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The Irreversibility of Inner Mitochondrial Membrane Permeabilization by Ca^{2+} plus Prooxidants Is Determined by the Extent of Membrane Protein Thiol Cross-linking

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We have previously shown that mitochondrial membrane potential ($\Delta\Psi$) drop promoted by prooxidants and Ca^{2+} can be reversed but not sustained by ethylene glycol-bis(β-aminoethyl-ether)-*N,N,N',N'*-tetraacetic acid (EGTA) unless dithiothreitol (DTT), a disulfide reductant, is also added [Valle, V. G. R., Fagian, M. M., Parentoni, L. S., Meinicke, A. R., and Vercesi, A. E. (1993). *Arch. Biochem. Biophys.* **307,** 1–7]. In this study we show that catalase or ADP are also able to potentiate this EGTA effect. When EGTA is added long after (12 min) the completion of swelling or $\Delta\Psi$ elimination, no membrane resealing occurs unless the EGTA addition was preceded by the inclusion of DTT, ADP, or catalase soon after $\Delta\Psi$ was collapsed. Total $\Delta\Psi$ recovery by EGTA is obtained only in the presence of ADP. The sensitivity of the ADP effect to carboxyatractyloside strongly supports the involvement of the ADP/ATP carrier in this mechanism. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of solubilized membrane proteins shows that protein aggregation due to thiol cross-linkage formed during $\Delta\Psi$ drop continues even after $\Delta\Psi$ is already eliminated. Titration with 5,5'-dithio-bis(2-nitrobenzoic acid) supports the data indicating that the formation of protein aggregates is paralleled by a decrease in the content of membrane protein thiols. Since the presence of ADP and EGTA prevents the progress of protein aggregation, we conclude that this process is responsible for both increased permeability to larger molecules and the irreversibility of $\Delta\Psi$ drop. The protective effect of catalase suggests that the continuous production of protein thiol cross-linking is mediated by mitochondrial generated reactive oxygen species.

KEY WORDS: Calcium; cyclosporin A; mitochondria; mitochondrial permeability transition pore; protein oxidation; reactive oxygen species.

INTRODUCTION

Inner mitochondrial membrane permeabilization caused by Ca^{2+} is generally referred to as mitochondrial membrane permeability transition and is assumed to be associated with the opening of a Ca^{2+}-induced pore, the mitochondrial membrane permeability transition pore (MTP) (for reviews, see Gunter and Pfeiffer, 1990; Bernardi *et al.*, 1994; Gunter *et al.*, 1994; Zoratti and Szabò, 1995). This Ca^{2+} effect is potentiated by various agents or conditions, such as inorganic phosphate, thiol, or pyridine nucleotide oxidants, low membrane potential, and oxidative stress (for reviews, see Gunter and Pfeiffer, 1990; Vercesi, 1993; Zoratti and Szabò, 1995).

It is known that mitochondrial membrane potential ($\Delta\Psi$) drop due to MTP opening is reversed by pyridine nucleotide reductants (Vercesi, 1984), by ethylene glycol-bis(β-aminoethyl-ether)-*N,N,N',N'*-tetraacetic acid (EGTA) (Al-Nasser and Crompton, 1986;

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Crompton *et al.*, 1987; Valle *et al.*, 1993; Bernardes *et al.*, 1994; Vercesi *et al.*, 1993), by the reductant dithiothreitol (DTT) (Valle *et al.* 1993), or by the immune supressor cyclosporin A in the presence of Mg^{2+} or ADP (Novgorodov *et al.*, 1994). Due to these characteristics, the MTP has been proposed to be a regulated channel, compatible with the maintenance of mitochondrial integrity and possibly related to specific functions not yet elucidated (for reviews, see Bernardi *et al.*, 1994 and Gunter *et al.*, 1994). However, results from our laboratory (Valle *et al.*, 1993; Castilho *et al.*, 1995a, b; Kowaltowski *et al.*, 1995, 1996a) indicate that MTP opening is the result of membrane damage due to the oxidative attack of reactive oxygen species (ROS) to membrane protein thiols. This causes thiol cross-linkage and high-molecular-weight protein aggregate production (Fagian *et al.*, 1990; Valle *et al.*, 1993; Castilho *et al.*, 1995a).

It has been shown that MTP opening takes place during various pathological states such as ischemia/reperfusion or prolonged hypoxia (Nazareth *et al.*, 1991; Pastorino *et al.*, 1993) and may be an important event in the mechanism of cell death. The aim of this work was to establish conditions that permit mitochondrial recovery from the state of membrane permeabilization and detect alterations that may lead to irreversible mitochondrial injury.

MATERIALS AND METHODS

Isolation of Rat Liver Mitochondria

Mitochondria were isolated by conventional differential centrifugation from the livers of adult Wistar strain rats fasted overnight.

Standard Incubation Procedure

The experiments were carried out at 30°C in a standard reaction medium containing 130 mM KCl, 10 mM Hepes (2-hydroxyethyl-1-piperazine ethanesulfonic acid) buffer, pH 7.2, 2 mM succinate, 10 μM Ca^{2+}, and 4 μM rotenone. Other additions are indicated in the figure legends. The results shown are representative of a series of at least three experiments.

Determination of Mitochondrial Swelling

Mitochondrial swelling was estimated from the decrease in the absorbance at 520 nm measured in an SLM Aminco DW2000 spectrophotometer.

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

Mitochondria were incubated in standard reaction medium containing 3 μM tetraphenylphosphonium (TPP^+). The concentration of TPP^+ in the extramitochondrial medium was continuously monitored with a TPP^+-selective electrode prepared in our laboratory according to Kamo *et al.* (1979). The membrane potential was then calculated assuming that the TPP^+ distribution between mitochondria and medium follows the Nernst equation (Muratsugu *et al.*, 1977). Corrections due to the binding of TPP^+ to the mitochondrial membranes were made according to Jensen *et al.* (1986). No corrections were made to compensate $\Delta\Psi$ values for the continuous alteration in mitochondrial volume that occurs during the experiments.

Sodium Dodecyl Sulfate-Polyacrylamide Slab Gel Electrophoresis (SDS-PAGE)

Aliquots of mitochondria were taken and the matrix proteins were released by three subsequent freeze–thawing procedures. Electrophoresis of the solubilized membrane proteins was performed according to Laemmli (1970) and as described by Fagian *et al.* (1990).

Determination of Protein Thiol Groups Content

The mitochondrial suspension incubated in standard reaction medium was submitted to three subsequent freeze–thawing procedures to release matrix proteins and centrifuged 2 min at 10,000 rpm. The pellet was treated twice with 200 μl of 6.5% trichloroacetic acid and centrifuged at 10,000 rpm during 2 min in order to precipitate the protein. The final pellet was resuspended in 1 ml of medium containing 100 μM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), 0.5 mM EGTA, and 0.5 M Tris, pH 8.3. Absorption was measured at 412 nm, using cysteine for calibration.

Oxygen Uptake Measurements

Oxygen concentration was measured using a Clark-type electrode (Yellow Springs Instruments Co.) in a glass cuvette equipped with magnetic stirring.

Chemicals

ADP, *t*-butyl hydroperoxide, butylhydroxytoluene, carboxyatractyloside, catalase, EGTA, Hepes, oligomycin, and rotenone were obtained from Sigma Chemical Company (St. Louis, Missouri). All other reagents were commercial products of the highest purity grade available.

RESULTS

Figure 1 shows that $\Delta\Psi$ dissipation induced by *t*-butyl hydroperoxide and Ca^{2+} (line a) can be recovered by the addition of EGTA (line e), but is only sustained if catalase (line b), ADP (line c), or DTT (line d) are also added. MTP closure by EGTA in the presence of ADP was previously reported by Maddaiah and Kumbar (1993).

The addition of EGTA 12 min after $\Delta\Psi$ was completely eliminated did not induce significant $\Delta\Psi$ recovery (Fig. 2, line h) unless the mitochondrial suspension was treated with DTT (line d) or catalase (line e) just after $\Delta\Psi$ disruption took place. Line d shows that the addition of DTT alone induced a small and temporary recovery of $\Delta\Psi$, and that the later addition of EGTA to this suspension induced important $\Delta\Psi$ formation. The addition of catalase after $\Delta\Psi$ dissipation also promoted significant $\Delta\Psi$ recovery after the addition of EGTA (line e). In contrast, the addition of butylhydroxytoluene (BHT, line f), a lipid peroxidation inhibitor, or bromophenacyl bromide (line g), which inhibits phospholipase A_2 activity, did not improve $\Delta\Psi$ recovery after the addition of EGTA. These results suggest that the transition from a reversible to irreversible mitochondrial membrane permeabilization to protons involves ROS activity and protein thiol group oxidation, and not lipid peroxidation or enhanced activity of phospholipase A_2. The immune supressor cyclosporin A did not restore $\Delta\Psi$ or potentiate the

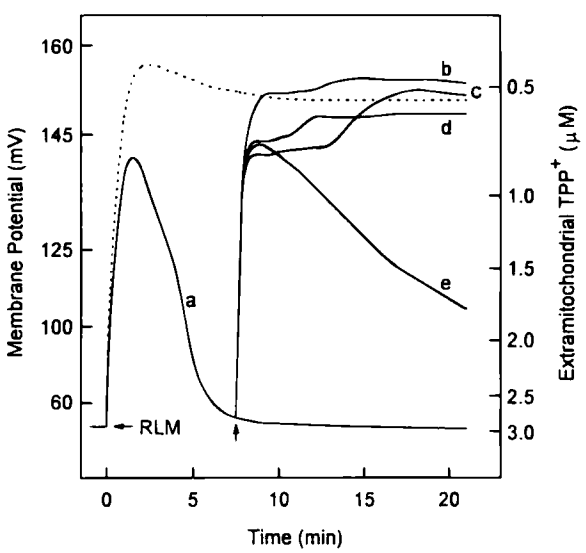

Fig. 1. Effect of EGTA, DTT, ADP, and catalase on $\Delta\Psi$ disruption induced by Ca^{2+} plus *t*-butyl hydroperoxide. Rat liver mitochondria (RLM, 1.0 mg/ml) were added to standard medium in the presence of 300 μM *t*-butyl hydroperoxide (line a). After $\Delta\Psi$ dissipation, 2 μM catalase plus 500 μM EGTA (line b), 200 μM ADP plus 500 μM EGTA (line c), 2 mM DTT plus 500 μM EGTA (line d), or 500 μM EGTA (line e) were added where indicated by the arrow. The dashed line represents a control experiment in the absence of *t*-butyl hydroperoxide and in the presence of 500 μM EGTA.

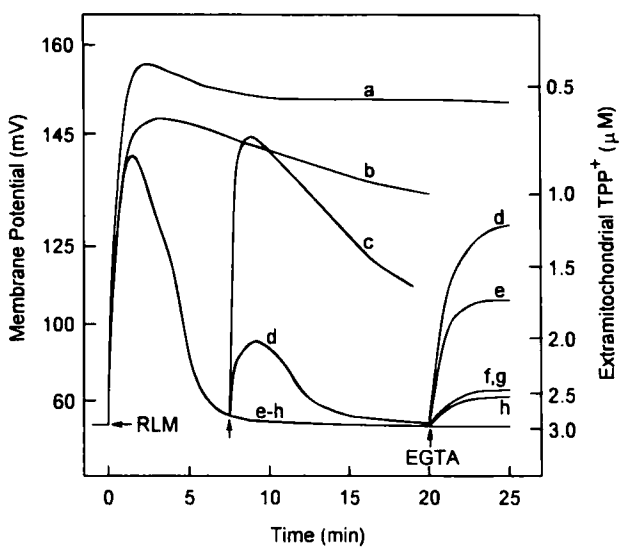

Fig. 2. EGTA-induced recovery of Ca^{2+} plus *t*-butyl hydroperoxide-induced $\Delta\Psi$ disruption: effect of antioxidants. RLM (1.0 mg/ml) were added to standard medium in the presence of 300 μM *t*-butyl hydroperoxide. Just after $\Delta\Psi$ dissipation, 500 μM EGTA (line c), 2 mM DTT (line d), 2 μM catalase (line e), 5 μM BHT (line f), or 20 μM bromophenacyl bromide (line g) were added where indicated by the arrow. EGTA (500 μM) was added 12 min after $\Delta\Psi$ dissipation was complete (lines d–h). Lines a and b represent control experiments in the presence and absence of 500 μM EGTA, respectively, in reaction medium not containing *t*-butyl hydroperoxide.

effect of EGTA under the conditions of Fig. 2 (result not shown). Indeed, literature data show that cyclosporin A can induce $\Delta\Psi$ recovery only in the presence of Mg^{2+} or ADP (Novgorodov *et al.*, 1994).

As previously reported, mitochondrial permeabilization by Ca^{2+} or Ca^{2+} plus prooxidants is associated with membrane protein aggregate formation (Fagian *et al.*, 1990; Valle *et al.*, 1993; Castilho *et al.*, 1995a). The possible participation of these protein alterations in the transition from reversible to irreversible permeabilization was assessed by electrophoresis analysis of solubilized membrane proteins. Figure 3 shows that simultaneously to membrane $\Delta\Psi$ dissipation induced by *t*-butyl hydroperoxide and Ca^{2+} there is formation of high-molecular-weight protein aggregates, which appear on the upper part of the running gel (lane b). These protein aggregates did not appear when mitochondria were incubated in the absence of *t*-butyl hydroperoxide and Ca^{2+} (lane a). Ten minutes after

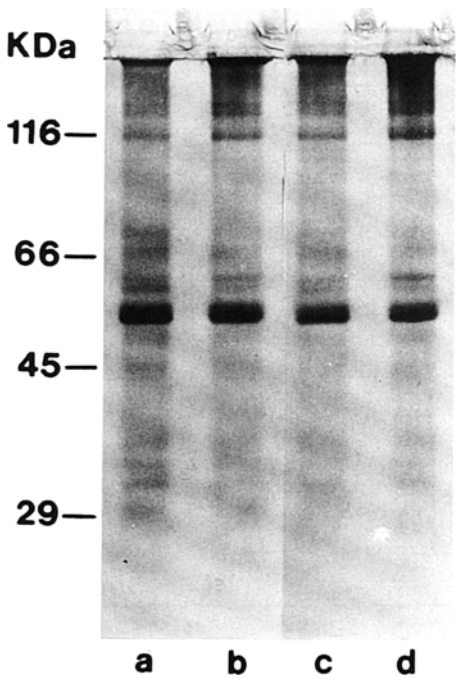

Fig. 3. SDS-polyacrylamide slab gel electrophoresis of membrane proteins of mitochondria treated with Ca^{2+} plus *t*-butyl hydroperoxide. In each lane, 10 μg protein were applied to a 10% acrylamide running gel after mitochondrial incubation under the conditions described for Fig. 1. Lane a: RLM (1.0 mg/ml) incubated 15 min in the presence of 200 μM EGTA. Lane b: RLM incubated 5 min in the presence of 300 μM *t*-butyl hydroperoxide. Lane c: RLM incubated 15 min in the presence of 300 μM *t*-butyl hydroperoxide, with the addition of 200 μM EGTA plus 200 μM ADP at 5 min. Lane d: RLM incubated 15 min in the presence of 300 μM *t*-butyl hydroperoxide.

$\Delta\Psi$ was dissipated, when recovery of $\Delta\Psi$ was not obtainable, the quantity of protein aggregates was significantly larger (lane d). The addition of EGTA and ADP just after completion of $\Delta\Psi$ dissipation prevented the progress and decreased the amount of protein aggregation (lane c). Table I shows that under the experimental conditions in which there is formation of protein aggregates, there is also a decrease in the content of membrane protein thiols (compare the experiment in the presence of EGTA with that in the presence of *t*-butyl hydroperoxide plus Ca^{2+} at 5 min). When protein aggregation is extensive, as in Fig. 3, lane d, and permeabilization irreversible, there is a large decrease in membrane protein thiols (*t*-butyl hydroperoxide plus Ca^{2+} at 15 min). This decrease in content of membrane protein thiols is inhibited by catalase, dithiothreitol, and ADP in a carboxyatractyloside-sensitive manner, added at 5 min. This suggests that the continuous production of ROS and membrane protein thiol oxidation, leading to formation of protein aggregates, is the cause of irreversible $\Delta\Psi$ decrease.

The effect of ADP on $\Delta\Psi$ recovery induced by a delayed addition of EGTA is shown in Fig. 4. Line b shows that the previous addition of ADP promoted a complete re-establishment of $\Delta\Psi$ after EGTA addition. This effect was potentiated by oligomycin that prevents ADP phosphorylation (line a). The presence of carboxyatractyloside, which stabilizes the ADP/ATP carrier in the c-conformation (Klingenberg, 1989), inhibited completely the effect of ADP (line c). If ADP was added just before EGTA, only a small improvement in $\Delta\Psi$ recovery was observed (line d).

Table I. Effect of ADP, Catalase, and Dithiothreitol on Oxidation of Mitochondrial Membrane Protein Thiol Groups by 500 μM *t*-Butyl Hydroperoxide (*t*-bOOH) and 10 μM Ca^{2+}[a]

Initial conditions	Additions at 5 min	-SH (nmols mg/protein)
1 mM EGTA		44.0 ± 1.1[b]
Ca^{2+}		37.5 ± 0.1
Ca^{2+} + *t*-bOOH[c]		30.0 ± 1.1
Ca^{2+} + *t*-bOOH		10.5 ± 0.2
Ca^{2+} + *t*-bOOH	200 μM ADP	32.7 ± 0.9
Ca^{2+} + *t*-bOOH	200 μM ADP + 10 μM carboxyatractyloside	12.0 ± 0.5
Ca^{2+} + *t*-bOOH	2 μM catalase	29.5 ± 0.7
Ca^{2+} + *t*-bOOH	2 mM dithiothreitol	33.8 ± 1.3

[a] The experimental conditions were similar to those of Fig. 3. The reactions were stopped at 15 min.
[b] Values represent average of 3 experiments ± S.D.
[c] This reaction was stopped at 5 min.

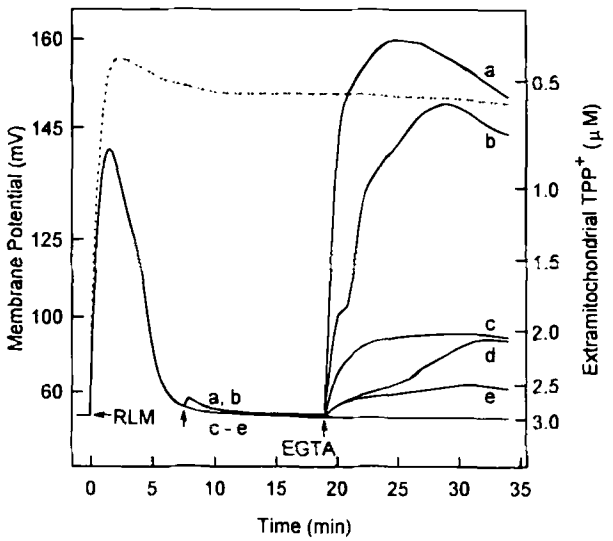

Fig. 4. EGTA-induced recovery of Ca^{2+} plus *t*-butyl hydroperoxide-induced $\Delta\Psi$ disruption: effect of ADP and carboxyatractyloside. RLM (1.0 mg/ml) were added to standard medium in the presence of 300 μM *t*-butyl hydroperoxide. Just after $\Delta\Psi$ dissipation, 200 μM ADP plus 1 μg/mg oligomycin (line a), 200 μM ADP (line b), or 200 μM ADP plus 20 μM carboxyatractyloside (line c) were added where indicated by the arrow. EGTA (500 μM) was added 12 min after $\Delta\Psi$ dissipation was complete (lines a–e). Line d represents an experiment in which 200 μM ADP was added just prior to the addition of EGTA. The dashed line represents a control experiment in the absence of *t*-butyl hydroperoxide and in the presence of 500 μM EGTA.

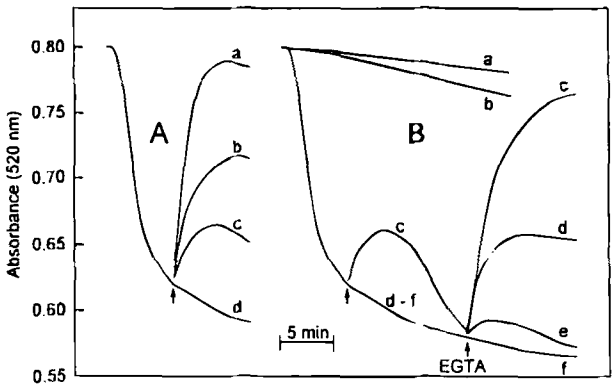

Fig. 5. Recovery of mitochondrial swelling induced by Ca^{2+} plus *t*-butyl hydroperoxide: effect of ADP, EGTA, and DTT. RLM (0.5 mg/ml) were added to standard medium in the presence of 300 μM *t*-butyl hydroperoxide. Panel A: ADP (200 μM) plus 500 μM EGTA (line a), 2 mM DTT plus 500 μM EGTA (line b), or 500 μM EGTA (line c) were added where indicated by the arrow, 5 min after the beginning of absorbance decrease. Line d represents an experiment in the presence of 300 μM *t*-butyl hydroperoxide, without further additions. Panel B: ADP (200 μM, line c) or 2 mM DTT (line d) were added where indicated by the arrow 5 min after the beginning of absorbance decrease. EGTA (500 μM) was added 15 min after the beginning of absorbance decrease, where indicated (lines c–e). Line f represents an experiment in the presence of *t*-butyl hydroperoxide, without further additions. Lines a and b represent control experiments in the presence and absence of 500 μM EGTA, respectively, in reaction medium not containing *t*-butyl hydroperoxide.

Nonspecific inner mitochondrial membrane permeabilization can be studied using the classical swelling technique to monitor the influx of osmotic support into the organelle (Gunter and Pfeiffer, 1990). Figure 5 shows that mitochondrial swelling induced by *t*-butyl hydroperoxide and Ca^{2+} can be partially reversed by the addition of EGTA soon after the decrease in absorbance (panel A, line c). This mitochondrial contraction was greatly enhanced if DTT was added together with EGTA (line b) and was complete if the suspension was treated with ADP plus EGTA (line a). This finding is compatible with membrane resealing (MTP closure) followed by extrusion of the osmotic support that penetrated before addition of EGTA and ADP. Mitochondrial swelling was not reversed by EGTA added 10 min after absorbance decrease (panel B, line e), unless ADP (line c) or DTT (line d) was previously added. These results are compatible with the recovery of $\Delta\Psi$ observed in Figs. 2 and 4.

The experiment of Fig. 6 was designed to investigate the correlation between MTP opening and closure and the degree of mitochondrial intactness, as indicated

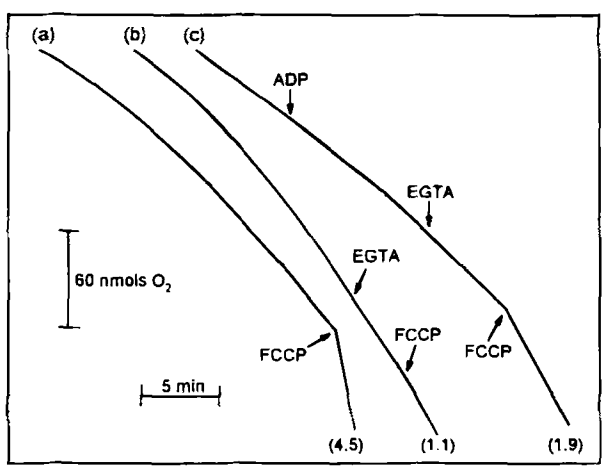

Fig. 6. Correlation between MTP opening and closure and mitochondrial respiratory control ratio. RLM (0.5 mg/ml) were incubated in standard medium containing 1 μM cytochrome *c*, in the presence of 500 μM EGTA (line a) or 300 μM *t*-butyl hydroperoxide (lines b and c). ADP (200 μM), 500 μM EGTA, or 1 μM FCCP were added where indicated. Oligomycin (1 μg/mg protein) was added to all experiments at 6 min. The numbers in parentheses indicate mitochondrial respiratory control ratios, calculated as the relation between the rates of uncoupled and state-4 O_2 consumption.

by FCCP-induced respiratory control. Line a shows that when mitochondria were incubated in the presence of EGTA, the respiratory control ratio after a 20-min incubation was 4.5. When Ca^{2+} and *t*-butyl hydroperoxide were present during 15 min (line b), a condition in which the addition of EGTA did not cause $\Delta\Psi$ recovery, the addition of FCCP caused a very small stimulation of respiration (respiratory control of 1.1). Even under the conditions in which there is a complete recovery of mitochondrial membrane potential (see Fig. 4, line a) and volume (Fig. 5B, line c), mitochondrial state-4 respiration was still released and the respiratory control ratio was significantly diminished (Fig. 6, line c).

DISCUSSION

The results presented in this (Figs. 1, 2, and 5) and previous communications (Valle *et al.*, 1993; Castilho *et al.*, 1995a) suggest that the enhanced and continuous production of ROS by mitochondria treated with Ca^{2+} or Ca^{2+} plus *t*-butyl hydroperoxide leads to membrane permeabilization via oxidation of membrane protein thiol groups producing protein aggregates of high molecular weight (Fagian *et al.*, 1990). This hypothesis is supported by the fact that inhibition of either lipid peroxidation or phospholipase A_2 activity does not promote significant mitochondrial protection under our experimental conditions (Fig. 2).

The electrophoresis (Fig. 3) and protein thiol determination (Table I) presented show that the amount of protein oxidation and cross-link increases with time even after $\Delta\Psi$ was already eliminated. This suggests that membrane permeabilization to small molecules or ions does not require extensive thiol cross-linkage, and may occur even with modification of single proteins via intrapeptide –SS– bond formation (Petronilli *et al.*, 1994), a process reversed by DTT. At this phase of the process, pore opening may be promoted by Ca^{2+} binding to these proteins and Ca^{2+} removal by EGTA would reseal the membrane (Fig. 1). When protein aggregation is extensive (Fig. 3, lane d), due to a high degree of oxidative stress, membrane permeabilization becomes irreversible. We propose that these situations represent different phases of the permeabilization process. Initially, intrapeptide bond formation would confer permeability to small ions and molecules. Progress of protein thiol cross-linking could also create an assembly, similar to the mitochondrial benzodiazepine receptor (McEnery *et al.*, 1992), which would open a cavity large enough to explain the properties of the MTP (Harbury *et al.*, 1993; Bernardes *et al.*, 1994). Finally, generalized protein aggregation, producing protein clusters, may cause a large and irreversible degree of permeabilization in which even matrix proteins can cross the membrane (Igbavboa *et al.*, 1989). It is important to emphasize that even when MTP closure is still obtained with complete restoration of mitochondrial membrane potential and volume (Fig. 4, line a), there was a significant impairment of mitochondrial functions, as detected through measurements of respiratory control (Fig. 6). The lower rate of oxygen consumption observed after FCCP addition in Fig. 6 (lines b and c) certainly results from the loss of respiratory chain components during mitochondrial swelling. This result indicates that MTP opening is paralleled by other mitochondrial alterations. Indeed, when inorganic phosphate ($P_i > 1$ mM) is present, high rates of lipid peroxidation parallel oxidation of protein thiols (Kowaltowski *et al.*, 1996b). In addition, unpublished results (Almeida, A.M., and Vercesi, A.E.) from this laboratory show that mitochondrial membrane permeabilization induced by Ca^{2+} and prooxidants is accompanied even by mtDNA fragmentation.

Interestingly, the carboxyatractyloside-sensitive inhibition by ADP of irreversible mitochondrial membrane permeabilization supports the proposed participation of the ADP/ATP carrier in this mechanism (for review, see Zoratti and Szabò, 1995). This carrier is the most abundant integral protein of the inner mitochondrial membrane and its interaction with various ligands (ADP, ATP, bongkrekate, carboxyatractyloside) induces conformation changes in the carrier intself (m or c conformation) (Klingenberg, 1989) and structural modification of the mitochondria (Scherer and Klingenberg, 1974). In this regard, Vercesi (1984) showed that bongkrekate or ADP, which shift mitochondria to a condensed configuration (Scherer and Klingenberg, 1974), strongly protect against membrane permeabilization induced by Ca^{2+} plus oxaloacetate or diamide. In contrast, carboxyatractyloside, which changes mitochondria to the orthodox configuration (Scherer and Klingenberg, 1974), had an opposite effect. It might be possible that binding of ADP to the carrier could grant protection against protein thiol oxidation either by (i) promoting alterations of the ADP/ATP carrier conformation that changes the position of its thiol groups (Klingenberg, 1989; Vignais and Vignais, 1972; Majima *et al.*, 1994), rendering them not accessible to oxidation by ROS or (ii) binding of ADP to the ADP/ATP carrier changes mitochondria

from the orthodox to condensed configuration, an alteration that may protect thiol groups of other membrane proteins against oxidation. The second hypothesis is supported by results of Novgorodov *et al.* (1994), showing indirect involvement of the ADP/ATP carrier in the mechanism of MTP opening.

The data presented support the hypothesis that permeability transition is a situation related to alterations in mitochondrial structure and functions (thiol oxidation, membrane protein aggregation, alterations of respiratory chain components, mtDNA breakage) that may progress and lead to a state of irreversible inner membrane permeabilization and generalized damage. These results are in agreement with a number of studies describing direct and indirect evidence that the energy loss that precedes lethal cell injury, under pathological conditions associated with cell Ca^{2+} overload, is linked to the state of mitochondrial permeability transition (Crompton, 1990; Nazareth *et al.*, 1991; Imberti *et al.*, 1992; Pastorino *et al.*, 1993). Although these alterations can be prevented or "reversed" within a short period of time, a prolonged exposition of mitochondria to these conditions may render the damage unrecoverable and result in cell death.

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