Inhibition of the Mitochondrial Permeability Transition by Cyclosporin A during Long Time Frame Experiments: Relationship between Pore Opening and the Activity of Mitochondrial Phospholipases†

Kimberly M. Broekemeier and Douglas R. Pfeiffer*

Department of Medical Biochemistry, College of Medicine, The Ohio State University, Columbus, Ohio 43210

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ABSTRACT: Inhibition of the mitochondrial permeability transition pore by cyclosporin A or trifluoperazine is transient on the time scale of cell injury studies (hours). However, these agents act synergistically and produce long-lasting inhibition when used in combination. The cause of this synergism has been investigated from the perspective of the known action of trifluoperazine as an inhibitor of mitochondrial phospholipase A_2. Free fatty acids, which are phospholipase reaction products, facilitate pore opening in a concentration-dependent manner (I_{50} ~2 nmol/mg of mitochondrial protein). Endogenous and exogenous fatty acids are similarly effective. Fatty acids of differing structure are also similarly effective, but long-chain alcohols and alkanes are ineffective. Free fatty acids accumulate in cyclosporin A-treated mitochondria when Ca^{2+} plus *tert*-butyl hydroperoxide or Ca^{2+} plus *N*-ethylmaleimide is present, but do not accumulate when Ca^{2+} plus inorganic phosphate is present. In the presence of cyclosporin A, bovine serum albumin markedly delays pore opening induced by *tert*-butyl hydroperoxide or *N*-ethylmaleimide, but has little effect on pore opening induced by inorganic phosphate, which is subject to long-lasting inhibition by cyclosporin A without trifluoperazine. Free fatty acid accumulation is thus a factor which limits pore inhibition by cyclosporin A. However, trifluoperazine has no effect on free fatty acid accumulation in intact, cyclosporin-inhibited mitochondria and thus does not act by inhibiting phospholipases. Comparing the actions of free fatty acids, trifluoperazine, long-chain acyl cations, and other effectors on the pore suggests that a more negative membrane surface potential favors pore opening and a more positive potential favors a closed pore. Expected surface potential effects of trifluoperazine can explain the synergism between this compound and cyclosporin A as pore inhibitors. Surface potential may influence the pore through the voltage-sensing element which responds to transmembrane potential. The present data also suggest that long-lived, solute-selective forms of the pore exist when it is opened in the presence of inhibitors. The implications of these findings for pore regulation and for the use of cyclosporin A to identify pore opening as a component of cell injury mechanisms are discussed.

Opening of the mitochondrial permeability transition pore (PTP)[1] is inhibited by low levels of cyclosporin A (CsA) (Fournier et al., 1987; Crompton et al., 1988; Broekemeier et al., 1989; Strzelecki et al., 1988; Novgorodov et al., 1990), possibly as a result of a high-affinity interaction with a matrix space protein (cyclophilin) having peptide bond isomerase activity (Halestrap & Davidson, 1990; McGuinnes et al., 1990).. CsA is the most potent inhibitor of PTP opening identified to date [see Gunter and Pfeiffer (1990) and Gunter et al. (1994) for a review] and, as such, is a powerful tool for investigating the mechanism of the transition and its possible roles in physiological and pathological processes. No physiological function has been identified so far, and, indeed, there are only indirect indications that the PTP actually opens in normal cells. These relate, in part, to the elevated *in situ* mitochondrial Ca^{2+} contents which arise in cardiac myocytes (Altschuld et al., 1992) and hepatocytes (Kass et al., 1992) treated with CsA. There are also reports that radiolabeled mannitol or sucrose administered to intact animals or isolated cells gains access to the mitochondrial matrix (Ostiling & Johanson, 1984; Tolleshaug & Seglen, 1984; Tolleshuag et al., 1985), possibly through an occasional opening of the PTP. On the other hand, fluorescence cell imaging studies have shown that cytoplasmic calcein is excluded from the mitochondrial matrix in hepatocytes for periods of hours, indicating that the PTP remains closed under specific conditions (Nieminen et al., 1995).

Regarding pathological processes, CsA pretreatment is known to improve the survival of animals subjected to surgically-induced hepatic ischemia (Hayashi et al., 1988; Kawano et al., 1989; Kurokawa et al., 1992). Crompton and Costi have proposed that opening of the PTP contributes to reperfusion injury in heart (Crompton & Costi, 1988, 1990). We have shown that CsA protects hepatocytes subjected to *tert*-butyl hydroperoxide under conditions where lipid peroxidation is inhibited by a high medium Ca^{2+} concentration

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* Address correspondence to this author at the Department of Medical Biochemistry, The Ohio State University, 1645 Neil Ave., 310A Hamilton Hall, Columbus, OH 43210-1218. Telephone: (614) 292-8774. Facsimile: (614) 292-4118.

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[1] Abbreviations: BSA, bovine serum albumin; CsA, cyclosporin A; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PTP, permeability transition pore.

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(Broekemeier et al., 1992). This finding indicates that the mechanism of cell killing by *tert*-butyl hydroperoxide can be dissected into peroxidation-dependent and -independent components, as does work by others (Rubin & Farber, 1984; Masaki et al., 1989), and implicates the mitochondrial permeability transition as an important component of the peroxidation-independent mechanism. More recently, a substantial number of publications have described protective effects of CsA on cells subjected to a variety of injurious conditions. Such studies have been conducted on hepatocytes (Kass et al., 1992; Snyder et al., 1992; Pastorino et al., 1993, 1994, 1995; Imberti et al., 1992, 1993), heart/myocytes (Nazareth et al., 1991; Griffiths & Halestrap, 1993, 1995), and other types of cells (Fujii et al., 1994; Botla et al., 1995). Collectively, these reports support a broad involvement of the permeability transition in mechanisms of cell injury and suggest that CsA may have potential as a pharmacological agent, useful for reducing the extent of tissue damage produced by a range of injurious conditions [see Gunter and Pfeiffer (1990), Crompton and Andreeva (1993), Gunter et al. (1994), Halestrap (1994), and Zoratti and Szabó (1995) for further review].

To fully interpret the effects of CsA on mechanisms of cell injury and to maximize the value of this inhibitor for revealing the mechanism of the transition *per se*, knowledge of the persistence of CsA action as an inhibitor of the PTP is required. This requirement seems pressing because cell injury studies are normally conducted on time frames of a few hours, whereas we have shown that the inhibitory action of CsA on PTP opening in isolated mitochondria is gradually lost as the time frame exceeds ~15 min (Broekemeier & Pfeiffer, 1989). Our initial report was restricted to PTP opening induced by Ca^{2+} plus *tert*-butyl hydroperoxide, however, which is not sufficient to allow general evaluation of the persistence of the CsA inhibitory action.

In this report, we describe the effectiveness of CsA as a long time frame inhibitor of pore opening, when it is induced by a wide range of conditions. The relative effectiveness of trifluoperazine used alone and together with CsA is also evaluated. Finally, the role of phospholipolysis in limiting the duration of the CsA action is examined, and apparent solute-selective forms of the PTP are demonstrated. Aspects of these findings have appeared in preliminary forms (Broekemeier & Pfeiffer, 1993; Bernardi et al., 1994).

EXPERIMENTAL PROCEDURES

Reagents. Common chemicals were obtained from commercial sources and were reagent grade or better. Crude ruthenium red was purchased from Sigma and used as provided because purification removes the primary component which inhibits the mitochondrial Ca^{2+} uniporter (Broekemeier et al., 1994). CsA was a gift from Dr. David L. Winter of the Sandoz Research Institute, East Hanover, NJ, whereas the hypolipidemic agent WY-14643 was a gift from Dr. Wolfgang Bauman of the Hormel Institute. Stock solutions of the latter compounds and others having poor water solubility were prepared in ethanol. The total ethanol concentration was kept at <1% by volume in all mitochondrial incubations.

Preparation and Incubation of Mitochondria. Liver mitochondria were prepared from male Sprague-Dawley rats which weighed approximately 250 g, using procedures described previously (Broekemeier et al., 1985). EGTA (0.5 mM) and bovine serum albumin (2 mg/mL) were present in the homogenizing medium but were omitted from the washing medium which contained 230 mM mannitol, 70 mM sucrose, and 3 mM Hepes (Na^+), pH 7.4. The final pellets were suspended at approximately 60 mg of protein/mL in washing media and were maintained on ice until use. Protein concentration was determined by the Biuret reaction in the presence of 1% deoxycholate (Na^+).

All incubations were conducted at 25 °C and 1.0 mg of protein/mL, in media which contained 10 mM succinate (Na^+) plus rotenone at 0.5 nmol/mg of protein and 3 mM Hepes (Na^+), pH 7.4, plus sufficient mannitol−sucrose (3:1 mole ratio) to give a total osmotic strength of 300 mOsM. When utilized, CsA, trifluoperazine, or other inhibitors of PTP opening were added to the incubations immediately following mitochondria. During long time frame incubations, vessels providing a substantial surface to volume ratio were employed, together with stirring, to maintain oxygen availability during the experiments. A continuing oxygen supply was indicated by retention of accumulated Ca^{2+}, or in some cases by measurements made with an oxygen electrode.

Some of the conclusions from this work are arrived at by comparing the time course of PTP opening under different conditions, with the individual incubations lasting for 1−3 h. In these experiments, all relevant incubations were conducted in parallel, rather than sequentially, because PTP opening occurs more readily as a mitochondrial preparation ages (e.g., Riley & Pfeiffer, 1985). Experiments of these types were repeated at least 3 times with the data shown being representative.

Indicators of the Permeability Transition and Related Measurements. Release of matrix space K^+, Mg^{2+}, and Ca^{2+} was determined by atomic absorption measurements made on supernatants, following rapid sedimentation of mitochondria in a microcentrifuge (Riley & Pfeiffer, 1985). Centrifugation at 13000*g* was continued for 2 min, whereas sedimentation requires ~20 s (Riley & Pfeiffer, 1985). In addition to cation release, the transition was monitored by swelling, which reflects a high permeability of the inner membrane to mannitol and sucrose. Swelling measurements were made with an Aminco DW2a spectrophotometer (Riley & Pfeiffer, 1985) and were continuous in the case of short time frame experiments (0 to ~20 min). For longer time frame experiments involving parallel incubations, these measurements were made discontinuously using a probe colorimeter (Beavis et al., 1985), by moving the probe from vessel to vessel and recording the readings. Rupture of the mitochondrial inner membrane was assessed by determining the release of malate dehydrogenase activity which was assayed as described by Bergmeyer (1974).

Determination of Free Fatty Acids and Lysophospholipids. Samples of mitochondrial incubations containing 3.0 mg of protein were extracted by a modified Folch technique (Broekemeier et al., 1985). Aliquots of the lower, lipid-containing phase were reacted with diazomethane to yield methyl esters of free fatty acids while leaving the acyl groups of complex lipids undisturbed (Schlenk & Gellerman, 1960). Prior to extraction and reaction with diazomethane, a known amount of heptadecanonic acid (17:0) was added to serve as an internal standard (Pfeiffer et al., 1979). Fatty acid methyl esters were separated from other lipids on silica gel mini-columns and were quantitated by gas−liquid chromatography, utilizing an instrument equipped with a computing

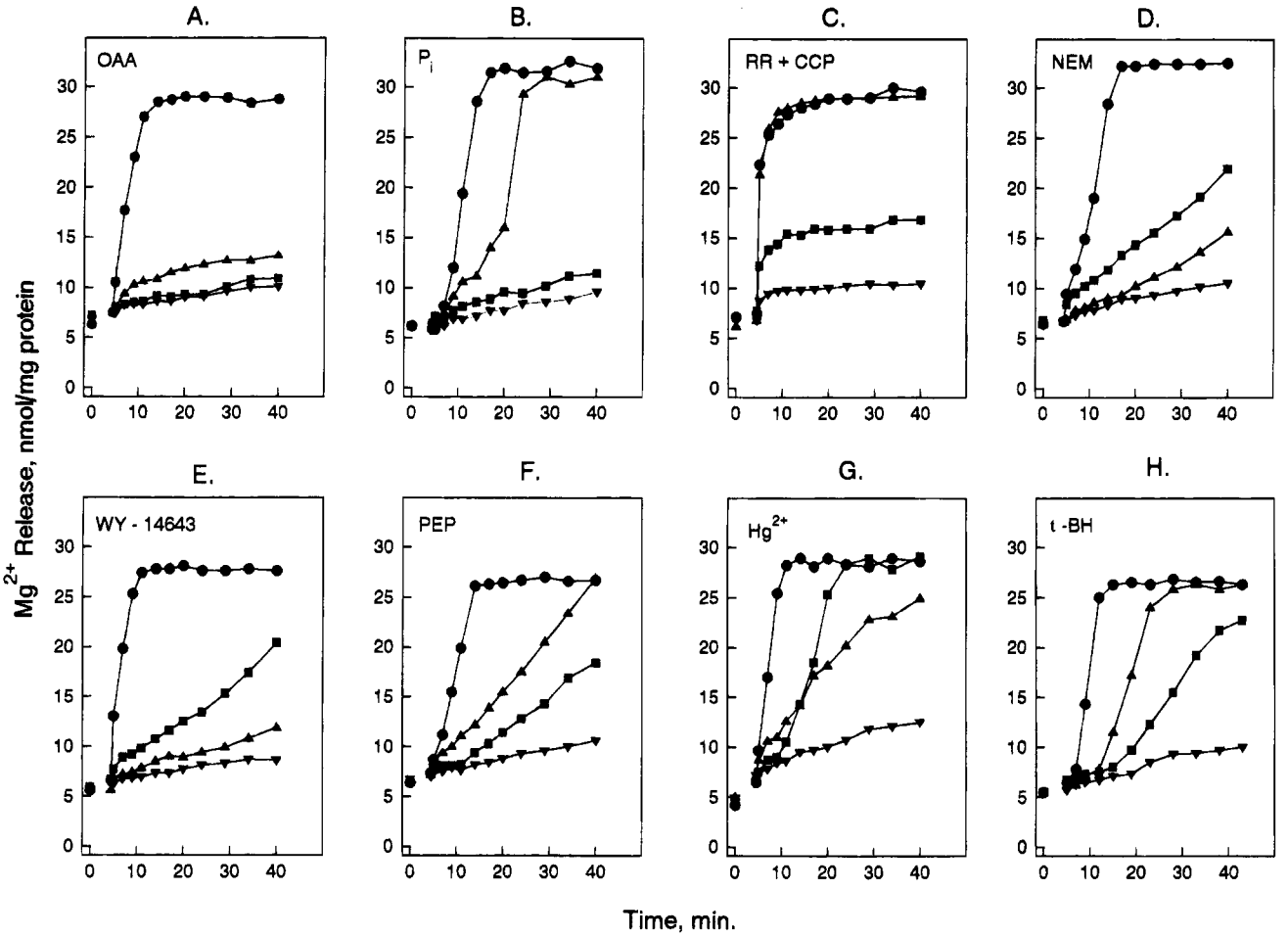

FIGURE 1: Inhibition of the permeability transition by CsA and trifluoperazine; Mg^{2+} release. Mitochondria were incubated and the release of Mg^{2+} was determined as described under Experimental Procedures. $CaCl_2$ (70 nmol/mg of protein) and the various transition-inducing agents were added at 2 and 4 min, respectively. For all panels: (●) no other addition; (■) CsA was present at 0.5 nmol/mg of protein; (▲) trifluoperazine was present at 60 μM; (▼) CsA and trifluoperazine were present at 0.5 nmol/mg of protein and 60 μM, respectively. The inducing agents were as follows: panel A, oxalacetate (2 mM) plus inorganic phosphate (200 μM); panel B, inorganic phosphate (3 mM); panel C, ruthenium red (1 nmol/mg of protein) plus carbonyl cyanide *p*-chlorophenylhydrazone (3 nmol/mg of protein); panel D, *N*-ethylmaleimide (200 μM); panel E, WY-14643 (100 μM); panel F, phosphoenolpyruvate (3 mM) plus inorganic phosphate (200 μM); panel G, $HgCl_2$ (2 nmol/mg of protein) plus dithiothreitol (3 nmol/mg of protein); panel H, *tert*-butyl hydroperoxide (200 μM).

integrator (Broekemeier et al., 1985; Pfeiffer et al., 1979). Peak areas were converted to units of nanomoles per milligram of mitochondrial protein by referring to the area of the internal standard peak and the equivalents of mitochondrial protein represented by the sample (Broekemeier et al., 1985; Pfeiffer et al., 1979).

To quantitate lysophosphatidylethanolamine (LPE), a separate aliquot of the lipid extract lower phase was reacted with fluorescamine to label amino group-containing components with a fluorescent chromophore (Schmid et al., 1981). Labeled LPE was then separated from other labeled components by thin-layer chromatography and quantitated by fluorescence spectroscopy (Schmid et al., 1981). Lysophosphatidylcholine (LPC) was quantitated by a literature procedure (Gurantz et al., 1981; Reers et al., 1984) after separating this component from other choline-containing lipids by thin-layer chromatography. Phospholipase D was employed to release esterified choline, which can then be acted upon by choline oxidase to produce H_2O_2. The latter product subsequently reacts with phenol plus antipyrene, in the presence of peroxidase, to produce a quinone which is determined spectroscopically at 500 nm (Gurantz et al., 1981; Reers et al., 1984).

RESULTS

CsA and Trifluoperazine Act Synergistically as Inhibitors of PTP Opening. Figure 1, which was obtained using isolated mitochondria, shows the effectiveness of CsA as an inhibitor of PTP opening over a time frame which approaches those used in cell injury studies. Mg^{2+} release was taken as the indicator of PTP formation, and the data show that persistence of the CsA effect is a function of the inducing agent employed. With oxalacetate, phosphate, or ruthenium red plus uncoupler (panels A, B, and C, respectively), inhibition is relatively complete and long-lasting. With *N*-ethylmaleimide, WY-14643, or phosphoenolpyruvate (panels D, E, and F, respectively), inhibition is substantial, with Mg^{2+} release limited to approximately 50% during the 40 min incubation period investigated. Hg^{2+} and *tert*-butyl hydroperoxide (panels G and H, respectively) cause complete release of Mg^{2+} in the presence of CsA, following an initial period of 10−15 min, during which inhibition is relatively complete.

The effectiveness of trifluoperazine as an inhibitor of PTP formation is also time-dependent in a manner which varies with the inducing agent employed. In some cases, trifluoperazine is more effective than CsA (*N*-ethylmaleimide, Hg^{2+}, WY 14643); in some cases, it is less or similarly effective

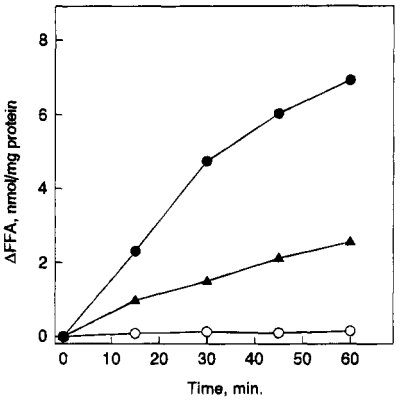

FIGURE 2: Accumulation of free fatty acids in mitochondria incubated in the presence of CsA. Conditions were as described under Experimental Procedures with CsA (0.5 nmol/mg of protein) present from the beginning of the incubation. $CaCl_2$ (70 nmol/mg of protein) was added at 2 min followed at 4 min by *tert*-butyl hydroperoxide (●), 400 μM; *N*-ethylmaleimide (▲), 200 μM; or inorganic phosphate (○), 2 mM. Samples were taken at the indicated times, and free fatty acids were determined as described under Experimental Procedures.

(*tert*-butyl hydroperoxide, oxalacetate, phosphate, phosphoenolpyruvate), whereas with ruthenium red plus uncoupler it is essentially ineffective. Most importantly, when CsA and trifluoperazine are used together, inhibition of PTP formation as judged by Mg^{2+} release is essentially complete for 40 min or longer, regardless of which inducing condition is employed. This apparent additive or synergistic effect of the two inhibitors is seen even though each one, when used alone, was present at a (near) maximally effective concentration (data not shown).

From the group of agents considered in Figure 1, phosphate, *N*-ethylmaleimide, and *tert*-butyl hydroperoxide were selected for further studies on the time dependency of PTP inhibition by CsA, and the synergism between CsA and trifluoperazine in maintaining a closed PTP. These agents were chosen because they are likely to promote PTP opening by different detailed mechanisms (Gunter & Pfeiffer, 1990) and because they bracket the range of the behaviors which is seen in the larger group.

Hydrolysis of Lipids in Mitochondria Treated with CsA and the Effect of Lipid Hydrolysis Products on PTP Opening. Trifluoperazine inhibits phospholipase A_2 in disrupted mitochondria (Broekemeier et al., 1985), and earlier studies showed that phospholipid hydrolysis products accumulate in mitochondria under conditions which produce the transition [see Gunter and Pfeiffer (1990)]. In view of these relationships, it seemed possible that the time-dependent loss of CsA activity as an inhibitor of PTP opening might actually represent an action of phospholipases exerted upon the membrane lipid phase, to produce PTP-independent pathways for solute movement across the inner membrane (Broekemeier & Pfeiffer, 1989). To determine the nature and extent of phospholipid hydrolysis in CsA-treated mitochondria, we followed the accumulation of free fatty acids and lysophospholipids during hour time frame incubations. When CsA is present, significant amounts of free fatty acids accumulate in mitochondria incubated with Ca^{2+} plus *tert*-butyl hydroperoxide or Ca^{2+} plus *N*-ethylmaleimide; however, no fatty acids accumulate in mitochondria incubated with Ca^{2+} plus phosphate (Figure 2). The free fatty acids arise primarily from phospholipid hydrolysis, as shown by parallel determinations of LPE and LPC, which gave summed values that

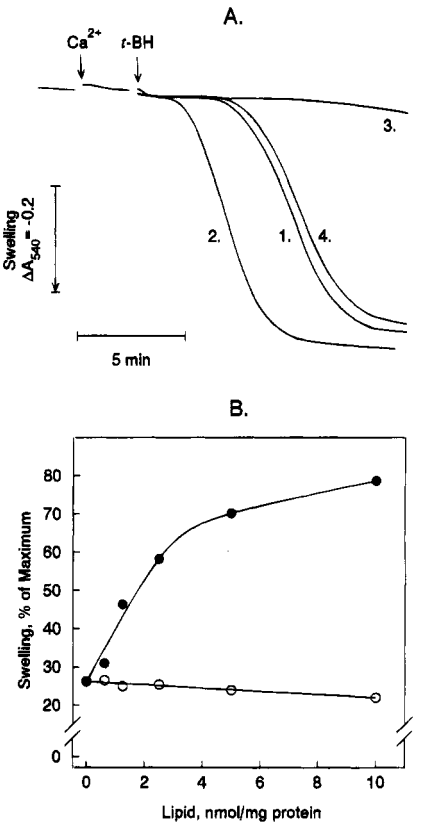

FIGURE 3: Stimulation of the permeability transition by palmitic acid: induction by *tert*-butyl hydroperoxide. Mitochondria were incubated and swelling was determined as described under Experimental Procedures. (Panel A) $CaCl_2$ (70 nmol/mg of protein) and *tert*-butyl hydroperoxide (200 μM) were added where indicated. Trace 1, no further additions; trace 2, same as trace 1 except that palmitic acid (10 nmol/mg of protein) was present from the beginning of the incubation; trace 3, same as trace 2 except that CsA (0.5 nmol/mg of protein) was also present from the beginning of the incubation; trace 4, same as trace 2 except that palmitoyl alcohol (10 nmol/mg of protein) was used instead of palmitic acid. (Panel B) The data were obtained from experiments like that shown in panel A, trace 2. (●) Palmitic acid was present at the indicated level from the beginning of the incubations. (○) Palmitoyl alcohol was used instead of palmitic acid. For both the acid and the alcohol, the percent of maximal swelling was determined at ~5 min following the addition of *tert*-butyl hydroperoxide.

are similar to the total levels of free fatty acids (data not shown). Since rat liver mitochondria contain approximately 175 nmol of phospholipid/mg of protein (Pfeiffer et al., 1979), the levels of hydrolysis products which accumulate are too low to cause a marked perturbation of the membrane lipid phase by acting as detergents (e.g., Kamp et al., 1993). However, within the range of levels observed, these products markedly stimulate opening of the PTP. Regardless of whether PTP opening is induced by *tert*-butyl hydroperoxide (Figure 3), *N*-ethylmaleimide (Figure 4), or inorganic phosphate (Figure 5), free fatty acids added at 0.2−10 nmol/mg of protein enhance PTP opening in a concentration-dependent manner. This action of free fatty acids is eliminated by CsA over short time frames, is not seen if either Ca^{2+} or the inducing agent is omitted, and may not be greatly dependent upon fatty acid chain length or the extent of unsaturation, because palmitic (16:0) and linoleic (18:2) acids are similarly effective (data not shown). An ionizable carboxylic acid moiety is required, however, because neither a long-chain alcohol (Figures 3−5) nor a long-chain alkane (data not shown) can duplicate the free fatty acid effect.

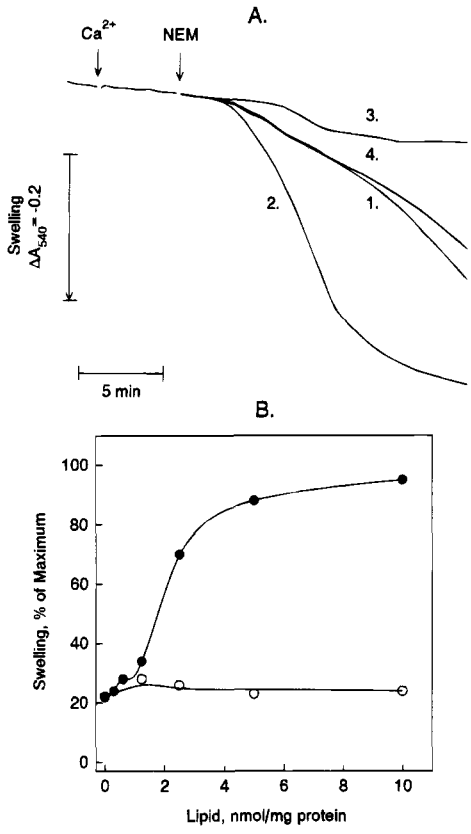

FIGURE 4: Stimulation of the permeability transition by palmitic acid: induction by *N*-ethylmaleimide. For both panels A and B, the experiments are analogous to those shown in Figure 3 except that the transition was induced by *N*-ethylmaleimide (300 μM) instead of *tert*-butyl hydroperoxide. In addition, for panel B, the percent of maximal swelling values were determined at ~8 min following the addition of *N*-ethylmaleimide.

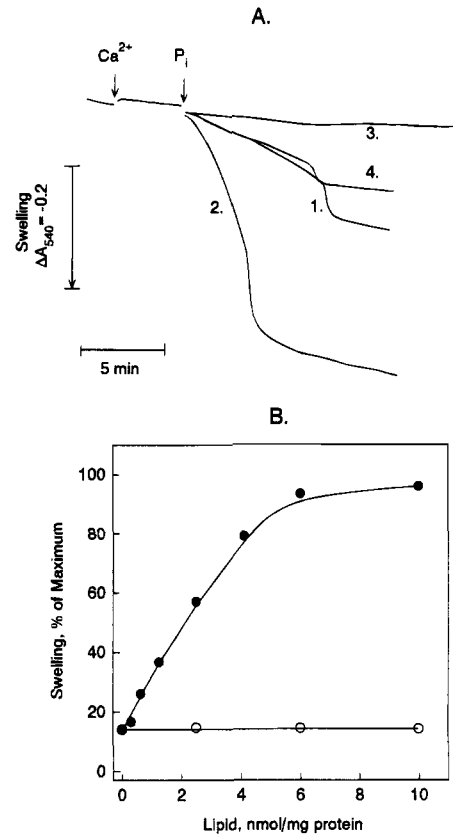

FIGURE 5: Stimulation of the permeability transition by palmitic acid: induction by inorganic phosphate. For both panels A and B, the experiments are analogous to those shown in Figure 3 except that the transition was induced by inorganic phosphate (2.5 mM) instead of *tert*-butyl hydroperoxide.

Exogenous lysophospholipids also stimulate PTP opening; however, on a mole basis, they are only 10−20% as active as the free fatty acids (data not shown). It is not clear at present if the minor actions of these compounds are produced by the lysophospholipids *per se*, or if they arise from small amounts of free fatty acids produced by hydrolysis of the exogenous compounds.

The Relationship between Free Fatty Acid Accumulation and the Loss of CsA Activity as an Inhibitor of the PTP. Since free fatty acids accumulate in mitochondria incubated with some inducers of PTP opening when CsA is present (Figure 2), and since exogenous fatty acids stimulate PTP opening during short time frame experiments when CsA is not present (Figures 3−5), it seemed possible that accumulating free fatty acids contribute to the loss of CsA activity during long time frame incubations. To test this possibility, the action of bovine serum albumin (BSA) on the maintenance of CsA inhibitory action was examined. The presence of 2 μM BSA markedly delays PTP opening induced by Ca^{2+} plus *tert*-butyl hydroperoxide or Ca^{2+} plus *N*-ethylmaleimide, regardless of whether K^+ release, Mg^{2+} release, or swelling is taken as the indicator of an open PTP (Figures 6 and 7). In contrast, there is little or no effect of BSA when PTP opening is induced by Ca^{2+} plus phosphate, and, indeed, induction of the PTP by phosphate is subject to long-lasting inhibition by CSA in the absence of trifluoperazine (Figure 8). Thus, under inducing conditions which allow free fatty acid accumulation (Figures 6 and 7, compare to Figure 2), BSA enhances the action of CsA, whereas BSA is not required to maintain the action of CsA when fatty acid accumulation does not occur (Figure 8, compare to Figure 2).

While the data in Figures 6−8 implicate free fatty acid accumulation as an important factor leading to PTP opening in the presence of CsA, they also show that marked differences in the absolute rate of opening are apparent depending upon whether K^+ release, Mg^{2+} release, or swelling is taken as the indicator. These differences become larger as the time course of PTP opening is lengthened by the presence of inhibitors, in a manner which is again dependent upon the inducing agent employed. As shown in Figure 9, which presents data obtained in the presence of CsA plus BSA, the half-times for K^+ release, Mg^{2+} release, and swelling, respectively, are progressively separated by 20 min intervals when the transition is induced by *tert*-butyl hydroperoxide (Figure 9A). When induction is by *N*-ethylmaleimide, these time separations are even greater (Figure 9B), whereas with phosphate (Figure 9C) the pattern is similar to what is seen under noninducing conditions (Figure 9D). When taken in sum, these data suggest that the short-lived PTP substates (precursors) which are seen by patchclamp techniques (e.g., Petronilli et al., 1989; Zorov et al., 1992) can become long-lived under conditions which antagonize PTP opening, resulting in partially opened forms of the PTP which are solute-selective (see further discussion below).

Trifluoperazine Does Not Inhibit the Hydrolysis of Phospholipids in Intact Mitochondria Treated with CsA. Based upon the above data and the reported activity of trifluoperazine as an inhibitor of mitochondrial phospholipase A_2 (Broekemeier et al., 1985), one might expect that synergism

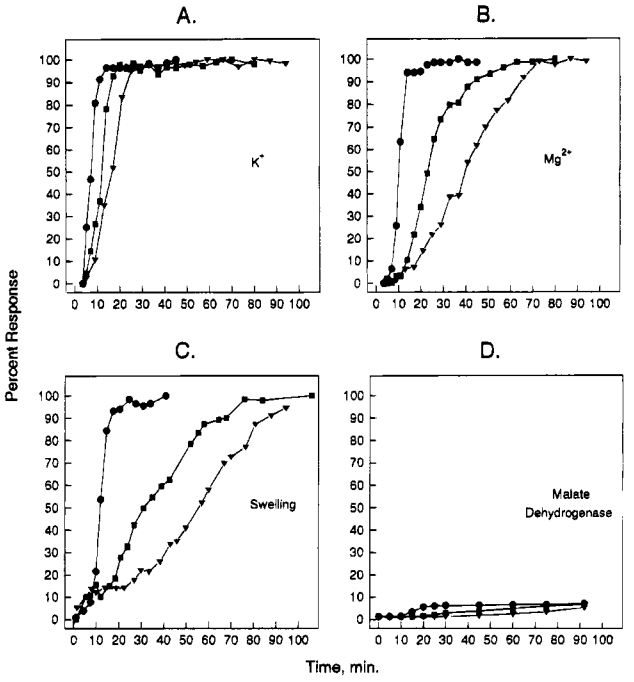

FIGURE 6: Inhibition of the permeability transition by CsA and BSA: induction by *tert*-butyl hydroperoxide. Mitochondria were incubated, and K^+ release (panel A), Mg^{2+} release (panel B), swelling (panel C), and malate dehydrogenase release (panel D) were determined as described under Experimental Procedures. All incubations were conducted in parallel using mitochondria from a single preparation so that the time courses shown are directly comparable. In all cases, $CaCl_2$ (60 nmol/mg of protein) was added at 2 min, and *tert*-butyl hydroperoxide (400 μM) was added 2 min later. For all panels: (●) no further additions; (■) CsA (0.5 nmol/mg of protein) was present from the beginning of the incubation; (▼) CsA (0.5 nmol/mg of protein) plus BSA (2 μM) was present from the beginning of the incubation.

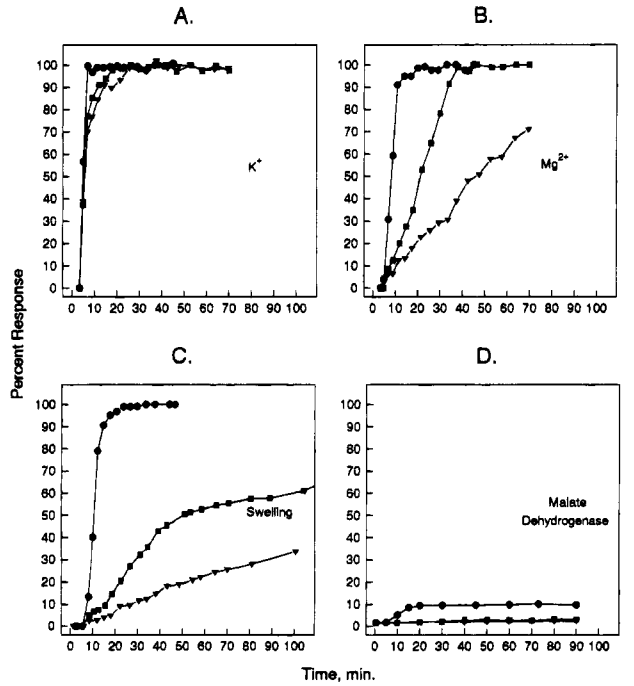

FIGURE 7: Inhibition of the permeability transition by CsA and BSA: induction by *N*-ethylmaleimide. For all panels, the experiments are analogous to those shown in Figure 6 except that the transition was induced by *N*-ethylmaleimide (300 μM) instead of *tert*-butyl hydroperoxide.

between the actions of CsA and trifluoperazine as inhibitors of PTP opening arises because the latter compound prevents

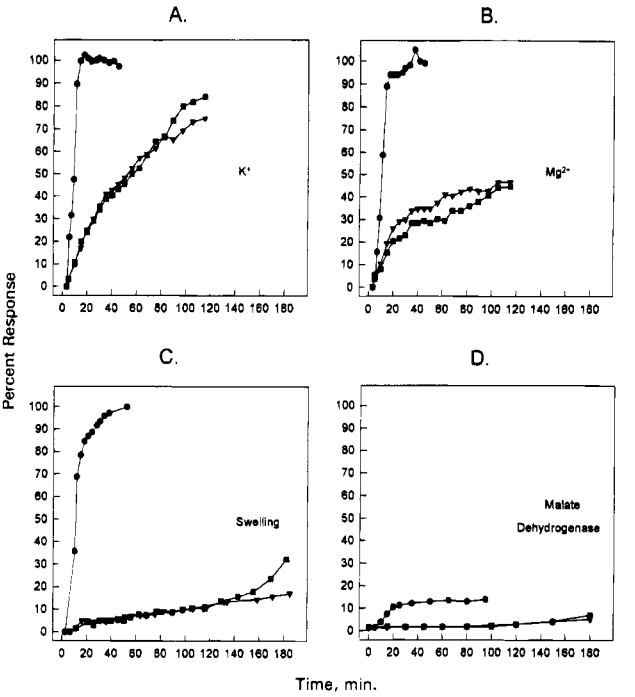

FIGURE 8: Inhibition of the permeability transition by CsA and BSA: induction by inorganic phosphate. For all panels, the experiments are analogous to those shown in Figure 6 except that the transition was induced by inorganic phosphate (2.5 mM) instead of *tert*-butyl hydroperoxide.

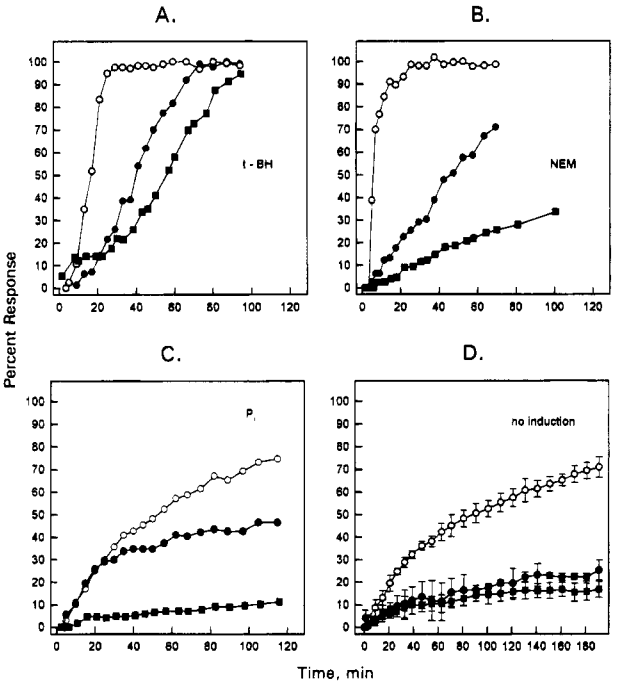

FIGURE 9: Apparent solute selectivity of the PTP induced in the presence of CsA plus BSA. The data in panels A–C are from the same experiments shown in Figures 6–8, CsA plus BSA present. For these panels and panel D: (○) K^+ release; (●) Mg^{2+} release; (■) swelling. For panel D, each point is a mean ± SD of three values obtained from three separate incubations in which CsA (0.5 nmol/mg of protein) and BSA (2 μM) were present. For one of these, neither Ca^{2+} nor an inducing agent was present. In another, Ca^{2+} only was present at 60 nmol/mg of protein. In the third, inorganic phosphate only was present at 2.5 mM.

fatty acid accumulation which would otherwise antagonize the action of CsA. However, when compared to Figure 2, Figure 10 shows that trifluoperazine has no inhibitory effect on free fatty acid accumulation in mitochondria incubated

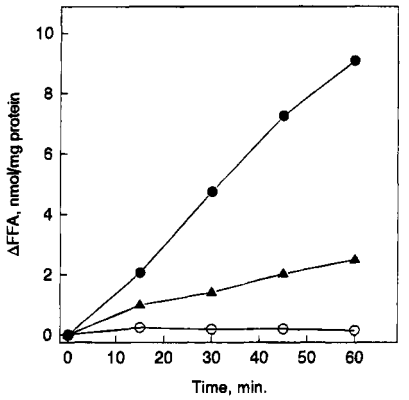

FIGURE 10: Accumulation of free fatty acids in mitochondria in the presence of CsA and trifluoperazine. Conditions were as described under Experimental Procedures with CsA (0.5 nmol/mg of protein) and trifluoperazine (60 μM) present from the beginning of the incubation. $CaCl_2$ (70 nmol/mg of protein) was added at 2 min followed at 4 min by *tert*-butyl hydroperoxide (●), 400 μM; *N*-ethylmaleimide (▲), 200 μM; or inorganic phosphate (○), 2 mM. Samples were taken at the indicated times, and free fatty acids were determined as described under Experimental Procedures.

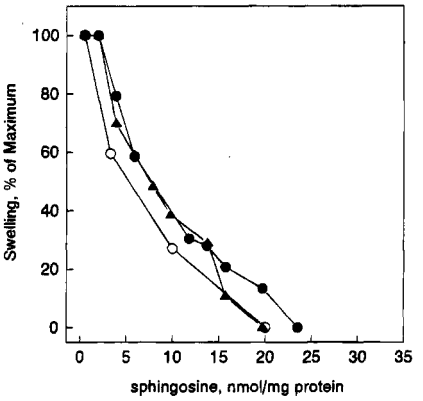

FIGURE 11: Inhibition of the permeability transition by sphingosine. Mitochondria were incubated and swelling was determined as described under Experimental Procedures with the medium containing the indicated level of sphingosine. $CaCl_2$ was added at 2 min followed at 4 min by (●) *tert*-butyl hydroperoxide (200 μM); (▲) *N*-ethylmaleimide (300 μM); or (○) inorganic phosphate (2 mM). The extents of swelling were determined at 12, 7, and 12 min following the addition of these agents, respectively.

with CsA in the presence of Ca^{2+} plus agents which induce the transition, and may even have a small stimulating effect. These data conflict with earlier results which indicated an inhibitory action of trifluoperazine on mitochondrial phospholipase A_2 (Broekemeier et al., 1985). The older data were obtained with an isolated membrane preparation instead of intact mitochondria, however, and thus conditions of Ca^{2+} concentration, exposure of the enzyme to other matrix components, and other factors are much different in the present experiments. These differences presumably explain the discrepancy, and, regardless of this discrepancy, the data shown here indicate that the presence or absence of fatty acid accumulation cannot explain the synergism between CsA and trifluoperazine in maintaining a closed PTP that was shown in Figure 1.

Some insight into why the two inhibitors act synergistically, as well as why low levels of free fatty acids favor an open PTP, arises from Figure 11. This figure shows that sphingosine inhibits PTP opening induced by Ca^{2+} plus any of the three inducing agents used alone. The concentration dependencies are similar to the concentration dependence of free fatty acids acting to promote an open PTP (compare Figure 11 with Figures 3−5). This action of sphingosine is not structurally specific because similar data are obtained with psychosine and stearylamine [see Bernardi et al. (1994) and data not shown]. Although having different structures, all three of these agents have similar physical properties because they are long-chain cationic lipids.

DISCUSSION

Use of CsA in Cell Injury Studies. Cyclosporin A is the most potent and generally applicable inhibitor of the mitochondrial permeability transition identified so far (Bernardi et al., 1994). However, the present data show that this inhibitory activity is transient when viewed on time scales longer than the few minutes which are normally involved when studying the transition in isolated mitochondria. This finding suggests that the use of CsA alone to evaluate the involvement of PTP opening in mechanisms of cell injury will cause the importance of this event to be underestimated because the time frames of cell injury studies usually exceed those for which CsA remains active. Thus, when CsA-treated cells die in response to injurious conditions, it cannot be assumed that the permeability transition was not responsible. A better test for the involvement of PTP opening in mechanisms of cell injury would seem to be the combined actions of CsA and trifluoperazine. Indeed, Lemasters and co-workers have already shown that trifluoperazine improves the effectiveness of CsA in protecting hepatocytes treated with *tert*-butyl hydroperoxide (Imberti et al., 1992, 1993), and Farber and co-workers have shown that butacaine has the same effect in hepatocytes subjected to anoxia or to inhibitors of the mitochondrial electron transport chain (Pastorino et al., 1993). Like trifluoperazine, local anesthetics such as butacaine can inhibit the mitochondrial permeability transition and phospholipase A_2 (e.g., Pfeiffer et al., 1979). Both of these agents display an array of pharmacological activities, however, and so it remains uncertain if they enhance the action of CsA solely by helping to maintain a closed PTP or if other actions are also involved. To further test this point, it would be useful to determine if more selective inhibitors of PTP opening can also be used to prolong the actions of CsA on mitochondria and intact cells.

Potential Mechanisms Leading to Loss of CsA Activity. It is important to determine why the actions of CsA on PTP opening are transitory because the answer could reveal fundamental aspects of how the PTP is regulated. Regarding possible explanations, several protease activities are found in mitochondria [see Figueiredo and Dugue-Magalhaes (1994) and references cited therein], and, therefore, a protease-mediated degradation of CsA might explain the loss of activity during extended experiments. We do not favor this possibility because raising the CsA concentration by severalfold above the 0.5 μM value used here has little or no effect on maintaining a closed PTP (data not shown). In addition, preliminary experiments using mass spectroscopic methods, conducted in collaboration with Drs. David Whitman and Larry Bowers at the University of Minnesota, Department of Laboratory Medicine and Pathology, did not show extensive degradation of CsA by mitochondria (data not shown). It is still possible that mitochondrial protease activities contribute to loss of CsA-dependent PTP inhibition, however, because the amphipathic peptides mastoparan and MP14 cause a CsA-insensitive opening of the PTP when they are present externally in the micromolar concentration range

(Pfeiffer et al., 1995). Picomole amounts of other amphipathic peptides produced in the matrix space by proteases thus might be a factor in the time-dependent loss of CsA activity as an inhibitor of the PTP.

Loss of matrix space adenine nucleotides and Mg^{2+} via the slow-acting ATP:Mg cotransporter (e.g., Nosek & Aprille, 1992; Joyal & Aprille, 1992) is another factor which might contribute to the time-dependent loss of PTP inhibition by CsA. This is because matrix space ADP, ATP, and Mg^{2+} enhance the effectiveness of CsA in causing PTP closure, and presumably contribute to inhibition by CsA of any initial opening of the PTP (Novgorodov et al., 1992, 1994; Crompton & Andreeva, 1994). Analysis of mitochondrial nucleotides by high-pressure liquid chromatography during extended incubations in the presence of CsA did show some loss of adenine nucleotides (data not shown). The interpretation of such findings is not straightforward, however, because solute-selective forms of the PTP (which are indicated by Figures 6−9), rather than the cotransporter, may have been responsible. In that case, nucleotide release, being after the fact, could not explain the initial failure of CSA to maintain a closed PTP. Other factors which might be involved in loss of PTP inhibition by CSA include a changing reduction state of mitochondrial pyridine nucleotides or pyridine nucleotide hydrolysis [see Richter and Kass (1991)], and a changing distribution of the protonmotive force between its electrical and chemical components [see Bernardi et al. (1994)].

Free fatty acid accumulation is one factor which clearly seems to be involved in promoting PTP opening when CsA is present. This is shown by Figures 3−5, which demonstrate that low amounts of exogenous free fatty acids facilitate PTP opening in the absence of CsA, and by Figures 6−8, which show that BSA delays the time-dependent loss of CsA activity as an inhibitor of the PTP.

Potential Mechanisms of PTP Regulation by Free Fatty Acids. In considering how free fatty acids might facilitate PTP opening, it is important to recall that even high levels applied alone to Ca^{2+}-loaded mitochondria fail to induce the transition [see Gunter and Pfeiffer (1990)]. They are, therefore, low activity inducers which can be thought of as increasing the sensitivity of mitchondria to more potent agents, such as phosphate and *tert*-butyl hydroperoxide, facilitating their actions which lead to an open PTP. This situation is generally consistent with free fatty acids acting by an indirect mechanism. Additional points to consider initially are the source of free fatty acids accumulating in CsA-treated mitochondria, and whether or not there are differences between exogenous fatty acids and fatty acids which arise from endogenous sources, with respect to PTP regulation. These questions are of interest because the data in Figures 3−5 (CsA absent) were obtained by applying exogenous fatty acids, whereas in Figures 1 and 6−8 (CsA present) fatty acids arose from endogenous sources. The predominant endogenous source is undoubtedly phospholipids because LPE and LPC accumulate in sum to levels which are similar to those of total free fatty acids (data not shown). Fatty acids derived from the acyl coenzyme A pool might also be contributing; however, the mitochondrial content of acyl coenzyme A is on the order of 0.5 nmol/mg (DiLisa et al., 1989), and so the potential importance of this source seems limited. Regardless of their source, there does not appear to be a significant difference between fatty acids generated from endogenous sources and exogenous fatty acids with respect to regulating the PTP. To test this point, we preincubated mitochondria with Ca^{2+} for increasing lengths of time, and then compared the time course of the transition in these mitochondria to the level of free fatty acids (which accumulate during preincubation), and further compared these parameters to the time course of the transition in normal mitochondria exposed to similar levels of exogenous free fatty acids. These experiments showed that endogenous and exogenous fatty acids are similarly effective (data not shown). These findings and the fact that phospholipids are the major source of endogenous free fatty acids restore a role for mitochondrial phospholipases in regulating the transition, as was proposed in early reports [see Gunter and Pfeiffer (1990)]. These findings also suggest that fatty acids arising from both mitochondrial and nonmitochondrial sources would be effective in limiting the PTP inhibition afforded by CsA *in vivo*.

Fatty acids are weak uncouplers in mitochondria because they rapidly distribute between opposing membrane surfaces as unionized carboxylic acids (Kamp & Hamilton, 1992; Kamp et al., 1993), and are transported from the matrix surface to the external surface of the inner membrane as carboxylate anions. The latter process is a charge-uncompensated reaction catalyzed (at least in part) by the adenine nucleotide translocase (Skulachev, 1991; Wojtczak & Schönfeld, 1993). The sum effect of these processes is a charge-uncompensated transport of H^+ down its electrochemical gradient which constitutes uncoupling. Because an open PTP is favored by reduced membrane potential (Bernardi, 1992; Petronilli et al., 1993), we considered the possibility that the uncoupling activity of fatty acids explains their influence on the PTP. Measurements carried out with a tetraphenylphosphonium cation electrode showed that the reductions in membrane potential produced by fatty acids at the levels employed here are small (~10 mV at 5 nmol/mg of protein). Adding small amounts of carbonyl cyanide *p*-chlorophenylhydrazone to match this degree of depolarization failed to significantly affect the rate of PTP opening (data not shown), and thus it seems possible to exclude uncoupling as a major factor by which fatty acids favor an open PTP.

Acyl coenzyme A might be produced in small quantities from accumulating free fatty acids, and these compounds are known to favor an open PTP (DiLisa et al., 1989; Siliprandi et al., 1992). However, no external acyl coenzyme A generating system was present during this work, and acyl coenzyme A synthesis within the matrix space should be minimal because of the medium chain length selectivity of the matrix space synthetases (Philipp & Parson, 1979). In addition, matrix space acyl coenzyme A would be subject to some oxidation during the extended experiments, in spite of the presence of excess succinate. Thus, formation of acyl coenzyme A is also an improbable explanation for the effects of free fatty acids which are seen under the present conditions.

When the effect of free fatty acids on PTP opening is considered together with the inhibitory effect of sphingosine (Figure 11), changes in membrane surface potential emerge as the probable explanation for the actions of both anionic and cationic lipids on the PTP. These polar lipids will associate with mitochondrial membranes with their acyl chain inserted into the membrane interior and with the charge-bearing moiety located at the interface. Thus, free fatty acids and sphingosine will increase and decrease the negative membrane surface potential, respectively. Because the PTP

is regulated by the transmembrane electrical potential, it is thought to have a voltage sensing element as a structural component (Petronilli et al., 1993, 1994). This element could be responsive to membrane surface potential as well as to bulk phase transmembrane potential, perhaps sensing these quantities as a vector sum (Bernardi et al., 1994). The magnitude of changes in surface potentials and summed potentials involved here could be substantial, but these changes are difficult to calculate. This is because the ionization states of free fatty acids and sphingosine associated with mitochondrial membranes are uncertain and the distribution of the agents between the various membrane surfaces is also uncertain. Thus, at present it can only be said that more positive surface potentials are associated with PTP inhibition while more negative potentials are associated with PTP activation.

Membrane Surface Potential and Other Regulators of the PTP. Free fatty acids and sphingosine influence components of cell signaling mechanisms and other processes, such that there are other ways by which they might regulate the PTP. Invoking changes in membrane surface potential as the central mechanism is attractive among these possibilities, because it also rationalizes the effects of additional PTP modulators without the need for agent-specific sites of interaction on a structure which already seems overpopulated with proposed effector sites. For example, an action through surface potential can explain the synergism between CsA and trifluoperazine in maintaining a closed PTP because the latter agent can be protonated to form an amphipathic cation that would make the membrane surface potential more positive upon association. Actions via surface potential predict a lack of structure specificity for PTP regulators which function through that mechanism. Thus, the actions of psychosine and stearylamine on the PTP are also explained (Bernardi et al., 1994). Spermine (Lapidus & Sokolove, 1992, 1993, 1994) and external divalent cations (Bernardi et al., 1993) inhibit PTP opening, and both would be expected to render surface potentials more positive through association. Shifting the adenine nucleotide translocase between its C and M conformers dramatically affects the open/closed probability of the PTP [see Halestrap (1994)] and is also known to substantially change the transmembrane surface potential gradient (Scherer & Klingenberg, 1974; Rottenberg & Marbach, 1990). Through these examples it is seen that enlarging the recognized role of transmembrane potential to include roles for surface potential and surface potential gradients in regulating the PTP helps to answer the long-standing question of how structurally diverse PTP effectors produce common regulatory actions (Gunter & Pfeiffer, 1990).

Solute-Selective Forms of the PTP. Along different lines, aspects of the present data strongly suggest that the PTP can exist in partially open states which are long-lived and solute-selective when CsA or CsA plus BSA is present. These forms may correspond to the short-lived reduced conductance states (substates) which are seen by patchclamp methods during opening and closure of the PTP (Zoratti & Szabó, 1994, 1995), since these forms become longer-lived in the presence of inhibitors [see Kinnally et al. (1992)]. Apparent solute selectivity of the PTP is shown most clearly in Figure 9 where it is seen that as the PTP opens throughout the mitochondrial population, when CsA and BSA are present, K^+ release occurs much faster than Mg^{2+} release, which in turn is faster than swelling (mannitol and sucrose permeation). Although there have been earlier suggestions of solute selectivity displayed by PTP substates [Riley & Pfeiffer, 1985; reviewed in Zoratti and Szabó (1995)], the time separation between permeation of specific solutes achieved here is particularly dramatic.

Several ion-conducting channels and other transport mechanisms move K^+ and Mg^{2+} across the inner mitochondrial membrane (Zoratti and Szabó, 1994, 1995; Kinnally et al., 1992), and so it is possible that one or more of these is involved in the early movement of cations, compared to mannitol/sucrose, when pore opening is slowed by inhibitors. However, two factors link the early cation movements to the PTP. The first is seen in Figure 9 when panels A−C are compared to panel D. The latter panel shows the pattern of solute movement under control (no induction) conditions, and the comparison shows that accelerated movement of all solutes is dependent upon conditions which induce the transition. Thus, all solute movements are likely to involve the PTP. The second factor is the progressive inhibition of all solute movements produced by CsA and CsA plus BSA (Figures 6−8). These agents inhibit the PTP but are not thought to inhibit other cation transport activities which could potentially contribute to the early release of K^+ and Mg^{2+}.

If PTP substates are indeed solute-selective and long-lived in the presence of CsA, then there are additional problems with using CsA in a diagnostic manner to implicate PTP opening in mechanisms of cell injury. Partially open forms may appear early and diminish mitochondrial function, even when the fully open form is attained much later. Again, this could cause the importance of the PTP in cell killing to be underestimated. Additional implications of long-lived and selective PTP substates relate to the mechanisms of PTP opening and closure, and to the way in which CsA may favor a closed PTP. The pathway for initial opening may require that the selective PTP substates be "visited", and the target of CsA (cyclophilin?) could catalyze the sequential structural conversions involved. This would explain why the substates become long-lived when CsA is present. However, rapid PTP closure can occur in the presence of CsA (e.g. Novgorodov et al., 1994). One way to reconcile this with a catalytic role for the CsA target during PTP opening would be if multiple closed states were attainable from the open state, with one or more of these being readily available without catalysis.

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