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Permeability Transition Pore of the Inner Mitochondrial Membrane Can Operate in Two Open States with Different Selectivities

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Prooxidants induce release of Ca^{2+} from mitochondria through the giant solute pore in the mitochondrial inner membrane. However, under appropriate conditions prooxidants can induce Ca^{2+} release without inducing a nonspecific permeability change. Prooxidant-induced release of Ca^{2+} is *selective*. Presumably, this is the result of the operation of a permeability pathway for H^+ coupled to the reversal of the Ca^{2+} uniporter, the latter generating the selectivity. The solute pore and prooxidant-induced Ca^{2+}-specific pathways exhibit common sensitivities to a set of inhibitors and activators. It is proposed that the pore can operate in two open states: (1) permeable to H^+ only and (2) permeable to solutes of $M_r < 1500$. Under some conditions, prooxidants induce the H^+-selective state which, in turn, collapses the inner membrane potential and permits selective loss of Ca^{2+} via the Ca^{2+} uniporter.

KEY WORDS: Mitochondria; permeability transition pore; Ca^{2+}; cyclosporin A; oxidants.

MECHANISMS BY WHICH Ca^{2+} ENTERS AND LEAVES MITOCHONDRIA

Extensive evidence indicates that change in mitochondrial $[Ca^{2+}]$ followed by dysfunction of mitochondrial metabolism is an essential component of the mechanisms leading to apoptosis, carcinogenesis, and to cell injury associated with ischemia/reperfusion [1–3]. Although appreciation of the mechanisms by which mitochondria transport Ca^{2+} has evolved considerably in 40 years of intensive investigation (for review see [1, 2]), the overall control of Ca^{2+} transport and its detailed mechanisms are far from a full understanding. Five dissimilar transporting systems are thought to be involved in the maintenance of cellular Ca^{2+} homeostasis by mitochondria. Diversity of these systems reflects multiplicity of functional links between mitochondria and biochemical processes in cytoplasm mediated by Ca^{2+}.

There is a general consensus that calcium transport across the inner mitochondrial membrane is mediated by separate influx and efflux pathways [1,2] that can be organized into three distinct groups:

1. Electrogenic Ca^{2+} uniporter. Accumulation of Ca^{2+} by energized mitochondria occurs via an electrophoretic Ca^{2+}-uniporter that is driven by $\Delta\Psi$. The Ca^{2+}-uniporter is activated by spermine, and can be inhibited by Mg^{2+} and ruthenium red [1,2].

2. Low capacity efflux porters. The maximum activity of these porters is negligible compared to the maximum activity of the Ca^{2+} uniporter. These are electroneutral $Ca^{2+}/2H^+$ and $Ca^{2+}/2Na^+$ porters that ensure precise tuning of matrix Ca^{2+} in the time course of oxidative phosphorylation. Operation of these porters at maximum rate does not dissipate the inner membrane potential ($\Delta\Psi$) to an extent sufficient for inhibition of ATP synthesis. These porters are active when mitochondria produce ATP and redox equivalents (e.g., NADPH, NADH, and GSH) for the needs

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of the cell and buffer cytoplasmic Ca^{2+}, thus maintaining the concentration of this important second messenger in the lower physiological range—less than 1 μM.

3. High-capacity efflux porters. This group comprises the prooxidant-induced Ca^{2+} release pathway, the mitochondrial permeability transition pore (PTP),[3] and the uniport pathway operating in reverse. When fully activated, these systems release Ca^{2+} instantaneously and completely dissipate $\Delta\Psi$, thereby uncoupling respiration from phosphorylation. In this state, mitochondria oxidize cytoplasmic redox equivalents and consume ATP, thus depriving the cell of energy resources. Depletion of cellular ATP stores (and, therefore, temporary or permanent inactivation of Ca^{2+}-ATPases), in conjunction with release of mitochondrial Ca^{2+}, produces a Ca^{2+} spike sufficient to trigger processes with low affinity for Ca^{2+} (activation of Ca^{2+}-dependent phospholipases, proteases, and endonucleases) [3,4] and contributes to irreversible cell injury under oxidative stress and toxemia [4–8].

Now that we have described Ca^{2+} transporting pathways in general, we will consider mechanisms of Ca^{2+} efflux in greater detail:

a. Na^+/Ca^{2+}-exchange. Sodium-dependent Ca^{2+} efflux occurs by the electroneutral exchange of matrix Ca^{2+} with extramitochondrial Na^+ in a reaction catalyzed by a 110-kDa inner membrane protein [9]. Na^+/Ca^{2+}-exchange can be inhibited by diltiazem, Mg^{2+}, tetraphenylphosphonium, and by increasing extramitochondrial [Ca^{2+}]. This mechanism is insensitive to ruthenium red and to cyclosporin A (CSA), the inhibitor of the mitochondrial permeability transition pore [2].

b. Permeability Transition Pore (PTP). Under a number of conditions, Na^+-independent Ca^{2+} efflux can be attributed to the opening of the Ca^{2+}-activated nonspecific channel (or PTP) in the inner membrane of mitochondria. PTP is activated by cooperative binding of 2 Ca^{2+} to the matrix aspect of the inner membrane [10]. The presence of a second agent referred to as a Ca^{2+}-releasing agent is required in addition to Ca^{2+} to open the pore [1,2]. Remarkably, pore opening is fully reversed when Ca^{2+} is removed. In the open state, PTP has a diameter of 2 to 3 nm and renders the inner membrane nonselectively permeable for low-M_r solutes <1500 Da [1,2]. PTP seems to open abruptly without intermediate stages to a form that permits free entry of sucrose and molecules of similar size [11]. In patch-clamp experiments, a 1.3-nS megachannel has been tentatively identified as PTP (for review, see [12,13]).

Opening of the pore uncouples oxidation from phosphorylation, allows diffusion of small molecules and ions, including Ca^{2+}, out of the mitochondrial matrix, and counterdiffusion of components of the incubation medium into the matrix space. These solute fluxes dissipate concentration gradients of small molecules, but matrix proteins do not permeate through the pore [14], and the osmotic imbalance thus formed results in high-amplitude swelling of mitochondria [2].

The nature of the components or subunits that participate in the formation of the PTP is still obscure. Recently, it has been proposed that VDAC (mitochondrial porin) [15] and/or the adenine nucleotide carrier [16] may be the pore-forming component(s) of the PTP. However, the available data are also consistent with the proposal that the PTP is formed from a distinct protein in the inner mitochondrial membrane [17].

c. CSA-insensitive, Na^+-independent mechanism of Ca^{2+} release. This Ca^{2+}-efflux mechanism provides a slow release of Ca^{2+} with V_{max} in the range of 1.0–1.4 nmol/mg/min [18]. Ca^{2+} release mediated by this mechanism is insensitive to ruthenium red, diltiazem, tetraphenylphosphonium, and to CSA. The mechanism of this efflux pathway, which was originally suggested to be a passive Ca^{2+}/nH^+ exchanger [19], has not been elucidated as yet. Rottenberg and Marbach [20] have presented evidence supporting electroneutral $Ca^{2+}/2H^+$ exchange. However, Gunter *et al.* [2] have reported that this mechanism transports Ca^{2+} out of the mitochondrial matrix against a Ca^{2+} gradient many times greater than that possible for the passive $Ca^{2+}/2H^+$ exchanger. This finding could only be explained if the stoichiometry for this putative Ca^{2+}/nH^+, exchange mechanism is 1:3 (Ca^{2+} to H^+) or if the additional energy is being supplied to the transporter from some source (e.g., from the electron transport chain) other than the energy contained in the electrochemical gradients of the exchanged ions [2].

d. Ca^{2+} release by reverse uniport. The electrophoretic Ca^{2+}-uniporter shows reversibility. Any intervention that dissipates $\Delta\Psi$ sufficiently allows net Ca^{2+} release by uniport reversal. Therefore, several investi-

[3] The acronym PTP [permeability transition pore, following Bernardi, P. (1992). *J. Biol. Chem.* **267**, 8834–8839] will be further used to define the pore instead of the commonly used acronyms MPTP, MCC, Ca^{2+}-dependent pore, nonspecific pore, etc.

gators maintain that Ca^{2+} release normally occurs by this mechanism following a decline in $\Delta\Psi$ [21,22]. The sensitivities of the forward and reverse reactions of the uniporter to ruthenium red are different; the latter is blocked by higher concentrations of the inhibitor [21]. Thus, loss of Ca^{2+} upon de-energization of ruthenium red-treated mitochondria could occur by backflow through the uniporter. This is in agreement with the observation of Bernardi *et al.* [23] that part of the uncoupler-induced Ca^{2+} efflux occurring via the uniporter is insensitive to ruthenium red.

e. Prooxidant-induced Ca^{2+} efflux. Enzyme-catalyzed (hydroperoxides, acetoacetate, divicine, and quinones) or nonenzymatic (alloxan) oxidation of matrix pyridine nucleotides has been reported to induce Ca^{2+} efflux from the mitochondrial matrix by an electroneutral $Ca^{2+}/2H^+$ exchange process that is not inhibited by ruthenium red [24,25].

Oxidant-induced Ca^{2+} release occurs without swelling, loss of K^+, or sucrose entry into the matrix space, providing that ruthenium red or EGTA, both of which inhibit Ca^{2+} reuptake via electrogenic uniporter [2], are included in the incubation media [26,27]. These observations strongly suggest that Ca^{2+} release observed in these studies cannot be accounted for by the opening of PTP.

Like PTP, the prooxidant-induced Ca^{2+}/H^+ exchange is inhibited by CSA [28] and, in the presence of ruthenium red, the reported values of maximum release velocity for this pathway fall between 12 and 30 nmol/mg/min [29,30]. In contrast to the low transport capacity, CSA- and Na^+-insensitive Ca^{2+}-efflux mechanism (described in Section c), the rapid Ca^{2+} cycling ensured by operation of the prooxidant-induced Ca^{2+}/H^+ exchanger considerably increases effective proton conductance of the inner membrane, and may result in complete collapse of the $\Delta\Psi$ [24,29,31].

An alternative explanation of the prooxidant-induced Ca^{2+} release has been put forward by Pfeiffer *et al.* [21]. It has been proposed that prooxidants induce a specific increase in proton conductance through the inner membrane. The resulting collapse of $\Delta\Psi$ would promote Ca^{2+} release by the reverse uniport pathway (see Section d). More specifically this model suggests that:

1. The proton conducting pathway in the membrane lipid phase is created by lysophospholipids and/or free fatty acids, arising from a Ca^{2+}-dependent increase in phospholipase A_2 activity, with a simultaneous inhibition of lysophospholipid acyltransferase activity (for example, by prooxidants).

2. Subpopulations of mitochondria undergo transient de-energization in heterogeneous rather than uniform fashion. Cyclic phospholipid deacylation/reacylation results in cyclic de-energization/recoupling of mitochondrial subpopulations due to the cyclic increase in the levels of the lipid degradation products.

3. Ruthenium red interrupts the exchange of Ca^{2+} between de-energizing and re-energizing mitochondria, resulting in a net Ca^{2+} release proceeding in the absence of nonspecific permeability changes.

The point is often made that prooxidant-induced Ca^{2+} efflux occurs when the membrane potential is maintained at a high level or even increases [24,27,29]. This observation appears to be inconsistent with Pfeiffer's model. However, the TPP^+ distribution method utilized to estimate $\Delta\Psi$ in these studies is only able to detect an average energy state of the whole mitochondrial population. In a heterogeneous population of mitochondria, when individual subpopulations differ one from the other with respect to their energy state, the mitochondria with high $\Delta\Psi$ dominate the TPP^+ readout (see [1,2,32] and references therein). Therefore, this method is not applicable to the accurate estimation of the $\Delta\Psi$ in subpopulations with low $\Delta\Psi$. Early studies of ultrastructural changes associated with the Ca^{2+}-induced transition in permeability [10], and more recent experiments utilizing a pulsed-flow solute-entrapment technique [33], reveal continuous interconversion between the closed and open states of the pore in permeabilized mitochondria. This interconversion results in coexistence of permeable and impermeable mitochondrial fractions. A key study of solute fluxes and TPP^+ redistribution in such heterogeneous mitochondrial population was made by Al-Nasser and Crompton [14], who have shown that at intermediate concentrations of matrix free Ca^{2+}, when cation-binding site of the pore is far from saturation, sucrose enters mitochondria continuously with time, despite maintenance of high $\Delta\Psi$ as monitored by the TPP^+ distribution method. The reason is that TPP^+ released from permeabilized (de-energized, $\Delta\Psi = 0$) mitochondria is reaccumulated by resealed (well-coupled) mitochondrial fractions that generate unimpaired $\Delta\Psi$. This behavior is consistent with continued interconversion of permeable ($\Delta\Psi = 0$) and impermeable (maximum $\Delta\Psi$) mitochondrial fractions. According to calculations of Crompton [1], 5% of the total mitochondrial population in the de-energized state ($\Delta\Psi = 0$) at any

instant would decrease $\Delta\Psi$ *calculated* from TPP^+ redistribution in the whole population by about 1% of the maximum $\Delta\Psi$. Moreover, the same 1% decrease in $\Delta\Psi$ calculated from the $TPP^+_{bound}/TPP^+_{free}$ ratio could result from partial de-energization ($0 < \Delta\Psi <$ maximum) of a larger fraction of the mitochondria. In the presence of ruthenium red or EGTA to prevent reaccumulation of Ca^{2+}, cyclic de-energization/resealing would eventually allow Ca^{2+} to leave, with time, the whole population, notwithstanding the maintenance of apparently high $\Delta\Psi$.

Thus, cyclic de-energization/recoupling of mitochondrial fractions has the potential to explain prooxidant-induced, ruthenium red-insensitive Ca^{2+} release without involvement of a distinct release carrier (e.g., oxidant-modulated $Ca^{2+}/2H^+$-exchanger).

Pfeiffer and co-workers hypothesized that transient increase in H^+ permeability is brought about by cyclic rise in the content of lipid-degradation products [21]. Although this explanation is a popular one, its validity is questionable for a number of reasons. Specifically, recent studies have shown a lack of phospholipid acylation/deacylation cycle in mitochondria, and that acyltransferase activity is associated with contaminating microsomes [34]. Since, within Pfeiffer's model, the steady state of lipid degradation products in the inner membrane is established by the interplay between phospholipase A_2 and the opposing lysophospholipid acyltransferase activity [21], the metabolic basis for cyclic mitochondrial depolarization becomes controversial. Beyond this, whereas CSA completely prevents prooxidant-induced Ca^{2+} release [28], there is no appreciable effect of CSA on Ca^{2+}-induced accumulation of free fatty acids and/or lysophospholipids [34,35]. These observations are incompatible with the proposal that the accumulation of lipid degradation products associated with the inner membrane could create specific proton-conducting channels resulting in the collapse of $\Delta\Psi$ and backflow of Ca^{2+} via uniporter. Overall, this model allows for the explanation of prooxidant-induced Ca^{2+} release within the context of a "reverse uniport" mechanism (Section d). However, the identity of the H^+ channel is now in question. Moreover, the mechanism of the transient inner membrane depolarization is also not easily attributable to the cyclic acylation/deacylation of mitochondrial phospholipids.

In this paper we will provide evidence that *fast Ca^{2+} efflux mechanisms* are mediated by one and the same structure of the inner membrane, namely the *permeability transition* pore operating in different selectivity states.

HYPOTHESIS: IN INTACT MITOCHONDRIA THE PERMEABILITY TRANSITION PORE CAN OPERATE IN TWO OPEN STATES WITH DIFFERENT SELECTIVITIES

Remarkably, both PTP formation and prooxidant-induced Ca^{2+} release have a common requirement for matrix Ca^{2+} and cyclosporin A-sensitivity [1–3,24,28]. Moreover, many agents and conditions that have been found to affect the PTP also have been shown to affect the prooxidant-induced, Ca^{2+}-selective release pathway (Table I), suggesting a common mechanism. There are indications that PTP-inducing agents function by a common mechanism despite their chemical diversity. For that reason, PTP opening is inhibited by pharmacological agents with the same relative potency under

Table I. A Comparison of the Properties of the Prooxidant-Induced Ca^{2+}-Selective Release Pathway and PTP

Prooxidant-induced Ca^{2+}-selective release pathway	PTP
High specificity for Ca^{2+}, Sr^{2+}, and Mn^{2+} are not released under identical conditions [30]	Activation by cooperative binding of 2 Ca^{2+} on some internal trigger site. Sr^{2+} and Mn^{2+} are competitive inhibitors with respect to Ca^{2+} [10]
Hyperbolic dependence of the release rate on Ca^{2+} load [29]	Very steep increase in Ca^{2+} efflux rate with increasing Ca^{2+} load [18].
Activation by oxidants of mitochondrial NAD(P)H [24]	Activation by oxidants of mitochondrial NAD(P)H and/or GSH [2]
Inhibition by CSA in concentration <0.5 nmol/mg protein [28]	Complete suppression of ion fluxes attributable to PTP operation by CSA in concentration <0.8 nmol/mg protein [39]
Inhibition by butylated hydroxytoluene (100 nmol/mg protein) [37]	Inhibition by butylated hydroxytoluene and some other free radical scavengers (25–50 nmol/mg protein) [40]
Inhibition by ATP [24]	Inhibition by ADP and ATP [2]
Inhibition by local anaesthetics [38]	Inhibition by local anesthetics [2]

varied experimental conditions regardless of which inducing agent is employed [1,2,36]. Because the same consideration is true with respect to prooxidant-induced Ca^{2+} efflux [1,2,24], it allows these transport systems to be qualitatively compared regardless of differences in experimental conditions.

The profound similarity of the inhibition profiles of the PTP and the prooxidant-induced Ca^{2+} efflux mechanism have led to continuous debates regarding whether or not mechanisms underlying these Ca^{2+} efflux pathways are identical. All things considered, we think that the answer is yes because the existing controversy about the pathways of prooxidant-induced mitochondrial Ca^{2+} release may be resolved by the following hypothesis:

a. The PTP can operate in the two open states: (1) a state permeable only to H^+; or (2) a state permeable to sucrose and other solutes of $M_r < 1500$, including H^+ and Ca^{2+}. Both H^+-selective and nonselective states of the PTP are Ca^{2+}-dependent. Decrease in matrix $[Ca^{2+}]$ shifts the PTP into closed state.

b. Transition of the PTP from the closed state to the sucrose-permeable state occurs through the H^+-selective state as an intermediate step. Dissipation of $\Delta\Psi$ resulting from activation of the H^+-selective state triggers transition of the pore into the sucrose-permeable state [41]. However, $\Delta\Psi$ is only one of the factors modulating the pore activity, and simply dissipating mitochondrial protonmotive force by addition of uncoupler is not sufficient to promote permeabilization [41–44]; the concentration of endogenous pore inhibitors (e.g., NAD(P)H, Mg^{2+}, ADP, H^+, etc.) must also be sufficiently decreased, and/or the concentration of $\Delta\Psi$ modulators (i.e., free fatty acids, acyl-CoA, lysophospholipids, Ca^{2+}) must be increased. Therefore, matrix concentrations of pore effectors will determine the probability of subsequent transition of the H^+ selective state into the nonselective state. Under appropriate conditions, these two open states of the pore could be completely dissociated.

c. Oxidation of redox couples (NAD(P)H/NAD(P), GSH/GSSG) of mitochondria loaded with Ca^{2+} converts the closed pore into a form permeable to H^+ only. Induction of the H^+-permeable pore in an individual mitochondrion leads to collapse of $\Delta\Psi$ below the critical value of 110–130 mV and allows the uniporter-mediated discharge of accumulated Ca^{2+} [44,45]. Since the pore conversion to sucrose-permeable form can be controlled independently of the absolute value of $\Delta\Psi$ by the matrix concentration of physiological effectors [46], under appropriate conditions, Ca^{2+} release by reverse uniport following dissipation of $\Delta\Psi$ does not lead immediately to further conversion of the PTP into nonselective form within any given mitochondrion.

d. Because both forms of the PTP are Ca^{2+} dependent, the lower matrix Ca^{2+} level in H^+-permeable mitochondria then leads to inactivation of the H^+-permeable form of the PTP. This, in turn, results in restoration of $\Delta\Psi$ and Ca^{2+} reuptake. A dynamic interconversion of the H^+-permeable and H^+-impermeable forms of the pore then occurs as Ca^{2+} is reaccumulated and lost. When Ca^{2+} reuptake is prevented by ruthenium red or EGTA, each mitochondrion would release Ca^{2+}, recouple, and remain recoupled (and generate high $\Delta\Psi$) since decrease in matrix $[Ca^{2+}]$ would result in dissociation of Ca^{2+} from the trigger site and PTP inactivation. In this case the apparent maintenance of $\Delta\Psi$ in the entire mitochondrial population as measured by TPP^+ redistribution is deceptive and would reflect the number of mitochondria in energized state at any point in time [1,2].

e. The H^+-permeable pore may be further converted to the sucrose-permeable state at a slower rate, perhaps due to loss of regulatory factors (e.g., adenine nucleotides via Ca^{2+}-regulated ATP-Mg^{2+}/P_i carrier [47]) that modulate the affinity of the pore for Ca^{2+} and/or to depression of $\Delta\Psi$ due to continuous cycling of Ca^{2+} between de-energizing and re-energizing mitochondria.

f. Accumulating evidence indicates that it is inappropriate to model the regulation of the PTP around the action of single activators or inhibitors. Rather, the PTP appears to be modulated through the combined action of diverse effectors which presumably act at a number of regulatory binding sites. These are *peptidel protein* binding site (s) [48]; benzodiazepine receptor [49]; multiple drug receptor [50]; adenine nucleotide binding sites that relay changes in cytosolic ATP/ADP ratio to mitochondrial enzymes and porters [51,52]; redox sensor [53]; cyclosporin A binding site (s) that is believed to be identical to mitochondrial cyclophilin [2,12,13]; and polyvalent cation (Ca^{2+}, Mg^{2+}, La^{3+}, and H^+) binding site of the pore that is a mitochondrial sensor for $Ca^{2+}/Mg^{2+}/H^+$ ratio in cytosolic and mitochondrial compartments [10]. Given the complex nature of PTP regulation, it is anticipated that stabilization of the PTP in a given permeability state can be achieved at multifarious combinations of the PTP regulators. Conversely, at two equal conditions (i.e., $[Ca^{2+}]$ and the extent of oxidation of redox sensor site) redis-

tribution of different permeability states of the pore would depend on the concentration of a third regulator. With such reservations in mind, it is possible to explain an apparent paradox why oxidation of redox couples of rat liver mitochondria converts the pore into a form permeable to H^+ only, while in beef heart mitochondria the PTP opens to H^+ only when the trigger is high Ca^{2+} and opens completely when the trigger is low Ca^{2+} plus oxidant, e.g., the reverse situation is taking place [17]. The basis for these polar effects of the PTP activators in beef heart vs. rat liver mitochondria is still to be established.

EVIDENCE IN FAVOR OF THE HYPOTHESIS

a. The strict exchange between 2 proton and 1 Ca^{2+} at the efflux carrier is obligatory for the model of "prooxidant-induced $Ca^{2+}/2H^+$ exchanger". The exchange of 1 Ca^{2+} to 2 H^+ was reported in earlier studies [25]. Subsequently, it has been shown that Ca^{2+}/nH^+ exchange stoichiometry varies with the pH of the incubation medium, and that no significant variations of Ca^{2+} efflux occurs when medium pH or ΔpH is varied [1,2]. Recently, modulation of ruthenium red-insensitive Ca^{2+} efflux by ΔpH was reported by Rottenberg and Marbach ([20], but see [2] for further discussion of this contention). However, this Ca^{2+} efflux mechanism was CSA-insensitive and, therefore, could not be attributed to prooxidant-induced $Ca^{2+}/2H^+$ exchange. This evidence argues against direct obligatory linkage of 1 Ca^{2+} and 2 H^+ fluxes in prooxidant-induced Ca^{2+} release, and indicates that these cations are transported via separate pathways. In the framework of our hypothesis Ca^{2+} moves via reversal of Ca^{2+} uniporter due to deenergization, and protons cross the membrane via the PTP operating in the H^+-selective state.

b. It is now widely accepted that P_i plus Ca^{2+}-induced increase in the permeability of the inner membrane results from PTP opening [1,2,27]. In this case, the loss of ΔpH nearly parallels the development of nonspecific permeability. However, Crompton *et al.* [39] have shown that a CSA-sensitive, H^+-specific permeability increase in liver mitochondria can be completely separated from Ca^{2+} plus P_i-induced nonspecific permeability changes. It seems likely that in liver mitochondria, P_i accelerates conversion of the H^+-permeable form of PTP to the sucrose-permeable form, resulting in overlapping changes in specific and nonspecific permeability. Recently separation of CSA-sensitive H^+-specific and CSA-sensitive nonspecific permeability changes was reported in beef heart mitochondria [17].

c. The mitochondrial megachannel, a plausible counterpart of PTP in patch-clamp experiments, has a maximum conductance of about 1.3 nS and a multitude of conductance substates [12,13,]. Half-maximum conductance steps, as well as of 100 pS and about 200 pS, were frequently observed as isolated events, especially as closures at positive potentials [12,13]. It reveals the existence of a molecular basis for operation of the pore in two states with different selectivities observed in intact mitochondria, e.g., the small-conductance substate of MCC, presumably 100 or 200 pS, corresponding to the H^+-state of the pore, and the maximum-conductance state corresponding to fully opened pore.

d. It has been proposed that PTP opening is secondary to prooxidant-induced Ca^{2+} cycling arising from continuous operation of Ca^{2+} uptake (ruthenium red-sensitive uniporter) and release ($Ca^{2+}/2H^+$ exchanger) pathways [24,27]. According to this model, CSA and some other pore inhibitors are seen as acting on $Ca^{2+}/2H^+$ exchange to interrupt Ca^{2+} cycling. The arrest of Ca^{2+} cycling, in turn, prevents the PTP opening. Thus, this model implies that the PTP and Ca^{2+}/H^+ exchange have different inhibition profile, and inhibitors of permeabilization do not affect the PTP directly. However, direct inhibition of PTP activity by CSA, amiodarone, ADP, and Mg^{2+} shown in recent patch-clamp experiments [12,13], in which the endogenous PTP effectors are presumed to have been completely removed, argues against this proposal, and indicates that inhibition of prooxidant-induced Ca^{2+} efflux by these compounds can be explained by suppression of the H^+-permeable form of the PTP within the context of the "reverse uniport" mechanism.

e. The PTP has been shown to possess a binding site that can nonselectively accept a variety of ligands, including butylated hydroxytoluene and local anesthetics, that inhibit the pore activity over the concentration range of 5–100 μM [49,50]. The finding that several compounds of dissimilar structure have qualitatively the same potency as inhibitors of the PTP and prooxidant-induced Ca^{2+} release is evidence that both pathways involve a common structure (according to our hypothesis the H^+-permeable form of the PTP), and is in line with the reported inhibitory profile of the PTP. However, given the structural alterations that might be associated with a change in permeability from a proton channel to the PTP, one might hypothesize that these

conformational rearrangements could affect binding affinity for the PTP modulators. In agreement with this idea, recent studies by Crompton and co-workers [39] have shown that low concentration of CSA prevents the PTP-mediated sucrose permeation into the matrix space but is without effect on membrane depolarization attributed to increased H^+ conductance. Further increase in CSA concentration prevents the development of H^+-specific conductance as would be expected if the structural alterations of the channel associated with transition from a H^+-selective form to the nonselective form changes binding affinity for this inhibitor.

DISCUSSION

The model we propose accounts for the common sensitivities of the prooxidant-induced Ca^{2+}-specific pathway and the PTP to a set of inhibitors and activators without implicating an unidentified Ca^{2+}-release carrier distinct from the well-established porters—electrogenic Ca^{2+}-uniporter and the PTP. Despite the fact that PTP and the prooxidant-induced Ca^{2+} release pathway share similar inhibition profile, more work is required to clarify the molecular mechanisms underlying the pathways for fast release of Ca^{2+}. In this respect, analysis of the structure–activity relationship of drugs of different pharmacological classes inhibiting the PTP-mediated solute transport and the prooxidant-induced Ca^{2+} release should provide a useful tool for further identifying the nature of these processes.

Despite the fact that many of the effects of mitochondrial ion-transport processes, with the exception of CSA, would be expected to act nonspecifically (i.e., local anesthetics) in the inhibiting activity of dissimilar ion porters, comparison of the relative potency of the ligands on the PTP-mediated transport and on the prooxidant-induced Ca^{2+} release under defined conditions allows one to overcome this problem. This approach has been proven useful in defining the nature of mitochondrial ion porters embedded into the inner membrane [54]. However, it would be difficult to confirm or refute unequivocally the hypothesis regarding the nature of the prooxidant-induced Ca^{2+} release without reconstitution of purified component(s) of the system of interest into the planar bilayer, or into proteoliposomes, and subsequent comparison of its electrophysiological properties and inhibition profile with characteristics ofthe ion porters in intact mitochondria. Recent purification of a functionally competent complex of three mitochondrial proteins (the inner membrane adenine nucleotide carrier, the outer membrane voltage-dependent anion channel (VDAC), and an 18-kDa protein of the outer membrane) displaying ion channel activity similar to the PTP seems to be the first step in this direction [55].

There is growing evidence that PTP opening makes an important contribution to the development of pathological processes associated with oxidative stress and toxemia [4–8]. In contrast, the induction of the H^+-permeable state of PTP could be a part of the physiological mechanism of Ca^{2+} release from mitochondria in intact cells. This is in line with the observations that CSA: (a) retards irreversible cell injury under oxidative stress [4–8], and (b) stimulates the selective accumulation of Ca^{2+} by mitochondria in apparently intact heart cells [56] and hepatocytes [7]. The long-term accumulation of Ca^{2+} by mitochondria associated with inactivation of the H^+-form of the PTP by CSA may account for the mechanism of cell toxicity of this widely employed immunosuppressant.

In the framework of our hypothesis the PTP is discussed as an individual component of the inner membrane distinct from other mitochondrial porters. Although this point of view prevails at the moment, our recent experiments suggest that the pore formation may relate to the malfunction of mitochondrial protein import mechanism; part of this mechanism could be converted into the large nonspecific channel under pathological conditions [48].

Regardless of the nature of the pore-forming components, an ideal protector against cell injury associated with disregulation of PTP activity is seen as blocking the transition of the pore from the H^+ permeable to the nonselectively permeable state. In this respect, discovery of the mitochondrial multiple drug receptor (MMDR) that readily binds drugs from different chemical and pharmacological classes with significant consequences to PTP activity [50] is of a special interest because it offers a suitable target for pharmacological interventions designed to correct abnormalities associated with disregulation of mitochondrial ion homeostasis observed under oxidative stress, toxemia, apoptosis, and malignant transformation.

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