Modulation of the Mitochondrial Permeability Transition Pore

EFFECT OF PROTONS AND DIVALENT CATIONS

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We have studied the induction of the mitochondrial cyclosporin A-sensitive permeability transition pore (PTP) by the bifunctional SH group reagent phenylarsine oxide (PhAsO). Addition of nanomolar concentrations of the electroneutral H+-K+ ionophore nigericin to nonrespiring mitochondria in sucrose medium determines a dramatic increase of the time required for PTP induction by PhAsO, while no effect of nigericin is apparent in KCl medium. Using mitochondria loaded with the internal pH indicator 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein, we show that the effect of nigericin is mediated by the ionophore-induced acidification of matrix pH. Indeed, experimental manipulation of pH, by a number of treatments indicates that PTP induction is directly related to matrix pH, in that the PTP induction process becomes slower as pH decreases at constant pH.

PTP induction by PhAsO in respiration-inhibited mitochondria is stimulated by Ca2+ and inhibited by a series of divalent cations. Since PhAsO induces the PTP even in the presence of excess EGTA and in the absence of respiration (Lenartowicz, E., Bernardi, P., and Azzone, G. F. (1991) J. Bioenerg. Biomembr. 23, 679–688), we have been able to study the Ca2+ dependence of the induction process. We show that the apparent K_m for Ca2+ activation is about 10^-6 M and that Ca2+, cyclosporin A, and inhibitory Me2+ ions behave as if they were competing for the same binding site(s) on the pore. Since similar results are obtained from patch-clamp experiments on the mitochondrial megachannel (Szabo, I., Bernardi, P., and Zoratti, M. (1992) J. Biol. Chem. 267, 2940–2946), we suggest that (i) the PTP and the mitochondrial megachannel are the same molecular structures and (ii) the same factors affect both the process of pore induction and its open-closed orientation.

Energy conservation by a chemiosmotic mechanism implies a very low permeability of the inner mitochondrial membrane to ions and solutes, allowing the buildup of a proton electrochemical gradient (\(\Delta G^*_H\)) of 200 mV that can be used for ATP synthesis and carrier-mediated ion and metabolite transport (1, 2). This has major implications for mitochondrial volume homeostasis, which is maintained by a tight control of monovalent cation fluxes via (i) cation influx down the cation electrochemical gradient, driven by the inside-negative membrane potential and mediated by regulated uniports (3–7) and (ii) cation efflux against the cation electrochemical gradient, driven by the pH gradient and mediated by electroneutral H+-K+ and H+-Na+ antiporters (8–10).

Since the beginning of work on oxidative phosphorylation in isolated mitochondria, conditions have been described that cause a Ca2+-dependent increase of mitochondrial permeability to ions and solutes with molecular masses up to 1200 daltons, leading to loss of the homeostatic mechanisms of volume control with matrix swelling and uncoupling of oxidative phosphorylation (11, 12) (see reviews by Saris (13) for early work and by Gunter and Pfeiffer (14) for more recent references). As evidence in favor of Mitchell’s chemiosmotic hypothesis accumulated, the Ca2+-dependent permeability transition has been widely considered as a “damaging” effect that may be more relevant to mitochondrial pathology than to mitochondrial physiology.

Two main mechanisms have been proposed to explain the permeability transition of the inner mitochondrial membrane: (i) opening of an unselective pore (PTP) with a minimum diameter of 2.8 nm (15) regulated by Ca2+ binding on the matrix side of the membrane and reversed by EGTA, as indicated by the pioneering work of Hunter and Haworth (16–20); (ii) induction of permeability defects in the membrane lipid phase due to accumulation of lysophospholipids following activation of phospholipase A2 (21, 22). Since scavengers of oxygen radicals prevent pore induction by oxidative stress (23–25), radical species are likely to be involved as well, possibly via oxidation of critical SH groups in the inner membrane (26–30). Strong support for the pore hypothesis came with the discovery that cyclosporin A inhibits the permeability transition (31–33) but not phospholipase A2 activity (33). However, both pathways may be involved in parallel and/or cooperate in maintaining the PTP in an “open” conformation (14, 34), adding complexity to the interpretation of a phenomenon that in the presence of Ca2+ can be induced by over 40 unrelated compounds (14). Since the high affinity inhibitor cyclosporin A allows for the first time unambiguous identification of pore operation, we have begun a systematic study aimed at understanding the role of the PTP in mitochondrial physiology and volume regulation through the requirements for pore induction and the effectors of pore modulation.

In this study we show that PTP induction by the bifunctional SH group reagent PhAsO has the following properties: (i) the process of pore induction is dramatically affected by...
matrix pH, in that PTP induction is efficiently prevented when pH falls below 7.0 independently of the agents or conditions used to modulate pH; (ii) PTP induction is stimulated by Ca\(^{2+}\) with an apparent \(K_m\) of \(10^{-6}\) M and competitively inhibited by several Me\(^{2+}\) ions with \(K_i\) values ranging between \(2 \times 10^{-5}\) and \(2 \times 10^{-4}\) M; (iii) Ca\(^{2+}\) counteracts cyclosporin A inhibition, suggesting that Ca\(^{2+}\) binding can influence the affinity of the cyclosporin A site for its ligand. Since similar results are obtained in patch-clamp experiments on modulation of MMC activity (35), we suggest that (i) the PTP and the MMC are the same molecular species, as indicated earlier from patch-clamp studies of MMC sensitivity to cyclosporin A (36); and (ii) the same factors affect both the process of pore induction and its open-closed probability. A portion of this work has already been presented in abstract form (37).

**MATERIALS AND METHODS**

Preparation of mitochondria and measurements of oxygen consumption were performed as described previously (38). The kinetics of matrix swelling caused by water fluxes was determined by following the changes in absorbance at 540 nm with either a Perkin-Elmer lambda 5 spectrophotometer or with a Hewlett-Packard 8452A diode array spectrophotometer. Tedeschi and Harris (39) have shown that rat liver mitochondria undergo osmotically induced swelling-shrinkage cycles in accordance with the Boyle-van’t Hoff equation and the reciprocal relation between mitochondrial volume and reciprocal of osmolarity. Massari et al. (40) then showed that a linear relation could also be obtained between average mitochondrial volume and the reciprocal of absorbance, provided that the protein concentration did not exceed 1 mg x ml\(^{-1}\). It is then possible to calculate the initial rate of water flux toward the matrix from the slope of the tangent to the absorbance change trace.

\[ V = a \text{ mg protein/Abs} \times \Delta \text{Abs/}\Delta t \]

where \(V\) is the rate of water translocation, \(a\) is the slope of the plot of volume/mg of protein versus mg of protein/absorbance, \(\Delta \text{Abs}\) is the initial absorbance of the mitochondrial suspension, and \(\Delta \text{Abs/}\Delta t\) is the absorbance change during the time interval \(\Delta t\). The rates and amounts of water translocation can then be converted into rates and amounts of solute translocation as described (40). Since for any set of swelling measurements Abs and mg of protein were constant, we have expressed rates as absorbance changes rather than solute fluxes for the sake of simplicity.

For loading with BCECF, 12.5 mg of mitochondria were incubated with 5 \(\mu\)g of BCECF in 0.5 ml of 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 0.1 mM EGTA-Tris (final volume 0.5 ml at room temperature). After 20 min in the dark, mitochondria were spun at 13,000 \(\times\) g at 4 °C, washed once with ice-cold medium without BCECF, and finally resuspended in 0.5 ml of 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 0.1 mM EGTA and stored on ice. BCECF fluorescence was followed with a Perkin-Elmer 650-40 spectrophotometer (excitation wavelength, 500 nm with 2-nm slit and emission wavelength, 525 nm with 5-nm slit). BCECF-loaded mitochondria (0.4 mg in a final volume of 2 ml) were added to a cuvette thermostatted at 25 °C (incubation conditions are specified in the figure legends). Calibration of the fluorescence signal with intramitochondrial pH was achieved by incubating BCECF-loaded mitochondria in 100 mM KCl, 30 \(\mu\)M EGTA-Tris, 2 \(\mu\)M rotenone, 0.8 \(\mu\)M etidium bromide, 5 \(\times\) 10\(^{-4}\) M carbonyl cyanide-m-trifluoromethoxyphenylhydrazone, 0.25 \(\mu\)M valinomycin, and 10 mM Tris-Mops buffer adjusted to final pH values ranging between 6.5 and 8.0. When fluorescence intensity was plotted against extramitochondrial pH (measured with a glass electrode) a straight line was obtained (cf. also Refs. 41 and 42), and this was used to convert fluorescence readings to pH values. This method rests on the consideration that in energized mitochondria freely permeable to K\(^+\) and H\(^+\) in KCl medium \(pH_e = p_{H+}\), as indicated by the study of Jung and Brierley (41). Incubation conditions are specified in the figure legends. Adventitious plus mitochondrial Ca\(^{2+}\) (as determined by atomic absorption spectroscopy on the supernatants obtained from mitochondria centrifuged at 14,000 \(\times\) g for 10 min at 4 °C while the PTP had been induced) were taken into account in calculating the final Ca\(^{2+}\) concentrations. All chemicals were of the highest purity commercially available, while cyclosporin A was a generous gift of Sandoz Pharma AG (Basel). Under all conditions and treatments that lead to matrix swelling referred to as “PTP induction” in the present paper it was ascertained that cyclosporin A had a fully inhibitory effect.

**RESULTS**

**Modulation of the PTP by Matrix Protons**—Fig. 1 shows an experiment in which the PTP was induced by the addition of PhAsO to nonrespiring mitochondria incubated at pH 7.4 in an isotonic, sucrose-based medium containing 5 \(\mu\)M Ca\(^{2+}\). After a lag of about 4 min, a phase of absorbance decrease ensued, reflecting sucrose diffusion into the matrix of mitochondria that have undergone the permeability transition (14). Available evidence indicates that PTP opening for the individual mitochondrion is an all-or-nothing event (18) and that sucrose equilibration in permeabilized mitochondria takes place with a t\(_{1/2}\) of 860 ms (43). Thus, the absorbance decrease induced by PhAsO reflects the contribution of increasing numbers of fully permeabilized mitochondria rather than the slow diffusion of sucrose in a synchronized population. In this paper we therefore consider the absorbance decrease as a measure of the rate of permeabilization (see also “Discussion”). Fig. 1 also shows that a pulse of HCl, shifting the medium pH to 6.8, caused an immediate inhibition of the permeabilization process, which could be reversed by a pulse of Tris base restoring the initial pH value of 7.4. The experiment confirms that H\(^+\) ions exert an inhibitory effect on the PTP, as first reported by Haworth and Hunter (18), and prompted us to investigate whether the inhibitory site is on the matrix or on the cytoplasmic side of the pore.

Fig. 2 illustrates the effect of the electroneutral H\(^+\)-K\(^+\) ionophore nigericin on induction of the PTP by PhAsO in nonrespiring mitochondria incubated in sucrose or KCl media. In sucrose medium (Fig. 2A) addition of nigericin caused a small absorbance increase, due to the volume contraction following depletion of matrix K\(^+\) in exchange for medium H\(^+\). Interestingly, as little as 0.7 nm nigericin delayed the onset of PhAsO-induced, PTP-mediated permeabilization, and the effect was maximal at about 3 nm nigericin. On the other hand, up to 25 nm nigericin had a very minor effect on the time of induction in KCl medium, while the rate of permeabilization was somewhat stimulated rather than inhibited (Fig. 2B). The experiment suggests that the nigericin-induced delay of the permeabilization response to PhAsO is not due to an effect of nigericin per se but is rather related to the nigericin-induced modifications of existing ionic gradients. Since in sucrose (but not in KCl) medium nigericin catalyzes net K\(^+\) efflux in exchange for H\(^+\) with matrix acidification, we addressed the question of whether matrix pH affects the process of PTP induction.

![Fig. 1. pH-dependent changes of mitochondrial permeabilization induced by phenylarsine oxide.](image-url)
point is strongly supported by additional experiments where constitutive H+-Na+ antiporter for PTP induction, which was considerably shortened already if protons inhibit the process of pore induction, the more acidic is the initial pH, the longer should the lag phase elapse between PhAsO addition and onset of permeabilization. The rationale is that if matrix addition with the lag phase elapsing between PhAsO addition and induction of the PTP, in minutes.

FIG. 2. Effect of nigericin on mitochondrial permeabilization induced by phenylarsine oxide in sucrose and KCl media. Experimental conditions were as in Fig. 1, except that the Tris-Mops buffer was 10 mM, and in traces (B) the sucrose was replaced with 0.1 M KCl. Where indicated, the concentrations of nigericin (Nig) labeling each trace were added, followed by 25 μM PhAsO. In the dashed traces, no nigericin was added. The initial absorbance readings in A and B were identical.

To monitor the variations of matrix pH we have used mitochondria loaded with the internal pH indicator, BCECF. Preliminary experiments, not shown here, indicated that the basic features of PTP activation (inducing agents, Ca2+ stimulation, cyclosporin A sensitivity) were not affected by BCECF. On the other hand, since PTP opening leads to BCECF release, we have correlated the steady-state pH value (from BCECF fluorescence readings) at the moment of PhAsO addition with the lag phase elapsing between PhAsO addition and onset of permeabilization. The rationale is that if matrix protons inhibit the process of pore induction, the more acidic is the initial pH, the longer should the lag phase be.

Nonrespiring heart mitochondria in sucrose medium maintain an acidic internal pH related to the Donnan potential (41). Fig. 3 shows that nonrespiring liver mitochondria incubated in isotonic sucrose maintain a pHi of about 6.5. As sucrose was replaced by increasing concentrations of Na+, pHi increased up to a value of 7.5 due to operation of the constitutive H+-Na+ antiporter (42, 44). A parallel measurement of PTP induction by PhAsO revealed that increasing concentrations of Na+ had a dramatic effect on the lag phase required for PTP induction, which was considerably shortened already at 10 mM Na+ (Fig. 3). The experiment suggests that the effect of Na+ may be mediated by the changes of pHi. This point is strongly supported by additional experiments where matrix pH was modified by different treatments: (i) addition of increasing concentrations of NH3; instead of Na+, to nonrespiring mitochondria; NH3 diffusion into the matrix is favored by an acidic pHi, since NH3 protonation traps the impermanent NH3 species inside the matrix with a pH shift toward more alkaline values; (ii) addition of succinate, causing H+ ejection on the redox H+/pumps coupled to electron flow; in this case, pHi increases because of the net H+ translocation, since H+ backflow toward the matrix via the lipid bilayer (the H+ leak) is much slower than H+ ejection via the H+ pumps; (iii) addition of malonate to mitochondria oxidizing succinate; malonate is a competitive inhibitor of succinate dehydrogenase and therefore slows down succinate oxidation without affecting the H+ leaks; this in turn lowers the H+ pump/H+ leak ratio and therefore the steady state matrix pH of respiring mitochondria. Data collected from these experiments with several preparations of mitochondria are shown in Fig. 4, which correlates the pHi values with the lag phase required for induction of the PTP. Irrespective of the method used to modify internal pHi, all of the data could be fitted by a single line indicating that pore induction is very effectively prevented as pHi falls below about 7.0. It is important to stress that these measurements must be performed in the presence of excess EGTA. Indeed, Ca2+ uptake driven either by respiration or by the H+ diffusion potential when pHi is lower than pHi (45) would partially counteract H+ inhibition.

Modulation of the PTP by Divalent Cations—It is widely believed that the permeability transition has a mandatory requirement for Ca2+ (14). Since the regulatory site(s) is internal (14), it has been difficult to correlate the mitochondrial Ca2+ load with the actual matrix Ca2+ concentration and therefore the affinity of Ca2+ for its putative regulatory site. Since PhAsO is able to induce the PTP even in the presence of excess EGTA in nonrespiring mitochondria (30), this inducer in principle allows the determination of the Ca2+ dependence of pore induction. Indeed, little or no Ca2+ concentration gradient is maintained by deenergized mitochondria since Ca2+ equilibrates between the two sides of the membrane.

FIG. 3. Effect of Na+ on intramitochondrial pH and on the induction time of mitochondrial permeabilization induced by phenylarsine oxide. The incubation medium contained the indicated concentrations of NaCl plus sucrose to give 0.2 osmolar, 10 mM Tris-Mops, pH 7.4, 0.5 mM EGTA-Tris, and 2 μM rotenone; final volume, 2 ml; 25 °C. The experiments were started by the addition of 0.4 mg of BCECF-loaded rat liver mitochondria. Filled symbols, matrix pH; open symbols, time elapsing between the addition of 30 μM phenylarsine oxide and induction of the PTP, in minutes.

FIG. 4. Correlation between intramitochondrial pH and induction time of mitochondrial permeabilization induced by phenylarsine oxide. The incubation medium contained 10 mM Tris-Mops, pH 7.4, 0.5 mM EGTA-Tris, 2 μM rotenone, and sucrose (squares), sucrose plus NaCl (circles), or sucrose plus (NH4)2SO4 (triangles). The Na+ or NH3 concentration was varied between 10 and 100 mM, with a proportional decrease of the sucrose concentration to maintain the sucrose plus salt osmolarity to 0.2 osmolar. In two determinations in sucrose medium, 1 mM succinate (diamond) and 1 mM succinate plus 1 mM malonate (asterisk) were added. Open, filled, or partially filled symbols refer to independent determinations on different batches of BCECF-loaded mitochondria. Values on the ordinate refer to the time elapsing between addition of 50 μM phenylarsine oxide and induction of the PTP as a function of matrix pH.
via the Ca\(^{2+}\) uniporter. Fig. 5 shows a double-reciprocal plot of the rate of PhAsO-induced absorbance decrease as a function of the Ca\(^{2+}\) concentration. In the range of [Ca\(^{2+}\)] considered a linear correlation was obtained, allowing extrapolation of an apparent \(K_a\) of about 10\(^{-6}\) M.

Fig. 6 shows an experiment where the permeability transition was induced with PhAsO in nonrespiring mitochondria incubated in the presence of 5 \(\mu\)M Ca\(^{2+}\). When the rate of permeabilization was nearly maximal, addition of Mn\(^{2+}\) caused an immediate inhibition. If excess Ca\(^{2+}\) was now added the process of permeabilization resumed, indicating that mitochondria had undergone a reversible inhibition and suggesting that Ca\(^{2+}\) and Mn\(^{2+}\) may be competing for the same binding site(s). To test this hypothesis we have measured the inhibition of the permeabilization rate by Mn\(^{2+}\) at different Ca\(^{2+}\) concentrations and analyzed the results according to a Dixon plot. As shown by the experiment reported in Fig. 7, inhibition was competitive with a \(K_i\) of about 20 pM. Similar results, not shown here, were obtained with Mg\(^{2+}\) (\(K_i\) 0.2 mM), Ba\(^{2+}\) (\(K_i\) 0.2 mM), and Sr\(^{2+}\) (\(K_i\) 21 \(\mu\)M).

Cyclosporin A is a powerful inhibitor of the PTP (31-33) and of the MMC (36). It was therefore of interest to assess whether the stimulatory effect of Ca\(^{2+}\) can overcome cyclosporin A inhibition, or rather the positive effector (Ca\(^{2+}\)) and the negative effector (cyclosporin A) act by binding to different targets on the inner membrane. We have therefore carried out experiments where the PTP was induced by PhAsO in nonrespiring mitochondria incubated in sucrose media containing 6 or 56 \(\mu\)M Ca\(^{2+}\) in the presence of increasing concentrations of cyclosporin A. Fig. 8 shows a Dixon plot for cyclosporin A inhibition of PTP induction from such an experiment. The data indicate that cyclosporin A inhibits the PTP with a \(K_i\) of 10 nM and that it displays a clear competitive pattern with respect to Ca\(^{2+}\), since the intercept on the abscissa increased 8-fold as the Ca\(^{2+}\) concentration was increased from 6 to 56 \(\mu\)M.
Swelling in sucrose media is a relatively unambiguous indicator of the fact that mitochondria have become permeable to sucrose. If swelling is inhibited by cyclosporin A, one may safely conclude that the permeability increase is mediated by the PTP (14). Although measurements of the swelling rate are commonly used to quantitate this phenomenon, e.g. in inhibitor titrations (33), the kinetics of swelling is complex; addition of the inducer is followed by a lag phase of variable length during which absorbance (i.e. mitochondrial volume) remains constant; only after this lag phase a process of absorbance decrease ensues. The rate of absorbance decrease is variable, but kinetics of several minutes are not uncommon (e.g. Figs. 1 and 6). This pattern suggests that in normal mitochondria the PTP is not functionally present and that the lag phase reflects the time needed to “assemble,” or activate, the PTP. The actual process of absorbance decrease, on the other hand, is open to two interpretations: (i) the rate of absorbance decrease reflects the rate of sucrose diffusion into the matrix of a synchronized population of permeable mitochondria; in this case, changes of the rate of absorbance decrease reflect changes in the probability that the PTP stays in the “open” configuration; (ii) the rate of absorbance decrease reflects the permeabilization of increasing numbers of mitochondria, where for the individual mitochondrion pore opening is an all-or-nothing process with virtually instantaneous sucrose diffusion into the matrix through a pore remaining in a high open probability state; in this case, the rate of absorbance decrease is a measure of how close to each other are the induction times for individual mitochondria: the closer the induction times, the faster the rate of absorbance decrease.

We favor the latter interpretation for the following reasons. (i) A morphological study of mitochondria fixed at various time intervals during the swelling process showed that the mitochondria were either in the condensed or in the swollen state, while intermediate states were never observed; rather than a continuous process, the absorbance changes thus appear to detect the various proportions of mitochondria in the swollen or condensed state (18); (ii) the process of sucrose diffusion into the matrix of permeabilized mitochondria, measured by rapid mixing techniques, has a t/2 of a mere 860 ms (43); (iii) patch-clamp studies on mitoplasts indicate that the MMC (which we identify with the PTP, see below) favors a high open probability state. We therefore think that studies on mitochondrial populations are more suited to understand the factors involved in pore induction, while the modulators of the pore-opened closed probability can be more fruitfully addressed by studies at the single channel level (35).

Modulation of the PTP—Data in this paper show, for the first time, that the process of pore induction is affected by matrix pH. This statement is supported by the finding that, irrespective of whether matrix pH is changed with nigericin, Na+, NH4+, succinate, or malonate, the time required for PTP induction correlates very well with pH (Figs. 3 and 4). Although the mechanism(s) of H+ interference with the induction process remain to be elucidated, it appears that PTP induction is slower at acidic matrix pH values. It must be noted that the acidic matrix pH maintained by nonrespiring mitochondria in sucrose can be dissipated by electrophoretic movement of counterions neutralizing the ΔpH-driven H+ diffusion potential, i.e. by uptake of medium cations and/or by efflux of matrix anions (45). While Ca2+ uptake can be very efficient under these conditions (45), in studies with BCECF, not shown here, we have observed that the nigericin-induced matrix acidification was transient even in the presence of EGTA, presumably because of a slow uptake of Tris (46).

The finding that the process of PTP induction is inhibited when pH falls below 7.0 has several implications. Mitochondria possess a Ca2+ unipporter, which equilibrates Ca2+ with its electrochemical gradient across the inner membrane (see the recent review by Gunter and Pfeiffer (14)). In respiring, coupled mitochondria Ca2+ uptake is accompanied by membrane depolarization (47), H+ ejection (13), and, in the absence of weak acids, by the buildup of an inside-alkaline pH gradient that can be as high as 100 mV (38), corresponding to a pHr > 8.5. Thus, Ca2+ uptake leads to an increase of the matrix Ca2+ concentration and to a decrease of matrix H+. For PTP induction, this means that the inhibitor (H+) is removed while the activator (Ca2+) is added. In 1981 (48) we had already noted that acetate, by preventing the pHr increase following Ca2+ uptake in the presence of N-ethylmaleimide, was able to prevent the increase of mitochondrial permeability and proposed (48) that the alkaline matrix pH, besides N-ethylmaleimide per se, contributed to the Ca2+-dependent permeability increase. The possibility that part of the Ca2+ requirement for pore induction may in fact be a requirement for H+ ejection is being actively investigated. On the other hand, the reason why phosphate has an inducing rather than inhibiting effect on the PTP despite its buffering effect on pH remains an open question.

Using the bifunctional SH group reagent PhAsO, which induces the PTP even in the absence of Ca2+ and respiration (30), we have been able to define the apparent Kd for Ca2+ stimulation of PTP induction in deenergized mitochondria to about 10^-7 M (Fig. 5). Inhibitory Me2+ ions compete with Ca2+ and exhibit Kd values ranging between 2 × 10^-5 and 2 × 10^-4 M. Inhibition of pore induction by cyclosporin A is affected by Ca2+, and higher concentrations of cyclosporin A are required as the Ca2+ concentration is increased (Fig. 8). These data suggest that either the Ca2+-binding site is part of the cyclosporin A-binding site or that Ca2+ binding can indirectly influence the affinity of the neighboring cyclosporin A site for its ligand. Our data are in excellent agreement with a recent study of radiolabeled cyclosporin A binding to mitochondria, where inhibition of pore induction appears to correlate with a low capacity, high affinity site with a Kd of less than 12 nM displaying competition with micromolar Ca2+ concentrations (49).

PTP and MMC—Patch-clamp experiments of the mitochondrial inner membrane have led to the discovery of several conductances (reviewed in Ref. 50). Two channels have been identified so far with this technique: a 107-picoSiemens channel slightly selective for anions (51, 52); and a 1.3-nanoSiemens megachannel, MMC (53, 54). The MMC is of particular relevance here; it is inhibited by cyclosporin A (35) and appears to be formed by units exhibiting a cooperative behavior (37, 54). Data in the companion paper (38) show that the MMC is activated by Ca2+, is competitively inhibited by Me2+ ions, is inhibited below pH 7.0, and cyclosporin A inhibition can be reversed by Ca2+ (35). These data are quite similar to those found here for the process of PTP induction in intact mitochondria, suggesting that (i) the PTP and the MMC are the same molecular species; and (ii) the same factors affect both the process of pore induction and its open-closed configuration.

Patch-clamp experiments of the mitochondrial inner membrane require preparation of mitoplasts by osmotic shock, and incubation in the presence of Ca2+ for a few minutes (between 3 and 15) is needed for seal formation. This leads to: (i) loss of matrix constituents that prevent pore opening in intact
mitochondria via direct interaction with the pore (ADP, Mg\(^2+)\), or by contributing to the establishment of an acidotic matrix pH (diffusible proteins contributing to a Donnan potential), or by as yet unrecognized interactions (e.g. inhibitory proteins); (ii) membrane stretch, that may itself represent an activation signal as is the case for the mitochondrial H\(^+-K^+\) antiporter (55), the mitochondrial Na\(^+\)-independent Ca\(^{2+}\) efflux pathway (56), and for the stretch-activated channels of yeast (57), Xenopus oocytes (58), and bacteria (59). Thus, by all probability induction of the PTP takes place before current recordings are initiated.

Future Directions—Induction of the PTP in vitro leads to loss of mitochondrial energy-linked functions, with collapse of the \(\Delta$$H\) and cessation of ATP synthesis, while substrate oxidation proceeds due to futile H\(^+\) cycling. Volume homeostasis is lost in parallel, and mitochondria undergo colloidosmotic swelling because endogenous transport mechanisms are overcome by solute equilibration through the pore. Thus, PTP induction in vitro may be expected to have dramatic consequences on cell viability, and it has been suggested that inappropriate pore opening during postischemic reperfusion may contribute to tissue damage under these conditions (43). But what is, then, the physiological role of the PTP? We believe that this question can only be answered by a concerted effort along several lines of research, including: (i) a functional characterization of pore induction and of the effectors of pore modulation; (ii) further characterization of the PTP by electrophysiology; (iii) purification of the pore components and reconstitution of pore activity in artificial membranes.

Findings in this and the companion paper (35) may open a new perspective in the isolation of the components of the PTP-MMC, since specific mitochondrial proteins can now be tested for their activity as a high conductance, cyclosporin A-sensitive channel in reconstituted systems with the patch-clamp technique. Studies in this direction are now in progress in our laboratories.

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