Hypothesis

Regulated and unregulated mitochondrial permeability transition pores: a new paradigm of pore structure and function?

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Abstract Cyclosporin A (CsA) inhibits the mitochondrial permeability transition (MPT), but not always. To characterize the CsA-sensitive and -insensitive MPT, rat liver mitochondria were exposed to low and high doses of various MPT inducers. Mitochondrial swelling, cyclophilin D membrane binding and permeability transition (PT) pore diameter were measured. The results indicate two conductance modes for the PT pore: one activated by Ca^{2+} and inhibited by CsA and Mg^{2+} and the other unregulated. We propose a new model of pore formation and gating in which PT pores form by aggregation of misfolded integral membrane proteins damaged by oxidant and other stresses. Chaperone-like proteins initially block conductance through these misfolded protein clusters; however, increased Ca^{2+} opens these regulated PT pores, an effect blocked by CsA. When protein clusters exceed chaperones available to block conductance, unregulated pore opening occurs.

Key words: Rat liver mitochondrion; Cyclophilin D; Cyclosporin A; Calcium; Amphipathic peptide; Oxidant chemical

1. Introduction

Opening of high conductance permeability transition (PT) pores induces the mitochondrial PT (MPT), which is characterized by mitochondrial swelling, uncoupling and inner membrane permeabilization to solutes up to 1500 Da [1]. Ca^{2+} induces pore opening, whereas Mg^{2+} and cyclosporin A (CsA) close the pore. PT pores play an important pathogenic role in both necrotic and apoptotic cell death (reviewed in [2,3]). The molecular composition and regulation of PT pores, however, remain poorly understood. A current model suggests that PT pores form at contact sites between the mitochondrial inner and outer membranes by association of the voltage-dependent anion channel (VDAC) in the outer membrane, the adenine nucleotide translocator (ANT) in the inner membrane, cyclophilin D (CypD) in the matrix, and possibly other proteins [4,5]. This working model is consistent with inhibition and activation of the MPT by bongkrekic acid and atractyloside, ligands of ANT, and blockade by CsA, which binds to CypD [6,7].

Several experimental findings are difficult to explain by this model of PT pore structure. First, although Ca^{2+}-dependent activation and CsA inhibition of the MPT are considered hallmarks of the MPT, the MPT can also occur in the absence of Ca^{2+} [8–10], and inhibition of the MPT by CsA can be transitory or ineffective [9]. Second, although oxidative stress is reported to induce CypD binding to mitochondrial membranes [11–13], VDAC and ANT bind tightly to CypD affinity columns in the absence of Ca^{2+} and exogenous oxidant [5]. Third, although PT pore-like conductance can be reconstituted with ANT alone, ANT in combination with VDAC and CypD, and ANT plus other proteins [5,14,15], other purified mitochondrial anion carriers, such as the aspartate/glutamate and phosphate carriers, can also be converted to unspecific pores [16–18]. Fourth, small exogenous amphipathic peptides and the mitochondrial matrix targeting sequence of cytochrome *c* oxidase subunit IV (COX IV) induce an MPT in mitochondria that is inhibited by CsA [8,19]. Lastly, although PT pores are often assumed to be a normal constituent of mitochondrial membranes, induction of the MPT typically requires mitochondrial membrane damage, such as from oxygen radical attack and protein disulfide cross-linking [20]. Because of the discrepant literature, we reconsider the basic properties of PT pore physiology. Here, we present data indicating that PT pores opened by any of several inducers operate in two modes, a regulated mode that is activated by Ca^{2+} and inhibited by CsA and Mg^{2+} and an unregulated mode that is Ca^{2+}-independent and insensitive to CsA and Mg^{2+}. To account for the two modes of pore conductance and other features of pore physiology, we propose a new model of PT pore formation and regulation.

2. Materials and methods

Rat liver mitochondria were isolated in 250 mM sucrose, 2 mM K^+-HEPES buffer, pH 7.4, as described [21]. Mitochondrial swelling was monitored at 25°C by absorbance at 540 nm with a ThermoMax 96-well plate reader (Molecular Devices, Sunnyvale, CA, USA) in incubation buffer containing 200 mM sucrose, 20 μM EGTA, 5 mM succinate, 2 μM rotenone, 1 μg/ml oligomycin, 20 mM Tris, 20 mM

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Abbreviations: ANT, adenine nucleotide translocator; COX IV, cytochrome *c* oxidase subunit IV; CsA, cyclosporin A; CypD, cyclophilin D; DTT, dithiothreitol; GSH, reduced glutathione; MPT, mitochondrial permeability transition; PEG, polyethylene glycol; PhAsO, phenylarsine oxide; PT, permeability transition; VDAC, voltage-dependent anion channel

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HEPES and 1 mM KH_2PO_4, pH 7.2. PT pore size was estimated by solute exclusion using polyethylene glycol (PEG) of various molecular weights (0.4–8 kDa), as described [8].

To chelate endogenous mitochondrial Ca^{2+}, mitochondria were first incubated with 100 μM EGTA-AM for 30 min at room temperature. The EGTA-loaded mitochondria were then centrifuged at $10\,000\times g$ and resuspended in incubation buffer. Fluorescence of the Ca^{2+} indicator, Fluo-5N (1 μM), and absorbance were determined in a fluorescence plate reader (BMG LabTechnologies GmbH, Offenburg, Germany), as described [22].

To determine CypD membrane binding, mitochondria were frozen in liquid nitrogen, thawed and sonicated on ice. The sonicate was centrifuged at $10\,000\times g$ for 20 min at 4°C, and the resulting supernatant was centrifuged at $150\,000\times g$ for 1 h at 4°C to pellet the membrane fraction. Membrane protein (20 μg) was resolved by 15% SDS–polyacrylamide gel electrophoresis and transferred to a PVDF membrane. Western blotting was carried out, and signals were detected using ECL Plus (Amersham, Piscataway, NJ, USA) and a gel imaging system (Molecular Dynamics, Sunnyvale, CA, USA). Primary antibody raised against the N-terminal peptide of the CypD that only recognizes mitochondrial cyclophilin [23] was custom-synthesized by Bethyl Laboratory (Montgomery, TX, USA). To assess equal loading of proteins, blots were stripped and reprobed with mouse anti-COX IV antibody (Molecular Probes, Eugene, OR, USA).

3. Results and discussion

3.1. Low doses of inducers cause a Ca^{2+}-dependent MPT that is inhibited by CsA and Mg^{2+}, whereas high doses induce a Ca^{2+}-independent and CsA- and Mg^{2+}-insensitive MPT

In the classically defined MPT, an inducer in the presence of Ca^{2+} initiates large amplitude mitochondrial swelling after a time interval of several seconds or minutes. Fig. 1 illustrates this behavior for $HgCl_2$. Although neither 5 μM $HgCl_2$ nor 50 μM $CaCl_2$ alone induced mitochondrial swelling (data not shown), $CaCl_2$ plus $HgCl_2$ produced marked mitochondrial swelling that reached a maximum after 8 min (Fig. 1A, trace a). CsA (1 μM), EGTA (0.5 mM) and $MgCl_2$ (5 mM) completely inhibited this swelling (traces b, c and d, respectively). However, at a higher dose of $HgCl_2$ (20 μM), large amplitude mitochondrial swelling occurred rapidly in the absence of added $CaCl_2$ (Fig. 1B, trace a) and was not prevented by CsA or EGTA (traces b and c, respectively). $MgCl_2$ also did not block swelling, although it did decrease the magnitude of the absorbance change by about a third (trace d). Similar results were obtained with low (100 nM) and high doses (200 μM) of phenylarsine oxide (PhAsO), a thiol cross-linker agent ([24], data not shown).

Pore-forming amphipathic peptides like alamethicin and mastoparan have been reported to induce mitochondrial swelling that resembles the MPT [8]. As shown in Fig. 1C, alamethicin at 50 nM induced swelling that required Ca^{2+} and was blocked by CsA, $MgCl_2$ and EGTA. By contrast, a higher dose of alamethicin (1 μM) caused swelling that did not require Ca^{2+} and which was not blocked by CsA, EGTA or $MgCl_2$, although $MgCl_2$ decreased the magnitude of the absorbance change by about 30% (Fig. 1D). Similar results were obtained with low (1 μM) and high (4 μM) doses of mastoparan (data not shown).

To assure that swelling after high-dose induction of the MPT was not caused by endogenous Ca^{2+}, mitochondria were ester-loaded with EGTA. As shown in Fig. 2A, in sham-loaded mitochondria (trace a), 20 μM $HgCl_2$ produced a small but rapid increase in extramitochondrial Fluo-5N

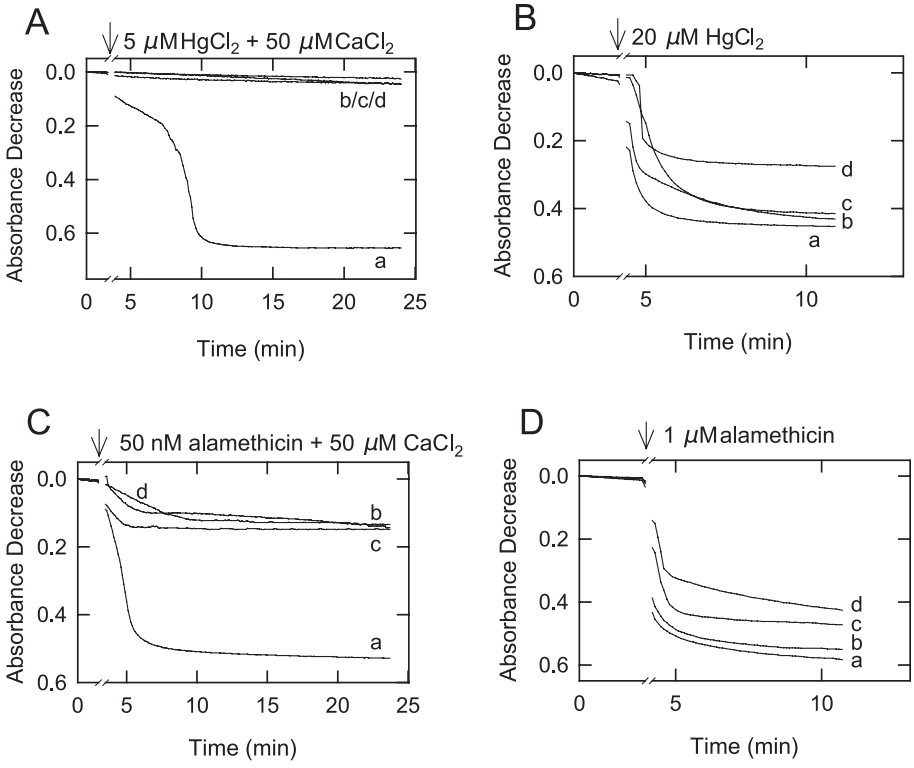


Fig. 1. Opening of regulated and unregulated PT pores by $HgCl_2$ and alamethicin. Mitochondria (0.5 mg protein/ml) were added to incubation buffer, and onset of the MPT was monitored by absorbance, as described in Section 2. After 2–3 min pre-incubation, 5 μM $HgCl_2$ plus 50 μM $CaCl_2$ (A), 20 μM $HgCl_2$ (B), 50 nM alamethicin plus 50 μM $CaCl_2$ (C), and 1 μM alamethicin (D) were added. From the beginning of the incubations, 1 μM CsA (traces b), 0.5 mM EGTA (traces c), or 5 mM $MgCl_2$ (traces d) was present or no additions were made (traces a). The data shown represent four independent experiments.

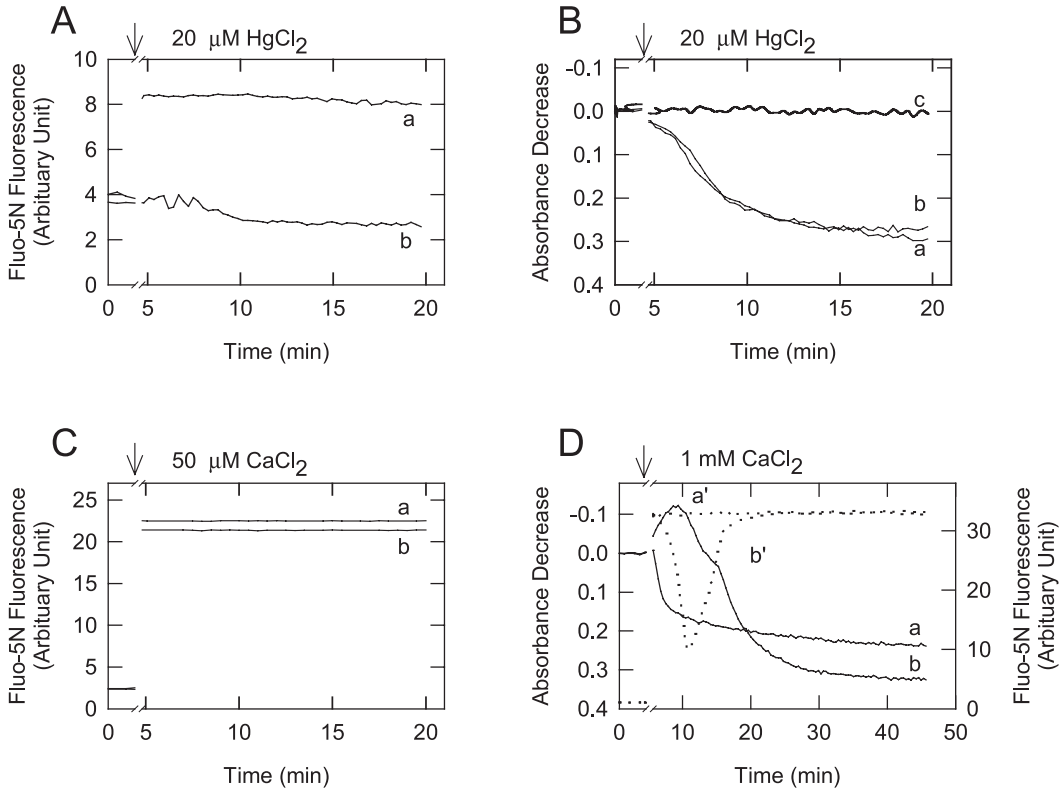


Fig. 2. Induction of PT pores by high-dose $HgCl_2$ after chelation of endogenous Ca^{2+} and by a high dose of $CaCl_2$ in the presence of CsA. In A–C, mitochondria (0.5 mg protein/ml) were sham-loaded or ester-loaded with EGTA and resuspended in incubation buffer. Free Ca^{2+} and mitochondria swelling were then measured simultaneously from Fluo-5N (1 μM) fluorescence (A) and absorbance (B), respectively, after addition of 20 μM $HgCl_2$ (traces a and b) or no addition (trace c), as described in Section 2. In C, mitochondria were pre-incubated for 1 min with 0.1 μM ruthenium red, and 50 μM $CaCl_2$ was added to show the response of Fluo-5N to Ca^{2+}. Traces a are sham-loaded mitochondria, and traces b and c are EGTA-loaded mitochondria. In D, 1 mM $CaCl_2$ was added to mitochondria in the absence (traces a and a′) or presence (traces b and b′) of 10 μM CsA. Mitochondrial swelling (solid lines) and extramitochondrial free Ca^{2+} (dotted lines) were measured simultaneously.

fluorescence, which reflects the release of endogenous Ca^{2+} after onset of the MPT. By contrast, EGTA-loaded mitochondria did not release Ca^{2+} (trace b), indicating that EGTA had chelated all measurable endogenous Ca^{2+}. Nonetheless, swelling occurred in EGTA-loaded mitochondria to the same extent as sham-loaded mitochondria after 20 μM $HgCl_2$, although swelling did not occur in the absence of $HgCl_2$ (Fig. 2B). These results confirm that the MPT induced by high-dose $HgCl_2$ was fully Ca^{2+}-independent. To assure that EGTA was loaded into the mitochondria matrix and not simply added to the extramitochondrial space, mitochondria were pre-incubated with 0.1 μM ruthenium red, which blocks Ca^{2+} uptake by the uniporter. In the presence of ruthenium red, the increase in Fluo-5N fluorescence after the addition of 50 μM $CaCl_2$ was the same in sham-loaded mitochondria as in EGTA-loaded mitochondria (Fig. 2C), indicating that incubation with EGTA-AM did not cause chelation of Ca^{2+} outside of the mitochondria.

In our incubation buffer, $CaCl_2$ alone at concentrations between 100 and 500 μM induced swelling that was completely blocked by 1 μM CsA (data not shown), whereas 1 mM $CaCl_2$ induced swelling that was not blocked by 1–10 μM CsA (Fig. 2D and data not shown). Swelling was not driven osmotically by CaPi uptake after high-dose $CaCl_2$, because in the presence of CsA mitochondria initially took up the added Ca^{2+} without swelling. Rather, swelling occurred as mitochondria released Ca^{2+}, as measured by Fluo-5N, which

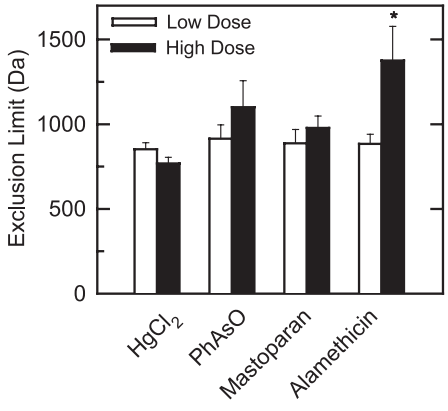


Fig. 3. PEG exclusion by regulated and unregulated PT pores. Mitochondria (0.5 mg protein/ml) were incubated at 25°C in a sucrose-based incubation buffer medium in which 40% of the total osmotic pressure was derived from PEGs of a molecular weight from 0.4 to 8 kDa. The molecular weight of PEG causing 50% inhibition of the absorbance change was determined. MPT was induced by 50 μM $CaCl_2$ plus low doses (white bars) of $HgCl_2$ (5 μM), PhAsO (100 nM), mastoparan (1 μM) and alamethicin (50 nM), and high doses (black bars) of $HgCl_2$ (20 μM), PhAsO (200 μM), mastoparan (4 μM) and alamethicin (1 μM) without added $CaCl_2$. Error bars are standard deviations from four or five determinations. $^*P < 0.01$ vs. low-dose alamethicin.

is consistent with MPT-induced swelling (Fig. 2D). Thus, Ca^{2+} at low and high doses was also able to induce CsA-sensitive and -insensitive PT pore opening.

Although the consensus of most reviews is that onset of the MPT is critically dependent on Ca^{2+} and is inhibited by CsA [25–27], our observations that the MPT can become independent of Ca^{2+} and insensitive to CsA actually confirm individual findings in several previous reports. For example, it was reported previously that a low concentration (1 μM) of mastoparan induced an MPT that was Ca^{2+}-dependent and CsA-sensitive, while a high concentration (3 μM) of mastoparan induced an MPT that was Ca^{2+}-independent and CsA-insensitive [8]. Alamethicin at a high concentration (1.8 μM) also caused Ca^{2+}-independent mitochondrial swelling. It was proposed that low-dose mastoparan directly caused PT pore opening, whereas high-dose alamethicin and mastoparan produced inner membrane permeabilization via a direct effect on the lipid bilayer [8]. In another study, low-dose (3 μM) ganglioside GD3 induced an MPT that required exogenous Ca^{2+} and was blocked by CsA, while high-dose (100 μM) ganglioside GD3 in the presence of succinate induced an MPT that was independent of elevated Ca^{2+} and not prevented by CsA [9]. Several studies showed non-classical MPT behavior after exposure of mitochondria to oxidant chemicals. For example, after exposure of mitochondria to *tert*-butyl hydroperoxide, CsA delayed but did not prevent onset of mitochondrial swelling [28]. In other studies, PhAsO at high concentrations (60 μM–1 mM) but not low concentrations (10–15 μM) induced an MPT that was independent of Ca^{2+}, although sensitivity to inhibition by CsA was partially if not fully retained [9,24]. Similarly, we observed that a high dose of $HgCl_2$ (20 μM) and PhAsO (200 μM) also produced Ca^{2+}-independent mitochondrial swelling (Fig. 1B and data not shown). Although CsA did not prevent the MPT in our experiments, close inspection of the traces reveals that CsA did delay slightly the onset of the MPT and decrease the initial rate of swelling both with high-dose $HgCl_2$ (Fig. 1B, trace b) and PhAsO (data not shown), which may be consistent with the earlier studies.

Taken together, these observations indicate that the classical description of PT pore behavior is incomplete. We propose that the PT pore has two functional modes: a regulated mode activated by Ca^{2+} and inhibited by CsA and Mg^{2+}, and an unregulated mode that is Ca^{2+}-independent and insensitive to CsA and Mg^{2+}. In general, a low level of MPT induction opens regulated pores, whereas stronger MPT induction opens unregulated pores. Therefore, a basic but generally unrecognized feature of the MPT is its ability to transform from a Ca^{2+}-dependent and CsA-sensitive event to a Ca^{2+}-independent and CsA-insensitive event as the strength of MPT induction increases.

3.2. Regulated and unregulated PT pores opened by the same inducers have similar PEG exclusion limits except for alamethicin

Regulated and unregulated pores after MPT induction could represent different functional states of the same pore or two altogether different pores. To address this issue, we determined the mean size of regulated and unregulated PT pores formed after different inducers based on exclusion of PEG of various molecular weights, as described by Pfeiffer et al. [8]. The molecular weight of PEG that caused 50% inhibition of swelling was plotted for each MPT inducer at a low dose in the presence of 50 μM $CaCl_2$ and at a high dose in the absence of $CaCl_2$ (Fig. 3). Low and high doses of $HgCl_2$, PhAsO, mastoparan and alamethicin produced PT pores with 50% PEG exclusion limits of 800–1400 Da. Analysis of variance and post hoc analysis using the Tukey–Kramer multiple comparisons test revealed that average pore size after low induction was not significantly different for the four different inducers ($P > 0.05$). Average pore size was also not different between low and high doses of the inducers, except for alamethicin, which produced a $\sim$50% larger pore at a high dose (1400 vs. 900 Da, $P < 0.01$). These results extend and confirm the earlier findings of Pfeiffer et al. [8] showing that PT pores induced by low (1 μM) and high (3 μM) concentrations of mastoparan are of similar average size, which is smaller than the average size of pores induced by high-dose alamethicin. Kinetic analysis of mitochondrial swelling after MPT induction also shows that increasing concentrations of PhAsO do not change mean pore size, although pore number increases [29]. The near identity of pore size after low- and high-dose MPT induction with $HgCl_2$, PhAsO and mastoparan is consistent with the idea that regulated and unregulated pores represent two functional modes of the same pore.

3.3. CypD binding to membranes accompanies regulated pore opening but is not required for unregulated pore conductance

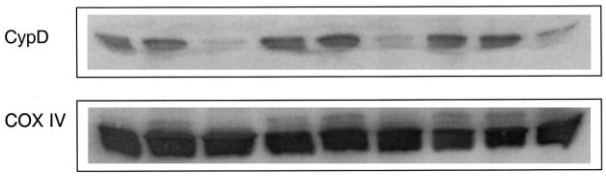
CypD binding to mitochondrial membranes is reported to increase with oxidative stress and to regulate pore opening [11–13,30]. To determine the role of CypD membrane binding during regulated and unregulated PT pore opening, membrane bound CypD was detected by Western analysis. As shown in Fig. 4A, when mitochondria were incubated at room temperature for 10 min without an MPT inducer, CypD was detected in the membrane fraction. Absorbance measurements confirmed that swelling had not occurred in these mitochondria. Similar CypD binding to membranes occurred in mitochondria that were briefly incubated on ice in incubation buffer containing 5 mM dithiothreitol (DTT) and 5 mM reduced glutathione (GSH). CypD binding to membranes was specific, since 1 μM CsA removed virtually all CypD from the membrane fraction.

CypD membrane binding was then determined before and after onset of the regulated MPT. After 30 s incubation with low-dose $HgCl_2$ (5 μM plus 50 μM $CaCl_2$) or alamethicin (50 nM plus 50 μM $CaCl_2$) before onset of the MPT, CypD binding was the same as after 10 min of incubation after swelling had occurred (Fig. 4A,B). Moreover, the amount of binding in the presence of low-dose inducers was not different from that after incubation without inducers. CsA (1 μM) also dissociated CypD from the membrane fraction after low-dose $HgCl_2$ and alamethicin. CypD also bound to membranes when mitochondria were incubated with high-dose $HgCl_2$ (20 μM) or alamethicin (1 μM). CsA (1 μM) dissociated CypD from membranes in the presence of high-dose inducers, even though CsA did not block swelling (Fig. 4A,B). The blots were stripped and reprobed for COX IV, which showed equal protein loading for each sample.

Similar results were obtained for mitochondria incubated with low and high doses of PhAsO and mastoparan with and without CsA (data not shown). We also incubated the mitochondria in a high ionic strength incubation buffer in

A

$HgCl_2$	-	-	-	Low	Low	Low	High	High	High
$CaCl_2$	-	-	-	+	+	+	-	-	-
CsA	-	-	+	-	-	+	-	-	+
Time (min)	0.5*	10	10	0.5	10	10	0.5	10	10



B

Alamethicin	-	-	-	Low	Low	Low	High	High	High
$CaCl_2$	-	-	-	+	+	+	-	-	-
CsA	-	-	+	-	-	+	-	-	+
Time (min)	0.5*	10	10	0.5	10	10	0.5	10	10

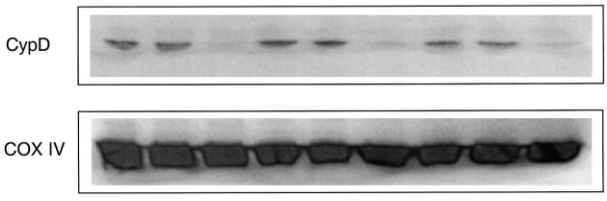


Fig. 4. Effect of PT inducers and CsA on CypD membrane binding. Mitochondria (0.5 mg protein/ml) were incubated at room temperature for 30 s or 10 min in buffers containing the indicated reagents, or on ice for 30 s in a buffer containing 5 mM DTT and 5 mM GSH (first lanes of A and B, 0.5*). Membrane fractions were prepared, and CypD content was detected by Western blotting, as described in Section 2. Blots were stripped and reprobed with COX IV to assess equal loading of protein. In A, mitochondria were incubated with 0, 5 μM (low dose) and 20 μM (high dose) $HgCl_2$ in the presence or absence of 1 μM CsA. In B, mitochondria were incubated with 0, 50 nM (low dose) and 1 μM (high dose) alamethicin in the presence or absence of 1 μM CsA. 50 μM $CaCl_2$ was added with low-dose $HgCl_2$ and alamethicin.

which 100 mM KCl replaced sucrose. The amount of CypD bound to membranes was decreased due to the increased ionic strength, but the pattern of CypD binding was the same as in sucrose-based buffer (data not shown). We also added the chaotropic agent KSCN immediately after the incubation of mitochondria and before freezing, as described [12,13], but we still failed to observe an increase of CypD binding to the membrane fraction after oxidant chemicals, including PhAsO (0.1–200 μM), *tert*-butyl hydroperoxide (0.1–5 mM), and diamide (0.1–1 mM) (data not shown). Although our data do not confirm some earlier reports of increased CypD membrane binding under oxidative stress [11–13], our findings do confirm other reports of CsA-sensitive CypD membrane binding in sucrose-based buffer without addition of MPT inducers [30] and of ANT/VDAC binding to CypD affinity matrices in the absence of Ca^{2+} or oxidant chemicals [5]. Taken together, these results indicate that CypD binds to mitochondrial membranes in the absence of MPT induction and that dissociation of CypD accompanies closure of regulated PT pores by CsA. In contrast, CsA-induced CypD membrane dissociation did not cause unregulated PT pore closure.

3.4. *New paradigm of the PT pore as a chaperone-gated cluster of misfolded amphipathic membrane proteins*

The current model of PT pore structure proposes that PT pores are composed of ANT, VDAC, CypD and possibly other proteins, but this model does not account for how increasing concentrations of inducers first cause opening of regulated PT pores and subsequently unregulated pores at higher concentrations (Fig. 1). Moreover, the current model provides no basis for understanding how pore-like activities can be reconstituted from different proteins and protein mixtures that do not depend on any single protein, including ANT [16–18]. Indeed, we confirm here that completely exogenous pore-forming peptides like alamethicin and mastoparan at low concentrations form regulated PT pores whose conductance properties are very similar to the PT pores induced by low concentrations of oxidant chemicals. Moreover, these PT pores switch to unregulated pores as do the pores induced by oxidants (Fig. 1) [8]. Finally, the current model does not explain how unregulated pore opening occurs even when CsA blocks CypD binding to the membrane (Fig. 4), and how agents causing direct protein modification and damage promote pore formation.

To better accommodate the experimental facts, we propose a new paradigm for pore composition, structure and regulation to guide future investigation (Fig. 5). In our model, damage after oxidative stress and other perturbations causes misfolding of native membrane proteins. This misfolding exposes hydrophilic residues to the bilayer phase. These hydrophilic surfaces cluster and enclose aqueous channels that conduct low molecular weight solutes (Fig. 5A). Alterations to thiol groups, particularly cross-linking of SH groups, may be critical for the initial misfolding of membrane proteins leading to clustering [31,32]. Similarly, exogenous amphipathic peptides,

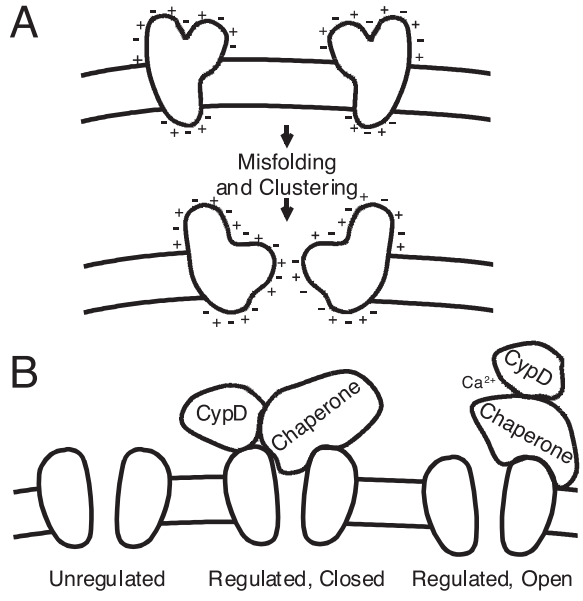


Fig. 5. New paradigm of PT pore structure and regulation. A: Misfolding and clustering of native membrane proteins after oxidative damage and other perturbations expose hydrophilic residues to the bilayer phase to enclose aqueous channels conducting low molecular weight solutes. Exogenous amphipathic peptides also assemble into pore-forming clusters. B: Chaperone-like proteins bind to the amphipathic protein clusters to block pore conductance. CypD also binds to the clusters, perhaps to refold proteins to their native state. Increased Ca^{2+} causes CypD to perturb the protein cluster/chaperone complex to open a regulated PT pore, an effect antagonized by CsA. Opening of unregulated pores occurs when protein clusters exceed chaperones available to block their conductance.

such as mastoparan and alamethicin, also assemble into pore-forming clusters. As the most abundant protein in the mitochondrial inner membrane on a molar basis, ANT is often involved in such amphipathic protein clustering. After the initial formation of amphipathic protein clusters, we propose that one or more chaperone-like proteins bind to the clusters to block pore conductance. CypD also binds to the clusters, perhaps to refold the proteins to their native state, which is consistent with the *cis–trans* proline peptidyl isomerase activity of CypD [6]. Ca^{2+} binding to the pore complex causes CypD to perturb the amphipathic protein cluster/chaperone complex to an open conductance state, an effect antagonized by CsA and Mg^{2+}. This complex of an amphipathic peptide cluster, CypD and the as yet unidentified chaperone constitutes the regulated PT pore. In addition to conferring opposing regulation by Ca^{2+} and Mg^{2+} and susceptibility to conductance blockade by CsA, binding of chaperones and CypD to misfolded protein clusters may confer other regulatory properties as well, such as voltage and pH dependence [31,33,34]. Future studies will be needed to determine whether transformation from regulated to unregulated PT pores also causes loss of voltage and pH dependence of pore conductance.

Although no direct evidence currently exists that a second chaperone protein besides CypD associates with PT pore-forming clusters, many studies indicate that chaperone proteins, such as Hsp10, Hsp60 and Hsp70, protect against apoptosis at the mitochondrial level by blocking cytochrome *c* release into the cytosol [35,36]. Since the MPT is an important event promoting apoptosis, these heat shock proteins represent candidate chaperones regulating PT pore function. Moreover, the association of PT pore formation with oxidative stress and membrane protein damage also supports the possible involvement of chaperones in PT pore formation and regulation. Future studies will be needed to determine which chaperone protein(s) interact with the PT clusters.

Opening of unregulated pores occurs when the number of amphipathic protein clusters exceeds the number of chaperones available to block their conductance (Fig. 5B). When this occurs, the amphipathic protein clusters remain in an open state unregulated by Ca^{2+}, CsA and Mg^{2+}. Alternatively, protein clusters may grow in size to the extent that chaperone inhibition is overcome. However, our data indicate that average diameters of regulated and unregulated pores with various inducers are relatively invariant, except for pores formed by alamethicin (Fig. 3). In this chaperone-gated model, amphipathic protein clusters do not constitute a single molecular entity but will be composed of different denatured protein or exogenous peptides depending upon the nature of MPT induction. Thus, conductance by individual PT pores may vary with their varying protein composition. This expectation is consistent with a kinetic analysis of mitochondrial swelling after the MPT, which concludes that the diameters of individual pores are heterogeneous [29].

In this model, Ca^{2+} has two roles in PT pore formation. First, Ca^{2+} directly induces opening of regulated pores once they are formed. Second, Ca^{2+} has an independent effect of inducing oxygen radical formation by mitochondria [20,37]. Oxygen radical generation then leads to protein cross-linking, misfolding and formation of amphipathic protein clusters. By these two different effects of Ca^{2+}, low- and high-dose Ca^{2+} can also produce regulated (CsA-sensitive) and unregulated (CsA-insensitive) PT pores (Fig. 2D).

Although our model is still speculative, it does account for puzzling aspects of PT pore behavior. These aspects include opening of regulated and unregulated PT pores by low and high concentrations of MPT inducers, the apparent involvement of a variety of unrelated proteins and peptides in PT pore formation, the ability of unregulated PT pores to form in the absence of CypD binding, and the association of PT pore formation with membrane protein damage. In cell injury, alternations of non-specific permeability of the plasma membrane also occur [38,39], and these may also be the result of clustering of misfolded membrane proteins. Our new paradigm provides an alternative model for pore composition and biology that may be helpful in guiding future study of the molecular mechanisms of cellular injury.

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