclobios oxygraph (Anton Paar, Innsbruck, Austria) in the respiration buffer (220 mM D-mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.4) and as indicated, in the presence of 2 μM cyclosporin A (+CspA), or 10 mM creatine (+Cr), 10 mM phosphocreatine, 10 mM cyclocreatine (+cyCr), 10 mM guanidinopropionic acid (+GPA). It is evident that 10 mM Cr and 10 mM cyclocreatine have a similar effect to 2 μM CspA by inhibiting the 5 μM Atr stimulation of oxygen consumption after the addition of 40 μM $CaCl_2$.

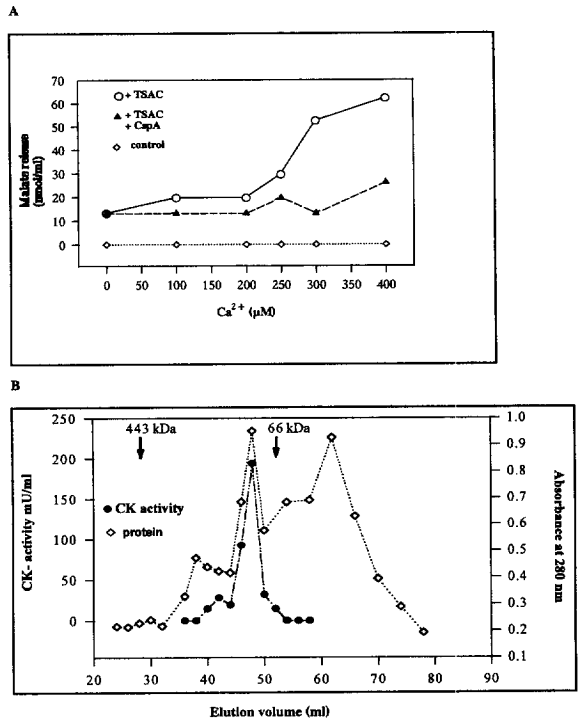

Fig. 2. (A) The isolated complexes incorporated into liposomes were loaded with malate and treated with concentrations of Ca^{2+} from 0 to 400 μM, and analysed for malate release (empty diamonds). These experiments were repeated after preincubation for 15 min at 25°C of the vesicles with 500 nM *N*-methylVal-4-cyclosporin (CspA) plus the transition state analogue complex, i.e. 5 mM $MgCl_2$, 20 mM creatine, 50 mM KNO_3 and 4 mM ADP (TSAC, inducing a Mi-CK dimer-octamer transition (filled triangles), or preincubated with TSAC alone, before exposure of isolated PTP complexes to the increasing calcium concentrations (empty circles). Note that dimerisation of Mi-CK led to maximal malate release (permeability transition-like pore opening). (B) The dissociation of the creatine kinase octamer was verified by Superdex 200 chromatography of the TSAC treated DEAE fractions. Nearly all the CK activity (filled circles) and protein (empty triangles), measured by absorption at 280 nm, spectrophotometrically eluted in a peak representing a molecular weight of approximately 80 kDa. Arrows indicate calibrated molecular weight elution volumes (ml).

4. Conclusion

The results presented in this study reveal that human Mi_a-CK expressed in the liver of transgenic mice behaves exactly as Mi_a-CK does in isolated brain mitochondria, where it is most commonly expressed [17]. The spectrophotometric analyses of mitochondrial swelling as well as respiration measurements of control and transgenic liver mitochondria revealed that permeability transition only occurred in the presence of both 40 μM $CaCl_2$ and 5 μM Atr. This suggests that calcium cycling is responsible for the initial increased rate of oxygen consumption over state 4, but is insufficient to induce permeability transition. Such $[Ca^{2+}]$ may have a priming effect of ANT in isolated mitochondria, which supports permeability transition in the presence of low levels of Atr (an ANT substrate), which has already been proposed [12]. The absence of EDTA or EGTA in our experiments explains the low amount of calcium and Atr necessary to induce permeability transition, and why millimolar quantities of Atr were necessary in the experiments of the Kreomer group, which were carried out in the presence of the above chelating agents [1,8]. The respiration rate increases 2.5-fold over state 4 due to permeability transition, indicating a collapse of the mitochondrial inner membrane potential. Furthermore, the respiration experiments in the presence of Ca^{2+} and Atr provide proof that the oxygen consumption induced by the Atr specific inducible permeability transition can be inhibited by Mi_a-CK in the presence of Cr and cycloCr (similar to CspA). Neither PCr nor GPA has any effect under the conditions we used. The isolated and reconstituted brain complexes showed that octameric Mi_a-CK is essential to inhibit the CspA sensitive release of malate in the presence of 0–400 μM Ca^{2+}. We believe that the complex isolation procedure from solubilised rat brain homogenates may selectively enrich Mi_a-CK bound to ANT, perhaps due to residually bound Cr. A recent small angle X-ray scattering study of Mi_b-CK and MM-CK size upon substrate binding showed that binding of Mg nucleotides led to a marked decrease of the radius of gyration of both MM-CK and Mi-CK, leading to a more compact molecular shape, whereas Cr had no observable effect [31]. Therefore, after binding of Cr or cycloCr by Mi_a-CK, an additional, subtle structural change must arise to facilitate the Mi_a-CK-ANT interaction to occur. GPA feeding for at least 6 weeks has been shown to lead to a doubling of BB-CK in mouse [32] and rat brains [17], and to increased resistance to hypoxia [32]. It would therefore appear from our results that the latter protective effect is due to the increased cytosolic BB-CK preventing ATP catabolism [32] and not related to permeability transition inhibition. Furthermore, permeability transition is implicated in myocardial cell, and hepatocyte, injury during ischaemia and reperfusion and cycloCr has been shown to be a potent inhibitor of such damage in cardiac tissue, whereas Cr was not [33,34]. We propose that this specific cycloCr effect may lock the Mi-CK-ANT complex into a permeability transition inhibitory conformation more effectively than Cr. This could occur as cycloCr is phosphorylated at a 30-fold slower rate than Cr [27,35]. The reported effect of Cr and cycloCr in inhibiting permeability transition opening and consequently the mitochondrial events initiating apoptosis [8] may be relevant clinically in the future, considering the potential of Cr supplementation as an adjuvant therapy for a number of neuromuscular degenerative diseases [36,37].

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