Cyclosporin A blocks 6-hydroxydopamine-induced efflux of Ca^{2+} from mitochondria without inactivating the mitochondrial inner-membrane pore

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Oxidative stress causes Ca^{2+}-loaded mitochondria to release Ca^{2+}. The mechanism of this efflux is unclear, but it appears to be associated with the opening of a pore in the mitochondrial inner membrane. Pore opening depolarizes the mitochondria, letting solutes enter the mitochondrial matrix, causing swelling. Cyclosporin A (CsA) prevents opening of this pore. The neurotoxin 6-hydroxydopamine (6HD) autoxidizes, producing free radicals, which cause oxidative stress. In this paper it is shown that 6HD-induced efflux from Ca^{2+}-loaded mitochondria was prevented by CsA. The 6HD-induced Ca^{2+} efflux was not accompanied by mitochondrial swelling, depolarization of the mitochondrial inner membrane or movement of radiolabelled sucrose into the mitochondrial matrix. In agreement with others [Schlegel, Schweizer and Richter (1992) Biochem. J. **285**, 65–69], these findings suggest that the mitochondrial pore remained closed during pro-oxidant-induced Ca^{2+} efflux. However, the implication that CsA blocks pro-oxidant-induced Ca^{2+} efflux by some mechanism other than inactivating the mitochondrial pore, suggests that the interaction of CsA with mitochondria may be more complex than is currently supposed.

INTRODUCTION

The neurotoxins 6-hydroxydopamine (6HD) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) cause lesions similar to idiopathic Parkinson's disease in primates by destroying catecholaminergic neurons in the substantia nigra (Gerlach et al., 1991). The toxicity of these neurotoxins is, at least in part, due to oxidative stress. Autoxidation of 6HD produces free radicals (Cohen and Heikkila, 1974) and MPTP induces free-radical production (Hasegawa et al., 1990; Ramsay and Singer, 1992; Zang and Misra, 1992), in addition to inhibition of respiration by its metabolite 1-methyl-4-phenylpyridinium (MPP) (Nicklas et al., 1985; Singer and Ramsay, 1990). The pathology and aetiology of idiopathic Parkinson's disease may involve both oxidative damage (Halliwell, 1992; Jenner, 1991) and neurotoxins (Langston and Young, 1992), therefore the mechanism by which oxidative stress caused by 6HD or MPP kills cells is important.

A major cause of cell damage during oxidative stress is disruption of mitochondrial Ca^{2+} homoeostasis (Richter and Frei, 1988; Thomas and Reed, 1989). Accumulation of Ca^{2+} by mitochondria in conjunction with oxidative stress opens a non-specific pore in the mitochondrial inner membrane. Opening of this pore is shown experimentally by depolarization of the mitochondria, release of accumulated Ca^{2+}, penetration of external solutes into the mitochondrial matrix and swelling of the mitochondria (Crompton et al., 1987, 1992; Crompton and Costi, 1988; Carbonera and Azzone, 1988; Gunter and Pfeiffer, 1990). Pore opening is regulated by mitochondrial Ca^{2+} concentration, pH and membrane potential (Bernardi, 1992; Bernardi et al., 1993; Petronilli et al., 1993). Low concentrations of Cyclosporin A (CsA) block pore opening, and this is presumed to follow the inhibition by CsA of a specific matrix peptidyl-prolyl *cis–trans* isomerase (Crompton et al., 1992). A recent report suggests that pro-oxidant-induced Ca^{2+} efflux may precede pore opening (Schlegel et al., 1992); therefore the mechanism of mitochondrial Ca^{2+} release is unclear, and the role of the mitochondrial pore in this efflux is uncertain.

The function of the mitochondrial pore is not known, but, as CsA protects cells against ischaemic and oxidative damage (Nazareth et al., 1991; Broekemeier et al., 1992; Crompton et al., 1992), pore opening may have a role in the pathological disruption of Ca^{2+} homoeostasis. Addition of 6HD and MPP together to Ca^{2+}-loaded mitochondria causes loss of accumulated Ca^{2+} (Frei and Richter, 1986), and in isolated hepatocytes MPP increases the overall cellular Ca^{2+} load (Di Monte et al., 1986) and decreases mitochondrial Ca^{2+} content (Kass et al., 1988). Furthermore, CsA protects hepatocytes against MPP toxicity (Snyder et al., 1992). Therefore disruption of mitochondrial Ca^{2+} homoeostasis, possibly by opening the mitochondrial pore, may account for some of the toxicity of MPP and 6HD.

This paper reports the effects of the neurotoxins 6HD and MPP on Ca^{2+} efflux from Ca^{2+}-loaded mitochondria. The effect of CsA on this efflux is discussed and experiments investigating the state of the mitochondrial pore during Ca^{2+} efflux, by a variety of criteria (membrane potential, sucrose permeation and swelling), are reported.

MATERIALS AND METHODS

Preparation of mitochondria

Fed female Wistar-derived rats (200–300 g) maintained on a standard diet were killed by cervical dislocation after stunning: liver mitochondria were prepared by homogenization, followed by differential centrifugation (Chappell and Hansford, 1972) in ice-cold medium containing 250 mM sucrose, 5 mM Tris and 1 mM EGTA adjusted to pH 7.4 (with HCl) at 25 °C. After the initial pelleting and washing, the mitochondria were washed in medium containing 250 mM sucrose and 5 mM Tris adjusted to pH 7.4 (with HCl) at 25 °C. Mitochondrial protein content was

Abbreviations used: CsA, Cyclosporin A; 6HD, 6-hydroxydopamine; MPP, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; TPMP, methyltriphenylphosphonium cation.

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determined by the biuret method, with BSA as a standard (Gornall et al., 1949). Mitochondria were stored on ice at 40–60 mg of protein/ml and used within 4 h of preparation.

Incubation of mitochondria with radioisotopes

Mitochondria (1 mg of protein) were suspended in 1 ml of incubation medium [195 mM mannitol, 65 mM sucrose and 3 mM Hepes (pH 7.4, with KOH)] in 1.5 ml plastic centrifuge tubes at 25 °C to which had been added rotenone (13 μM), $CaCl_2$ (70 μM), succinate (10 mM), various radioisotopes {$^{45}CaCl_2$, [^{3}H]methyltriphenylphosphonium cation ([^{3}H]TPMP) or ^3H_2O and [^{14}C]sucrose} and CsA (0.5 μM) or ethanol vehicle. The energized mitochondria accumulated Ca^{2+} for 2.5 min before the additions were made. After a further period of incubation, the mitochondria were pelleted by centrifugation at 10000 *g* for 1 min. Supernatant (400 μl) was removed, the mitochondrial pellet was dried and resuspended with 40 μl of Triton X-100 (20 %, v/v), and 350 μl of water was added to equalize quenching. Scintillant (3.8 ml of LKB Optiphase Hisafe II) was added to the samples, which were then counted for radioactivity on an LKB Rack-Beta liquid-scintillation counter by using appropriate quench and cross-over corrections.

Measurement of Ca^{2+} efflux

To measure mitochondrial Ca^{2+} efflux, $^{45}CaCl_2$ (210000–230000 d.p.m./ml) was added to the incubation medium and the $^{45}Ca^{2+}$ remaining in the mitochondrial pellet at various times was measured. Negligible Ca^{2+} was added with the radioisotope; the Ca^{2+} content of the mitochondrial preparation was 3.8 ± 0.4 nmol/mg of protein (mean ± S.E.M. for 3 preparations, determined by induction-coupled plasma optical-emission spectroscopy), and Ca^{2+} contamination of the incubation medium was negligible. Therefore the total Ca^{2+} content of an incubation was 73.8 nmol/ml. The amount of non-matrix Ca^{2+} in the pellet, which was determined by control experiments in the presence of excess carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; 333 nM) or Ruthenium Red (1 nM), was small (< 5 nmol of Ca^{2+}), does not affect the conclusions of this paper, and has not been subtracted from the data presented here.

Measurement of mitochondrial membrane potential

The mitochondrial membrane potential was determined from the distribution of the lipophilic cation TPMP across the mitochondrial inner membrane (Brown and Brand, 1985). After adding 5 μM TPMP containing 50 nCi/ml [^{3}H]TPMP to the incubation medium, the concentration of TPMP in the supernatant and pellet was determined as outlined above. Mitochondrial matrix volumes were determined in parallel as outlined below and were used to calculate the intramitochondrial concentration of TPMP. The membrane potential was calculated from the Nernst equation, with a correction for the 60 % non-specific intramitochondrial binding of TPMP (Brown and Brand, 1985).

Measurement of mitochondrial volume and sucrose permeation

The mitochondrial pellet spaces accessible to sucrose and water were determined by incubating mitochondria with [^{14}C]sucrose (100 nCi/ml) and ^3H_2O (0.5 μCi/ml) (Brown and Brand, 1985). Estimation of the mitochondrial matrix volume was based on the assumption that the mitochondrial inner membrane is impermeable to sucrose.

Measurement of mitochondrial swelling

Large-amplitude mitochondrial swelling was measured by monitoring the decrease in light-scattering of a mitochondrial suspension at 540 nm (Broekemeier et al., 1989). Mitochondria were suspended (1 mg/ml) in incubation medium containing rotenone (13 μM) and succinate (10 mM) in a 3 ml cuvette at 25 °C in a Pharmacia–LKB Ultrospec II u.v./visible spectrophotometer. After the absorption stabilized, swelling was assessed by adding 210 nmol of $CaCl_2$, followed by further additions of other compounds, as described in the Results and discussion section. The initial increase in 540 nm absorption due to autoxidation of 6HD was corrected for by using data from appropriate control experiments.

Materials

[^{3}H]TPMP bromide was supplied by NEN–Du Pont, and ^3H_2O, $^{45}CaCl_2$ and [^{14}C]sucrose were supplied by Amersham. MPP iodide was supplied by Research Biochemicals Inc. and 6HD hydrochloride was from Fluka. The 10 mM stock solutions of 6HD in water (pH 4–4.5) were prepared fresh each day, stored on ice and used within 3 h, over which time decomposition due to autoxidation was negligible. CsA was kindly given by Sandoz Pharma, Basel, Switzerland. All other chemicals were from Sigma, BDH or Boehringer–Mannheim.

RESULTS AND DISCUSSION

Efflux of mitochondrial Ca^{2+}

Addition of 6HD to Ca^{2+}-loaded mitochondria caused efflux of about 40 % of the mitochondrial Ca^{2+} within 7.5 min (Figure 1a). MPP enhanced the rate of 6HD-induced Ca^{2+} efflux, but MPP alone had no effect (Figure 1a). When this experiment was repeated with CsA present, the efflux of Ca^{2+} decreased substantially. Only the combined presence of MPP and 6HD resulted in detectable Ca^{2+} efflux (Figure 1b).

Since Ca^{2+} released by the mitochondria can be re-accumulated, it is likely that in these experiments Ca^{2+} was cycling across the mitochondrial inner membrane, dissipating the membrane potential. To prevent this, EGTA was added simultaneously with the neurotoxins (Figures 2a and 2b). Addition of EGTA alone caused a slow loss of Ca^{2+} from the mitochondria, presumably by reversal of Ca^{2+} uptake and the action of the normal Ca^{2+}-efflux pathway (Gunter and Pfeiffer, 1990). MPP had no effect on this efflux; however addition of 6HD, or 6HD and MPP together, caused a rapid efflux of Ca^{2+} (Figure 2a), similar to the effect in the absence of EGTA (Figure 1a). In the presence of EGTA, CsA completely prevented Ca^{2+} efflux caused by 6HD or MPP and 6HD (Figure 2b), in contrast with the partial inhibition obtained in its absence (Figure 1b).

These data (Figures 1 and 2) indicate that 6HD rapidly stimulates Ca^{2+} efflux from Ca^{2+}-loaded mitochondria. This finding contrasts with an earlier report that 6HD alone does not lead to Ca^{2+} efflux under Ca^{2+}-cycling or non-cycling conditions. The reason for the discrepancy is unclear, as the conditions used in the earlier study (Frei and Richter, 1986) appear similar to those reported here. As addition of a range of pro-oxidants to Ca^{2+}-loaded mitochondria leads to Ca^{2+} efflux (Crompton et al., 1987, 1992; Crompton and Costi, 1988; Carbonera and Azzone,

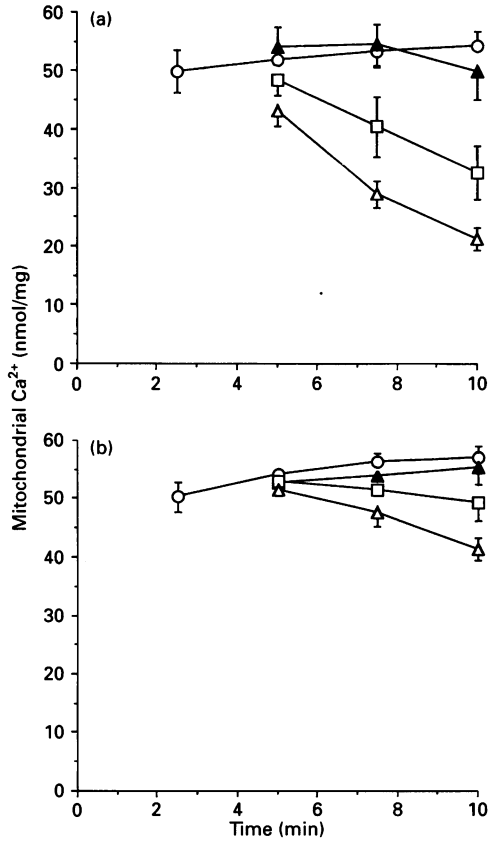

Figure 1 Effect of CsA on neurotoxin-induced release of mitochondrially accumulated Ca^{2+}

In (**a**) mitochondria were incubated as described in the Materials and methods section, and after 2.5 min either no addition was made (○), or MPP (0.5 mM; ▲), 6HD (0.5 mM; □) or MPP and 6HD together (both 0.5 mM; △) was added, and samples were taken at the time intervals indicated to measure the amount of Ca^{2+} remaining in the mitochondria. In (**b**) this experiment was repeated in the presence of CsA (0.5 μM). Each time point was done in triplicate, and the data shown here are means ± S.E.M. for five mitochondrial preparations.

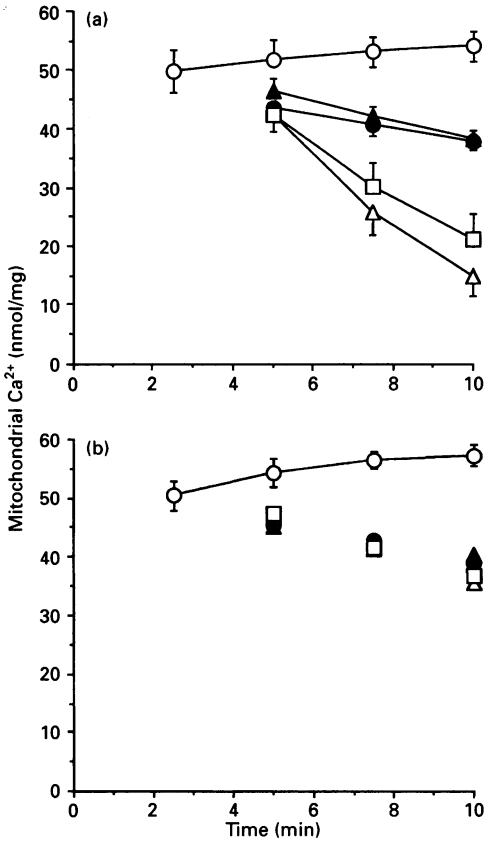

Figure 2 Effect of EGTA and CsA on neurotoxin-induced release of mitochondrially accumulated Ca^{2+}

In (**a**) mitochondria were incubated as described in the legend to Figure 1(a), and after 2.5 min incubation either no addition was made (○), or EGTA (potassium salt, pH 7; 0.5 mM; ●) on its own, MPP + EGTA (both 0.5 mM; ▲), 6HD + EGTA (both 0.5 mM; □) or MPP, 6HD + EGTA together (all three 0.5 mM; △) was added. In (**b**) this experiment was repeated in the presence of CsA (0.5 μM). For clarity, some error bars and interconnecting lines were omitted from this Figure. Each time point was done in triplicate, and the data shown here are means ± S.E.M. for five mitochondrial preparations.

1988; Gunter and Pfeiffer, 1990) and 6HD autoxidizes, producing superoxide, H_2O_2 and hydroxyl radicals (Cohen and Heikkila, 1974), induction of Ca^{2+} efflux by 6HD is consistent with the action of other pro-oxidants. The finding that addition of MPP and 6HD together, but not MPP alone, stimulated Ca^{2+} efflux is in agreement with the earlier report (Frei and Richter, 1986). The basis of MPP stimulation of 6HD-induced calcium efflux is unclear; it may be related to the decrease in membrane potential (Table 1) which occurred in the presence of MPP.

Our data showed that during Ca^{2+} cycling CsA blocked much of the stimulation of Ca^{2+} efflux by 6HD, or 6HD and MPP together, but, when Ca^{2+} cycling was prevented (by adding EGTA), CsA blocked efflux completely. Pro-oxidants cause Ca^{2+}-loaded mitochondria to lose Ca^{2+} and induce pore opening, both of which can be prevented by CsA (Crompton et al., 1987, 1992; Crompton and Costi, 1988; Carbonera and Azzone, 1988; Gunter and Pfeiffer, 1990). Therefore it seemed reasonable to assume that the Ca^{2+} efflux (Figures 1a and 2a) followed 6HD-induced opening of the mitochondrial pore and that the inhibition of Ca^{2+} efflux by CsA (Figures 1b and 2b) followed occlusion of this pore. To test this possibility, experiments were carried out to determine the state of the pore during Ca^{2+} efflux.

Mitochondrial swelling

Pro-oxidants such as t-butyl hydroperoxide open the mitochondrial pore in Ca^{2+}-loaded mitochondria, allowing entry of solutes, which causes them to swell (Broekemeier et al., 1989). Addition of phosphate (1 mM) to Ca^{2+}-loaded mitochondria induced pore opening, causing large amplitude swelling (half an absorbance unit over 3 min; results not shown) which was prevented by adding CsA (0.5 μM), in agreement with other reports (e.g. Broekemeier et al., 1989). If, instead of phosphate, MPP (1–10 mM), 6HD (0.5 mM) or MPP and 6HD together (both 0.5 mM) was added, there was no swelling (results not shown), suggesting that during Ca^{2+} efflux the mitochondrial pore was closed.

Measurement of mitochondrial membrane potential

Opening the pore depolarizes the mitochondrial inner membrane (e.g. Al-Nasser and Crompton, 1986). The mitochondrial membrane potential after 2.5 min accumulation of Ca^{2+} was relatively low, presumably due to Ca^{2+} cycling and accumulation, and was unaffected by CsA (Table 1). When Ca^{2+} cycling was prevented by EGTA, the membrane potential increased, as expected. MPP

Table 1 Effect of MPP, 6HD and CsA on mitochondrial membrane potential

Mitochondria were incubated in the presence or absence of CsA (0.5 μM) as described in the Materials and methods section, and after 2.5 min incubation, during which time the mitochondria accumulated Ca^{2+}, the membrane potential was measured. At this point, nothing, MPP (0.5 mM), 6HD (0.5 mM), MPP and 6HD together (both 0.5 mM), EGTA (K salt, pH 7; 0.5 mM) alone, or EGTA with MPP (0.5 mM), 6HD (0.5 mM) or MPP and 6HD together (both 0.5 mM) was added and the membrane potential was measured after a further 7.5 min incubation. For some experiments Ca^{2+} was omitted from the incubation medium. Each determination was the mean of three incubations, and the data here are the mean ± S.E.M. for three mitochondrial preparations. The mitochondrial matrix volumes used to calculate membrane potentials in the presence of calcium were taken from Table 2. For calculating the membrane potential in the absence of Ca^{2+}, the mitochondrial matrix volume was assumed to be 0.5 μl/mg of protein. The significance of the effect of CsA was tested by Student's *t* test: $^*P < 0.05$.

		Membrane potential (mV)			
		$+Ca^{2+}$		$-Ca^{2+}$	
Additions	Incubation time (min)	−CsA	+CsA	−CsA	+CsA
None	2.5	96 ± 7	97 ± 9	180 ± 7	179 ± 6
None	10	129 ± 6	121 ± 5	182 ± 8	181 ± 5
MPP	10	115 ± 4	112 ± 3	165 ± 7	163 ± 7
6HD	10	103 ± 5	111 ± 8	158 ± 8	160 ± 7
MPP + 6HD	10	96 ± 4	98 ± 8	150 ± 9	150 ± 7
EGTA	10	141 ± 2	144 ± 1	175 ± 2	180 ± 3
EGTA + MPP	10	129 ± 2	126 ± 5	163 ± 1	163 ± 2
EGTA + 6HD	10	139 ± 3	137 ± 3	178 ± 1	179 ± 3
EGTA + MPP + 6HD	10	137 ± 2	*127 ± 3	163 ± 3	162 ± 2

lowered the membrane potential slightly in the presence or absence of Ca^{2+} cycling (Table 1), but this decrease was also seen in the absence of Ca^{2+} loading (Table 1). 6HD also lowered the membrane potential under conditions of Ca^{2+} cycling, but only had a marginal effect when EGTA was present (Table 1). This effect was also seen in mitochondria not subjected to calcium loading (Table 1). MPP and 6HD together had a substantial effect on the membrane potential in the presence or absence of EGTA. However, Table 1 shows that this also occurred in the absence of Ca^{2+} loading.

It is noteworthy that in the presence or absence of Ca^{2+} cycling CsA had no effect on membrane potential under conditions where 6HD caused Ca^{2+} efflux (Table 1 and Figures 1a and 2a). This suggested that the CsA block of 6HD-induced Ca^{2+} efflux (Figures 1b and 2b) occurred without preventing inactivation of the mitochondrial pore. In summary, measurements of mitochondrial membrane potential indicate that 6HD-induced Ca^{2+} efflux occurs when the mitochondrial pore is closed and therefore that the inhibition of efflux by CsA does not follow prevention of pore opening.

Mitochondrial volumes

In measuring the membrane potential, it was assumed that the mitochondria comprised a homogeneous population. Ca^{2+} efflux may result from transient opening of the pore, followed rapidly by closing of the pore and repolarization of the mitochondria. Bulk measurements of membrane potential might not reveal such transient changes. To test the possibility of transient pore opening, the exclusion of radiolabelled sucrose by mitochondria was examined. Since sucrose is known to equilibrate rapidly across the mitochondrial inner membrane through the open pore (Crompton and Costi, 1988), radiolabelled sucrose added to a

Table 2 Effects of MPP, 6HD and CsA on mitochondrial volume

Mitochondria were incubated with [^{14}C]sucrose and ^3H_2O as described in the Materials and methods section. Each determination was mean of six individual incubations, and the data here are means ± S.E.M. for experiments on three separate mitochondrial preparations.

		Mitochondrial volume (μl/mg of protein)	
Additions	Incubation time (min)	−CsA	+CsA
No addition	2.5	0.95 ± 0.03	0.90 ± 0.06
No addition	10	0.74 ± 0.14	0.77 ± 0.09
MPP	10	0.83 ± 0.24	0.84 ± 0.20
6HD	10	0.80 ± 0.12	0.95 ± 0.16
MPP + 6HD	10	0.88 ± 0.10	0.99 ± 0.14
EGTA	10	0.65 ± 0.05	0.79 ± 0.06
EGTA + MPP	10	0.87 ± 0.03	0.88 ± 0.06
EGTA + 6HD	10	0.65 ± 0.10	0.81 ± 0.07
EGTA + MPP + 6HD	10	0.50 ± 0.06	0.87 ± 0.13

mitochondrial incubation in which the pore opens transiently would be gradually accumulated into the mitochondrial matrix. An apparent decrease in mitochondrial volume (measured by sucrose exclusion) would ensue even if the pore opened only transiently during Ca^{2+} efflux (Crompton and Costi, 1988).

Mitochondrial [^{14}C]sucrose- and ^3H_2O-accessible spaces were measured under the same conditions as for Ca^{2+} efflux and membrane potential. Table 2 shows that during calcium cycling the effects of MPP, 6HD or MPP and 6HD together on volume were marginal and that CsA did not have a substantial effect on the volume. When Ca^{2+} cycling was prevented by addition of EGTA, CsA caused small volume increases both in the control incubation and in the presence of 6HD, indicating that CsA prevented Ca^{2+} efflux without affecting the permeation of sucrose into the mitochondrial matrix. The volume measurements show that Ca^{2+} efflux induced by 6HD occurred without an increase in the permeation of sucrose into the mitochondrial matrix. There is still the possibility of pore opening to a minor extent during Ca^{2+} efflux induced by 6HD, or by 6HD and MPP together.

Summary and general discussion

The efflux of Ca^{2+} from Ca^{2+}-loaded mitochondria is increased by 6HD, both alone and in the presence of MPP, in a CsA-sensitive manner. These data suggest that the protective effect of CsA against MPP in hepatocytes (Snyder et al., 1992) may have been due in part to prevention of loss of mitochondrial Ca^{2+} homoeostasis. The efflux and blockage occurred when Ca^{2+} cycled across the mitochondrial inner membrane, and more so when Ca^{2+} cycling was prevented by addition of EGTA. The efflux occurred in the absence of mitochondrial swelling, depolarization or accumulation of sucrose and, although CsA blocked Ca^{2+} efflux, it did not affect these parameters. This surprising and novel finding indicates the likelihood that CsA prevents Ca^{2+} efflux, not by preventing pore opening but by some other mechanism.

It is known that addition of the pro-oxidant t-butyl hydroperoxide to Ca^{2+}-loaded mitochondria causes Ca^{2+} release in the absence of pore opening (Schlegel et al., 1992); however, the effect of CsA on this efflux was not tested. The findings shown here are consistent with the report that the release of Ca^{2+} from Ca^{2+}-loaded mitochondria induced by the pro-oxidant *N*-acetyl-*p*-benzoquinone imine could be blocked by CsA (Weis et

al., 1992), whereas ATP was retained, suggesting that the pore was closed during Ca^{2+} efflux. These reports, and the data from the present study, are consistent with the recent proposal that mitochondrial pore opening requires Ca^{2+} and is influenced by matrix pH, and that collapse of the membrane potential is a cause, not a consequence, of pore opening (Bernardi, 1992; Bernardi et al., 1993; Petronilli et al., 1993). However, the interaction of CsA with the pore and the mechanism by which it prevents pro-oxidant-induced calcium efflux is unclear. Perhaps the mitochondrial matrix peptidyl-prolyl *cis–trans* isomerase (Connern and Halestrap, 1992) inhibited by CsA has separate targets; the pore and additional membrane protein(s) which allow Ca^{2+} efflux without depolarizing mitochondria. Alternatively, the mitochondrial pore may have a number of conformations, intermediate between fully open and closed, which allows Ca^{2+}, but not other solutes, to pass through the membrane (Novgorodov et al., 1992).

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