

# Phosphate Is Essential for Inhibition of the Mitochondrial Permeability Transition Pore by Cyclosporin A and by Cyclophilin D Ablation\*

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Energized mouse liver mitochondria displayed the same calcium retention capacity (a sensitive measure of the propensity of the permeability transition pore (PTP) to open) irrespective of whether phosphate, arsenate, or vanadate was the permeating anion. Unexpectedly, however, phosphate was specifically required for PTP desensitization by cyclosporin A (CsA) or by genetic inactivation of cyclophilin D (CyP-D). Indeed, when phosphate was replaced by arsenate, vanadate, or bicarbonate, the inhibitory effects of CsA and of CyP-D ablation on the PTP disappeared. After loading with the same amount of  $\text{Ca}^{2+}$  in the presence of arsenate or vanadate but in the absence of phosphate, the sensitivity of the PTP to a variety of inducers was identical in mitochondria from wild-type mice, CyP-D-null mice, and wild-type mice treated with CsA. These findings call for a reassessment of conclusions on the role of the PTP in cell death that are based on the effects of CsA or of CyP-D ablation.

An increased permeability of the mitochondrial inner membrane, the permeability transition, is a key event in cell death (1). The permeability transition is due to opening of the permeability transition pore (PTP),<sup>2</sup> a high conductance channel of unknown molecular structure that is modulated by cyclophilin D (CyP-D) (2). The PTP can be desensitized by the CyP inhibitor cyclosporin A (CsA) (3–6) or by ablation of CyP-D (7–10), and CyP-D-null mice are strikingly resistant to ischemic heart (7, 9) and brain (10) damage and to experimental autoimmune encephalomyelitis (11). Treatment with CsA cured a mouse model of collagen VI muscular dystrophy through PTP inhibi-

tion (12) and normalized mitochondrial function and apoptosis in patients with collagen VI muscular dystrophies (13, 14). CyP-D ablation led to recovery from muscle pathology in other mouse models of muscular dystrophy, suggesting that PTP opening may play a role in more than one form of myopathy (15). The mechanism through which treatment with CsA and lack of CyP-D affects the PTP remains unsolved, however; and the extent to which it is possible to apply results obtained from *in vitro* studies to the status of the PTP *in situ* remains an open question (2). Here, we show that ablation of CyP-D or treatment with CsA does not directly cause PTP inhibition, but rather unmasks an inhibitory site for  $\text{P}_i$ . Indeed, we found that the inhibitory effects of CsA and of CyP-D ablation disappeared when  $\text{P}_i$  was replaced by vanadate ( $\text{V}_i$ ), arsenate ( $\text{As}_i$ ), or bicarbonate and that, in the absence of  $\text{P}_i$ , the PTP sensitivity to  $\text{Ca}^{2+}$  and oxidative stress was identical in wild-type and CyP-D-null mitochondria. Our results indicate that the PTP is not sensitive to CsA or to CyP-D ablation unless  $\text{P}_i$  is present.

## EXPERIMENTAL PROCEDURES

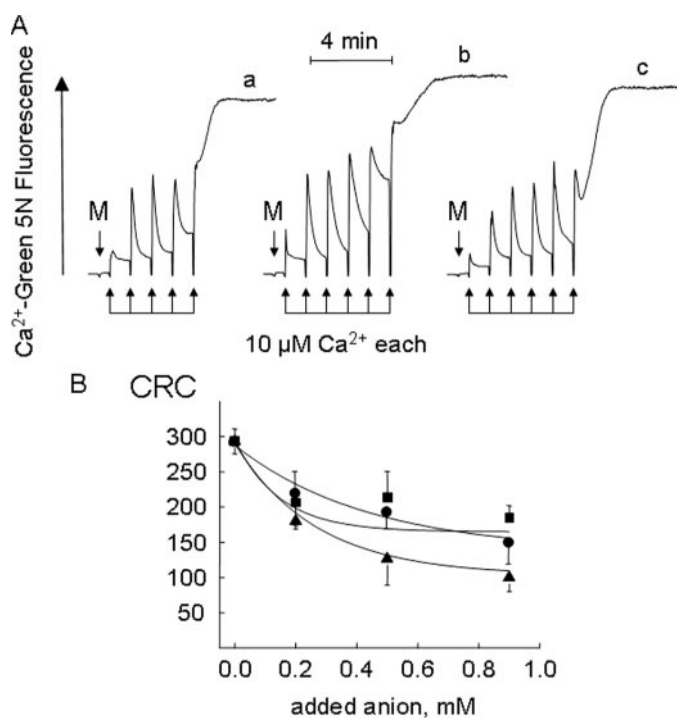
Mitochondria were isolated from the livers of C57BL/6J (wild-type) and *Ppif*<sup>-/-</sup> C57BL/6J (CyP-D-null) mice by standard differential centrifugation. The properties of the CyP-D-null mitochondria have been described elsewhere (8). Protein concentration was determined using the biuret method, and the mitochondrial suspension was kept on ice and used within 4 h of preparation.

The incubation medium contained 120 mM KCl, 10 mM Tris/MOPS, 5 mM Tris glutamate, 2.5 mM Tris malate, 20  $\mu\text{M}$  Tris/EGTA, and  $\text{P}_i$ ,  $\text{V}_i$ , or  $\text{As}_i$  (as specified in the figure legends) at pH 7.4. For all measurements, the concentration of mitochondrial protein was 0.5 mg/ml at 25 °C. Oxygen consumption was measured with a Clark-type oxygen electrode in a closed 2-ml vessel equipped with magnetic stirring. Membrane potential was estimated based on fluorescence quenching of rhodamine 123 (16) in 2-ml stirred cuvettes with a PerkinElmer Life Sciences LS50B spectrofluorometer (0.3  $\mu\text{M}$  rhodamine 123; excitation and emission wavelengths of 503 and 525 nm, respectively). The mitochondrial calcium retention capacity (CRC) (17) was determined in medium supplemented with 1  $\mu\text{M}$  Calcium Green-5N (Molecular Probes) either with the PerkinElmer Life Sciences spectrofluorometer (excitation and emission wavelengths of 505 and 535 nm, respectively) or with a Fluoroskan Ascent FL (Thermo Electron Corp.) equipped with a plate shaker (excitation and emission wavelengths of 485 and 538 nm, respectively, with a 10-nm band-pass filter) in a final volume of 0.2 ml. Ten micromolar  $\text{CaCl}_2$  pulses were added at 1-min intervals until onset of the permeability transition, which is marked by a precipitous release of the accumulated  $\text{Ca}^{2+}$ . All chemicals were of the highest purity commercially available. Reported results are the means of at least three experiments for each condition, and error bars refer to S.E.

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<sup>2</sup> The abbreviations used are: PTP, permeability transition pore; CyP-D, cyclophilin D; CsA, cyclosporin A;  $\text{V}_i$ , vanadate;  $\text{As}_i$ , arsenate; MOPS, 4-morpholinopropanesulfonic acid; CRC, calcium retention capacity.

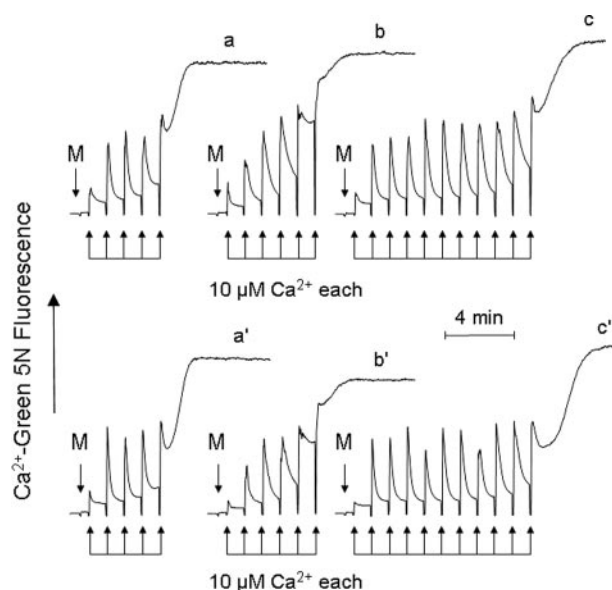


**FIGURE 1. Effect of anions on the mitochondrial permeability transition.** A, the incubation medium was supplemented with 1 mM  $As_4$  (trace a), 1 mM  $V_i$  (trace b), or 1 mM  $P_i$  (trace c). The conditions were as follows: 2-ml final volume, pH 7.4, and 25 °C. Where indicated, 1 mg of mouse liver mitochondria (M) was added, followed by a train of  $Ca^{2+}$  pulses of 10  $\mu$ M each (arrows). B, the experimental conditions were as described for A, except that the medium contained 0.1 mM  $P_i$  plus the indicated concentrations of  $As_4$  (▲),  $V_i$  (■), and  $P_i$  (●). The CRC is expressed in nanomoles of  $Ca^{2+}$ /mg of protein.

## RESULTS

Preliminary experiments were carried out to test whether substitution of  $P_i$  with  $As_4$  or  $V_i$  affects basic mitochondrial properties. Resting membrane potential was identical irrespective of the anion, and cycles of depolarization-repolarization were observed during the train of  $Ca^{2+}$  pulses, with the precipitous depolarization expected of PTP opening occurring at lower  $Ca^{2+}$  loads with  $As_4$  than with  $P_i$  and  $V_i$  (Fig. 1). As expected, (i) in the presence of  $V_i$ , ADP did not stimulate respiration; and (ii) because of the lability of ADP- $As_4$ , which immediately regenerates ADP, in the presence of  $As_4$ , basal respiration was higher, and ADP-stimulated respiration did not return to state 4 levels unless oligomycin was added (data not shown). Thus, replacement of  $P_i$  with  $As_4$  or  $V_i$  does not impair the ability of mitochondria to develop and maintain the membrane potential, which is a prerequisite for energy-dependent  $Ca^{2+}$  uptake.

We then investigated whether the PTP would open when  $P_i$  was replaced by  $As_4$  or  $V_i$ , which both support uptake of  $Ca^{2+}$  by energized mitochondria (Fig. 1A). Mitochondria incubated in the presence of 1 mM  $As_4$  (trace a),  $V_i$  (trace b), or  $P_i$  (trace c) readily accumulated a train of  $Ca^{2+}$  pulses until a threshold matrix  $Ca^{2+}$  was reached, causing opening of the PTP, which was detectable as the precipitous release of the accumulated  $Ca^{2+}$ .  $As_4$  was slightly more potent than  $P_i$  or  $V_i$ , but no major differences among the three anions were observed when the CRC (a sensitive measure of the propensity of the PTP to open



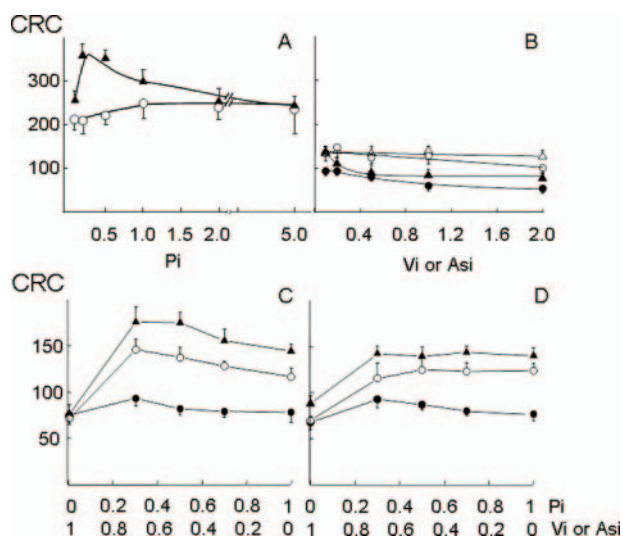
**FIGURE 2. Desensitization of the PTP by CsA or by CyP-D ablation depends on  $P_i$ .** Where indicated (M), 1 mg of wild-type mitochondria treated with 0.8  $\mu$ M CsA (traces a–c) or of CyP-D-null mitochondria (traces a'–c') was added to medium containing 1 mM  $As_4$  (traces a and a'), 1 mM  $V_i$  (traces b and b'), or 1 mM  $P_i$  (traces c and c') under conditions otherwise identical to those described in the legend to Fig. 1A.

(17)) was studied as a function of the anion concentration (Fig. 1B), indicating that  $V_i$  and  $As_4$  can replace  $P_i$  as PTP inducers.

Treatment with CsA or ablation of CyP-D desensitizes the PTP to  $Ca^{2+}$  *in vitro*, as shown by experiments that are routinely performed in the presence of  $P_i$  (7–10). Surprisingly, when  $Ca^{2+}$  uptake was studied in the presence of  $As_4$  (Fig. 2, traces a and a') or  $V_i$  (traces b and b'), the CRC of mitochondria was unaffected by treatment with CsA (traces a and b) or by ablation of CyP-D (traces a' and b'), whereas the expected increase of the CRC (*i.e.* PTP desensitization to  $Ca^{2+}$ ) was readily observed in the presence of  $P_i$  (traces c and c'). Results similar to those seen with  $V_i$  and  $As_4$  were also obtained with bicarbonate (data not shown). These experiments suggest that  $P_i$  is the actual inhibitor of the PTP when CsA is present or CyP-D is absent.

We determined the concentration dependence of PTP inhibition by  $P_i$ . In the absence of CyP-D, the peak CRC increase was observed between 0.2 and 0.5 mM  $P_i$  (Fig. 3A, closed symbols). The desensitizing effect of CsA in wild-type mitochondria was lower and reached its maximal value at 1 mM  $P_i$  (Fig. 3A, open symbols). Neither  $V_i$  (Fig. 3B, open symbols) nor  $As_4$  (closed symbols) increased the CRC in wild-type (circles) or CyP-D-null (triangles) mitochondria.

Because accumulation of anions decreases matrix free  $[Mg^{2+}]$  and  $[Ca^{2+}]$  (18), we also studied the CRC of mitochondria incubated in media containing increasing concentrations of  $P_i$  and decreasing concentrations of  $V_i$  or  $As_4$  so as to maintain the total anion concentration at 1 mM. These experiments confirmed that only  $P_i$  desensitized the PTP to  $Ca^{2+}$  in CsA-treated wild-type mitochondria and in CyP-D-null mitochondria and that neither  $V_i$  (Fig. 3C) nor  $As_4$  (Fig. 3D) could substitute for  $P_i$  in PTP inhibition. These findings demonstrate that the inhibitory site unmasked by pharmacological inhibition or



**FIGURE 3. Desensitization of the PTP by CsA or by CyP-D ablation depends on  $P_i$ .** *A*, wild-type mitochondria treated with  $0.8 \mu\text{M}$  CsA ( $\circ$ ) or CyP-D-null mitochondria ( $\blacktriangle$ ) were incubated in the presence of the indicated millimolar concentrations of  $P_i$ . *B*, wild-type (circles) or CyP-D-null (triangles) mitochondria were incubated in the presence of the indicated millimolar concentrations of  $V_i$  (open symbols) or  $As_i$  (closed symbols). *C* and *D*, wild-type mitochondria ( $\bullet$ ), CyP-D-null mitochondria ( $\blacktriangle$ ), or wild-type mitochondria treated with  $0.8 \mu\text{M}$  CsA ( $\circ$ ) were incubated with the indicated millimolar concentrations of  $P_i$  and  $V_i$  (*C*) or  $P_i$  and  $As_i$  (*D*). The incubation conditions were otherwise identical to those described in the legend to Fig. 1*A*, and the CRC is expressed in nanomoles of  $\text{Ca}^{2+}$ /mg of protein.

by genetic ablation of CyP-D is strikingly selective for  $P_i$  over its analogs  $As_i$  and  $V_i$ .

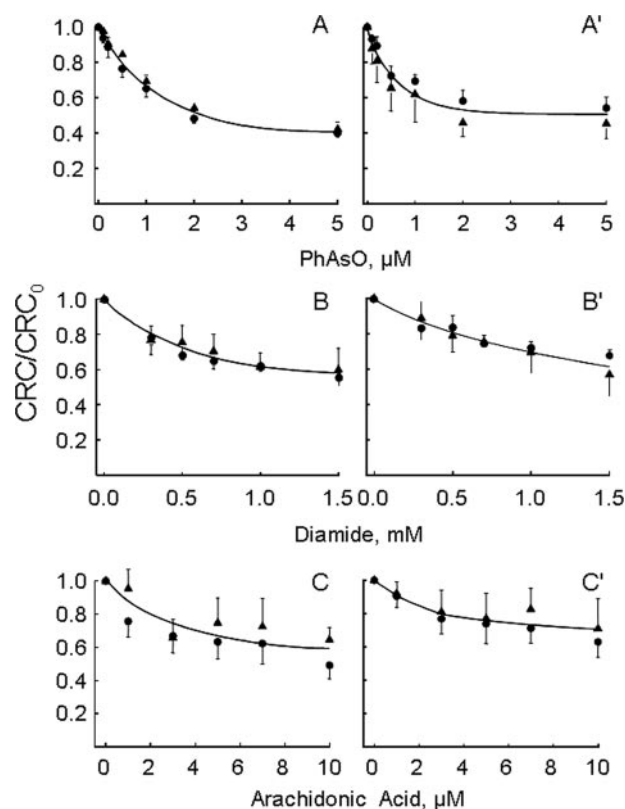
Because the CRC of wild-type and CyP-D-null mitochondria is the same when  $P_i$  is replaced by  $V_i$  or  $As_i$ , we could test the sensitivity to PTP inducers under exactly the same  $\text{Ca}^{2+}$ -loading conditions. Strikingly, the response of the PTP to phenylarsine oxide, diamide, and arachidonic acid was indistinguishable for the two genotypes (Fig. 4). This finding is remarkable because it indicates that the basal sensitivity of the PTP to effectors of pathophysiological relevance is not affected by the lack of CyP-D unless the concentration of  $P_i$  is high enough for PTP inhibition.

## DISCUSSION

**$\text{Ca}^{2+}$  and  $P_i$  Uptake in Energized Mitochondria**—Mitochondria isolated from a wide variety of tissues have a remarkable capacity to accumulate  $\text{Ca}^{2+}$  (19–22).  $\text{Ca}^{2+}$  uptake is an electrophoretic process driven by the  $\text{Ca}^{2+}$  electrochemical gradient,  $\Delta\tilde{\mu}_{\text{Ca}}$  (Equation 1).

$$\Delta\tilde{\mu}_{\text{Ca}} = zF\Delta\psi + RT \ln[\text{Ca}^{2+}]_i/[\text{Ca}^{2+}]_o \quad (\text{Eq. 1})$$

In respiring coupled mitochondria, the inside-negative  $\Delta\psi$  favors accumulation of  $\text{Ca}^{2+}$ , which is transported with a net charge of 2 (23, 24).  $\text{Ca}^{2+}$ -dependent depolarization is compensated by  $\text{H}^+$  extrusion, causing matrix alkalinization that prevents the recovery of  $\Delta\psi$ , limiting the further ability to accumulate  $\text{Ca}^{2+}$  (25–27). Uptake of substantial amounts of  $\text{Ca}^{2+}$  therefore requires (i) buffering of matrix pH to allow regeneration of the  $\Delta\psi$  and (ii) buffering of matrix  $\text{Ca}^{2+}$  to prevent the buildup of a  $\text{Ca}^{2+}$  concentration gradient (25–27). Buffering of matrix pH is achieved by the simultaneous uptake of protons and anions via diffusion of the undissociated acid (as in the case



**FIGURE 4. Sensitivity of PTP to inducers is unaffected by CyP-D ablation in the absence of  $P_i$ .** Wild-type ( $\bullet$ ) or CyP-D-null ( $\blacktriangle$ ) mitochondria were incubated in the presence of  $1 \text{ mM}$   $V_i$  (*A–C*) or  $1 \text{ mM}$   $As_i$  (*A'–C'*) in the presence of the indicated concentrations of phenylarsine oxide (*PhAsO*), diamide, or arachidonic acid. Experimental conditions were otherwise identical to those described in the legend to Fig. 1*A*, and the CRC is normalized to the value measured in the absence of PTP inducers ( $\text{CRC}_0$ ).

of acetate) or of  $\text{CO}_2$  (which regenerates bicarbonate and  $\text{H}^+$  in the matrix) or through transport proteins (like the  $\text{H}^+$ - $P_i$  symporter) (28); buffering of matrix  $\text{Ca}^{2+}$  depends on the cotransported anion and is maximal for  $P_i$ , so in its presence, matrix free  $[\text{Ca}^{2+}]$  becomes invariant with matrix  $\text{Ca}^{2+}$  load (18). In mitochondria energized in the presence of  $P_i$  (which is by far the most used anion *in vitro* and is likely to play a major role *in situ*), the  $\Delta\tilde{\mu}_{\text{Ca}}$  thus favors the accumulation of large loads of both  $\text{Ca}^{2+}$  and  $P_i$  (29). Because  $\text{Ca}^{2+}$  is essential for PTP opening (30) and because  $P_i$  is a classic inducer of the permeability transition (31), it has been difficult to sort the effects of  $P_i$  from those of  $\text{Ca}^{2+}$  and to understand why, despite its effect of lowering matrix free  $[\text{Ca}^{2+}]$ ,  $P_i$  behaves as a PTP inducer (18). The results of the present work contribute to clarifying these long-standing problems and identify a novel feature of  $P_i$  as an inhibitor of the PTP that mediates the effects of CsA and of CyP-D ablation.

**$P_i$  as a PTP Inducer**—Increasing  $P_i$  concentrations decrease matrix free  $[\text{Ca}^{2+}]$ , which should in turn decrease the probability of PTP opening (30). We suspect that the inducing effect of  $P_i$ , which is shared by its analogs  $As_i$  and  $V_i$ , is due in part to the decrease in matrix free  $[\text{Mg}^{2+}]$ , which, like all divalent cations but  $\text{Ca}^{2+}$ , decreases the probability of PTP opening (32), and in part to buffering of matrix pH at  $\sim 7.3$ , *i.e.* the optimum for opening of the PTP, the probability of opening of which declines at both lower and higher matrix pH values (33). An

additional possibility is that matrix  $P_i$  gives rise to the formation of polyphosphate, which promotes the permeability transition (34). Because the inducing properties of  $P_i$  are shared by  $As_i$  and  $V_i$  with the same concentration dependence, it will be interesting to test if the latter can substitute for  $P_i$  in the formation of complexes with  $Ca^{2+}$  and polyhydroxybutyrate, which mimic the ion-conductive properties of the PTP (35).

*$P_i$  as a PTP Inhibitor*—The most important result of this study is the demonstration that  $P_i$  has a *desensitizing* effect on the PTP. This can be detected as a shift of the threshold matrix  $Ca^{2+}$  required for pore opening at higher  $Ca^{2+}$  loads, *provided that CsA is added or CyP-D is absent*.  $As_i$ ,  $V_i$ , and bicarbonate could not replace  $P_i$ , suggesting that the effect is not mediated by changes of matrix free  $Mg^{2+}$  and/or pH. Indeed, desensitization was observed at increasing  $P_i$  even when the total [ $P_i + As_i$ ] or [ $P_i + V_i$ ] was kept constant, indicating that only  $P_i$  is able to bind a PTP regulatory site that is not accessible when CyP-D is present. This finding suggests that CyP-D may not be a PTP inducer *per se*, but rather a factor that prevents the inhibitory action of  $P_i$  from being exerted. We have documented an inhibitory effect of  $P_i$  also after treatment of wild-type mitochondria with CsA, which is known to detach CyP-D from its membrane binding site(s) (36, 37). Thus,  $P_i$  rather than CsA appears to be the actual PTP desensitizer also in the presence of the drug. Inhibition by CsA can also be observed in de-energized mitochondria incubated in thiocyanate-based medium (6). Under these conditions, diffusion of  $SCN^-$  provides the driving force for  $Ca^{2+}$  uptake (38), which is then readily followed by PTP opening (6). We suspect that the lipophilic  $SCN^-$  anion may have access to the  $P_i$ -binding site and substitute for  $P_i$ , particularly at the very high concentrations (typically 150 mM) used in this type of swelling assays.

*A Role for the  $P_i$  Carrier?*—Quite recently, it has been proposed that the  $P_i$  carrier itself may constitute the PTP, possibly in association with the adenine nucleotide translocator (39). Although this is certainly a possibility, we think that the present results do not directly bear on the issue. Indeed, (i) although  $P_i$ ,  $As_i$ , and  $V_i$  are all inducers of the PTP (Fig. 1), only  $P_i$  behaves as a PTP inhibitor (Fig. 3), yet all these anions are transported by the  $P_i$  carrier. (ii)  $SCN^-$  shares the inhibitory properties of  $P_i$  in the presence of CsA, yet it does not need a carrier to cross the lipid bilayer. (iii) Ubiquinone 0 and Ro 68-3400 are reported to inhibit the  $P_i$  carrier in de-energized mitochondria incubated with 40 mM  $P_i$  (39), but this is probably not relevant to PTP inhibition in energized mitochondria that accumulate  $Ca^{2+}$  at the physiological concentration of 1 mM  $P_i$  (40, 41); indeed and as invariably observed with the  $P_i$  carrier inhibitors *N*-ethylmaleimide (26) and mersalyl (29), inhibition of  $P_i$  uptake should have resulted in a dramatic decrease in the rate and extent of  $Ca^{2+}$  uptake (see also the first paragraph under “Discussion”), which instead is never observed in mitochondria treated with any inhibitory ubiquinone analog (17, 42, 43) or with Ro 68-3400 (44). (iv) Ubiquinone 0 (17) and Ro 68-3400 (44), which inhibit the PTP independent of CyP-D (8), desensitize the PTP to  $Ca^{2+}$  even when  $P_i$  is replaced by  $As_i$  or  $V_i$  (data not shown). Thus, our results do not necessarily implicate the  $P_i$  carrier as the mediator of the complex effects of  $P_i$  on the PTP.

*Implications for in Vivo Studies*—CyP-D inhibition is an important pharmacological target for PTP desensitization, as demonstrated by the striking therapeutic efficacy of CsA in many disease models (see Ref. 2 for review), in patients with collagen VI muscular dystrophies (13, 14), and in heart infarction (45) and by the effect of CyP-D ablation in murine models of multiple sclerosis (11) and muscular dystrophy (15). By cautious extrapolation of the present results to the conditions prevailing *in vivo*, we can conclude that the concentration of  $P_i$  in the animal models, in collagen VI muscular dystrophy patients, and in heart infarction is high enough for CsA to be effective, in keeping with current estimates of cellular free  $P_i$  in the 1 mM range (40, 41). This may not be the case in all conditions, however. For example, in perfused rabbit hearts during KCl arrest, tissue free  $P_i$  drops below 0.1 mM (40). It is also remarkable that the pharmacological effect of CsA is not as pronounced as that of CyP-D ablation (Fig. 3), suggesting that extreme caution should be exerted before concluding that the PTP is not involved in a given paradigm of mitochondrial dysfunction only because CsA does not exert a protective effect.

It is fortunate that  $P_i$  was included in mitochondrial swelling assays of PTP *in vitro* so that the desensitizing effect of CsA was not missed. On the other hand, the  $P_i$  dependence of PTP inhibition by CsA may represent just one example of the factors that control the PTP sensitivity to inhibitors *in situ*. It is very striking that, in the absence of  $P_i$ , the sensitivity of the PTP to  $Ca^{2+}$  and inducers is identical in wild-type and CyP-D-null mitochondria, confirming our earlier conclusion that the basic features of the PTP are not affected by the presence of CyP-D (8). Thus, extreme prudence should be exerted in extrapolating results from *in vitro* studies to the status of the PTP *in vivo*, and we suspect that the PTP may be involved in even more paradigms of cell death than is currently believed.

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