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The Composition of the Incubation Medium Influences the Sensitivity of Mitochondrial Permeability Transition to Cyclosporin A

Edmundo Chávez,[1,2] Noemí García,[1] Cecilia Zazueta,[1] Francisco Correa,[1] César Avilés,[1] Gerardo García,[1] and Eros O. Balam[1]

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The aim of this work was to study permeability transition, and the influence of the composition of the incubation medium, on the inhibitory action of cyclosporin A. It was found that cyclosporin inhibited the opening of a nonspecific pore, as induced by the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone, provided K^+ was present in the incubation medium, but failed to do so if mitochondria are incubated in sucrose or Na^+-based medium. It was also found that the sensitivity of mitochondria to the uncoupler depended on the incubation mixture, being more sensitive when sucrose was the osmotic support. Matrix Ca^{2+} release, large amplitude swelling, and drop in transmembrane electric gradient revealed permeability transition. The titration of membrane thiol groups shows them to be increased in mitochondria incubated in sucrose medium, in comparison with the values found in mitochondria incubated in KCl or NaCl medium. Our proposal is that the incubation in sucrose medium propitiated a conformational change of membrane proteins in such a way that cyclosporin was unable to bind to its target site.

KEY WORDS: Mitochondria; Cyclosporin A; Potassium; Permeability transition; Mitochondrial calcium.

INTRODUCTION

Membrane permeability transition is a mitochondrial process, brought about as a consequence of matrix Ca^{2+} overload, in addition to an inductor agent (Bernardi, 1999; Zoratti and Szabo, 1995). Mitochondria undergo such a process through the opening of a nonspecific transmembrane pore, which allows the release of matrix solutes with a molecular mass <1500 Da (Crompton *et al.*, 1987). A series of inductors have been introduced into the field of Ca^{2+}-induced membrane leakiness, among them the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Gunter and Pfeiffer, 1990; Lim *et al.*, 2001), which collapses the pH gradient and switches the membrane permeability from specific to nonspecific. Regardless of the condition imposed to induce permeability transition, the immunosuppressive agent cyclosporin A (CSA) proved shown to be an effective inhibitor (Broekemeier *et al.*, 1989). The inhibitory action of CSA requires special conditions, i.e., the addition of ADP (Novgorodov *et al.*, 1994; Zazueta *et al.*, 1994), or phosphate (Chávez *et al.*, 1997). Furthermore, the effect of CSA is dependent on the energy state of mitochondria (Chávez *et al.*, 2000), and the kind of oxidizable substrate (Chávez *et al.*, 2002).

There are evidences that the ionic composition of the incubation medium influences the induction, or inhibition, of permeability transition. It was found by our group (Chávez *et al.*, 1991) that pore opening, as induced by carboxyatractyloside, is largely stimulated by K^+. On the other hand, several authors have shown that Mg^{2+}, per se, inhibits nonspecific permeability (Hunter and Haworth, 1979), and is required for the inhibitory effect of CSA (Novgorodov *et al.*, 1994). Therefore, the experiments in this work were designed to investigate the

[1] Departamento de Bioquímica, Instituto Nacional de Cardiología Ignacio Chávez, 014080 México, D. F., México.

[2] To whom correspondence should be addressed; e-mail: echavez@ssa.gob.mx.

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ability of K^+ to modulate the sensitivity of mitochondrial permeability transition to CSA, as well as the role of K^+ on the sensitivity of mitochondria to CCCP-induced permeability transition. The results obtained indicate that K^+ is an important prerequisite for the inhibitory effect of CSA on CCCP-induced matrix Ca^{2+} release, mitochondrial swelling, and collapse of the transmembrane potential. In contrast, CSA fails to inhibit permeability transition when mitochondria are incubated in sucrose or sodium medium. In addition, it is also shown that by incubating mitochondria in KCl and NaCl medium, a higher concentration of CCCP is required to promote Ca^{2+} efflux, in contrast with that when mitochondria are incubated in sucrose medium. We also found that incubation of mitochondria in sucrose medium cause an increase in the titration of membrane thiol groups; this suggests a conformational change in membrane proteins, following a steric hindrance of the target site of cyclosporin.

MATERIALS AND METHODS

Kidney cortex mitochondria were prepared after homogenization of the tissue in 0.25 M sucrose–1 mM EDTA adjusted to pH 7.3, following the standard centrifugation procedure. The last washing was carried out in an EDTA-free sucrose medium. Potassium-depleted mitochondria were prepared according to the method described by Gómez-Puyou *et al.* (1969) by incubating mitochondria in a medium that contained 125 mM NaCl, 10 mM phosphate, 10 mM glutamate, and 10 mM EDTA. After 10 min incubation at room temperature, mitochondria were centrifuged at 10,000 rpm, and washed twice with 250 mM sucrose free of EDTA. Mitochondrial protein was determined by the Lowry method (Lowry *et al.*, 1951). Mitochondrial potassium content was determined by atomic absorption in acid extracts. The uptake and release of mitochondrial Ca^{2+} were analyzed spectrophotometrically at 675–685 nm, using the metallochromic indicator Arsenazo III (Scarpa *et al.*, 1978). The transmembrane electric gradient was estimated in a spectrophotometer at 511–535 nm, by using the hydrophobic cation Safranine (Akerman, and Wikström, 1976). Thiol groups of membrane proteins were titrated by adding 300 μM of the Ellman's reagent 5,5′-dithiobis 2-nitrobenzoic acid to mitochondria incubated in KCl, NaCl, or sucrose medium; after 10 min incubation to mixture was spun down at 12,000 rpm, and the resulting supernatant was used for the spectrophotometric measurement at 412 nm. Cysteine was used as a standard. Changes in mitochondrial volume were followed at 540 nm. Other experimental conditions as were described in the respective legends of figures.

RESULTS

Effect of the Composition of Incubation Mixture on CCCP-Induced Permeability Transition

In a previous work we showed that carboxyatractyloside effectively opens transmembrane pore provided K^+ is added to the incubation medium (Chávez *et al.*, 1991). Thus, the purpose of the experiments in Fig. 1 became focused on the study of the role of K^+ on CCCP-induced permeability transition. The data in Fig. 1(A) show the response of mitochondria incubated in 125 mM KCl, as osmotic support, to the addition of increasing concentrations of the uncoupler. As observed, 0.25 μM CCCP induced a slight efflux of the accumulated Ca^{2+}. However, the efflux rate was considerably stimulated after the addition of 0.5 μM CCCP. In contrast, when mitochondria were incubated in 250 mM sucrose-based medium (Fig. 1(B)), the picture was quite different, i.e., 0.25 μM CCCP induced a fast matrix Ca^{2+} discharge, which was notably increased with 0.375 μM CCCP. Searching for some cation selectivity, the experiment in Fig. 1(C) was performed by incubating mitochondria in 125 mM NaCl. Under these conditions, mitochondrial membrane proved to be more resistant to the uncoupler since 0.25 μM CCCP did not induce Ca^{2+} release, this reaction only took place until the concentration of CCCP reached 0.5 μM, being faster with 0.75 μM CCCP. However, the rate was slower than that obtained in KCl or sucrose media. From the above, it is evident that mitochondria are less sensitive to the uncoupler when incubated in KCl or NaCl, than mitochondria incubated in sucrose medium.

Effect of the Composition of the Incubation Medium on the Protective Action of CSA

The latter findings open the possibility of modifications in the sensitivity to CSA, for pore closure, by changing the composition of the incubation mixture. It is clear, from the results shown in Fig. 2 that the inhibitory behavior of CSA depends on the presence of K^+. As observed in Fig. 2(A), in mitochondria incubated in 125 mM KCl, 0.5 μM CSA completely arrested Ca^{2+} release reaction induced after the addition of 0.375 μM CCCP. In contrast, Fig. 2(B) shows that CSA was unable to do so when the medium contained 250 mM sucrose instead of KCl. With the aim to carry out a comparison with K^+, mitochondria were incubated in 125 mM NaCl. In Fig. 2(C) it is shown that under these conditions CSA also failed to inhibit Ca^{2+} efflux, as induced by 0.5 μM CCCP. It should be noted that this concentration was used to reach a significant efflux rate of Ca^{2+} (see Fig. 1(C)).

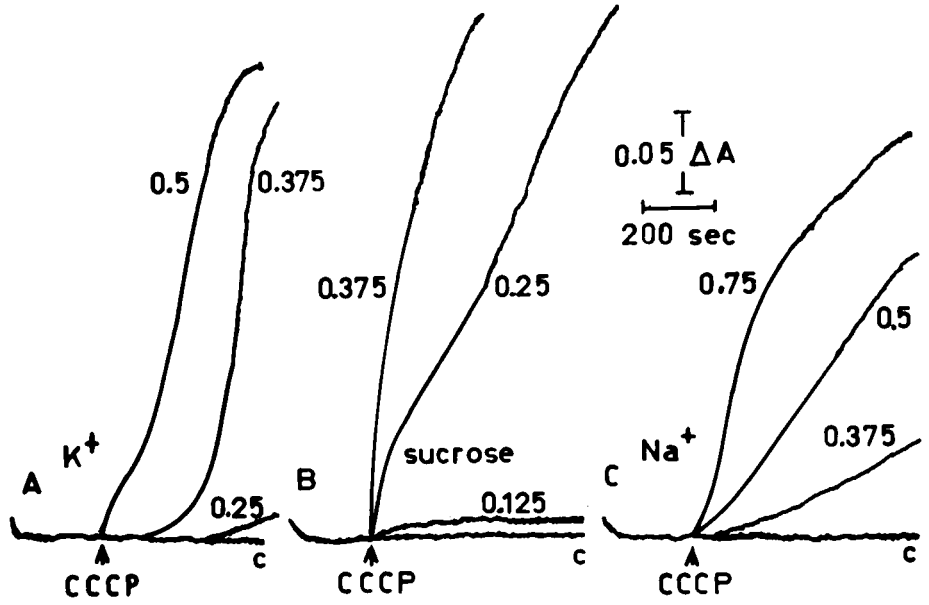

Fig. 1. CCCP-induced matrix Ca^{2+} release in mitochondria incubated under different conditions. Two milligrams of mitochondrial protein were incubated in media that contained, as osmotic support, in A, 125 mM KCl; in B, 250 mM sucrose; and in C, 125 mM NaCl. In addition, the media contained 50 μM $CaCl_2$, 10 mM succinate, 10 mM HEPES, 2 mM phosphate, 100 μM ADP, 5 μg rotenone, 5 μg oligomycin, and 50 μM Arsenazo III. The numbers at the side of the traces indicate the micromolar concentrations of CCCP added. The pH level was adjusted to pH 7.3 with Tris base. Final volume: 3 mL. Temperature: 22°C.

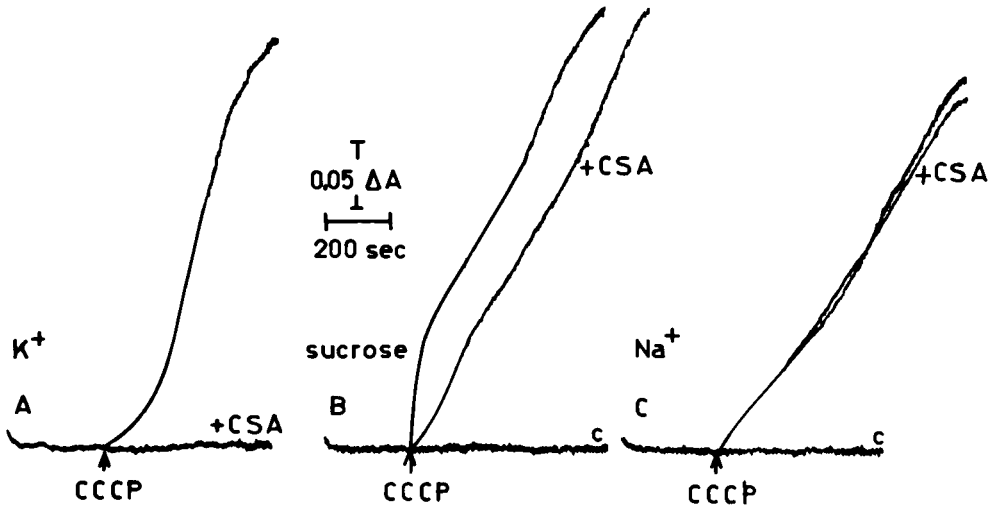

Fig. 2. The effect of the composition of the incubation medium on the inhibitory action of CSA. Two milligrams of mitochondrial protein were added to incubation media that contained in A, 125 mM KCl; in B, 250 mM sucrose; and in C, 125 mM NaCl. The additions of CCCP were as follows: in A and B, 0.375 μM; and in C, 0.5 μM. CSA was added at a concentration of 0.5 μM. Other conditions as indicated in Fig. 1.

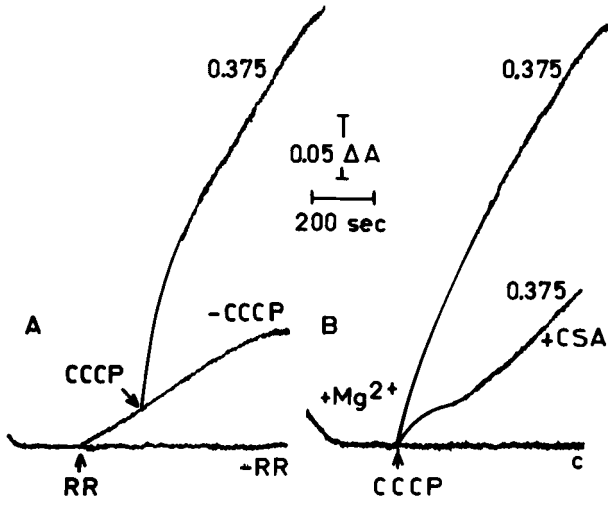

Fig. 3. The effect of ruthenium red and magnesium on CCCP-induced Ca^{2+} efflux in sucrose medium. The incubation media contained 250 mM sucrose, in addition to the basic reagents indicated in Fig. 1. Where indicated, 0.166 μM ruthenium red (RR), 160 μM $MgCl_2$, or 0.375 μM CCCP were added. Other conditions as indicated in Fig. 1.

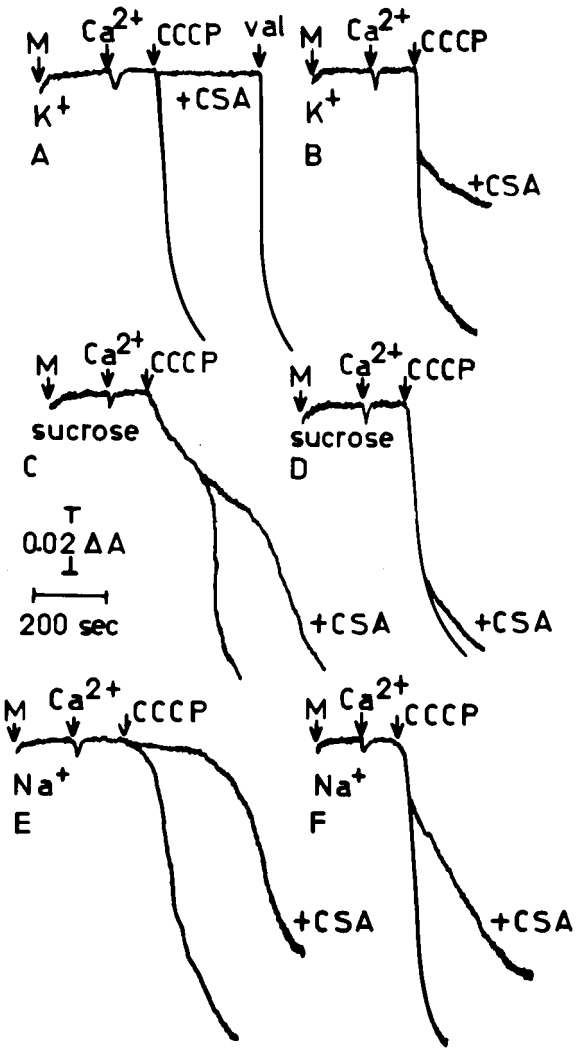

Fig. 4. The influence of the incubation medium on the effect of CSA on CCCP-induced collapse of the transmembrane potential. Two milligrams of mitochondrial protein were incubated under the following conditions: in A and B the media contained 125 mM KCl; in C and D, 250 mM sucrose; and in E and F, NaCl. The basic media were similar to that indicated in Fig. 1, except that 10 μM safranine was added instead of Arsenazo III. The added concentrations of CCCP were as follows: in A, C, and E, 0.125 μM; in B, D, and F, 0.25 μM. In addition, 50 μM Ca^{2+}, 0.5 μM CSA, and 5 μg valinomycin were added. Final volume: 3 mL. Temperature: 22°C.

The Effect of Ruthenium Red on CCCP-Induced Permeability Transition in Mitochondria Incubated in Sucrose Medium, and the Effect of Mg^{2+} on CSA Inhibition

Considering the lack of inhibition by CSA, the possibility emerges that in sucrose medium CCCP-induced Ca^{2+} efflux could be accomplished through the reversible pathway of the calcium uniporter, a reaction that is inhibited by ruthenium red (Kroner, 1992). Figure 3(A) shows that 0.166 μM ruthenium red did not inhibit matrix Ca^{2+} depletion after the addition of 0.375 μM CCCP. On the other hand, it has been previously reported that Mg^{2+} strengthens the effect of CSA on permeability transition (Novgorodov *et al.*, 1994). Thus, the sucrose medium was supplemented with 160 μM $MgCl_2$, a concentration that does not inhibit Ca^{2+} uptake. Figure 3(B) shows that, certainly, in the presence of magnesium, CSA considerably retards the rate of Ca^{2+} release.

Effect of the Composition of the Incubation Medium on the Protective Effect of CSA on CCCP-Induced Collapse of the Transmembrane Potential

The collapse of the transmembrane electric gradient ($\Delta\Psi$) by CCCP is also an evidence indicating the opening of the nonselective pore. Therefore, it was decided to analyze the role of CSA in this reaction, under the different incubation conditions. As shown in Fig. 4(A), the addition of 0.5 μM CSA to mitochondria incubated in KCl medium avoids the drop in $\Delta\Psi$ induced by the addition of 0.125 μM CCCP. Figure 4(B) shows, however, that at a high concentration of CCCP, i.e., 0.25 μM, CSA only slightly inhibited the decrease in $\Delta\Psi$. Contrasting with that observed in KCl medium, when mitochondria were incubated in sucrose medium (Fig. 4(C)), CSA partially decreased the rate of collapse

of $\Delta\Psi$, as stimulated by 0.125 μM CCCP. Figure 4(D) illustrates that CSA failed to inhibit the effect of 0.25 μM on $\Delta\Psi$. In NaCl medium CSA considerably delayed the collapse of membrane potential after the addition of 0.125 μM CCCP (Fig. 4(E)). Figure 4(F) shows that, in NaCl medium, the rate in the drop of $\Delta\Psi$, by the addition of 0.25 μM CCCP is slightly modified by CSA. These results reinforce the assumption on the requirement of K^+ for the protective effect of CSA on permeability transition.

The Role of the Composition of the Incubation Medium on the Effect of CSA on CCCP-Induced Mitochondrial Swelling

To further ascertain the effect of the incubation medium on CSA pore closure, mitochondrial swelling, as a parameter to estimate permeability transition, was analyzed. Figure 5(A) shows that, in KCl medium, CSA indeed completely inhibited the large amplitude swelling stimulated by 0.375 μM CCCP. As shown in Fig. 5(B), when mitochondria were incubated in sucrose medium, CSA inhibited, by about 40%, mitochondrial swelling induced by 0.375 μM CCP. Figure 5(C) shows that swelling of mitochondria as induced by the addition of 0.5 μM CCCP was inhibited by approximately 50% with CSA, when the incubation mixture contained NaCl. It should be noted that at the indicated concentration the uncoupler induced an appreciable increase in mitochondrial volume.

Effect of the Composition of the Incubation Medium on CCCP-Induced Ca^{2+} Efflux From Potassium-Depleted Mitochondria

Mitochondrial inner membrane is usually exposed to a high concentration of matrix K^+. Thus, with the aim to discard a possible effect of this cation on the influence that the incubation mixture exerts, the experiments shown in Fig. 6 were carried out with K^+-depleted mitochondria. It should be noted that control mitochondria contained 58.15 nmol K^+/mg protein; after depletion this value was diminished to 10.9 nmol/mg protein. Figure 6, Panel A illustrates the effect of different concentrations of CCCP on Ca^{2+} content in mitochondria incubated in 125 mM KCl. A particular characteristic observed was

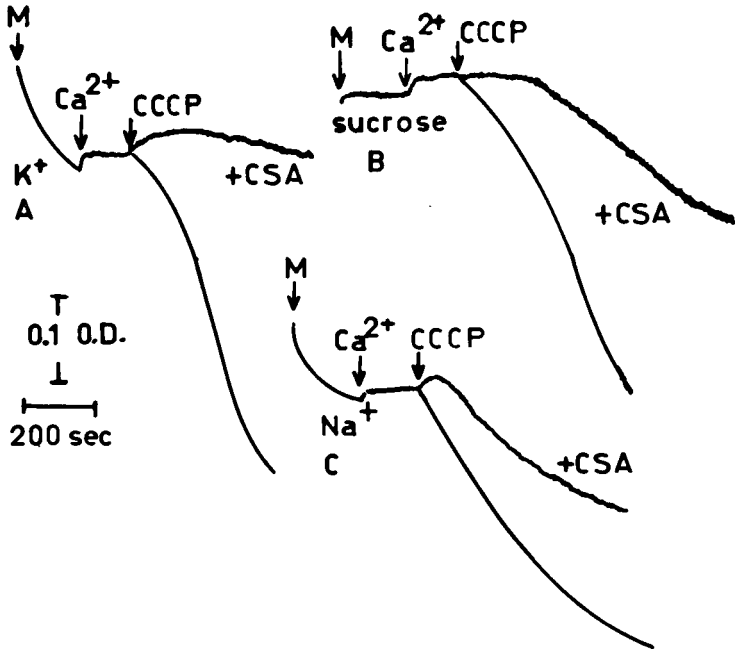

Fig. 5. The effect of the incubation medium on the inhibition by CSA of CCCP-induced mitochondrial swelling. Two milligrams of mitochondrial protein were incubated in a medium containing in A, B, and C respectively, 125 mM KCl, 250 mM sucrose or 125 mM NaCl, in addition to the basic medium described in Fig. 1. In A and B 0.375 μM CCCP were added, and in C, 0.5 μM CCCP. Furthermore, 50 μM Ca^{2+} and 0.5 μM CSA were added where indicated. Volume: 3 mL. Temperature: 22°C.

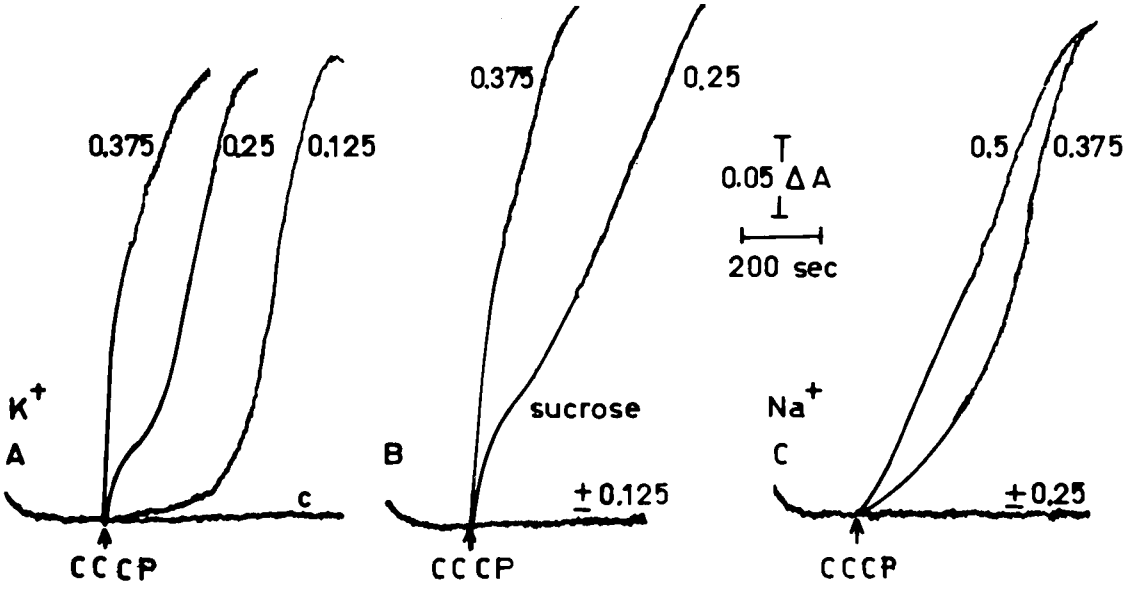

Fig. 6. Influence of the composition of the incubation mixture on Ca^{2+} efflux from potassium-depleted mitochondria as induced by increasing concentrations of CCCP. Two milligrams of protein from K^+-depleted mitochondria were incubated in medium containing in A, 125 mM KCl, in B, 250 mM sucrose, and in C, 125 mM NaCl, in addition to the basic mixture described in Fig. 1. The numbers at the side of the traces indicate μM concentrations of CCCP. Volume: 3 mL. Temperature: 22°C.

the fact that mitochondria becomes more sensitive to the action of the uncoupler, in comparison to that observed in control mitochondria (Fig. 1). A similar behavior was observed when mitochondria were incubated in sucrose or NaCl media, Panels B and C, respectively.

Effect of CSA on CCCP-Induced Ca^{2+} Efflux From K^+-Depleted Mitochondria

The data in Fig. 7(A) were obtained from mitochondria incubated in KCl medium. As expected, in K^+-

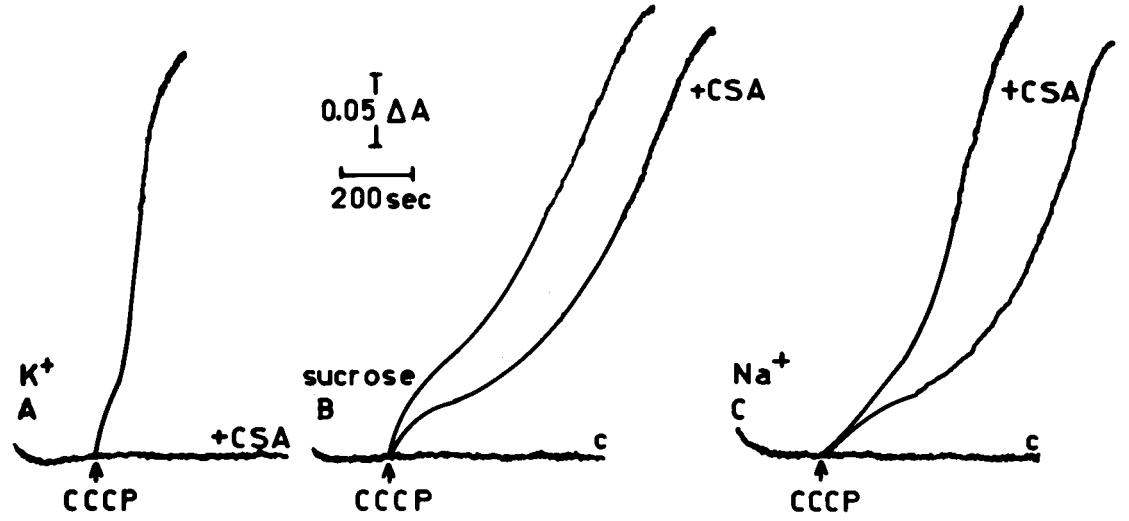

Fig. 7. Influence of the composition of the incubation medium on the inhibition by CSA of CCCP-induced Ca^{2+} release from potassium-depleted mitochondria. Two milligrams of K^+-depleted mitochondria were incubated in a medium containing in A, 125 mM KCl; and in B, 250 mM sucrose; and in C, 125 mM NaCl. The media contained, in addition to the basic mixture described in Fig. 1, the following concentrations of CCCP: in A and B, 0.375 μM; and in C, 0.5 μM. Where indicated, 0.5 μM CSA was added. Volume: 3 mL. Temperature: 22°C.

depleted mitochondria CSA completely eliminated Ca^{2+} efflux promoted after the addition of 0.375 μM CCCP. Figure 7(B) shows, however, that when the medium contained sucrose as osmotic support, CSA only slightly delayed, for a few minutes, the Ca^{2+} efflux phase, and following that, a complete release occurred. A similar result was obtained with mitochondria incubated in Na^+ medium (Fig. 7(C)).

Effect of the Incubation Mixture on the Titration of Membrane Thiol Groups

Differences in the sensitivity to CSA could be explained as occuring from a structural change in membrane proteins. To assess such possibility, titration of membrane thiol groups was carried out. The titration indicated an increase in the exposed −SH groups when mitochondria were incubated in sucrose medium, i.e., 53.45 ± 0.05 nmol/mg, in relation with those found when mitochondria were incubated in KCl or NaCl medium, 36.3 ± 1.3, and 35.7 ± 0.4, respectively. The values represent averages of three different experiments plus/minus standard deviation.

DISCUSSION

The inhibitory effect of CSA on nonspecific permeability is mainly related to its interaction with the matrix enzyme peptidyl–prolyl cis–trans isomerase (Tanveer, *et al.*, 1996). However, several reports indicate that, indeed, CSA may be bound to membrane proteins different from that of isomerase (McGuinness *et al.*, 1990). To accomplish the interaction of CSA with cyclophilin or a putative membrane protein some conditions are necessary, say, the presence of ADP or Mg^{2+}. Regarding the requirement for ADP, to reach the highest effectiveness of inhibition, Novgorodov *et al.* (1994) assert that the nucleotide acts synergistically with CSA through its binding to a membrane site, insensitive to carboxyatractyloside (CAT), whereas Andreeva and Crompton (1994) have shown that ADP selectively increases the binding of CSA to a membrane protein of approximately 10 kDa. Our results are consistent with the role of ADP, when mitochondria are exposed to a K^+ medium; however, as we found, ADP did not influence the action of CSA in sucrose medium. In reference to the role of Mg^{2+}, Novgorodov *et al.* (1994) discuss that the cation may form a complex with ADP, favoring the binding of the nucleotide to a site that regulates the opening/closure cycles of the pore. In turn Bernardi *et al.* (1992) proposed that Mg^{2+} interacts directly with the pore, closing it, whereas Andreyev *et al.* (1994) argue that Mg^{2+} acts in a competitive fashion, with carboxyatractyloside regulating pore opening. Yet, the data presented here indicate that, under the conditions of sucrose incubation, the addition of Mg^{2+} partially improves the effect of CSA on CCCP-induced Ca^{2+} depletion. It is possible that we cannot observe an improvement effect of the divalent cation on account of the low concentration used; but it should be recognized that under our experimental conditions a high concentration of Mg^{2+} inhibited Ca^{2+} uptake. Moreover, as discussed by Novgorodov *et al.* (1994), the Mg^{2+}–CSA pair may close the pore in some mitochondria but not in others.

With regard to the effect of monovalent cations on permeability transition, our group has been engaged in the study of the role of K^+ on the activity of the transmembrane nonspecific pore. We have shown that K^+ improves the effect of CAT on permeability transition (Chávez, *et al.*, 1991), and also abolishes the protective action of phosphate on Ca^{2+}-induced membrane leakage (Chávez, *et al.*, 1997). The data presented in this paper are in fact the first indication that K^+ is a necessary prerequisite for a complete inhibitory action by CSA, when permeability transition is brought about by CCCP. The effect of the cation appears not to be merely due to an increase in the ionic strength, since as already shown, Na^+ cannot substitute the effect of K^+. We have previously shown that K^+ increases the free Ca^{2+} fraction (Chávez *et al.*, 1991). Thus, it is provocative to assume that the binding of CSA to its membrane target site would depend on free matrix Ca^{2+} concentration. Actually, in some models, Ca^{2+} is required for the effect of CSA; for example, Ryffel *et al.* (1993) have shown that, in Jurkat cells, the formation of a ternary complex between CSA, calcineurin, and cyclophilin is dependent on the presence of Ca^{2+}. There are a number of published data about the failure of CSA to inhibit permeability transition. Dissimilar causes are attributed to such a failure, i.e, Nepomuceno and Pereira-da-Silva (1993) have shown that CSA only transiently inhibits mitochondrial swelling when induced by lipid peroxidation. By the same token, Fortes *et al.* (2001) demonstrated that, in potato mitochondria, when membrane damage is induced by oxidative stress, CSA does not inhibit it. Brustovetsky and Dubinsky (2000) showed that in brain mitochondria CSA fails to inhibit membrane leakage as induced by large Ca^{2+} load, palmitic acid, or the protonophore carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone. Our results appear to be in agreement with the work of Malkevitch *et al.* (1997), who demonstrated that CSA fails to block nonspecific pore when its opening is induced by thyroxine; it should be pointed out that mitochondria were incubated in sucrose medium. Nevertheless, the lack of inhibition by

CSA can be ascribed to a structural change of membrane proteins.

There is a growing body of evidence arguing that the nonspecific pore may be formed by the adenine nucleotide translocase (ANT; for a review see Bernardi, 1999). After the model of Halestrap *et al.* (1997), the aperture of the pore, besides Ca^{2+} and an assortment of promoters, requires the binding of matrix cyclophilin to ANT, whereas the closure depends on it being unbound, because of the interaction with CSA. The model was firmly established by experiments of Woodfield *et al*. (1998). A conformational change of membrane proteins would result in a steric hindrance of the binding of CSA to its target site. In fact, Ricchelli *et al.* (1999) demonstrated that permeability transition induces modifications in membrane fluidity, which can be attributed to conformational changes of pore-forming proteins. Moreover, Campo *et al.* (1997) argue that FCCP and CCCP, at nanomolar concentrations, may act as sulfhydryl reagents do since their effects on mitochondria can be reversed by dithiothreitol. Thus, the lack of CSA inhibition in sucrose medium may evoke a change in the structure of membrane proteins. Our findings about the increased titration of membrane thiols in mitochondria incubated in sucrose medium are in agreement with that assumption.

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