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On the Protection by Inorganic Phosphate of Calcium-Induced Membrane Permeability Transition

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The role of inorganic phosphate as inhibitor of mitochondrial membrane permeability transition was studied. It is shown that in mitochondria containing a high phosphate concentration, i.e., 68 nmol/mg, Ca^{2+} did not activate the pore opening. Conversely, at lower levels of matrix phosphate, i.e., 38 nmol/mg, Ca^{2+} was able to induce subsequent pore opening. The inhibitory effect of phosphate was apparent in sucrose-based media, but it was not achieved in KCl media. The matrix free Ca^{2+} concentration and matrix pH were lowered by phosphate, but they were always higher in K^+-media. In the absence of ADP, phosphate strengthened the inhibitory effect of cyclosporin A on carboxyatractyloside-induced Ca^{2+} efflux. Acetate was unable to replace phosphate in the induction of the aforementioned effects. It is concluded that phosphate preserves selective membrane permeability by diminishing the matrix free Ca^{2+} concentration.

KEY WORDS: Mitochondrial calcium; inorganic phosphate; membrane permeability transition; calcium transport.

INTRODUCTION

Activation of membrane permeability transition can be accomplished by a massive matrix Ca^{2+} accumulation, in addition to an inducing agent (for a review see Gunter and Pfeiffer, 1990). From the wide assortment of inducing agents perhaps the most used is inorganic phosphate. The damaging action of phosphate on membrane permeability has been ascribed to the depletion of the matrix adenine nucleotide pool (Wilson and Asimakis, 1987; Lapidus and Sokolove, 1994; Savage and Reed, 1994), prevention of matrix acidification (Petronilli *et al.*, 1993), and diminution of intramitochondrial free Mg^{2+} (Jung *et al.*, 1990).

Brustovetsky and Klingenberg (1996) proposed that Ca^{2+} induces pore opening through its binding to the cardiolipin core of the adenine nucleotide translocase (ANT). In this regard, we have recently provided evidence that the lipid composition of the inner membrane modulates Ca^{2+}-induced pore opening (Chávez *et al.*, 1996). On the other hand, Halestrap and Davidson (1990) argue that Ca^{2+} binds directly to ANT which in turn is converted to a nonspecific hydrophilic channel. It is known that from the total Ca^{2+} accumulated, the free fraction accounts for pore opening (Nichols and Akerman, 1982; Chávez *et al.*, 1989). In this respect we have previously shown that the free Ca^{2+} fraction depends on the amount of intramitochondrial K^+, which masks negative charges to which Ca^{2+} binds (Chávez *et al.*, 1991); thus, K^+ increases the Ca^{2+} active fraction. Considering the above, it is conceivable that the damaging Ca^{2+} fraction may also be regulated by the change in the internal phosphate concentration, i.e., an increase in matrix inorganic phosphate may hinder the activating effect of Ca^{2+} on membrane pore opening.

The ability of the immunosuppressant cyclosporin A (CSA)[2] to inhibit membrane permeability transition (Fournier *et al.*, 1987; Crompton *et al.*, 1988; Davidson

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[2] Abbreviations used: Pi, inorganic phosphate; ANT, adenine nucleotide translocase; CSA, cyclosporin A; BCECF, 2',7'-bis (carboxyethyl)-5(6)carboxyfluorescein; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; $[Ca^{2+}]_m$, mitochondrial free Ca^{2+} concentration; $\Delta\psi$, transmembrane potential.

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and Halestrap, 1990) has been well established. This effect of cyclosporin A is improved by ADP (Novgorodov *et al.*, 1990; Andreeva and Crompton, 1994; Zazueta *et al.*, 1994), but inhibited by Ca^{2+} (Crompton and Andreeva, 1994; Andreeva *et al.*, 1995). The observation that Ca^{2+} influences both pore opening and the inhibitory effect of CSA prompted us to investigate whether phosphate, by affecting the mitochondrial matrix active Ca^{2+} fraction, may operate as modulator of both the membrane permeability transition and the action of CSA on pore closure. The results show that, indeed, phosphate diminishes intramitochondrial free Ca^{2+} concentration and strengthens the effect of CSA on pore closure. Such effects of phosphate are dependent on the osmotic support, being apparent in sucrose media, while they are absent in KCl media.

MATERIAL AND METHODS

Mitochondria were isolated from rat kidney by the conventional differential centrifugation method; the tissue was homogenized in 250 mM sucrose-1 mM EDTA, pH 7.3; after the final centrifugation the pellet was suspended in EDTA-free sucrose. Mitochondrial protein concentration was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Calcium movements were followed spectrophotometrically, at 675–685 nm, using the metallochromic indicator Arsenazo III (Scarpa *et al.*, 1978). Mitochondrial transmembrane potential was determined by using the dye Safranine, at 524–554 nm (Zanotti and Azzone, 1979). The uptake of phosphate was measured by incubating mitochondria in 5 mM [^{32}Pi]-H_3PO_4-Tris, pH 7.3 (sp. act. 600 cpm/nmol); after 5 min, an aliquot of 0.2 ml was filtered through a Millipore filter of 0.45 μm pore diameter, and the radioactivity retained in the filter was measured. Intramitochondrial free Ca^{2+} concentration was determined as previously described (Moreno-Sánchez *et al.*, 1979), using the Ca^{2+} fluorescent indicator Fluo 3AM. Mitochondria (45–60 mg) were loaded with 5 μM Fluo 3AM by incubation during 30 min at 25°C in 250 mM sucrose, 10 mM HEPES, 0.5 mM EGTA, 0.5% fatty acid-free bovine serum albumin, 1 mM ADP, and 1 mM $MgCl_2$, pH 7.4. Mitochondria were washed by dilution, centrifuged, and suspended in the isolation medium supplemented with 0.2% fatty acid-free albumin; fluorescence of Fluo 3-loaded mitochondria was monitored at an excitation wavelength of 500 nm; emission was taken at 530 nm. Minimal fluorescence was determined by addition of 500 pmol A23187 and 5 mM EGTA/30 mM Tris, pH 10.5. Maximal fluorescence was attained by adding 10 mM $CaCl_2$. The dissociation constant of the Fluo 3-Ca was assumed to be 400 nM (Kao *et al.*, 1989). Intramitochondrial H^+ concentration was measured in mitochondria loaded with 5 μM BCECFAM following the protocol described for Fluo 3-loaded mitochondria. The fluorescence obtained at 500 nm from BCECF-loaded mitochondria excited at two wavelengths (500/450 nm) was measured in an Aminco-Bowman spectrofluorometer Series 2. Calibration of matrix BCECF fluorescence was achieved at the end of each experiment by equilibrating external and matrix pH with 1.1 μM CCCP and 0.005% (v/v) Triton X-100 (Davis *et al.*, 1987; Jung *et al.*, 1989). Other experimental conditions were as described in the corresponding legends to the figures.

RESULTS

The experiments shown in Fig. 1 were carried out with the aim to analyze the influence of different phos-

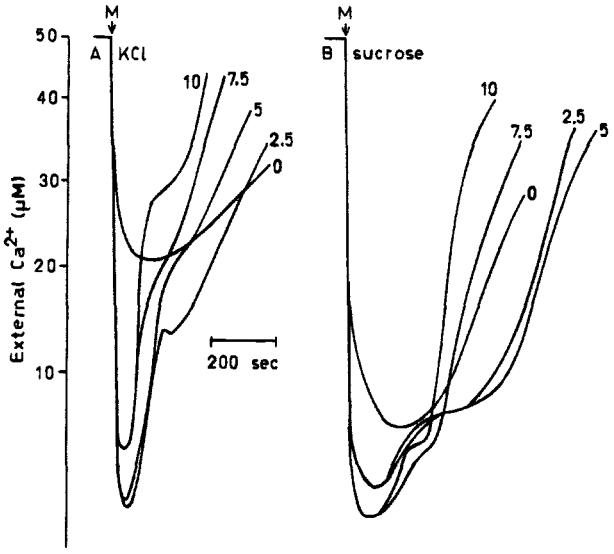

Fig. 1. Effect of increasing concentrations of phosphate on mitochondrial Ca^{2+} accumulation. (**A**) 2 mg of protein from mitochondria were incubated in 125 mM KCl, 10 mM succinate, 10 mM HEPES, 50 μM $CaCl_2$, 50 μM Arsenazo III, 5 μM rotenone, and 5 μg oligomycin. The media were adjusted to pH 7.3 with Tris. (**B**) Mitochondria (2 mg protein) were incubated in similar conditions except that 250 mM sucrose was used as osmotic support instead of 125 mM KCl. The numbers at the side of the traces indicate the concentrations, in mM, of phosphate added. Final volume, 3 ml. Temperature 25°C.

phate concentrations on matrix Ca^{2+} accumulation in mitochondria incubated in 125 mM KCl (Fig. 1A) or 250 mM sucrose (Fig. 1B). As illustrated, mitochondria incubated in KCl media accumulated Ca^{2+} at a fast rate as the concentration of phosphate was increased from 2.5 to 10 mM. However, matrix Ca^{2+} was not retained, and a rapid release reaction takes place; notably, the rate of Ca^{2+} efflux was still enhanced by increasing the phosphate concentration from 5 to 10 mM, concentrations commonly used to induce permeability transition (Wilson and Asimakis, 1987; Lapidus and Sokolove, 1994; Bravo *et al.*, 1997). The accumulation of Ca^{2+} by mitochondria incubated in sucrose media (Fig. 1B) followed a similar kinetics to that observed by mitochondria incubated in KCl media, although the efflux phase was performed at a lower rate, even at 10 mM phosphate. It is noteworthy that at a concentration of 5 mM phosphate the time of Ca^{2+} retention was more apparent.

Considering the latter results, 5 mM phosphate was chosen to study the significance of this anion on the modulation of membrane permeability transition. As well as Ca^{2+} efflux, pore opening can be estimated by following the kinetics of discharge of $\Delta\psi$. Figure 2 illustrates the role of phosphate on the transmembrane electric gradient in mitochondria incubated in isotonic KCl or sucrose media. Panel A (trace a) shows that in mitochondria incubated in KCl, in the absence of Pi, the addition of 50 μM Ca^{2+} produces a decrease in the transmembrane potential, which eventually results in its total collapse. In the presence of 5 mM inorganic phosphate the addition of 50 μM Ca^{2+} caused a fast drop in $\Delta\psi$ (trace b). In the experiment shown in panel B mitochondria were incubated in sucrose media, and as indicated (trace a), in the absence of Pi the addition of 50 μM Ca^{2+} induced a transient deenergization; nevertheless, after a partial recovery, $\Delta\psi$ remains at a high level. Interestingly, in the presence of 5 mM phosphate, the addition of 50 μM Ca^{2+} did not induce a discharge in $\Delta\psi$ (trace b), although it must be mentioned that in these conditions Ca^{2+} efflux takes place (Fig. 1B). It should be noted that in the absence of Pi and Ca^{2+}, mitochondria incubated in either potassium (Panel A, trace c) or sucrose (Panel B, trace c) media are able to build up and maintain a high level of membrane potential.

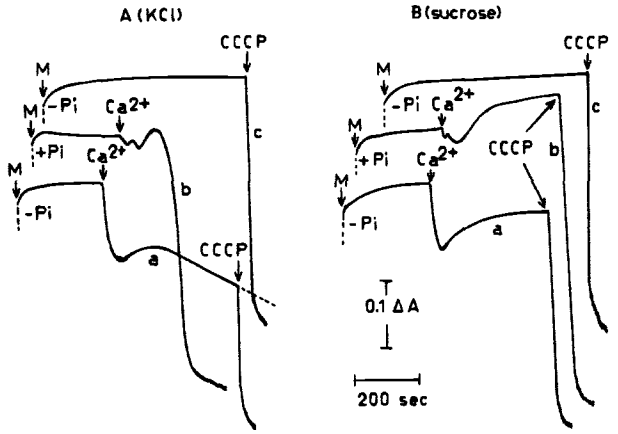

Fig. 2. Effect of phosphate on transmembrane potential of mitochondria incubated in KCl or sucrose. 2 mg mitochondrial protein were added to media containing (**A**) 125 mM KCl, 10 mM succinate, 10 mM HEPES, 10 μM Safranine, 5 μg rotenone, and 5 μg oligomycin, and (**B**) 250 mM sucrose instead of 125 mM KCl. Where indicated, 5 mM phosphate, 50 μM $CaCl_2$, or 1.0 μM CCCP were added. Final volume 3 ml. Temperature 25°C.

The experiments shown in Fig. 3 were carried out to explore the influence of phosphate and cyclosporin A on Ca^{2+} accumulation by mitochondria incubated in 125 mM KCl as osmotic support. Figure 3A (trace a) illustrates that in the absence of ADP and phosphate, Ca^{2+} was taken up to a limited extent, and then a rapid release of the cation followed. The addition of 0.5 μM CSA (trace b) did not promote the uptake of a larger amount of Ca^{2+} although its release occurred at a slightly lower rate. In Fig. 3B (trace a) it is shown that mitochondria incubated in the presence of 5 mM Pi accumulated Ca^{2+} at a faster rate and to a greater extent; however, due to the opening of the nonspecific pore the cation was rapidly released. Figure 3B (trace b) shows that in the absence of ADP, the combined action of 0.5 μM CSA and 5 mM Pi induced a massive Ca^{2+} accumulation. The cation was retained for approximately 6 min and then it gradually began to be released. In the presence of Pi and CSA, Ca^{2+} was retained for a longer time, provided that 10 μM mersalyl (mers) was added, which maintained Pi inside mitochondria (trace c). In agreement with previous reports (Andreeva and Crompton, 1994; Flores-Herrera *et al.*, 1995; Bravo *et al.*, 1997), trace d shows that when the K^+ medium was supplemented with 50 μM ADP in addition to 5 mM Pi, membrane integrity was preserved and Ca^{2+} remained inside the mitochondria.

The experiments depicted in Fig. 4 were performed to investigate the effect of Pi on Ca^{2+} retention, using 250 mM sucrose as osmotic support instead of 125 mM KCl. As observed (Fig. 4A, trace a), similarly to the incubation in KCl media, mitochondria were unable to retain Ca^{2+} in the absence of Pi, even after the addition of 0.5 μM CSA (trace b). Figure 4B (trace

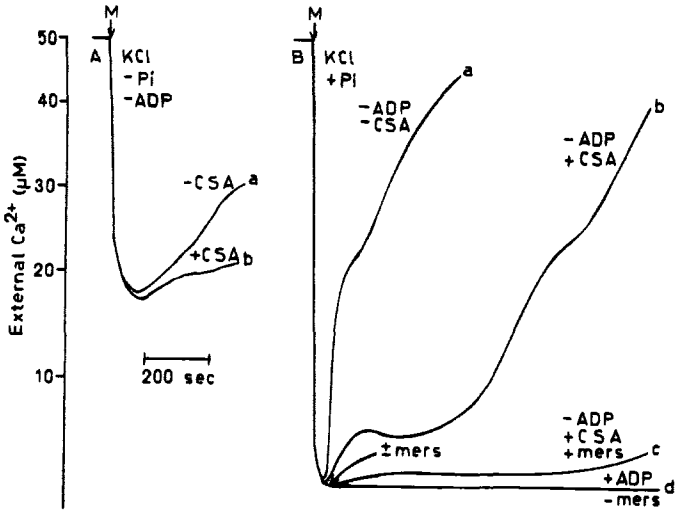

Fig. 3. Effect of phosphate, ADP, and cyclosporin A on Ca^{2+} retention by mitochondria incubated in KCl. Experimental conditions as described in Fig. 1A. Where indicated, 0.5 μM cyclosporin A (CSA), 50 μM ADP, 10 μM mersalyl (mers), or 5 mM phosphate (Pi) was added.

a) shows that 5 mM phosphate promotes Ca^{2+} retention for a longer time than it does in KCl medium. Interestingly, trace b indicates that after the addition of 5 mM Pi, CSA was able to inhibit Ca^{2+}-dependent nonspecific membrane leak, inducing the retention of the accumulated Ca^{2+}, although ADP was not added.

According to Novgorodov *et al.* (1990, 1991, 1994) and Zazueta *et al.* (1994), ADP reinforces the inhibitory effect of CSA on carboxyatractyloside-induced membrane permeabilization. Nevertheless, from the results in Figs. 3 and 4, it could be assumed that, similarly to ADP, phosphate may potentiate the effect of CSA. To add support to this proposal the experiment shown in Fig. 5 was performed. Figure 5A shows that such a statement appears to be correct since when the medium was supplemented with 5 mM Pi (trace a), 5 μM CAT did not promote the efflux of accumulated Ca^{2+}. Conversely, in a phosphate-free medium (trace b), CAT induced the efflux of accumulated Ca^{2+} regardless of the addition of 50 μM ADP plus 0.5 μM cyclosporin. Furthermore, Fig. 5B shows that in sucrose media CAT was unable to promote Ca^{2+} efflux from mitochondria incubated in the absence of ADP but in the presence of CSA as well as phosphate (trace a). However, in mitochondria incubated in KCl, plus CSA and phosphate, carboxyatractyloside induced Ca^{2+} release (trace b). Thus, it can be concluded that, at least in sucrose media, phosphate instead of ADP was required for the protective effect of CSA on the calcium-releasing action of carboxyatractyloside.

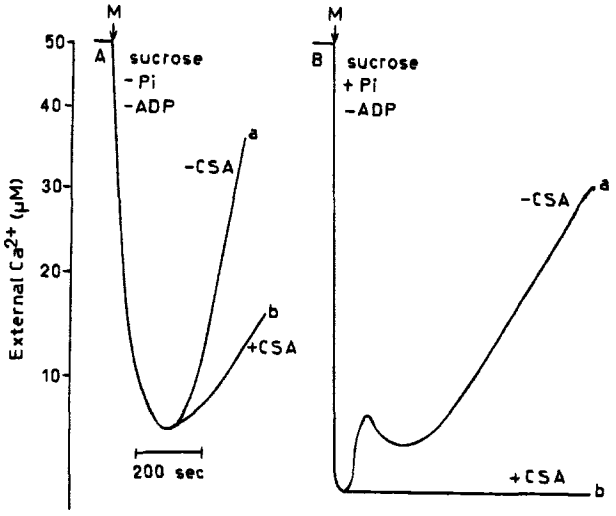

Fig. 4. Effect of phosphate and cyclosporin A on Ca^{2+} retention by mitochondria incubated in sucrose. Experimental conditions were similar to those in Fig. 3, except that 250 mM sucrose was used instead of 125 mM KCl to maintain the osmolarity. Where indicated, 5 mM phosphate and 0.5 μM cyclosporin A (CSA) were added.

The assumption that phosphate specifically enhances the action of CSA on permeability transition was supported by the observation that acetate does not reproduce the effect of phosphate (Fig. 6). For instance, in a K^+ medium (Panel A), when Pi was replaced by 5 mM acetate, Ca^{2+} was not retained regardless of the addition of ADP and CSA (trace a). In mitochondria incubated in sucrose medium using also acetate as permeant anion (Panel B), CAT

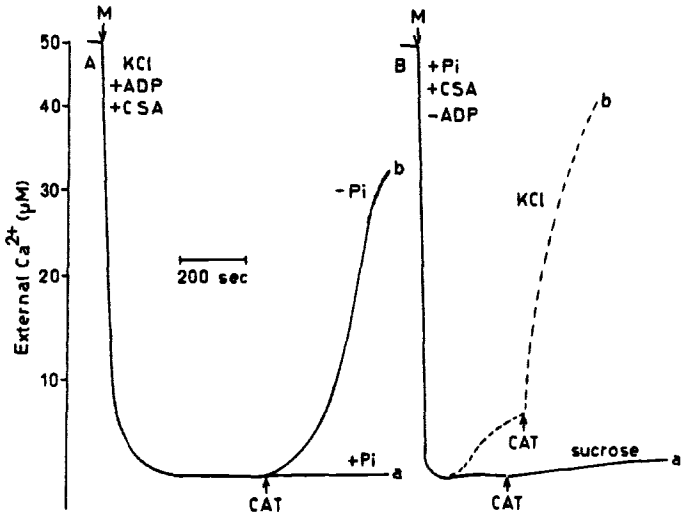

Fig. 5. Influence of phosphate on cyclosporin-induced Ca^{2+} retention. (**A**) Mitochondria were incubated in KCl-based media. (**B**) mitochondria were incubated in sucrose-based media, except for trace b, which shows the results obtained from mitochondria incubated in KCl-based medium. Where indicated, 5 mM phosphate (Pi), 0.5 μM cyclosporin (CSA), 50 μM ADP, or 5 μM carboxyatractyloside (CAT) were added.

induced the release of Ca^{2+} even in the presence of ADP and CSA (trace a). In contrast to phosphate, acetate was unable to induce retention of Ca^{2+} even in the presence of CSA (trace b). Nevertheless, the addition of ADP plus CSA to mitochondria conferred them the ability to retain Ca^{2+} in the presence of acetate as a permeant anion (trace c).

The observation that phosphate may inhibit membrane permeability transition, depending on the incubation mixture, prompted us to analyze the uptake of Pi in different conditions. The accumulation of Pi was similar in sucrose media with or without ADP or CSA, i.e., 61–68 nmol/mg (Table I). However, in KCl media the amount of matrix Pi was dependent on the presence of ADP, i.e., with 50 μM ADP the uptake of phosphate

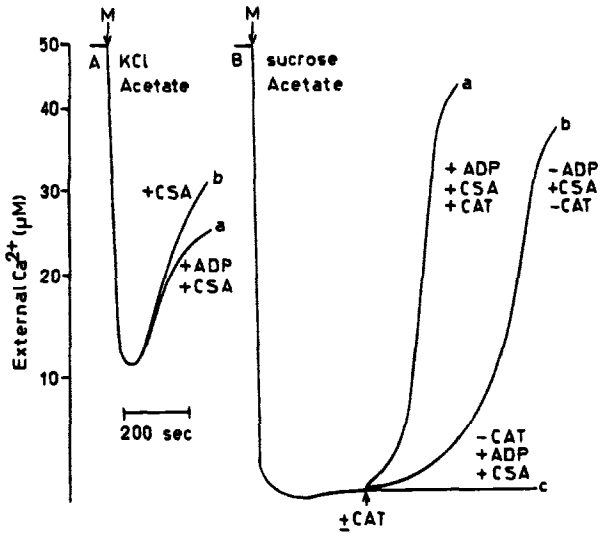

Fig. 6. Failure of acetate to strengthen the effect of cyclosporin A on Ca^{2+} retention. For the experiments shown in Fig. 6A, mitochondria were incubated in 125 mM KCl. The results shown in Fig. 6B were obtained with mitochondria incubated in 250 mM sucrose. 5 mM acetate instead of phosphate was added as permeant anion. Other additions were carried out similarly to those described in the other figures.

Table I. Phosphate Uptake[a]

	Osmotic support	
Conditions	KCl	Sucrose
+ ADP	69.4	65.16
+ CSA	26.9	61.6
–CSA –ADP	38.1	68.03

[a] 2 mg protein from mitochondria were incubated in media containing in addition to 125 mM KCl or 250 mM sucrose, 10 mM succinate, 5 mM [^{32}P] –H_3PO_4 (sp. act. 600 cpm/nmol), 50 μM $CaCl_2$, 10 mM HEPES, 5 μg rotenone, and, where indicated, 50 μM ADP or 0.5 μM CSA was added. After 5 min of incubation an aliquot of 0.2 ml was filtered through a filter of 0.45 μm pore diameter and the radioactivity retained was measured in a scintillation counter.

reached 69 nmol/mg; this value was diminished to 38 nmol/mg in the absence of ADP. The addition of CSA did not increase Pi uptake in mitochondria incubated in either KCl or sucrose media.

At this stage of the experimental work it could be assumed that the protective role of Pi appeared to be related to variations in the concentration of matrix free Ca^{2+}, and that it depended on the osmotic support of the incubation media. To place these assumptions on firmer ground the experiment in Table II was performed. In the absence of added Pi, the concentration of free Ca^{2+} ($[Ca^{2+}]_m$) was higher in KCl (533 nM) than in sucrose medium (390 nM). Although the addition of phosphate diminished $[Ca^{2+}]_m$ in both K^+ and sucrose media, to 346 and 273 nM respectively, $[Ca^{2+}]_m$ remained higher in K^+ medium. Interestingly however, after the addition of ADP and CSA, $[Ca^{2+}]_m$ diminished; but it did to a lower value in sucrose (194 nM) than in K^+ media (324 nM).

The uptake of phosphate influences both $[Ca^{2+}]_m$ (see Gunter *et al.*, 1994, for a review) and intramitochondrial pH (Hoek *et al.*, 1971). In this respect, Petronilli *et al.* (1993) and Brustovetsky and Klingenberg (1996) stated that pore opening is inhibited by increasing matrix H^+ concentration. Accordingly, Table III shows that in sucrose medium, in which phosphate protects against membrane leakage, intramitochondrial pH values are lower that in KCl medium, in which Pi does not promote pore closure.

DISCUSSION

A growing body of evidence supports the notion that phosphate is an inductor of the nonspecific increased permeability (for a review see Gunter and Pfeiffer, 1990; Zoratti and Szabò, 1995). In contrast, the results found in this work, when mitochondria were incubated in sucrose media, suggest that inorganic phosphate has a dual opposing effect: it may be an efficient prerequisite to prevent membrane leakage, and it may strengthen the ability of CSA to block the opening of the nonspecific pore. The hyperpermeable state is characterized by a fast matrix Ca^{2+} release, mitochondrial swelling, and collapse of the transmembrane electric gradient. These effects are prevented by cyclosporin A, being amplified by ADP (Novgorodov et al., 1990; Andreeva and Crompton, 1994; Zazueta *et al.*, 1994).

In a previous work by Crompton *et al.* (1988), it is indicated that mitochondrial Ca^{2+} is retained by the combined action of phosphate and CSA. The authors explain their results in terms of a CSA inhibition of the inducing action of Pi on pore opening. Our findings, however, indicate that Pi is required for the protective effect of CSA. This assumption is based on the fact that in the absence of Pi, cyclosporin was unable to retain accumulated Ca^{2+} and did not inhibit CAT-induced pore opening regardless of the addition of 50 μM ADP. The synergistic relationship between phosphate and CSA as inhibitors of nonspecific leakiness, described here, is similar to that shown by ADP-CSA (Novgorodov *et al.*, 1990; Zazueta *et al.*, 1994). In fact, the model of Halestrap and Davidson (1990) proposed that phosphate may replace ADP in binding to the adenine nucleotide translocase when the carrier is in the cytosolic (C) conformation. Accordingly, Pi, by interacting with the translocase in the C conformation, would prevent the binding of cyclophilin to translocase, which in turn would facilitate the binding of CSA to cyclophilin, removing the isomerase and closing the pore.

Table II. Intramitochondrial Free Ca^{2+} Concentrations (nM Ca^{2+})[a]

Additions	KCl	Sucrose
—	201	247
+ 1.4 μM Ca^{2+}	533	390
Ca^{2+} + Pi	346 → 216	273 → 124
Ca^{2+} + Pi + ADP + CSA	324 → 264	194 → 278

[a]Experimental conditions as described in Materials and Methods. The arrows indicate that the addition of external Ca^{2+} induced an initial change in the $[Ca^{2+}]_m$ which stabilized toward a different value after a few minutes. Similar results were obtained in two other mitochondrial preparations. The additions were as follows: 5 mM phosphate; 50 μM ADP, and 0.5 μM cyclosporin A.

Table III. Intramitochondrial H^+ Concentration as Depending on the Incubation Conditions[a]

	Matrix pH	
Additions	KCl	Sucrose
Succinate	7.52	7.18
Succ + ADP	7.40	7.17
Succ + ADP + Pi	7.32	7.11
Succ + ADP + Pi + Ca^{2+}	7.15	7.13 → 7.00
Succ + CSA	7.45	7.18
Succ + ADP + CSA	7.37	7.13

[a]Experimental conditions as described in Materials and Methods. Where indicated, 10 mM succinate (succ), 50 μM ADP, 5 mM phosphate (Pi), 50 μM Ca^{2+}, and 0.5 μM cyclosporin A (CSA) were added. The arrow indicates that the addition of external Ca^{2+} induced an initial change in internal pH which stabilized toward a different value after a few minutes. Similar results were obtained in two other mitochondrial preparations.

Crompton and Andreeva (1994) and Andreeva *et al.* (1995) have reported that Ca^{2+} inhibits the binding of CSA to a membrane component which Tanveer *et al.* (1996) have identified as a peptide of 21 kDa. Considering that phosphate diminishes $[Ca^{2+}]_m$, the addition of such anion would be associated, on the one hand, with the pore remaining fixed in the closed conformation, and on the other hand, if the pore is open, with increasing the pore-closing action of CSA. The free Ca^{2+} fraction depends on the intramitochondrial negative charges (Chávez *et al.*, 1991, 1996); thus, phosphate, by providing a high density of such a charge, can diminish the matrix active Ca^{2+} concentration available to cause membrane leakage. Accordingly, the experiment with the fluorescent Ca^{2+} indicator Fluo 3AM indicates that the concentration of matrix free Ca^{2+} was diminished in the presence of Pi.

The protective effect of phosphate on pore opening was more apparent in sucrose than in KCl media. This finding is in concordance with a previous report indicating that K^+ increases the free Ca^{2+} concentration (Chávez *et al.*, 1991). In addition, the differential effect of phosphate, by amplifying the protective action of CSA in sucrose versus KCl medium, can be explained by the finding that the concentration of matrix phosphate was higher in the former medium.

Bernardi *et al.* (1992), Petronilli *et al.* (1993), and Lapidus and Sokolove (1994) have demonstrated that the opening of the transmembrane nonspecific pore is inhibited by matrix acidification. These authors suggest that protonation of some component of the pore system, in the matrix side, changes the pore to a closed configuration. Thus, the difference in the role of phosphate in sucrose or K^+ medium can also be explained by the fact that matrix pH was found to be lower in sucrose than in K^+ medium.

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