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Mitochondrial membrane protein thiol reactivity with *N*-ethylmaleimide or mersalyl is modified by Ca^{2+}: correlation with mitochondrial permeability transition

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Abstract

The content of mitochondrial membrane protein thiol groups accessible to react with the monofunctional thiol reagents mersalyl or *N*-ethylmaleimide (NEM) was determined using Ellman's reagent. Deenergized mitochondria incubated in the presence of Ca^{2+} (0–500 μM) undergo a very significant decrease in the content of membrane protein thiols accessible to NEM, and an increase in the content of thiols accessible to mersalyl. This process is time-dependent and inhibited by Mg^{2+}, ruthenium red and ADP, but not by cyclosporin A. This suggests that Ca^{2+} binding to the inner mitochondrial membrane promotes extensive alterations in the conformation of membrane proteins that result in location changes of thiol groups. The relationship between these alterations and mitochondrial membrane permeability transition was studied through the effect of NEM and mersalyl on mitochondrial swelling induced by Ca^{2+} plus *t*-butyl hydroperoxide (*t*-bOOH) or Ca^{2+} plus the thiol cross-linkers 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) or phenylarsine oxide (PhAsO). We observed that the hydrophobic thiol reagent NEM inhibits the effects of *t*-bOOH, DIDS and PhAsO, while the hydrophilic thiol reagent mersalyl inhibits only the effect of DIDS. Permeability transition in all the situations studied is accompanied by a significant decrease in the total membrane protein thiol content. In addition, mitochondrial membrane permeabilization induced by PhAsO is inhibited by EGTA, but not by ruthenium red. This result suggests that PhAsO leads to permeability transition through a mechanism independent of intramitochondrial Ca^{2+}-induced alterations of thiol group reactivity, but dependent on Ca^{2+} binding to an extramitochondrial site. This site is sensitive to extramitochondrial Ca^{2+} concentrations in range of 1–50 μM.

Keywords: Calcium ion; Cyclosporin A; Mitochondrion; Mitochondrial membrane permeability transition; Protein oxidation; Reactive oxygen species

Abbreviations: *t*-bOOH, *t*-butyl hydroperoxide; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone; Hepes, 2-hydroxyethyl-1-piperazineethanesulfonic acid; Mers, mersalyl; MPT, mitochondrial membrane permeability transition; NEM, *N*-ethylmaleimide; PhAsO, phenylarsine oxide; PTP, mitochondrial membrane permeability transition pore; RLM, rat liver mitochondria; ROS, reactive oxygen species; RR, ruthenium red.

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1. Introduction

Ca^{2+}-dependent inner mitochondrial membrane permeabilization is currently referred to as mitochondrial membrane permeability transition (MPT), and believed to be mediated by the opening of a proteinaceous pore sensitive to the immune suppressor cyclosporin A (for reviews, see Refs. [1,2]). Despite extensive studies, the role of the mitochondrial membrane permeability transition pore (PTP) remains unknown. It has been proposed that the PTP is a highly regulated pore that may participate in physiological mitochondrial functions [2–4]. Alternatively, evidence has accumulated that PTP is a form of mitochondrial damage that may play a role in cell impairment and death in situations of oxidative stress such as ischemia/reperfusion or prolonged hypoxia [5–8].

Permeability transition is accelerated by a variety of compounds (inducers) [1,2] which include pyridine nucleotide or glutathione oxidants, inorganic phosphate (P_i), arsenate, uncouplers and thiol cross-linking reagents such as phenylarsine oxide (PhAsO) and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) [9]. Because of the inhibitory effect of antioxidants, such as catalase, on permeability transition induced by the uncoupler FCCP [10], P_i [10,11] or pro-oxidants [12,13], and due to the similarity between the permeabilization caused by these agents and thiol cross-linkers [9,13], our group has proposed that permeability transition may be caused by mitochondrial-generated reactive oxygen species (ROS) that oxidize protein thiol groups leading to thiol cross-linkage and the formation of non-specific membrane pores. Indeed, we have demonstrated that a burst in the mitochondrial production of H_2O_2 [10,11] and carbon centered radicals [13] precedes PTP opening. MPT induced by thiol cross-linkers is independent of ROS, and occurs due to thiol cross-linkage promoted directly by the inducer [9,13,14].

The role of Ca^{2+} in MPT is still poorly understood. The inhibitory effect of ruthenium red (RR), which blocks Ca^{2+} influx [15], indicates that the presence of intramitochondrial Ca^{2+} is necessary for PTP opening [1,2]. Our group has shown that Ca^{2+} acts in MPT by increasing mitochondrial ROS generation [10,11,13] and mobilizing intramitochondrial Fe^{2+} [13], thus increasing the generation of the hydroxyl radical. Previous results from our laboratory have shown that mitochondrial permeabilization induced by DIDS is independent of the presence of ROS and is accompanied by a Ca^{2+}-dependent production of thiol cross-linkages [9]. These results suggest that Ca^{2+} may alter the reactivity of membrane protein thiol groups, promoting their reaction with DIDS. In this report we study the relationship between Ca^{2+} and the reactivity of thiol groups with thiol reagents, in order to further understand the role of Ca^{2+} in MPT.

2. Materials and methods

2.1. Isolation of rat liver mitochondria

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

2.2. Standard incubation procedure

The experiments were carried out at 30°C in a standard reaction medium containing 125 mM sucrose, 65 mM KCl, 10 mM Hepes buffer (pH 7.2), 10 μM Ca^{2+}, 1 μM FCCP and 200 nM antimycin A. Other additions are indicated in the figure legends. The results shown are representative or averages of a series of at least three experiments.

2.3. Determination of membrane protein thiol content

The mitochondrial suspension, incubated in standard reaction medium, was submitted to three subsequent freeze-thawing procedures to release matrix proteins and was then centrifuged during 2 min at 10 000 rpm in Eppendorf Centrifuge 5415 C. The pellet was treated with 200 μl of 6.5% trichloroacetic acid and centrifuged at 10 000 rpm during 2 min in order to precipitate the protein. This procedure was repeated two times. The final pellet was suspended in 200 μl of a medium containing 0.5 mM EDTA and 0.5 M Tris (pH 8.3) and protein concentration was determined according to Lowry et al. [16]. 50 μg of protein were added to 1 ml of a solution containing 100 μM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) [17], 0.5 mM EDTA and 0.5 M Tris (pH 8.3). Absorption was measured at 412 nm, using glu-

tathione for calibration. Suspensions incubated in the presence of dithiothreitol, NEM or mersalyl were washed extensively with standard incubation medium before the release of matrix proteins.

2.4. Determination of mitochondrial swelling

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

2.5. Materials

ADP, *t*-bOOH, carboxyatractyloside, DTNB, EGTA, Hepes, mersalyl, NEM, PhAsO and RR were obtained from Sigma Chemical Company (St. Louis, MO). All other reagents were commercial products of the highest purity grade available.

3. Results

In Fig. 1, deenergized mitochondria were incubated in the presence of the hydrophilic monofunctional thiol reagent mersalyl [18,19]. Mitochondrial membrane protein was then extracted, and the content of the free thiol groups (not accessible to reaction with mersalyl) was determined using Ellman's reagent (DTNB). We noted that the content of membrane protein thiol groups not accessible to reaction with mersalyl decreases with increasing concentrations of added Ca^{2+} (▲). This decrease was slightly stimulated by the presence of the Ca^{2+} ionofore A_{23187} in the incubation medium (■) and was inhibited by Mg^{2+} (●), an inhibitor of MPT [1,2]. Increasing concentrations of Mg^{2+} (2–10 mM) promoted progressively larger inhibitions (30–73%) of 400 μM Ca^{2+}-induced membrane protein thiol reactivity alterations. When the mitochondrial suspension was treated with the hydrophobic monofunctional thiol reagent *N*-ethylmaleimide (NEM) [18,19], after incubations in the presence of increasing concentrations of added Ca^{2+}, the content of free thiol groups (not accessible to reaction with NEM) increases with increasing Ca^{2+} concentrations (▼). NEM was added after 4 min of incubation to allow for Ca^{2+}-induced membrane protein thiol reactivity alterations before this treatment. Table 1 shows that Ca^{2+}-induced decrease in thiols not accessible to reaction with mersalyl can be inhibited completely by ruthenium red (RR), indicating that this effect requires intramitochondrial Ca^{2+}, as suggested by the stimulating effect of the Ca^{2+} ionophore (Fig. 1). The Ca^{2+} effect is also partially inhibited by ADP, in a carboxyatractyloside-sensitive manner, suggesting that changes

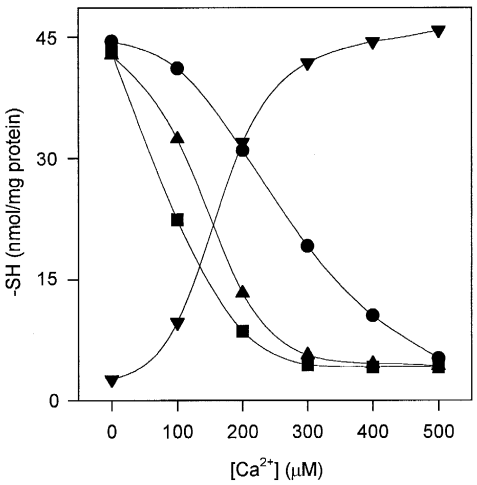

Fig. 1. Ca^{2+}-induced alterations of mitochondrial membrane protein thiol reactivity. Rat liver mitochondria (RLM, 0.4 mg/ml) were incubated 5 min in reaction medium containing (▲) 20 μM mersalyl and 0–500 μM Ca^{2+}, (■) 20 μM mersalyl, 0–500 μM Ca^{2+} and 1 μM A_{23187}, (●) 20 μM mersalyl, 0–500 μM Ca^{2+} and 1 mM Mg^{2+}, (▼) 0–500 μM Ca^{2+}. For (▼), 20 μM NEM was added 4 min after the beginning of the incubation. The membrane protein thiol content was determined as described in Section 2.

Table 1
Ca^{2+}-induced decrease in membrane protein thiols not accessible to mersalyl: effect of permeability transition inhibitors

Conditions	-SH (nmol/mg protein)
no other additions	4.3 ± 0.3 [a]
500 μM EGTA [b]	44.2 ± 2.9
1 μM RR	48.7 ± 2.6
500 μM ADP	23.2 ± 0.5
500 μM ADP + 2 μM carboxyatractyloside	6.2 ± 0.5
1 μM Cyclosporin A	4.2 ± 0.4

The experimental conditions were similar to those in Fig. 1. RLM (0.4 mg/ml) were incubated 5 min in the presence of 20 μM mersalyl and 500 μM Ca^{2+}. Other additions are indicated in the table. Membrane protein thiol content was determined as described in Section 2.

[a] Values represent average of 3 experiments ± S.D.

[b] No Ca^{2+} was added.

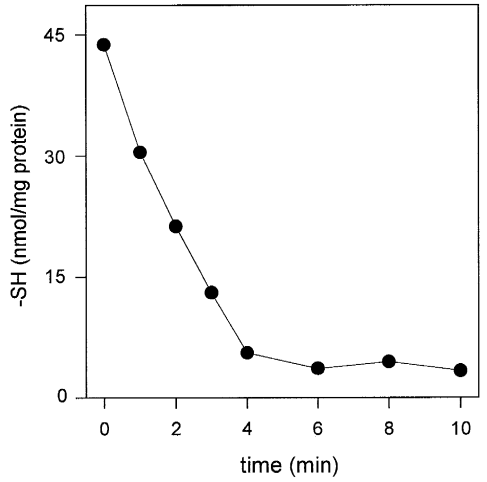

Fig. 2. Time-dependence of Ca^{2+}-induced decrease in thiols not accessible to mersalyl. RLM (0.4 mg/ml) were incubated in reaction medium containing 20 μM mersalyl and 500 μM Ca^{2+}. The reactions were stopped at the time indicated.

in the conformation of the ADP/ATP translocator are involved in the alteration of thiol group reactivity. It is well known that the change from the m- to the c-ADP/ATP carrier configuration changes the position of the thiol groups of this protein [20,21]. These alterations are insensitive to cyclosporin A, a well-known inhibitor of MPT [1,2]. In Fig. 2, the membrane protein thiol group content not accessible to reaction with mersalyl was determined at different incubation times in the presence of 500 μM Ca^{2+}. It can be observed that the decrease in this content is time-dependent, and is complete after 4 min incubation in the presence of Ca^{2+}.

In order to study the relationship between Ca^{2+}-induced alterations of thiol group reactivity and MPT, the effect of NEM and mersalyl on mitochondrial swelling induced by Ca^{2+} plus *t*-bOOH or Ca^{2+} plus the thiol cross-linker reagents 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and phenylarsine oxide (PhAsO) was studied. In these conditions, mitochondrial swelling is the result of non-specific inner mitochondrial membrane permeabilization. Fig. 3, panel A, shows that mitochondrial swelling induced by the hydrophilic disulfide reagent DIDS (line d) may be inhibited when either 20 μM NEM (line b) or 20 μM mersalyl (line c) are added before Ca^{2+}. If NEM or mersalyl were added after Ca^{2+} (panel B), a situation in which most thiol groups are not reactive with NEM (see Fig. 1), only mersalyl (line a) promoted a protective effect. Higher concentrations of mersalyl or NEM in the incubation medium were deleterious to the mitochondria (results not shown).

Mitochondrial swelling induced by the hydrophobic disulfide reagent PhAsO (Fig. 4) may be inhibited by NEM added before or after Ca^{2+} (lines a) and is not inhibited by mersalyl (panel A, line c and panel B, line b). This indicates that PhAsO-induced MPT occurs via cross-linkage of thiol groups reactive with NEM, and is not influenced by Ca^{2+}-induced thiol reactivity alterations. In Fig. 4A, the addition of Ca^{2+} before PhAsO does not alter the effects of NEM or mersalyl on mitochondrial swelling (results not shown).

Fig. 5 shows that mitochondrial swelling induced by the prooxidant *t*-bOOH and Ca^{2+} is inhibited by NEM added before or after Ca^{2+} (lines a) and is not inhibited by mersalyl (panel A, line c and panel B, line b), as occurs with mitochondrial swelling in-

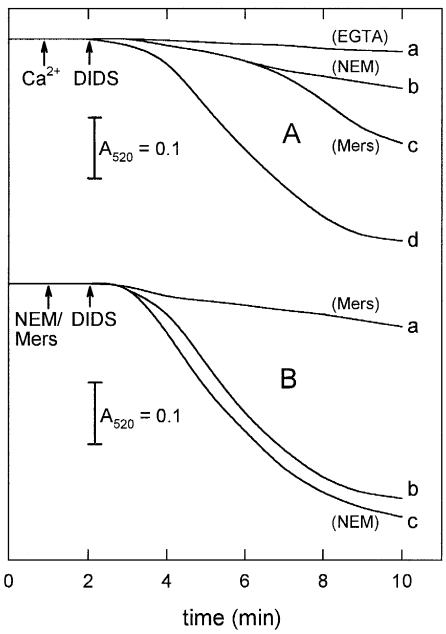

Fig. 3. Ca^{2+} and DIDS-induced mitochondrial swelling: effect of NEM and mersalyl (Mers). In panel A, RLM (0.3 mg/ml) were added to reaction medium containing 500 μM EGTA (line a), 20 μM NEM (line b), 20 μM mersalyl (line c) or no additions (line d). Ca^{2+} (300 μM) and 300 μM DIDS were added where indicated in all experiments. In panel B, RLM were added to reaction medium in the presence of 300 μM Ca^{2+}. 300 μM DIDS (lines a–c), 20 μM mersalyl (line a) and 20 μM NEM (line c) were added where indicated.

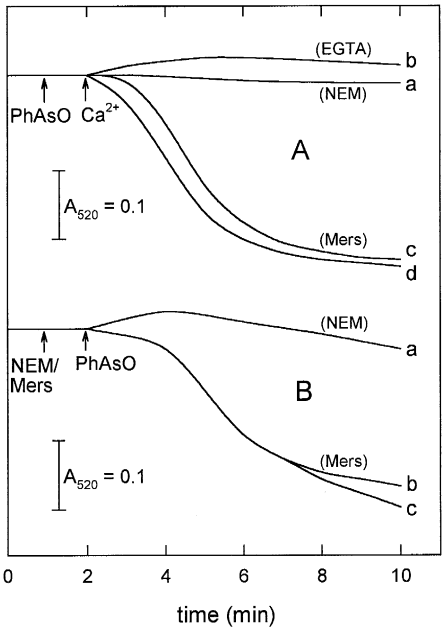

Fig. 4. Ca^{2+} and PhAsO-induced mitochondrial swelling: effect of NEM and mersalyl (Mers). In panel A, RLM (0.3 mg/ml) were added to reaction medium containing 20 μM NEM (line a), 500 μM EGTA (line b), 20 μM mersalyl (line c) or no additions (line d). Ca^{2+} (300 μM) and 15 μM PhAsO were added where indicated in all experiments. In panel B, RLM were added to reaction medium in the presence 300 μM Ca^{2+}. 15 μM PhAsO (lines a–c), 20 μM NEM (line a) and 20 μM mersalyl (line b) were added where indicated.

duced by PhAsO. Interestingly, the mitochondrial swelling observed with either *t*-bOOH or PhAsO is significantly slower when Ca^{2+} is added at the beginning of the run (Figs. 4 and 5, panels B, compare with panels A), probably due to Ca^{2+}-induced alterations of protein thiol group reactivity, decreasing the access of these thiol groups to PhAsO or reactive oxygen species accumulated in the presence of *t*-bOOH [12,13].

We have previously shown that MPT is accompanied by the oxidation and cross-linkage of protein thiol groups [8,9,12]. In order to verify if this process was involved in MPT in the situations of Figs. 3–5, we determined the total membrane protein thiol content in the presence of Ca^{2+} and MPT inducers (Table 2). It can be observed that mitochondrial membrane permeabilization induced by Ca^{2+} and either *t*-bOOH, PhAsO or DIDS is accompanied by a decrease in the membrane protein thiol content. The disulfide reductant dithiothreitol (2 mM) totally reverts the effect of *t*-bOOH, but has no effect on PhAsO or DIDS-induced decrease in protein thiol content (results not shown). If PhAsO or *t*-bOOH are incubated in the presence of DIDS, there is a further decrease in the thiol content, when compared to PhAsO or *t*-bOOH alone, but if PhAsO and *t*-bOOH are incubated together, the decrease in the thiol content is similar to that observed in the incubation in the presence of *t*-bOOH alone. In Table 2, it may also be noted that the decrease of the total thiol content induced by PhAsO is not sensitive to EGTA, which inhibits mitochondrial permeabilization.

The experiments depicted in Fig. 6 were conducted to identify the role of Ca^{2+} in PhAsO-induced mitochondrial permeabilization. The mitochondrial suspension was treated with EGTA, in order to eliminate free Ca^{2+} present in mitochondrial preparation, and then treated with RR to block Ca^{2+} uptake. Under these conditions, DIDS and added extramitochondrial Ca^{2+} (panel A, line b) do not induce mitochondrial

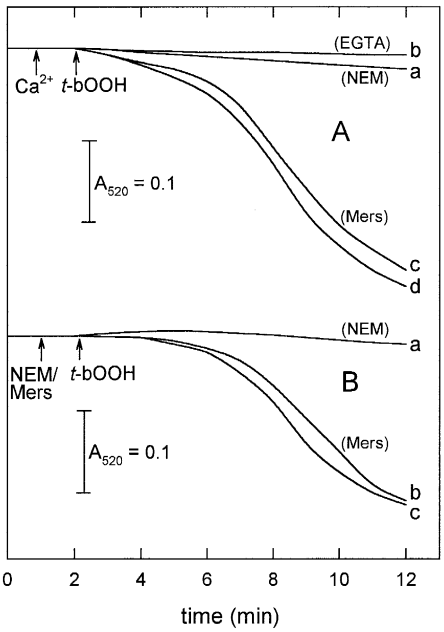

Fig. 5. Ca^{2+} and *t*-bOOH-induced mitochondrial swelling: effect of NEM and mersalyl (Mers). In panel A, RLM (0.3 mg/ml) were added to reaction medium containing 20 μM NEM (line a), 500 μM EGTA (line b), 20 μM mersalyl (line c) or no additions (line d). Ca^{2+} (300 μM) and 500 μM *t*-bOOH were added where indicated in all experiments. In panel B, RLM were added to reaction medium in the presence 300 μM Ca^{2+}. 500 μM *t*-bOOH (lines a–c), 20 μM NEM (line a) and 20 μM mersalyl (line b) were added where indicated.

Table 2
Effect of 500 μM *t*-bOOH, 300 μM DIDS and 15 μM PhAsO on total mitochondrial [-SH]

Conditions	-SH (nmol/mg protein)
EGTA	57.1 ± 0.6 [a]
Ca^{2+}	53.9 ± 1.1
EGTA + *t*-bOOH	56.4 ± 0.4
EGTA + DIDS	56.5 ± 0.7
EGTA + PhAsO	41.4 ± 1.6
Ca^{2+} + *t*-bOOH	36.5 ± 0.7
Ca^{2+} + DIDS	39.1 ± 0.5
Ca^{2+} + PhAsO	41.5 ± 0.9
Ca^{2+} + *t*-bOOH + DIDS	31.4 ± 1.0
Ca^{2+} + DIDS + PhAsO	34.9 ± 0.8
Ca^{2+} + *t*-bOOH + PhAsO	36.2 ± 1.1

RLM (0.4 mg/ml) were incubated 15 min in standard reaction medium containing 500 μM EGTA or 500 μM Ca^{2+} as indicated in the table. Total [-SH] concentration was determined as described in Section 2.
[a] Values represent average of 3 experiments ± S.D.

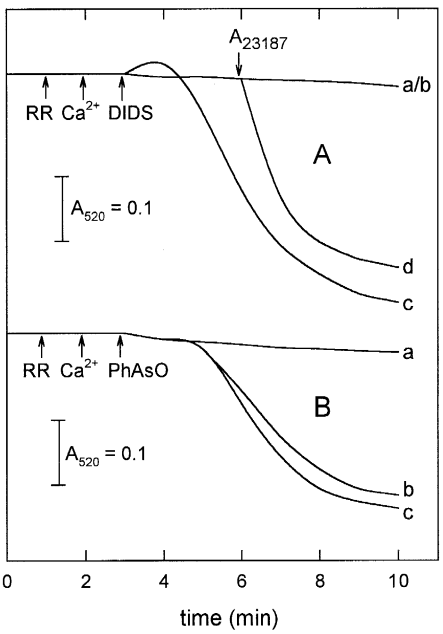

Fig. 6. Effect of intramitochondrial Ca^{2+} on DIDS or PhAsO-induced mitochondrial swelling. RLM (0.3 mg/ml) were added to reaction medium containing 100 μM EGTA. 5 μM RR (lines b and d), 400 μM Ca^{2+} (lines b, c and d) and 1 μM A_{23187} (line d) were added where indicated by arrows. 300 μM DIDS (panel A) or 15 μM PhAsO (panel B) were added where indicated in order to initiate mitochondrial swelling. Lines a represent control experiments without the addition of Ca^{2+}.

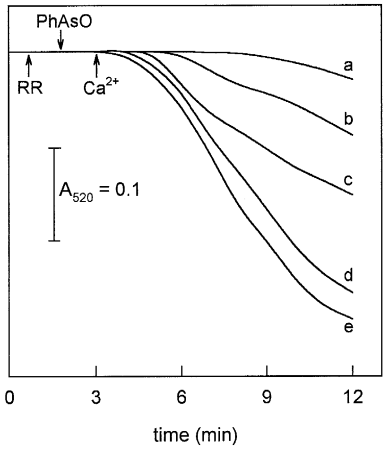

Fig. 7. Effect of extramitochondrial Ca^{2+} on PhAsO-induced mitochondrial swelling. RLM (0.3 mg/ml) were added to reaction medium containing 50 μM EGTA. 5 μM RR (lines a–e), 15 μM PhAsO (lines a–e) and 10 μM Ca^{2+} (line a), 30 μM Ca^{2+} (line b), 40 μM Ca^{2+} (line c), 50 μM Ca^{2+} (line d) or 100 μM Ca^{2+} (line e) were added where indicated. Free Ca^{2+} concentration obtained: 0.2 μM (line a), 0.5 μM (line b), 2.5 μM (line c), 10 μM (line d), 60 μM (line e).

swelling, unless a Ca^{2+} ionophore (line d) is added, but surprisingly mitochondrial swelling is observed in the presence of PhAsO and added extramitochondrial Ca^{2+} (panel B, line b). This indicates that PhAsO-induced MPT depends on extramitochondrial Ca^{2+}. Fig. 7 shows that extramitochondrial Ca^{2+} concentrations of 1–50 μM are sufficient to lead to mitochondrial permeabilization in the presence of PhAsO. A half maximum effect was observed in the presence of 2.5 μM Ca^{2+} (line c), suggesting the presence of an external Ca^{2+} binding site, sensitive to low Ca^{2+} concentrations, which triggers PTP opening. Bernardi et al. [22] have demonstrated that PhAsO may promote mitochondrial permeabilization in the absence of Ca^{2+}; however, higher PhAsO concentrations (75 μM) were used.

4. Discussion

It has been proposed that one of the mitochondrial permeability transition-inducing roles of Ca^{2+} is binding to the inner mitochondrial membrane and changing the conformation of membrane proteins and the location and reactivity of their thiol groups [9,13]. Membrane protein thiol groups can be either buried

in the lipophilic membrane phase or oriented toward the hydrophilic phase. The experiments presented in this paper show that high Ca^{2+} concentrations render membrane protein thiols more accessible to react with hydrophilic mono and bifunctional thiol reagents, such as mersalyl (Fig. 1) and DIDS (Fig. 3). PhAsO and *t*-bOOH-induced MPT are not influenced by this Ca^{2+} effect, and occur due to the oxidation of thiol groups accessible to react with the hydrophobic thiol reagent NEM (Figs. 4 and 5). Experiments conducted with iodoacetic acid, a polar monofunctional thiol reagent, showed effects similar to those observed with mersalyl (results not shown). The inhibitory effect of NEM in MPT has been reported previously [14,18,19,23], but the relationship between NEM, Ca^{2+} and protein thiol group reactivity has not been explored.

Previous results from our group show that *t*-bOOH-induced MPT is mediated by an increased production of reactive oxygen species at the level of coenzyme Q [13]. These reactive oxygen species (mainly the hydroxyl radical) certainly promote the oxidation of protein thiol groups buried in the lipid phase of mitochondrial membrane, close to their production sites. The results presented here (Figs. 3–5 and Table 2) suggest that PhAsO and *t*-bOOH induce membrane permeabilization via oxidation of thiol groups with different reactivity than those oxidized by DIDS. Therefore, the decrease in the content of membrane protein thiols caused by PhAsO or *t*-bOOH and that caused by DIDS is partially additive (Table 2). In contrast, the decrease in thiols caused by PhAsO and *t*-bOOH is not additive. These results show that different MTP inducers may react with distinct mitochondrial membrane protein thiol groups.

The complete inhibition by RR of Ca^{2+}-induced alterations in protein thiol group reactivity (Table 1) and the stimulation observed in the presence of the Ca^{2+} ionophore (Fig. 1) suggest that alterations in thiol group reactivity are caused by intramitochondrial Ca^{2+}. The Ca^{2+} effect on protein thiol group reactivity may be explained by alterations caused by this cation on the mitochondrial membrane structure. Indeed, recent results from our group (Grijalba, M.T., personal communication), using electron paramagnetic resonance, demonstrated that Ca^{2+} binding to cardiolipins induces lateral phase separation in heart submitochondrial particles. This certainly affects lipid/protein interactions, and may result in unmasking of membrane protein thiol groups. In this regard, it has been proposed that changes of the secondary structure and activity of the ADP/ATP carrier, an important protein involved in MPT [1,2,8], may be modulated by its interactions with cardiolipin [24]. Interestingly, cyclosporin A, a widely used MPT inhibitor [1,2], did not have any effect on Ca^{2+}-induced alterations in membrane protein thiol group reactivity. This result supports the idea that cyclosporin A inhibits directly PTP opening.

The experiments depicted in Figs. 6 and 7 show that PhAsO-induced MPT is dependent on extramitochondrial Ca^{2+} concentrations, which act as a trigger for membrane permeabilization. This external Ca^{2+} binding site could be involved in the regulation of PTP opening in pathological situations, such as ischemia and reperfusion, when cytosolic Ca^{2+} concentrations attain levels over 1 μM ([25] and refs. therein). Based on experiments in which MPT was induced by uncouplers, Bernardi et al. [26] have proposed that PTP opening may be inhibited by high external Ca^{2+} concentrations ($I_{50} = 0.2$ mM).

Altogether, the data presented here support and add to previous data from ourselves [9,12,13,27] and other groups [14,28,29] indicating that MPT occurs due to the oxidation and cross-linkage of membrane protein thiol groups. This results in protein aggregation that, when extensive, determines an irreversible state of mitochondrial membrane permeabilization [8]. This membrane protein thiol cross-linkage may be caused by thiol cross-linkers [9,14], respiratory chain-generated reactive oxygen species [10,11,13] or exogenous reactive oxygen species [27,30], and leads to the formation of the PTP, triggered by Ca^{2+} [1,2,9,27].

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