The Mitochondrial Phosphate Carrier Interacts with Cyclophilin D and May Play a Key Role in the Permeability Transition

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The mitochondrial permeability transition pore (MPTP) plays a key role in cell death, yet its molecular identity remains uncertain. Although knock-out studies have confirmed critical roles for both cyclophilin-D (CyP-D) and the adenine nucleotide translocase (ANT), given a strong enough stimulus MPTP opening can occur in the absence of either. Here we provide evidence that the mitochondrial phosphate carrier (PiC) may also be a critical component of the MPTP. Phenylarsine oxide (PAO) was found to activate MPTP opening in the presence of carboxyatractyloside (CAT) that prevents ANT binding to immobilized PAO. Only four proteins from solubilized CAT-treated beef heart inner mitochondrial membranes bound to immobilized PAO, one of which was the PiC. GST-CyP-D pull-down and co-immunoprecipitation studies revealed CsA-sensitive binding of PiC to CyP-D; this increased following diamide treatment. Co-immunoprecipitation of the ANT with the PiC was also observed but was insensitive to CsA treatment. N-ethylmaleimide and ubiquinone analogues (UQ₀ and Ro 68-3400) inhibited phosphate transport into rat liver mitochondria with the same concentration dependence as their inhibition of MPTP opening. UQ₀ and Ro 68-3400 also induced the “m” conformation of the ANT, as does NEM, and reduced the binding of both the PiC and ANT to the PAO column. We propose a model for the MPTP in which a calcium-triggered conformational change of the PiC, facilitated by CyP-D, induces pore opening. An interaction of the PiC with the ANT may enable agents that bind to either transporter to modulate pore opening.

The mitochondrial permeability transition pore (MPTP)³ is a nonspecific pore, permeable to all molecules of less than 1.5 kDa, which opens in the inner mitochondrial membrane (IMM) under conditions of calcium overload. Opening is greatly enhanced by adenine nucleotide depletion, elevated phosphate, and oxidative stress, conditions known to accompany exposure to some toxins and reperfusion following a period of ischemia (1–4). Indeed, the opening of the MPTP is now recognized as a major cause of the necrotic cell death occurring under such conditions and inhibiting MPTP opening with drugs such as cyclosporin A analogues, and Sanglifehrin A offers substantial protection (4–7).

In view of its critical role in cell death, it is important to understand the molecular mechanism of the MPTP. A widely held model places the adenine nucleotide translocase (ANT) as the pore-forming component in the inner mitochondrial membrane with the peptidyl-prolyl cis-trans isomerase (PPIase) activity of cyclophilin-D (CyP-D), facilitating a calcium-induced conformational change (2, 3, 8). This model was initially proposed by us largely on the basis of the effects of known ligands of CyP-D and the ANT (9). Cyclosporin A (CsA), first shown to inhibit pore opening by Crompton et al. (10), was the key to identifying the role for CyP-D, and we subsequently showed that the potency of several CsA analogues as inhibitors of the pore correlated with their ability to inhibit the activity of a matrix peptidyl-prolyl cis-trans isomerase (PPIase) (11). The importance of this protein has now been confirmed by the demonstration that mitochondria from CyP-D knock-out mice do not exhibit CsA-sensitive MPTP opening (12–14).

A role for the ANT in MPTP formation was implied by the observation that pore opening is enhanced by agents such as carboxyatractyloside (CAT) that induce the “c” conformation of the ANT and inhibited by agents such as bongkrekic acid (BKA) that enhance the “m” conformation (9, 15–17). In addition, the ability of nucleotides to inhibit pore opening correlates with their ability to act as substrates for the ANT (17). Furthermore, oxidative stress can modify a matrix cysteine on the ANT (Cys¹⁶⁰ in rat ANT1) preventing adenine nucleotide binding and so accounting for the sensitization of the MPTP to calcium by oxidative stress (17, 18). More direct evidence was obtained

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The abbreviations used are: MPTP, mitochondrial permeability transition pore; AK₂, adenylate kinase 2; ANT, adenine nucleotide translocase; BKA, bongkrekic acid; BSA, bovine serum albumin; CAT, carboxyatractyloside; CsA, cyclosporin A; CyP-D, cyclophilin-D; IMM, inner mitochondrial membrane; NEM, N-ethylmaleimide; NTA, nitrilotriacetic acid; PAO, phenylarsine oxide; PiC, mitochondrial phosphate carrier; PPIase, peptidyl-prolyl cis-trans isomerase; ROS, reactive oxygen species; UQ₀, ubiquinone; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PEG, polyethylene glycol; MOPS, 4-morpholinepropanesulfonic acid; GST, glutathione S-transferase; IP, immunoprecipitate.
by the demonstration by us and others that the ANT binds tightly to GST-CyP-D (19, 20). Finally, the purified and reconstituted adenine nucleotide translocase of *Neurospora crassa* can form nonspecific channels at high calcium concentrations (21), and the opening probability of these pores is increased at high membrane potential by the presence of cyclophilin from *Neurospora crassa* (22).

Despite the strong evidence summarized above, an absolute requirement for the ANT in MPTP formation is unlikely because liver mitochondria of mice genetically modified to lack the two major isoforms of ANT (ANT1 and ANT2) still exhibit CsA-inhibitable MPTP opening (23). It should be noted that although these mitochondria show very little ANT activity, they do contain ANT4 (24), which probably explains how the animals survive (6, 25). Nevertheless the MPTP of the ANT-deficient mitochondria is no longer sensitive to ligands of the ANT and requires much higher calcium concentrations to open. These data are most easily interpreted in one of two ways. Either the ANT is not the major pore-forming component of the MPTP, but rather plays a regulatory role, or the ANT is the normal pore-forming component, but in its absence, other membrane transporters present in the IMM at much lower concentrations may fulfill the same role (6). An alternative possibility has been suggested by He and Lemasters (27) in which the MPTP forms as a result of aggregation of misfolded integral membrane proteins damaged by oxidant and other stresses. CyP-D blocks conductance through these protein aggregates, but when protein clusters exceed CyP-D, unregulated pore opening occurs that is stimulated by calcium and inhibited by CsA binding to CyP-D. Because the ANT is the most abundant protein in the inner mitochondrial membrane and is susceptible to oxidative damage, this may account for its apparent involvement in MPTP formation. However, the activation and inhibition of MPTP opening by CAT and BKA, respectively, argue against such a nonspecific effect because both ligands stabilize the ANT (8).

In this study, we report that the mitochondrial phosphate carrier (PiC) can associate with the ANT and binds to CyP-D in a CsA-sensitive manner. We also show that phenylarsine oxide (PAO), N-ethylmaleimide (NEM), and ubiquinone analogues, all known modulators of MPTP opening, can bind to both proteins. Our current results, taken together with published data, are consistent with a model for the MPTP in which the pore formation involves a calcium-triggered, CyP-D-facilitated conformational change of the PiC that is modulated by the conformational state of a closely associated ANT molecule.

**EXPERIMENTAL PROCEDURES**

*Materials—* All reagents were obtained from Sigma unless otherwise stated. Ro 68-3400 was a kind gift of Hoffmann-La Roche Ltd, Basel, Switzerland. Monoclonal antibodies against CyP-D and ANT1 were obtained from Mitoscience (Eugene, Oregon 97403), while rabbit polyclonal antibodies against the C terminus of the ANT and the whole ANT were those we raised previously (18, 19). A rabbit polyclonal antibody against the PiC was raised commercially (Sigma-Genosys) using the C-terminal 11 amino acids (ESLKKKLGLLTE) conjugated to bovine serum albumin (BSA). A rabbit polyclonal antibody against adenylate kinase 2 (AK2) was obtained from Abcam (Cambridge CB4 0FW, UK).

*Preparation of Mitochondria—* Mitochondria were prepared from rat heart and liver following homogenization in sucrose isolation buffer (ISA; 300 mM sucrose, 10 mM Tris–HCl, 2 mM EGTA, pH 7.4) using a Polytron PT10 (heart) or Dounce Potter (liver) homogenizer, and purified by Percoll gradient centrifugation as described previously (28, 29). For preparation of beef heart mitochondria, fresh hearts were collected from the abattoir, stored on ice during transit for 30 min and then, following removal of connective tissues, fats, and coagulated blood, cut into small pieces (~25 g) and washed in sucrose isolation buffer. Homogenization was achieved using a meat grinder with plate holes of 4 mm followed by a Waring blender as described elsewhere (30). The pH of the final homogenate was adjusted to pH 7.4 with Tris base. Isolation Buffer B (ISB; ISA + 0.5% (w/v) BSA) was added to the homogenate (500 ml of ISB/liter homogenate), and the mixture was centrifuged at 1,000 × g for 5 min. The supernatant was decanted through a double layer of cheese cloth and then centrifuged at 12,000 × g for 5 min. Subsequent steps were performed as described for rat heart mitochondria. Mitochondrial protein concentration was determined by Biuret assay using BSA as a standard (31).

*MPTP Opening and Mitochondrial Phosphate Transport: Swelling Assays—* For routine measurement of MPTP opening mitochondrial swelling (decrease in A520) was measured under de-energized conditions at 25 °C in KSCN buffer (150 mM KSCN, 20 mM MOPS, 10 mM Tris, 0.5 mM rotenone, 0.5 mM antimycin; pH 7.2) containing 2 mM nitrolotriacetic acid (NTA) and 2 mM A23187 (9, 17). Swelling was initiated by addition of 1.4 mM CaCl₂ (180 μM free [Ca²⁺]). For comparison of the sensitivity of MPTP opening and phosphate transport to inhibitors, KCl buffer was employed (125 mM KCl, 20 mM MOPS, 10 mM Tris, 0.5 mM rotenone, 0.5 mM antimycin pH 7.2) and was supplemented with 2 mM NTA, 2 mM A23187, and 20 mM NaPi. For measurement of phosphate transport, the KCl buffer was supplemented with 0.5 mM EGTA, 4 μM nigericin, and, unless otherwise stated, 0.5 μM cyclosporin-A (CsA). Ubiquinone analogues were added at the required concentration to the KCl buffer, whereas for NEM treatment, mitochondria (40 mg/ml) were pretreated in isolation buffer (ISA) at 0 °C for 2 min with the required NEM concentration prior to dilution into the assay buffer. Following addition of mitochondria (7 mg of protein) to 7 ml of assay buffer, aliquots (3.5 ml) were incubated at 25 °C (MPTP assay) or 6 °C in the sample and reference cuvettes of a split-beam spectrophotometer for 5 min. The assay was then initiated by the addition of either 70 μl of 2 mM NaPi, (pH 7.2 neutralized with Tris) for phosphate transport or 16 μl of 0.2 mM CaCl₂ (final free [Ca²⁺] 45 μM) for MPTP opening. A₅₂₀ was monitored continuously with on-line data acquisition at 10 or 2 data points per second, respectively.

*Shrinkage of Preswollen Mitochondria—* The sensitivity of the MPTP to inhibition by ADP was determined by the shrinkage of preswollen mitochondria (increase in A₅₂₀) upon addition of polyethylene glycol (PEG-2000) as described previously (17). Mitochondria were preswollen by incubating for 20 min at 2 mg/ml and 30 °C in KSCN buffer containing 1 mM CaCl₂ and where required CAT and PAO. Swelling was terminated by the
addition of 1.2 mM EGTA and centrifugation at 12,000 \( \times g \) for 10 min to pellet the swollen mitochondria, which were then resuspended at 20 mg/ml in KSCN buffer containing 2 mM NTA and 2 \( \mu M \) A23187. Preswollen mitochondria (1 mg) were incubated in the sample cuvette of the split-beam spectrophotometer at 25 \(^\circ\)C in 3 ml of KSCN buffer supplemented with 2 mM NTA, 2 \( \mu M \) A23187, and the required concentration of Ca\(^{2+}\) and ADP. After 1 min, shrinkage was initiated by the rapid addition of 0.5 ml of 50% (w/v) PEG 2000 to the sample cuvette through the injection port followed by vigorous mixing with an overhead stirrer. \( A_{520} \) was monitored continuously (10 data points/second).

**ANT Conformational Changes**—Changes in light-scattering (\( A_{520} \)) of energized beef heart mitochondria were detected upon addition of ligands of the ANT and submicromolar [Ca\(^{2+}\)]. These light-scattering changes occur in the absence of any change in mitochondrial volume and reflect conformational changes of the ANT (29, 32). Bovine heart mitochondria were incubated at 2 mg/ml and 30 \(^\circ\)C in 7 ml of buffer containing 125 mM KCl, 20 mM MOPS, 10 mM Tris, 0.5 mM EGTA, 5 mM L-glutamate, 2 mM L-malate, and 7.5 mM oligomycin, pH 7.2. Aliquots, (3.5 ml) were added to both cuvettes of a split-beam spectrophotometer and additions made to the sample cuvette as required.

**Binding of Solubilized IMM Proteins to Immobilized Phenylarsine Oxide**—4-Amino-phenylarsine oxide (Toronto Research Chemicals, Ontario, Canada, M3128) was conjugated with Affi-Gel 10 (Bio-Rad) as described previously (17). After incubation with 0.3 \( M \) ethanolamine to block unreacted groups, the PAO column material was collected by centrifugation at 500 \( \times g \) for 30 s and washed three times with 10 volumes of column wash buffer (PCB; 150 mM Na\(_2\)SO\(_4\), 50 mM HEPES, 1 mM EDTA, 0.25% (w/v) Triton X-100, pH 7.2). Mitochondria and IMM were purified as described previously (18, 19). For the preparative column (Fig. 4), columns (0.5 ml) were poured and washed with 20 volumes of PCB and IMMs solubilized at 10 mg/ml in PCB containing 3% (w/v) Triton X-100 for 15 min at 4 \(^\circ\)C. Insoluble material was removed by centrifugation at 16,000 \( \times g \) and 4 \(^\circ\)C for 10 min. Solubilized sample (1 ml) was passed through a 0.5-ml column twice, and any unbound material was washed through with 20 volumes of PAO column buffer. Bound protein was then eluted with PAO column buffer containing 10 mM dithiothreitol. All the column steps were performed at 4 \(^\circ\)C. For analytical binding studies (Figs. 2 and 9) IMMs were solubilized at 0.5 mg/ml in PCB containing 0.5% (w/v) Triton X-100 for 15 min at 4 \(^\circ\)C and following removal of insoluble material a sample (1 ml) was incubated with 100 \( \mu \)l of PAO beads (50% slurry) for 30 min at 4 \(^\circ\)C. The beads were washed five times with 1 ml of PCB. Bound protein was then eluted by incubation of beads in 100 \( \mu \)l of PCB containing 10 mM dithiothreitol for 30 min at 4 \(^\circ\)C. The beads were collected by centrifugation, and the protein concentration of the eluted supernatant determined by Bradford assay prior to analysis by SDS-PAGE and Western blotting.

**RESULTS**

**An Antibody Raised against Rat Liver ANT Recognizes a Protein Other Than ANT**—In previous studies that investigated whether the MPTP could be detected in proteoliposomes containing reconstituted ANT (37), we used ANT purified from rat...
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FIGURE 1. The ANT from beef heart mitochondria is not detected by a polyclonal antibody previously raised against purified rat ANT. Beef heart SMPs (5 mg/ml) were solubilized in N-dodecyl β-D-maltoside supplemented with 5 μM atractyloside for 30 min at 4°C. Extracts were passed through an anion exchange column (RESOURCEQ, the flow-through of which was then applied to a cation exchange column (RESOURCETM S), as described under “Experimental Procedures.” Proteins were separated by SDS/PAGE and revealed by Coomassie Blue staining. ANT was detected by Western blotting using three different antibodies (see “Experimental Procedures”). Fractions are labeled across the bottom of the figure: SMP; Sol, SMP solubilized SMPs; Q, Resource-Q flow-through fraction applied to the Resource-S column; S, Resource-S flow-through fraction.

liver mitochondria according to the method developed by Rojo and Wallimann (38). We also used this preparation to raise an antibody against the whole ANT that reacted strongly with an inner membrane protein of 30 kDa as expected for the ANT (18). However, recently we suspected the purity and stability of this ANT preparation and thus purified the more stable CAT-bound form of bovine ANT1 using the method of Smith et al. (36). In Fig. 1, we demonstrate that the flow-through fraction of the S-Sepharose contains a major band at 30 kDa that was confirmed to be ANT1 by sequencing with mass spectrometry. Furthermore, this band was detected by Western blotting using both a commercial monoclonal antibody against ANT1 (MitoScience) and our own polyclonal antibody raised against the C terminus of rat ANT1. However, the polyclonal antibody that we previously raised against whole rat liver ANT (18) failed to detect a protein in this fraction and also showed different levels of the immunoreactive protein in the other fractions. These data imply that our polyclonal antibody against rat liver ANT is not detecting ANT1. Although they do not totally exclude the possibility that it might detect ANT2, which is present at low levels in heart mitochondria, this is unlikely in view of the large sequence identity between the two isoforms (8).

Phenylarsine Oxide Activates the MPTP Independently of the ANT—We have previously shown that the ANT present in rat liver mitochondria binds to immobilized PAO, a vicinal thiol reagent, and we identified Cys160 and Cys257 on two matrix-facing loops of the ANT as the residues cross-linked by the reagent (18). Our initial studies using the C-terminal ANT antibody demonstrated that CAT pretreatment of mitochondria abolished ANT binding to the PAO column (17). This is consistent with the published structure of ANT1 with CAT bound that reveals that Cys160 and Cys257 are too far apart to be cross-linked by PAO (39). However, subsequent experiments using the polyclonal antibody raised against the whole ANT were in conflict with the earlier data and showed that CAT pretreatment did not abolish binding of the protein to the PAO column (18). The data shown in the inset of Fig. 2B confirm these data and demonstrate that the monoclonal antibody against ANT1 behaves like the C-terminal antibody in detecting a decrease in ANT binding to the PAO column following CAT treatment. BKA treatment also abolished ANT binding (Fig. 2B). Thus it can be concluded that our polyclonal antibody raised against the whole rat ANT is detecting a protein other than the ANT and that this protein binds to the PAO column in a CAT-insensitive manner.

These data led us to question whether the ANT was the locus of the activation of MPT opening by PAO. First, we investigated the effects of inhibitors of the ANT on the ability of PAO to activate MPT opening. In panel A of Fig. 2 we confirm our earlier observations (17) that PAO treatment strongly activates MPT opening under de-energized conditions while CAT alone has only a modest effect and BKA a modest inhibitory effect. Because CAT inhibits ANT binding to the PAO column, it might be expected that pretreatment of mitochondria with CAT prior to PAO treatment would abolish the activation of MPT opening by PAO. However, the data of Fig. 2A show that PAO still caused a large activation of MPT opening under these conditions. BKA gave a slight inhibition compared with PAO alone, but again MPT opening was still strongly activated by PAO. When added after the PAO, CAT gave a slight further activation of MPT opening and BKA a slight inhibition (Fig. 2B). Taken together, the data of Fig. 2 imply that PAO can activate MPT opening independently of its binding to the ANT.

We have previously concluded that one mechanism by which PAO activates MPT opening is to prevent the inhibition by ADP, and we proposed that this effect was mediated by modification of Cys160 of the ANT (17). The data of Fig. 2 cast doubt on this conclusion, and thus we investigated the effects of PAO on the ability of ADP to inhibit pore opening in mitochondria pretreated with CAT. For this purpose the shrinkage assay of preswollen mitochondria was used as described under “Experimental Procedures,” and data are presented in Fig. 3. As we observed previously, CAT treatment of mitochondria greatly diminished the sensitivity of the MPTP to inhibition by ADP, but an even greater effect was observed with PAO treatment. However, this effect of PAO was maintained in the presence of CAT, which, as noted above, prevented the ANT binding to immobilized PAO. These data imply that PAO must be reducing the sensitivity of MPT opening to ADP inhibition, at least in part, independently of its binding to the ANT. It seemed probable that PAO could be exerting this effect by modifying a protein from CAT-treated mitochondria that still binds to the PAO column and is detected by the antibody against the whole ANT. Subsequent experiments were directed at identifying this protein.

Identifying Proteins Other Than the ANT That Bind to Immobilized Phenylarsine Oxide—Inner mitochondrial membranes from control and CAT-treated beef heart mitochondria were solubilized in detergent, applied to a PAO column, and, following extensive washing, bound proteins were eluted using dithiothreitol and separated by SDS-PAGE. Protein staining revealed, as expected, a major protein of 30 kDa whose binding was prevented by CAT as shown in Fig. 4. Confirmation that this was ANT1 was provided by mass spectrometry (supple-
mental Table S1) and Western blotting with the monoclonal antibody against ANT1. In addition to ANT1, four other protein bands were clearly visible whose binding was unaffected by CAT treatment. Three of these were in the 28–35-kDa region characteristic of mitochondrial membrane transporters and mass spectrometry identified them as matching Q3SWX4_BOVIN (the hypothetical bovine homolog of human NIPSNAP-2 (Swiss-Prot O75323), a protein of unknown function also identified in mouse liver mitochondria (24)), adenylate kinase 2 (AK2-an intermembrane space enzyme), and the mitochondrial phosphate carrier (PiC). The identities of PiC and AK2 were confirmed by Western blotting. The fourth protein band was somewhat larger (45 kDa) and mass spectrometry identified this as ubiquinol-cytochrome c reductase core protein II precursor. Mass spectrometry data are provided in supplemental Table S1.

Of these four proteins, the most likely candidates to be components of the MPTP would seem to be the PiC and AK2. AK2 binds both ADP and ATP and thus might be a potential regulatory site for adenine nucleotide inhibition of the pore. However, we were unable to detect any effect on MPTP opening of the inhibitor of AK2, P1,P5-di(adenosine-5') pentaphosphate at 50 μM, a concentration sufficient to inhibit AK2 activity by more than 90% (supplemental Fig. S1). A role for the PiC would be consistent with the long-standing observation that phosphate is a potent activator of MPTP opening (41).

The Mitochondrial Phosphate Carrier Binds to CyP-D in a CsA-sensitive Manner—The PiC shares common structural motifs with the ANT (41) and thus might also bind CyP-D (6). This was investigated further in the experiments reported in Fig. 5. In Fig. 5A, we compare the binding of PiC and ANT to immobilized GST-CyP-D. Using the monoclonal ANT antibody we were unable to detect any CsA-sensitive ANT binding; nor did diamide treatment of mitochondria enhance binding although CAT treatment abolished binding. By contrast, the PiC antibody revealed that the PiC did bind to GST-CyP-D in a CsA-sensitive manner and that binding was increased by diamide treatment consistent with activation of MPTP opening by this agent (17). The polyclonal antibody raised against the whole ANT gave almost identical results to the PiC antibody, providing further evidence that this antibody is detecting the PiC rather than the ANT. Overall, these data imply that it is the PiC that is binding to CyP-D in a CsA-sensitive manner rather than the ANT.
In Fig. 5B we present the results from a separate experiment in which the C-terminal ANT antibody was used rather than the monoclonal antibody. The results of this experiment were similar to those using the monoclonal ANT antibody with one notable exception. Using this antibody we were able to demonstrate CsA-sensitive ANT binding to the GST-CyP-D thus reproducing our earlier data using this antibody (19). The reason for this difference probably reflects differences in isomform specificity of the ANT antibodies. The C-terminal sequence used for the polyclonal antibody is conserved across ANT1 and ANT2, and this antibody works equally well with both heart (mainly ANT1 but some ANT2) and liver (only ANT2). However, the monoclonal antibody is raised against ANT1 and in our hands detects very little ANT in liver mitochondria compared with heart mitochondria (supplemental Fig. S2). This discrimination by the monoclonal antibody between heart and liver mitochondria was batch-dependent with some batches almost totally inactive against liver mitochondria. These data lead us to conclude that it may be ANT2 that is binding in a CsA-sensitive manner to GST-CyP-D rather than ANT1.

Further evidence for the CsA-sensitive binding of the PiC to CyP-D was provided by co-immunoprecipitation experiments. In the experiments reported in Fig. 6A, the PiC antibody was used for the immunoprecipitation of detergent-solubilized proteins from rat liver and heart mitochondria. The presence of CyP-D in the IP was confirmed by Western blotting with the monoclonal CyP-D antibody and CsA treatment greatly reduced this. As a control, the presence of PiC in the IP was confirmed by Western blotting (Fig. 6), and this sometimes revealed a doublet that may reflect the two known isoforms of the PiC (42). The antibody against the whole ANT appeared to detect the same two proteins, consistent with the PiC rather than the ANT being the major target of this antibody. The presence of the ANT in the IP was detected with either the monoclonal or the C-terminal antibody, and this was not affected by CsA treatment. However, treatment with CAT greatly reduced the ANT present in the IP, perhaps suggesting a conformational-
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A

IP with PiC antibody

Liver mitochondria

Heart mitochondria

30 kDa

32 kDa

18 kDa

Cont CAT CsA CAT + CsA CsA + CAT

Monoclonal ANT

Polyclonal whole ANT

Monoclonal PiC

Monoclonal CyP-D

C-terminal ANT

B

IP with ANT C-terminal antibody

Liver mitochondria

Heart mitochondria

30 kDa

32 kDa

18 kDa

Cont CAT CsA CAT + CsA CsA + CAT

Monoclonal ANT

Polyclonal whole ANT

Monoclonal CyP-D

Monoclonal PiC

C-terminal ANT

FIGURE 6. CsA-sensitive co-immunoprecipitation of CyP-D with the PiC and ANT. Mitochondria (2 mg/ml) were treated at room temperature with 2 μM CsA or 2 μM CAT for 10 min, before their solubilization and incubation with immunoprecipitating antibodies raised against the C terminus of the PiC (A) or ANT (B), as described under “Experimental Procedures.” Where CsA and CAT were added in combination, mitochondria were incubated with CsA for 10 min at 22 °C before the addition of CAT and further incubation for 10 min. Immunocomplexes were separated by SDS/PAGE and analyzed by Western blotting with the antibodies indicated.

dependent interaction between the ANT and the PiC. BKA appeared to have a similar but lesser effect. One explanation for these data is that some of the ANT denatures during solubilization and binds nonspecifically to the immunopellet. Because CAT and to a lesser extent BKA may stabilize the ANT against denaturation (43), this may be the reason why they reduce the amount of ANT detected in the immunopellet. Indeed, when an immunoprecipitation experiment was performed using an antibody against the plasma membrane monocarboxylate transporter MCT1 that is absent in our mitochondrial preparations (44), Western blotting detected some ANT in the IP that was not present in CAT-treated samples (data not shown). Despite the complications of ANT denaturation because neither CAT nor BKA affected the amount of CyP-D in the immunoprecipitate, these data provide further evidence that CyP-D binds to the PiC rather than the ANT.

For completion, we also performed immunoprecipitation with the C-terminal antibody against the ANT (Fig. 6B). Here too we found that CAT treatment (and to a lesser extent BKA) reduced the amount of ANT detected in the IP, especially in liver mitochondria. This again implies that a significant proportion of the ANT present in the pellet was denatured and nonspecifically bound to the immunopellet. However, PiC was also found in the pellet, and the amount was independent of CAT, BKA, and CsA suggesting it was binding to the native ANT in either conformation and independently of CyP-D. A small amount of CyP-D was also present in the IP, and this was reduced by CsA treatment consistent with it being bound to the PiC associated with the ANT rather than the ANT itself.

Comparing the Effects of N-Ethylmaleimide and Ubiquinone Analogues on MPTP Opening and Phosphate Transport—Chernyak and Bernardi (45) demonstrated that low concentrations of the thiol reagent NEM can inhibit MPTP opening, and we have confirmed this (18). It is also known that the PiC is very sensitive to inhibition by this reagent (46), and thus it is possible that the PiC is the locus of action of NEM as an inhibitor of MPTP opening. To investigate this further, we compared the sensitivity of phosphate transport and pore opening to NEM under identical conditions by using a light scattering assay for phosphate transport in de-energized mitochondria. The principle of this assay, illustrated in Fig. 7A, is that in the presence of nigericin, potassium can enter de-energized mitochondria when accompanied by phosphate and so cause swelling that is accompanied by a decrease in light scattering. Sufficient nigericin must be added to ensure that it is the activity of the PiC rather than the nigericin that limits phosphate-dependent swelling. In preliminary experiments (not shown), it was established that maximal rates of phosphate-induced swelling were induced by 4 μM nigericin and that the temperature of the assay had to be lowered to 6 °C to resolve initial rates of transport. Typical traces are shown in Fig. 7B. Note that there was an initial instantaneous decrease in light scattering due to volume dilution of the mitochondria prior to osmotic contraction and then swelling as the phosphate entered the matrix. As shown in Fig. 7B, in the presence of NEM at 20 nmol per mg protein, sufficient to prevent MPTP opening to the same extent as CsA (Fig. 7C), phosphate transport (B) was totally abolished. Now only the first two phases of the light scattering change were observed with the final decrease in light scattering that is caused by P$_i$ entry being abolished. The presence of 0.5 μM CsA totally abolished MPTP opening (Fig. 7C) but was without effect on P$_i$ transport. Indeed, in subsequent experiments, 0.5 μM CsA was present in all phosphate transport assays to ensure that no swelling occurred that was due to MPTP opening following CsA addition. To measure true initial rates of transport at increasing NEM concentrations, each time course (Fig. 7D) was corrected by subtracting the data for the fully inhibited transporter, and this yielded the corrected traces shown in Fig. 7E. True initial rates of transport were then calculated from these traces by using first order regression analysis as described previously (47). The data of Fig. 7F compare the effects of increasing NEM concentrations on these initial rates with those on the rate of MPTP-induced swelling under the same conditions. It can be seen that the concentration dependence of both processes is the same, providing further evidence for a critical role of the PiC in MPTP formation.

Another class of inhibitors of the MTPD described by Bernardi and co-workers (48–51) are ubiquinone (UQ) analogues of which UQ$_0$, and Ro 68-3400 have been most studied. In panel A of Fig. 8, we show the effects of increasing concentrations of Ro 68-3400 on the rate of phosphate transport by liver mitochondria using the swelling assay, and confirm that this agent is a potent inhibitor of the PiC. The initial rates of phosphate...
transport at increasing inhibitor concentrations were calculated and compared with data for the inhibition of MPTP opening. Panel B presents the mean data (±S.E. from three separate mitochondrial preparations) for the inhibitory effect of Ro 68-3400, while panel C provides similar data for UQo. As was found for NEM treatment, the sensitivity of both processes to the inhibitors was very similar. The slightly greater inhibition of MPTP opening than phosphate transport at the lowest concentrations of Ro 68-3400 may reflect an additional inhibitory effect exerted through its binding to the ANT as outlined below.

If the PiC is a major target for the inhibitory effects of UQo and Ro 68-3400 on MPTP opening, then it might be anticipated that the two processes would show similar sensitivity to NEM treatment. The slight difference in sensitivity to NEM in the two processes (Fig. 5) is consistent with this prediction. Parallel experiments with Ro 68-3400 and UQo show similar inhibitory behaviors, with a slightly higher inhibition of MPTP opening than phosphate transport at low concentrations of Ro 68-3400. This may suggest an additional inhibitory effect exerted through its binding to the ANT as outlined below.

FIGURE 7. Concentration dependence of the inhibition of MPTP opening and mitochondrial phosphate transport by N-ethylmaleimide. Panel B shows light scattering traces for phosphate transport following treatment with increasing concentrations of Ro 68-3400 for 5 min prior to addition of 40 mM NaPi to initiate transport. These data and similar data for UQo were corrected by subtraction of the maximally inhibited trace and initial rates determined in order to calculate the percentage inhibition of transport as described in the text. To calculate initial rates of transport, first order regression analysis was performed using GraphPad Prism to the equation $A_{200} = \text{initial rate}/k \times \exp(-kt) + \text{plateau}$, where $k$ is the rate constant, $t$ is time in seconds, and plateau the final $A_{200}$ once swelling is completed. The initial rates were used to calculate the percentage inhibition of transport at each NEM concentration and mean data (±S.E.) of three separate experiments are given in F (squares) where data for MPTP inhibition are also given (circles).

FIGURE 8. Concentration dependence of the inhibition of MPTP opening and mitochondrial phosphate transport by UQo and Ro 68-3400. Experiments were performed as described in the legend to Fig. 7. A shows light scattering traces for phosphate transport following treatment with increasing concentrations of Ro 68-3400 for 5 min prior to addition of 40 mM NaPi to initiate transport. These data and similar data for UQo were corrected by subtraction of the maximally inhibited trace and initial rates determined in order to calculate the percentage inhibition of transport as described in the text. Mean data (±S.E.) of three separate experiments are given (closed squares) for Ro 68-3400 in B and for UQo in C. Parallel data for MPTP inhibition are also presented (closed circles).
Mitochondrial Phosphate Carrier and Permeability Transition

Ubiquinone Analogues That Inhibit MPTP Opening Induce the m Conformation of the ANT—It is well established that BKA is a potent inhibitor of the MPTP and that it exerts its effect by inducing the m conformation of the ANT (8). Because UQo and Ro 68-3400 both inhibited the binding of the ANT to the PAO column (Fig. 9A), it seemed possible that, in addition to their effect on the PiC, these agents might also bind to the ANT in a similar manner to BKA. We investigated whether this might be the case by using a light-scattering technique to detect the change from the c to the m conformation of the ANT in heart mitochondria (52, 53). Energized heart mitochondria treated with 1.0 μM free [Ca²⁺] undergo a small decrease in light scattering that is independent of the permeability transition and can be reversed by ADP or BKA that induce the m conformation of the ANT. If CAT is added after ADP addition, the light scattering reverts to the value before ADP addition, representing the c conformation (9, 29, 32). In Fig. 10A, we show that the ability of 8 μM BKA to fully induce the m conformation is mimicked by addition of either 30 μM Ro 68-3400 or 100 μM UQo. In Fig. 9B we show a more detailed concentration dependence of the effect of Ro 68-3400 on the conformational change of the ANT, which reveals a half-maximal effect at about 5 μM, which is not dissimilar to its effect on MPTP opening (Fig. 7E). Further confirmation that Ro 68-3400 binds to and inhibits the ANT in a manner similar to BKA is provided by its effects on State 3 respiration by heart mitochondria as shown in Fig. 11. When succinate (plus rotenone) was the respiratory substrate, ADP-stimulated respiration was blocked by either 5 μM Ro 68-3400 or 50 μM Ro 68-3400, consistent with inhibition of ADP transport into the mitochondria. Furthermore, addition of 0.04 μM FCCP, an uncoupler, overcame the effects of the inhibitors on respiration as predicted. Although similar effects of BKA were observed when glutamate plus malate were used as substrates, following addition of Ro 68-3400 respiration could not be restored by uncoupler. Ubiquinone analogues have been shown to act as inhibitors of complex 1 of the respiratory chain (44, 49), and thus our data are readily explained by such an effect at the concentrations of Ro 68-3400 used. These high concentrations are required because the low flux control coefficient of the ANT for ADP-stimulated respiration under these conditions means that the ANT must be grossly inhibited before state 3 respiration is affected (54).

DISCUSSION

The MPTP May Involve CyP-D Binding to the PiC in Association with the ANT—In this study, we have shown that the antibody we previously raised against the purified ANT does not bind to the ANT, but rather to the PiC (Figs. 1, 4, 5, and 6). The protein against which the whole ANT antibody was raised was purified by a published protocol (37, 38) and was of the right size for the ANT. However, it was never sequenced to confirm its identity. In retrospect, it seems probable that a major component of the protein was the PiC rather than the ANT and that it was this to which the rabbit raised antibodies. Furthermore, co-immunoprecipitation studies with a specific PiC carrier antibody and GST-CyP-D pull-down experiments both demonstrate that the PiC binds CyP-D in a CsA-sensitive manner (Figs. 5 and 6). Co-immunoprecipitation of the ANT anticipated that these agents inhibit the PiC binding to the PAO affinity matrix and the data of Fig. 9A show that this is indeed the case. Both agents prevented binding of the PiC from solubilized bovine heart IMM to immobilized PAO; ANT binding was also reduced. This is in contrast to CAT and BKA, which had no effect on the PiC binding but did block ANT binding. Importantly, pretreatment of mitochondria with UQo and Ro 68-3400 not only prevented PiC binding to the PAO column, but also prevented PAO from activating MPTP opening as shown in Fig. 9B. This contrasts with the inability of CAT and BKA pretreatment to do this (Fig. 2A) providing further evidence that the PiC is an important locus of both PAO activation and UQo/Ro 68-3400 inhibition of MPTP opening.

FIGURE 9. Ubiquinone analogues inhibit PiC and ANT binding to the PAO column and PAO activation of MPTP opening. In A, rat liver mitochondria (5 mg/ml) were pretreated with 0.1 mM PAO, 20 μM CAT, 20 μM BKA, 50 μM UQo, or 20 μM Ro 68-3400 for 10 min at room temperature prior to solubilization with Triton X-100 and application to a PAO column. Subsequent washing and elution of bound proteins was performed as described under "Experimental Procedures." In B, MPTP opening was assayed under de-energized conditions in KSCN buffer as described in the legend to Fig. 2. For the top 6 traces, the mitochondrial suspension was pretreated for 1 min with 50 μM UQo, 20 μM Ro 68-3400, or without further addition prior to the start of recording and subsequent additions of PAO (20 μM) or Ca²⁺ (180 μM free [Ca²⁺]) to the sample cuvette. In the bottom 3 traces, the mitochondrial suspension was pretreated with PAO (20 μM) for 1 min prior to the start of recording and additions of 20 μM UQo, 20 μM Ro 68-3400, and 180 μM Ca²⁺.
with the PiC and vice versa (Fig. 6) implies that these two proteins interact, although the large reduction in the co-immunoprecipitation that occurs in the presence of CAT suggests that a major portion of this interaction may be through nonspecific binding of the denatured solubilized ANT. Nevertheless, the binding that remains in the presence of CAT or BKA could represent a specific interaction between the two proteins, and there is evidence that the PiC and ANT may interact together when associated with the ATP synthase within “the ATP synthasome” (55, 56). Our data provide some evidence that ANT2 association with the PiC is more important in MPTP opening than ANT1. Thus the monoclonal ANT antibody raised against ANT1 did not detect any reduction by CsA in the ANT binding to GST-CyP-D whereas the C-terminal ANT antibody that binds equally well to ANT1 and ANT2 does show a CsA-induced reduction in ANT binding (Fig. 5). This could provide a possible explanation for the greater sensitivity of liver mitochondria (ANT2) to MPTP opening than heart mitochondria (mainly ANT1). It would also explain why Crompton et al. (20) using solubilized heart mitochondrial inner membranes found that the binding of ANT of to CyP-D was not CsA-sensitive, whereas in our similar experiments with liver mitochondria, ANT binding was CsA-sensitive (19).

The very similar concentration dependence of the inhibition of the MPTP and PiC by NEM, UQ0, and Ro 68-3400 (Figs. 8 and 9) is most consistent with a model for the MPTP in which it is the PiC that provides the pore-forming component following a calcium-triggered conformational change facilitated by the peptidyl-prolyl cis-trans isomerase activity of CyP-D. An interaction with the c conformation of the ANT (primarily ANT2) would enhance the sensitivity of the PiC to this conformational change whereas the m conformation would either exert no such effect or inhibit the process. This would explain how pore opening can be demonstrated in mitochondria containing no ANT1 or ANT2, but with reduced sensitivity to calcium and no sensitivity to ligands of the ANT (23). An additional inhibitory effect of Ro 68-3400 and UQ0 may be exerted through their binding to the ANT and inducing the m conformation like BKA (Fig. 10) as discussed below.

A role for the PiC in pore formation is also consistent with the observation that the PiC of yeast mitochondria can be converted into a nonspecific anion channel by a dithiol cross-link between two Cys\textsuperscript{28} residues within transmembrane helix 1 to form a PiC dimer (57). An equivalent cysteine residue (Cys\textsuperscript{27}) is also present in bovine PiC and could account for the observed binding of the PiC to the PAO column that is CAT-insensitive (Figs. 4 and 9A). We have previously proposed that the site of action of PAO to activate the MPTP is on the ANT where it blocks ADP binding and so prevents the ADP-mediated inhibition of pore opening (17). However, the data in Figs. 2 and 3 demonstrate that PAO activates pore opening in the presence of CAT despite CAT preventing PAO binding to the ANT. Thus an alternative/additional site of PAO action is required, and the PiC is an obvious candidate because CAT does not prevent the binding of PAO to the PiC.
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NEM and Ubiquinone Analogues May Inhibit the Pore by Binding to Both the ANT and PiC—It should be noted that at low concentrations of NEM, which inhibit MPTP opening, phosphate transport is inhibited by covalently modifying Cys\(^{42}\) of the rat PiC, which is not present in the NEM-insensitive yeast PiC (58, 59), while Cys\(^{27}\) is unreactive to this thiol reagent. However, at such concentrations, NEM also binds to Cys\(^{57}\) of the ANT, and this prevents the ANT from assuming the c conformation (60), which may provide an additional site of NEM inhibition of pore opening as proposed previously (18). Thus, there are two potential sites for NEM inhibition of pore opening just as there are for UQo and Ro 68–3400. Importantly, PAO did not inhibit phosphate transport at concentrations up to 100 \(\mu M\) (data not shown), which is consistent with its ability to activate rather than inhibit the MPTP.

The inhibition of MPTP opening by ubiquinone analogues (48, 49) was shown to be associated with labeling of a 32-kDa protein of the inner mitochondrial membrane by \([^{3}^{3}P]Ro 68-3400\) (51). This protein was originally identified as the voltage-dependent anion channel (VDAC1) (51). However, it was subsequently demonstrated that Ro 68-3400 labels the same 32-kDa protein in mitochondria lacking VDAC1 thus revealing that VDAC1 was not the target of ubiquinone analogues (61). Furthermore, mitochondria lacking all isoforms of VDAC show normal pore opening, demonstrating that VDAC is not an essential component of the MPTP (62). Because the PiC runs at 32 kDa on SDS-PAGE and we have shown an interaction between the PiC and Ro 68-3400 (Figs. 8 and 9), it is possible that the 32 kDa-labeled protein detected by Bernardi and co-workers is the PiC.

Other Evidence That the PiC Is an Important Component of the MPTP—Two final pieces of circumstantial evidence support a critical role for the PiC in MPTP formation. First, it has been known for many years that phosphate is a potent activator of the MPTP (40), and thus it could be that the phosphate-bound form of the PiC is more susceptible to the conformational change that occurs during MPTP formation. Conversely, when NEM inhibits the PiC the conformational change is inhibited, accounting, at least in part, for the inhibition of the MPTP by this reagent. Second, a role for the PiC in MPTP opening is consistent with the observation (63) that knockdown of the MPC in HeLa cells reduces their sensitivity to apoptosis induced by staurosporine in which MPTP opening has been implicated (26). Furthermore, overexpression of the PiC has been shown to induce apoptosis (63). However, to prove the role of both the ANT and PiC together with CyP-D in the formation and regulation of the MPTP will require their reconstitution into proteoliposomes and demonstration of calcium-mediated, CsA-sensitive pore opening. Work to this end is underway but is hampered by the instability of mitochondrial membrane carriers during purification (8, 43).

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