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Inactivation of Mitochondrial Permeability Transition Pore by Octylguanidine and Octylamine

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Mitochondrial permeability transition occurs through a Ca^{2+}-dependent opening of a transmembrane pore, whose identity has been attributed to that of the adenine nucleotide translocase (ANT). In this work, we induced permeability transition by adding 0.5 μM carboxyatractyloside. The process was evaluated analyzing Ca^{2+} efflux, a drop in transmembrane electric gradient, and swelling. We found that the amphiphyllic cations octylguanidine and octylamine, at the concentration of 100 μM, inhibited, almost completely, nonspecific membrane permeability. Hexylguanidine, hexylamine, as well as guanidine chloride and hydroxylamine failed to do so. The inhibition was reversed after the addition of 40 mM Li^+, Na^+ K^+, Rb^+, or Cs^+; K^+ was the most effective. We propose that the positive charge of the amines interact with negative charges of membrane proteins, more likely the ADP/ATP carrier, while the alkyl chain penetrates into the hydrophobic milieu of the inner membrane, fixing the reagent.

KEY WORDS: Mitochondria; octylguanidine; octylamine; carboxyatractyloside; permeability transition, kidney mitochondria; nonspecific pore; calcium; mitochondrial calcium; mitochondrial membrane.

INTRODUCTION

Permeability transition occurs through a nonspecific inner membrane pore whose chemical structure has not, as yet, been well determined. Certainly, a general consensus ascribes to adenine nucleotide translocase (ANT) the ability to convert itself into the Ca^{2+}-induced transmembrane pore (LêQuôc and LêQuôc, 1989; Novgorodov *et al.*, 1990; Tikhonova *et al.*, 1990; Brustovetsky and Klingenberg, 1996; Rück *et al.*, 1998; Zazueta *et al.*, 1998). However, the question about the electrical environment prevailing in the aqueous channel has been ignored. Indeed, it is accepted that adenine nucleotide transport, as well as the binding of the inhibitors require at least three positive charges, located in the protein system (Klingenberg, 1985; Majima *et al.*, 1994). Nevertheless, since that the pore permits the efflux of matrix Ca^{2+} (Gunter and Pfeiffer, 1990; Zoratti and Szabò, 1995), the possibility arises that it may also contain a negative environment. This assumption appears to be true, considering the fact that polyamines, such as spermine and spermidine, among other amines, block the nonspecific pore (Tassani *et al.*, 1995; Rustenbeck *et al.*, 1998a). Conceivably, the efficiency of polyamines to inhibit the increased permeability must depend on both, the positive charges, as well as on the methylene carbon chain.

In another context, there are several previous reports on the interaction between negatively charged membrane sites and alkyl guanidines. An early work by Gómez-Puyou *et al.* (1977), indicated that octylguanidine inhibited electron transport on site I of the respiratory chain, through its binding to a negative

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membrane site. Additional information about the effects of guanidines on mitochondria was supplied by Beaty *et al.* (1986), who reported that octylguanidine inhibits mitochondrial K^+ influx. Considering the above, this work was designed to obtain further insight into the ionic structure of the pore and to establish the role of the membrane lipid milieu on the inhibition by alkyl amines. Hence, we studied the effect of alkyl cations, namely, hexyl- and octylguanidine and hexyl- and octylamine on permeability transition. Our results indicate that the amphiphyllic cations octylguanidine and octylamine inhibit permeability transition induced by 0.5 μM carboxyatractyloside (CAT). This inhibition was reversed by monovalent cations with the specificity: $K^+ > Rb^+ > Cs^+ = Li^+ = Na^+$. A noteworthy observation was that hexylguanidine and hexylamine failed to inhibit permeability transition. We propose that the positive bulk of octylguanidine and octylamine is bound on the cytosol side of the pore, being retained through the solubilization of the alkyl chain in the lipid phase of the membrane.

MATERALS AND METHODS

Octyl- and hexylguanidine compounds were synthesized by a member of our team, according to a previously reported method (Phillips and Clarke, 1923). Hexylamine and octylamine were obtained from Sigma-Aldrich Co. Kidney cortex mitochondria were prepared by centrifugation of the tissue homogenized in 0.25 M sucrose—1 mM EDTA, pH 7.3. Protein was measured by the Lowry method (1951). Calcium movement was determined spectrophotometrically at 675–685 nm using the indicator arsenazo III (Scarpa *et al.*, 1978). Alternatively, matrix Ca^{2+} content was measured using $^{45}CaCl_2$ (sp. act. 700 cpm/nmol) under the conditions described in the Results section. Transmembrane potential was monitored at 524–554 nm by using the dye safranine (Akerman and Wikström, 1976). Mitochondrial swelling was followed by changes in the absorbency at 540 nm. The basic medium (3 ml), adjusted to pH 7.3 with Tris, contained 250 mM sucrose, 5 mM KCl, 10 mM succinate, 4 mM phosphate, 10 mM HEPES, 50 μM $CaCl_2$, 200 μM ADP, 5 μg rotenone, and 2 μg oligomycin. ADP exchange reaction was assayed by incubating 1 mg of mitochondrial protein in 1 ml of 125 mM KCl, 10 mM HEPES, pH 7.3, 5 μg rotenone, 5 μg oligomycin, 20 nmol [^{3}H]ADP (sp. act. 800 cpm/nmol), and increasing concentrations of octylguanidine or octylamine, from 50 to 150 μM each. Fluorescence polarization was measured in mitochondria as described elsewhere (Chávez *et al.*, 1996).

RESULTS

Several reports indicate that carboxyatractyloside induces membrane permeability transition that is characterized, among other variables, by matrix Ca^{2+} efflux. (Chávez *et al.*, 1991, 1998). Figure 1A shows that the addition of 100 μM octylamine (OA) abolished membrane leakage as induced by 0.5 μM CAT. Notably, hexylamine (HA) was unable to inhibit Ca^{2+} release. Figure 1B shows that, similarly to OA, 100 μM octylguanidine (OG) inhibits matrix Ca^{2+} depletion, initiated after the addition of 0.5 μM CAT. It should be noted that, as occurred with hexylamine, hexylguanidine (HA) failed to inhibit pore opening. Guanidine or hydroxylamine, which lack an alkyl chain, did not have any inhibitory effect (not shown). Significantly, when OG and OA were added to the incubation medium, previous to the addition of mitochondria, they did not inhibit Ca^{2+} uptake (not shown). This finding points out that alkyl amines accomplish the closure of the nonspecific pore, without inhibiting the calcium uniporter.

Figure 2 summarizes experiments performed to study the concentration dependence of OG and OA on CAT-induced Ca^{2+} efflux. As observed, octylguanidine at low concentrations: 10–40 μM appears to be more effective than octylamine. Although, as indicated, both reagents reach their maximum effect, *i.e.*, 85% inhibition, with 100 μM. It is important to mention that higher concentrations, such as 200 μM, of OG or OA, did not increase the inhibition (not shown).

Mitochondria undergo a rapid permeability transition when loaded with Ca^{2+} in the absence of ADP (Hunter and Haworth, 1979; Chávez and Jay, 1987; Halestrap *et al.*, 1997). In agreement, Fig. 3 shows that kidney mitochondria were unable to retain Ca^{2+} when incubated in an ADP-free medium. Interestingly, Ca^{2+} was kept inside mitochondria when the medium was supplemented with 100 μM octylguanidine or octylamine. This finding is similar to that reported by Lapidus and Sokolove (1994) indicating that the polyamine spermine partially inhibits permeability transition in ADP-depleted mitochondria.

Gómez-Puyou et al. (1977) demonstrated that K^+ competes with octylguanidine, releasing the inhibition of this reagent on site I of the respiratory chain. Mayan

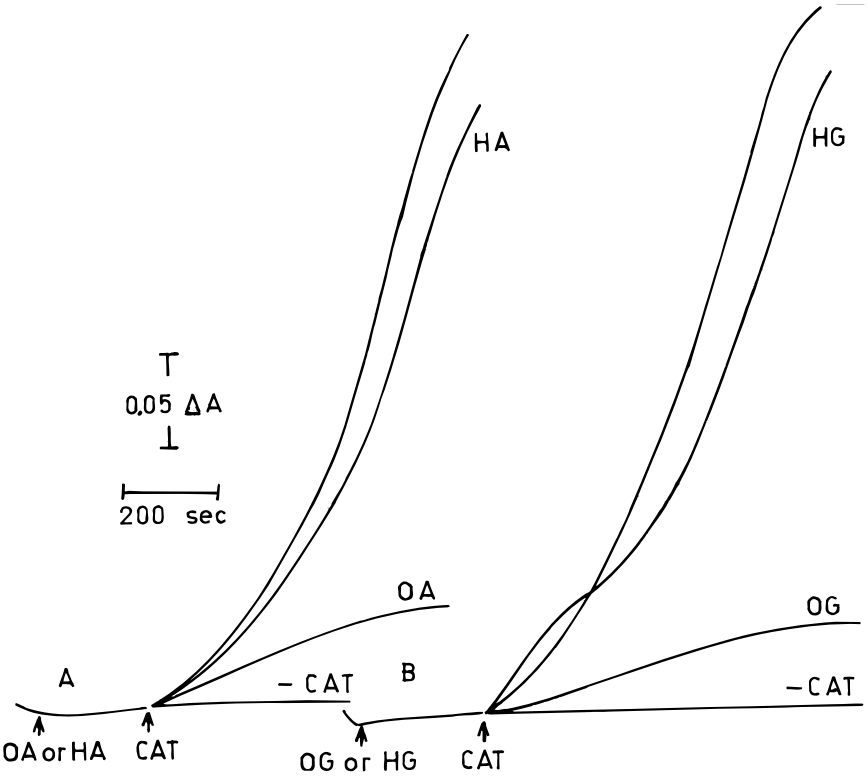

Fig. 1. The effect of alkyl amines on Ca^{2+} efflux as induced by carboxyatractyloside. Mitochondrial protein (2 mg) were added to 3 ml of medium described in Section on Materials and Methods. Where indicated, 0.5 μM carboxyatractyloside (CAT) and 100 μM each octylamine (OA), hexylamine (HA), octylguanidine (OG), and hexylguanidine (HG) were added. Temperature 23°C

et al. (1992) have shown that various alkyl- and arylguanidinium derivatives act as competitors of Na^+ or Rb^+ for the cation sites on kidney Na^+, K^+-ATPase. Considering the latter, we examined the ability of Li^+, Na^+, K^+, Rb^+, and Cs^+ to revert the inhibition of octylamine on CAT-induced permeability transition. Table I shows that all the monovalent cations examined reversed the inhibition. Remarkably, however, K^+ appears to be the most effective.

Inhibition of the increased permeability to Ca^{2+} efflux runs in parallel with an inhibition of mitochondrial swelling. However, we found that, notwithstanding the 85% inhibition by of OG on Ca^{2+} release, mitochondrial swelling was inhibited by about 50% (not shown). This atypical response is similar to that observed by Beatrice *et al.* (1980). These authors showed that addition of oxaloacetic acid initiates a fast osmotic swelling, while Ca^{2+} remains inside mitochondria.

The efficiency of OG and OA to maintain the nonspecific pore closed was also explored by analyzing $\Delta\Psi$. We found that OG and OA completely inhibited membrane depolarization caused by 0.5 μM CAT. Furthermore, after the addition of 0.166 μM ruthenium red, OG was able to partially restore the transmembrane electric gradient, as does cyclosporin A (Petronilli *et al.*, 1994; Novgorodov *et al.*, 1994; Kushnareva *et al.*, 1995) (not shown).

At this stage of the experimental results, we decided to explore the possibility that OG and OA might modify membrane fluidity. It was found that the reagents do not influence such a membrane property, *i.e.*, the order parameter values were 0.231, 0.225, and 0.223, in controls, plus 100 μM OG and plus 100 μM OA, respectively.

DISCUSSION

In this study we present results indicating that the amphiphyllic cations octylguanidine and octylamine hinder the opening of the nonspecific transmembrane

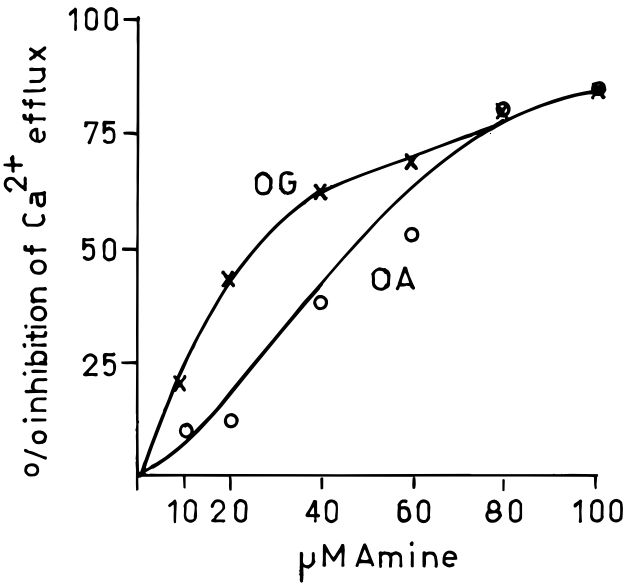

Fig. 2. Inhibition of permeability transition by increasing concentrations of octylguanidine and octylamine. Experimental conditions similar to those described for Fig. 1, except for the indicated concentrations of the amines that were added.

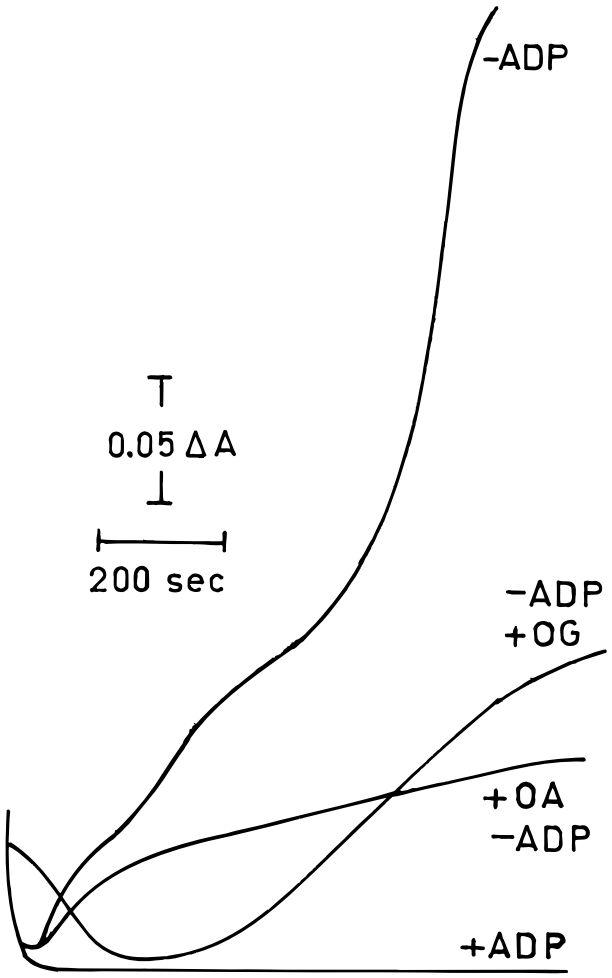

Fig. 3. Effect of octylguanidine and octylamine on mitochondrial Ca^{2+} retention in the absence of ADP. Experiments were carried out as described for Fig. 1, except that, where indicated, ADP was not added. Where indicated 100 μM octylguanidine (OG) and 100 μM octylamine (OA) were added.

pore. In previous reports the inhibition of membrane permeability transition by polycations as spermine and spermidine has been documented (Tassani *et al.*, 1995; Rustenbeck *et al.* 1998a, 1998b). In general, the authors discuss that the action of these compounds depends on the number of positive charges as well as on the nature of the alkyl chain. It is proposed that the inhibition is achieved through the binding of the cationic moiety to negative charges of membrane phospholipids (Rustenbeck *et al.*, 1998b).

Regarding the mechanism by which OG and OA block the pore, it might be by interacting, through their positively charged group, with a negative site. However, unlike that proposed for spermine and spermidine (Rustenbeck *et al.*, 1998b), our hypothesis is that the target site would be negative amino acid residues of a membrane protein. Such a protein would be the adenine nucleotide translocase. This latter notion emerges from two findings: (1) the alkyl amines block the effect of CAT on membrane leakage, and (2) OG and OA may replace ADP to allow the permanence of Ca^{2+} inside the mitochondria.

Certainly, ANT possesses 15 glutamate and 6 aspartate residues (Klingenberg, 1989). We would like to speculate that some of these charges might form a negative vestibule to accommodate the positive charge of octylguanidine or octylamine. The negative pocket must have a sufficiently large cavity to accommodate the guanidine headgroup with a maximum volume of 79.25 $Å^3$, or the amino group with a maximum volume of 23.25 $Å^3$. Considering the above, nearly three amine headgroups may be bound inside the negative pocket per one of guanidine. However, pore closure would also result from an unspecific screening of the membrane negative surface charges, as proposed by Broekemeier and Pfeiffer (1995) for the protonated form of trifluoperazine, which acts synergistically with CSA.

The observed effect of monovalent cations to release the inhibition of alkylamines would be in agreement with the proposal of Broekemeier and Pfeiffer (1995), about the screening of negative charges. Nevertheless, a cationic selectivity was demonstrated. The

Table I. Mitochondrial Ca^{2+}[a]

	Additions	nmol/mg
a.	Control	54.1
b.	CAT	4.4
c.	OA + CAT	47.1
d.	OA + CAT + Li^+	16.3
e.	OA + CAT + Na^+	15.6
f.	OA + CAT + K^+	6.8
g.	OA + CAT + Rb^+	9.4
h.	OA + CAT + Cs^+	12.1

[a] The role of different monovalent cations on the protective effect of octylamine on CAT-induced Ca^{2+} efflux. Mitochondrial protein (2 mg) was incubated in a medium similar to that described for Fig. 1, except that it contained 50 μM $^{45}CaCl_2$. Under control conditions: (a) after 12 min incubation, an aliquot of 0.2 ml was withdrawn and filtered through a 0.45-μm pore size filter. In (b), 0.5 μM CAT was added after a 3-min incubation and the aliquot was filtered at minute 12. Where indicated (c to h) the media contained 100 μM octylamine (OA), and the protocol was as follows: 3 min after the addition of mitochondria, an aliquot of 0.2 ml was filtered to estimate $^{45}Ca^{2+}$ accumulation; 3 min later, 0.5 μM CAT was added, followed, 1 min later, by the addition of 40 mM of the indicated cation. The filtration was performed after 12 min of total incubation time. The values are the average of three experiments with different mitochondrial preparations.

following order of effectiveness was found: $K^+ > Rb^+ > Cs^+ = Li^+ = Na^+$. The high effect of K^+ may possibly be due to its ability to increase free-matrix Ca^{2+} concentration activating CAT-induced membrane leakage, as previously demonstrated (Chávez *et al.*, 1991).

The importance of the hydrophobic chain is sustained by the finding that the reagents lacking it, *i.e.*, guanidine chloride and hydroxylamine failed to inhibit permeability transition. It should be noted that the longitude of an alkyl chain of 8C is 7.62 Å, while the longitude of a 6C chain is 5.72 Å. This signifies that the penetration of 5.72 Å does not suffice to maintain reagents like hexylguanidine, or amine, anchored to their target sites.

Inhibition of permeability transition was not due to a diminution in membrane fluidity, since it was found that the order parameter of fluorescence polarization did not change. Likely, these reagents do not penetrate sufficiently into the membrane to modify the fluidity.

Finally, it is important to note that alkyl amines do not displace CAT from its binding site, since these compounds fail to reverse CAT-induced inhibition of ADP exchange reaction (not shown).

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