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Effects of Decreasing Mitochondrial Volume on the Regulation of the Permeability Transition Pore

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The permeability transition pore (PTP) is a Ca^{2+}-sensitive mitochondrial inner membrane channel involved in several models of cell death. Because the matrix concentration of PTP regulatory factors depends on matrix volume, we have investigated the role of the mitochondrial volume in PTP regulation. By incubating rat liver mitochondria in media of different osmolarity, we found that the Ca^{2+} threshold required for PTP opening dramatically increased when mitochondrial volume decreased relative to the standard condition. This shrinkage-induced PTP inhibition was not related to the observed changes in protonmotive force, or pyridine nucleotide redox state and persisted when mitochondria were depleted of adenine nucleotides. On the other hand, mitochondrial volume did not affect PTP regulation when mitochondria were depleted of Mg^{2+}. By studying the effects of Mg^{2+}, cyclosporin A (CsA) and ubiquinone 0 (Ub_0) on PTP regulation, we found that mitochondrial shrinkage increased the efficacy of Mg^{2+} and Ub_0 at PTP inhibition, whereas it decreased that of CsA. The ability of mitochondrial volume to alter the activity of several PTP regulators represents a hitherto unrecognized characteristic of the pore that might lead to a new approach for its pharmacological modulation.

KEY WORDS: Mitochondria; volume; permeability transition pore; magnesium; cyclosporin; ubiquinone.

INTRODUCTION

The mitochondrial permeability transition pore (PTP) is a mitochondrial inner membrane channel, whose opening alters mitochondrial physiology (Zoratti and Szabo, 1995). Usually closed in order to allow ATP synthesis, the PTP plays a key role in different models of cell death after extended opening (Green and Reed, 1998; Desagher and Martinou, 2000). The relevance of the PTP in the commitment to cell death is supported (i) by the finding of a protective effect of different PTP inhibitors in several models of cell death (Lemasters *et al.*, 1998; Dumont *et al.*, 1999; Crompton, 2000; Gastman *et al.*, 2000; Kroemer and Reed, 2000; Pastorino and Hoek, 2000; Vande Velde *et al.*, 2000; Chauvin *et al.*, 2001), (ii) by the demonstration that PTP opening occurs in intact cells (Nieminen *et al.*, 1995; Petronilli *et al.*, 1999; Chauvin *et al.*, 2001), and (iii) by the fact that PTP opening leads to the release of proapoptotic intermembrane space proteins both in vitro and in vivo (Kantrow and Piantadosi, 1997; Scarlett and Murphy, 1997; Petronilli *et al.*, 2001; De Giorgi *et al.*, 2002).

PTP regulation has been extensively studied over the past 20 years. Although PTP regulation exhibits a number of tissue-specific characteristics (Fontaine *et al.*, 1998), matrix Ca^{2+} is the single most important factor for opening of the pore. The PTP can be also modulated by a variety of drugs and physiological factors that are classified

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Key to abbreviations: PTP, permeability transition pore; CRC, Ca^{2+} retention capacity; EGTA, ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; DMO, 5,5-dimethyloxazolidine-2,4-dione; $TPMP^+$, triphenylmethylphosphonium ion; PPi, pyrophosphatidic acid; MOPS, 4-morpholinepropanesulfonic acid; CsA, cyclosporin A; Ub_0, ubiquinone 0; DNP, 2,4-dinitrophenol.

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as PTP inhibitors or PTP activators depending on whether they cause an increase or a decrease, respectively, of the amount of Ca^{2+} required for pore opening (Zoratti and Szabo, 1995). Physiological factors such as Pi, oxidation of pyridine nucleotides, alkalinization of matrix and decrease of the electrical transmembrane potential favor PTP opening, whereas Mg^{2+} and adenine nucleotides prevent it. Among the drugs that modulate the permeability transition, the PTP inhibitor cyclosporin A (CsA) has become the standard diagnostic tool for the characterization of the PTP. In a series of recent studies, we have shown that the PTP is also regulated by specific binding of several ubiquinone analogs presumably via a unique binding-site (Fontaine *et al.*, 1998; Walter *et al.*, 2000, 2002).

Mitochondrial volume is modulated in vivo by glucogenic hormones such as glucagon, vasopressin and epinephrine (Quinlan *et al.*, 1983; Halestrap, 1989), and by drugs such as the mitochondrial K_{ATP}, channel opener diazoxide (Kowaltowski *et al.*, 2001; Dos Santos *et al.*, 2002). Moreover, hyperthyroidism, polyunsaturated fatty acid deficiency and partial hepatectomy have been shown to induce larges changes in liver mitochondrial volume (Murray *et al.*, 1981; Horrum *et al.*, 1991; Fontaine *et al.*, 1996; Nogueira *et al.*, 2002). Because the matrix concentration of PTP regulatory factors will depend in part upon matrix volume, we sought to investigate the influence of the mitochondrial volume in PTP regulation. We found that the amount of Ca^{2+} required for PTP opening dramatically increased when matrix volume decreased relative to the standard isoosmotic condition. This phenomenon was not related to the observed changes in bioenergetic parameters, persisted when mitochondria were depleted of adenine nucleotides but disappeared when mitochondria were depleted of Mg^{2+}. Moreover, we found that the ability of Mg^{2+}, CsA or ubiquinone 0 (Ub_0) to inhibit the PTP varied according to matrix volume. The finding that the effects of Ca^{2+}, Mg^{2+}, CsA and Ub_0 on the PTP are modified by matrix volume represents a hitherto unrecognized feature of the PTP.

MATERIALS AND METHODS

Rat liver mitochondria were prepared according to standard differential centrifugation procedures in a medium containing 250 mM sucrose, 10 mM Tris–HCI (pH 7.4), and 0.1 mM EGTA–Tris. Unless otherwise specified, mitochondria were incubated in a medium containing 5 mM Pi–Tris, 10 mM Tris–MOPS, 5 mM glutamate–Tris, 2.5 mM malate–Tris of which the osmolarity was set at 250, 300 or 400 mOsM with sucrose.

Mitochondrial oxygen consumption was measured polarographically at 25°C using a Clark-type oxygen electrode. For protonmotive force measurements (Devin *et al.*, 1996) matrix space was estimated using ^3H_2O and [^{14}Cl-mannitol, while $\Delta\Psi_m$ and ΔpH were calculated based on the distribution of [^{3}H]-TPMP$^+$ and [^{14}C]-DMO, respectively. In preliminary experiments, we checked that [^{14}C]-sucrose, [^{14}C]-dextran and [^{32}P]Pi in the presence of mersalyl gave results comparable to those measured with [^{14}C]-mannitol (data not shown). We also verified that the distribution of [^{3}H]-TPMP$^+$ was not influenced by mitochondrial volume (Devin *et al.*, 1996, 1997).

Alternatively, $\Delta\Psi_m$ was measured fluorimetrically in the presence of 0.2 μM rhodamine 123 as described in (Emaus *et al.*, 1986) (excitation–emission: 503–525 nm). Extramitochondrial Ca^{2+} concentration was measured fluorimetrically in the presence of 1 μM Calcium Green-5N (excitation–emission: 506–532 nm), while pyridine nucleotide oxidation–reduction status was estimated based on endogenous fluorescence of NAD(P)H (excitation–emission, 340–460 nm) as previously described (Koretsky and Balaban, 1987). Fluorimetric assays were carried out at 25°C with either a PTI Quantamaster C61 or a Kontron SFM 23 spectrofluorimeter equipped with magnetic stirring and thermostatic control. For pyridine nucleotide oxidation–reduction status results are expressed as the percent reduction, where 100% reduction refers to the signal measured after the addition of 1.25 μM rotenone and 100% oxidation to the signal measured after the addition of 500 nM CCCP.

For adenine nucleotide depletion, mitochondria were incubated for at least 5 min in the presence of 1 mM PPi before the addition of the first Ca^{2+} pulse. Under this condition, rat liver mitochondria lose more than 85% of their adenine nucleotide content (Asimakis and Aprille, 1980). In preliminary experiments, we ensured that longer incubation time did not affect the Ca^{2+} retention capacity, indicating that further loss of adenine nucleotide did not affect PTP opening.

A23187, EGTA, DNP, Tris, HCl, glutamic acid, $CaCl_2$, ruthenium red, CsA and Ub_0 were purchased from Sigma–Aldrich. Pi, PPi and malic acid were purchased from Merck while all radiolabeled compounds were from Amersham.

Results are expressed as mean ± SE. Statistical significant differences were assessed by ANOVA followed by Fisher's protected least significant difference (PLSD) post hoc test or by paired student's *t* test, (Stat View, Abacus concepts, Inc., Berkeley, CA, 1992).

RESULTS

In the experiments depicted in Fig. 1A, energized rat liver mitochondria were incubated in a medium whose

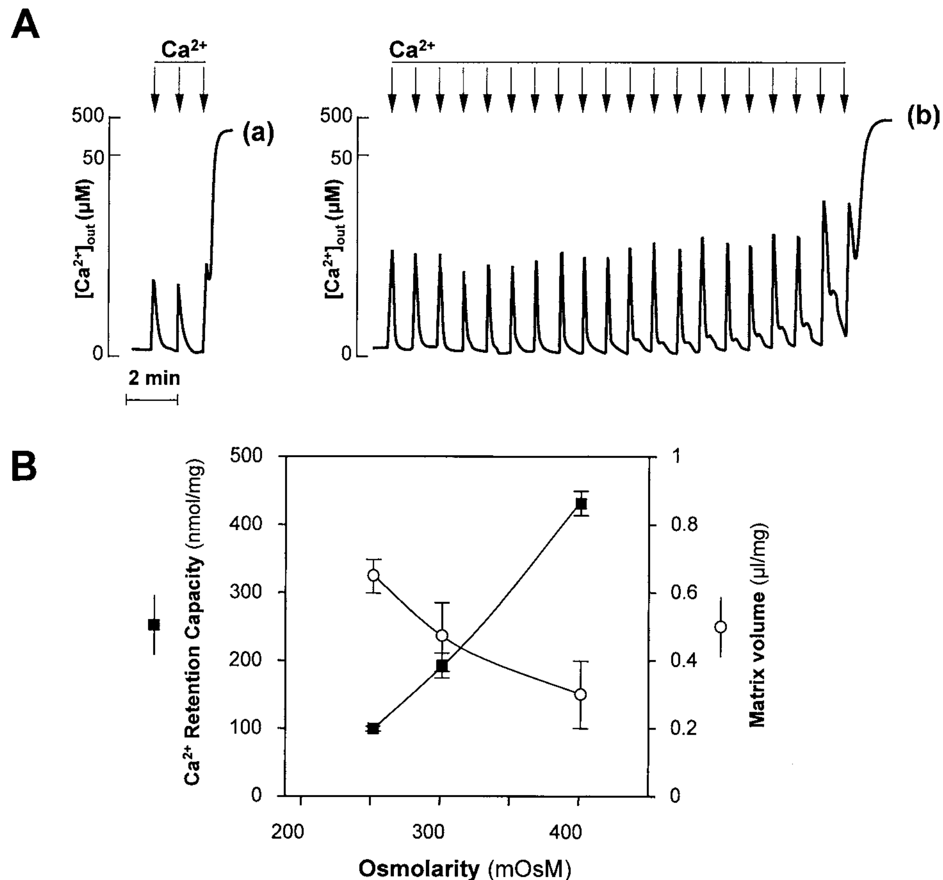

Fig. 1. Effect of sucrose concentration on matrix volume and on the Ca^{2+} retention capacity of rat liver mitochondria. The incubation medium contained 5 mM Pi–Tris, 10 mM Tris–MOPS, 5 mM glutamate–Tris, 2.5 mM malate–Tris, osmolarity was adjusted at the indicated values with sucrose (see Material and Methods section). The medium was supplemented either with ^3H_2O plus [^{14}C]mannitol for matrix volume determination or with 1 μM Calcium Green-5N for Ca^{2+} retention capacity (CRC) determination. The final volume was 2 mL, pH 7.4, 25°C. Panel A: the osmolarity was set at 250 (trace a) or 400 mOsM (trace b). Experiments were started by the addition of 2 mg of mitochondria (not shown). The arrows indicate individual pulses, which represent serial additions of 25 μM Ca^{2+}. Panel B: the CRC, which denotes the minimal Ca^{2+} load required for PTP opening was measured by adding trains of 25 μM Ca^{2+} pulses at 1 min intervals exactly as detailed in panel A. The values presented are mean ± SE of four and six experiments for matrix volume and CRC, respectively. Statistical significant differences were assessed by ANOVA for repeated measure, $p < 0.05$ for matrix volume and CRC.

osmolarity had been set at 250 or 400 mOsM. Mitochondria were then loaded with a train of 25 μM Ca^{2+} pulses at 1 min intervals. Under this protocol, mitochondria took up and retained Ca^{2+} until the load reached a threshold value that induced PTP opening, which was followed by a fast process of Ca^{2+} release (Fig. 1A) accompanied by membrane depolarization and mitochondrial swelling (not shown). As shown in Fig. 1A, the CRC (i.e. the Ca^{2+} load required to induce PTP opening) was increased fourfold at 400 mOsM (trace b) relative to 250 mOsM (trace a). The relationship between the CRC and the osmolarity is detailed in Fig. 1 (panel B, closed squares), which also reports the change of matrix volume as a function of medium osmolarity (Fig. 1B, open circles; $p < 0.05$, PLSD post hoc test between each value).

In order to establish the basis for the observed changes in PTP modulation, we next measured a set of bioenergetics parameters known to affect the PTP. As shown in Fig. 2, the protonmotive force components (namely, $\Delta\Psi_m$ and ΔpH) and the NAD(P)H reduction state varied linearly with osmolarity. Since increasing osmolarity (i.e. decreasing matrix volume) led to oxidation of pyridine nucleotides and alkalinization of matrix, which are known to favor PTP opening, the PTP inhibition

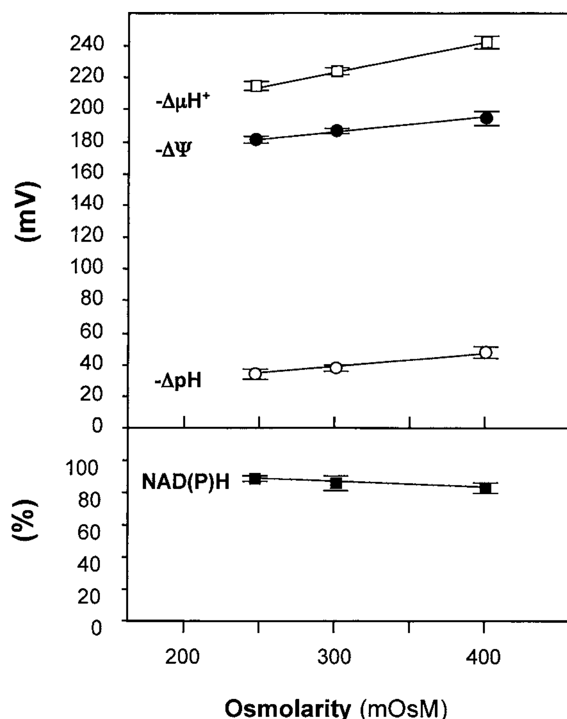

Fig. 2. Effect of osmolarity on the bioenergetics parameters. Experimental conditions were the same as in Fig. 1. Medium was supplemented with [^{3}H]TPMP$^+$ for $\Delta\Psi_m$ determination, or [^{14}C]DMO for ΔpH determination. The values presented are mean $\pm$ SE of four experiments. Statistical significant differences were assessed by ANOVA for repeated measure, $p < 0.05$ for $\Delta\Psi_m$, ΔpH and NADH.

observed at high osmolarity was obviously not related to such changes. To determine whether the higher $\Delta\Psi_m$ in hypertonic medium could be the basis for PTP inhibition, we compared the depolarization induced by increasing concentration of DNP in the presence of EGTA (i.e. with a closed PTP) to that obtained after the uptake of a small Ca^{2+} load that was unable to open the pore per se. As expected, when mitochondria were incubated at 250 mOsM (Fig. 3A), the DNP-induced depolarization was much more pronounced after Ca^{2+} loading (open symbols) than in the presence of EGTA (closed symbols), revealing a major contribution of the PTP to depolarization under this particular condition. Figure 3B shows that under the same condition of Ca^{2+} loading, the contribution of the pore to depolarization was much less pronounced when mitochondria were incubated at 400 mOsM. Hence, the comparison of Figs. 3A with 3B shows that for identical values of $\Delta\Psi_m$ when the pore was closed (e.g. 160 mV), the same Ca^{2+} load induced PTP opening only when mitochondria were incubated at 250 mOsM, indicating that matrix volume regulated the pore independently of $\Delta\Psi_m$.

In another set of experiments, mitochondria were incubated at 400 mOsM in the presence of a concentration of DNP adjusted to make $\Delta\Psi_m$ identical to that of the control condition at 250 mOsM (i.e. 180 mV). The experiments showed that a Ca^{2+} load that opened the pore at 250 mOsM was still unable to do so at 400 mOsM (data not shown), further confirming that matrix shrinkage inhibited the pore independently of the absolute value of $\Delta\Psi_m$.

Matrix adenine nucleotides and Mg^{2+} are well-recognized PTP inhibitors. Although some efflux is possible as matrix volume decreases, mitochondrial shrinkage is likely to increase their matrix concentrations and thus decrease the PTP open probability. Therefore, we studied the effect of mitochondrial shrinkage on PTP regulation after mitochondria had been supplemented with either 1 mM PPi (in order to deplete the adenine nucleotides pool) or with 0.1 nmol/mg protein A23187 (a concentration at which this ionophore depleted matrix Mg^{2+} without affecting mitochondrial Ca^{2+} content (Pfeiffer *et al.*, 1976)). It is important to stress that at the concentration used, A23187 did not affect mitochondrial volume (as assessed by mitochondrial absorbance at 540 nm), $\Delta\Psi_m$, or the kinetics of Ca^{2+} uptake assessed fluorimetrically with rhodamine 123 and Calcium Green-5N, respectively (data not shown).

As shown in Fig 4, treatment with PPi (i.e. depletion of the mitochondrial adenine nucleotides pool) favored PTP opening: the amount of Ca^{2+} inducing PTP opening was half that of the control condition for the given osmolarity. However, the osmolarity of the medium still affected PTP regulation after adenine nucleotides depletion, the CRC at 400 mOsM in the presence of 1 mM PPi remaining higher than that at 250 mOsM without PPi. These findings indicate that the effect of mitochondrial volume on PTP regulation was not due to the resulting change in adenine nucleotide matrix concentration alone. On the other hand, in mitochondria treated with A23187 (i.e. depleted of Mg^{2+}), the CRC was no longer affected by the decrease in matrix volume, suggesting that the observed PTP inhibition at low matrix volume in non-depleted mitochondria could be explained by the increase in matrix Mg^{2+} concentration.

To further test this hypothesis (knowing that Mg^{2+} regulates PTP opening via two sites, one inside one outside (Bernardi *et al.*, 1993)), we studied PTP opening in mitochondria where either external Mg^{2+} concentration alone or both external and internal Mg^{2+} concentration were changed. As shown in Fig. 5A, the CRC increased with the external Mg^{2+} concentration both at 250 and 400 mOsM. On the contrary, when mitochondria were exposed to Mg^{2+} in the presence of A23187

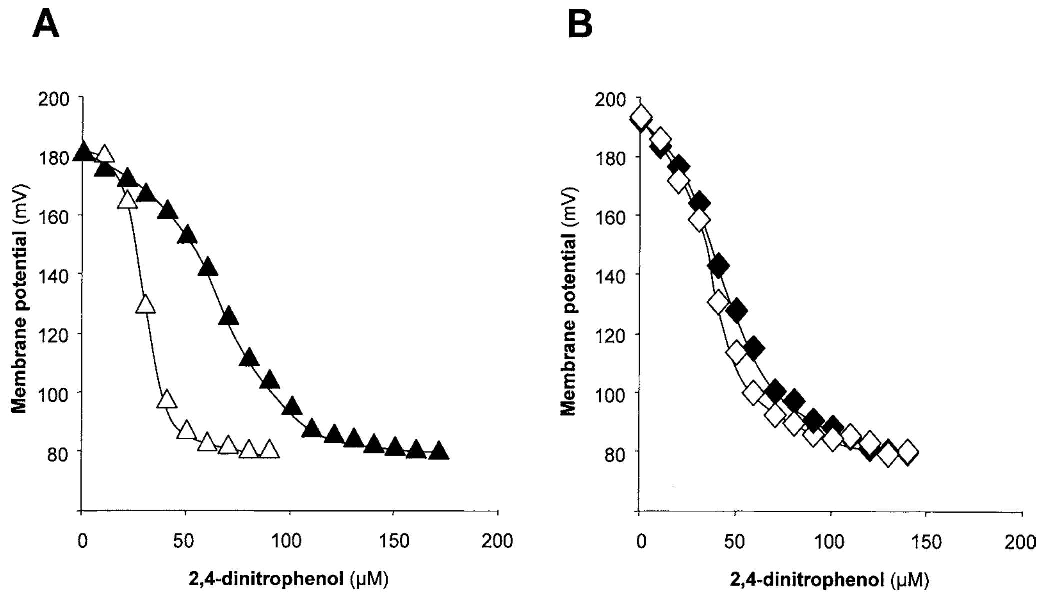

Fig. 3. Effect of mitochondrial volume on PTP opening induced by depolarization. Experimental conditions were as in Fig. 1 except that the incubation medium was supplemented with 0.2 μM Rhodamine 123. Osmolarity was set at 250 mOsM (panel A) or 400 mOsM (panel B), mitochondria were incubated in the presence of 100 mM EGTA (closed symbols) or loaded with 25 μM Ca^{2+} after which Ca^{2+} uniport was blocked by the addition of 1 μM ruthenium red (open symbols). Membrane potential values were measured two minutes after each addition of DNP to allow $\Delta\Psi_m$ stabilization.

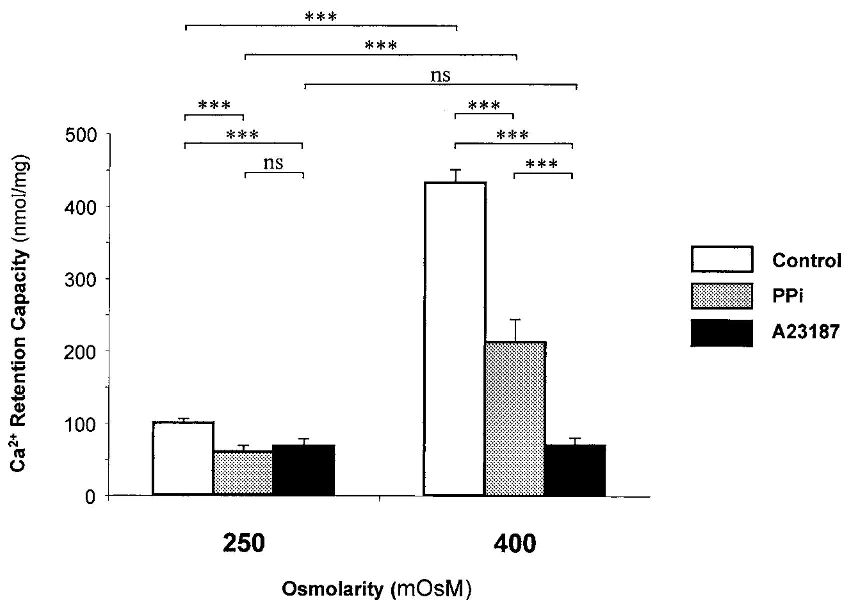

Fig. 4. Effect of mitochondrial volume on the Ca^{2+} retention capacity after depletion of adenine nucleotides or Mg^{2+}. Experimental conditions were the same as in Fig. 1. Osmolarity was adjusted at the indicated values with sucrose. When indicated, mitochondria were incubated with 1 mM PPi or 0.1 nmol/mg protein A23187. The Ca^{2+} retention capacity was determined exactly as shown in Fig. 1. The values presented are mean ± SE from at least six independent experiments carried out with six different mitochondrial preparations. $^{***}p < 0.01$, paired student's t test.

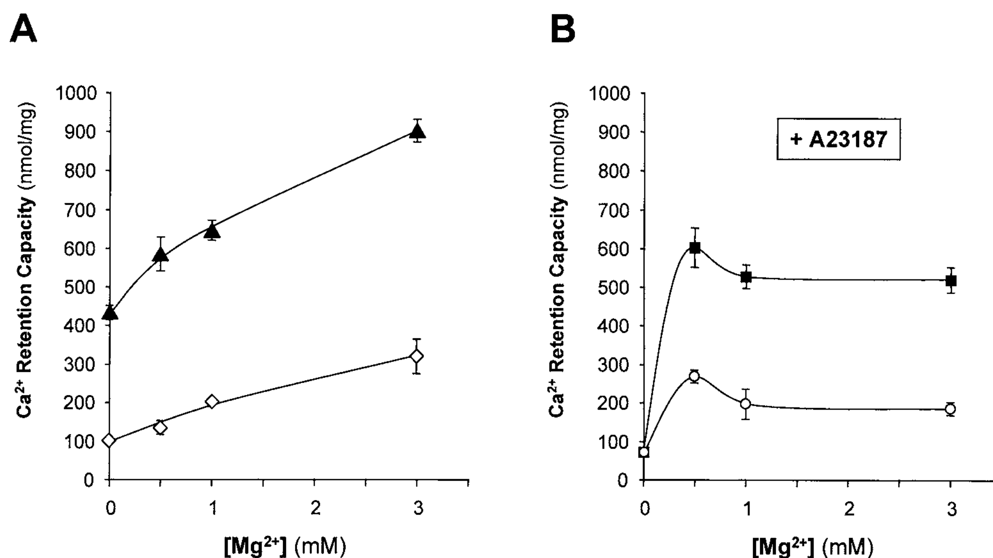

Fig. 5. Effect of mitochondrial volume on the efficacy of Mg^{2+} at PTP inhibition. Experimental conditions were the same as in Fig. 1. Osmolarity was adjusted at 250 (open symbols) or 400 mOsM (closed symbols) with sucrose. The medium was supplemented with the indicated amount of Mg^{2+} in the absence (panel A) or presence (panel B) of 0.1 nmol/mg protein A23187. The Ca^{2+} retention capacity was determined exactly as shown in Fig. 1. The values presented are mean ± SE from three independent experiments carried out with three different mitochondrial preparations.

(i.e. when external Mg^{2+} concentration equilibrates with matrix Mg^{2+} concentration (Reed and Lardy, 1972)), the CRC dramatically increased when Mg^{2+} concentration varied from 0 to 0.5 mM but slightly decreased above 0.5 mM (Fig. 5B). For a given concentration of Mg^{2+} the CRC was always higher at 400 mOsM than at 250 mOsM. In other words, when external and internal Mg^{2+} concentrations were set independently of matrix volume, the CRC was higher at 400 mOsM than at 250 mOsM, indicating that matrix volume modulated the efficacy of Mg^{2+} at PTP regulation. Obviously, in the absence of Mg^{2+}, such a mechanism of regulation could not work. Interestingly, the fact that the CRC slightly decreased above 0.5 mM Mg^{2+} when both external and internal concentrations varied (Fig. 5B), contrarily to what occurred when the internal Mg^{2+} concentration was constant (Fig. 5A), suggests that matrix Mg^{2+} concentrations above 0.5 mM actually favor PTP opening.

To test whether mitochondrial volume affected other PTP regulators, we next studied the effect of hypertonic conditions on the effects of CsA and Ub_0. As shown in Fig. 6A, in the presence of CsA the CRC was the same at 250 and 400 mOsM. Importantly, CsA did not significantly increase the CRC at 400 mOsM suggesting that CsA was no longer effective at low mitochondrial volume. As shown in Fig. 6B, in the presence of A23187 (i.e. in the absence of matrix Mg^{2+}) CsA became almost ineffective at 400 mOsM. Finally, as shown in Fig. 6, either in the presence (panel A) or in the absence of matrix Mg^{2+} (panel B), Ub_0 efficacy was increased by mitochondrial shrinkage.

DISCUSSION

In this study, we have shown that (i) mitochondrial volume dramatically affects the Ca^{2+} load required for PTP opening in a matrix Mg^{2+}-sensitive manner, with an effect that can be dissociated from the resulting changes in the bioenergetics parameters and the matrix concentration of adenine nucleotides, and (ii) the regulatory effects of Mg^{2+}, CsA and Ub_0 on the PTP are modified with mitochondrial volume.

Mitochondrial volume plays a key role in mitochondrial metabolism (Devin *et al.*, 1996) and mitochondrial shrinkage affects several physiological parameters known to regulate PTP opening. On the one hand, mitochondrial shrinkage led to matrix alkalinization and pyridine nucleotides oxidation, which would have had to favor PTP opening. On the other hand, it led to increase in $\Delta\Psi_m$, matrix ADP and Mg^{2+} concentrations, which could have participated to the observed PTP inhibition. However, the fact that PTP inhibition at low matrix volume persisted after ADP depletion (see Fig. 4), or when $\Delta\Psi_m$ was

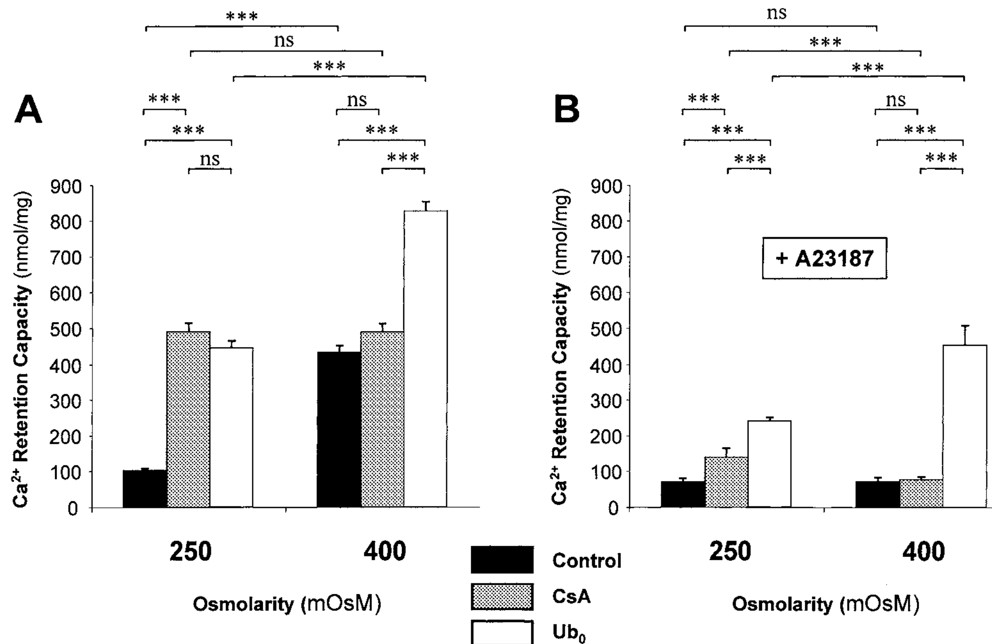

Fig. 6. Effect of mitochondrial volume on the efficacy of CsA and Ub_0 at PTP inhibition. Experimental conditions were the same as in Fig. 1. Osmolarity was adjusted at the indicated values with sucrose. Experiments were performed in the absence (panel A) or presence (panel B) of 0.1 nmol/mg protein A23187. The Ca^{2+} retention capacity was determined exactly as shown in Fig. 1 in the absence of inhibitor or in the presence of 1 μM CsA or 50 μM Ub_0. The values presented are mean ± SE from at least six independent experiments carried out with six different mitochondrial preparations. $^{***}p < 0.01$, paired student's *t* test.

decreased (see Fig. 3) indicates that these parameters did not play a key role in mitochondrial shrinkage-induced PTP inhibition.

Conversely, the fact that mitochondrial volume did not directly affect the CRC after Mg^{2+} depletion (see Fig. 4) indicates that matrix Mg^{2+} is essential for PTP regulation by mitochondrial volume. Free matrix Mg^{2+} concentration is normally close to 0.4 mM in isoosmotic condition (Corkey *et al.*, 1986) and is assumed to increase when matrix volume decreases. It must however be kept in mind that results from Fig. 5 suggest that increasing matrix Mg^{2+} concentration over 0.5 mM favors PTP opening. Consistent with this explanation, at 3 mM Mg^{2+}, the CRC was lower in the presence than in the absence of A23187 (compare Figs. 5A and 5B). Regardless of the effect of matrix Mg^{2+} concentration on PTP regulation, Fig. 5B shows that for a given Mg^{2+} concentration, PTP is more potently inhibited in hyperosmotic than isoosmotic condition, indicating that matrix volume per se modulated the efficacy of Mg^{2+} at PTP regulation.

The finding that the efficiency of exogenous PTP regulators such as CsA and Ub_0 depends on matrix volume represents a second hitherto unrecognized characteristic of the pore. These events are most easily observed after Mg^{2+} depletion but persist in the presence of Mg^{2+} inside mitochondria. Of special interest since it is widely used as a standard diagnostic tool for PTP characterization is the observation that CsA can become almost ineffective at low matrix volume. It should be noted that this lack of efficiency could not be overcome using higher concentrations of CsA (data not shown). It is generally accepted that CsA regulates the PTP by displacing the endogenous activator cyclophilin D from the pore (Connern and Halestrap, 1996; Nicolli *et al.*, 1996). Moreover, it has been reported that cyclophilin D binding decreases with mitochondrial volume in de-energized mitochondria (Connern and Halestrap, 1996), which is consistent with the fact that CsA became almost ineffective at 400 mOsM (see Fig. 6). It must be noted, however, that such displacement of cyclophilin D does not inhibit PTP opening in the absence of matrix Mg^{2+} (see Fig. 6B), suggesting either that PTP regulation by cyclophilin D requires Mg^{2+}, or that the efficacy of cyclophilin D at PTP regulation changes with matrix volume.

Although the permeability transition is primarily an inner membrane event, it has been shown that proteins located in the outer membrane affect PTP opening (Pastorino *et al.*, 1999; Kowaltowski *et al.*, 2000; Kim

et al., 2002). The mechanisms by which these outer membrane proteins regulate the permeability transition are unknown. We note, however, that Bcl-2 overexpression affects mitochondrial volume (Kowaltowski *et al.*, 2002). Moreover, it has been recently shown that several PTP inhibitors (including Ub_0) bind to the outer membrane protein $VDAC_1$ (Cesura *et al.*, 2003), which may account for their effect on permeability transition. Together, these data strongly suggest that permeability transition is regulated by complex and dynamic interactions between the inner and outer membrane components. Since the folding of the inner membrane changes with matrix volume, matrix volume affects the number or the feature of the contact sites between the two membranes. It is therefore conceivable that matrix volume may directly affect the interactions between the inner and outer membrane components of the PTP.

It must be kept in mind that mitochondrial volume does not regulate PTP opening after Mg^{2+} depletion (see Fig. 4). This finding suggests either that Mg^{2+} directly affects the interactions between the inner and outer membrane components of the PTP or that PTP regulation by such interactions require the presence of Mg^{2+} inside mitochondria.

Up to now, the regulation of the PTP by outer membrane proteins has been demonstrated by modulating the amount of several proteins such as BAX and Bcl-2 (Pastorino *et al.*, 1999; Kowaltowski *et al.*, 2000; Kim *et al.*, 2002). Although presently speculative, our findings suggest an alternative possibility to change the interactions between the inner and outer membrane proteins without changing their quantity. Whether or not this mechanism occurs in vivo remains to be established but such a demonstration might represent a new approach for modulating the PTP in intact cells.

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