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Induction of Mitochondrial Permeability Transition by the DNA-intercalating Cationic Dye Ethidium Bromide

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This work shows that the DNA cationic probe, ethidium bromide (EtBr), induces the transition from selective to non-selective mitochondrial permeability. This statement is based on the findings, indicating: (i) EtBr induced the release of accumulated Ca^{2+} through a mechanism sensitive to cyclosporin A and octylguanidine; (ii) EtBr induced the release of cytochrome c and (iii) EtBr induced mitochondrial swelling. Interestingly, mersalyl inhibited, in a non-competitive fashion, EtBr uptake, which would indicate that the uptake may be carried out through a protein membrane system. This work also shows that the effect of the dye on permeability transition was stimulated by carboxyatractyloside. Taking into account the facts that EtBr inhibited the ADP exchange reaction and increased the binding of the fluorescent probe eosin-5-maleimide to adenine nucleotide translocase, it is tempting to assume a possible interaction between EtBr and the ADP/ATP carrier.

Key words: ADP/ATP-translocase, calcium, ethidium bromide, kidney mitochondria, permeability transition.

Abbreviations: ANT, adenine nucleotide translocase; CAT, carboxyatractyloside; CSA, cyclosporin A; EMA, eosin-5-maleimide; EtBr, ethidium bromide; NEM, N-ethylmaleimide; OG, octylguanidine; RC, respiratory control.

Lipophilic cations, among them ethidium bromide (EtBr), have been used to modify mitochondrial functions. This reagent, which is the most commonly used to stain nucleic acids, is actively accumulated into the mitochondrial matrix (*1*), having DNA as the main target (*2*, *3*). The above produces changes in the mitochondrial genome generating mutations, *i.e*. in Drosophila cells (*4*), and modifications in the mitochondrial ultrastrucure of a human leukaemia T-cell line (*5*). Besides, accumulation of EtBr induces uncoupling of oxidative phosphorylation (*6*) and inhibition of the respiratory chain (*7*, *8*). However, the effect that this reagent may have on membrane permeability has not been studied.

This work was addressed at determining the effect of EtBr on mitochondrial permeability transition. It was found that increasing concentrations of EtBr induced the efflux of accumulated Ca^{2+} through a mechanism that is sensitive to the immunosuppressant, cyclosporin A (CSA), and the hydrophobic cation, octylguanidine (OG). EtBr-induced Ca^{2+} release was stimulated after the addition of carboxyatractyloside (CAT). In the presence of Ca^{2+}, EtBr induced a fast and large amplitude osmotic swelling and the efflux of cytochrome c. In turn, Ca^{2+} induced the release of accumulated EtBr, through a mechanism inhibited by CSA and OG. In addition, accumulation of the dye was inhibited in a non-competitive fashion by mersalyl. Another important finding was that EtBr inhibited the ADP exchange reaction and increased the binding of eosin-5-maleimide (EMA) to adenine nucleotide translocase (ANT), suggesting that the dye interacts with the carrier to exert its effects.

MATERIALS AND METHODS

Mitochondria from the kidney cortex of Wistar rats was prepared by homogenizing the tissue in 0.25 M sucrose-1 mM EDTA adjusted to pH 7.3, and following the standard centrifugation pattern. Protein content was determined according to Lowry *et al*. (*9*). Mitochondrial Ca^{2+} movements were followed spectrophotometrically at 675–685 nm, using the indicator Arsenazo III. Swelling of mitochondria was analysed at 540 nm. Accumulation of EtBr into the mitochondrial matrix was determined fluorometrically at 530 nm excitation-590 nm emission after incubation of mitochondria, during 8 min, in 3 ml of the medium. After the incubation time, the mixture was centrifuged and the pellet was dissolved with 0.2 ml sodium dodecyl sulphate, and 2 ml distilled water was added. The concentration of EtBr was obtained from a standard calibration curve. ADP exchange reaction was carried out by incubating 1 mg of mitochondrial protein in 1 ml of the standard incubation medium during 5 min; immediately, 0.2 ml of the mixture was withdrawn and filtered through a 0.45-μm pore size filter. The radioactivity contained in the filter was measured in a scintillation counter. Fluorescent labelling of ANTwas carried out

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by incubating mitochondria during 30 min in the presence of 20 nmol EMA per milligram of protein, according to Majima *et al*. (*10*). Cytochrome c was determined according to Correa *et al*. (*11*). Briefly, 2 mg of mitochondrial protein was added to 3 ml of a basic medium containing 30 μM EtBr; after 8 min of incubation, 1.5 mg of mitochondrial protein was withdrawn and the samples were centrifuged for 10 min at 18,000*g*, the supernatants were precipitated with trichloroacetic acid, and the pellets were washed once. Protein (15 μg) was loaded onto 15% acrylamide SDS–PAGE gels and transferred to a PVDF membrane for immunodetection. Cytochrome c content in mitochondria was evaluated using a primary monoclonal antibody against cytochrome c (1 : 1,000 dilution) and an alkaline phosphate-conjugated secondary antibody was used. Mitochondrial oxygen consumption was followed polarographically using a Clark type electrode. The standard incubation medium contained 125 mM KCl, 10 mM succinate, 3 mM phosphate, 10 mM HEPES at pH 7.3, 100 μM ADP, 5 μg rotenone and 2 μg oligomycin.

RESULTS

Figure 1 examines the effect of EtBr on mitochondrial Ca^{2+} content. In (A), it is observed that an increase in the concentration of EtBr, from 20 to 50 μM, corresponds to an increase in the rate of mitochondrial Ca^{2+} release. It should be noted that, after the addition of 10 μM EtBr, Ca^{2+} still remained within the matrix. Control trace (C) indicating Ca^{2+} uptake, without addition of EtBr is shown. The experiment shown in (B) was performed to know if EtBr-induced Ca^{2+} release occurred through the opening of the permeability transition pore (PTP). As observed, the addition of CSA, a specific inhibitor of pore opening, to the mixture completely abolished the Ca^{2+} release reaction. The result depicted in (C) seems to reinforce the assumption that EtBr opens the non-specific pore since, as observed, increasing concentrations of OG, which has been reported to inhibit such a reaction (*12*), circumvent the effect of EtBr. In (D), trace a, it is shown that CAT, a well-known inducer of PTP (*13*, *14*), at the concentration of 0.2 μM was unable to promote Ca^{2+} efflux. Interestingly, however, the addition of 10 μM EtBr, a concentration unable to open the pore, induced PTP opening and, therefore, Ca^{2+} efflux took place (trace b).

Permeability transition can also be followed through mitochondrial swelling. In this regard, Fig. 2 shows the modifications in the volume of mitochondria treated with EtBr. Figure 2A, trace a, shows that, in the absence of EtBr, the addition of Ca^{2+} did not induce mitochondrial swelling. In turn, trace b illustrates that in the absence of Ca^{2+}, EtBr, *per se*, was unable to increase mitochondrial volume. In contrast, trace c indicates that, in the presence of EtBr, after the addition of Ca^{2+}, mitochondria undergo a fast and large amplitude swelling. Figure 2B, trace a, shows that the swelling, as induced by EtBr–Ca^{2+}, was considerably inhibited when CSA was present in the incubation medium. Trace b shows that OG partially inhibited the EtBr–Ca^{2+} induced mitochondrial volume increase.

As known from previous reports, EtBr is accumulated in the mitochondrial matrix depending on the internal negative membrane potential (*1*, *15*). Table 1 indicates that, indeed, control mitochondria, using oxidizing succinate as the substrate, accumulated 43.02 ± 1.02 nmol/mg protein of EtBr. However, the addition of Ca^{2+} restrained this accumulation by ~80%, *i.e*. to 10.97 ± 2.98 nmol/mg. As observed, CSA and OG, to some extent, avoided the effect of Ca^{2+}. An interesting finding was the fact that Sr^{2+} did not inhibit EtBr accumulation. Next, the question emerged about the pathway followed by EtBr to get access into the mitochondrial matrix. To answer such a question, uptake of EtBr was assayed in the presence of transport inhibitors, such as mersalyl, *N*-ethylmaleimide (NEM) and CAT. Interestingly, the uptake of EtBr was inhibited by ~50% with mersalyl, which is a reagent for thiols of membrane surface; in contrast, the hydrophobic thiol reagent, NEM, did not inhibit it. Similarly CAT, a specific inhibitor for the adenine nucleotide carrier failed to inhibit EtBr transport. Results also showed that phosphate (Pi) was not required for the accumulation of EtBr.

As a consequence of the latter result, the following questions emerged: Is the uptake of EtBr inhibited by the presence of Ca^{2+}? Or well, did the uptake of Ca^{2+} induce the release of EtBr? To answer these questions, an experiment analysing time-dependent kinetics of EtBr uptake, with and without Ca^{2+}, was performed. Figure 3A shows that the maximal amount of EtBr accumulation, regardless of the addition of Ca^{2+}, was attained at 2 min. Nevertheless, as shown, EtBr started to be released after two more minutes from those mitochondria incubated in Ca^{2+}-containing medium; whereas, EtBr remained fully accumulated in mitochondria incubated in Ca^{2+}-free medium even after 10 min incubation. Now the question arises about which ion is released at first instance, *i.e*. EtBr or Ca^{2+}? To answer this, the experiment shown in Fig. 3B was performed. As observed, 2 min after the addition of EtBr, accumulated Ca^{2+} started to be released. These results indicate that EtBr and Ca^{2+} were released at the same time.

The fact that CAT turns mitochondria more sensitive to the effect of EtBr on the opening of the non-specific pore, prompted us to explore the possibility of an interaction between the ANT and EtBr, in such a way that the ADP-exchange reaction would result inhibited. Certainly, the reaction was inhibited by ~50%, since control mitochondria exchanged 6.7 ± 0.4 nmol ADP/mg, whereas EtBr-containing mitochondria exchanged 3.0 ± 0.6 nmol ADP/mg. This would point out an interaction between the carrier and the dye. The latter results appear to be mirrored with those found when the effect of EtBr on respiratory control (RC) was analysed, *i.e*. RC was diminished from 3.0 in control mitochondria to 2.0 in EtBr-treated mitochondria.

Considering that mersalyl inhibited the accumulation of EtBr, it was decided to explore the inhibition kinetics by using different concentrations of EtBr, as well as mersalyl. Figure 4 shows that the control Lineweaver–Burk plot revealed a V_{max} of 100 nmol EtBr taken up per 8 min/mg protein, with a Michaelis constant of 50 nmol/mg. In the presence of 10 μM mersalyl, V_{max} was reduced to 76.9 nmol EtBr taken up per 8 min/mg,

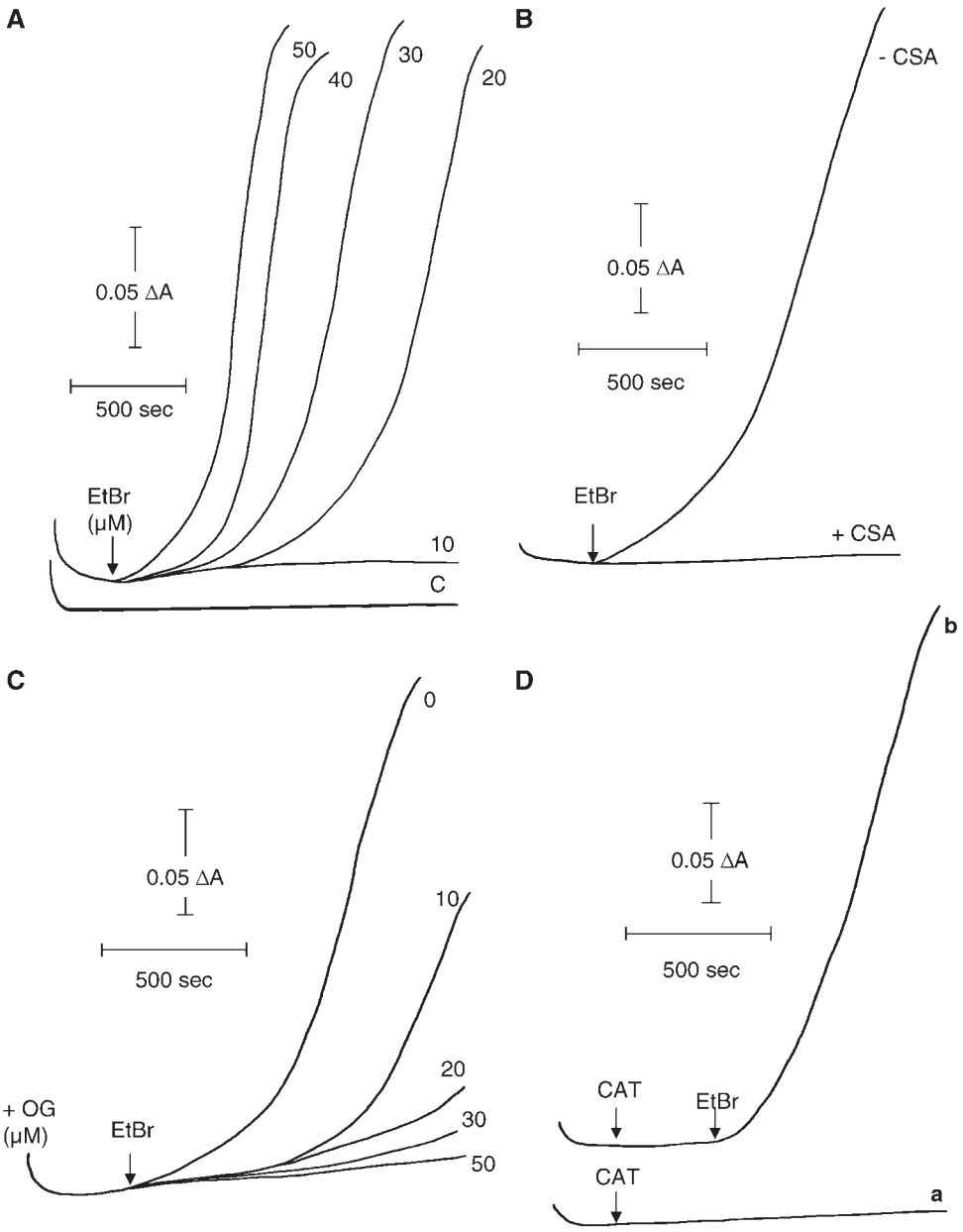

Fig. 1. **Effect of increasing concentrations of ethidium bromide on mitochondrial Ca^{2+} content.** Mitochondrial protein (2 mg) was added to 3 ml of the incubation mixture, as described under MATERIALS AND METHODS section. (A) The indicated concentrations of EtBr were added. In control, trace (C), EtBr was not added. Where indicated in (B), in addition to 30 μM EtBr, 0.5 μM CSA was added. As indicated in (C), the medium contained the indicated concentrations of OG, and 30 μM EtBr. In (D), additions were, in trace a, 0.2 μM Cat, and in trace b, 0.2 μM CAT and 10 μM EtBr. Temperature 25°C.

and, in the presence of 20 μM mersalyl, V_{max} was reduced to 50 nmol per 8 min/mg. Since the K_m did not change after adding mersalyl, the conclusion was that the thiol reagent behaves as a classical non-competitive inhibitor.

The experiment shown in Fig. 5 was aimed at determining the amount of –SH groups, titrated by mersalyl, essential to inhibit EtBr accumulation into the matrix. As illustrated, blockage of nearly 15 nmol thiol groups per milligram of protein suffices to inhibit by ~50% the uptake of EtBr.

At this stage of the experimental work, the possible interaction of EtBr with ANT was further explored. For this purpose, the binding of the fluorescent marker, EMA, to ANT was analysed. As depicted in Fig. 6, lane 4, by incubating mitochondria with EtBr plus Ca^{2+}, labelling of ANT by the fluorescent probe was increased; as shown in lane 5 such a reaction was partially inhibited after the addition of CSA. Plausibly, EtBr promoted a configurational change in ANT exposing buried thiol groups that resulted more accessible for EMA. It is worthwhile to mention that additional bands labelled with the fluorescent probe come into view. To explain the above, it should be taken into account that the amount of fast reacting –SH groups titrated with Ag

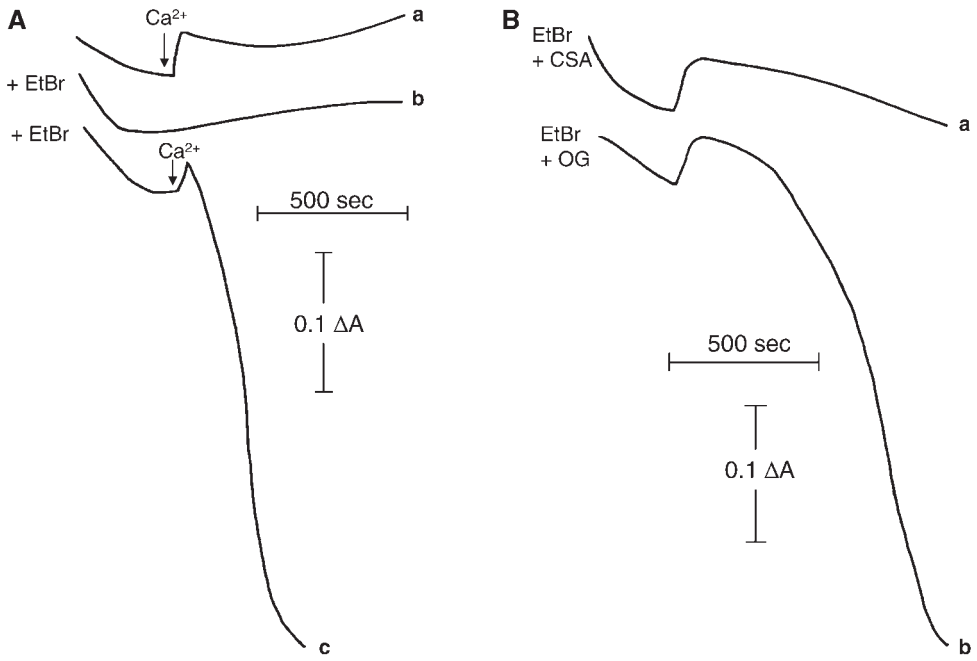

Fig. 2. **Effect of ethidium bromide on mitochondrial swelling.** (A) trace a, 50 μM Ca^{2+} was added; in trace b, the medium contained 30 μM EtBr; in trace c, the medium contained 30 μM ethidium bromide and, as indicated, 50 μM Ca^{2+} was added. (B) trace a, in addition to 30 μM EtBr, the medium contained 0.5 μM CSA. In trace b, the medium contained 50 μM OG and 30 μM EtBr; where indicated, 50 μM Ca^{2+} was added; 2 mg of mitochondrial protein was incubated in 3 ml of the basic medium. Temperature 25°C.

Table 1. **Ethidium bromide uptake under different incubation conditions.**

Additions	(nmol/mg)
Control	43.02 ± 1.02
50 μM Ca^{2+}	10.97 ± 2.98
Ca^{2+} + 0.5 μM CSA	28.45 ± 1.29
Ca^{2+} ± 50 μM OG	25.2 ± 1.35
50 μM Sr^{2+}	36.95 ± 1.75
30 μM mersalyl	20.12 ± 3.93
30 μM NEM	41.77 ± 2.05
5 μM CAT	40.27 ± 1.36
−Pi	41.95 ± 2.47

Mitochondiral protein (2 mg) was incubated in 3 ml of the basic medium, as described under MATERIALS AND METHODS section. In addition, the mixtures contained 30 μM EtBr and the indicated additions were made. After 8 min of incubation time, the mixtures were centrifuged and EtBr was determined in the pellets, as described. The results represent the average of four different experiments ± SD. ANOVA Newman–Kuels test was used for the statistical analysis (Prism 4.0 software).

and *p*-chloromercuryphenylsulphonate are in the order of 10–20 nmol/mg (*16*). Thus, there are various membrane proteins susceptible to be labelled with EMA. Nevertheless, there is a major labelled band at 30 kDa corresponding to the position of ANT. This fluorescent profile is similar to that reported by McStay *et al*. (*17*).

Then, we examined the release of the proapoptotic protein, cytochrome c, from mitochondria in response to the effect of EtBr. Figure 7 shows that, indeed, the mutagen induced the release of cytochrome c. It should be noted that Ca^{2+} was required for the EtBr-induced cytochrome c release (lane 4). The inhibition by CSA (lane 5) clearly indicates that the release of the respiratory chain protein was caused by the induction of permeability transition by EtBr.

DISCUSSION

Mitochondria undergo a highly non-specific permeability characterized by the release of matrix molecules weighing up to 1,500 Da. This process, named permeability transition, requires matrix Ca^{2+} overload, as well as an inducer agent (*18*). The results presented in this study clearly demonstrate that the mitochondrial genotoxic EtBr behaves as an inducer of permeability transition, *i.e*. it induces the discharge of accumulated matrix Ca^{2+}, promotes mitochondrial swelling, and cytochrome c release. Certainly, it has been previously reported that EtBr acts as inhibitor (*19*, *20*), as well as uncoupler (*6*), of some mitochondrial functions. Therefore, through an uncoupling mechanism, EtBr would induce the described results. However, the data presented in this work provide evidence that our findings were not due to an uncoupling effect of the dye. First of all, EtBr-induced membrane leakage was inhibited by CSA, a specific inhibitor of permeability transition (*21*) and, second, according to the current hypothesis adduced for the opening of the non-specific pore, matrix Ca^{2+} load was required. It is important to mention that CSA

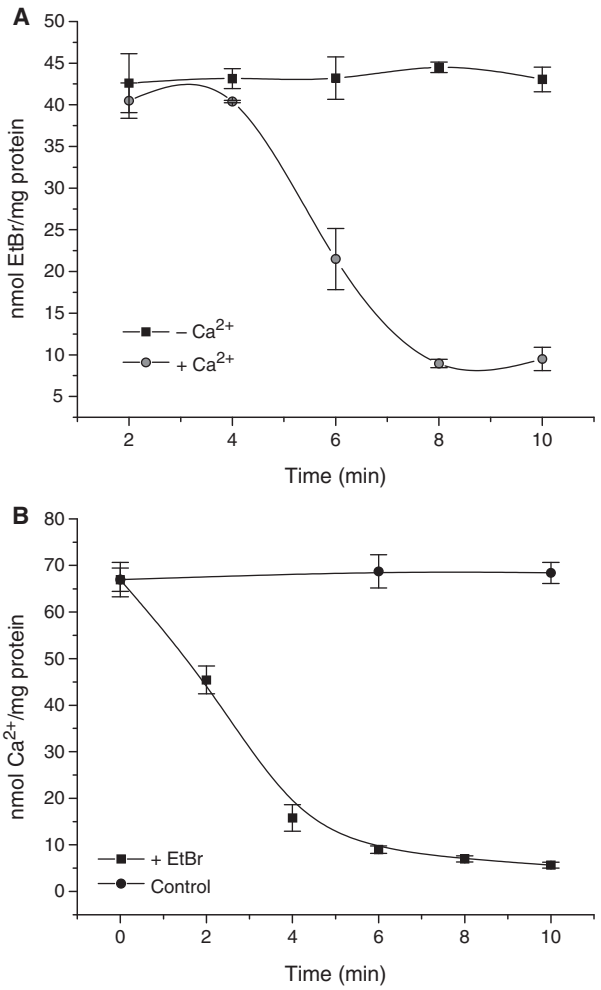

Fig. 3. **Induction of ethidium bromide release by Ca^{2+}, (A) induction of Ca^{2+} release by EtBr, (B).** Experimental conditions as described for Figure 1. In (A) 2 mg of mitochondrial protein was incubated in the presence of 30 μM EtBr and 50 μM Ca^{2+}; at the indicated times, the mixtures were centrifuged at 10,000 r.p.m., and the amount of EtBr was determined in the pellet as described under MATERIALS AND METHODS section. In (B) 2 mg mitochondrial protein was preincubated during 5 min in mixtures containing 50 μM $^{45}Ca^{2+}$ (specific activity, 3,775 c.p.m./nmol), and as indicated, at zero time, 30 μM EtBr was added. At the indicated times, aliquots of 0.2 ml were withdrawn and filtered through a 0.45 μm filter. The radioactivity contained in the filter was estimated. The values represent the average of six different experiments, the bars correspond to SD. ANOVA Newman–Kuels was used for statistical analysis.

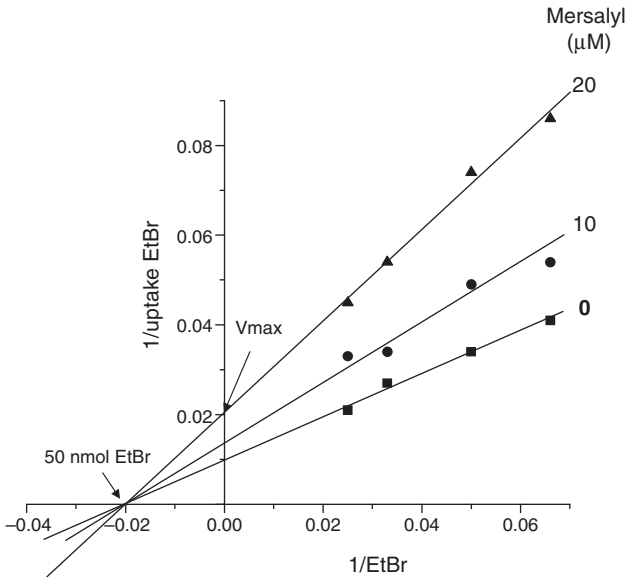

Fig. 4. **Lineweaver–Burk plot of the inhibition of EtBr uptake by mersalyl.** Mitochondiral protein (2 mg) was incubated in 3 ml of a basic medium as described for Figure 1, and in the presence of 10, 15, 20, 30 and 40 μM EtBr. In trace a, mersalyl was not added; in trace b, the medium contained 10 μM mersalyl, and, in trace c, mersalyl was added at the concentration of 20 μM. After 8 min incubation, the mixtures were centrifuged and the amount of ethidium bromide was estimated as described under MATERIALS AND METHODS section. The values represent the average of three different experiments.

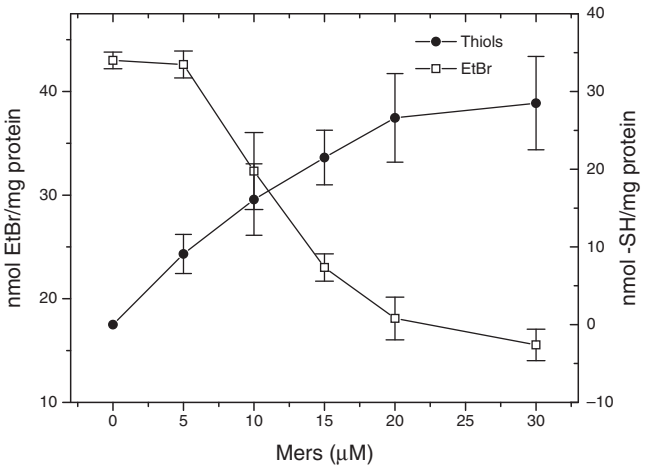

Fig. 5. **Relationship between the titrated membrane sulphydryl groups by mersalyl and the inhibition of EtBr uptake.** Mitochondrial protein (2 mg) was incubated during 8 min in 3 ml of the basic medium containing 30 μM EtBr and the indicated concentrations of mersalyl. After the incubation time, the mixtures were centrifuged to determine matrix EtBr, as described in MATERIALS AND METHODS section. In parallel, after the incubation time, 100 μM DTNB was added to the incubation mixtures and, after centrifugation, the free –SH groups titrated by DTNB were estimated at 412 nm; a standard cysteine curve was used. The values represent the average of six different experiments ± SD ANOVA Newman–Kuels test, using Prism 4.0 software, was used for the statistical analysis.

does not inhibit the effect of classical uncouplers and that Ca^{2+} load is not a requirement for the action of uncouplers.

Regarding the characteristics of EtBr accumulation, previous reports indicate that EtBr is accumulated into the matrix through an energy-dependent mechanism (*1*). This is in agreement with our results, indicating that addition of Ca^{2+} induces release of the dye. This would mean that the energy used to accumulate EtBr may, in turn, be used for Ca^{2+} accumulation; thus EtBr efflux must take place. However, taking into account that this

process was inhibited by CSA, this reaction should be considered as the inducer of pore opening. To add further evidence to the above, the finding indicating that EtBr was accumulated regardless of the addition of strontium, a cation whose accumulation is also energy dependent but that it fails to induce pore opening, rather than inhibiting, must be kept in mind (*22*).

In spite of the widespread use of EtBr as mutagen of mitochondrial DNA (*23*), to our knowledge, not enough attention has been devoted to know the mechanism by which this hydrophobic cation is transported across the mitochondrial membrane to reach DNA. This important issue was solved by the results of this work, mainly regarding the membrane pathway that might be followed by EtBr. As demonstrated, titration of thiol groups by mersalyl inhibited the uptake of EtBr. In fact, a titration of ~15 nmol thiols/mg was required to inhibit 50% of the uptake of the dye. Remarkably, these titrated thiols must be located on the membrane surface, since the hydrophobic thiol reagent, NEM, even at the concentration of 30 μM, failed to inhibit the transport of EtBr.

It is worthwhile to recognize that the chemical nature of the molecule implicated in the delivery of EtBr through the inner membrane into the matrix is far from being established in this work. However, it can be proposed that EtBr would be taken up by mitochondria in a selective way by a similar transport membrane system that seems to exist in plasma membrane of yeasts for potassium and EtBr (*24*, *25*). To reinforce such a proposal, previous reports indicating an interaction between thiol reagents and mitochondrial K^+ transport system (*26–28*) can be taken into account.

About the mechanism involved in the EtBr-induced permeability transition, it is tempting to assume that it

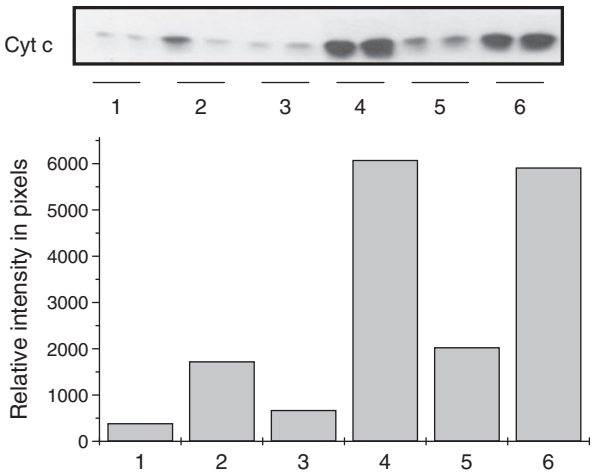

Fig. 7. **Cytochrome *c* release from EtBr-treated mitochondria.** Experimental conditions as described in MATERIALS AND METHODS section. Control mitochondria are shown in lane 1, the incubation mixtures contained, in lane 2, 30 μM EtBr; in lane 3, 50 μM Ca^{2+} was added; in lane 4, the medium contained 30 μM EtBr plus 50 μM Ca^{2+}; in lane 5, in addition to Ca^{2+} and EtBr, 0.5 μM CSA was added, and in lane 6, the medium contained Ca^{2+}, EtBr and 50 μM OG

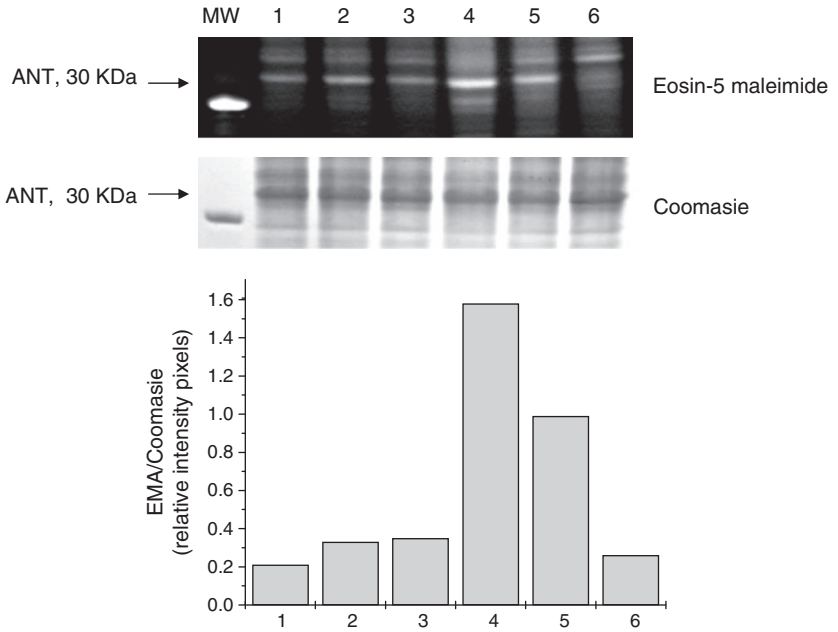

Fig. 6. **The increased labelling, by EMA, of the ADP/ATP carrier as induced by EtBr.** Mitochondrial protein (2 mg) was suspended in 3 ml of the basic medium. The additions were as follows: in lane 2, 50 μM Ca^{2+}; in lane 3, 30 μM EtBr plus 20 μM EGTA; in lane 4, 50 μM Ca^{2+} and 30 μM EtBr; in lane 5, 50 μM Ca^{2+}, 0.5 μM CSA, and 30 μM EtBr; in lane 6, 0.2 μM CAT, 30 μM EtBr and 20 μM EGTA; to all samples 20 nmol/mg of EMA was added. (A) shows the fluorescence profile. Control is shown in lane 1. The mixtures were incubated during 30 min at 4°C in the dark, and the reaction was stopped by adding 30 mM DTT; 300 μg protein were then subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis in 10% polyacrylamide under non-reducing conditions, and the fluorescence intensity was estimated with an UV lamp. (B) Shows the profile of ANT stained with Coomasie blue. (C) Illustrates the relative intensity of the fluorescence profile in pixels.

could be exerted through an interaction with the ANT. Our proposal on the interaction between EtBr and ANT would be supported on three facts: (i) EtBr inhibited the ADP exchange reaction, (ii) CAT stimulated the effect of EtBr and (iii) EtBr increased the binding of EMA to ADP/ATP. Regarding the binding of EMA to the ANT carrier, Majima *et al*. (*10*) discuss that the fluorescent probe labels Cys^{159} very fast, Cys^{56} slowly, Cys^{256} very slowly and does not label Cys^{128}. Considering the above, we can speculate that the increased labelling, as induced by EtBr, would be caused by an EtBr-induced configurational change of the carrier, in such a way that Cys^{256} would be exposed to the probe. We are aware that, recently, some doubts have arisen about the central role of ANT as constituent of the pore (*29*); however, it appears that the consensus indicates that ANT may play a role as regulator of pore opening (*30*, *31*). Finally, we would like to add that, taking into account that EtBr promotes the release of cytochrome c, this reagent would be a potential inducer of apoptosis. This should be kept in mind since the dye is frequently used to induce mutations in mitochondrial DNA, and the survival of the cell must be preserved to be successful (*32*).

CONFLICT OF INTEREST

None declared.

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