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# Calcineurin-independent inhibition of mitochondrial $Ca^{2+}$ uptake by cyclosporin A

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**1** Cyclosporin A (CsA) is a widely used compound because of its potent immunosupressive properties, derived mainly from the inhibition of calcineurin, and also because of its ability to block the mitochondrial permeability transition pore (PTP). This second effect has been involved in the protection against apoptosis mediated by release of mitochondrial factors. We show here that CsA (1–10$\mu$M) has an additional effect on $Ca^{2+}$ homeostasis in mitochondria that cannot be attributed to inhibition of PTP.

**2** By measuring specifically mitochondrial [$Ca^{2+}$] with targeted aequorin, we show that CsA inhibited $Ca^{2+}$ entry into mitochondria both in intact and in permeabilized cells, and this effect was stronger when $Ca^{2+}$ entry was triggered by low cytosolic [$Ca^{2+}$], below 5 $\mu$M.

**3** Inhibition of mitochondrial $Ca^{2+}$ uptake required micromolar concentrations of CsA and was not mimicked by other inhibitors of calcineurin such as FK-506 or cypermethrin, nor by a different inhibitor of the PTP, bongkrekic acid.

**4** CsA blocked the increase in mitochondrial $Ca^{2+}$ uptake rate induced by the mitochondrial $Ca^{2+}$ uniporter activator SB202190.

**5** Our results suggest that CsA inhibits $Ca^{2+}$ entry through the $Ca^{2+}$ uniporter by a mechanism independent of the inhibition of PTP or calcineurin. This effect may contribute to reduce depolarization and $Ca^{2+}$ overloading in mitochondria after cell stimulation, and thus cooperate with the direct inhibition of PTP to prevent apoptosis.
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**Keywords:** aequorin, mitochondria; histamine; cyclosporin A; permeability transition pore; $Ca^{2+}$ uniporter; HeLa; apoptosis

**Abbreviations:** $[Ca^{2+}]_c$, cytosolic [$Ca^{2+}$]; $[Ca^{2+}]_M$, mitochondrial [$Ca^{2+}$]; CsA, cyclosporin A; PTP, permeability transition pore

## Introduction

Mitochondrial $Ca^{2+}$ homeostasis is controlled by several mechanisms of $Ca^{2+}$ transport present in the inner membrane of the organelle (Bernardi, 1999; Duchen, 2000; Rizzuto *et al.*, 2000). The main pathway for $Ca^{2+}$ influx into mitochondria is the $Ca^{2+}$ uniporter, a specific and reversible $Ca^{2+}$ transporter that under physiological conditions mediates entry of $Ca^{2+}$ into mitochondria driven by the negative electrical potential of the matrix. This uniporter becomes activated only in the presence of micromolar concentrations of $Ca^{2+}$ in the cytosolic side, and is therefore closed under resting conditions. The presence of a different mechanism, named rapid uptake mode, adapted to sequester $Ca^{2+}$ from trains of physiological $Ca^{2+}$ transients has also been suggested (Gunter *et al.*, 1998), although no direct evidence for this mechanism has been obtained in intact cells. Both the $Ca^{2+}$ uniporter and the rapid uptake mode are inhibited by ruthenium red.

$Ca^{2+}$ exit from mitochondria is carried out by both a $Na^+$/$Ca^{2+}$ exchanger and a $H^+$/$Ca^{2+}$ exchanger, and under some conditions, by the opening of the permeability transition pore (PTP). This is a complex system whose molecular structure is still unclear and may include a series of constituents such as the voltage-dependent anion channel, the adenine nucleotide transporter, cyclophilin D and others (Bernardi, 1999; Crompton, 1999; Kim *et al.*, 2003). Cyclosporin A (CsA) is a well known and potent inhibitor of the PTP, producing full inhibition at concentrations in the submicromolar range (Bernardi *et al.*, 1994). It binds to cyclophilin D with high affinity and probably prevents its association with the adenine nucleotide transporter (Crompton, 1999). However, this effect of CsA is not specific, and this compound affects other cellular processes, some of them also related to $Ca^{2+}$ homeostasis. It is well known, for example, that immunosupression induced by CsA depends on the inhibition of calcineurin by complexes of CsA and cytosolic cyclophilins (Ivery, 2000; Matsuda & Koyasu, 2000). This results in inhibition of $Ca^{2+}$-dependent signal transduction pathways in a range of cell types, including transcription of early response cytokines, such as IL-2, responsible for initiating the early proliferative signal in helper T cells. In addition, CsA may produce other effects probably via binding to different proteins of the family of the cyclophilins (Matsuda & Koyasu, 2000; Tran *et al.*, 2000; Cristillo & Bierer, 2002). Some of these effects appear at higher, micromolar, concentrations of CsA, and in some

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studies clearly different effects are obtained when low (submicromolar) or high (micromolar) concentrations of CsA are used (Griffiths *et al.*, 2000).

The aim of this study has been to test if some of these multiple effects of CsA could be explained by a direct action of this compound on mitochondrial $Ca^{2+}$ uptake. We show here that micromolar concentrations of CsA inhibit $Ca^{2+}$ entry into mitochondria through the $Ca^{2+}$ uniporter, an effect that was not mimicked by other inhibitors of calcineurin. This effect is important because it may contribute to, or even mimic, some of the consequences of the direct inhibition of the PTP, such as inhibition of mitochondrial depolarization after stimulus or indirect inhibition of PTP opening due to reduced $Ca^{2+}$ entry into mitochondria.

# Methods

### *Cell culture and transfection*

The construction strategy of the mutated mitochondrially targeted aequorin chimera has been described previously (Montero *et al.*, 2000). HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. The HeLa cell clone MM5, which stably expresses mitochondrially targeted mutated aequorin, has been described previously (Montero *et al.*, 2002). Similar data were obtained using wild-type cells transiently transfected with the mitmutAEQ/pcDNA3.1 plasmid. The construct for cytosolic aequorin has also been described previously (Montero *et al.*, 2002). It was cloned into the pcDNA3.1. plasmid and used to transfect wild-type HeLa cells. Transfections were carried out using Metafectene (Biontex, Munich, Germany).

### *Mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_M$) and cytosolic $[Ca^{2+}]$($[Ca^{2+}]_c$) measurements*

The HeLa cell clone MM5 was used for $[Ca^{2+}]_M$ measurements. $[Ca^{2+}]_c$ measurements were carried out using HeLa cells transiently transfected with the plasmid for cytosolic aequorin. Cells were plated onto 13 mm round coverslips. For aequorin reconstitution, HeLa cells expressing cytosolic aequorin were incubated for 1–2 h at room temperature with 1 $\mu$M of wild-type coelenterazine, and cells expressing mitochondrially targeted mutated aequorin were incubated for 1–2 h at room temperature with either 1 $\mu$M of wild-type coelenterazine or 1 $\mu$M of coelenterazine n, in standard medium containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES, pH 7.4. Cells were then placed in the perfusion chamber of a purpose-built luminometer thermostatized at 37°C. For the experiments with permeabilized cells, mitochondrially targeted mutated aequorin was reconstituted with coelenterazine n, in order to reduce its $Ca^{2+}$-affinity and allow measurement of higher $[Ca^{2+}]_M$. Then, standard medium containing 0.5 mM EGTA instead of $Ca^{2+}$ was perfused for 1 min, followed by 1 min of intracellular medium (130 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM $K_3PO_4$, 0,5 mM EGTA, 1 mM ATP, 20 $\mu$M ADP, 2 mM succinate, 20 mM HEPES, pH 7) containing 100 $\mu$M digitonin. Then, intracellular medium without digitonin was perfused for 1 min, followed by buffers of known $[Ca^{2+}]$ between 2,5 and 10 $\mu$M, prepared in intracellular medium using HEDTA/$Ca^{2+}$/$Mg^{2+}$ mixtures.

### *Materials*

Wild-type coelenterazine and coelenterazine n were obtained from Molecular Probes (O, U.S.A.). CsA was from Novartis (Basil, Switzerland). SB202190 and cypermethrin were from Tocris, Bristol (U.K.). Bongkrekic acid was from Sigma (Madrid). FK-506 was from LC Laboratories (Woburn, MA, U.S.A.). Other reagents were from Sigma (Madrid) or Merck, (Darmstadt).

### *Statistical analysis*

Averaged data are means $\pm$ s.e.m. Analysis of variance (ANOVA) was applied to see differences between groups. The level of significance is indicated as: *, $P<0.05$; **, $P<0.005$; ***, $P<0.0005$.

# Results

We have studied the effects of CsA on both the mitochondrial and the cytosolic $[Ca^{2+}]$ transient induced by histamine in HeLa cells. Figure 1A shows that the $[Ca^{2+}]_M$ transient was reduced in the presence of 10 $\mu$M CsA. In seven similar experiments, the $[Ca^{2+}]_M$ peak in cells treated with CsA was $46\pm6\%$ (mean$\pm$s.e.m.) of the control peak. Instead, as shown in Figure 1B, the $[Ca^{2+}]_c$ peak was not modified by CsA. In 10 similar experiments, the $[Ca^{2+}]_c$ peak in cells treated with CsA was $105\pm7\%$ (mean$\pm$s.e.m.) of the control peak. These results cannot be explained by inhibition by CsA of the PTP. Rather, in case the PTP opens during histamine stimulation, we would expect CsA to increase the $[Ca^{2+}]_M$ peak or perhaps

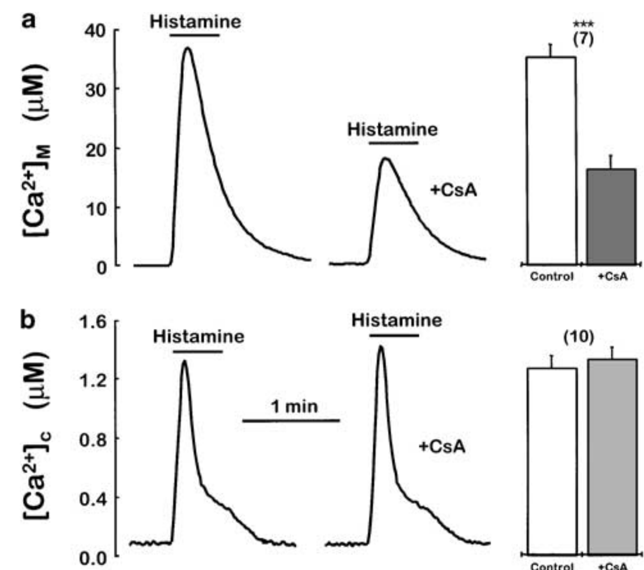


**Figure 1** Effect of CsA on the $[Ca^{2+}]_M$ and $[Ca^{2+}]_c$ peaks induced by histamine. HeLa cells expressing either mutated mitochondrially targeted aequorin (upper panels) or cytosolic aequorin (lower panels) were reconstituted with native coelenterazine. Then, they were stimulated with 100 $\mu$M histamine either in control cells or in cells incubated with 10 $\mu$M CsA for 5 min prior and during stimulation (+CsA). Experiments are representative of seven to 10 similar ones. The right part of the figure shows mean results indicating number of experiments and significance (***, $P<0.0005$).

to reduce the rate of return to resting levels. The inhibition of the $[Ca^{2+}]_M$ peak observed suggest instead that CsA is reducing $Ca^{2+}$ uptake into mitochondria.

To obtain further information on this phenomenon, we studied directly the effect of CsA on mitochondrial $Ca^{2+}$ uptake in permeabilized cells. Figure 2 shows that perfusion of a controlled $[Ca^{2+}]$ in permeabilized cells induced an increase in $[Ca^{2+}]_M$, and that subsequent addition of CsA was able to stop $[Ca^{2+}]_M$ increase and reduce $[Ca^{2+}]_M$. This effect was only partially reversible after washing of CsA, perhaps because of the strong lipophilic character of the CsA molecule. Figure 3 shows that CsA inhibited $Ca^{2+}$ uptake by mitochondria induced by a range of $[Ca^{2+}]_c$ concentrations, from 2.5 to 10 $\mu$M, although the effects were stronger at lower $[Ca^{2+}]_c$. It can be appreciated that CsA produced a large decrease in the steady-state $[Ca^{2+}]_M$ when solutions containing 2.5–3.5 $\mu$M $[Ca^{2+}]_c$ were perfused, but a much smaller effect at $[Ca^{2+}]_c$ above 5 $\mu$M. Similar findings are obtained by measuring the rates of $Ca^{2+}$ uptake at each $[Ca^{2+}]_c$. While CsA reduced the uptake rate by 80% at 2.5 $\mu$M $[Ca^{2+}]_c$, only a 42% inhibition was observed at 10 $\mu$M $[Ca^{2+}]_c$. Figure 4 shows the mean rates of uptake obtained in several experiments similar to those of Figure 3, and the percentages of inhibition of the $Ca^{2+}$ uptake rate obtained at each $[Ca^{2+}]$.

The effects of CsA shown above required micromolar concentrations of the inhibitor. Figure 5 shows the dose–response effect of CsA on the inhibition of mitochondrial $Ca^{2+}$ uptake. The upper panel shows a typical experiment in which the effect of several CsA concentrations was tested on mitochondrial $Ca^{2+}$ uptake in permeabilized cells. The lower panel shows the mean inhibition of the final steady-state $[Ca^{2+}]_M$ obtained for every CsA concentration in several similar experiments. Considering the inhibition obtained at 10 $\mu$M CsA as the maximum inhibition, the $IC_{50}$ was about 1 $\mu$M.

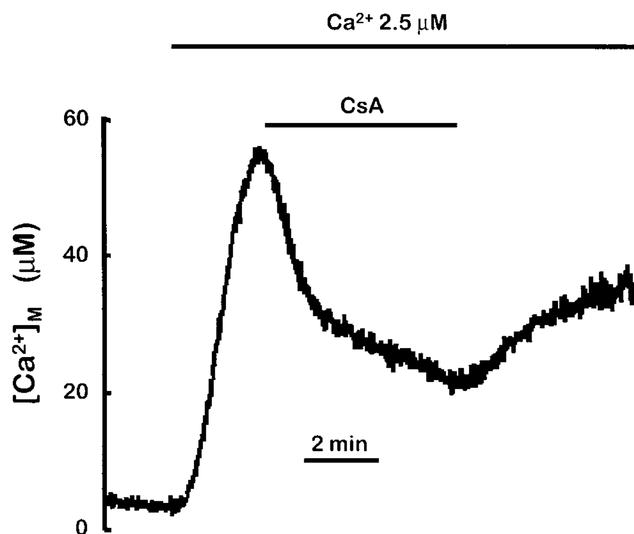


**Figure 2** Effect of CsA on $[Ca^{2+}]_M$ in permeabilized cells. Cells expressing mutated mitochondrially targeted aequorin were reconstituted with coelenterazine n. Then they were permeabilized as described in Methods and a $Ca^{2+}$ buffer containing 2.5 $\mu$M $[Ca^{2+}]$ was perfused as indicated in the figure. Then, after 2 min, 10 $\mu$M CsA was perfused in the same buffer as indicated. This experiment is representative of eight similar ones.

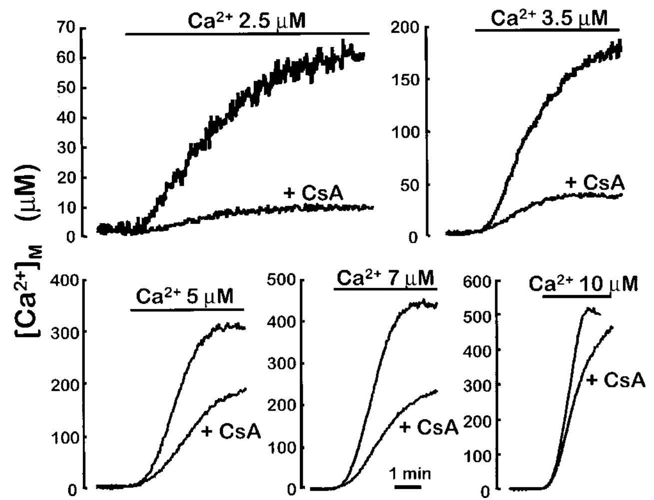


**Figure 3** Effect of CsA on $[Ca^{2+}]_M$ in permeabilized cells at different $[Ca^{2+}]_c$. Cells expressing mutated mitochondrially targeted aequorin were reconstituted with coelenterazine n. Then they were permeabilized as described in Methods and $Ca^{2+}$ buffers containing 2.5–10 $\mu$M $[Ca^{2+}]$ were perfused as indicated in the figure. In the traces labelled '+CsA', cells were treated with 10 $\mu$M CsA for 5 min prior and during perfusion of the $Ca^{2+}$ buffer. Experiments are representative of five to nine similar ones of each kind, and statistics is given in Figure 4.

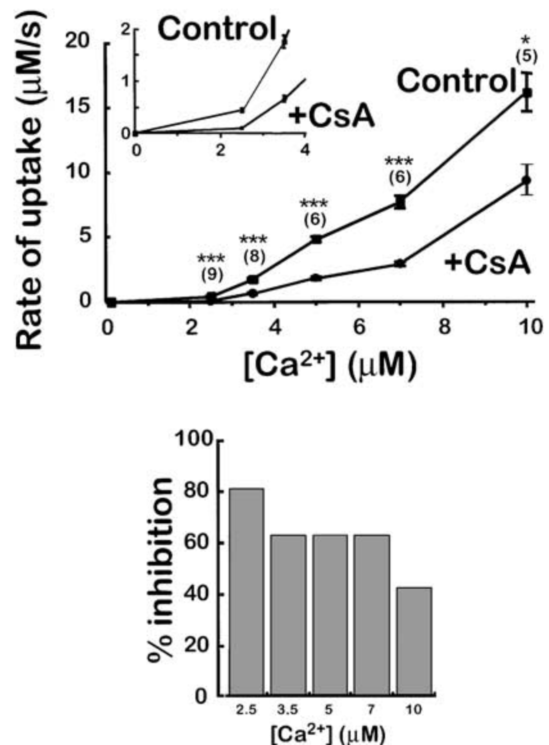


**Figure 4** Dependence on $[Ca^{2+}]_c$ of the inhibition of $Ca^{2+}$ uptake by CsA. The figure shows mean $Ca^{2+}$ uptake rates obtained from five to nine experiments similar to those of Figure 3, performed at each $[Ca^{2+}]_c$. The inset shows expanded effects of the lower $[Ca^{2+}]_c$. Error bars show s.e.m. Number of experiments for each condition and significance of the inhibition by CsA with respect to the controls in each case are also indicated (*, $P<0.05$; ***, $P<0.0005$). The lower part of the figure shows the percentage of inhibition of the $Ca^{2+}$ uptake rate obtained at each $[Ca^{2+}]$.

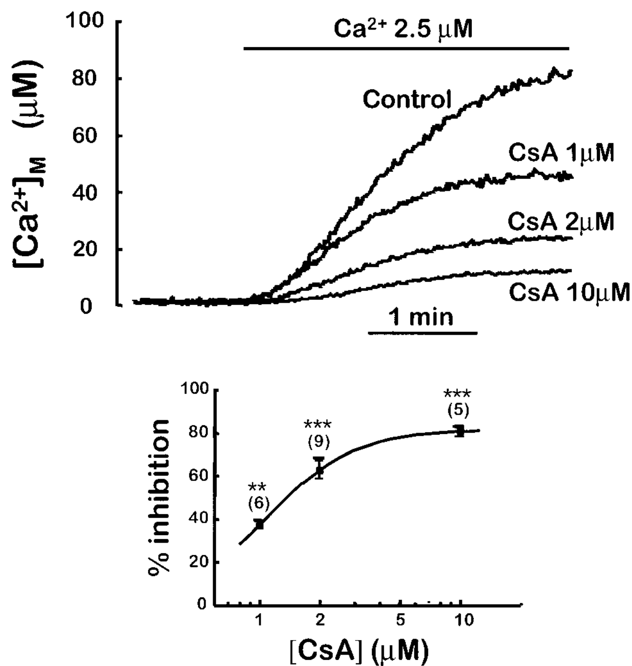


**Figure 5** Dose dependence of the inhibition of $[Ca^{2+}]_m$ uptake by CsA. Cells expressing mutated mitochondrially targeted aequorin were reconstituted with coelenterazine n. Then, they were permeabilized as described in Methods and a $Ca^{2+}$ buffer containing 2.5 $\mu$M $[Ca^{2+}]$ was perfused as indicated in the figure. The upper panel shows typical experiments of $Ca^{2+}$ uptake in the absence or in the presence of different concentrations of CsA. The traces labelled 'CsA' show experiments in which cells were treated with either 1, 2 or 10 $\mu$M CsA for 5 min prior and during perfusion of the $Ca^{2+}$ buffer. Experiments are representative of five to nine similar ones of each kind. The lower panel shows the mean data obtained from all of these experiments. Error bars show s.e.m. Number of experiments for each condition and significance of the inhibition by each CsA concentration with respect to the control are also indicated (**, $P<0.005$; ***, $P<0.0005$).

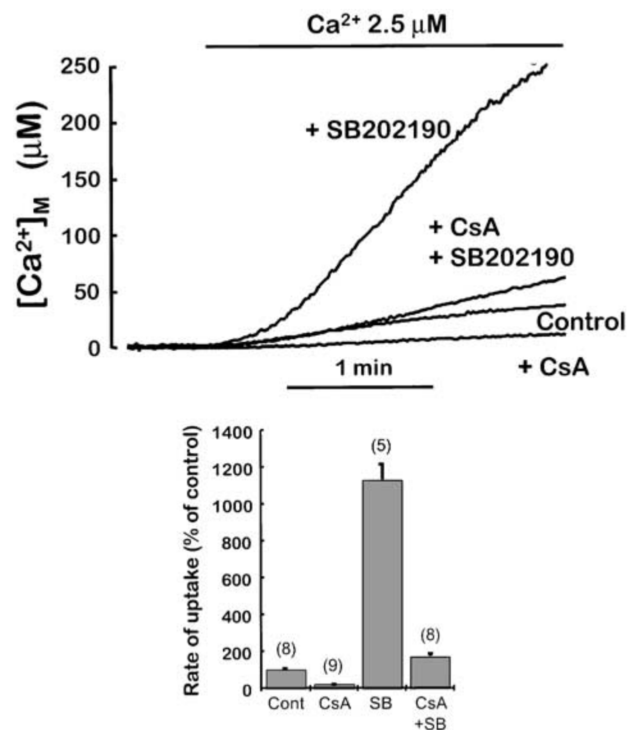


**Figure 6** Effect of CsA and SB202190 on $[Ca^{2+}]_m$ uptake. Cells expressing mutated mitochondrially targeted aequorin were reconstituted with coelenterazine n. Then, they were permeabilized as described in Methods and a $Ca^{2+}$ buffer containing 2.5 $\mu$M $[Ca^{2+}]$ was perfused as indicated in the figure. The traces labelled '+SB202190', '+CsA' and '+CsA+SB202190' were treated with either 10 $\mu$M SB202190, 10 $\mu$M CsA or both for 5 min prior and during perfusion of the $Ca^{2+}$ buffer. Experiments are representative of eight to nine similar ones of each kind. The lower part of the figure shows mean results indicating number of experiments and s.e.m.

Finally, we compared the effect of the simultaneous treatment of the cells with CsA and with the activator of the mitochondrial $Ca^{2+}$ uniporter SB202190. Figure 6 shows that SB202190 produced a large increase in the mitochondrial $Ca^{2+}$ uptake, as previously reported (Montero *et al*., 2002), and CsA reduced mitochondrial $Ca^{2+}$ uptake, as shown above. Then, when both compounds were added together, $Ca^{2+}$ uptake by mitochondria returned to be close to control values. SB202190 is a protein kinase inhibitor, apparently specific for p38 mitogen-associated protein kinase (Davies *et al*., 2000). Thus, we have suggested that a protein kinase may constitutively be reducing the activity of the uniporter, so that in the presence of the inhibitor a phosphatase would reverse the effect and increase $Ca^{2+}$ uptake (Montero *et al*., 2002). However, the phosphatase activity of calcineurin was not involved in this process, as other inhibitors of calcineurin different from CsA, such as FK506 or cypermethrin, produced no significant effect on mitochondrial $Ca^{2+}$ uptake in permeabilized cells. The rate of mitochondrial $Ca^{2+}$ uptake was $101\pm4\%$ (mean$\pm$s.e.m, $n=3$) of the controls in the presence of 10 $\mu$M FK506, and $108\pm7\%$ (mean$\pm$s.e.m, $n=6$) of the controls in the presence of 2 $\mu$M cypermethrin. Similarly, other inhibitors of the PTP such as bongkrekic acid also produced no effect on $[Ca^{2+}]_m$ uptake (the rate of uptake was $104\pm11\%$ (mean$\pm$s.e.m, $n=6$) of the controls in the presence of 5 $\mu$M bongkrekic acid), showing that inhibition of the PTP is neither involved in the inhibition of $[Ca^{2+}]_m$ uptake by CsA.

## Discussion

CsA is a cyclic undecapeptide that has been widely used in the treatment of allograft rejection and graft *versus* host disease because of its potent immunosuppresive properties. There is evidence that this effect relies mainly on the inhibition of calcineurin by a complex of CsA and cytosolic cyclophilins, although other mechanisms may also be involved (Ivery, 2000; Matsuda & Koyasu, 2000). However, in spite of its efficacy for prevention of organ transplant rejection, the use of CsA as an immunosuppressant is limited by the presence of severe side effects including nephrotoxicity, neurotoxicity, hepatotoxicity and promotion of cancer progression (Matsuda & Koyasu, 2000). These effects could depend on the action of CsA on other intracellular targets, such as the mitochondrial permeability transition pore PTP. Inhibition of PTP by CsA has been shown to prevent release of proapoptotic factors from mitochondria and so protect cells against apoptosis induced by different mechanisms. In fact, CsA is widely used today to

test the involvement of the PTP in apoptosis (Di Lisa & Bernardi, 1998; Crompton, 1999; Duchen, 2000; Bernardi *et al.*, 2001).

We show in this paper an additional effect of CsA on mitochondria, which is the inhibition of $Ca^{2+}$ uptake through the $Ca^{2+}$ uniporter. This transport system constitutes the main $Ca^{2+}$ influx pathway of mitochondria and its activation is strongly dependent on the cytosolic $[Ca^{2+}]$ in the low micromolar range, which is the physiological range of $[Ca^{2+}]$ reached during cell stimulation. CsA shifts the curve of activation by $Ca^{2+}$ of the uniporter to the right, reducing $Ca^{2+}$ entry into mitochondria during $[Ca^{2+}]_c$ transients. This effect cannot be explained by the inhibition of PTP, because this pathway can only mediate $Ca^{2+}$ release from mitochondria and not $Ca^{2+}$ influx. In fact, inhibition of PTP should increase mitochondrial membrane potential and reduce $Ca^{2+}$ efflux from mitochondria, leading to increased levels of $Ca^{2+}$ in mitochondria. On the other hand, the $Ca^{2+}$ entry into mitochondria we measure is fully blocked by ruthenium red (Montero *et al.*, 2000), while the PTP is not sensitive to this compound (see Bernardi, 1999), and a different inhibitor of PTP, bongkrekic acid, had no effect on $Ca^{2+}$ entry. Finally, the inhibition of $Ca^{2+}$ entry that we show in this paper occurs at micromolar concentrations of CsA, with an $IC_{50}$ around 1 $\mu$M, while inhibition by CsA of the PTP takes place at concentrations at least one order of magnitude lower (see Bernardi *et al.*, 1994 and references therein). As a conclusion, CsA inhibits $Ca^{2+}$ entry by a mechanism clearly different than that responsible of inhibition of PTP. Regarding that mechanism, we show that CsA antagonized the effect of SB202190. This inhibitor of p38 mitogen-associated protein kinase strongly activates $Ca^{2+}$ uptake by mitochondria at low $[Ca^{2+}]_c$ (Montero *et al.*, 2002), an effect which is opposite to that found here for CsA. We reasoned that, if the effect of SB202190 was mediated by protein kinase inhibition, that of CsA could be mediated by inhibition of a phosphatase such as calcineurin. However, this was not the case, as the concentrations of CsA required to inhibit $Ca^{2+}$ entry were higher than those necessary to block calcineurin, and other inhibitors of calcineurin, such as FK-506 or cypermethrin produced no effect on $Ca^{2+}$ entry into mitochondria.

The new effect of CsA described here may be relevant to interpret some of the actions of this compound. Although inhibition of the PTP by CsA occurs at lower concentrations, most studies dealing with the effect of CsA on PTP use concentrations in the micromolar range (Ichas *et al.*, 1997; Smaili *et al.*, 2001; Brustovetsky *et al.*, 2002; Levy *et al.*, 2003). Under these conditions, CsA may be acting both as PTP blocker and as inhibitor of $Ca^{2+}$ entry. Interestingly, inhibition of $Ca^{2+}$ entry would be expected to produce some effects similar to those of inhibition of PTP, for example, to reduce mitochondrial depolarization after cell stimulation (that induced by $Ca^{2+}$ entry) and to inhibit indirectly the opening of PTP *via* a reduction in the $[Ca^{2+}]_M$ levels. In contrast, direct inhibition of PTP should inhibit also mitochondrial depolarization (that induced by PTP opening), but should lead to an increase in the $[Ca^{2+}]_M$ levels. This double effect of CsA, inhibition of PTP at low concentrations and of $Ca^{2+}$ entry at higher concentrations, may be responsible for the discrepancies in the effects of CsA on $[Ca^{2+}]_M$ found by different authors. It has been shown that low concentrations of CsA increased $[Ca^{2+}]_M$ after reoxygenation injury of cardiomyocytes, while high doses reduced it (Griffiths *et al.*, 2000). In contrast, 5–10 $\mu$M CsA has been found to increase $[Ca^{2+}]_m$ uptake and buffering (Smaili *et al.*, 2001; Levy *et al.*, 2003). An additional factor that may contribute to these discrepancies is the $[Ca^{2+}]_c$ dependence of the effect we show in this paper. The inhibition of $Ca^{2+}$ entry by CsA occurs mainly at low physiological $[Ca^{2+}]_c$, and may not be detectable when $[Ca^{2+}]_m$ uptake is triggered by higher $[Ca^{2+}]_c$. In conclusion, we show here a new effect of CsA that should be taken into account to interpret the effects of this compound on $[Ca^{2+}]_m$ homeostasis, PTP opening and apoptosis.

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