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Action of Local Anaesthetics on Passive and Energy-Linked Ion Translocation in the Inner Mitochondrial Membrane

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Abstract

The effect of dibucaine on passive and respiration-driven ion translocation and oxidative phosphorylation in submitochondrial particles from beef-heart has been studied.

Dibucaine inhibited the nigericin-mediated H^+/K^+ exchange diffusion and the electrogenic, valinomycin-mediated K^+ translocation in submitochondrial particles.

The local anaesthetic exerted a direct stimulatory effect on the respiration-driven proton uptake and on the passive proton-diffusion reactions. The increase of the respiration-linked proton turnover caused by dibucaine was accompanied by uncoupling of oxidative phosphorylation. It is concluded that spontaneous noncoupled as well as ionophore-mediated K^+ translocation in mitochondria occurs across phospholipid bilayer regions of the membrane whilst other components of the membrane would be specifically involved in active and passive proton translocation across the membrane.

The results indicate that polar groups of membrane phospholipids play an important role in energy conservation and transfer in the mitochondrial membrane.

Introduction

A useful approach to the elucidation of the molecular mechanism of solute translocation across biological membranes is given by the study of the response of the system to agents which specifically interact with membrane components. This paper describes the action of the local anaesthetic dibucaine on proton and K^+ translocation in submito-

chondrial particles. Dibucaine is a cationic, lipid-soluble, complex amine derivative of quinoline; it competes, like other related anaesthetics, with protons and cations for binding to membrane phospholipids [1-3]. Local anaesthetics have been found to be competitive inhibitors of the binding of cations to mitochondria and submitochondrial particles [4]. Furthermore these drugs inhibit the spontaneous and valinomycin-mediated K^+ translocation in mitochondria [5] and artificial phospholipid bilayer membranes [6].

In previous papers [7-9] it was shown that passive proton diffusion in submitochondrial particles consists of two parallel processes: a fast reaction, mediated by a proton-monovalent cation exchange system of the membrane and a slow noncoupled reaction. Evidence was also presented showing that active, respiration-linked proton translocation consists of a single electrogenic flux. It is shown here that dibucaine causes a marked acceleration of the two proton diffusion processes and a consequent decrease of the steady-state aerobic proton uptake. A direct stimulatory effect of dibucaine on the respiration-linked proton current could also be demonstrated. These effects of dibucaine on proton translocation were accompanied by uncoupling of oxidative phosphorylation.

Materials and Methods

Dibucaine chloride was obtained from Pfaltz and Bauer Inc., Flushing, N.Y.; Valinomycin from Calbiochem, Luzern, Switzerland; Nigericin was kindly given by Dr. H. A. Lardy.

Valinomycin and nigericin were added as alcoholic solutions, dibucaine was added dissolved in H_2O-alchohol, 70 : 30, v : v.

Preparation of Submitochondrial Particles

Mg, ATP-submitochondrial particles were prepared according to Löw and Vallin [10]; sonication of heavy beef-heart mitochondria was carried out using an Ultrasonic Branson Sonifier, Mod. W 185, for 60 sec at 70 watt.

Incubation Procedure

Incubation of submitochondrial particles (1-4.8 mg protein/ml) was carried out, under a constant N_2 flow. The incubation mixture was thermostated to $\pm 0.01^{\circ}C$. The basic reaction mixture contained: 250 mM sucrose and 0.2 mg purified catalase/ml; for other additions see the legends to figures. Respiration was activated by repetitive pulses of 0.4-3% H_2O_2 (5 μl/ml).

Measurements of Respiration and Ion Translocation

The concentration of H^+, K^+ and O_2 was monitored potentiometrically by electrodes immersed in the reaction mixture. Respiration was measured with a Clark oxygen electrode (Yellow Springs Instrument Co. Inc., Mod. 5331). The pH was monitored with fast responding glass electrodes, Ingold KG, Frankfurt/Main, Germany or Beckman combination Electrode (n. 39030), Beckman Instruments International, Geneve, Switzerland. For other details see ref. [9]. The K^+ concentration was measured with a Beckman cationic electrode (n. 39047). Under the experimental conditions used, the response of this electrode to the pH changes was negligible.

Oxidative Phosporylation

Oxygen uptake was measured manometrically. Phosphate esterification was measured enzymically with glucose-6-phosphate dehydrogenase, correction being made for adenylate kinase activity.

Mathematical Analysis

Potentiometric measurements of H^+ translocation were analysed as described previously [8, 9].

Effect of Dibucaine on Proton Translocation

Figure. 1 illustrates the effect of dibucaine on proton translocation induced by oxygen pulses of succinate-supplemented, anaerobic Mg-ATP particles, suspended in a K^+- or a choline-medium. In both media dibucaine depressed the extent of the respiration-driven proton uptake and accelerated the anaerobic proton release. The initial rate of proton uptake was depressed by dibucaine in the K^+ medium, but stimulated in the choline medium. This differential effect of dibucaine is clearly shown by Fig. 2 which gives the concentration dependence of the effect of the drug on the respiration-induced proton translocation in submitochondrial particles.

The respiration-driven proton uptake by submitochondrial particles is three-fourfold stimulated by valinomycin *plus* K^+ [9, 11].

Figure 3 shows that dibucaine caused a very marked depression of both the initial rate and the extent of the respiration-driven proton uptake by submitochondrial particles supplemented with valinomycin *plus* K^+. Dibucaine at the concentration of 250-300 nmol/mg protein practically abolished the stimulatory effect of the ionophore on the aerobic proton uptake. Nigericin, which induces electroneutral exchange of protons with potassium ions [12], collapsed the aerobic proton

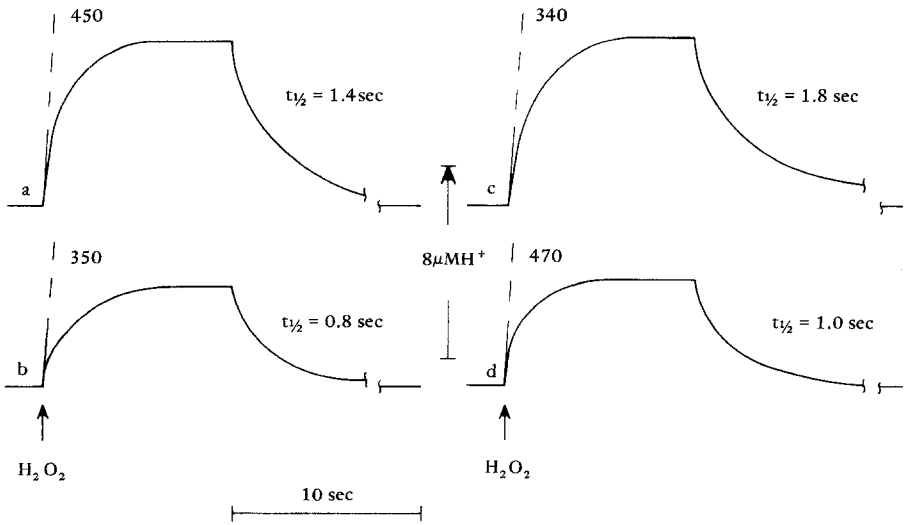

Figure 1. Effect of dibucaine on respiration-induced proton translocation in submitochondrial particles in the presence of K^+ or choline. The reaction mixture (1.5 ml) contained, in addition to the basic components: 15 mM succinate, 1 mM EDTA and submitochondrial particles (1.6 mg protein/ml). The final pH was adjusted to 8.0 with KOH or Choline; experiments a and b: K^+ medium; experiments c and d: choline medium. Dibucaine (experiments b and d) was added at a final concentration 150 μM. Incubation temperature, 30°C. The net initial rates given in the Figure, are expressed as ngion H^+/(mg protein.min).

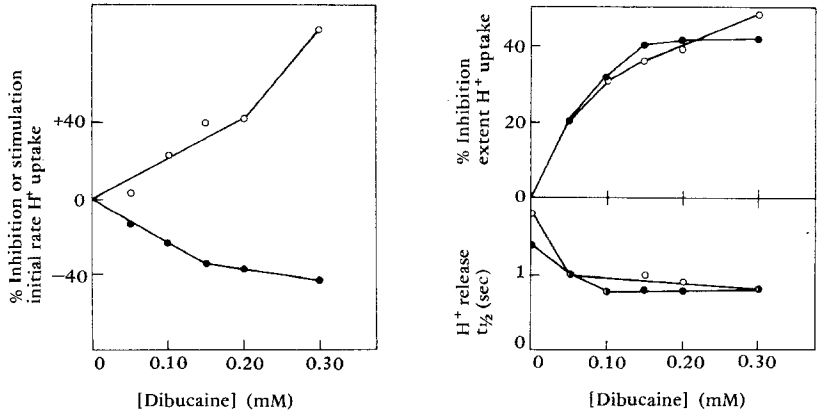

Figure 2. Concentration dependence of the effect of dibucaine on proton translocation in a K^+- and a choline-medium in submitochondrial particles. For experimental conditions see legend to Fig. 1 and Methods. Closed symbols refer to the K^+ medium; open symbols to the choline medium.

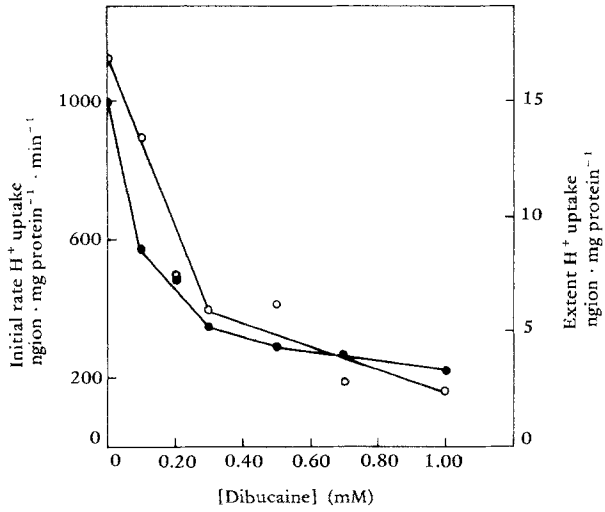

Figure 3. Effect of dibucaine on the extent and the initial rate of respiration-driven proton uptake in submitochondrial particles treated with valinomycin *plus* K^+. The reaction mixture (1.5 ml) contained, in addition to the basic components: 13.3 mM K-succinate, 30 mM KCl, valinomycin (0.1 μg/ml) and submitochondrial particles (1.7 mg protein/ml). Final pH, 7.0. Incubation temperature, 30° C. Initial rate: o—o; Extent: ●—●.

gradient established across the membrane of valinomycin-supplemented submitochondrial particles by respiration (Fig. 4; see refs. 9, 11, 12). 200 μM dibucaine prevented, almost completely, the opposite effects of the two artificial potassium-carriers on the respiration-driven proton uptake (Fig. 4).

It has been shown that thiocyanate, as well as other permeating anions, are as effective as valinomycin *plus* K^+ in stimulating the respiration-driven proton uptake [7-9, 11].

In the experiment of Fig. 5 the effect of dibucaine on the respiration-driven proton influx and the energy-linked proton turnover at the steady state, in a system supplemented with thiocyanate, was examined. The incubation mixture contained an excess of EDTA and EGTA to chelate divalent cations present in submitochondrial particles (see ref. 9). In the presence of thiocyanate dibucaine, in the 100-500 μM concentration range, stimulated the initial rate but depressed the extent of the aerobic proton uptake. The reduction of the extent of proton uptake was evidently due to stimulation of the proton back-flow. Dibucaine caused, in fact, a significant increase of the initial rate of the anaerobic proton release. Respiration was unaffected up to 200 μM dibucaine, then it was slightly inhibited. Consequently the H^+/O quotient, measured as the

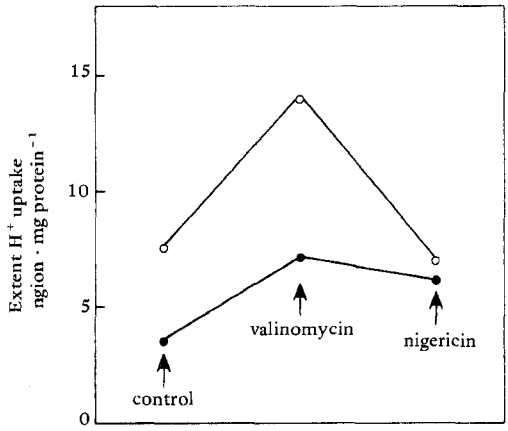

Figure 4. Effect of dibucaine on the extent of aerobic proton uptake in the presence of ionophores. The reaction mixture (1.5 ml) contained, in addition to the basic copmonents: 15 mM K-succinate and submitochondrial particles (1.7 mg protein/ml). Final pH, 7.0. Valinomycin: 0.2 μg/ml; nigericin: 0.2 μg/ml; closed symbols refer to the samples treated with 200 μM dibucaine. Incubation temperature, 30° C.

ratio between the initial rate of proton release and the steady-state respiratory rate, was increased by dibucaine.

In Fig. 6 the effect of dibucaine on the kinetics of the anaerobic release of the protons taken up by submitochondrial particles during respiration is illustrated. Protons were released with a biphasic kinetics, which could be resolved, by applying the double exponential equation, in two first-order plots [7-9]. The slopes of the two lines give the respective velocity constants, the intercepts with the ordinate the amount of protons translocated by the two processes. 400 μM dibucaine caused a marked increase of the velocity constants of both the proton-diffusion reactions. The stimulation was however greater for the slow than for the fast process and this resulted in an increase of the percentage of proton translocated by the slow process. Figure 7 gives a titration of the stimulatory effect of dibucaine on the two proton-diffusion reactions in submitochondrial particles at two protein concentrations. The stimulatory action of dibucaine depended upon the drug/protein ratio. For both proton-diffusion reactions half maximal effect was obtained at approximatively 100 nmol of dibucaine/mg protein, saturation at 290 nmol. Over all the concentration range of dibucaine used the slow process of proton diffusion was more profoundly stimulated than the fast process.

In Table I data are presented on the effect of dibucaine on the activation energy of the two proton diffusion processes in submitochon-

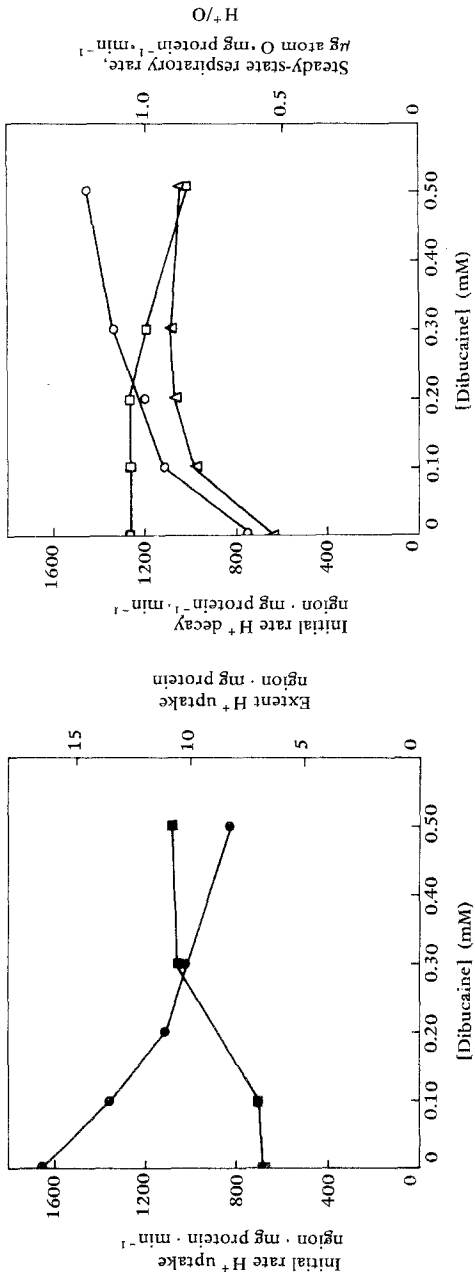

Figure 5. Effect of dibucaine on proton translocation and steady-state H^+/O quotient in submitochondrial particles. The reaction mixture (1.5 ml) contained, in addition to the basic components: 15 mM choline-succinate, 5 mM KSCN, 3 mM choline-EDTA, 3 mM choline-EGTA and submitochondrial particles (1.6 mg protein/ml). Final pH, 7.0. Incubation temperature, 30 °C. Initial rate H^+ uptake: ■—■; Extent H^+ uptake: ●—●; Steady-state respiratory rate: □—□; Initial rate H^+ decay: △—△; H^+/O quotient: ○—○.

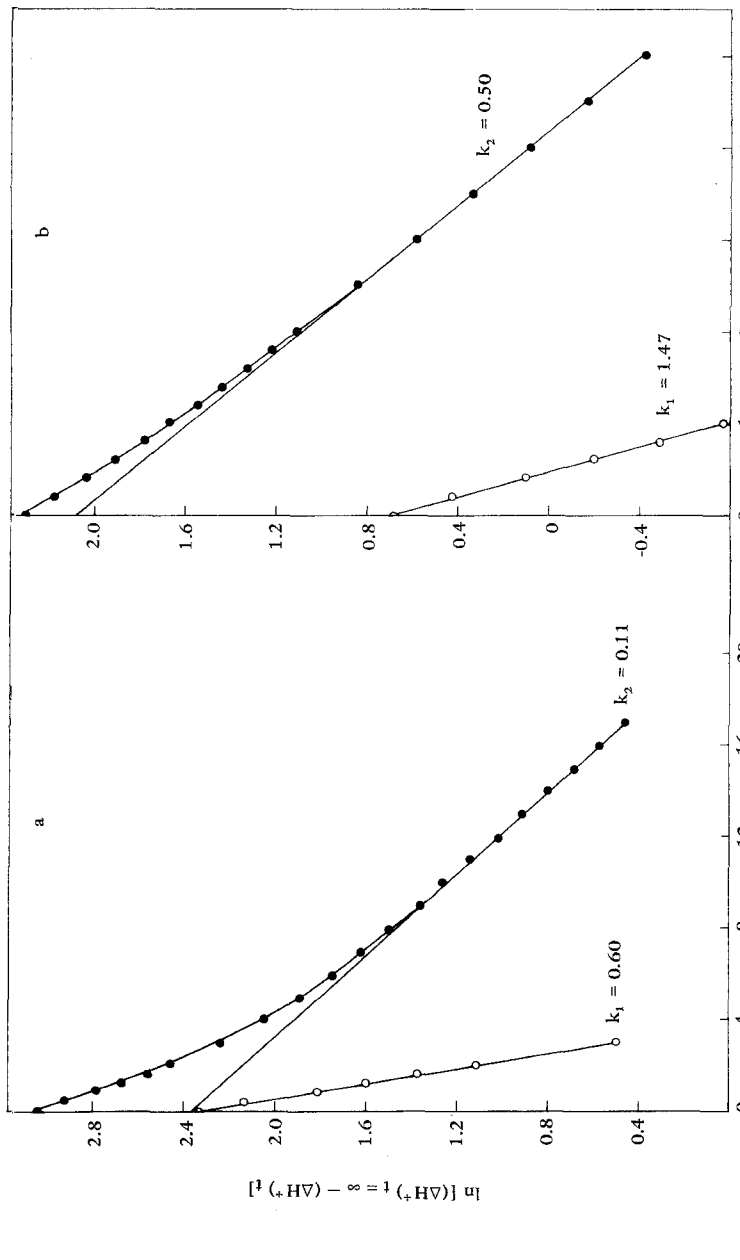

Figure 6. Effect of dibucaine on the kinetics of the anaerobic proton release from submitochondrial particles. The reaction mixture (2.0 ml) contained, in addition to the basic components: 15 mM K-succinate, 10 mM KSCN and submitochondrial particles (2.4 mg protein/ml). Final pH 7.0. Dibucaine (experiment b) was added at a final concentration of 400 μM. Incubation temperature, 30 °C. The proton release was analysed as previously described [9], by the double exponential equation:

$$(\Delta H^+)_{t=\infty} - (\Delta H^+)_t = (\Delta H_1^+) e^{-k_1 t} + (\Delta H_2^+) e^{-k_2 t},$$

where $(\Delta H^+)_{t=\infty}$ stands for the amount of protons taken up by respiring particles at the steady-state and $(\Delta H^+)_t$ represents the amount of protons retained by the particles at time = t, during the phase of anaerobic release.

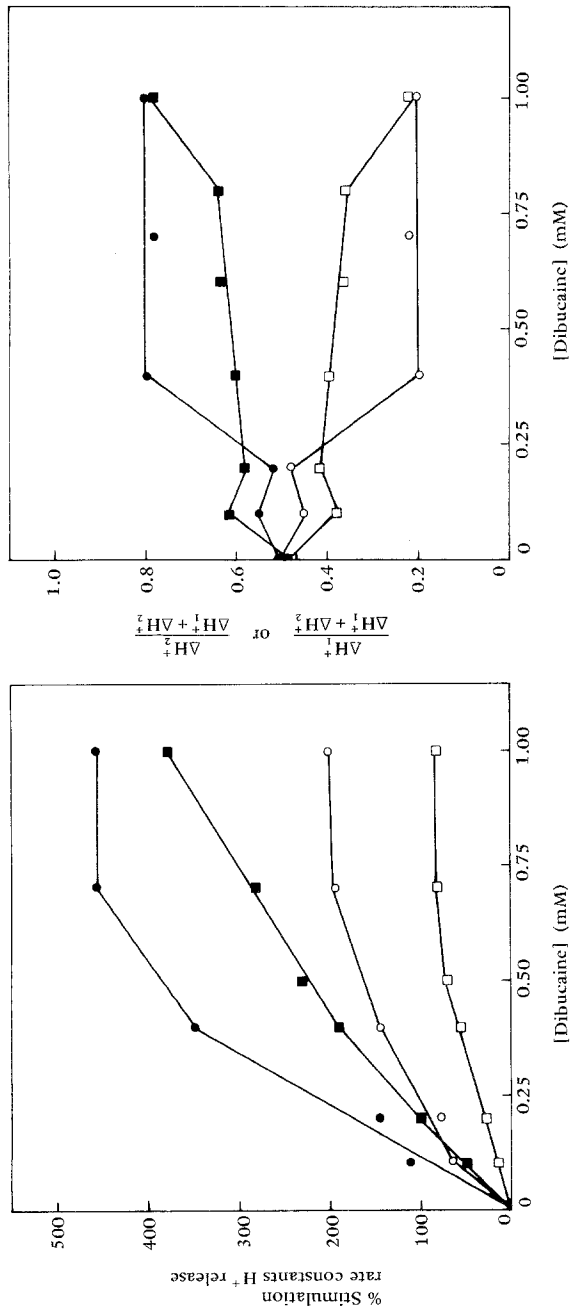

Figure 7. Titration of the dibucaine effect on the anaerobic proton release from submitochondrial particles at two protein concentrations. For the experimental conditions see legend to Fig. 6. Submitochondrial particles: 2.4 mg protein/ml (circles) or 4.8 mg protein/ml (squares). Open and closed symbols refer respectively to the fast and the slow proton diffusion process. The amount of protons translocated by the fast process (ΔH_1^+) and the slow process (ΔH_2^+) were obtained as described in the legend to Fig. 6 and text.

TABLE 1. Effect of dibucaine on the activation energy for the proton diffusion processes in submitochondrial particles

Additions	Kcalories / gion H^+	
	E_1	E_2
none	8.1 ± 0.8	13.1 ± 0.9
200 μM Dibucaine	12.5 ± 1.0	14.4 ± 0.8

The reaction mixture (1.5 ml) contained, in addition to the basic components: 15 mM choline-succinate, 10 mM KSCN and submitochondrial particles (1.7 mg protein/ml). Final pH, 7.0. Temperature range 20-40° C. Subscripts 1 and 2 refer to the fast and to the slow process of the anaerobic proton release. The values represent the mean of three determinations. The Arrhenius plot gave for the two processes, straight lines in the temperature range examined (see refs. 7, 9).

drial particles. Dibucaine increased the activation energy of both the processes; the increase was particularly significant in the case of the fast proton-diffusion reaction. Thus the increase of the velocity constants of the two proton diffusion reactions caused by dibucaine is not due to a decrease of their activation energies (see Eq. (1)).

$$\ln k = \ln A - \frac{E}{RT} \tag{1}$$

It is possible that the increase of the velocity constants is due to an increase of the coefficient A of the Arrhenius equation (1). In fact it could be calculated from equation (1) that dibucaine increased by a factor of four orders of magnitude the coefficient A of the fast proton-diffusion process and by a factor of 1 order the coefficient of the slow process.

Effect of Dibucaine on K^+ Translocation

The respiration-linked proton uptake by submitochondrial particles is accompanied in the presence of permeating anions by a significant K^+ uptake [7-9, 13, 14]. The aerobic K^+ uptake is greatly enhanced, at the expense of H^+ uptake, by nigericin [9, 13]. Dibucaine inhibited K^+ uptake both in the absence and presence of nigericin (Fig. 8). In the second case, however, the process was more sensitive to the inhibitory action of dibucaine. The concentration of dibucaine giving 50% inhibition of K^+ uptake was, in fact, 80 μM in the presence and 160 μM in the absence of dibucaine (see Fig. 8). At a concentration of 250-300 nmol/mg particle protein and above this dibucaine practically abolished the nigericin-induced promotion of the aerobic K^+ uptake by the particles.

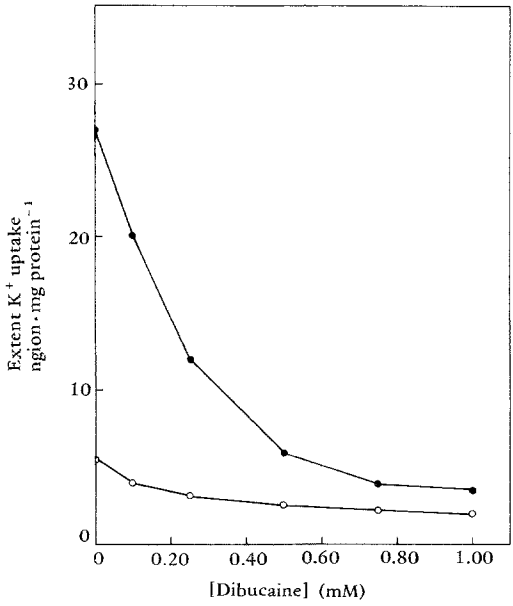

Figure 8. Effect of dibucaine on the spontaneous and nigericin-promoted aerobic K^+ uptake by submitochondrial particles. The reaction mixture (8.0 ml) contained, in addition to the basic components: 20 mM Tris-succinate, 30 mM Tris-nitrate, 500 μM K^+ and submitochondrial particles (2.7 mg protein/ml). Final pH, 7.0. Incubation temperature, 30° C. Nigericin (closed symbols): 0.3 μg/ml.

The electrometric traces of Fig. 9 illustrate the effect of dibucaine on the valinomycin-mediated K^+ translocation. Valinomycin, added at the aerobic steady-state to submitochondrial particles, supplemented with nitrate and nigericin, gave a rapid and extensive discharge of the K^+ taken up during the respiratory phase. Dibucaine caused a marked inhibition of the initial rate and increased the $t_{1/2}$ of the valinomycin induced K^+ discharge.

Effect of Dibucaine on Oxidative Phosphorylation

The experiment of Fig. 10 shows that dibucaine, in the same concentration range at which enhanced the steady-state H^+/O ratio, caused a marked decrease of the P/O ratio in submitochondrial particles. This was due to inhibition of oxidative phosphorylation since respiration was not significantly affected by dibucaine. Similar results, not shown, were obtained in intact rat-liver mitochondria.

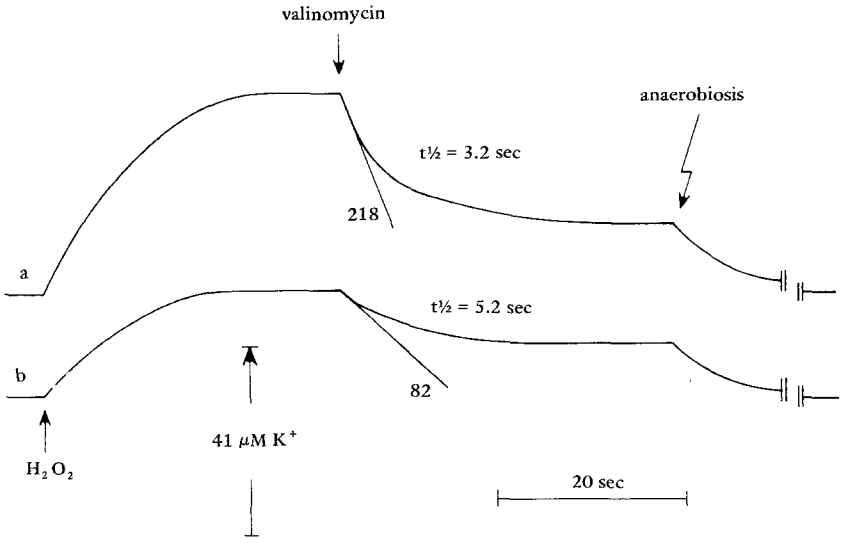

Figure 9. Effect of dibucaine on the valinomycin-mediated K^+ translocation in submitochondrial particles. The reaction mixture (8.0 ml) contained, in addition to the basic components: 20 mM Tris-succinate, 50 mM Tris-nitrate, 1 mM K^+, nigericin 0.13 μg/ml and submitochondrial particles (1.3 mg protein/ml). Final pH, 7.0. Incubation temperature, 30° C. Dibucaine (expt. b) was added at a final concentration of 300 μM; valinomycin: 0.3 μg/ml. The net initial rates of K^+ decay, given in the Figure, are expressed as ngion K^+/(mg protein.min).

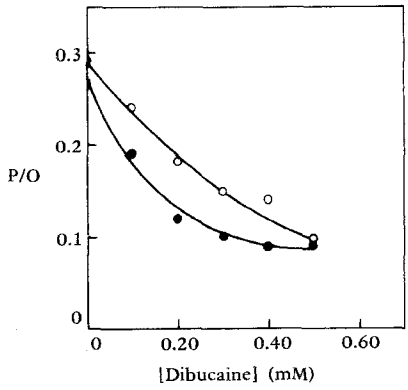

Figure 10. Effect of dibucaine on the P/O ratio in submitochondrial particles, in a K^+ and a choline medium. The reaction mixture (1.0 ml) contained: 200 mM sucrose, 10 mM succinate, 20 mM glucose, 5 mM H_3PO_4, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM ADP, 7 units hexokinase and submitochondrial particles (1.0 mg protein/ml). The final pH was adjusted to 7.5 with KOH or choline. Incubation temperature, 30° C. Incubation time 14 min. Closed symbols refer to the K^+ medium, open symbols to the choline medium. For experimental details see under Methods.

Discussion

The present investigation shows that dibucaine has a two-fold action on ion translocation in submitochondrial particles: it enhances the energy-linked cyclic flow of protons and inhibits the ionophore-mediated K^+ translocation. Various lines of evidence show that the respiration-driven proton translocation in mitochondria is accompanied by the development of a membrane potential [7-9, 15, 16]. Electrogenic proton translocation is rapidly inhibited by the membrane potential unless this is dissipated by co-migration of permeant anions or counterflux of permeant cations or K^+ in the presence of valinomycin [7, 9, 15, 16]. Dibucaine depressed the initial rate of the respiration-driven proton uptake by submitochondrial particles when the process was charge-compensated by valinomycin-mediated electrophoretic K^+ migration but did not inhibit, rather enhanced, this rate when electrophoretic cation migration was minimized by replacing K^+ in the medium with impermeant choline or when the proton current was compensated by co-migration of thiocyanate. Thus the inhibition of proton uptake observed in the first case must be due to the inhibition by dibucaine of the valinomycin-mediated K^+ efflux from the particles (Fig. 9; cf. ref. 5). The explanation for the inhibition by dibucaine of the aerobic H^+ influx observed in the K^+ medium in the absence of valinomycin is that electrogenic proton influx is, in this case, charge-compensated, at least in part, by spontaneous K^+ influx. It is concluded that dibucaine exerts a direct stimulatory action on the oxido-reduction linked proton translocation.

The stimulatory effect exerted by the local anaesthetic on the respiration-driven proton uptake is accompanied by an even more marked enhancement of the passive proton back-flow. In fact, also when the rate of proton influx was enhanced, the amount of protons taken up by submitochondrial particles at the steady-state was reduced by dibucaine. Stimulation by dibucaine of both proton influx and efflux resulted in acceleration of the respiration linked proton turnover at the steady-state (Fig. 5). The increased proton-turnover led to energy dissipation and inhibition of oxidative phosphorylation (Fig. 10).

Kinetic analysis of the effect of dibucaine on the anaerobic proton release shows that the local anaesthetic stimulated both the fast proton-cation exchange-diffusion and the slow non-coupled proton diffusion in submitochondrial particles (Fig. 6; see refs. 7-9). The extent of stimulation of the latter process was however considerably higher. Dibucaine inhibited the aerobic K^+ uptake which accompanied, in the absence or presence of nigericin, the respiration-driven proton uptake by submitochondrial particles (Fig. 8). We have presented evidence that, in the absence of nigericin, the aerobic K^+ uptake by submitochondrial particles is mediated by a proton-cation exchange system of the

membrane which, in anaerobiosis, catalyzes the fast exchange component of the proton diffusion [9]. Since this system is apparently uninhibited by dibucaine it is conceivable that the depression of the spontaneous aerobic K^+ uptake is due to the reduction of the aerobic proton gradient caused by the anaesthetic. However the experiment of Fig. 8 shows that dibucaine has a direct inhibitory action on the nigericin-mediated K^+ translocation.

According to the fluid-mosaic structure [17], a model which is apparently supported by various experimental approaches [18-21], the mitochondrial membrane is made up of a continuous phospholipid bilayer with proteins embedded in the lipid matrix. The similarity of the effects of ionophores and of local anaesthetics on K^+ translocation in artificial phospholipid membranes and mitochondrial membranes [5, 6] suggests that spontaneous, noncoupled as well as valinomycin and nigericin mediated K^+ translocation in mitochondria take place across lipid-bilayer regions of the membrane. The lipophilic K^+-ionophore complexes facilitate ion diffusion in the hydrophobic phase. On the contrary positively charged local anaesthetics bind to the phospholipid polar heads exposed on the membrane surface, cause a positive change in the surface potential and hence inhibit K^+ migration across the phospholipid bilayer. Dibucaine on the other hand does not inhibit rather stimulates both the respiration-driven proton translocation as well as the passive proton-diffusion reactions. This indicates that molecular components, different from the phospholipids reactive towards local anaesthetics, are involved in proton translocation across the mitochondrial membrane.

It has been shown that local anaesthetics react with phosphodiester groups of cephalins and that this reaction is characterized by proton release from the phospholipids [1, 2]. It is conceivable that protonation of basic groups of membrane phospholipids depresses proton translocation across the mitochondrial membrane by lowering the proton concentration in the vicinity of the active sites of carrier-molecules. The inhibitory effect of the phospholipids will be relieved by the binding of local anaesthetics to their basic groups. This explanation is supported by the observation that the concentration of dibucaine required to give maximal stimulation of the proton diffusion reactions in submitochondrial particles amounted to about 300 nmol/mg membrane protein. This figure roughly corresponds to the amount of cephalins present in the inner mitochondrial membrane [19, 22]. In line with the view that proton binding to membrane phospholipids hinders proton translocation would also be the possibility that the increase of the velocity constants of the two proton diffusion reactions caused by dibucaine is due to an increase of the frequency factor of the two reactions (coefficient A in equation (1)). The anaesthetic by impeding proton binding to structural phospholipids might increase the number of useful collisions of protons

with molecular components of the membrane directly involved in their translocation.

The energy state of mitochondria is affected by proton and electrolyte flows across the mitochondrial membrane. The low efficiency of oxidative phosphorylation, and other energy transfer reactions in submitochondrial particles, appears to be the consequence of an increased energy-linked proton turnover across the mitochondrial membrane [23]. The present study indicates that membrane phospholipids, by controlling proton and electrolyte translocation across the mitochondrial membrane, may play a prominent role in energy conservation and transfer in mitochondria (see also refs. 19, 24).

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