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PROTECTIVE EFFECT OF TRIFLUOPERAZINE ON THE MITOCHONDRIAL DAMAGE INDUCED BY Ca^{2+} PLUS PROOXIDANTS

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Abstract—Isolated rat liver mitochondria undergo extensive swelling and disruption of membrane potential when they accumulate Ca^{2+} in the presence of a prooxidant such as diamide or *t*-butylhydroperoxide. The phenothiazinic drug trifluoperazine, at concentrations (15–35 μM) which do not inhibit respiration or the influx of Ca^{2+} into mitochondria, significantly protected mitochondria against the deleterious effects of Ca^{2+} plus a prooxidant. In contrast, at concentrations higher than 100 μM the drug potentiated these deleterious effects of Ca^{2+} and prooxidants and had a damaging effect *per se* on the inner mitochondrial membrane. It is proposed that the protection conferred by the drug is mediated by changes in membrane protein structure that decrease the production of protein thiol cross-linkings which occur when mitochondria accumulate calcium under oxidant stress conditions.

During a study of the stoichiometry between Ca^{2+} accumulation, H^+ extrusion and O_2 uptake by rat heart mitochondria, Vercesi *et al.* [1] observed that Ca^{2+} is not retained in the matrix when mitochondria are oxidizing NAD-linked substrates. In contrast, Ca^{2+} is retained when these mitochondria are respiring on succinate in the presence of rotenone which prevents net electron flow from the endogenous pyridine nucleotides to oxygen. A more detailed study [2] indicated that Ca^{2+} efflux from isolated mitochondria from rat heart and liver, as well as from Ehrlich ascites tumor cells, could be stimulated by the oxidized state of mitochondrial pyridine nucleotides. Thus, the use of succinate as substrate, in the presence of rotenone, permits the maintenance of a more reduced steady-state level of mitochondrial NAD(P)H, and consequently a higher capacity of the organelle to retain the accumulated calcium [3]. Additional studies from our [4, 5] and other laboratories [reviewed in Ref. 6] have indicated that this Ca^{2+} release is associated with a nonspecific increase in mitochondrial membrane permeability. This increase in permeability is believed to account for the so-called permeability transition of mitochondria, a condition that might arise from the operation of a giant pore [6], now characterized as the cyclosporin A-sensitive permeability transition pore [6–8]. It has been proposed that these alterations in mitochondrial membrane permeability may be a critical step in the pathogenesis of cell injury associated with perturbations of intracellular calcium homeostasis caused by such factors and conditions as chemical toxins, ischemia and oxidative stress [reviewed in Ref. 6].

More recently we provided evidence that the perturbation of the inner mitochondrial membrane caused by Ca^{2+} plus prooxidants is associated with protein polymerization due to thiol cross-linking [9]. The reversibility of both the permeability increase [9] and the protein aggregation† by treatment of the mitochondrial preparation with dithiothreitol permits us to propose that the permeability transition pore may arise from the cross-linking via disulfide bridges of membrane proteins.

Previous reports about the effect of trifluoperazine on Ca^{2+} flux in mitochondria have indicated that the drug inhibits: (i) Ca^{2+} uptake by mitochondria of guinea pig peritoneal macrophages [10]; (ii) Na^+-induced Ca^{2+} efflux from different kind of mitochondria [11–13]; and (iii) *N*-ethylmaleimide-stimulated ruthenium red-induced Ca^{2+} release and related swelling of rat liver mitochondria [14]. Some of these studies [11, 12, 14] have implicated the inhibition of mitochondrial phospholipase A_2 in the mechanism of trifluoperazine action. It is also known that trifluoperazine inhibits the peroxidation of microsomal phospholipids [15, 16] and low density lipoproteins [17] and protects mitochondria against the damage caused by ischemia and reperfusion [18]. The mechanisms underlying these last effects are not elucidated but seem to be independent of the reaction of trifluoperazine with free radicals [15–17]. It is also proposed that this and other phenothiazine derivatives induce membrane structural changes secondary to alterations in membrane protein structure that convert surface sites (-SH) groups to buried sites [19]. In view of these properties of phenothiazinic drugs, it is reasonable to hypothesize that they may interfere with the production of protein thiol cross-linking induced by Ca^{2+} plus prooxidants, thus preventing the formation of the permeability transition pore.

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† Valle VGR, Parentoni LS, Fagian MM, Meinicke AR and Vercesi AE, unpublished results.

The present study describes the effects of trifluoperazine on the mitochondrial damage induced by Ca^{2+} plus prooxidants. The results show that trifluoperazine, at low concentrations, decreases the production of membrane protein aggregates and protects against the damage induced by Ca^{2+} plus oxidative stress in isolated rat liver mitochondria.

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. The homogenate was prepared in 250 mM sucrose, 1.0 mM EGTA*/ and 5.0 mM HEPES buffer (pH 7.2). The mitochondrial suspension was washed twice in the same medium containing 0.1 mM EGTA and the final pellet was diluted in 250 mM sucrose to a protein concentration of 80–100 mg/mL.

Standard incubation procedure. The experiments were carried out at 30° in a basic medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES buffer (pH 7.2), and 5.0 μM rotenone. Other additions are indicated in the figure legends.

Sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis. Aliquots of mitochondria incubated for 10 min in the basic medium were taken and the matrix proteins were released by three subsequent freeze–thawing procedures. Electrophoresis of the membrane-solubilized proteins was performed by SDS–PAGE in a discontinuous system according to Laemmli [20] and as described by Fabian *et al.* [9]. The running gel was 12% in polyacrylamide and the stacking gel was 3.5%.

Oxygen uptake measurements. Oxygen consumption was measured with a Clark-type electrode (Yellow Springs Instruments Co.) in a 1.0-mL thermostatized glass chamber equipped with magnetic stirring.

Determination of Ca^{2+} movements. Variations in free Ca^{2+} concentrations were followed by measuring the changes in the absorbance spectrum of arsenazo III [21], using an SLM Aminco DW2000 spectrophotometer at the wavelength pair 675–685 nm.

Measurements of mitochondrial transmembrane electrical potential ($\Delta\psi$). Mitochondria were incubated in the reaction medium containing 3 μM tetraphenylphosphonium (TPP^+). The concentration of TPP^+ in the extramitochondrial medium was monitored continuously with a TPP^+-selective electrode prepared in our laboratory according to Kamo *et al.* [22]. The membrane potential was then calculated assuming that the TPP^+ distribution between mitochondria and medium follows the Nernst equation [23]. Corrections due to the binding of TPP^+ to the mitochondrial membranes were made according to Jensen *et al.* [24].

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

Chemicals. Trifluoperazine, rotenone, diamide, *t*-butylhydroperoxide, HEPES, and EGTA were obtained from the Sigma Chemical Co., and TPP^+ was from the Aldrich Chemical Co. All other reagents were commercial products of the highest available grade of purity.

RESULTS AND DISCUSSION

Effect of increasing concentrations of trifluoperazine on mitochondrial swelling by Ca^{2+} plus prooxidants. The experiments depicted in Fig. 1 show the rates of absorbance decrease (mitochondrial swelling) of a suspension of respiring mitochondria incubated in the presence or absence of 10 μM Ca^{2+}, 300 μM diamide (panel A) or 300 μM *t*-butylhydroperoxide (panel B) and different concentrations of trifluoperazine. Line a shows that no swelling occurred in the absence of Ca^{2+} and oxidants. Line b indicates the existence of a slow rate of swelling in the presence of Ca^{2+} alone but line c illustrates the occurrence of a fast and extensive swelling when Ca^{2+} and an oxidant were present simultaneously. Lines d and e show an increasing degree of inhibition, in both rate and extent, of mitochondrial swelling when the concentration of trifluoperazine in the medium increased from 15 to 35 μM, respectively. In contrast, line f indicates that by increasing the trifluoperazine concentration to 45 μM, the inhibition of swelling decreased. This suggests that, at higher concentrations, trifluoperazine may be deleterious to the mitochondrial membrane. This was confirmed by the finding in line g indicating that a 250 μM concentration of the drug not only increased the mitochondrial swelling induced by Ca^{2+} plus a prooxidant but it also induced, *per se*, a large amplitude mitochondrial swelling (dashed line). At a concentration of 100 μM trifluoperazine, significant mitochondrial swelling was observed (data not shown). This damaging effect of high concentrations of trifluoperazine is in agreement with previous data showing similar results of the drug on other membranes [25] and on the inner mitochondrial membrane of *Trypanosoma cruzi* [26]. The previous observations that trifluoperazine inhibits mitochondrial respiration [25, 27] prompted us to study the effect of this drug on the respiration of rat liver mitochondria, under our experimental conditions. This may indicate whether or not the dual effect of trifluoperazine, observed in Fig. 1, is related to different energy states of mitochondria due to inhibition of respiration.

Inhibition by trifluoperazine of succinate-supported O_2 consumption by rat liver mitochondria. Figure 2 shows the concentration–response relationship between trifluoperazine and the rate of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP)-stimulated respiration. It can be observed that a marked inhibition began at a concentration close to 70 μM trifluoperazine and increased rapidly

* Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)*N,N'*-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; TTP^+, tetraphenylphosphonium; and $\Delta\psi$, transmembrane electrical potential.

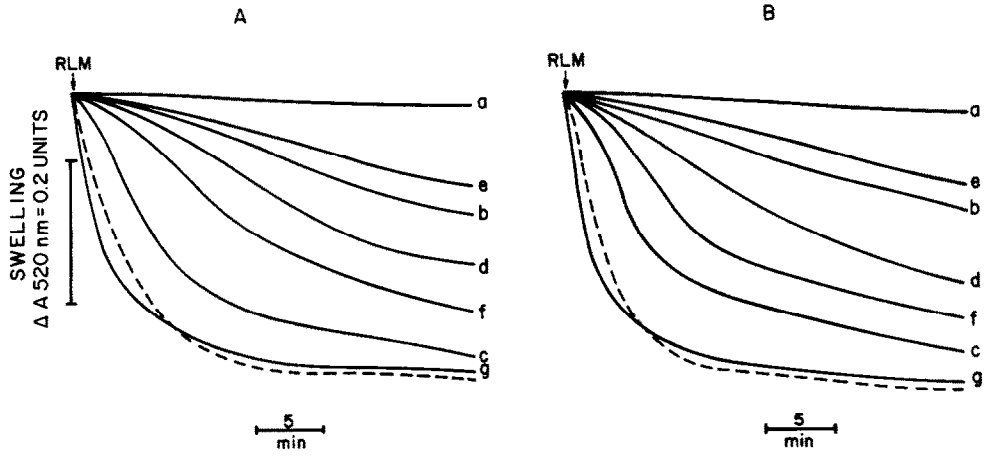

Fig. 1. Effect of trifluoperazine on mitochondrial swelling induced by Ca^{2+} and diamide (A) or *t*-butylhydroperoxide (B). Rat liver mitochondria (RLM, 0.5 mg/mL) were incubated in the basic medium containing: (a) 1.0 mM EGTA; (b) 10 μM Ca^{2+}; (c) 10 μM Ca^{2+} plus 300 μM diamide (A) or 300 μM *t*-butylhydroperoxide (B); and (d–g) identical to c plus 15, 35, 45 and 250 μM trifluoperazine, respectively. The dashed line represents an experiment in the absence of prooxidant and in the presence of 10 μM Ca^{2+} and 250 μM trifluoperazine.

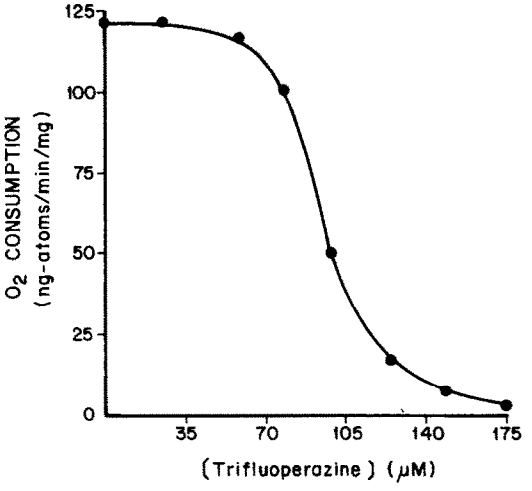

Fig. 2. Inhibition of respiration by trifluoperazine. Rat liver mitochondria (1.0 mg/mL) were preincubated in the basic medium containing 1.0 μM FCCP and different concentrations of trifluoperazine in the absence of succinate. After 3 min, respiration was initiated by the addition of 2.0 mM succinate. Each point is the average of three different determinations.

as the concentration increased. At 175 μM, the inhibition was almost 100%. This curve is displaced slightly to the right when compared to that obtained by Vale *et al.* [27] although they used completely different experimental conditions and dinitrophenol, instead of FCCP, as the uncoupler. Similar results were obtained when mitochondria were energized by NAD-linked substrates or by the electron-donating system ascorbate + *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), in the presence of rotenone and antimycin-A (data not shown). In the case of NAD-linked substrates, inhibition started at a trifluoperazine concentration of 25 μM and was almost complete at 100 μM trifluoperazine.

These results indicate that trifluoperazine, in the concentration range that protects mitochondria against the deleterious effects of Ca^{2+} (succinate as substrate), does not inhibit mitochondrial respiration. Therefore, this protection is not related to the energy state of mitochondria and may be the result of direct interactions between the drug and membrane proteins involved in the mechanism of membrane permeabilization by Ca^{2+} plus prooxidants. Interactions of trifluoperazine with specific membrane proteins have been demonstrated for the Ca^{2+}-ATPase of sarcoplasmic reticulum [28, 29] and mitochondrial F_0F_1-ATPase [29], for example.

Effect of trifluoperazine on the stimulation of mitochondrial Ca^{2+} *release induced by prooxidants.* Figure 3 shows that when mitochondria were added to a reaction medium containing succinate in the presence of rotenone and 10 μM Ca^{2+}, a fast decrease in Ca^{2+} concentration started immediately and continued until the ambient Ca^{2+} concentration was lowered to less than 1.0 μM. In the absence of prooxidants (line a), the accumulated Ca^{2+} was retained by mitochondria during the experimental observation time (10 min). However, in the presence of diamide (line b) or *t*-butylhydroperoxide (line c) all of the Ca^{2+} [accumulated plus endogenous (5–10 nmol/mg)] was released after periods of about 5 and 8 min, respectively. This release of mitochondrial calcium was almost completely abolished by 35 μM trifluoperazine when the oxidant was diamide (line d), and was completely abolished when the oxidant was *t*-butylhydroperoxide (line e). Line f shows an experiment performed in the absence of prooxidants

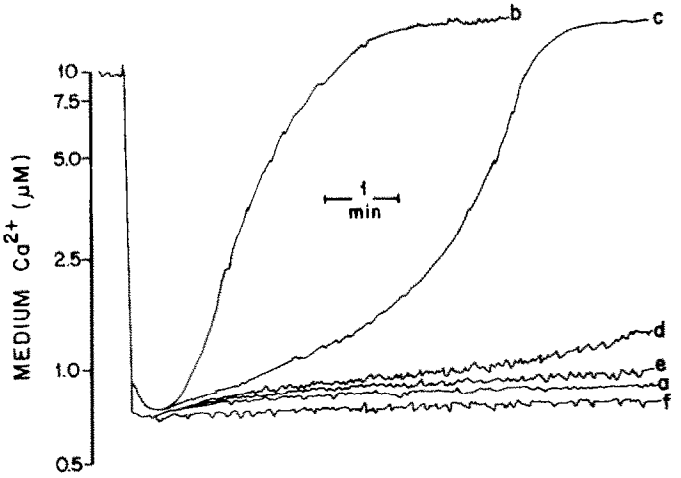

Fig. 3. Inhibition by trifluoperazine of the expontaneous mitochondrial Ca^{2+} release induced by prooxidants. Rat liver mitochondria (1.0 mg/mL) were added to the basic medium containing: (a) 10 μM Ca^{2+}; (b) 10 μM Ca^{2+} and 300 μM diamide; (c) 10 μM Ca^{2+} and 300 μM *t*-butylhydroperoxide; (d) 35 μM trifluoperazine under the conditions of line b; (e) 35 μM trifluoperazine under the conditions of line c; and (f) 10 μM Ca^{2+} and 35 μM trifluoperazine.

and in the presence of 35 μM trifluoperazine, indicating that, at this concentration, the drug does not affect either the rate or the extent of Ca^{2+} accumulation by mitochondria. It can also be observed that the presence of trifluoperazine did not change the initial concentration of free Ca^{2+} in the medium. These results clearly eliminate the possibility that the protective action of trifluoperazine on the mitochondrial damage, under these conditions, is due to an inhibition of Ca^{2+} accumulation by mitochondria. Spectrophotometric determinations of Fe^{2+} and Fe^{3+} in the presence of trifluoperazine indicated that the drug does not chelate this metal as well (data not shown).

Effect of trifluoperazine on the disruption of membrane potential induced by Ca^{2+} *plus prooxidants.* The previous experiments (Figs. 2 and 3) indicate that trifluoperazine abolishes the deleterious effects of Ca^{2+} on mitochondria at concentrations which do not inhibit either the respiration or the uptake of the cation. This suggests that trifluoperazine may prevent the Ca^{2+}-induced permeabilization of the inner mitochondrial membrane that takes place in the presence of prooxidants. This is followed by mitochondrial swelling and disruption of membrane potential [4]. This possibility was assessed by measurements of $\Delta\psi$ supported by succinate oxidation in medium containing Ca^{2+} plus diamide in the presence or absence of trifluoperazine (Fig. 4). Line a, obtained in the presence of Ca^{2+} alone, shows that a $\Delta\psi$ of the order of 150 mV was built and sustained during the time of observation. In contrast, when diamide was added, both the magnitude and maintenance of $\Delta\psi$ decreased markedly (see line b). In addition, it is also shown that the inclusion of dithiothreitol or EGTA caused restoration of $\Delta\psi$. These results are in agreement with previous data [9, 30] indicating that this increase in membrane permeability can be reversed by thiol reductants or Ca^{2+} chelators. In contrast, the addition of trifluoperazine at different times, under the conditions of line b, failed to restore $\Delta\psi$. However, when the drug was present from the beginning of the experiment, mitochondria were able to build up and sustain a membrane potential of about 130 mV in medium containing Ca^{2+} and diamide (line c). This indicates that the nature of the trifluoperazine effect on this mechanism is different from those of dithiothreitol and EGTA. Very similar results were obtained, when we examined the effect of trifluoperazine on the disruption of $\Delta\psi$ caused by *t*-butylhydroperoxide (data not shown).

Effect of trifluoperazine on the production of mitochondrial membrane protein aggregates by Ca^{2+} *plus prooxidants.* We have shown previously [9] that the permeabilization of the inner mitochondrial membrane by Ca^{2+} plus prooxidants is associated with the formation of protein aggregates due to the production of thiol cross-linking. To ascertain whether the protective effect of trifluoperazine on this process of membrane permeabilization could be related to a decrease in protein polymerization, we performed electrophoresis of solubilized mitochondrial membrane proteins. Figure 5 shows the results of electrophoresis when samples were taken after incubation under the conditions of Fig. 4. Lanes a, b and d show protein bands obtained in the presence of Ca^{2+} alone (control experiment), Ca^{2+} plus *t*-butylhydroperoxide and Ca^{2+} plus diamide, respectively. It can be seen that the presence of the prooxidants increased the amount of proteins of molecular mass greater than 116 kDa. In the case of diamide this was more pronounced and a protein band which did not enter the running gel was observed (lane d). That trifluoperazine diminishes the production of these high molecular weight proteins can be observed in lanes c and e

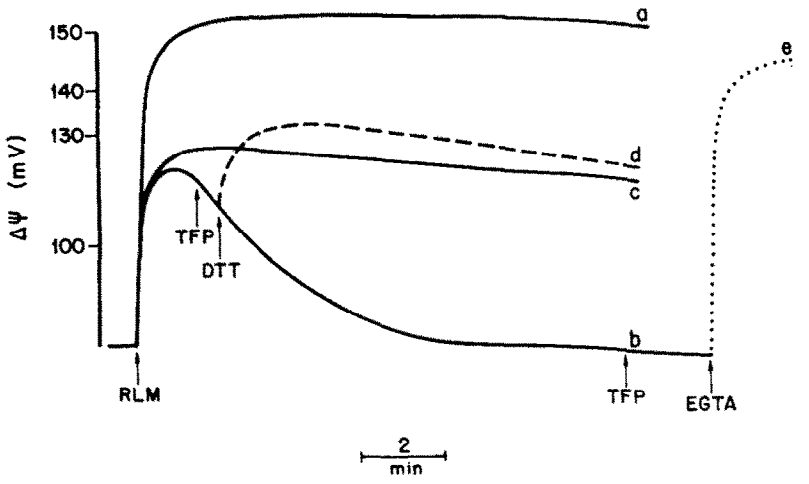

Fig. 4. Effect of trifluoperazine (TFP) on $\Delta\psi$ disruption induced by Ca^{2+} and prooxidants. Rat liver mitochondria (RLM, 1.0 mg/mL) were added to the basic medium containing: (a) 10 μM Ca^{2+}; (b) 10 μM Ca^{2+}, 300 μM diamide and 35 μM trifluoperazine (added where shown); and (c) 10 μM Ca^{2+}, 300 μM diamide and 35 μM trifluoperazine. Dithiothreitol (DTT, 1.0 mM) and EGTA (1.0 mM) were added where shown (dashed and dotted lines, respectively). TPP^+ (3.0 μM) was present in all experiments.

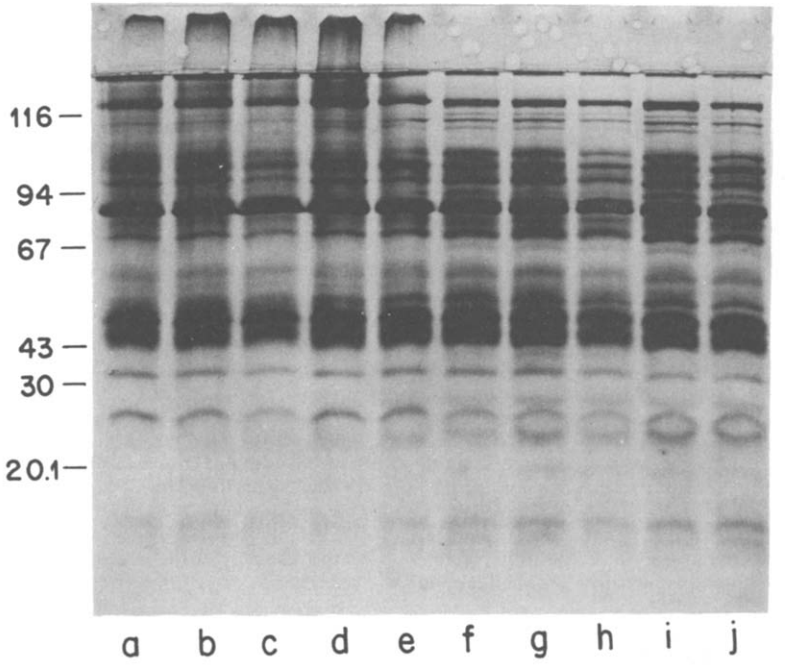

Fig. 5. SDS–PAGE of membrane protein from rat liver mitochondria incubated in the presence of Ca^{2+} and *t*-butylhydroperoxide or diamide: Effect of trifluoperazine. In each lane, 10 μg of protein was applied to a 12% polyacrylamide running gel after incubation for 10 min under the conditions described for Fig. 4. Lane a, 10 μM Ca^{2+}; lane b, 10 μM Ca^{2+} and 300 μM *t*-butylhydroperoxide; lane c, 10 μM Ca^{2+}, 300 μM *t*-butylhydroperoxide and 35 μM trifluoperazine; line d, 10 μM Ca^{2+} and 300 μM diamide; line e, 10 μM Ca^{2+}, 300 μM diamide and 35 μM trifluoperazine. Lanes f–j, identical to a–e except that the samples were boiled with 0.5% β-mercaptoethanol before application to the gels.

where *t*-butylhydroperoxide or diamide was present, respectively. Lanes f–j were assays identical to lanes a–e respectively, except that the samples were boiled in 0.5% β-merceptoethanol before applying them to the gels. This treatment eliminated all of the proteins above the 116 kDa band, indicating that they were aggregates that resulted from thiol cross-linking. The presence of some of these proteins in lane a (Ca^{2+} alone), which disappeared after the treatment with β-mercaptoethanol, could be due to a stimulation of mitochondrial production of reactive oxygen species in response to Ca^{2+} accumulation [31], as discussed previously [9]. These oxyradicals can attack protein thiols, thus explaining the deleterious effects of high Ca^{2+} concentrations alone [6, 9] and the inhibition by trifluoperazine of the mitochondrial swelling induced by the cation [32]. With respect to the Ca^{2+} requirement for significant membrane damage induced by prooxidants, we have proposed [9] that Ca^{2+} stimulates the production of protein aggregates due to its binding to certain membrane proteins. This binding would induce conformational changes in these proteins that decrease the distance between critical sulfhydryl groups and favors the formation of dithiols under oxidative stress conditions. On the basis of this rationale, we also propose that oxidative stress-induced protein thiol cross-linking is inhibited by trifluoperazine which induces changes in membrane proteins that convert surface into buried thiols, decreasing the probability of dithiol formation [19]. This would prevent the formation of the permeability transition pore and the consequent mitochondrial damage.

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