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On the Role of the Respiratory Complex I on Membrane Permeability Transition

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In this work we studied permeability transition by incubating mitochondria in the presence of 50 μM Ca^{2+} and malate/glutamate as substrates. This condition, besides inducing the release of pyridine nucleotides, promotes the generation of reactive oxygen-derived species by the complex I of the respiratory chain. The latter leads to the opening of the mitochondrial permeability transition pore. Ca^{2+} release, mitochondrial swelling and collapse of the transmembrane electric potential, were analyzed to assess this process. We propose that the mechanism for pore opening, in addition to the oxidative stress, involves the uncoupling effect of fatty acids providing activation of phospholipase A2, lipid peroxidation, and the oxidation of membrane thiols. This proposal emerges from the data indicating the protective effect of bovine serum albumin and *N*-ethylmaleimide. The key role of reactive oxygen species was implied based on the fact that the scavenger α-phenyl-*tert*-butyl nitrone inhibited pore opening.

KEY WORDS: Mitochondria; permeability transition; mitochondrial calcium; complex I of the respiratory chain; oxidative stress; reactive oxygen species.

INTRODUCTION

Matrix Ca^{2+} overload leads mitochondria to a membrane permeability transition characterized by the opening of a high conductance pore, of approximately 3 nm of diameter, that allows the efflux of metabolites and ions (Crompton *et al*., 1987; Zoratti and Szabo, 1995; Bernardi, 1999). The chemical nature of the pore has been ascribed to be the adenine nucleotide translocase (Brustovetsky and Klingenberg, 1996; Woodfield *et al*., 1998; Haworth and Hunter, 2000; Zazueta *et al*., 2000). This assumption is based on the fact that ADP and to a lesser extent ATP inhibit the opening of the non-specific pore, while atractyloside and carboxyatractyloside open it (Haworth and Hunter, 2000). A fast matrix Ca^{2+} discharge, mitochondrial swelling, and collapse of the transmembrane electric gradient characterize the hyperpermeable state. In addition to Ca^{2+} overload, pore opening requires of inducing agents that have a very dissimilar chemical structure, such as sulfhydryl reagents, *N*-ethylmaleimide, and phenylarsine oxide (Kowaltowski *et al*., 1997); heavy metals, Pb^{2+} and Hg^{2+} (Chávez *et al*., 1987; Chávez and Holguín, 1988); metabolites as inorganic phosphate and fatty acids (Bravo *et al*., 1997; Kushnareva *et al*., 1999; Wieckowski *et al*., 2000); prooxidants, like *t*-butyl hydroperoxide, and oxygen-derived reactive species (Broekemeier and Pfeiffer, 1995; Kushnareva and Sokolove, 2000). Further, non-specific permeability transition pore can be opened through pathological processes such as ischemia/reperfusion (Arteaga *et al*., 1992; Halestrap *et al*., 2004), and hyperthyroidism (Kalderon *et al*., 1995). Regarding the oxygen-derived reactive species (ROS) these reagents can be generated chemically by different mechanisms, i.e., the addition of Fe^{2+} or Fe^{3+} plus citrate (Castilho *et al*., 1995). Nevertheless, mitochondrial electron flow through complexes I and III becomes the principal physiologic generator of superoxide and H_2O_2 (Herrero and Barja, 2000; McLennan and Esposti, 2000). Indeed, the activity of superoxide

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dismutase, catalase and glutathione peroxidase protects the cell from the low amount of mitochondrial ROS that are generated under physiologic conditions. However, under pathologic circumstances, for example reperfusion-induced Ca^{2+} overload, the activity of the protective systems appears to be insufficient.

Several reports have demonstrated that the mechanism by which the oxidative stress open the pore would be related to lipid peroxidation, and thiol oxidation of membrane proteins (Fagian *et al*., 1990; Castilho *et al*., 1995; Castilho *et al*., 1998; Cosso *et al*., 2002). ROS also activates phospholipase A2 (Madesh and Balasubramanian, 1997) that catalyzes fatty acid hydrolysis from membrane phospholipids. Broekemeier and Pfeiffer (1989) postulate that activation of mitochondrial phospholipase A2 leads to an intramembranous accumulation of lysophospholipids and free fatty acids, that in turn are thought to be responsible for the increased permeability.

In this paper we show that incubation of mitochondria in the presence of supraphysiological Ca^{2+} concentrations promotes dysfunction of mitochondrial Complex I. At the first glance such a dysfunction would be attributed to the loss of pyridine nucleotides (Vinogradov *et al*., 1972). However, it must be recognized that the interruption of electron transport in Site I, i.e., by rotenone (Li *et al*., 2003), brings about the generation of reactive oxygen species, which in turn leads mitochondria to undergo permeability transition. It is proposed that the mechanism involved, in which ROS play a central role, is the production of free fatty acids and oxidation of membrane thiols. This assumption is based on the fact that pore opening was inhibited by the addition of the fatty acids chelator bovine serum albumin, as well as by the addition of the thiol-modifier *N*-ethylmaleimide, and the ROS scavenger α-phenyl-*N*-*tert*-butyl nitrone.

MATERIALS AND METHODS

Mitochondria from the cortex of rat kidney were prepared by homogenizing the tissue in 0.25 M sucrose-1 mM EDTA, adjusted to pH 7.3 with Tris, and following the standard centrifugation pattern. The last wash was performed in EDTA-free sucrose medium. Mitochondrial protein was measured with the Lowry method (Lowry *et al*., 1951). Calcium movement was followed using the metallochromic indicator Arsenazo III at 675–685 nm (Scarpa *et al*., 1978). Mitochondrial transmembrane electric gradient was assayed at 511–533 nm by using Safranine as indicator (Akerman and Wikström, 1976). Changes in optical density at 540 nm were used as indicators of mitochondrial swelling. Matrix NADH release was measured fluorometrically at 340 nm excitation and 460 nm emission, in the supernatant obtained after centrifugation of mitochondria incubated in the presence of Ca^{2+}. Lipid peroxidation was analyzed by measuring the thiobarbituric acid reactive species formed after incubation of mitochondria in media containing malate/glutamate as the substrates and 50 μM $CaCl_2$ (Buege and Aust, 1978). The phenazine methosulfate (PMS) and 2,6-dichlorophenolindophenol (DCPIP) method was followed to measure succinate dehydrogenase activity (Mowery *et al*., 1977). Briefly, 2 mg mitochondrial protein were preincubated in the basic media containing malate/glutamate as the substrates, with or without 5 μg rotenone; in addition the media contained 0.5 mM PMS, and 50 μM DCPIP. After 5 min 5 mM succinate was added and the changes in absorbency at 600 nm were measured. The basic incubation medium, adjusted to pH 7.3, contained 125 mM KCl, 10 mM malate, 10 mM glutamate, 3 mM inorganic phosphate, 10 mM HEPES, 3 μg oligomycin and 200 μM ADP. Where indicated the NAD-dependent substrates were substituted by 10 mM succinate plus 5 μg rotenone. The ANOVA analysis was used to compare the data of NADH released and TBARS generated. Values are expressed as means $\pm$ S.D.; significance was set at $P < 0.05$.

RESULTS

Ca^{2+} is rapidly accumulated into mitochondria when supported by the oxidation of a respiratory substrate. However, as illustrated in Fig. 1A, in malate/glutamate oxidizing mitochondria, accumulation of the cation depends on its concentration in the incubation mixture. Trace a shows that mitochondria were inefficient to take up a large amount of Ca^{2+} when the concentration of this metal attained 50 μM; whereas, as seen in trace b, a fast accumulation and retention was achieved when Ca^{2+} concentration was diminished to 25 μM. The failure of mitochondria to accumulate the cation would be attributed to the Ca^{2+}-induced loss of pyridine nucleotides (Vinogradov *et al*., 1972). However, Fig. 1A, trace a, also shows that the addition of 5 mM succinate did not induce Ca^{2+} retention. With the purpose to discard the possibility that succinate dehydrogenase were inhibited by the oxaloacetate formed in the absence of rotenone, the activity of this enzyme was measured, the values obtained with or without rotenone were very similar, i.e., 106.9 ± 26, and 108.6 ± 17, respectively. From the latter, arises the possibility that, together with the release of pyridine nucleotides, the inefficacy to accumulate Ca^{2+} would be due to the opening of the non-specific pore, through a mechanism involving the function of the respiratory Complex I, as has been

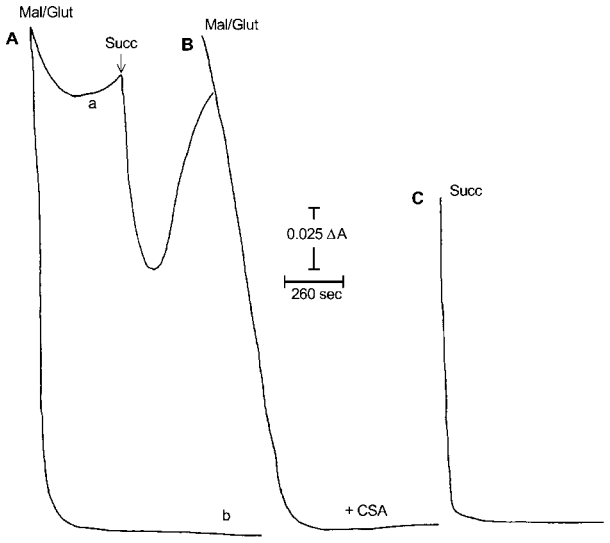

Fig. 1. The inability of malate/glutamate-oxidizing mitochondria to accumulate calcium. Mitochondrial protein (2 mg) was added to 3 ml of the basic incubation mixture described under Materials and Methods. Where indicated 10 mM malate and 10 mM glutamate (Mal/Glut), or 10 mM succinate (Succ), plus 5 μg rotenone, were added as substrates. In Panel (A) trace a, the medium contained 50 μM $CaCl_2$, in trace b the medium contained 25 μM $CaCl_2$. In Panel (B) 0.5 μM cyclosporin A (CSA) was added. In Panel (C) the medium contained 100 μM $CaCl_2$. The media contained in addition 50 μM Arsenazo III. Where indicated 5 mM succinate was added. Temperature 25°C.

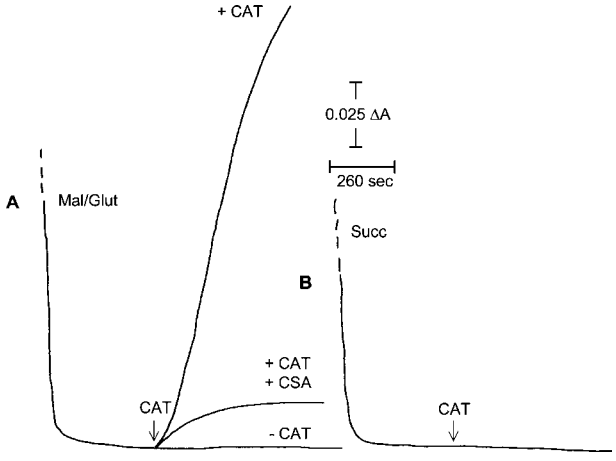

Fig. 2. The calcium-releasing effect of carboxyatractyloside on malate/glutamate-oxidizing mitochondria. Mitochondrial protein (2 mg) was incubated in similar conditions to those described for Fig. 1. In (A) the medium contained 25 μM $CaCl_2$, and where indicated 0.3 μM carboxyatractyloside (CAT), or 0.5 μM CSA were added. In (B) the medium contained 100 μM $CaCl_2$, and where indicated 1 μM carboxyatractyloside (CAT) was added.

proposed by Fontaine *et al*. (1998). This latter was confirmed by the result shown in Fig. 1B which indicates that the addition of cyclosporin A (CSA) restored the ability of mitochondria to accumulate Ca^{2+} with NAD-dependent substrates. Furthermore, Fig. 1C shows that, even in the presence of 100 μM Ca^{2+}, succinate oxidation promoted the accumulation of the cation.

To assess NADH release, this nucleotide was measured and the values indicated that, in our conditions the addition of 50 μM Ca^{2+} to malate–glutamate oxidizing mitochondria induced NADH release, i.e., 0.999 ± 0.075 nmol/mg protein. The NADH efflux was inhibited to 0.038 ± 0.033 nmol/mg protein after adding cyclosporin A (CSA).

As shown, malate/glutamate supported Ca^{2+} accumulation when the concentration of this cation attained 25 μM. Nevertheless, mitochondria seem to be more susceptible to membrane leakage as is illustrated by the experiment in Fig. 2A. As observed, a fast matrix Ca^{2+} efflux followed after the addition of 0.3 μM carboxyatractyloside (CAT); such a reaction was inhibited by CSA. This latter contrasts the events occurring in succinate-oxidizing mitochondria. As seen in Fig. 2B, Ca^{2+} remained accumulated in mitochondria even after adding of 1 μM CAT.

Analysis of membrane energization ($\Delta\psi$) is useful in assessing the intactness of the inner membrane after Ca^{2+} accumulation. Figure 3A, trace a, shows that the addition of 50 μM Ca^{2+} to malate/glutamate oxidizing mitochondria initiated a rapid decrease in $\Delta\psi$. Accordingly to the result obtained with Ca^{2+} movement (see Fig. 1A) the membrane potential was not re-established by the addition of succinate. Nevertheless, trace b indicates that a complete protection against the deleterious effect of calcium was attained by the addition of CSA. Figure 3B illustrates that, as expected, $\Delta\psi$ remained unchanged (at a high level) in succinate-oxidizing mitochondria, despite the addition of 100 μM Ca^{2+}. The inefficacy of succinate to reverse the collapse of $\Delta\psi$ must be due to the opening of the hyperconductance pore by Ca^{2+} cycling, this is demonstrated by the fact that, after addition of 0.5 mM EGTA, succinate clearly reversed the drop of membrane potential (Fig. 3C). In addition to Ca^{2+} cycling, the loss of pyridine nucleotides must be taken into account to explain the drop in $\Delta\psi$. Fig. 3D shows that $\Delta\psi$ can be partially restored by supplementing the medium with 100 μM NADH and 0.5 mM EGTA.

Swelling of mitochondria also reflects permeability transition process. Fig. 4A, trace a, reveals that the membrane leakage promoted by Ca^{2+} addition became evident when mitochondria oxidized malate/glutamate. Trace a also shows that succinate was unable to reverse the swelling. Trace b shows that CSA inhibited the Ca^{2+}-induced opening of the non-specific pore. Fig. 4B

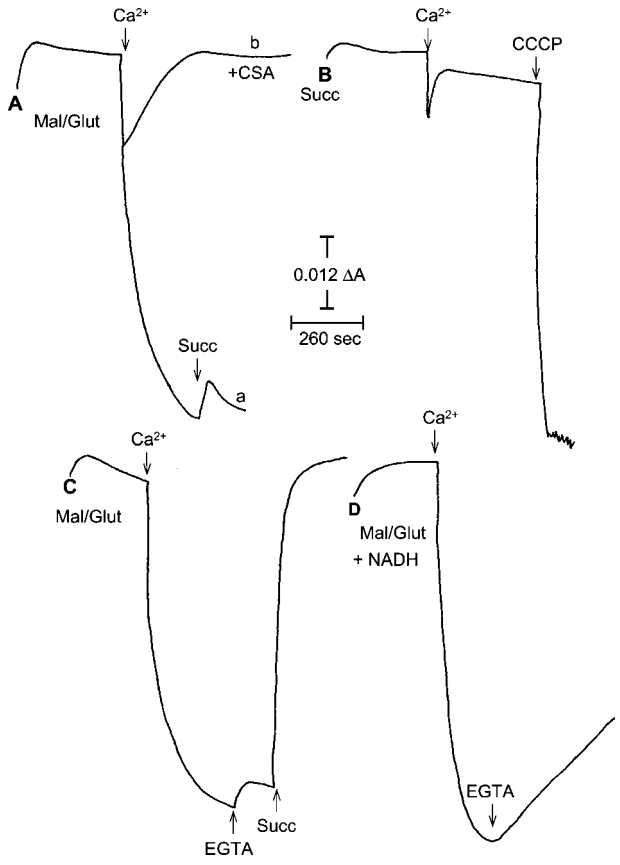

Fig. 3. The effect of calcium on the transmembrane potential in malate/glutamate or succinate-oxidizing mitochondria. Mitochondrial protein (2 mg) was incubated in similar conditions to those described for Fig. 1 except that the medium contained 10 μM Safranine instead of Arsenazo III. As indicated in traces a, c, and d the oxidizable substrates were malate/glutamate and the concentration of $CaCl_2$ added was 50 μM. In (B) the substrate was succinate and the concentration of $CaCl_2$ added was 100 μM. Where indicated in (A) 0.5 μM CSA and 5 mM succinate were added. In (B) 0.5 μM CCCP was added. In (C) 0.5 mM EGTA and 5 mM succinate were added. In (D) 100 μM NADH and 0.5 mM EGTA were added.

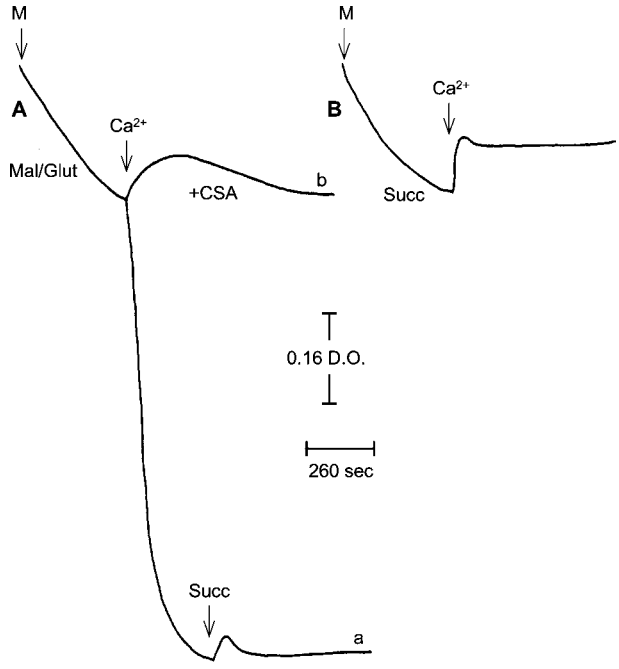

Fig. 4. The effect of calcium on the swelling of malate/glutamate or succinate-oxidizing mitochondria. Mitochondrial protein (2 mg) was incubated in similar conditions to those described for Fig. 1. In (A) the medium contained malate/glutamate and in (B) the medium contained succinate. Where indicated in (A) 50 μM $CaCl_2$, 0.5 μM CSA and 5 mM succinate were added. In (B) 100 μM $CaCl_2$ was added.

illustrates that 100 μM Ca^{2+} did not induce swelling when added to succinate-oxidizing mitochondria.

As has been pointed out, exposure of mitochondria to extremely high Ca^{2+} concentration, i.e., 50 μM as in this work, leads to phospholipase A2 activation (Broekemeier and Pfeiffer, 1989), and ROS formation by Site I of the respiratory chain (Herrero and Barja, 2000). Thus, we explored the protective effect of the scavengers for fatty acids and for ROS, i.e., bovine serum albumin (BSA) and α-phenyl-*N-tert*-butyl nitrone (PBN) respectively, on the ability of mitochondria to accumulate Ca^{2+}, by maintaining a membrane potential as well as preserving their volume. Figure 5A shows that the addition of 0.1% BSA restored the ability of malate–glutamate-oxidizing mitochondria to take up Ca^{2+}. That the latter was not an artifact, due to a possible binding of Ca^{2+} to BSA, was demonstrated by the fact that the uncoupler carbonylcyanide *m*-chlorophenylhydrazone (CCCP) induced a fast Ca^{2+} release that was slightly inhibited by CSA. Fig. 5B shows that although PBN promoted Ca^{2+} uptake to a larger extent, this cation was not retained for a longer time; however, interestingly the addition of succinate promoted a fast uptake and retention of the cation, indicating that PBN inhibited the opening of the non-specific pore.

Figure 6A shows that neither BSA nor PBN inhibited the Ca^{2+}-induced swelling of malate–glutamate-oxidizing mitochondria. A similar result was obtained when the effect of PBN on $\Delta\psi$, generated by the oxidation of malate/glutamate, was analyzed (Fig. 6B). Although, it should be mentioned that in the presence of PBN, succinate induced a transitory recovery of $\Delta\psi$ that was totally restored after EGTA addition.

In agreement to the reports by the group of Vercesi (Fagian *et al.*, 1990; Castilho *et al.*, 1995; Castilho *et al.*, 1998), oxidative stress also induces thiol oxidation of membrane proteins leading to membrane leakage. Therefore, we explored whether *N*-ethylmaleimide

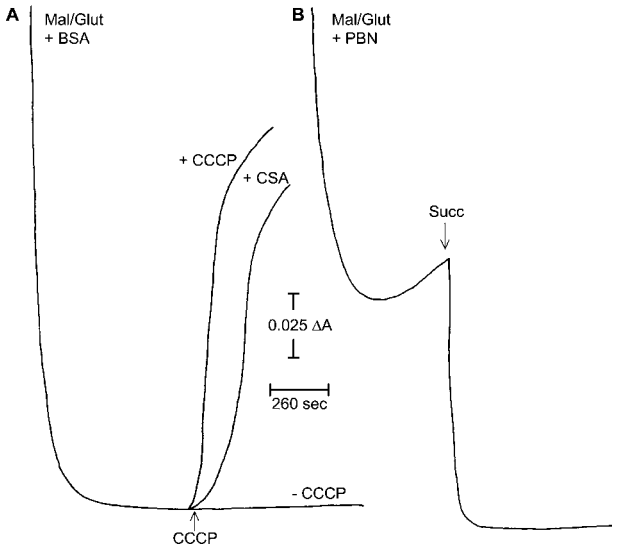

Fig. 5. The protective effect of BSA and PBN on the calcium uptake by malate/glutamate-oxidizing mitochondria. Mitochondria (2 mg protein) were incubated under similar conditions to those described in Fig. 1. In Panel (A) the medium contained 0.1% BSA, and where indicated 0.5 μM CCCP and 0.5 μM CSA were added. In Panel (B) the medium contained 200 μM PBN, and 5 mM succinate was added.

(NEM) would protect from the injuring effect of Ca^{2+}. As is shown in Fig. 7A, 50 μM NEM induced a better Ca^{2+} uptake than that in its absence (see Fig. 1A). However, NEM completely protected from Ca^{2+}-induced swelling

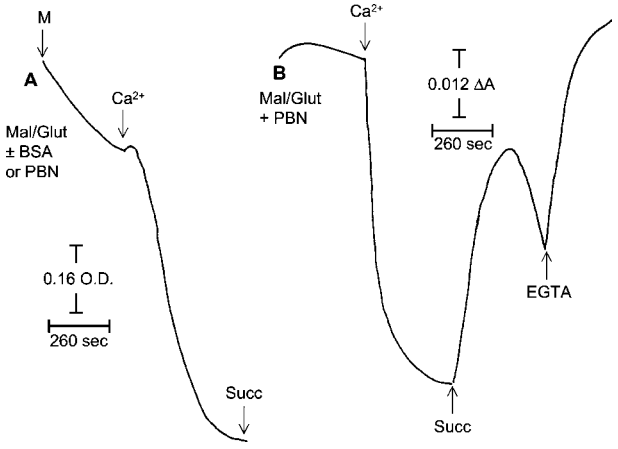

Fig. 6. The effect of BSA and PBN on mitochondrial swelling and transmembrane potential. Mitochondrial protein (2 mg) was incubated under similar conditions to those described for Figs. 1 and 3. In (A) the medium was supplemented with 0.1% BSA or 200 μM PBN, mitochondrial swelling was stimulated by the addition of 50 μM $CaCl_2$. In (B) the medium contained 200 μM PBN and collapse of $\Delta\psi$ was induced by the addition of 50 μM $CaCl_2$. Where indicated, 5 mM succinate and 0.5 mM EGTA were added.

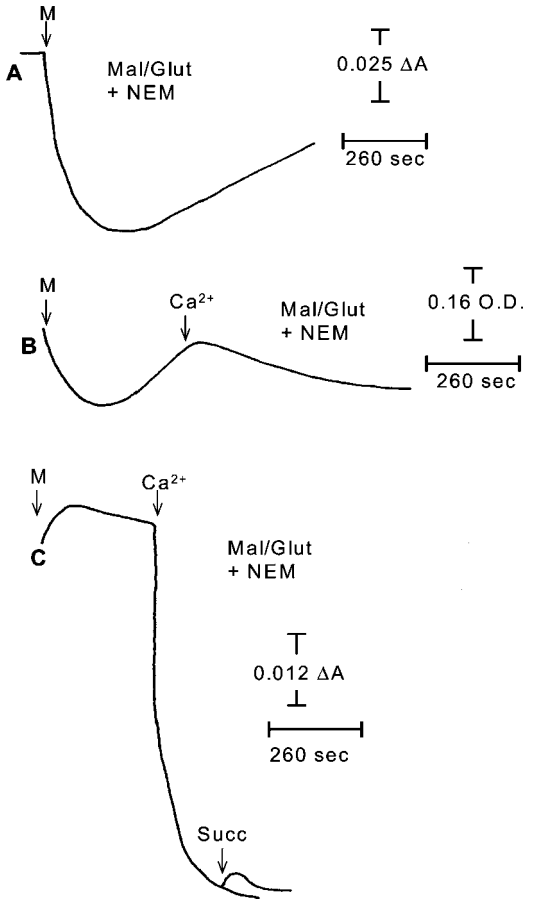

Fig. 7. The effect of *N*-ethylmaleimide on the effect of calcium in several functions of malate/glutamate-oxidizing mitochondria. Mitochondrial protein (2 mg) was added to incubation mixtures to analyze: in (A) calcium uptake; in (B) mitochondrial swelling; and in (C) transmembrane potential. The oxidizable substrates were malate and glutamate. The concentration of $CaCl_2$ added was 50 μM. *N*-ethylmaleimide (NEM) was added at a concentration of 50 μM. Where indicated 5 mM succinate was added.

(Fig. 7B). Figure 7C shows that NEM failed to avoid the collapse of the transmembrane electric gradient.

Figure 8 shows the production of thiobarbituric acid-reactive species (TBARS) by malate/glutamate-oxidizing mitochondria. The analysis indicated an increase of approximately 40% in the production of lipid peroxidation products by the addition of 50 μM Ca^{2+}, as well as an inhibition of 100% in the amount of TBARS generated in the presence of PBN. The addition of CSA inhibited by around 40% the Ca^{2+}-induced production of TBARS.

DISCUSSION

In agreement with previous reports (Fontaine *et al.*, 1998; Walter *et al.*, 2000) the results in this work show that

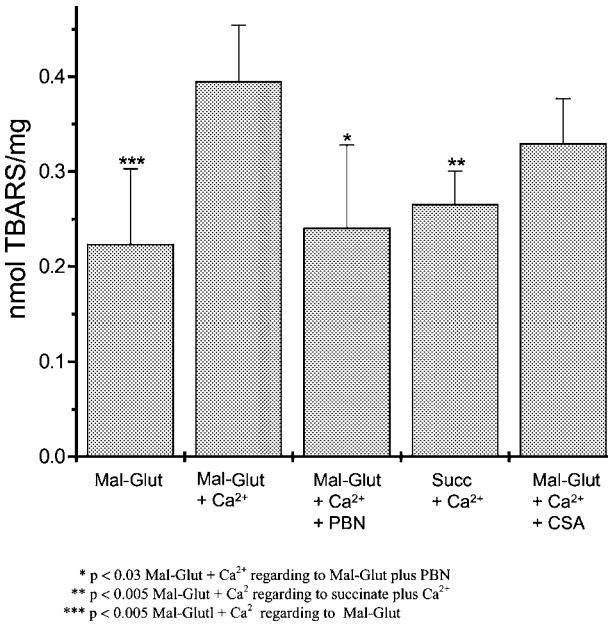

Fig. 8. The inhibitory effect of PBN on the Ca^{2+}-induced production of TBARS. Mitochondrial protein (2 mg) was added to the basic media as indicated under Methods. Where indicated the media contained 10 mM malate and 10 mM glutamate (Mal-Glut) or 10 mM succinate (Succ). Where indicated the media contained also 50 μM Ca^{2+}, 200 μM PBN and 0.5 μM CSA. After 10 min of incubation, 0.2% butyrated hydroxytoluene and 1 ml of potassium phosphate 0.15 M was added and the mixture was incubated during 20 min at 37°C. After this time 1.5 ml of 8% thiobarbituric acid and 1 ml of 20% acetic acid were added. After cooling 1 ml of 2% KCl and 4 ml butyl alcohol were added. TBARS were analyzed in the organic phase at 532 nm.

mitochondria oxidizing NAD-dependent substrates were unable to accumulate Ca^{2+} when they were incubated in a high concentration of the cation. Such a mitochondrial dysfunction has been attributed to a Ca^{2+}-induced loss of pyridine nucleotides (Vinogradov *et al*., 1972). However, as shown in this work, the addition of succinate did not support the accumulation of Ca^{2+}. According to the results shown, the failure of this substrate oxidation cannot be ascribed to the inhibition of succinate dehydrogenase activity by the accumulated oxaloacetate. Therefore it was assumed that succinate was unable to bring about Ca^{2+} accumulation due to the opening of the non-specific pore. This assumption is based on previous data from Maciel *et al*. (2001) and Batandier *et al*. (2004) who reported that oxygen-derived reactive species induce permeability transition.

The Ca^{2+}-induced permeability transition can be triggered by the generation of superoxide anion in Site I of the respiratory chain. Superoxide is produced as the result of single electron leaks to oxygen, increased by the interruption of the electron transport in the NADH dehydrogenase by the loss of pyridine nucleotides. An extensive oxidation or leak of matrix pyridine nucleotides conduces to a decrease in the level of GSH which in turn causes an improvement in the mitochondrial damage as induced by oxygen-derived reactive species (Reed and Savage, 1995). The oxidative stress products $O_2^{-\bullet}$, H_2O_2, and $^{\bullet}OH$ start a sequence of reactions implicating fatty acids production, lipoperoxidation, and thiol oxidation of membrane proteins. The activation of phospholipase A2 by superoxide radicals (Madesh and Balasubramanian, 1997) plays a key role in a cascade of events that initiate the opening of the non-specific pore. The increased level of fatty acids leads to an uncoupling-induced drop in $\Delta\psi$, mitochondrial swelling and non-specific pore opening. Skulachev (1998), Schönfeld and Bohnensack (1997), and Wojtczak *et al*. (1998) established that long-chain fatty acids, unlike the classic protonophores, uncouple oxidative phosphorylation by passing into mitochondria their protonated form through the lipid milieu of the membrane, while its dissociated form leaves the matrix to the cytosol side through the adenine nucleotide translocase. It is well established that uncoupling of mitochondria result in membrane permeability transition (Scorrano *et al*., 1997). The protective effect of BSA emphasizes the fact that the Site I-dependent oxidative stress generated fatty acids, which in turn open the transition pore. It should be noted that lipid peroxidation and thiol oxidation were also involved in the membrane leakage process. This latter was mirrored through the increase in the TBARS generation, which was inhibited by PBN, and the inhibition induced by NEM.

Although PBN did not restore totally the ability of mitochondria to accumulate Ca^{2+} with malate/glutamate as the substrates, it promoted succinate-supported Ca^{2+} uptake. The fact that PBN allows the Ca^{2+} accumulation by succinate indicates that the closure of the pore does not avoid the release of pyridine nucleotides. To this regard Schlegel *et al*. (1992) have shown that the release of pyridine nucleotides as induced by Ca^{2+} does not require the opening of the non-specific pore. These authors imply that the release of pyridine nucleotides may take place through a specific pathway.

Fontaine and coworkers (1998) showed that the opening of the high conductance pore is facilitated by the oxidation of NAD-dependent substrates. The proposal of this group is that a physical interaction exists between the non-selective pore and the NADH-CoQ reductase span in such a way that the rate of electron flow in this segment of the respiratory chain would modulate the probability of pore opening. This is a very provocative hypothesis, even more considering the data by Chen *et al*. (2004) about the co-purification of the F_1-ATPase together with

adenine nucleotide translocase and phosphate carrier; hence, it would not be surprising if in a near future the respiratory complex I appears to be located in the synthasome. Nevertheless, at this stage of the experimental work our proposal is that the interaction may be rather chemical than physical. In the putative chemical interaction the first messenger would be the superoxide anion initiating the overflow of reactions.

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