Action of Propranolol on Mitochondrial Proton Fluxes

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The action of propranolol, a β -blocking adrenergic agent, on mitochondrial proton fluxes in non-energized and energized conditions has been studied. 1. The drug inhibited the actions of valinomycin, nigericin and FCCP on the inner mitochondrial membrane. 2. It decreased the rate and extent of active energized proton expulsion and the passive collapse of the proton gradient so formed. 3. Propranolol was able to increase the permeation of chloride ion through the inner mitochondrial membrane in non-energized and energized conditions. 4. The drug inhibited mitochondrial contraction but stimulated swelling in various conditions.

It is suggested that propranolol is able to change the proton and chloride permeabilities of mitochondria by perturbing the structure of inner membrane phospholipids, thus enlarging the water-lipid interface.

Propranolol (1-isopropylamino-3-(1-naphthol-oxy)-propan-2-ol hydrochloride) is a widely used β -blocking adrenergic drug that is also known as a potent local anaesthetic. Its effects on membranes have been related to its lipophilicity.¹ The actions of local anaesthetics on liposome and mitochondrial membranes are rather controversial. It has been shown that they inhibit ion transport processes,²⁻⁵ but stimulatory effects have also been reported.⁴⁻⁸ Quite recently we have found that there are apparent differences in the action of propranolol on the inner mitochondrial membrane depending on the energetic state of the mitochondria.^{6,8} In non-energized conditions the permeation of potassium and chloride into mitochondria was stimulated as judged by swelling techniques. In energized oscillatory conditions in the presence of potassium phosphate plus valinomycin the rates of ion fluxes were decreased by propranolol. However, the extent of the ion uptake was increased by the drug if the external

potassium phosphate was more than *ca.* 15 mM at pH 7.2. The inhibition of the rates of ion fluxes was abolished by raised pH or nigericin suggesting that propranolol decreased the passive permeation of protons through the inner mitochondrial membrane.

In order to elucidate the reason for these apparent differences in the action of propranolol we began this work on the effects of propranolol on mitochondrial proton fluxes coupled to potassium fluxes in non-energized and energized conditions. To record the simultaneous pH changes in the membrane phase we used bromothymol blue as a probe. Even if energization⁹ and local anaesthetics¹⁰ change the binding of BTB^{*} on mitochondria, the absorbance changes of BTB were mainly in line with the observed ion movement.

MATERIAL AND METHODS

Rat liver mitochondria were prepared, and pH changes recorded mainly as described earlier.^{8,11} Mitochondrial swelling was followed by an Aminco DW 2 spectrophotometer at 630 nm.^{6,8} Mitochondrial protein was determined by a biuret method.¹² When the energized proton translocation was studied anaerobic mitochondria were energized with hydrogen peroxide in the presence of succinate, mainly as described by Mitchell and Moyle.¹³ In these experiments a Beckman pH electrode (39045) and a calomel electrode with an agar-KCl bridge were used, and malonate was added to decrease the rate of the respiration-induced pH

* Abbreviations used: BTB, bromothymol blue; FCCP, carbonylcyanide-*p*-trifluoromethoxy phenylhydrazine; HOQNO, 2-heptyl-4-hydroxy-quinone *N*-oxide; Hepes, *N*-2-hydroxymethylpiperazine-*N'*-2-ethane-sulfonic acid; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

changes. Oxygen consumption was simultaneously followed with a Clark type electrode connected through an Aminco amplifier to a recorder.⁸ The changes of the absorbance of BTB were monitored at 618 nm using 700 nm as the reference wavelength with an Aminco DW 2 spectrophotometer.

In order to minimize the movements of divalent cations, 1 mM EDTA was present in all the media used. Propranolol was a gift from I.C.I., Macclesfield, England. It was dissolved in ethanol and an equal amount of ethanol was added in the control experiments. BTB was obtained from British Drug Houses, Ltd, Poole, England, and recrystallized.¹⁴ The source of other reagents was as described elsewhere.⁵

RESULTS

Effects on non-energized ion fluxes. The ion conductors valinomycin, nigericin, and FCCP all increase the penetration of potassium through the inner mitochondrial membrane.^{15,16} Valinomycin induces an electrogenic uptake of potassium, nigericin mediates an electroneutral exchange of protons and potassium, and FCCP increases the permeability of the membrane to protons, thus decreasing the resistance to H^+/K^+ -exchange.

When fresh non-energized mitochondria are incubated in sucrose, an exchange of internal potassium for external protons can be induced by the above mentioned lipophilic ion carriers.

The action of propranolol on the uptake of protons was studied in these conditions. The drug inhibited the rate of the uptake induced by each of the mentioned ion conductors (Fig. 1). The parallel contraction induced by the ion efflux was also inhibited by propranolol (Fig. 1C).

When the external medium was changed to potassium chloride, the three ion carriers again caused an uptake of protons (Fig. 2). This uptake was also inhibited by propranolol, but when nigericin was added in the presence of propranolol, the proton uptake seen in the control experiment was reversed, and a proton release occurred (Fig. 2A). This was seen over a wide range of nigericin concentrations.

In potassium chloride propranolol further stimulated the swelling of mitochondria that was induced by valinomycin, nigericin, or FCCP (Fig. 3). The stimulation was most conspicuous when nigericin was used (3A). Since in these conditions the swelling is thought to be limited

Fig. 1. The effect of propranolol on mitochondrial proton uptake and contraction in sucrose. The mitochondria were incubated in 0.15 M sucrose, 1 mM EDTA, then 7 μ M rotenone and 10 μ g oligomycin were added. The first arrow indicates the addition of 4 μ M HOQNO, (A) mitochondrial pH change, the second arrow indicates the addition of 0.2 μ g/ml valinomycin, the concentration of propranolol 260 μ M, mitochondrial protein 3.7 mg/ml. (B) as in (A) except 1.7 μ M FCCP and not valinomycin was added. (C) mitochondrial contraction, the second arrow indicates the addition of 0.18 μ g/ml valinomycin. The concentration of mitochondrial protein was 2.6 mg/ml. — indicates the control and - - - propranolol added before HOQNO. The pH was 7.2 before each addition of the ion conductors. The final volume in (A) and (B) was 1.5 ml.

Fig. 2. The effect of propranolol on proton fluxes in potassium chloride. The mitochondria were incubated in 0.1 M KCl, 1 mM EDTA, then 10 μ M rotenone and 10 μ g oligomycin were added. Propranolol when added was 200 μ M, HOQNO 4 μ M; mitochondrial protein was 4.8 mg/ml. (A) the first arrow indicates the addition of propranolol, the second that of HOQNO, and the third that of nigericin 0.3 μ g/ml. (B) and (C) the first arrows indicate the additions of HOQNO and the second those of 0.2 μ g/ml valinomycin (B) or 1.7 μ M FCCP. — is control and - - - propranolol present. The pH was 7.2 before each addition of the ion conductors. The final volume was 1.5 ml.

Fig. 3. The effect of propranolol on non-energized mitochondrial swelling in potassium chloride. The mitochondria were incubated in the presence of 0.1 M KCl, 1 mM EDTA, then 10 μ M rotenone, 10 μ g oligomycin and 4 μ M HOQNO were added. The first arrow indicates the addition of 160 μ M propranolol, the second that of 0.2 μ g/ml nigericin in (A) or that of 0.1 μ g/ml valinomycin in (B), or that of 1.2 μ M FCCP in (C) in 10 μ l of ethanol. Mitochondrial protein was 5.7 mg/ml.

by anion entry it is evident that chloride permeation was stimulated. The slight swelling seen in the control was preceded by an initial contraction, and this contraction phase was totally abolished by propranolol.

Propranolol clearly decreased the functioning of all the ion conductors. However, although propranolol decreased the rate of respiration in the presence of small concentrations of FCCP the inhibition was abolished by increasing amounts of FCCP, when the respiration was limited by the capacity of respiratory chains and not by the proton permeability (Fig. 4). We also found earlier that propranolol inhibited the rate of swelling of energized mitochondria in the presence of potassium acetate plus valinomycin.⁸

Effects on active ion fluxes. Mitochondria eject protons when energized in the absence of ADP. Propranolol decreased the rate of proton release induced by energization (H_2O_2 plus catalase).¹¹ It also depressed the extent of this release and the rate of passive uptake of protons that followed exhaustion of the oxygen (Fig. 5A). Valinomycin could not abolish the inhibition caused by propranolol (Fig. 5B), even at very high concentrations. This suggests, in

Fig. 4. The effect of propranolol on the uncoupling of mitochondrial respiration by FCCP. The mitochondria were incubated in the presence of $10 \mu\text{M}$ rotenone. The respiration was started by the addition of 10 mM Tris-succinate. The effect of FCCP was titrated by addition of small concentrations of FCCP. The points are means of two determinations. The medium in (A) 0.1 M KCl, 0.02 M Hepes, 1 mM EDTA, pH to 7.2 with Tris, and in (B) 0.2 M sucrose, 0.02 M Hepes, 1 mM EDTA, pH to 7.2 with Tris. — the control, - - - $150 \mu\text{M}$ propranolol.

agreement with our earlier findings,⁸ that the drug indeed inhibits proton translocation *per se*.

Proton pulses induced by small amounts of ATP were similarly inhibited by propranolol, but the translocation of ATP and ADP at small

concentrations was also inhibited by propranolol [not shown (*cf.* Refs. 17, 18)].

Using beef heart submitochondrial particles we could not find any oligomycin-like coupling effect¹⁹ with propranolol (not shown).

Fig. 5. The effect of propranolol on the proton ejection of energized mitochondria (A) in the absence of valinomycin and (B) in the presence of valinomycin. The mitochondria were incubated in the presence of 0.1 M KCl, 1 mM EDTA, $15 \mu\text{M}$ rotenone, 12 mM K-succinate, 2 mM K-malonate and catalase at 28°C . The respiration was initiated with H_2O_2 ($300 \mu\text{M}$) see also the method). (A) $280 \mu\text{M}$ propranolol, mitochondrial protein 5.4 mg/ml . (B) $0.17 \mu\text{g/ml}$ valinomycin, $200 \mu\text{M}$ propranolol, mitochondrial protein 4.0 mg/ml . — the control, - - - propranolol. The pH was 7.2 before each addition of H_2O_2 ; the final volume was 2.5 ml .

Fig. 6. The effect of propranolol on energized mitochondrial swelling in the presence of potassium chloride and valinomycin. The mitochondria were incubated in the presence of 7 μ M rotenone, 0.1 M KCl, 1 mM EDTA, 0.02 M Hepes, pH 7.2 with Tris. The first arrow indicates the addition of 160 μ M propranolol, the second that of 8 mM K-succinate, the third that of 0.1 μ g/ml valinomycin. — the control, - - - propranolol.

In the absence of valinomycin propranolol did not affect the swelling of energized mitochondria. In the presence of valinomycin the extent of swelling was increased and contraction inhibited (Fig. 6). The increase of swelling was not caused by succinate uptake, because we found no difference in the swelling if succinate was changed to ascorbate plus TMPD. It is concluded that propranolol increased the uptake of chloride in these conditions.

Changes in the absorbance of BTB. BTB has been used in the study of the pH changes occurring during ion uptake by mitochondria.⁷ Yet propranolol and local anaesthetics are known to change the binding of BTB.⁸

When mitochondria were incubated in the presence of BTB in KCl, the addition of propranolol caused a drastic change in the BTB absorbance ("alkalinization") (Fig. 7, the first arrow). The change was partially abolished by valinomycin (7A) and totally by nigericin (7B)

Fig. 7. The effect of propranolol on the extinction of bromothymol blue. The mitochondria were incubated in 0.1 M KCl, 0.02 M Hepes, 1 mM EDTA, pH 7.2, in the presence of 2 μ M BTB and 7 μ M rotenone. Mitochondrial protein was 2.0 mg/ml. The first arrow indicates the addition of 160 μ M propranolol, the second in (A) 0.1 μ g/ml valinomycin and in (B) 0.2 μ g/ml nigericin. The third arrow indicates the addition of 8 mM succinate and the fourth that of 4 μ M HOQNO.

and FCCP (not shown). If nigericin was added before propranolol the addition of propranolol did not change the absorbance of BTB. This suggests that the changes in BTB absorbance at least partly followed the pH changes in the membrane environment of BTB. The data with non-energized mitochondria were identical.

When the mitochondria were energized in the presence of BTB and valinomycin the changes in the absorbance were greater in the presence of propranolol than in the control (Fig. 7A), suggesting an increased alkalinity in the membrane. This is in line with the swelling experiment results (Fig. 6) where propranolol increased the ion uptake. When nigericin was present energization had no effect on the absorbance of BTB (Fig. 7B).

DISCUSSION

Propranolol inhibited all the proton fluxes that followed the addition of the ionophores nigericin or valinomycin or the uncoupler FCCP (see Ref. 4). The inhibition is probably related to the binding of propranolol to mitochondrial phospholipids.⁹ It is clear that propranolol changes the surface potential of the membrane when bound, but its lipophilicity may also change the solubility of the lipophilic ion conductors or increase the viscosity of the membrane *per se*.

In contrast to the effects of dibucaine reported by Papa *et al.*⁴ propranolol decreased the rates and extent of mitochondrial proton fluxes (Fig. 5). Earlier we have found that propranolol only slightly affected the mitochondrial ADP/O-ratio. Thus propranolol does not act as an uncoupler in mitochondria.

However, it is not likely that propranolol is a specific inhibitor of proton transfer. The inhibition is more likely to result from a retardation of proton transfer from one polarizable group (phospholipid) to another.²⁰ This retardation might be related to the increase of mitochondrial buffering groups that propranolol generates in non-energized conditions.¹¹ Because the concentrations of propranolol used are of the same order as the changes of proton concentrations we have studied, it is even possible that movements of propranolol in or through the inner mitochondrial membrane may change the proton gradients involved. Even if the

concentration of uncharged propranolol is low at pH 7.2, the affinity of the lipid membrane may be higher for the uncharged form of the drug.²¹

It is obvious that propranolol does not increase the permeability of the inner mitochondrial membrane to potassium in energized conditions. This suggests that the re-ordering that occurs in the inner mitochondrial membrane²² on energization changes the mechanism of the permeation of potassium through the inner membrane, although no definite evidence for the existence of a monovalent cation pump in mitochondria has been reported so far.²³⁻²⁷

We found earlier that propranolol stimulated mitochondrial swelling in 0.1 M NH_4Cl at pH 7.2 in the presence of FCCP.⁵ In these conditions the rate of mitochondrial swelling depends on the rate of anion permeability only, because NH_3 and protons easily penetrate mitochondrial membranes.

In line with the earlier findings propranolol increased the uptake of chloride into mitochondria (Fig. 3A and 6). Yet, immediately after the addition of nigericin, the anion permeability did not seem to be rate-limiting, since an increase of pH and a contraction were needed before the mitochondria began to swell (Figs. 2A and 3A). Propranolol seemed to abolish both the pH increase and the contraction, and the mitochondria were able to take up potassium and chloride immediately after the addition of nigericin.

Propranolol may increase the permeability of mitochondria to chloride in at least two ways. The drug has one positive charge and, because it is bound to the phospholipids of mitochondria,¹⁰ it could act as a carrier of chloride either into or through the inner membrane of mitochondria (*cf.* Ref. 28). Another possibility is a novel suggestion²⁸ that chloride might consume protons of the membrane water, creating a localized intramembrane pH difference. Local anaesthetics and propranolol expand biological membranes increasing the membrane water (for a review, see Ref. 30). As a result of this the accessibility of chloride to the membrane water would be easier, and the penetration could be stimulated. Whatever the mechanism is, this stimulation of chloride permeation can partly explain the rapid consumption of acid

that follows the acidification caused by the addition of propranolol to a mitochondrial suspension (Fig. 2A), and the reverse action of nigericin (seen in the same figure).

Energization seems to change the structure of mitochondria so that propranolol is no longer able to increase the permeability of the inner membrane to potassium. Yet, the drug inhibits the translocation of protons and stimulates that of chloride, stressing the important role of phospholipids in mitochondrial ion transport.

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