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Inhibition of the Mitochondrial Permeability Transition by Creatine Kinase Substrates

REQUIREMENT FOR MICROCOMPARTMENTATION*

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Mitochondria from transgenic mice, expressing enzymatically active mitochondrial creatine kinase in liver, were analyzed for opening of the permeability transition pore in the absence and presence of creatine kinase substrates but with no external adenine nucleotides added. In mitochondria from these transgenic mice, cyclosporin A-inhibited pore opening was delayed by creatine or cyclocreatine but not by β-guanidinopropionic acid. This observation correlated with the ability of these substrates to stimulate state 3 respiration in the presence of extramitochondrial ATP. The dependence of transition pore opening on calcium and magnesium concentration was studied in the presence and absence of creatine. If mitochondrial creatine kinase activity decreased (*i.e.* by omitting magnesium from the medium), protection of permeability transition pore opening by creatine or cyclocreatine was no longer seen. Likewise, when creatine kinase was added externally to liver mitochondria from wild-type mice that do not express mitochondrial creatine kinase in liver, no protective effect on pore opening by creatine and its analog was observed. All these findings indicate that mitochondrial creatine kinase activity located within the intermembrane and intercristae space, in conjunction with its tight functional coupling to oxidative phosphorylation, via the adenine nucleotide translocase, can modulate mitochondrial permeability transition in the presence of creatine. These results are of relevance for the design of creatine analogs for cell protection as potential adjuvant therapeutic tools against neurodegenerative diseases.

Most vertebrate cell types express cytosolic as well as mitochondrial isoforms of the enzyme creatine kinase (CK).[1] CK catalyzes the reversible transphosphorylation of phosphocreatine (PCr) to ATP. The findings of distinct subcellular localizations of creatine kinases have led to the formulation of the PCr circuit concept, proposing that sites of energy production (mitochondria and glycolysis) are tightly linked via Cr/PCr shuttling to sites of energy consumption (various cellular ATPases) (1–6). ATP generated by oxidative phosphorylation in mitochondria reacts with creatine (Cr) to produce PCr, a reaction mediated by mitochondrial CK (mtCK) located in the intermembrane and intercristae space. Phosphocreatine then diffuses to the cytosol to locally regenerate ATP via cytosolic CK from ADP. Cr produced during this reaction is shuttled back to the mitochondria for recharging it to PCr. This energy shuttling system is particularly efficient in tissues with a very high and fluctuating energy demand like skeletal and cardiac muscle, as well as in brain and neural tissues (7). Thus, in addition to the generally accepted temporal energy buffering function, PCr also provides a means to spatially buffer energy reserves (3). This holds especially true for highly polar cells like spermatozoa where the diffusion of ADP is the limiting factor (8).

Studies with cultured rat hippocampal neurons have shown recently that creatine protects against glutamate and β-amyloid toxicity (9), as well as against energetic insults in striatal neurons (10). Similarly, creatine and in some cases the related analog cyclocreatine (CyCr) exert protective effects in several animal models of neurodegenerative diseases, like Huntington's disease (11), amyotrophic lateral sclerosis (12), and a form of Parkinsonism (13). Likewise, Cr pretreatment of cultured myotubes from dystrophic *mdx* mice enhanced myotube formation and survival (14). Recent data indicate that Cr reduces muscle necrosis and protects mitochondrial function *in vivo* in *mdx* mice (15). Treatment of patients with Cr or Cr analogs has, therefore, been proposed as a possible adjuvant therapy for such diseases (16). The protection observed with Cr in these cell and animal models may be explained partially by its function as a cellular energy buffer and transport system via the PCr shuttle as outlined above. An additional potential mechanism of Cr protection may be linked to direct effects on mitochondrial permeability transition (MPT) (17), which has been suggested to be a causative event in different *in vivo* and *in vitro* models of cell death (18–24).

In an attempt to define the role of mitochondrial creatine kinase on MPT, we have shown in an earlier study that isolated liver mitochondria from transgenic mice containing mtCK did not respond by MPT pore opening upon treatment with Ca^{2+} plus atractyloside if Cr or CyCr were present in the medium

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[1] The abbreviations used are: CK, creatine kinase; ANT, adenine nucleotide translocase; Ap_5A, P^1,P^5-di(adenosine 5′)-pentaphosphate (an inhibitor of adenylate kinase); BB-CK, cytosolic brain-type creatine kinase; Cr, creatine; CsA, cyclosporin A; CyCr, cyclocreatine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; GPA, β-guanidinopropionic acid; IMS, intermembrane space; MPT, mitochondrial permeability transition; mtCK, mitochondrial creatine kinase; PCr, phosphocreatine; Mops, 4-morpholinepropanesulfonic acid.

(17). In the absence of Cr and CyCr, however, MPT pore opening could be fully induced by Ca^{2+} plus atractyloside. On the other hand, in liver mitochondria from control mice without mtCK, pore opening induced by Ca^{2+} plus atractyloside was independent on the presence or absence of Cr or CyCr. In the present study we investigated these effects in more detail, asking the question of whether inhibition of MPT pore opening by CK substrates depends on mtCK activity and whether the tight functional coupling between the CK reaction and oxidative phosphorylation, demonstrated recently to take place *in situ* (25), would lead to cycling of mitochondrial adenine nucleotides, thus resulting in net production of phosphorylated CK substrates. Our results indicate that substrate channeling between mtCK and adenine nucleotide translocase (ANT) takes place in a tight functionally coupled microcompartment that seems absolutely required for protection of MPT pore opening by creatine.

EXPERIMENTAL PROCEDURES

Source and Preparation of Mitochondria—Transgenic mice expressing the ubiquitous mitochondrial creatine kinase isoform in their liver mitochondria (transgenic liver-mtCK mice) were kindly provided by Dr. Alan P. Koretsky (National Institutes of Health, Bethesda). Mice were killed, and the liver was quickly removed and placed in ice-cold isolation buffer for mitochondria (10 mM Tris-HCl, pH 7.4, 250 mM sucrose) supplemented with 1 mM EDTA and 0.1% bovine serum albumin. Livers were homogenized, and the homogenate was centrifuged at $700 \times g$ for 10 min. The supernatant was filtered through two layers of nylon gauze and centrifuged at $7000 \times g$ for 10 min. The mitochondrial pellet was washed twice with isolation buffer (without EDTA and bovine serum albumin), centrifuged, and kept on ice.

Respiration and Swelling Measurements—Mitochondrial oxygen consumption was measured with a Cyclobios oxygraph (Anton Paar, Innsbruck, Austria) at 25 °C. The standard medium (2 ml) consisted of 10 mM Tris/Mops, pH 7.4, 250 mM sucrose, 10 mM phosphate/Tris, 2 mM $MgCl_2$, 0.5 mM EGTA/Tris, 2 μM rotenone, and 50 μM Ap_5A. State 4 respiration was stimulated by addition of 5 mM succinate/Tris. Creatine kinase substrates (Cr, CyCr, and GPA) were present at 10 mM concentration, and state 3 respiration was induced by adding 1 mM ATP. Mitochondrial protein concentration in all assays was 0.5 mg/ml. Deviations from these conditions are specified in the figure legends.

Mitochondrial MPT pore opening was measured by the convenient swelling assay and carried out in a UV4 UV/Vis spectrometer (Unicam) connected to a computer. Swelling curves were recorded at 540 nm, and data points were acquired every 1/8 s using Vision© software (version 3.10, Unicam Ltd.). Cuvettes containing the mitochondrial suspension were thermostated to 25 °C. Incubation conditions and induction of MPT are indicated in the legends to the figures.

Measurement of PCr Production by Quantitative Thin Layer Chromatography—Mitochondria were incubated under different conditions (see legend to Fig. 4) in the presence of $^{32}P_i$. Reactions were stopped after defined time intervals by addition of 1% (final concentration) SDS and centrifuged, and the supernatants were applied to Silica Gel 60 thin layer plates (Merck). Running solvent was a mixture of isopropyl alcohol, ethanol, and 25% ammonia (6:1:3, by volume). Thin layer plates were air-dried, exposed to a Kodak storage phosphorscreen SO230, and analyzed with a PhosphorImager (Storm 820, Amersham Biosciences). The position of PCr in thin layer chromatograms was identified in control experiments using recombinant brain-type BB-CK incubated in the presence of radioactive [^{14}C]creatine and cold ATP.

Binding of Exogenously Added CK to Mitochondria—Mouse liver mitochondria (0.5 mg/ml) were incubated in standard medium at pH 7.4 with 5 mM glutamate and 2.5 mM malate and without rotenone. Specified amounts of recombinant brain-type BB-CK or ubiquitous human mtCK (prepared as described elsewhere (26, 27)) were added. After a 5-min incubation, an aliquot of the suspension was removed (to measure total CK activity). The rest was centrifuged (5 min at $7000 \times g$) to separate mitochondria. CK activity was measured separately in the supernatants and mitochondrial pellets by a coupled enzymatic assay (28).

Other Methods—Protein concentrations were determined by Bradford assay (Bio-Rad) using bovine serum albumin as a standard. Mitochondrial adenine nucleotide content was measured by reversed phase chromatography of acid extracts according to Kay *et al.* (25).

TABLE I

Oxygen consumption rates of liver mitochondria from transgenic liver-mtCK mice in the presence of different creatine kinase substrates

Incubation conditions are specified under "Experimental Procedures." State 4 respiration was measured in the presence of 5 mM succinate/Tris, and state 3 was stimulated by addition of 1 mM ATP. Oxygen consumption values are means ± S.D. *n,* number of experiments. Note that transgenic mtCK containing mitochondria show Cr- and CyCr-stimulated respiration in contrast to liver mitochondria from control mice (not shown, see Ref. 17).

CK substrate	State 4	State 3	State 3/state 4	*n*
	nmol O·min^{-1}·mg^{-1} protein			
None	55.7 ± 2.6	64.9 ± 2.3	1.17	6
10 mM Cr	56.5 ± 2.9	167.9 ± 7.9	2.97	8
10 mM CyCr	57.7 ± 2.2	164.7 ± 7.0	2.86	8
10 mM GPA	55.3 ± 1.2	64.5 ± 2.0	1.17	8

RESULTS

To correlate the effects of the different creatine kinase substrates on MPT pore opening with their ability to stimulate oxidative phosphorylation, we first measured stimulation of state 3 respiration by ATP and CK substrates in mitochondria oxidizing succinate. The data of these experiments are summarized in Table I. In mtCK containing mitochondria from transgenic mice, substantial stimulation (about 3-fold over state 4) was observed only with Cr and CyCr (both 10 mM), but not with GPA, in the presence of externally added ATP, due to endogenous production of ADP by mitochondrial CK. The absence of a detectable stimulation with 10 mM GPA agrees with the inability of mitochondrial CK to phosphorylate this creatine analog (29, 30). No Cr- or CyCr-stimulated respiration was seen with mitochondria from control mice, which do not express mtCK in liver (17). These data confirm earlier findings with the same substrates given to heart mitochondria (30). In general, with respect to creatine-stimulated respiration, mitochondria from transgenic liver-mtCK mice are behaving comparably to mtCK containing heart mitochondria (see also Ref. 17).

Next, we measured, in transgenic mtCK-liver mitochondria, the opening of the permeability transition pore induced by Ca^{2+} in the presence of CK substrates, but in the complete absence of external adenine nucleotides, and under different of conditions. To avoid any effects caused by adenylate kinase activity, the specific adenylate kinase inhibitor Ap_5A was included in the medium in all experiments. In the experiments shown in Fig. 1*A*, mitochondria were energized with glutamate and malate in the absence of rotenone. Due to the presence of 2 mM Mg^{2+} (but no EGTA) in the medium, a rather high (120 μM) Ca^{2+} pulse had to be administered to overcome MPT inhibition by magnesium. As shown in Fig. 1*A*, Cr and CyCr effectively inhibited pore opening within the time frame of the experiment. In the absence of CK substrates or with GPA present, MPT pore opening occurred in a significant fraction of the mitochondrial population. These effects of CK substrates were independent, at least qualitatively, on how the MPT was triggered and of the respiratory substrates used as documented in Fig. 1, *B* and *C*. In the experiments shown in Fig. 1*B*, mitochondria were again energized with complex I substrates (glutamate and malate), but exposed to only 40 μM Ca^{2+} (which did not open the MPT *per se*). Subsequent depolarization with 0.2 μM FCCP led to rapid swelling of the mitochondria, both in the absence and presence of CK substrates. Swelling was again sensitive to CsA, as well as to 50 μM ubiquinone 0, a novel and general MPT inhibitor (not shown) (31), indicating opening of the MPT pore. Remarkably, however, Cr and CyCr exerted MPT protection in a significant subpopulation of mitochondria even in the absence of external adenine nucleotides and under these very strongly pore-promoting conditions (absence of EGTA, high phosphate, depolarization of mitochondria by

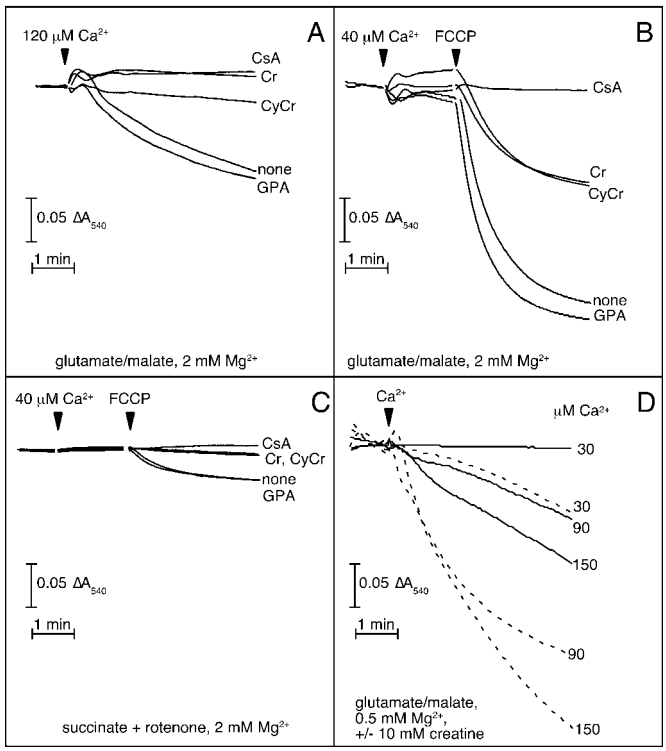

FIG. 1. **Swelling measurements showing the effect of creatine kinase substrates on MPT pore opening of liver mitochondria from transgenic liver-mtCK mice under different conditions.** The incubation medium (1 ml) contained 10 mM Tris/Mops, pH 7.4, 250 mM sucrose, 10 mM phosphate/Tris, 2 mM (*A–C*) or 0.5 mM (*D*) $MgCl_2$, and 50 μM Ap_5A. Mitochondria were energized either with 5 mM glutamate/Tris and 2.5 mM malate/Tris (*A*, *B*, and *D*) or with 5 mM succinate/Tris in the presence of 2 μM rotenone (*C*). CK substrates were present at 10 mM concentration. In the uppermost traces of *A–C*, the medium was supplemented with 1 μM CsA. Reactions were started by addition of 0.5 mg of mitochondria (not shown), and MPT was induced by Ca^{2+} or Ca^{2+} plus FCCP as indicated. *D*, *solid* and *dashed traces* represent conditions with and without 10 mM Cr, respectively.

FCCP, and presence of complex I substrates). Again, GPA did not protect mitochondria from MPT pore opening. Fig. 1*C* shows the same set of experiments but with succinate-energized mitochondria in the presence of rotenone. Here, the same qualitative conclusions can be drawn as from the data presented in Fig. 1, *A* and *B*. In accordance with the finding of Fontaine *et al.* (32), conditions are more restrictive for MPT pore opening with complex II-linked substrates due to decreased electron flux through complex I, a general property of the MPT. With succinate as the electron donor, Cr and CyCr, but not GPA, fully protected from MPT pore opening.

Next we analyzed MPT protection by creatine at different calcium concentrations. In the experiments displayed in Fig. 1*D,* the Mg^{2+} concentration was reduced to 0.5 mM to compare better the effect of Cr as a function of the calcium load. Under these conditions, 30 μM Ca^{2+} did not open the MPT pore if Cr was present. Pore opening was, however, observed at higher Ca^{2+} concentrations (90 and 150 μM, *solid curves* in Fig. 1*D*). Nevertheless, Cr still inhibited significant fractions of mitochondria when compared with conditions without Cr but equal Ca^{2+} concentrations (*dashed curves* in Fig. 1*D*).

Based on these observations and the data presented in Table I, we suggested that the protective effect on MPT pore opening seen with Cr and CyCr as compared with GPA could be related to the kinetic properties of these substrates, *i.e.* their rate of phosphorylation by mtCK. In contrast to GPA, Cr and CyCr are rapidly converted by mtCK via ATP to their respective phosphorylated compounds, PCr and CyPCr (30). If substrate phosphorylation by mtCK (with internally available ATP) were responsible for the observed effects, protection of MPT by Cr and CyCr should have been abolished under conditions where CK is inactive as an enzyme. As there is no absolutely specific CK inhibitor available, we omitted Mg^{2+} instead, which is an essential cofactor for the CK reaction (33), from the medium. With no Mg^{2+} present, creatine-stimulated respiration with ATP was almost completely absent as shown in Fig. 2*A* (compare slopes at *arrow* in *trace a* with that of *trace b*). Importantly, in the absence of exogenous Mg^{2+}, state 3 respiration was still observed after addition of ADP (disodium salt, Fig. 2*A*, *trace a*) indicating that sufficient matrix Mg^{2+} is available for phosphorylation of ADP by F_0F_1-ATP synthase, but this Mg^{2+} is not accessible to mtCK located in the intermembrane space. As shown in Fig. 2*B*, in the absence of Mg^{2+}, mitochondrial swelling induced by 20 μM Ca^{2+} and 0.2 μM FCCP occurred to the same extent regardless of whether CK substrates were present or not, in contrast to what was observed in the presence of Mg^{2+} (see the corresponding traces in Fig. 1*B*). MPT pore opening, however, was still fully blocked by 1 μM CsA, even in the absence of Mg^{2+}.

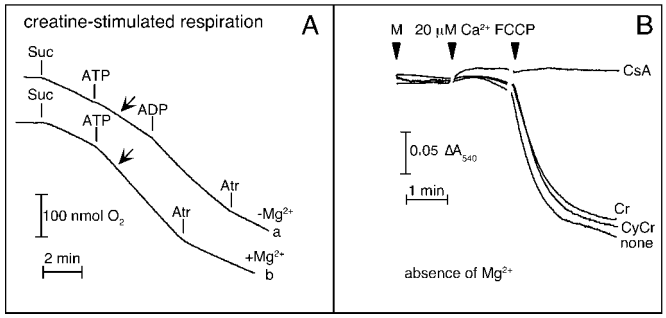

FIG. 2. **Effect of Mg^{2+} on respiration and swelling of liver mitochondria from transgenic liver-mtCK mice in the presence of creatine kinase substrates.** *A*, respiration measurements without (*trace a*) and with 2 mM $MgCl_2$ (*trace b*). Where indicated, 5 mM succinate/Tris (*Suc*), 1 mM ATP, 50 μM ADP, or 20 μM atractyloside (*Atr*) was added. Note that CK-mediated, Cr-stimulated state 3 respiration with ATP in the presence of Mg^{2+} is no longer observed in the absence of Mg^{2+} (compare slopes at *arrows* in *traces b* and *a*, respectively). *B*, swelling measurements. Incubation conditions were as specified in the legend to Fig. 1*A*, except that $MgCl_2$ was omitted. CK substrates (Cr or CyCr) were present at 10 mM concentration. In the *uppermost trace,* the medium was supplemented with 1 μM CsA. Reactions were started by addition of 0.5 mg of mitochondria (*M*) to 1 ml of medium followed by Ca^{2+} and FCCP as indicated.

In a further set of experiments, we analyzed the effect of Mg^{2+} and Cr on MPT in more detail by varying the concentrations of these substrates and measuring mtCK activity under identical conditions. Representative swelling measurements at two different Mg^{2+} concentrations with (*solid curves*) and without (*dashed curves*) Cr are displayed in Fig. 3*A*. From such curves we determined the absorption difference (ΔA_{540}) before and 4 min after triggering the MPT with 120 μM Ca^{2+} (same conditions as in the experiments of Fig. 1*A*). The ΔA_{540} values were used to calculate the fraction of swollen mitochondria with reference to the ΔA_{540} measured with 5 μM of the channel-forming peptide alamethicin, which resulted in (not MPT-caused) swelling of 100% of the mitochondria (not shown). As shown in Fig. 3*C*, the fraction of swollen mitochondria, *i.e.* the fraction having undergone a permeability transition, decreased at increasing Mg^{2+} concentrations, also in the absence of Cr (*open circles* in Fig. 3*C*). This was to be expected, as the MPT is regulated by an external inhibitory Me^{2+}-binding site (34). However, in the presence of 10 mM Cr, an additional protection starting at around 0.5 mM Mg^{2+} is observed (*closed circles* in Fig. 3*C*). At this Mg^{2+} concentration, mtCK activity, as measured in the pH-stat in the forward reaction with ATP plus Cr,

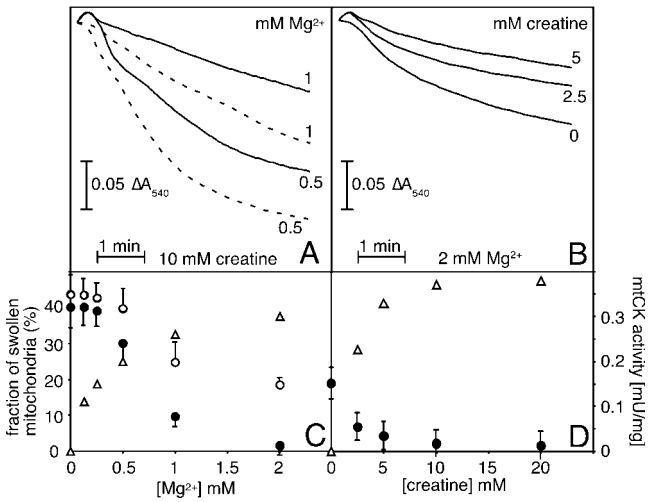

FIG. 3. **Correlation of mtCK activity with MPT inhibition at variable substrate concentrations.** Incubation conditions for the swelling experiments (*A* and *B*) were as in Fig. 1*A*. *A*, effect of $[Mg^{2+}]$ on MPT in the presence (*solid traces*) and absence (*dashed traces*) of 10 mM Cr. *B*, effect of Cr on MPT at constant $[Mg^{2+}]$ (2 mM). *C* and *D*, fraction of swollen mitochondria (calculated from ΔA_{540}, see text for details) in the presence (*filled circles*) and absence (*open circles*) of Cr, and mtCK activity (*triangles*) was measured in the forward reaction at pH 7.4 with a pH-stat (65).

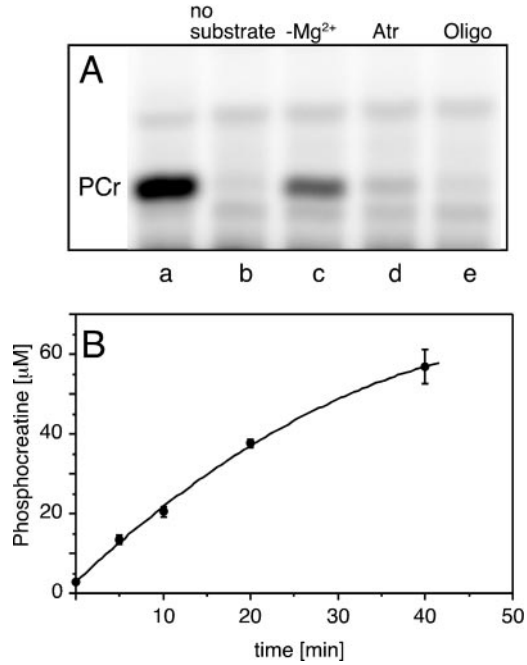

FIG. 4. **Phosphocreatine production by mitochondria from transgenic liver-mtCK mice in the absence of exogenous adenine nucleotides.** *A*, thin layer chromatography of solubilized mitochondria after a 40-min incubation of intact mitochondria in the presence of $^{32}P_i$ (1 μCi per assay). The incubation medium (50 μl) consisted of 10 mM Tris/Mops, pH 7.4, 250 mM sucrose, 10 mM phosphate/Tris, 2 mM $MgCl_2$ (except in *lane c*), 5 mM succinate/Tris (except in *lane b*), 2 μM rotenone, and 50 μM Ap_5A. In *lane d*, the medium was supplemented with 20 μM atractyloside (*Atr*) and in *lane e* with 1 μM oligomycin (*Oligo*). *B*, time course of PCr production by mitochondria incubated as described for *lane a* in *A*.

was at about 70% of the maximum value (*open triangles* in Fig. 3*C*). Therefore, MPT protection by Cr shows up at relatively high Mg^{2+} concentrations only (>0.5 mM) with corresponding high mtCK activities. A similar analysis carried out by variation of the Cr concentration at constant $[Mg^{2+}]$ (2 mM) revealed that MPT protection by Cr is clearly a function of mtCK activity (Fig. 3, *B* and *D*).

These data are in strong support of the idea that the rate of phosphorylation of CK substrates by active mtCK is related to their effect on MPT. Phosphorylation of these CK substrates occurs in microcompartments formed by mtCK and ANT (see below) (35). Because we did not add external adenine nucleotides, phosphorylation must occur via internally available ATP inside mitochondria, suggesting continuous cycling of internal ADP and ATP between matrix and intermembrane space mediated by ANT, if Cr or CyCr are present. As a consequence, if Cr is present, we should expect a net production of PCr even in the absence of exogenously added adenine nucleotides. This is indeed the case as shown in Fig. 4*A*. Mitochondria were incubated in the presence of $^{32}P_i$, Mg^{2+}, and 10 mM creatine. Only with energized mitochondria and fully active mtCK, as well as with a working oxidative phosphorylation system, we could observe a generation of PCr (Fig. 4, *lane a*). These are exactly the conditions used in the swelling experiments where MPT protection by Cr was observed (Fig. 1). With de-energized mitochondria (Fig. 4, *no substrate, lane b*), blocked ANT (20 μM atractyloside, *lane d*), or blocked F_0F_1-ATP synthase (1 μM oligomycin, *lane e*), no PCr was produced. Note in *lane c* (absence of Mg^{2+}), some PCr is still generated due to residual Mg^{2+} (probably bound to mtCK). Fig. 4*B* shows a time course experiment of net PCr production by energized mitochondria in the presence of external Cr plus Mg^{2+}, but no adenine nucleotides. The measured adenine nucleotide content is 0.11 ± 0.01 nmol per 25 μg of isolated mitochondria from transgenic liver-mtCK mice. After a 40-min incubation of succinate-energized mitochondria, 2.85 nmol of PCr were produced by 25 μg of mitochondria (Fig. 4*B*). Recalling that one ATP is consumed for every molecule of PCr produced, we can estimate that this is over 25 times more than the measured adenine nucleotide content. This quantitative consideration is a strong argument for internal nucleotide cycling.

Mitochondrial CK forms a microcompartment with ANT at the contact sites (together with outer membrane porin), as well as along the cristae (with ANT only (36, 37)). This compartmentation allows efficient transphosphorylation of ATP to PCr and export of the latter to the cytosol at peripheral contact sites. The reaction product ADP is fed back via ANT to the matrix for rephosphorylation resulting in an overall lowering of the apparent K_m values of ADP for oxidative phosphorylation (38). It is conceivable that microcompartmentation of mtCK and ANT is also responsible for the observed MPT inhibition by Cr and CyCr (48). To test this idea, we measured MPT pore opening in liver mitochondria from control mice lacking mtCK but with exogenously added recombinant human brain-type dimeric CK (BB-CK). Under the conditions used, especially at pH 7.4, this cytosolic isoform does not bind to mitochondrial outer membranes (Fig. 5*A*). The amount of BB-CK enzyme activity added in these experiments was equivalent to that found (based on mtCK activity) within the mitochondria of transgenic liver-mtCK mice. Under these conditions, no protection of MPT pore opening of control mitochondria with externally added CK by any of the CK substrates was seen, even in the presence of Mg^{2+} (Fig. 5*B*, shown only for Cr). By contrast, with the same amount of ubiquitous mtCK (the isoform expressed in the liver mitochondria of the transgenic mice), significant binding of the enzyme to the surface of mitochondria was observed (Fig. 5*A*). However, even with mtCK bound to the outside of mitochondria, no noticeable MPT protection by Cr was observed (Fig. 5*C*) as was also the case for BB-CK (Fig. 5*B*). Even increasing the total amount of mtCK by 10-fold in the medium did not bring about detectable MPT protection by Cr, although absolute binding of mtCK to the mitochondrial sur-

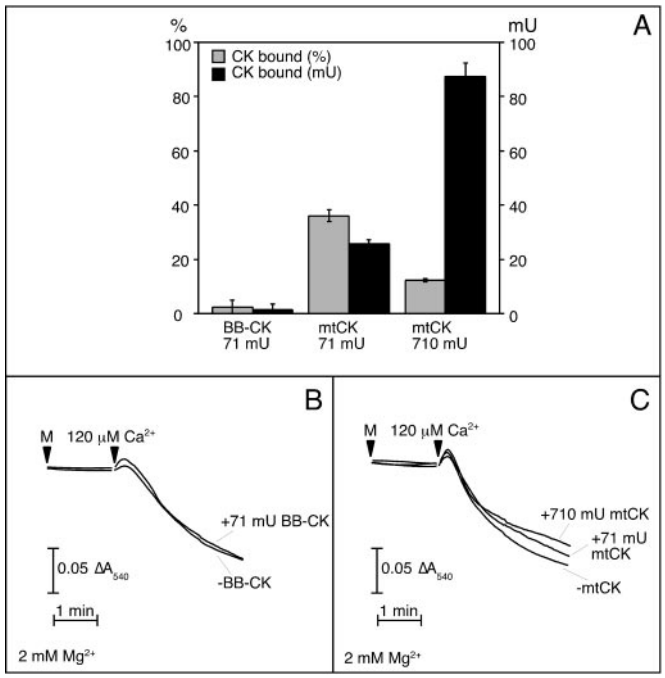

FIG. 5. **Effect of externally added creatine kinase on MPT pore opening of liver mitochondria from control mice in the presence of 10 mM Cr.** *A*, binding of different CK isoforms added externally to mitochondria from control mice. Conditions were as specified in the legend of Fig. 1*A*. Relative percentage (*%*, *gray bars*) and absolute amounts (milliunits (*mU*), *black bars*) of bound CK are given after incubation of mitochondria with either 71 milliunits BB-CK, 71 milliunits, or 710 milliunits mtCK. *B*, swelling of liver mitochondria from control mice with 71 milliunits externally added BB-CK present or in the absence of BB-CK. *C*, same as in *B*, but in the presence of different amounts of externally added mtCK.

face increased by more than 3-fold under these conditions (Fig. 5*A*). Thus, addition of CK externally or even mtCK bound to the outer membrane was not able to confer significant protection against MPT.

DISCUSSION

The present study was carried out to provide a mechanistic basis for the observation that certain substrates of creatine kinase efficiently inhibit the MPT (17). Because opening of the MPT pore appears to be causally related to cell death in several models (18–24), these studies may provide the basis for novel cytoprotective drugs. We have measured MPT pore opening in isolated mitochondria by the convenient swelling assay in sucrose-based medium and under different conditions, and we compared the response of transgenic mitochondria containing active or inactive CK with that of control mitochondria to which CK was added externally. These experiments revealed a major difference in MPT behavior. With liver mitochondria from transgenic mice, expressing mtCK in these organelles (39), but not with liver mitochondria from wild-type mice, we have observed MPT inhibition (based on measurements of uncoupled respiration) in the presence of Cr or CyCr in an earlier study (17). Here, by using mitochondrial swelling assays, we show that MPT protection by these CK substrates critically depends on two key factors.

The most striking new findings from the present study are that Cr protection of MPT requires magnesium, an essential cofactor for the CK reaction, and active mtCK at its proper location in the IMS. These observations together with the finding of net PCr production by isolated mitochondria, even in the absence of exogenous adenine nucleotides, suggest that endogenous adenine nucleotides are permanently cycling via ANT between matrix and IMS, if CK substrates are present that can efficiently be phosphorylated. This is the case for Cr and CyCr but not for GPA.

The consequences for MPT modulation by CK substrates could then be explained by the influence of nucleotide binding to ANT and conformational changes of this carrier (40, 41). To visualize this (see Fig. 6), during adenine nucleotide exchange, the common transport site for ADP and ATP on the ANT faces alternatively the matrix (m) and cytosolic (c) side (42, 43). Accordingly, the ANT changes its conformation between m- and c-state, if conditions allow adenine nucleotide transport. In the absence of a protonmotive force, the matrix ATP/ADP ratio of isolated mitochondria is low (40, 44), and it is entirely conceivable that adenine nucleotides are enzyme-bound (*e.g.* to the ATP synthase). Certain (unknown) fractions of transport units (shown as ANT dimers in Fig. 6, see also Ref 45) are locked either into the m- or c-state (Fig. 6*A*). Upon energization, the matrix ATP/ADP ratio rises, and ATP will be liberated and transported to the IMS. There, ATP either gets diluted in the medium or is trapped by mtCK (Fig. 6*B*). As a consequence, more transport units would now be in the c-state than before energization. Under these conditions, MPT pore opening should be favored, as seen with no CK substrate present. This situation is likely to be similar to the effect of the strong ANT inhibitor, atractyloside, known to stabilize the c-conformation and being an inducer of the MPT (46). If, however, Cr (or CyCr) is present, the trapped ATP will be transphosphorylated and the generated ADP transported to the matrix for rephosphorylation (Fig. 6*C*). As this process proceeds, the time-averaged fraction of transport units occupied with adenine nucleotides and being in the m-state is expected to be higher, and therefore, conditions for MPT pore opening are less favorable. In the presence of GPA, the ATP delivered to the active site of mtCK is not used up because GPA is not phosphorylated by mtCK (29). Consequently, the ANT is largely locked into the c-conformation, again favoring MPT pore opening. At present, it is still unclear how ANT conformation would affect the MPT. An indirect effect, *e.g.* via the surface potential, has been proposed (41, 47). Note the emphasis on microcompartmentation and functional coupling of mtCK and ANT as an essential part of the model shown in Fig. 6. This is corroborated by the fact that similar amounts of either cytosolic BB-CK or mtCK added externally to the outside of control mitochondria, as is present as mtCK in transgenic mitochondria, did not result in any significant Cr protection of MPT.

We have clearly demonstrated that in the presence of Cr, by measuring net production of PCr, oxidative phosphorylation proceeds even without adding external adenine nucleotides, although at a slow rate only. Nevertheless, besides the effect on the conformational state of the ANT, an additional contribution to MPT protection by Cr could be caused by variation of the matrix ATP/ADP ratio in favor of ADP (25), which is a strong MPT inhibitor (40, 44). On the other hand, the accumulating PCr is not believed to exert MPT inhibition (*e.g.* via the membrane potential) as we have shown earlier (17).

As mtCK functionally interacts with ANT at mitochondrial contact sites, as well as along the cristae membranes (36), a second possibility is that the two proteins may interact and that modulation by CK substrates may affect pore formation by the ANT. The known property of ANT to form an unspecific pore showing some of the characteristics of the MPT pore (48–50) in *in vitro* reconstituted systems has been taken by several authors as evidence that the ANT represents the central element of an MPT pore complex (51–59). However, it should be considered that pore formation is not unique to ANT but has also been described for other members of the mitochon-

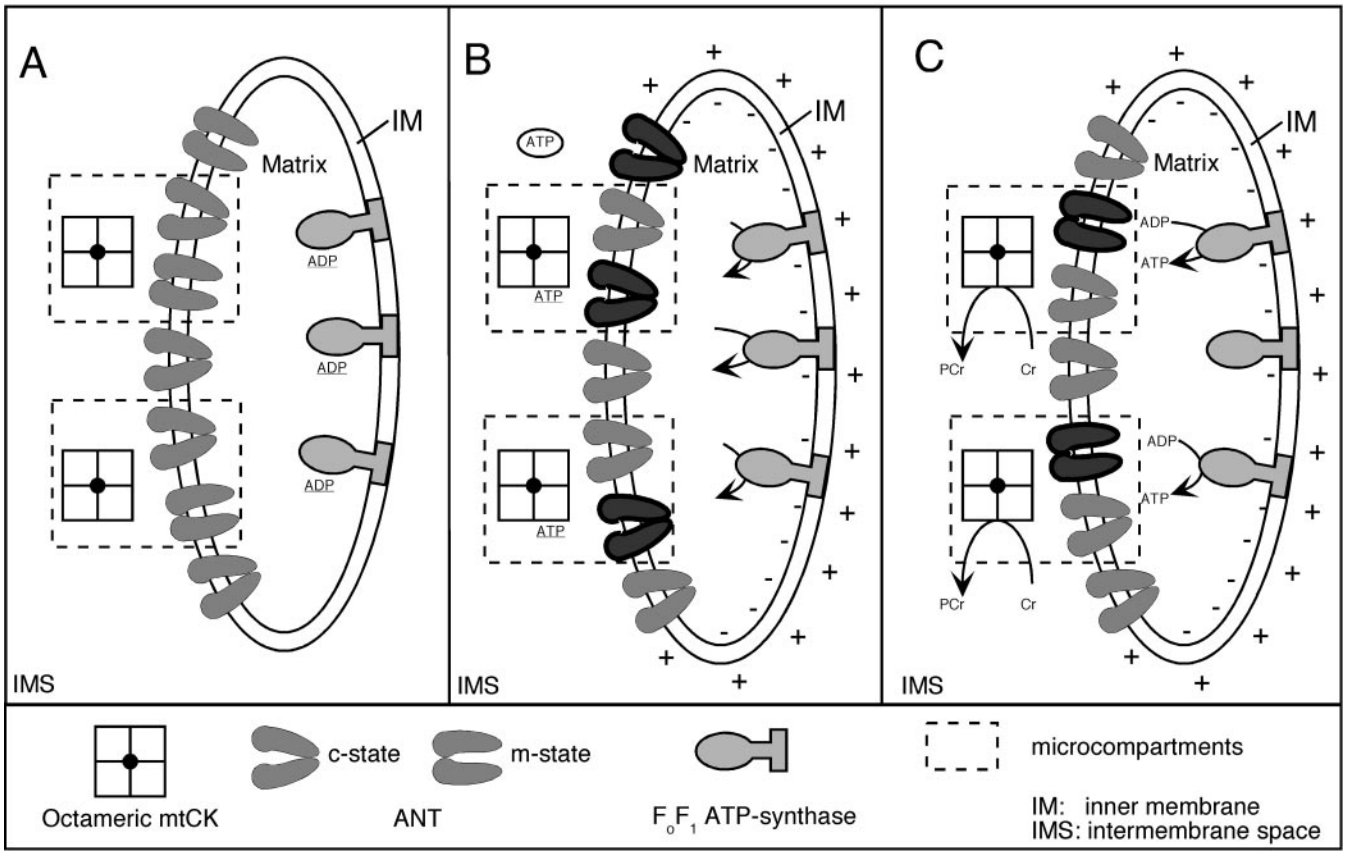

FIG. 6. **Model for MPT protection by Cr.** For clarity, the outer mitochondrial membrane has been omitted from the scheme, and only the inner membrane is shown. *A*, isolated mitochondria (de-energized). Matrix ATP/ADP ratio is expected to be low and ADP presumably mostly enzyme-bound (*e.g.* to the ATP synthase, *underlined*). ANT-dimers are in m- as well as c-conformation. *B*, energization (symbolized by *plus* and *minus signs* of membrane potential). Matrix ATP/ADP ratio is expected to be high. Formerly bound ADP is now converted to ATP (*arrows*). ATP is transported via ANT to the IMS. Some ATP (*underlined*) is trapped by octameric mtCK (symbolized as *squares*), and another fraction of ATP (*encircled*) is diluted into the medium. Some of the ANT dimers did change from m- to c-conformation (*outlined, dark*). *C*, in the presence of creatine, conversion of Cr to PCr by mtCK takes place. ADP produced in mtCK/ANT microcompartments is transported back into the matrix and is rephosphorylated there by the ATP synthase. Some ANT dimers have now switched from c- to m-conformation (*outlined, dark*). The *dashed rectangles* in *A–C* outline mtCK-ANT assemblies forming functionally coupled microcompartments. Further details are given in the text.

drial carrier family (60, 61). Furthermore, mitochondria from ANT-deficient yeast still exhibit a mitochondrial multiconductance channel which is believed to be the electrophysiological counterpart of the MPT (62). A recent study by Linder *et al.* (63), showing that arginine modification has pronounced influences on MPT pore opening that are not modulated by the ANT ligands atractyloside and bongkrekic acid, further questions a direct involvement of the ANT in pore formation. Finally, the structural interactions between mtCK and ANT that were postulated in models of mitochondrial contact sites (64) still await experimental proof.

Creatine has been shown to exert strong protective effects against glutamate and β-amyloid toxicity and energetic insults in neuronal cell cultures (9, 10), as well as in several animal models of neurodegenerative diseases (11–13). It is interesting to note that in the study of Brustovetsky *et al.* (10), Cr did not prevent MPT in isolated brain mitochondria, despite protection from energetic insults in cultured striatal neurons. This could be either due to the low magnesium concentration (0.5 mM) used by these authors in their assays or to the only weak coupling of the CK reaction to oxidative phosphorylation in these particular preparations. For the latter, however, no data were provided. In any case, both interpretations are fully in line with the model concerning the effect of Cr on MPT as proposed above. Thus, the beneficial effects of Cr seen in animal disease models can be attributed to at least two mechanisms that are ultimately linked by the PCr shuttle: 1) ATP levels and local ATP/ADP ratios in the cytosol are kept high, thus sustaining membrane ion gradients and other vital energy-consuming processes at a highly efficient level, and 2) mitochondria are protected from MPT pore opening via functional coupling of the mtCK reaction to oxidative phosphorylation.

Taken together, our findings offer important clues on MPT regulation by mitochondrial ADP phosphorylation and may have interesting implications for the design of creatine analogs to treat patients with neurodegenerative diseases. In order to fully exploit the advantages of the PCr shuttle for cell survival, such analogs would have to be substrates for CK in both the forward and reverse direction of the reaction.

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