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Ca^{2+} Induces a Cyclosporin A-Insensitive Permeability Transition Pore in Isolated Potato Tuber Mitochondria Mediated by Reactive Oxygen Species[1]

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Oxidative damage of mammalian mitochondria induced by Ca^{2+} and prooxidants is mediated by the attack of mitochondria-generated reactive oxygen species on membrane protein thiols promoting oxidation and cross-linkage that leads to the opening of the mitochondrial permeability transition pore (Castilho *et al.*, 1995). In this study, we present evidence that deenergized potato tuber (*Solanum tuberosum*) mitochondria, which do not possess a Ca^{2+} uniport, undergo inner membrane permeabilization when treated with Ca^{2+} (>0.2 mM), as indicated by mitochondrial swelling. Similar to rat liver mitochondria, this permeabilization is enhanced by diamide, a thiol oxidant that creates a condition of oxidative stress by oxidizing pyridine nucleotides. This is inhibited by the antioxidants catalase and dithiothreitol. Potato mitochondrial membrane permeabilization is not inhibited by ADP, cyclosporin A, and ruthenium red, and is partially inhibited by Mg^{2+} and acidic pH, well known inhibitors of the mammalian mitochondrial permeability transition. The lack of inhibition of potato mitochondrial permeabilization by cyclosporin A is in contrast to the inhibition of the peptidylprolyl *cis–trans* isomerase activity, that is related to the cyclosporin A-binding protein cyclophilin. Interestingly, the monofunctional thiol reagent mersalyl induces an extensive cyclosporin A-insensitive potato mitochondrial swelling, even in the presence of lower Ca^{2+} concentrations (>0.01 mM). In conclusion, we have identified a cyclosporin A-insensitive permeability transition pore in isolated potato mitochondria that is induced by reactive oxygen species.

KEY WORDS: Calcium; cyclosporin A; mitochondrial permeability transition; plant mitochondria; oxidative stress; *Solanum tuberosum.*

INTRODUCTION

It is well known that, under certain pathological conditions, mitochondrial matrix Ca^{2+} may reach a concentration that triggers the state of membrane permeability transition (MPT) in mammalian mitochondria (for reviews, see, Halestrap *et al.*, 1997; Lemasters, 1998; Kowaltowski and Vercesi, 1999; Crompton, 1999). MPT is defined as a Ca^{2+}-dependent, nonspecific permeabilization of the mitochondrial inner membrane, inhibited by submicromolar concentrations of cyclosporin A (Zoratti and Szabò, 1995). This process begins as a mitochondrial membrane permeabilization to protons and small ions and progresses as a permeabilization to small sugars and osmotic support, finally resulting in permeabilization to low-molecular mass proteins (<1500 Da), accompanied by irreversible mitochondrial dysfunction (Zoratti and Szabò, 1995; Castilho *et al.*, 1996). MPT can be enhanced by various agents (inducers) including prooxidants, thiol cross-linking reagents, inorganic phosphate (P_i) and uncouplers

[1] Key to abbreviations: $\Delta\Psi$, membrane potential; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; FCCP, carbonyl cyanide-4-trifluoromethoxyphenylhydrazone; MPT, mitochondrial permeability transition; ROS, reactive oxygen species.

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(Zoratti and Szabò, 1995; Kowaltowski and Vercesi, 1999).

Studies conducted in our laboratory (Valle *et al.*, 1993; Castilho *et al.*, 1995; Kowaltowski and Vercesi, 1999) and others (Zoratti and Szabò, 1995) have demonstrated that MPT, induced by prooxidants such as diamide or *t*-butyl hydroperoxide, is triggered by a Ca^{2+}-stimulated production of reactive oxygen species (ROS) (Castilho *et al.*, 1995; Grijalba *et al.*, 1999), which accumulates due to exhaustion of mitochondrial antioxidants GSH and NAD(P)H. Mitochondrially generated ROS promote the oxidation and cross-linkage of thiol groups on mitochondrial membrane proteins, leading to MPT (Valle *et al.*, 1993; Castilho *et al.*, 1995, 1996). Partial inhibition promoted by ruthenium red, an inhibitor of the Ca^{2+} uniport, indicates that these processes are dependent on intramitochondrial Ca^{2+} (Vercesi *et al.*, 1988).

Ca^{2+} transport by plant mitochondria varies in many aspects between different plant species (Chen and Lehninger, 1973; Dieter and Marmé, 1980) and between different tissues and ages in the same species (Dieter and Marmé, 1980). The data available also show that Ca^{2+} transport in plant mitochondria operates differently from that in animals (Moore and Åkerman, 1984). For example, mitochondria isolated from the hypocotyls of plants such as coffee (*Coffea arabica*), soybean (*Glycine max*), bean (*Phaseolus vulgaris*), and corn (*Zea mays*) have the ability to transport Ca^{2+} (Martins and Vercesi, 1985; Carnieri *et al.*, 1986). Therefore potato tubers (*Solanum tuberosum*), red beet roots (*Beta vulgaris*), and white cabbage leaves (*Brassica oleraceae*) mitochondria are unable to accumulate Ca^{2+}, despite the ability to build up a high transmembrane electrical potential, the driving force for Ca^{2+} uptake (Martins and Vercesi, 1985; Silva *et al.*, 1992). This suggests that these mitochondria do not possess the electrophoretic Ca^{2+} uniporter and constitute good models to study the mechanism by which Ca^{2+} affects mitochondrial membrane permeability. Other interesting data obtained in these studies showed that potato tuber mitochondria possess a Mg^{2+}-insensitive inner membrane anion channel (PIMAC) (Beavis and Vercesi, 1992), an uncoupling protein (PUMP) (Vercesi *et al.*, 1995) and an alternative oxidase (for a recent review see, Sluse and Jarmuszkiewicz, 1998) that could potentially participate in the modulation of the MPT via their effects on mitochondrial membrane potential ($\Delta\Psi$) and, hence, on the mitochondrial generation of ROS.

In this paper, we report the effect of Ca^{2+}, diamide, and thiol reagents on the permeability of potato tuber mitochondrial membrane, and its relationship with extramitochondrial Ca^{2+}. Our results indicate that potato mitochondrial membrane permeabilization occurs in a cyclosporin A-insensitive mechanism and is due the oxidation of protein thiol groups by mitochondrially generated ROS.

MATERIALS AND METHODS

Isolation of Potato Tuber Mitochondria

Potato tuber mitochondria were isolated from potatoes (*Solanum tuberosum* L., cv. "Bintje") obtained from the local market using a sucrose medium as described by Beavis and Vercesi (1992). The extraction medium contained 0.25 M sucrose, 10 mM HEPES-K^+, 2 mM EGTA, 3 mM cysteine, and bovine serum albumin (1 mg/ml); the pH was adjusted to 8.0 at 4°C. The wash medium contained 0.25 M sucrose, 2 mM HEPES-K^+, and 0.1 mM EGTA and was adjusted to pH 7.1 at 4°C. Potatoes (1 kg) are peeled, diced into 1 to 2 cm cubes, transferred to 2 liters of extraction medium at 4°C, and disrupted in a Waring Blendor at high speed for 15 s. The filtrate is then centrifuged at 1000 × *g* for 15 min and the pellet discarded. The mitochondria are then collected by centrifugation at 10,000 × *g* for 15 min, resuspended in about 40 ml wash medium, and then centrifuged twice at 250 × *g* for 10 min, the starch pellets are discarded after each spin. The mitochondria are then collected by centrifugation at 6000 × *g* for 15 min. The final pellet consists of a firm pellet with a "fluffy" layer on the top. The "fluffy" layer is discarded and the pellet resuspended in about 2 ml of 0.25 M sucrose to yield a stock suspension of about 20 to 30 mg/ml.

For purification of potato tuber mitochondria, portions (1 ml) of the final pellet were layered on top of gradients consisting of 40 ml of buffer containing 250 mM sucrose, 10 mM HEPES-KOH, pH 7.2, 0.3 mM EGTA, and 21% Percoll. The mixture was centrifuged at 39,000 × *g* for 30 min. Two bands of membranes were recovered from the gradient: a dense band recovered from approximately two-thirds down the tube, corresponded to purified mitochondria, while most contaminants were isolated from the Percoll gradient from a band above the mitochondria. The purified mitochondria were removed with a Pasteur pipet, diluted with 30 ml of 0.25 M sucrose, 2 mM HEPES-K^+, pH 7.1, and 0.1 mM EGTA and washed by centrifugation at 19,000 × *g* for 15 min to remove the Percoll. The pellet was resuspended in about 1 ml of 0.25 M sucrose to yield a stock suspension of about 15 to 20 mg/ml.

Isolation of Rat Liver Mitochondria

Mitochondria were isolated by conventional differential centrifugation of the livers of adult Wistar strain rats fasted overnight, as described by Castilho *et al.* (1995).

Standard Incubation Procedure

The experiments were carried out at 30°C in a standard reaction medium containing 300 mM sucrose, 10 mM HEPES-Na^+ buffer (pH 7.2), 0.5 μM carbonyl cyanide-4-trifluoromethoxyphenylhydrazone (FCCP), and 1 μM antimycin A. In the experiments conducted at different pHs, the buffer used was 10 mM MOPS/Tris instead HEPES. Other additions are indicated in the figure legends. The results shown are representative of a series of at least three experiments, using different mitochondrial preparations. The results were reproduced within 15% of variation.

Determination of Mitochondrial Swelling

Mitochondrial swelling was estimated from the decrease in the absorbance of the mitochondrial suspension at 520 nm measured in a Hitachi U-3000 spectrophotometer (Beavis *et al.*, 1985; Garlid and Beavis, 1985). This technique generates a light-scattering variable, "β," which normalizes reciprocal absorbance for mitochondrial protein concentration, "P" (mg/ml), according to the formula:

$$\beta = \frac{P}{P_s}(A^{-1} - a)$$

where a is a machine constant and P_s (equals 1 mg/ml) is a constant introduced to make β dimensionless.

Measurements of Mitochondrial Transmembrane Electrical Potential ($\Delta\psi$)

Mitochondrial membrane potential ($\Delta\psi$) was monitored by measuring the fluorescence changes of safranin O (5.0 μM), using a model F-4010 Hitachi spectrofluorometer (Hitachi, Ltd., Tokyo, Japan) operated at excitation and emission wavelengths of 495 and 586 nm, respectively, and slit widths of 5 nm (Åkerman and Wikström, 1976).

Determination of Ca^{2+} Movements

Variations in the concentration of free extramitochondrial Ca^{2+} were followed by measuring the changes in the absorbance spectrum of arsenazo III (40 μM), using a SLM Aminco DW2000 spectrophotometer (SLM Instruments, Inc., Urbana, Illinois) set at the 675–685 nm wavelength pair (Scarpa, 1979).

Measurement of Peptidylprolyl *cis-trans* Isomerase Activity

The peptidylprolyl *cis–trans* isomerase activity was measured by the procedure of Fischer *et al.* (1989). This assay was based on *cis–trans* isomerization of the peptide *N*-succinyl-alanyl-alanyl-prolyl-phenylalanyl 4-nitroanilide. Chymotrypsin hydrolyzes the nitroanilide only when the Ala–Pro is *trans*. This reaction is followed by an increase in absorbance at 390 nm. Mitochondria (0.25 mg/ml) were preincubated for 2 min at room temperature in reaction medium containing 40 mM HEPES-K^+ buffer (pH 8.0) and 0.05% Triton X-100, followed by 30s of preincubation with 20 μM chymotrypsin. Absorbance measurements were initiated 2s after the addition of 60 μM *N*-succinyl-alanyl-alanyl-prolyl-phenylalanyl 4-nitroanilide at 390 nm using a SLM Aminco DW2000 spectrophotometer.

Chemicals

Most of the chemicals, including ADP, catalase, chymotrypsin, cyclosporin A, diamide, dithiothreitol, EGTA, HEPES, MOPS, safranin O, succinate, ruthenium red, and *N*-succinyl-Ala-Ala-Pro-Phe 4-nitroanilide were obtained from Sigma Chemical Company (St. Louis, Missouri). All other reagents were commercial products of the highest purity grade available.

RESULTS

The study of inner mitochondrial membrane permeabilization induced by Ca^{2+} can be assessed by using the classic mitochondrial swelling technique to follow the net influx of the osmotic support associated with nonspecific increase in membrane permeability (Gunter and Pfeiffer, 1990). The experiments shown in Fig. 1 were conducted using deenergized mitochondria, a condition under which the Ca^{2+} influx can be driven by a gradient of the cation activity across the inner membrane if a Ca^{2+} uniport or a Ca^{2+} ionophore is present. This experimental condition has the advantage of eliminating the involvement of respiration, membrane potential, and Ca^{2+} cycling across the inner membrane in the mitochondrial swelling (Vercesi *et al.*, 1988). The results of Fig. 1 illustrate that the decrease in the absorbance of the mitochondrial suspension, induced by increasing Ca^{2+} concentrations (200–800 μM), occurs in a dose-dependent manner (lines b–e). The *dotted* line shows that in the presence of 300 μM

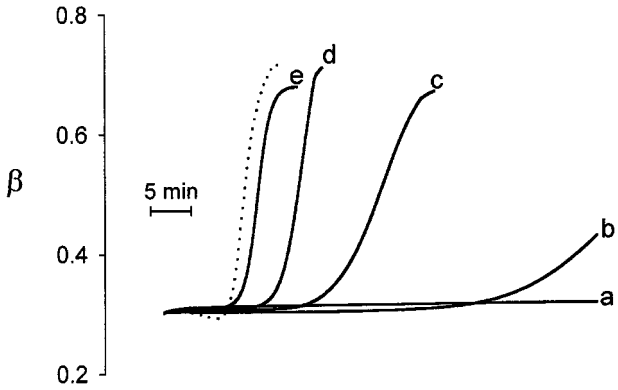

Fig. 1. Ca^{2+}-induced potato mitochondrial swelling: Effect of diamide. Potato tuber mitochondria (0.2 mg/ml) were incubated in standard reaction medium containing 200 μM Ca^{2+} (line b), 400 μM Ca^{2+} (line c), 600 μM Ca^{2+} (line d), 800 μM Ca^{2+} (line e) and 600 μM Ca^{2+} and 300 μM diamide (dotted line). Line a represents a control experiment without additions of Ca^{2+} and diamide.

diamide, a thiol oxidant (Kosower *et al.*, 1969), the lag phase that precedes swelling induced by 600 μM Ca^{2+} is shorter than when Ca^{2+} was added alone (line d).

Figure 2 (Panel A) confirms that potato tuber mitochondria (line a) are not able to take up Ca^{2+} (free [Ca^{2+}] = 20 μM) under our experimental conditions. Under the same conditions, rat liver mitochondria (line b) accumulated and retained the Ca^{2+} present in the medium, until the protonophore FCCP was included. Panel B shows that isolated potato tuber (line a) and rat liver (line b) mitochondria respiring on succinate were able to create a membrane potential ($\Delta\Psi$) and mantain it for 10 min. The addition of ADP caused a transient decrease in safranin fluorescence (lines a and b) during ADP phosphorylation. In contrast to rat liver mitochondria, no change in $\Delta\psi$ was observed in potato mitochondria when 20 μM of Ca^{2+} was added, confirming the absence of a Ca^{2+} transport system dependent on $\Delta\Psi$ in these mitochondria (line a).

The results shown in Fig. 3 (Panel A) indicate that 2 μM ruthenium red (line c), an inhibitor of the mitochondrial Ca^{2+} uniporter, and 5 μM ionomycin (line d), an ionophore with a high selectivity for Ca^{2+}, did not significantly change the patterns of potato mitochondrial swelling induced by 600 μM Ca^{2+} and 300 μM diamide (line b). In contrast, in rat liver mitochondria (Panel B) the addition of ruthenium red (line c) partially inhibits, while ionomycin (line d) stimulates Ca^{2+} and diamide-induced mitochondrial swelling.

In Fig. 4, the effects of different mitochondrial permeability transition inhibitors were tested on potato tuber mitochondrial swelling induced by diamide in the presence of Ca^{2+}. It was observed that dithiothreitol (A, line c), a disulfide reductant, and catalase (A, line d), an enzyme that degrades H_2O_2, completely inhibit potato mitochondrial swelling (A, line b). On the other hand, cyclosporin A (B, line c) and ADP (B, line e), classical inhibitors of the permeability transition pore in mammalian mitochondria (Gunter and Pfeiffer, 1990), had no inhibitory effect. Mg^{2+}, which competes with Ca^{2+} for binding sites on the mitochondrial membrane (Kowaltowski *et al.*, 1998), partially inhibits this potato mitochondrial swelling (B, line d). Since crude potato mitochondria are well-known to be contaminated with intact peroxisomes and amyloplast ghosts, we have also carried out experiments with purified potato tuber mitochondria (Fig. 4C). It was observed that dithiothreitol (C, line c) and catalase (C, line d) completely inhibit Ca^{2+} and diamide-induced swelling of purified potato tuber mitochondria (C, line b), while cyclosporin A (C, line e) had no inhibitory effect. These results showed that purified mitochondria give the same results to that obtained with crude potato tuber mitochondria.

The results depicted in Fig. 5 show the effect of medium pH (pH 6.6–7.6) on Ca^{2+} and diamide-induced potato mitochondrial swelling. We observed that by increasing pH, the lag phase that precedes mitochondrial swelling was progressively decreased (pH 6.6–7.6, respectively, lines a–f). These results suggest that acidic pH inhibits Ca^{2+}-induced potato mitochondrial swelling in the same way that it occurs with rat liver mitochondria (Halestrap, 1991; Bernardi *et al.*, 1992).

Inhibition of mammalian MPT by cyclosporin A is correlated with binding of cyclosporin A to cyclophilin with inhibition of peptidylprolyl *cis–trans* isomerase activity (for reviews, see Halestrap *et al.*, 1997; Cromptom, 1999). Since we observed no effect of cyclosporin A on Ca^{2+}-induced potato mitochondrial swelling, we investigated the presence of peptidylprolyl *cis–trans* isomerase activity in our preparations and its sensitivity to cyclosporin A. Figure 6 shows typical absorbance changes beginning 2 s after the assay of peptidylprolyl *cis–trans* isomerase activity was started, using the test peptide *N*-succinyl-alanyl-alanyl-prolyl-phenylalanyl 4-nitroanilide. Isomerization occurred in the absence of mitochondrial protein (lines c), but it was stimulated when rat liver (Panel A) or potato tuber (Panel B) mitochondria were present (lines a). Cyclosporin A (2 μM) inhibited the isomerase activity (lines b) in both types of mitochondria. These results show that there is no direct connection between the ability of cyclosporin A to inhibit peptidylprolyl *cis–trans* isomerase activity and potato mitochondrial permeabilization.

In order to further investigate the participation of protein thiol groups in Ca^{2+}-induced potato mitochondrial permeabilization, we tested the effects of monofunctional

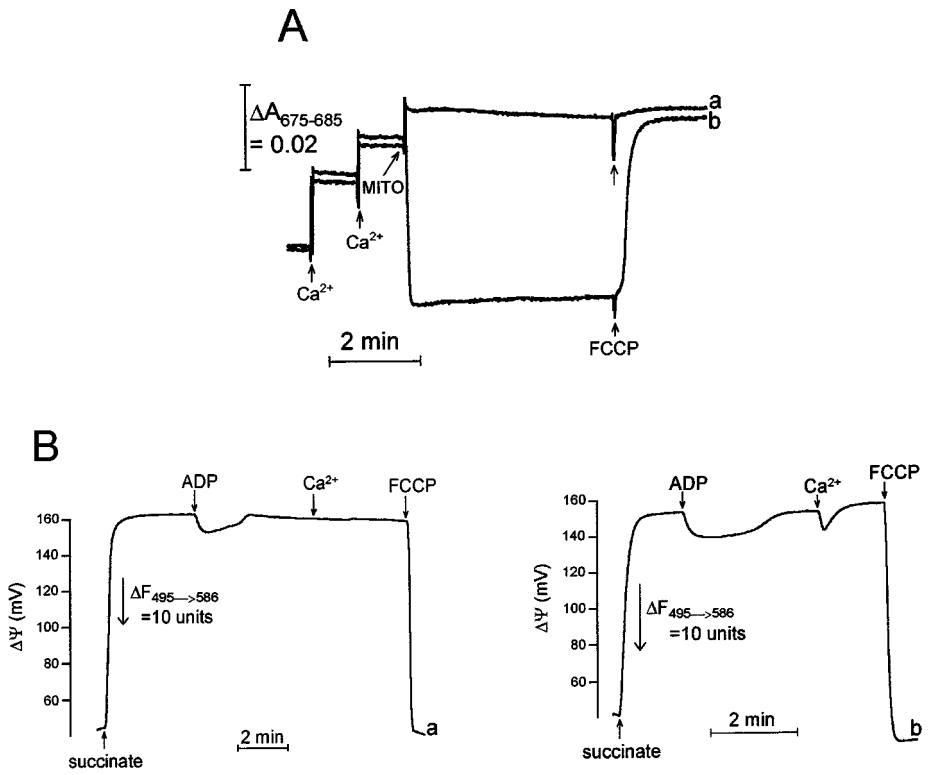

Fig. 2. Characteristics of Ca^{2+} uptake and membrane potential of potato tuber and rat liver mitochondria. Potato tuber (lines a) and rat liver mitochondria (lines b) were incubated in reaction medium containing 250 mM sucrose, 10 mM HEPES, pH 7.2, 1 mM P_i, 0.1 mg/ml BSA, 2 mM succinate, and 5 μM rotenone supplemented with 20 μM arsenazo III (A) or 5 μM safranin O (B). In (A), Ca^{2+} (two serial additions of 10 μM), 1 mg/ml mitochondria (MITO), and 1 μM FCCP were added where indicated (arrows). In (B), 100 μM ADP, 20 μM Ca^{2+}, and 1 μM FCCP were added where indicated (arrows).

thiol reagents and thiol cross-linker reagents on potato mitochondrial swelling (Fig. 7). When the experiments were conducted in the presence of EGTA (free $[Ca^{2+}] < 0.01$ μM) (Panel A), mersalyl (line a), but not phenylarsine oxide, DIDS, or *N*-ethylmaleimide, induced a slow mitochondrial swelling. Interestingly, mersalyl-induced mitochondrial swelling was strongly stimulated by 10 μM Ca^{2+} (Panel B, line a). In the presence of a higher Ca^{2+} concentration (600 μM; Panel C), all thiol reagents tested stimulated potato mitochondrial swelling. Mersalyl and Ca^{2+}-induced potato mitochondrial swelling was insensitive to cyclosporin A and ADP and was only partially inhibited by Mg^{2+} (5.0 mM) (results not shown).

DISCUSSION

The formation of the mammalian MPT pore can be mediated by a concerted action between Ca^{2+} and ROS leading to oxidation of membrane protein thiols (for a recent review see Kowaltowski and Vercesi, 1999). According to this model, Ca^{2+} ions are involved in the mechanism of MPT pore opening by: (1) binding to inner membrane cardiolipins and stimulating the production of O_2^- (superoxide anion radical) and, hence, H_2O_2, by the respiratory chain (Valle *et al.*, 1993; Dykens, 1994; Castilho *et al.*, 1995; Grijalba *et al.*, 1999), (2) stimulating the Fenton reaction through matrix Fe^{2+} mobilization (Castilho *et al.*, 1995), and (3) binding to membrane proteins that regulate MPT pore opening (Bernardi *et al.*, 1992; Crompton, 1999; Zoratti and Szabò, 1995). The results presented here indicate that high extramitochondrial Ca^{2+} concentrations promote permeabilization of the inner membrane of potato mitochondria in a mechanism sensitive to exogenous catalase and dithiothreitol (Fig. 4), suggesting the participation of ROS and protein thiol oxidation in this process. Moreover, the potentiation of Ca^{2+}-induced potato mitochondrial swelling by diamide (Fig. 1), a thiol oxidant, and mersalyl (Fig. 7), a monofunctional thiol reagent, confirms that protein thiol groups are important targets to ROS inducing potato mitochondrial membrane permeabilization.

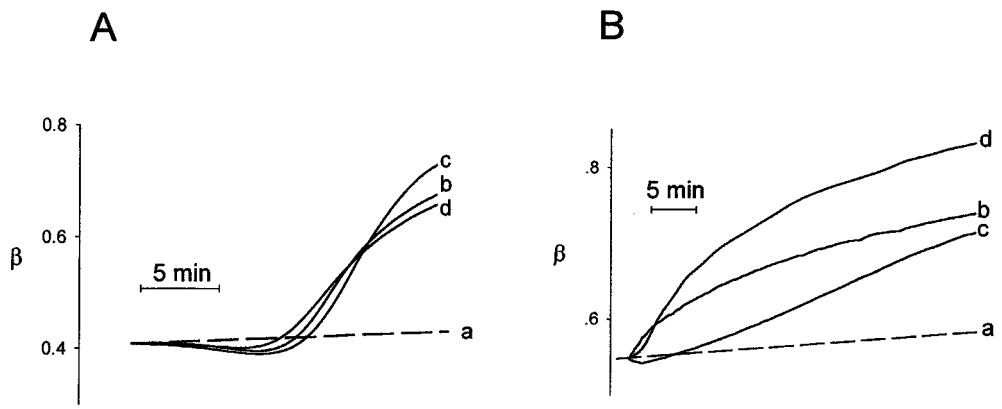

Fig. 3. Effect of ruthenium red and ionomycin on Ca^{2+} and diamide-induced mitochondrial swelling. Potato tuber (A) and rat liver mitochondria (B) (0.2 mg/ml) were incubated in standard reaction medium containing 300 μM diamide and 600 μM Ca^{2+} (lines b–d). Ruthenium red (2 μM) and 5 μM ionomycin were present in the experiments represented by lines c and d, respectively. The dashed lines represent control experiments without additions of Ca^{2+} and diamide.

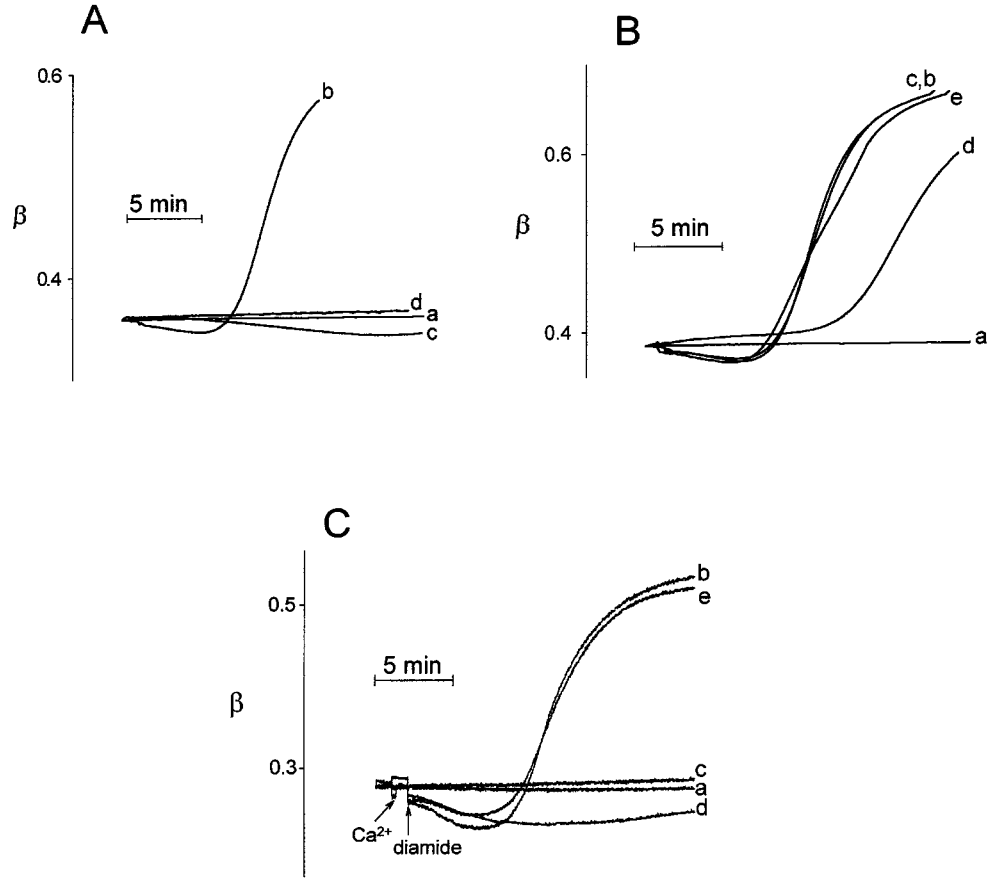

Fig. 4. Effect of antioxidants and permeability transition pore inhibitors on Ca^{2+} and diamide-induced potato tuber mitochondria swelling. Potato tuber mitochondria (0.2 mg/ml; crude mitochondria for (A and B), purified mitochondria for (C) were incubated in standard reaction medium in the presence of 300 μM diamide plus 600 μM Ca^{2+} (A–C, lines b–e). In (A), the experiments were conducted in reaction medium containing 2 mM DTT (line c) or 5 μM catalase (line d). In (B), the experiments were conducted in reaction medium containing 1 μM CsA (line c), 5 mM Mg^{2+} (line d) or 200 μM ADP (line e). In (C), (using purified potato tuber mitochondria), the experiments were conducted in reaction medium containing 2 mM DTT (line c), 5 μM catalase (line d) or 1 μM CsA (line e). Lines a represent control experiments.

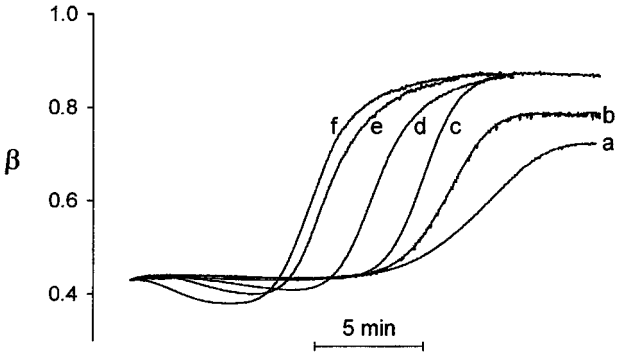

Fig. 5. Effect of pH on Ca^{2+} and diamide-induced potato tuber mitochondrial swelling. Potato tuber mitochondria (0.2 mg/ml) were incubated in the standard reaction medium buffered with 10 mM MOPS/Tris, in the presence of 300 μM diamide and 600 μM Ca^{2+}, at different pH (6.6, line a; 6.8, line b; 7.0, line c; 7.2, line d; 7.4, line e; 7.6, line f).

The presence of the Ca^{2+} ionophore ionomycin, that potentiates Ca^{2+}-induced rat liver MPT under deenergized conditions (Fig. 3B; Vercesi *et al.*, 1988), did not significantly stimulate swelling of potato mitochondria, which do not possess Ca^{2+} uniport. This result demonstrates that intramitochondrial Ca^{2+} is not necessary to induce potato MPT. In this regard, it is important to stress that ruthenium red did not totally inhibit opening of the MPT pore in deenergized rat liver mitochondria incubated in the presence of high Ca^{2+} concentrations (see Fig. 3B; Vercesi *et al.*, 1988). This suggests that, at high concentrations, the binding of Ca^{2+} to the external face of the inner membrane induces alterations similar to those induced by the high Ca^{2+} concentrations accumulated in the matrix of energized mitochondria. These alterations increase electron leakage and ROS production in mammalian mitochondria and seem to be mediated by disorganization in the electron-transferring components of the respiratory chain (Grijalba *et al.*, 1999). Indeed, the electron paramagnetic resonance (EPR) technique (Grijalba *et al.*, 1999) showed that Ca^{2+}, in the concentration range used in this work, induces lateral phase separation in "inside-out" heart submitochondrial particles.

Interestingly, in the present work, we have shown that potato mitochondria permeabilization induced by Ca^{2+} is poorly inhibited by the well-known inhibitors of the mammalian mitochondrial permeability transition, such as ADP and cyclosporin A, and it is only partially inhibited by Mg^{2+} and acidic pH. It is known that the inhibitory property of cyclosporin A on mammalian MPT is related to its binding to inner mitochondrial membrane cyclophilin D in a process associated with inhibition of the peptidylprolyl *cis–trans* isomerase activity (Nicolli *et al.*, 1996; Halestrap *et al.*, 1997). This implies that cyclophilin is a structural component of the mammalian MPT pore (Beutner *et al.*, 1998; Nicolli *et al.*, 1996) or catalyzes mammalian MPT opening via its peptide bond isomerase activity (Broekemeier and Pfeiffer, 1995; Novgorodov *et al.*, 1994). Cyclophilins are a large family of highly conserved proteins found in eukaryotes (Connern and Halestrap, 1992) and some plants (Mattoo, 1998; Breiman *et al.*, 1992). The dissociation between the property of cyclosporin A to inhibit peptidylprolyl *cis–trans* isomerase activity (Fig. 6) and to inhibit MPT pore opening in potato mitochondria (Fig. 5) suggests that peptidylprolyl *cis–trans* isomerase activity of cyclophilins may have other functions in potato mitochondria. Our results with potato mitochondria corroborate the recent proposal by Jung *et al.* (1997) that cyclophilin is a relatively recent phylogenetic component of the MPT, based on the observation of a similar cyclosporin A-insensitive MPT in yeast. Finally, the partial inhibition of potato MPT by acidic pH shows

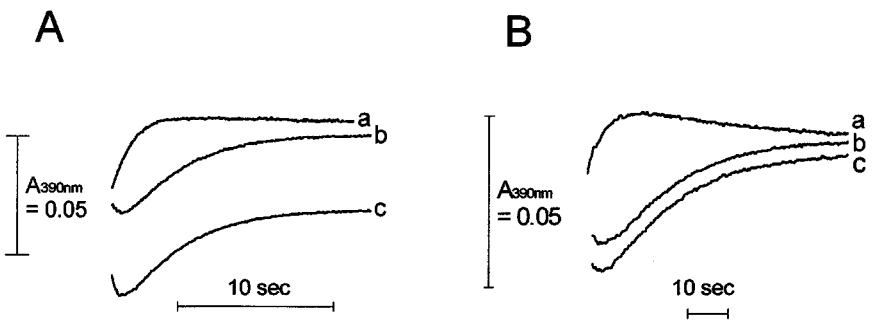

Fig. 6. Cyclosporin A inhibition of peptidylprolyl *cis–trans* isomerase in rat liver (A) and potato tuber mitochondria (B). Absorbance measurements were initiated 2 s after addition of chymotrypsin to assay buffer containing 0.25 mg/ml mitochondria (lines a), mitochondria plus 2 μM cyclosporin A (lines b) or no additions (lines c).

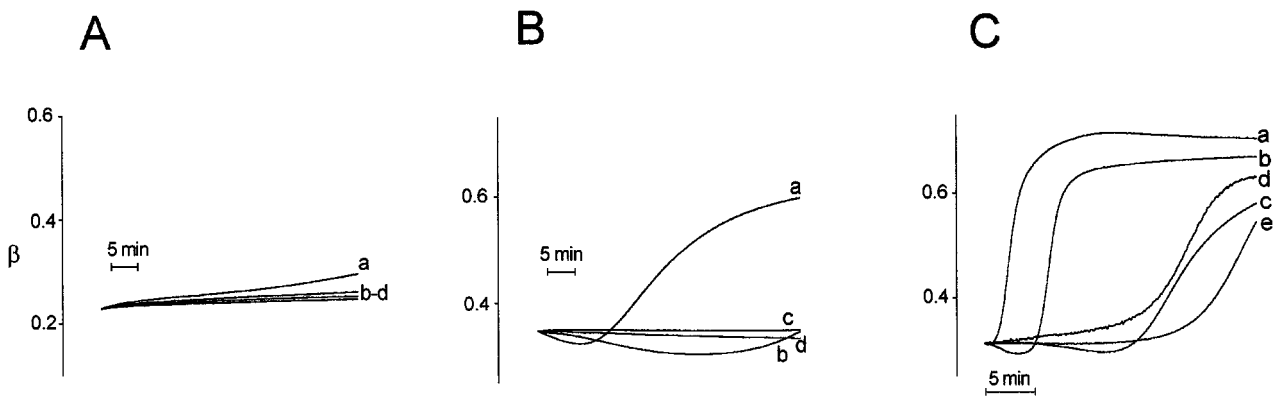

Fig. 7. Thiol reagent-induced potato mitochondrial swelling: Effect of Ca^{2+}. Potato tuber mitochondria (0.2 mg/ml) were incubated in standard reaction medium containing 1 mM EGTA (A), 10 μM Ca^{2+} (B) or 600 μM Ca^{2+} (C). Experiments were conducted in the presence of 50 μM of the thiol reagents: mersalyl (lines a), phenylarsine oxide (lines b), DIDS (lines c) or *N*-ethylmaleimide (lines d). Line e (C) represents a control experiment in the presence of 600 μM Ca^{2+} and without addition of thiol reagents.

that protonation of a critical histidyl residue of cyclophilin with its dissociation from the binding site on the MPT pore complex (Nicolli *et al.*, 1996) is not the only mechanism of MPT inhibition under this condition. Indeed, we have recently demonstrated that the inhibition of mammalian MPT observed at lower incubation medium pH is mediated by a decrease in membrane protein thiol reactivity due to the protonation of protein histidyl residues (Teixeira *et al.*, 1999).

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