

RESEARCH COMMUNICATION

Recruitment of mitochondrial cyclophilin to the mitochondrial inner membrane under conditions of oxidative stress that enhance the opening of a calcium-sensitive non-specific channel

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Binding of mitochondrial matrix cyclophilin (CyP) to the rat liver mitochondrial membranes was detected by SDS/PAGE and Western blotting with suitable antipeptide antibodies. Binding was not affected by prior exposure of mitochondria to Ca^{2+} , adenine nucleotides or inhibitors of the adenine nucleotide translocase, but was greatly increased by t-butyl hydroperoxide (tBH), phenylarsine oxide or diamide. These all sensitized the opening of the non-specific mitochondrial pore to $[\text{Ca}^{2+}]$, and the

effect of tBH was shown to be maintained after washing away the tBH, consistent with it being caused by the enhanced CyP binding. The bound CyP did not demonstrate peptidyl-prolyl *cis-trans* isomerase activity. CyP-binding was prevented by 5 μM cyclosporin A, but not reversed by cyclosporin treatment of the membranes. The effect of tBH on binding was concentration-dependent and maximal within 30 s.

INTRODUCTION

Supraphysiological $[\text{Ca}^{2+}]$ damages mitochondria, especially under conditions of oxidative stress, by inducing the opening of a non-specific pore in the mitochondrial inner membrane. This allows rapid equilibration of low-molecular-mass (< 1500 Da) solutes, resulting in massive swelling of the mitochondria [1–3]. The consequent damage may be a major contributor to the irreversible reperfusion injury that follows periods of ischaemia [4–6]. Pore formation is independent of phospholipase A_2 activity and is inhibited by cyclosporin A (CsA) at $< 1 \mu\text{M}$ [4,7–10]. The immunosuppressant activity of CsA involves binding of the drug to cyclophilin (CyP), a small (17 kDa) cytosolic protein which possesses peptidyl-prolyl *cis-trans* isomerase (PPIase) activity [11,12]. However, there is a family of cyclophilins, all exhibiting PPIase activity, whose normal functions are unknown [11,12]. We have provided strong evidence that a mitochondrial matrix isoform [13], which appears to be encoded by a separate gene [14], is involved in the formation of the non-specific pore. Detailed studies on the inhibition of pore opening and mitochondrial matrix PPIase by CsA and its analogues led us to propose that this mitochondrial CyP might bind to an integral membrane protein, the adenine nucleotide translocase (ANT) and, in the presence of Ca^{2+} , trigger a conformational change that induces pore formation [4,7]. Andreeva and Crompton [15], however, have provided evidence from binding studies and mitochondrial protein labelling using a photoactivatable analogue of CsA that a membrane-bound CsA-binding protein of 10 kDa may be involved in pore opening.

Many factors are known to sensitize the permeability transition to $[\text{Ca}^{2+}]$ including adenine nucleotide depletion, oxidative stress such as that induced by t-butyl hydroperoxide (tBH), some thiol reagents such as phenylarsine oxide and diamide, and agents such as carboxyatractyloside that enhance the 'c' conformation

of the ANT. Factors enhancing the 'm' conformation such as bongkrekate inhibit the transition [1,16–19]. In the present paper we report that oxidative stress induced by tBH, phenylarsine oxide and diamide leads to binding of CyP to the mitochondrial inner membrane, consistent with its role in pore opening. This is the first direct demonstration of CyP responding to an external stimulus and supports the hypothesis that a function of cyclophilins may be to interact with integral membrane proteins to modulate their function in response to an appropriate signal.

EXPERIMENTAL

Materials

Rat liver mitochondria were prepared with the inclusion of Percoll gradient centrifugation to remove microsomal and plasma membrane contamination as described previously [7]. They were stored on ice at 50 mg of protein/ml in isolation buffer and either used within 4 h of preparation or stored overnight (aged mitochondria). Antipeptide antibodies were raised to the synthetic N-terminal peptide of the mitochondrial matrix CyP [13], CAQNPLVYLDVGADGQPL, conjugated to keyhole-limpet haemocyanin (KLH) using *m*-maleimido-benzoic acid N-hydroxysuccinimide ester [20]. The residues CA were added to facilitate coupling to KLH. IgG was purified from immune serum by a combination of octanoic acid/ammonium sulphate precipitation [21] followed by removal of anti-KLH antibodies using an Affi-gel 10–KLH affinity column. The antipeptide antibody to the matrix CyP did not cross-react with the cytosolic CyP as would be expected from the lack of the relevant peptide sequence in the cytosolic form [13]. Polyethylene glycol (PEG) with an average molecular mass of 2000 Da (PEG 2000) was obtained from BDH/Merck Ltd. (Lutterworth, Leics., U.K.). The sources of all other chemicals and biochemicals were as described previously [7,13,22].

Abbreviations used: ANT, adenine nucleotide translocase; CsA, cyclosporin A; CyP, cyclophilin; ECL, enhanced chemiluminescence; KLH, keyhole limpet haemocyanin; NTA, nitrilotriacetic acid; PEG, polyethylene glycol; PPIase, peptidyl-prolyl *cis-trans* isomerase; tBH, t-butyl hydroperoxide;

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Methods

Measurements of mitochondrial non-specific pore opening

Mitochondrial swelling. Mitochondria were incubated at 25 °C and 1 mg of protein/ml in 3.5 ml of buffer containing 150 mM KSCN, 20 mM Mops, 10 mM Tris, 2 mM nitrilotriacetic acid (NTA), 0.1 μ M rotenone, 0.1 μ M antimycin, and 2 μ M A23187, pH 7.2. The calcium ionophore A23187 was added to ensure complete equilibration of Ca^{2+} across the mitochondrial inner membrane [22]. A_{520} was monitored continuously (2 data points/s) in a spectrophotometer with computerized data acquisition and averaging [7,22]. After 1 min, 0.45 mM CaCl_2 was added to give a free $[\text{Ca}^{2+}]$ of 30 μ M and data acquisition continued. Mitochondrial pore opening is associated with swelling that is accompanied by a decrease in light scattering, measured as a decrease in A_{520} .

Shrinkage of pre-swollen mitochondria. Mitochondria were pre-swollen by incubating at 3 mg protein/ml and 30 °C for 20 min in the KSCN buffer described above but without NTA or A23187 and containing 1 mM CaCl_2 in the absence (control) or presence of 1 mM tBH (tBH-pretreated). Measurements of light scattering and the A_{260} of mitochondria extracted in 5% (w/v) HClO_4 showed that this treatment led to maximal pore opening whether or not tBH was present. This is important, since it enables total loss of matrix nucleotides through the pore during the swelling and thus ensures that any effects of tBH treatment on the sensitivity of pore opening towards $[\text{Ca}^{2+}]$ cannot be accounted for by differences in matrix nucleotides, especially ADP, which inhibit pore opening [23]. Before sedimentation of the swollen mitochondria by centrifugation at 10000 g for 10 min, pores were closed by addition of 1.2 mM EGTA to chelate the added Ca^{2+} . In parallel experiments it was confirmed that pores were closed under these conditions (free $[\text{Ca}^{2+}] < 0.5 \mu\text{M}$). The mitochondria were resuspended in KSCN buffer containing both 2 mM NTA and 5 μ M A23187 at a final protein concentration of 50 mg/ml and stored on ice. For the shrinkage assay, swollen mitochondria (1 mg of protein) were added to 3 ml of KSCN buffer containing CaCl_2 at the concentration calculated to give the required free $[\text{Ca}^{2+}]$. This was calculated using a value for the K_d for the Ca^{2+} -NTA complex of 1.78×10^{-4} M at pH 7.2. The A_{520} was constantly monitored using a home-built spectrophotometer with computer-aided data acquisition at 10 data points/s. After exposure for 1 min to Ca^{2+} , 0.5 ml of 50% (w/v in water) PEG 2000 was injected through the addition port and rapidly mixed (< 1 s) using an integral paddle-stirrer. Rates of shrinkage were calculated by differentiation of the data over 0.5 s intervals, ignoring the first second where mixing artefacts distorted the signal.

Cyclophilin binding to mitochondrial membranes

Mitochondria (3 mg of protein/ml) were incubated for the required time at 25 °C in KSCN buffer without NTA or A23187 and with other additions as required. The mitochondrial suspension was then subjected to five cycles of rapid freeze-thawing (liquid N_2) before recovering the disrupted membranes by centrifugation for 30 min at 150000 g. The membranes were resuspended in the original volume of 300 mM sucrose, 2 mM EGTA, 10 mM Tris/HCl, pH 7.4 and again sedimented by high-speed centrifugation before resuspension in sucrose buffer at a final protein concentration of 1 mg/ml. Aliquots containing 30 μ g of protein were subjected to SDS/15% (w/v) PAGE and blotted onto Immobilon-P membranes. Mitochondrial cyclophilin (12 μ g), partially purified using Q-Sepharose followed by

S-Sepharose chromatography as described previously [13], was used as a standard. Bound CyP was detected using antipeptide antibodies raised to the N-terminal peptide of the mitochondrial matrix CyP raised as described under 'Materials'. The Western blots were developed using an enhanced chemiluminescence (ECL) detection kit (Amersham, Bucks., U.K.).

RESULTS AND DISCUSSION

This effect of tBH, phenylarsine oxide and diamide on pore opening under conditions used to investigate CyP binding to the mitochondrial membranes is illustrated in Figure 1. If pore opening is associated with CyP binding to a membrane protein, then it would be expected that such treatments should increase the binding, and this is shown to be the case in Figure 2(a). Membranes prepared from mitochondria incubated with these reagents were subjected to SDS/PAGE and Western blotting with polyclonal anti-CyP antibodies raised to an N-terminal peptide of the rat mitochondrial matrix CyP. Although a small amount of CyP was detected in control membranes, a large increase was noted following treatment with each of the three reagents. No measurable citrate synthase activity was detected in the membrane fraction, implying that the CyP detected was not due to matrix contamination. Furthermore, parallel Western blotting with an antibody to mitochondrial NAD^+ -dependent isocitrate dehydrogenase (provided by Professor R. M. Denton, University of Bristol) did not detect binding of this enzyme to the membranes under any conditions, confirming the specificity of CyP binding (results not shown).

This conclusion is further strengthened by the ability of CsA to prevent the binding of CyP, provided that it is added before the tBH (Figure 2b). When CsA was added to the medium used to wash the membranes from mitochondria incubated with tBH, the CyP bound could not be displaced. This may reflect a very slow dissociation of CyP from its membrane-binding protein, since we have previously demonstrated [13] that the second-order rate constant for association of CsA with CyP is only $0.9 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ and this value, together with the dissociation constant of 5 nM, would lead to a half-life for dissociation of 2.3×10^{13} s. If it is assumed that CyP binds to a membrane

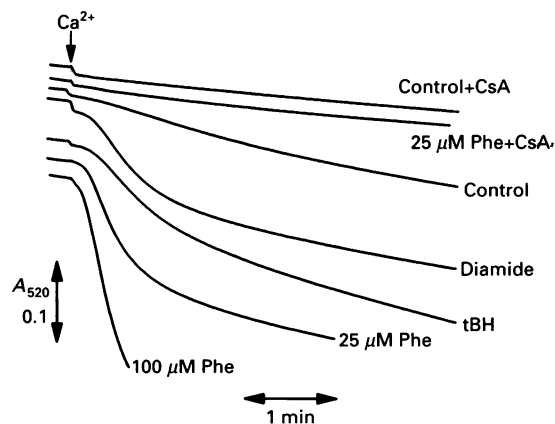


Figure 1 Enhancement of Ca^{2+} -dependent mitochondrial pore opening induced by tBH, diamide and phenylarsine oxide

Mitochondrial swelling was measured as described in the Experimental section and was initiated by addition of 0.45 mM CaCl_2 to give a free $[\text{Ca}^{2+}]$ of 30 μ M. Further additions of 1 μ M CsA, 0.5 mM diamide, 1 mM tBH or phenylarsine oxide (Phe, 25 or 100 μ M) were made at the start of the incubation as indicated. Control is in the absence of these further additions.

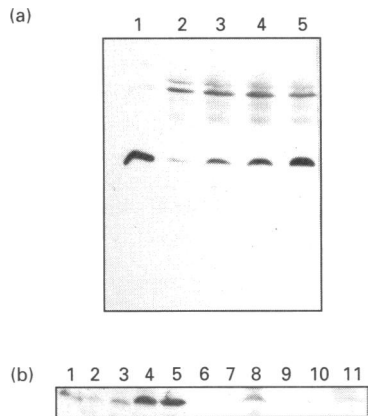


Figure 2 CyP binding to the mitochondrial inner membrane is induced by tBH, diamide and phenylarsine oxide (a) and prevented by prior exposure to CsA (b)

Rat liver mitochondria (50 mg of protein/ml in sucrose isolation buffer) were stored on ice for 18 h before use. In (b), lanes 6–8, 5 μ M CsA was present during the storage whereas in (b), lanes 9–11, 5 μ M CsA was added to the mitochondria 10 min before use. Mitochondria were incubated at 25 $^{\circ}$ C for (a) 30 s or (b) 10 min in KSCN buffer before preparation of membranes by freeze-thawing and analysis by SDS/PAGE and Western blotting as described in the Experimental section. In (a), further additions to the mitochondrial incubations were 2 mM EGTA (lane 2), 1 mM tBH (lane 3), 0.1 mM phenylarsine oxide (lane 4) or 0.5 mM diamide (lane 5). The proteins of higher molecular mass than CyP (shown in lane 1, which contained partially purified matrix CyP) were detected to varying degrees with the anti-CyP antibodies, but only the band corresponding to CyP (18 kDa) was reduced by pre-exposure of the antibody to the synthetic peptide (results not shown). In (b), only the band corresponding to CyP is shown. Further additions to the incubation were 2 mM EGTA (lanes 2, 6 and 9), 500 μ M Ca^{2+} (lanes 3, 7 and 10) or 1 mM tBH (lanes 4, 5, 8 and 11). In lane 5, the mitochondrial membranes were washed in sucrose buffer containing 5 μ M CsA.

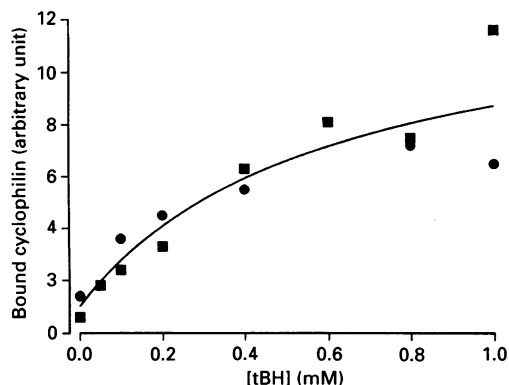


Figure 3 Concentration dependence of the tBH-induced increase in CyP binding to the mitochondrial inner membrane

The experimental protocol used was the same as that for Figure 2 but with the addition of tBH at the concentrations shown. The Western blots were developed by ECL and the film scanned for bound cyclophilin using a densitometer for calculation of the peak area of the band corresponding to CyP. Two separate experiments performed on different mitochondrial preparations are shown with exposure to tBH for 30 s (■) or 10 min (●).

protein in a similar manner to its interaction with CsA, the rate of dissociation is also likely to be very low. Such tight binding of CyP may explain how excised patches of the inner mitochondrial membrane maintain Ca^{2+} -dependent, CsA-inhibitable, high-conductance channels that are believed to be a manifestation of pore

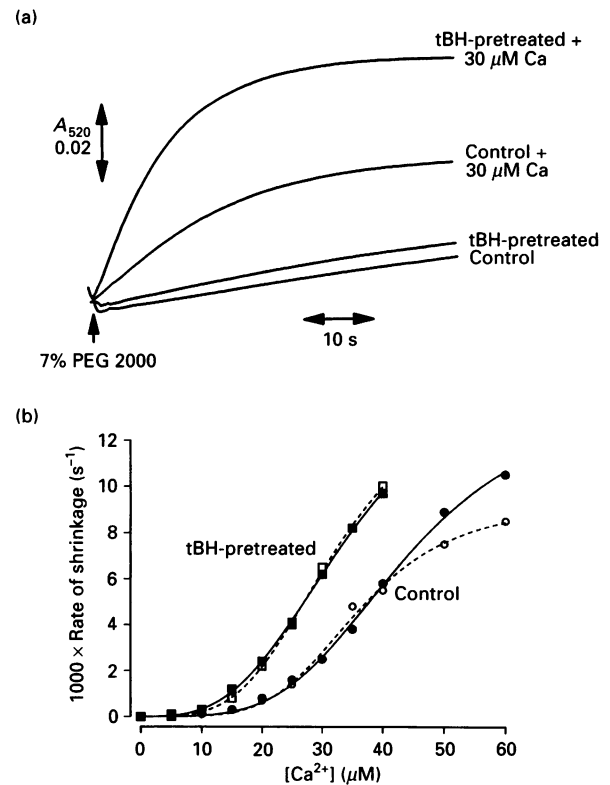


Figure 4 Effect of pretreatment of mitochondria with tBH on the sensitivity to $[Ca^{2+}]$ of mitochondrial pore opening

Pore opening in mitochondria pre-swollen for 10 min in the absence (control, ●, ○) or presence of 1 mM tBH (tBH-pretreated, ■, □) was determined at the $[Ca^{2+}]$ shown by measuring the rate of shrinkage ($\Delta A_{520}/\text{time}$) in response to the addition of 7% PEG 2000 as described in the Experimental section. Mitochondria were used within 3 h of preparation in (a) and (b, solid symbols and lines) or stored overnight on ice (b, open symbols and broken lines) before pre-swelling. In (b), the maximal rates of PEG-induced shrinkage at different $[Ca^{2+}]$ were determined by differentiation of the traces.

formation [24]. The binding of CyP to the inner membrane is unlikely to involve any covalent bond formation, since the only band reacting specifically with the anti-CyP antibody on Western blots has the same mobility as the purified CyP. However, the bound CyP showed no PPIase activity (measured as in [7]), implying that binding probably occurs at the active site of the enzyme.

The rate at which CyP binds to the mitochondrial membrane is rapid, since preparation of membranes by freezing 30 s after addition of the tBH gave as much CyP binding as did freezing after exposure for 10 min to the reagent (Figure 3). The concentration dependence of the tBH effect is also presented in Figure 3. The abilities of other agents known to activate or inhibit pore opening were tested in both the presence and absence of 1 mM tBH. Addition of 200 μ M ADP, 10 μ M bongkrekate or 10 μ M carboxyatractyloside were all without consistent effects on CyP binding (results not shown), suggesting that these agents influenced the pore opening independently of changes in CyP binding. Thus it is likely that pore opening requires CyP binding to activate the Ca^{2+} -trigger site, while adenine nucleotides and the conformation of the ATP/ADP carrier act independently to change the affinity of the trigger site for $[Ca^{2+}]$, as suggested by others [17,23]. This contrasts with the results of Andreeva and Crompton [15], who demonstrated that CsA binding to an inner-

membrane 10 kDa component thought to be associated with pore opening was increased by ADP.

In Figure 4 we provide data that confirm the ability of tBH treatment of mitochondria to increase the sensitivity of pore opening to $[Ca^{2+}]$. Addition of 7% (v/v) PEG 2000 causes shrinkage of mitochondria previously swollen as a result of pore opening, provided that the pores remain open; the rate of shrinkage (estimated as the increase in light scattering) gives an estimate of the extent of pore opening [2,23]. The ability of tBH to sensitize the opening of the pore to $[Ca^{2+}]$ was only observed when it was added to mitochondria during the initial swelling, and not when it was added to the pre-swollen mitochondria prior to shrinking. This suggests that some low-molecular-mass component essential for eliciting the effect of tBH might be lost through the pore during the swelling procedure. One possible candidate is GSH, which is oxidized by tBH treatment and is known to be lost through the open pore [25]. GSSG is able to form mixed-disulphide conjugates with membrane proteins such as the membrane component of the pore, and so might enhance its sensitivity to $[Ca^{2+}]$ [26]. However, we have been unable to mimic the effects of tBH by adding either GSH or GSSG to pre-swollen mitochondria (results not shown).

CONCLUSIONS

The data that we present in this paper clearly demonstrate that addition to mitochondria of thiol reagents or tBH (to induce oxidative stress) stimulates the binding of CyP to the inner mitochondrial membrane at the same time as it stimulates pore opening. This is consistent with our hypothesis that CyP interacts with some membrane protein, perhaps the ANT in the 'c' conformation, to induce pore opening [4,7,27]. It is interesting to note that oxidative stress has been reported to modify the ANT [28] and that this protein has some reactive thiol groups whose modification in the reconstituted carrier can induce channel-like properties [29].

The normal role of the CyPs within the cell is unclear, although their involvement in protein folding and trafficking has been suggested [11,12]. Although PPIases can greatly enhance protein folding, especially in the presence of protein disulphide isomerase [30], protein-folding reactions may well occur with sufficient speed in the absence of PPIase for normal cellular function. The data we present in this paper suggest another potential mode of action of CyPs in signal transduction; binding to membrane proteins under the influence of a suitable signal (oxidative stress in the case of the mitochondrial pore) and subsequent modulation of their activity. In this context, it is of interest that Thalhammer et al. [31] have described the presence of membrane-associated CyPs that can be released by urea or 0.5% (w/v) CHAPS in several subcellular organelles of rat liver. Furthermore, members of another class of proteins with PPIase activity, the FK506-binding proteins [11,12], have been shown to interact with the Ca^{2+} -release channel of the ryanodine receptor, modifying its gating properties and stabilizing the closed form of the channel [32]. Other members of this family and of the CyP family have been shown to be tightly associated with steroid hormone-receptor complexes [33,34]. The rate constant for dissociation of

CyP from the mitochondrial inner-membrane component of the pore appears from our data to be very low, since washing of the membranes does not remove the bound CyP, implying that removal of the CyP from its membrane target requires an additional factor *in vivo*. This would be akin to the hydrolysis of GTP that is required to dissociate the GTP-binding subunit of G-proteins from their target proteins [35]. The opening of the mitochondrial pore may well provide a model system for studying such CyP-membrane protein interactions.

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