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Original Article

Investigations of the Inhibitory Effect of Propranolol, Chlorpromazine, Quinine, and Dicyclohexylcarbodiimide on the Swelling of Yeast Mitochondria in Potassium Acetate. Evidences for Indirect Effects Mediated by the Lipid Phase

Xavier Roucou,[1] Stéphen Manon,[1] and Martine Guérin[1]

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The mode of action of propranolol, chlorpromazine, and quinine, three cationic drugs inhibiting swelling of yeast mitochondria in potassium acetate, was investigated by looking at their effect on fluorescent probes of the polar heads and of the nonpolar moiety of the membranes, under inhibitory conditions of swelling. As expected, propranolol and chlorpromazine exhibited specificity for anionic phospholipids since they increased the binding of the anionic probe 1-anilino 8-naphthalenesulfonate (ANS). Although propranolol did not release 1,6-diphenyl-1,3,5-hexatriene (DPH) from the hydrophobic moiety of the membrane, it increased the excimer/monomer fluorescence ratio of 10-(1-pyrene)decanoate, suggesting that it induced a limitation in the movements of the aliphatic chains of phospholipids. Opposite to propranolol, chlorpromazine removed DPH from the membrane, suggesting that it bound essentially to the hydrophobic moiety. However, chloramphenicol, which was also able to remove DPH but did not increase the binding of ANS, did not inhibit swelling. Inhibition by chlorpromazine therefore appeared to be related to its binding to the hydrophobic moiety of anionic phospholipids. Quinine had no effect on membrane properties: at inhibitory concentrations of swelling in potassium acetate, it did not inhibit swelling in ammonium phosphate (mediated by the phosphate/H^+ cotransporter), whereas propranolol and chlorpromazine did, suggesting a more specific effect of quinine on (a) protein(s) involved in the K^+/H^+ exchange. Dicyclohexylcarbodiimide (DCCD), which irreversibly inhibits the swelling in potassium acetate, bound to ethanolamine heads; despite this effect, DCCD had no major consequences on the binding of the probes. Consequently, propranolol and chlorpromazine are of no help for characterizing protein(s) catalyzing the K^+/H^+ exchange, although their effect on lipids seems to involve limited zones of the inner mitochondrial membrane. Quinine and DCCD, although they also bind to lipids, may inhibit the activity by acting on a limited number of proteins.

KEY WORDS: K^+/H^+ exchange; cationic drugs; dicyclohexylcarbodiimide; membrane fluidity; yeast mitochondria.

INTRODUCTION

The function of mitochondrial metabolism requires a fine regulation of the matricial volume (Halestrap, 1989; Halestrap *et al.*, 1992). The high transmembrane potential created by the respiratory chain may drive the matricial accumulation of cytoplasmic

[1] Institut de Biochimie et de Génétique Cellulaires du Centre National de la Recherche Scientifique, Université de Bordeaux II, 1 rue Camille Saint-Saëns, 33077 Bordeaux cedex, France.

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K^+; in addition, various acids are accumulated in the matrix for the needs of metabolism; the subsequent salt accumulation is expected to increase the matricial volume up to the rupture or, at least, to irreversible damages of the mitochondrial membrane. For these reasons, the existence of a K^+-exit pathway, catalyzed by an electroneutral K^+/H^+ antiporter, has been postulated in the chemio-osmotic theory (Mitchell, 1966).

Since mitochondria are nearly perfect osmometers (Chappel and Crofts, 1966), the method of isoosmotic swelling has been largely used for studying mitochondrial transports, including the putative K^+/H^+ exchange, although this activity is measured against the expected physiological direction (Dordick *et al.*, 1980; Garlid, 1980). By this method, a number of characteristics have been evidenced: (i) in mammalian mitochondria, this system is not spontaneously active but requires a depletion of divalent cations, such as Mg^{2+}, although its precise role remains unclear (Dordick *et al.*, 1980; Jung and Brierley, 1986); (ii) it is inhibited by both cytoplasmic and matricial H^+ concentrations (Beavis and Garlid, 1990); (iii) it is sensitive to dicyclohexylcarbodiimide (DCCD),[2] a hydrophobic carboxyl reagent (Martin *et al.*, 1984, 1986); (iv) it is sensitive to a number of amphiphilic amines (Nakashima and Garlid, 1982; Garlid and Nakashima, 1983; Garlid, 1988), some of them being already known to inhibit plasmalemmal systems of K^+-transport. The fact that the irreversible inhibition by DCCD could be prevented by the presence of reversible inhibitors (Mg^{2+}, amphiphilic amines) allowed the group of Garlid to identify (Martin *et al.*, 1984) and purify (Li *et al.*, 1990) an 82-kDa protein which, when reconstituted in liposomes, transported K^+ following an electroneutral mode and in a way sensitive to inhibitors of the native activity (Kakar *et al.*, 1989; Jezek *et al.*, 1990, Li *et al.*, 1990). However, at the present time, nothing is known about the structure of this protein.

The method of isoosmotic swelling in potassium acetate allows one to identify, in yeast mitochondria, an activity with characteristics very similar to those described in mammalian mitochondria (Villalobo *et al.*, 1981; Dabadie *et al.*, 1986; Manon and Guérin, 1992; Welihinda *et al.*, 1993). The main differences were that yeast mitochondria swelled spontaneously in potassium acetate and that this swelling was insensitive to Mg^{2+} but inhibited by Zn^{2+}.

[2] Abbreviations: ANS: 1-anilino 8-naphthalene sulfonate; DCCD: dicyclohexylcarbodiimide; DPH: 1,6-diphenyl-1,3,5-hexatriene; PDA: 10-(1-pyrene)decanoate.

Unfortunately, the identification in yeast of a protein similar to the 82-kDa protein from mammalian mitochondria revealed the following: the inhibition by DCCD could not be prevented by any of the reversible inhibitors nor incubation conditions we used, and all our attempts to reconstitute the activity from mitochondrial proteins, solubilized and fractionated, broke down (Roucou *et al.*, unpublished results).

In order to achieve the identification of the support of the activity, we have used the only tools we had, i.e., activity inhibitors, and have investigated thoroughly their mode of action. The results obtained with Zn^{2+} are reported elsewhere (Manon and Guérin, 1995). The present paper reports our investigations with the amphiphilic amines propranolol, chlorpromazine, quinine, and the carboxyl-reactive DCCD. It is concluded that, in agreement with previous reports about their binding to artificial and biological membranes, propranolol and chlorpromazine most probably act by the intermediate of the lipid phase. Despite the fact that they also bind to lipids, a direct effect of quinine and dicyclohexylcarboddiimide on a limited number of proteins cannot be rejected.

MATERIALS AND METHODS

The wild-type diploid *Saccharomyces cerevisiae* strain Yeast Foam was grown aerobically at 28°C in a 1% Yeast Extract (Difco), 0.12% ammonium sulfate, 0.1% potassium dihydrogen phosphate, 2% DL-lactate medium (pH 5.0) and harvested in the mid-exponential growth phase ($O.D._{550\ nm}$ = 4–5). Mitochondria were isolated from spheroplasts obtained by cytohelicase digestion according to Guérin *et al.* (1979). Mannitol and sorbitol used in isolation buffers were routinely deionized before use. Mitochondria were finally resuspended at 40–50 mg proteins/ml in a 0.6 M mannitol, 2 mM EGTA, 10 mM Tris/maleate buffer (pH 6.8) and could be frozen as small beads in liquid nitrogen, stored at −80°C for up to 3 months, and rapidly thawed without significant alterations of their osmotic properties. Protein concentration was measured by the biuret method with bovine serum albumin as a standard.

Swelling was measured at 25°C as the decrease of the apparent absorbance of a mitochondrial suspension at 520 nm in a Secomam S1000 spectrophotometer. Routinely, mitochondria were suspended at 0.5 mg/ml in a 0.3 M potassium acetate, 10 mM Tris/maleate buffer (pH 7.4) containing antimycin A (0.2–0.5 μg/mg).

The binding of cationic drugs to the polar and nonpolar moieties of the membrane was estimated by the capacity of the drugs to alter binding of the probes 1-anilino 8-naphthalene sulfonic acid (ANS) and 1,6-diphenyl-1,3,5-hexatriene (DPH) according to the general protocol reported by Joshi *et al.* (1989). In both cases, mitochondria (1 mg/ml) were suspended in 0.3 M potassium acetate, 10 mM Tris/maleate (pH 7.4) in the presence of 16.7 μM ANS or 5.4 μM DPH. Fluorescence measurements were done at 468 nm (excitation at 380 nm) for ANS and 440 nm (excitation at 368 nm) for DPH in a Kontron SPF25 spectrofluoromoter. ANS and DPH were nearly nonfluorescent in the aqueous phase and fluoresced strongly in the presence of mitochondrial membranes. When the fluorescence signal was stabilized, which took several seconds in the case of ANS but up to 20 min in the case of DPH, the different drugs were added at various concentrations and the resulting effect on the binding of the fluorescent probes was measured after the signal was stabilized again. The results are given as relative fluorescence intensity, the unity being the fluorescence intensity in the absence of any drugs. This value varied within less than 5% from one experiment to another.

The qualitative estimation of the degree of fluidity of the mitochondrial membrane was performed by measuring the fluorescence of 10-(1-pyrene) decanoic acid (PDA). This probe incorporates in the phospholipid bilayer and exists under two forms, monomeric and dimeric, having distinct maximal fluorescence wavelengths; the ratio between the two forms depends on the local concentration of the probe as well as on the rate of lateral diffusion (Galla and Sackmann, 1975; Galla and Luisetti, 1980; Galla and Hartmann, 1981). The advantage of using PDA over DPH for this type of experiment is that PDA can be used at higher concentration due to its higher water solubility; this allowed us to add it at a sufficiently high concentration to visualize a significant fluorescence signal corresponding to the dimeric form (see Results). Mitochondria were incubated as for the other probes in the presence of 11.5 or 63.5 μM PDA. Fluorescence measurements were done at 396 nm for the monomer and 470 nm for the dimer (excitation at 343 nm).

Extraction of mitochondrial lipids was done as follows: after incubation in the absence or in the presence of DCCD (see legends of figures), mitochondria were resuspended in 100 μl of water. Then 1 ml of chloroform/methanol 1/1 (v/v) was added and gentle agitation was maintained overnight. After a 10.000*g* centrifugation for 10 min, chloroform and water were added to the supernatant up to the proportion chloroform/methanol/water = 8/4/3 by vol.; after decantation, the organic phase was dried under a nitrogen stream, and the pellet was dissolved in 0.1 ml of chloroform/methanol 2/1 (v/v). Then 0.4 ml diethylether was added and the mixture was kept at −20°C for 1 h to precipitate remaining proteins. The supernatant was dried under a nitrogen stream and lipids were dissolved in 0.2 ml of chloroform/methanol 2/1 (v/v) and stored at −20°C.

Resolution and revelation of lipids were done according to Heape *et al.* (1985, 1986). Lipidic samples were spotted onto Silicagel plates (60 F254 from Merck, 10 cm × 10 cm) and developed using the mixture methyl acetate/1-propanol/ chloroform/ methanol/ KCl 0.25% (25/25/18/10/7 by vol.); 2 μg MGDG was migrated on the same plates as an internal standard. Revelation of the lipids was done by soaking the plates for several seconds in an 8% phosphoric acid, 3% cupric acetate solution, drying overnight at room temperature, and heating at 110°C for 30 min. Phospholipids were identified by comparison with commercial standards. Coloration intensity was measured with a Camag densitometer, and the lipids were quantified as compared to the coloration of MGDG spotted on the same plates.

All the drugs and probes were from Sigma/Aldrich and dissolved in water, methanol, or tetrahydrofuran depending on their solubility characteristics.

RESULTS

Correlation Between the Inhibitory Effect of Propranolol on the Swelling in Potassium Acetate and Its Binding on the Polar Moiety of the Membrane

In yeast mitochondria, in addition to the spontaneous swelling in potassium acetate, driven by the putative K^+/H^+ antiporter (Manon and Guérin, 1992; Welihinda *et al.*, 1993), propranolol was shown to inhibit a number of different ionic pathways such as a Mg^{2+}-sensitive Cl^--uniport (Manon and Guérin, 1993) and an ATP-induced anion channel (Guérin *et al.*, 1994). The same feature was observed in mammalian mitochondria where propranolol was shown to inhibit the $K^+(Na^+)/H^+$ exchange (Garlid, 1980), the Na^+/H^+ exchange (Nakashima and Garlid, 1982), the anion channel (Garlid and Beavis, 1986), and different conductances (see, e.g., Kinnally *et al.*, 1992). Such a lack

of specificity, although possibly revealing structural relationships between all these systems (see the discussion by Garlid and Beavis, 1986), was most likely due to an unspecific effect of propranolol on the membrane. Moreover, it had been shown that propranolol is able to bind to anionic phospholipids, particularly cardiolipins, of rat-liver mitochondria (Huunan-Seppälä, 1972). We therefore wondered whether the inhibitory effect of the drug on the swelling was directly related to its binding on the inner membrane.

Figure 1 reports a titration by propranolol of the spontaneous swelling of yeast mitochondria suspended in potassium acetate. Dixon representation of this inhibition (inset) allowed us to determine an IC_{50} around 130 μM (assuming a noncompetitive inhibition).

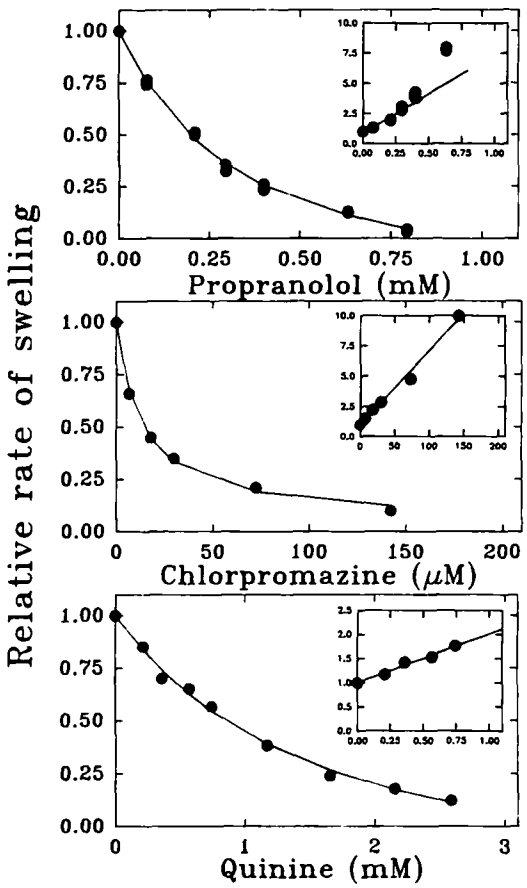

Fig. 1. Inhibition by cationic drugs of the swelling of yeast mitochondria suspended in potassium acetate. Mitochondria (0.5 mg/ml) were suspended in a 0.3 M potassium acetate, 10 mM Tris/maleate buffer (pH 7.4) in the presence of a range of propranolol, chlorpromazine, or quinine concentrations. Free concentration of the drugs was determined from their absorbance in the UV light determined in the supernatant after mitochondria centrifugation. Swelling rates were measured on the quasi linear part of the swelling curves. Insets: Dixon representation.

It had been shown on various systems that propranolol bound much better to anionic phospholipids than to neutral lipids (Huunan-Seppälä, 1972; Surewicz and Leyko, 1981). If propranolol binds to the polar heads of anionic phospholipids, it is expected to screen the charge of the membrane and to favor the binding of an anionic probe like ANS. This has already been observed on lung lamellar bodies (Joshi *et al.*, 1989), and we have observed the same property on yeast mitochondria (Fig. 2). On the other hand, no effect of propranolol on the binding of DPH was detected (Fig. 2), indicating that propranolol did not interact directly with the hydrophobic moiety of the membrane. The anionic pyrene probe PDA was used in evaluating if propranolol could have any effect on membrane dynamics. At a low probe concentration, we could detect only a low amount of the excimer form (I_e/I_m = 0.15), and propranolol slightly increased this amount (Fig. 3). The low I_e/I_m we found, opposite to, e.g., endoplasmic membranes (Kaihovaara *et al.*, 1991), may reveal the high fluidity of mitochondrial membranes. To ensure the fact that propranolol increased the I_e/I_m ratio, the amount of probe was

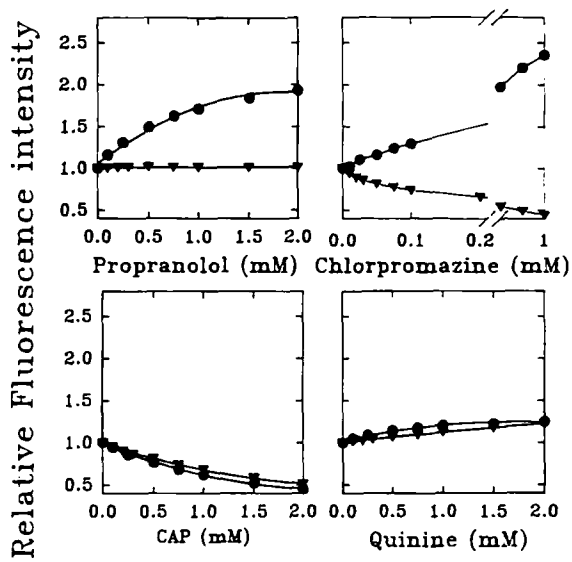

Fig. 2. Effect of cationic drugs on the binding of ANS and DPH. Mitochondria (0.5 mg/ml) were suspended in a 0.3 M potassium acetate, 10 mM Tris/maleate buffer (pH 7.4) at 25°C in the presence of 16.7 μM ANS (circles) or 5.4 μM DPH (triangles). The fluorescence intensity was measured at 468 nm (excitation at 380 nm) for ANS and 440 nm (excitation at 368 nm) for DPH. After stabilization of the signal, increments of drugs were added and the fluorescence signal was measured after 5 min (for ANS) and 20 min (for DPH). Fluorescence intensities are given as relative values, 1 being the value measured without the drug. This value did not change by more than 5% from experiment to experiment.

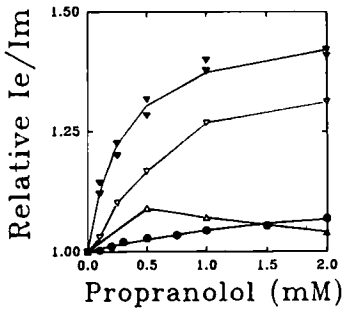

Fig. 3. Effect of propranolol on the fluorescence of PDA. Mitochondria (0.5 mg/ml) were suspended in a 0.3 M potassium acetate, 10 mM Tris/maleate buffer (pH 7.4) in the presence of 11.5 μM (circles) or 63.5 μM (triangles) PDA. Fluorescence intensities were measured at 396 nm (monomer I_m) and 470 nm (excimer, I_e) (excitation at 343 nm). The *y*-axis gives the relative I_e/I_m ratios with 1 as the ratio in the absence of propranolol. Actual values were 0.15 in the presence of 11.5 μM PDA and 0.8 in the presence of 63.5 μM PDA. Full symbols: controls without DCCD; Open triangles: mitochondria preincubated for 30 min (downward) or 2h (upward) at 4°C in the presence of 50 μg DCCD/mg.

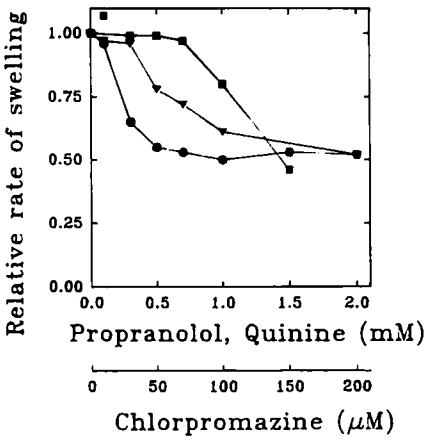

Fig. 4. Effect of cationic drugs on the swelling of mitochondria suspended in ammonium phosphate. Mitochondria (0.5 mg/ml) were suspended in a 0.3 M ammonium phosphate, 10 mM Tris/maleate buffer (pH 6.8) in the presence of the given concentrations of propranolol (circles), quinine (squares), or chlorpromazine (triangles). The *y*-axis corresponds to the initial rates of swelling.

enhanced up to a basal I_e/I_m ratio of 0.8: under these conditions, the effect of propranolol was without doubt, it markedly increased the I_e/I_m ratio, suggesting a decrease of lipid movements within the hydrophobic moiety of the membrane. From these data, it can be suggested that the inhibitory effect of propranolol, which occurred via binding on the polar moiety of anionic phospholipids, involved a rigidification of the mitochondrial membrane. This rigidifying effect may have two consequences: one on the general permeability of the membrane (e.g., to acetic acid and water), the other on the dynamics of putative intrinsic protein(s) catalyzing the exchange.

Finally, the inhibitory effect of propranolol on the swelling of mitochondria suspended in potassium acetate was compared to its effect on a well-characterized swelling, the phosphate entry via the Pi^-/H^+ cotransporter associated to the electroneutral diffusion of NH_3 (Chappel and Crofts, 1966). From data reported in Fig. 4, it can be seen that propranolol did have an inhibitory effect on this swelling within the same range of concentrations as for the swelling in potassium acetate (as compared to Fig. 1), but that the inhibition never reached more than 50%. It was checked that, under these conditions, mersalyl completely inhibited the swelling (not shown). Thus, propranolol had an inhibitory effect on transport different from the K^+/H^+ exchange (or any K^+ transport), suggesting an unspecific effect on the lipid phase, but the putative proteins(s) catalyzing the K^+/H^+ exchange appeared to be more sensitive to this unspecific effect than the phosphate carrier.

Inhibitory Effect of Phenothiazines on the Swelling in Potassium Acetate Is Correlated with Their Binding to the Hydrophobic Moiety of Anionic Phospholipids

We looked at the effect of three phenothiazines on the swelling: promazine, plus the two halogenated compounds, chlorpromazine and trifluoperazine. These three drugs had a strong inhibitory effect on the swelling (the effect of chlorpromazine is shown in Fig. 1). The IC_{50}, determined from Dixon curves, was 16 μM for chlorpromazine (inset), and within the range 15–30 μM for the other phenothiazines (not shown). They were therefore more efficient than propranolol.

Chlorpromazine had the same effect as propranolol on the binding of ANS (Fig. 2), within the same range of concentrations (EC_{50} estimated to be 600 μM), i.e., far above the inhibitory concentration, suggesting that, as for propranolol (and even more obviously), although a charge-screening effect of chlorpromazine did occur, it was not involved in the inhibitory effect. Opposite to propranolol, chlorpromazine induced a significant release of DPH (Fig. 2). Chlorpromazine removed PDA from the membrane as well as DPH (not shown); it was therefore not possible to investigate the effect of this drug on membrane dynamics.

As propranolol, chlorpromazine inhibited swelling of mitochondria suspended in ammonium phosphate within the same range of concentrations as for swelling in potassium acetate, but this inhibition did not reach more than 50% (Fig. 4).

Effect of a Noninhibitory Hydrophobic Drug: Chloramphenicol

From data obtained with chlorpromazine, we could conclude that the inhibitory effect of this drug was related to its ability for interacting with the hydrophobic moiety of the membrane. We therefore assayed other hydrophobic drugs, chosen for their similar effect to chlorpromazine on the binding of DPH. Chloramphenicol was of interest because its effect on the binding of DPH resembled that of chlorpromazine (Fig. 2). However, when assayed on swelling, chloramphenicol did not exhibit any inhibitory effect (not shown), showing that the interaction with the hydrophobic moiety was not a sufficient requirement to inhibit the swelling of mitochondria in potassium acetate. The difference can be identified by looking at the binding of ANS: not only did chloramphenicol fail to increase but it decreased the amount of ANS bound to the membrane (Fig. 2), showing that, opposite to chlorpromazine, it did not bind to anionic phospholipids. Amiodarone, at less than 50 μM, was also able to release DPH, but this drug was not an inhibitor of the swelling (not shown).

Taken together, these results suggested that the inhibitory effect of chlorpromazine was related both to its ability to bind to the hydrophobic moiety of the membrane and to its affinity for anionic phospholipids.

Effect of Quinine

Quinine, which is the first inhibitor of the K^+/H^+ exchange identified in rat-liver mitochondria (Garlid, 1980), is of special interest due to its specificity: (i) in rat-liver mitochondria, it inhibited the (unspecific) $K^+(Na^+)/H^+$ exchange but not the (specific) Na^+/H^+ exchange (Nakashima and Garlid, 1982) and (ii) in yeast mitochondria, it did not inhibit the ATP-induced anion channel (Guérin *et al.*, 1994) and only poorly inhibited the Mg^{2+}-sensitive Cl^--channel (Manon and Guérin, 1993).

The characteristics of inhibition of quinine were very close to those of propranolol (Fig. 1) except that the IC_{50} was much higher (1 mM).

We found that quinine had almost no effect on the binding of both ANS and DPH (Fig. 2) (the slight increase of fluorescence intensities was mostly due to quinine itself), showing that quinine had no general effect on the membrane. It may be hypothesized that its inhibitory effect would involve a limited number of proteins, although its primary binding occurred, as for propranolol and phenothiazines, via the lipidic phase. Because of its intrinsic fluorescence within the zone of fluorescence measurements, we could not assay the effect of quinine on the fluorescence of PDA.

Effect of DCCD on Phosphatidylethanolamine

DCCD is an efficient inhibitor of the swelling of yeast mitochondria in potassium acetate (Manon and Guérin, 1992). However, the inhibition requires higher concentrations and longer times of preincubation than in mammalian mitochondria (Welihinda *et al.*, 1993). A question arises: is DCCD, under the conditions where it inhibits the swelling in potassium acetate, acting also by the intermediate of the lipid phase?

Lipids were extracted from mitochondria treated or not with DCCD and separated by thin layer chromatography with a developer allowing the separation of each class of phospholipids (Heape *et al.*, 1986) (Fig. 5A). As compared to the control, two bands appeared having higher Rf's than any phospholipids. Amounts of the different phospholipids were estimated from the intensity of coloration of the different bands. DCCD induced a decrease of the peak corresponding to phosphatidylethanolamine but did not modify significantly those corresponding to cardiolipin and phosphatidylcholine. Figure 5B shows the evolution of the amounts of the different classes of phospholipids as a function of DCCD concentration. It appears that the decrease of the amount of PE was compensated by the increase of the two new bands. We also note the decrease of the peak containing PS and lyso-PE, which may be compensated by the increase of the band containing PI and PG, possibly containing the products of addition of DCCD on lyso-PE. Identification of the new bands was not undertaken since reaction of DCCD with primary amines is complex and gives rise to at least two kinds of products (Hassinen and Vuokila, 1993) which could correspond to the two new products we observed. Note also that the maximal amount of phosphatidyleth-

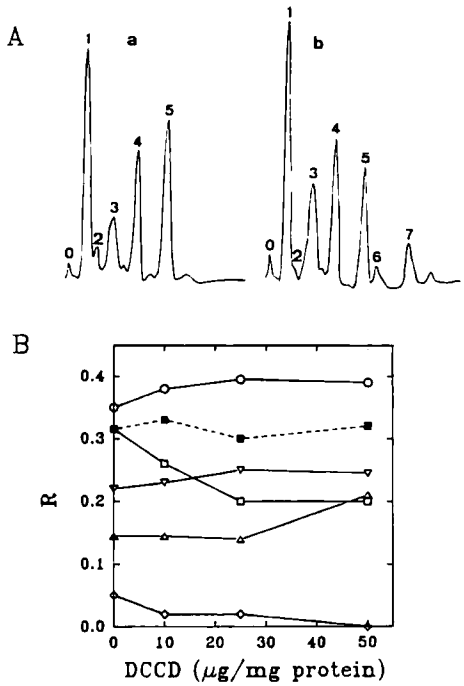

Fig. 5. Effect of DCCD on mitochondrial phospholipids. Mitochondria were preincubated with DCCD and phospholipids were extracted and separated as described in Materials and Methods. (A) Densitometric profile of lipids from (a) untreated mitochondria and (b) DCCD-treated mitochondria on HPTLC plates. DCCD migrated at the front (not shown here) under these conditions. (B) Evolution of the amount of the different bands as a function of DCCD concentration during the treatment. Circles, 1: PC; downward triangles, 4: CL; upward triangles, 3: PI + PG; diamonds, 2: PS + lysoPE; open squares, 5: PE; full squares: PE + bands 6 and 7.

anolamine bound to DCCD is around 40%, corresponding to the amount of ethanolamine heads directly accessible from the outside of the inner membrane (Krebs *et al.*, 1979).

There was no evidence that the modification of ethanolamine heads by DCCD altered the physical properties of the membrane: DCCD did not significantly change the binding of ANS and DPH, the I_e/I_m ratio of PDA, nor the effect of propranolol on the binding of ANS, nor the effect of chlorpromazine on the binding of DPH (not shown). Therefore, the dramatic effect of DCCD on PE did not alter the general properties of the membrane, such as its ability to bind amphiphilic and hydrophobic drugs and the dynamics of hydrophobic tails. On the other hand, we observed that DCCD partially or fully prevented (depending on the time of preincubation) the effect of propranolol on the I_e/I_m ratio of PDA (Fig. 3). We cannot attribute this effect of DCCD to its reaction with PE and/or with proteins but, in any case, this observation lends support to the fact that the charge-screening effect of propranolol is not the only factor responsible for its rigidifying effect.

DISCUSSION

We have investigated in this paper the specificity of the inhibitors of the mitochondrial K^+/H^+ exchange. In rat-liver mitochondria, cationic drugs inhibit a wide diversity of mitochondrial systems such as anion channels (Garlid and Beavis, 1986), K^+-uniporters (Bernardi *et al.*, 1989; Diwan and Moore, 1990), and the conductances depicted by patch-clamp (Kinnally *et al.*, 1992, Sorgato and Moran, 1993 for reviews). A similar feature was observed in yeast mitochondria (Manon and Guérin, 1992, 1993; Guérin *et al.*, 1994). Such a wide range of targets could reveal a lack of specificity of these drugs for any transport system. From data reported herein, it is very likely that the target of propranolol and chlorpromazine is the lipid phase: their inhibitory effect shows a very good correlation with their effect on the binding of ANS (for propranolol) and of DPH (for chlorpromazine).

Comparison of the effect of propranolol and chlorpromazine on swellings in potassium acetate and ammonium phosphate showed clearly that both drugs altered the membrane in a way able to affect the activity of a well-known carrier by 50%. The remaining question was to know why the swelling in potassium acetate was fully inhibited.

Three hypotheses could be proposed.

(i) The swelling in potassium acetate was only dependent on the lipid phase. Indeed, liposomes of pure phospholipids are able to catalyze a K^+/H^+ exchange in response to a K^+ gradient (Cooper *et al.*, 1990). It could therefore be hypothesized that permeability changes induced by cationic drugs induced a general loss of inner membrane permeability (including to K^+, H^+, acetic acid and water). However, this is unlikely if we refer to other observations (not reported herein) such as the characteristics of Zn^{2+}-sensitiviy of the spontaneous swelling, which could not be explained by an effect on the lipid phase (Manon and Guérin, 1995).

(ii) An 82-kDa protein able to catalyze a DCCD/quinine-sensitive electroneutral K^+-transport in liposomes was isolated from rat-liver mitochondria (Li *et al.*, 1990). Despite doubts concerning the true inhibitory effect of quinine and DCCD (especially at high concentrations on a small amount of proteoliposomes), one can consider that this protein alone is the K^+/H^+

antiporter, responsible for the swelling of mammalian mitochondria suspended in potassium acetate. The yeast system could also be catalyzed by such a unique protein, but this protein should be particularly sensitive to the structure of its surrounding lipids. Such a feature has been demonstrated for the adenine nucleotides carrier versus cardiolipins (Hoffman *et al.*, 1994) and strongly suggested for most of the mitochondrial transporters reconstituted versus cardiolipins (Krämer and Palmieri, 1989). It should, however, be noted that cardiolipins have a stabilizing effect on the lipid bilayer itself (Shibata *et al.*, 1994) and that the apparent cardiolipin requirement for the activity of the reconstituted carriers is not a sufficient argument for proposing a direct involvement in the activity of the carriers since cardiolipin could be required for the reconstitution step.

(iii) If we have been able to reconstitute an (apparent) K^+/H^+ exchange activity from solubilized inner membrane proteins (Manon and Guérin, 1992), it turns out that we were reproducing such an activity after fractionation of these proteins (by a number of different chromatographic methods). Although the extreme lability of the K^+/H^+ antiporter could be an explanation, another possibility could be the involvement of more than one protein in the activity. Such a hypothesis would fit with the ridigifying effect of propranolol on the mitochondrial membrane since the limitation of lateral diffusion of the proteins could prevent the formation of an active multiproteic arrangement. The hypothesis for a transient formation of a multiproteic arrangement has already been proposed for the mammalian permeability transition pore (Halestrap *et al.*, 1992; Pereira *et al.*, 1993; Valle *et al.*, 1993).

Despite the fact that the target of the inhibitors appears to be the lipid phase, there is a specificity of their action. There is a clear correlation between the potency of phenothiazines in inhibiting the swelling and their ability in releasing DPH, a probe of the hydrophobic moiety of the membrane. However, a high hydrophobicity is not a sufficient requirement for the inhibition: chloramphenicol released DPH with the same efficiency as phenothiazines without inhibiting the swelling. We observed that chloramphenicol, although interacting also with the hydrophilic moiety of the membrane, acted differently as phenothiazines since it released ANS. This could be an indication that the strong inhibitory effect of phenothiazines on swelling, although primarily occurring via a binding of the drug on the nonpolar moiety of the membrane, could be related to a better affinity for anionic phospholipids than for neutral lipids. On the other hand, propranolol only increased the binding of ANS without any effect on the binding of DPH: it therefore only bound to the hydrophilic moiety of the membrane, and more precisely to the negatively charged phospholipids. But, this binding appeared to have two distinct consequences: (i) a screen effect on negative charges, revealed by the increase of the binding of ANS, which was not prevented by DCCD, and (ii) a rigidifying effect of the membrane, prevented by DCCD. The difference between these two effects versus DCCD sensitivity is an argument for the hypothesis that the charge-screening effect of propranolol is not the only factor responsible for its ridifying effect. In addition, high concentrations (i.e., in the millimolar range) of divalent cations, such as Mg^{2+}, which are known to have a screening effect on negative charges of biological membranes, did not inhibit the swelling of yeast mitochondria suspended in potassium acetate (Manon and Guérin, 1992). Conversely, drugs having a similar effect as propranolol on ANS-binding did not inhibit the swelling [such as pindolol (not shown)], whereas quinine did (although with a high IC_{50}) without any effect on ANS binding. The rigidifying effect of propranolol we observed is not a general feature. The binding of propranolol, together with the binding of other cationic drugs (such as dibucaine or tetracaine) has largely been investigated on artificial lipid vesicles (see, e.g., Lee, 1977, 1978; Cullis *et al.*, 1978; Surewicz and Leyko, 1981; Luxnat and Galla, 1986; Zachowski and Durand, 1988): it has generally been concluded that these drugs decreased the lipid phase-transition temperature and then fluidified artificial membranes [but see controversial data reviewed by Goldstein (1984)]. In addition, we did not observe any effect of, e.g., dibucaine (which did not inhibit the swelling) on ANS binding to mitochondria, demonstrating that the effect of a drug may depend on the type of membrane. The high protein/lipid ratio of the inner mitochondrial membrane may considerably affect their response toward these drugs, as compared to artificial membranes or biological membranes with lower protein/lipid ratios. It should also be noted that the lower efficiency of cationic drugs on yeast mitochondria, as compared to mammalian mitochondria, may come from the lower fluidity of yeast mitochondrial inner membrane due to the near absence of polydesaturated fatty acids and the presence of ergosterol (Daum, 1985).

In conclusion, the lack of specific inhibitors (of a protein) is a major trouble in the investigation of the

function, regulation, and structure of mitochondrial ionic pathways. This does not apply only to the K^+/H^+ exchange but also to all the other pathways evidenced for several years [with the major exception of cyclosporin A on the permeability transition pore/megachannel (Fournier *et al.*, 1987; Gunter and Pfeiffer, 1990)]. Since we are now very suspicious concerning the use of cationic drugs for this type of study, we are looking for other types of effectors that are undoubtedly acting on proteins. We have already found divalent cations, such as Zn^{2+}, whose effect was prevented by thiol reagents (Manon and Guérin, 1995). The characterization of other types of effectors of the K^+/H^+ exchange, and the other ionic pathways evidenced in yeast (and mammalian) mitochondria, is under way.

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REFERENCES

Beavis, A. D., and Garlid, K. D. (1990). *J. Biol. Chem.* **265**, 2538–2545.
Bernardi, P., Angrilli, A., Ambrosin, V., and Azzone, G. F. (1989). *J. Biol. Chem.* **264**, 18902–18906.
Chappell, J. B., and Crofts, A. R. (1966). In *Regulation of Metabolic Processes in Mitochondria (Tager, J. M., et al.*, eds.,) Vol. 7, Elsevier, Amsterdam, pp. 293–312.
Cooper, C. E., Wrigglesworth, J. M., and Nicholls, P. (1990). *Biochem. Biophys. Res. Commun.* **173**, 1008–1012.
Cullis, P. R., Verklej, A. J., and Ververgaert, P. H. J. T. (1978). *Biochim. Biophys. Acta* **513**, 11–20.
Dabadie, P., Mazat, J. P., Rigoulet, M., and Guérin, B. (1986). 4th EBEC reports, p. 288.
Daum, G. (1985). *Biochim. Biophys. Acta* **822**, 1–42.
Diwan, J. J., and Moore, C. (1990). *Biochem. Int.* **22**, 843–848.
Dordick, R. S., Brierley, G. P., and Garlid, K. D. (1980). *J. Biol. Chem.* **255**, 10299–10305.
Fournier, N., Ducet, G., and Crevat, A. (1987). *J. Bionerg. Biomembr.* **19**, 297–303.
Galla, H. J., and Sackmann, E. (1975). *J. Am. Chem. Soc.* **97**, 4114–4120.
Galla, H. J., and Luisetti, J. (1980). *Biochim. Biophys. Acta* **596**, 108–117.
Galla, H. J., and Hartmann, W. (1981). *Methods Enzymol.* **72**, 471–479.
Garlid, K. D. (1980). *J. Biol. Chem.* **255**, 11273–11279.
Garlid, K. D. (1988). In *Integration of Mitochondrial Function* (Lemasters, J. J., Hackenbrock, C. R., Thurman, R. G., and Westerhoff, H. V., eds.), Plenum, New York, pp. 259–278.
Garlid, K. D., and Beavis, A. D. (1986). *Biochim. Biophys. Acta* **853**, 187–204.
Garlid, K. D., and Nakashima, R. A. (1983). *J. Biol. Chem.* **258**, 7974–7980.
Goldstein, D. B. (1984). *Annu. Rev. Pharmacol. Toxicol.* **24**, 43–64.
Guérin, B., Labbe, P., and Somlo, M. (1979). *Methods Enzymol.* **55**, 49–59.
Guérin, B., Bunoust, O., Rouqueys, V., and Rigoulet, M. (1994). *J. Biol. Chem.* **269**, 25406–25410.
Gunter, T. E., and Pfeiffer, D. R. (1990). *Am. J. Physiol.* **C258**, 755–786.
Halestrap, A. P. (1989). *Biochim. Biophys. Acta* **973**, 355–382.
Halestrap, A. P., Connern, C. P., and Griffiths, E. J. (1992). In *Molecular Mechanisms of Transport* (Quagliariello, E., and Palmieri, F., eds.), Elsevier, Amsterdam, pp. 259–266.
Hassinen, I. E., and Vuokila, P. T. (1993). *Biochim. Biophys. Acta* **1144**, 107–124.
Heape, A. M., Juguelin, H., Boiron, F., and Cassagne, C. (1985). *J. Chromatogr.* **322**, 391–395.
Heape, A. M., Juguelin, H., Fabre, M., Boiron, F., Garbay, B., Fournier, M., Bonnet, J., and Cassagne, C. (1986). *Dev. Brain Res.* **25**, 173–180.
Hoffman, B., Stöckl, A., Schlame, M., Beyer, K., and Klingenberg, M. (1994). *J. Biol. Chem.* **269**, 1940–1944.
Huunan-Seppälä, A. (1972). *Acta Chem. Scand.* **26**, 2713–2733.
Jezek, P., Mahdi, F. and Garlid, K. D. (1990). *J. Biol. Chem.* **265**, 10522–10526.
Joshi, U. M., Rao, P., Kodavanti, S., Lockard, V. G., and Mehendale, H. M. (1989). *Biochim. Biophys. Acta* **1004** 309–320.
Jung, D. W., and Brierley, G. P. (1986). *J. Biol. Chem.* **261**, 6408–6415.
Kakar, S. S., Mahdi, F., Li, X., and Garlid, K. D. (1989). *J. Biol. Chem.* **264**, 5846–5851.
Kaihovaara, P., Raulo, E., and Kinnunen, P. K. J. (1991). *Biochemistry* **30**, 8380–8396.
Kinnaly, K. W., Antonenko, Y. N., and Zorov, D. B. (1992). *J. Bioenerg. Biomembr.* **24**, 99–110.
Krämer, R., and Palmieri, F. (1989). *Biochim. Biophys. Acta* **974**, 1–23.
Krebs, J. J. R., Hauser, H., and Carafoli, E. (1979). *J. Biol. Chem.* **254**, 5308–5316.
Lee, A. G. (1977). *Moles. Pharmacol.* **13**, 474–487.
Lee, A. G. (1978). *Biochim. Biophys. Acta* **514**, 95–104.
Li, X., Hegazy, M. G., Mahdi, F., Jezek, P., Lane, R. D., and Garlid, K. D. (1990). *J. Biol. Chem.* **265**, 15316–15322.
Luxnat, M., and Galla, H. J. (1986). *Biochim. Biophys. Acta* **856**, 274–282.
Manon, S., and Guérin, M. (1992). *Biochim. Biophys. Acta* **1108**, 169–176.
Manon, S., and Guérin, M. (1993). *J. Bioenerg. Biomembr.* **25**, 671–678.
Manon, S., and Guérin, M. (1995). *Biochem. Mol. Biol. Internat.* **35**, 585–593.
Martin, W. H., Beavis, A. D., and Garlid, K. D. (1984). *J. Biol. Chem.* **259**, 2062–2065.
Martin, W. H., DiResta, D. J., and Garlid, K. D. (1986). *J. Biol. Chem.* **261**, 12300–12305.
Mitchell, P. (1966) *Biol. Rev.* **41**, 445–502.
Nakashima R. A., and Garlid, K. D. (1982) *J. Biol. Chem.* **257**, 9252–9254.
Pereira, R. S., Bertocchi, A. P. F., and Vercesi, A. E. (1993). *Biochem. Pharmacol.* **44**, 1795–1801.

Shibata, A., Ikawa, K., Shimooka, T., and Terada, H. (1994). *Biochim. Biophys. Acta* **1192**, 71–78.
Sorgato, M. C., and Moran, O. (1993). *Crit. Rev. Biochem. Mol. Biol.* **18**, 127–171.
Surewicz, W., and Leyko, W. (1981). *Biochim. Biophys. Acta* **643**, 387–397.
Szabo, I., and Zoratti, M. (1992). *J. Bionerg. Biomembr.* **24**, 111–117.
Valle, V. G. R., Fagian, M. M., Parentoni, L. S., Meinicke, A. R., and Vercesi, A. E. (1993). *Arch. Biochem. Biophys.* **307**, 1–7.
Villalobo, A., Briquet, M., and Goffeau, A. (1981). *Biochim. Biophys. Acta* **637**, 124–129.
Welihinda, A. A., Trumbly, R. J., Garlid, K. D., and Beavis, A. D. (1993). *Biochim. Biophys. Acta* **1144**, 367–373.
Zachowski, A., and Durand, P. (1988). *Biochim. Biophys. Acta* **937**, 411–416.