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Uncoupling effects of local anesthetics on rat liver mitochondria

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We demonstrate in this paper that bupivacaine, a local anesthetic, can act alone as an uncoupler of rat liver mitochondria. It stimulates state 4 respiration, induces a swelling in potassium acetate (in the presence of valinomycin), and collapses the transmembrane potential. Lidocaine, another local anesthetic, requires the presence of a lipophilic anion such as TPB^- to produce the same effects. TPB^- can also reinforce the action of bupivacaine. These differences in action of the two local anesthetics can be explained by the difference in their liposolubility.

Mitochondria; Uncoupler; Local anesthetic; Electron transport chain; (Rat liver)

1. INTRODUCTION

Although local anesthetics are normally viewed as reagents for use in nerve blockade, these compounds may also modify a large variety of non-neuronal processes. Among the latter, it has been shown that they affect mitochondrial metabolism. In particular, local anesthetics have been reported to stimulate [1], or inhibit electron transport [2], and to uncouple oxidative phosphorylation [1,3].

In some instances, there are conflicting reports concerning the existence of these effects. A relevant case is the question of whether local anesthetics uncouple oxidative phosphorylation in mitochondria. This problem was discussed by Garlid and Nakashima [1]. They demonstrated that hydrophobic amines with local anesthetic properties can mimic weak acid uncouplers in both respiring and non-respiring mitochondria, and that this drug effect necessarily requires a lipophilic anion such as TPB^-, or SCN^-. To explain this observation, they proposed a new mechanism for uncoupling, in which the protonated, cationic amine forms a lipophilic ion pair with the appropriate anion.

The present study was undertaken to characterize better the uncoupler-like action of local anesthetic molecules. We used two local anesthetics: bupivacaine and lidocaine. Both are amines, and are positively charged at physiological pH (pK 8.1 and 7.9, respectively [4]). However, bupivacaine is highly liposoluble (n-heptane/pH 7.4 buffer partition coefficient = 27.5 [5]), whilst

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Abbreviations: CCCP, carbonyl cyanide m-chlorophenylhydrazone; TPB^-, tetraphenylboron; bupivacaine, 1-butyl-N-(2,6-dimethylphenyl)-2-piperidinecarboxamide; lidocaine, 2-(diethylamino)-N-(2,6-dimethylphenyl)acetamide

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lidocaine is only slightly liposoluble (*n*-heptane/pH 7.4 buffer partition coefficient = 2.9 [5]). We demonstrate in this paper that, contrary to the conclusions of Garlid and Nakashima [1], the uncoupling effect of local anesthetics depends on their liposolubility and does not always require the presence of a lipophilic anion.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Sucrose was purchased from Merck. Bupivacaine and lidocaine were gifts from Laboratoires Roger Bellon. Other chemicals were from Sigma.

2.2. *Preparation of mitochondria*

Mitochondria were isolated from rat liver (Wistar) by differential centrifugation as described by Kligenberg and Slenczka [6]. The buffer composition was 0.25 M sucrose, 2 mM EDTA, 20 mM Tris-HCl, pH 7.2. Proteins were determined by the biuret method, using bovine serum albumin as the standard.

2.3. *Rate of respiration*

Oxygen consumption was measured polarographically at 28°C, using a Clark electrode. The composition of the respiration buffer was 0.2 M sucrose, 15 mM KCl, 5 mM Tris phosphate, 20 mM Tris-HCl, pH 7.2. Mitochondria (2 mg/ml) were incubated in the presence of rotenone (2.5 μg/mg) and 2 mM succinate.

2.4. *Swelling*

Osmotic swelling was recorded at 546 nm and 28°C, using an Eppendorf spectrophotometer. Mitochondria were incubated in 0.1 M potassium acetate with 10 mM Tris-maleate, pH 7.2, in the presence of antimycin (0.2 μg/mg) and oligomycin (25 μg/mg).

2.5. *Measurements of* $\Delta\psi$

The matrix space was determined using tritiated water and the inner-membrane-impermeable [^{14}C]mannitol, $\Delta\psi$ being determined from the distribution of ^{86}Rb (in the presence of valinomycin) [7]. Routinely, after equilibration (2 min), mitochondria were separated from the medium by rapid centrifugation (20 s) through a silicone oil layer (silicone AR 200 fluid).

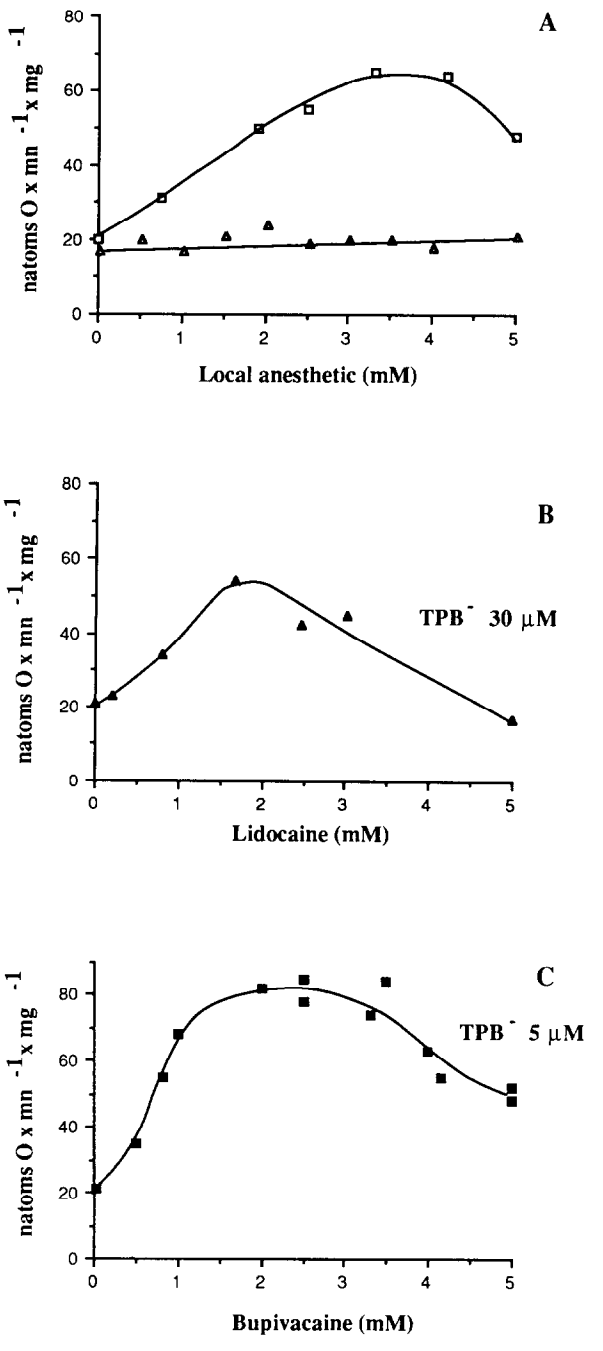

Fig.1. Local anesthetic effects on mitochondrial respiration. Mitochondria (2 mg/ml) were incubated in respiration buffer, in the presence of 2 mM succinate and 2.5 μg/mg rotenone. Effect of (A) bupivacaine (□) and lidocaine (△); (B) lidocaine in the presence of 30 μM TPB^-; (C) bupivacaine in the presence of 5 μM TPB^-.

In all the experiments, it was verified that TPB^- alone had no effect at the concentration used.

3. RESULTS

3.1. *Effect of local anesthetics on state 4 respiration*

Fig.1 shows the effects of bupivacaine and lidocaine on state 4 respiration. Lidocaine (fig.1A) has no effect on the respiratory rate at concentrations ranging between 0 and 5 mM. In contrast, the curve as a function of bupivacaine concentration is biphasic, exhibiting stimulation of respiration at low concentrations, followed by inhibition at higher levels. This behaviour is also observed with lidocaine, but only in the presence of 30 μM TPB^- (fig.1B). TPB^- also decreases the concentration at which this behaviour is observed with bupivacaine (fig.1C). In this case, 5 μM TPB^- is sufficient to demonstrate a significant effect. This biphasic behaviour indicates the existence of at least two effects or of a pleiotropic action of the drug. The inhibition of respiration is probably caused by the inhibition of one or more constituents of the respiratory chain. This hypothesis can be conveniently checked by studying the effect of the drug (with or without TPB^-) on the uncoupled respiration. Under these conditions, variations in membrane permeability do not interfere with inhibition of the respiratory chain, because the permeability is always maximum. Fig.2 illustrates the inhibition of uncoupled respiration by bupivacaine in the presence of 0.33 μM CCCP. The inhibition is reinforced in the presence of 5 μM TPB^-. These results suggest a direct effect of bupivacaine on a component of the respiratory chain. On the other hand, the stimulation of respiration could be due to a protonophore-like effect of the local anesthetics, affecting the H^+ permeability and, thus, the proton-motive force $\Delta\mu H^+$. The H^+ permeability was studied using the osmotic swelling method and $\Delta\psi$, the main component of $\Delta\mu H^+$, was evaluated as a function of local anesthetic concentration.

3.2. *Swelling in potassium acetate*

When rat liver mitochondria are incubated in 0.1 M potassium acetate, swelling necessitates simultaneous permeabilization to K^+ (valinomycin) and to H^+ (by addition of a protonophore such as CCCP) (fig.3A). Fig.3B,C shows that bupivacaine can take the place of CCCP to promote swelling in the presence of valinomycin (added before or after bupivacaine), demonstrating the role of this drug as a protonophore. As in fig.1, lidocaine alone has only a slight effect on swelling and requires the presence of TPB^- in order to play a protonophoric role (not shown). As expected 5 μM TPB^- reinforces the role of bupivacaine (fig.3B,C).

3.3. *$\Delta\psi$ measurements*

Table 1 demonstrates that $\Delta\psi$ decreases with increasing bupivacaine concentration. In the presence of 5 μM TPB^- the decrease in $\Delta\psi$ is enhanced. These results are in accordance with the stimulation of state 4 respiration and the swelling in potassium acetate.

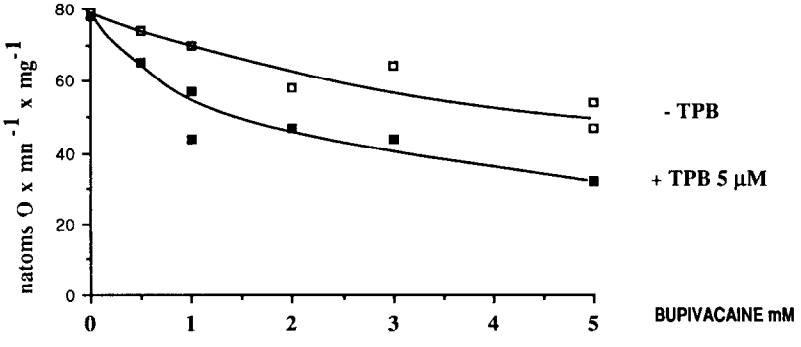

Fig.2. Inhibition of uncoupled respiration by bupivacaine. Experimental conditions as in fig.1 except that 0.33 μM CCCP was added. (□) Without TPB^-, (■) with 5 μM TPB^-.

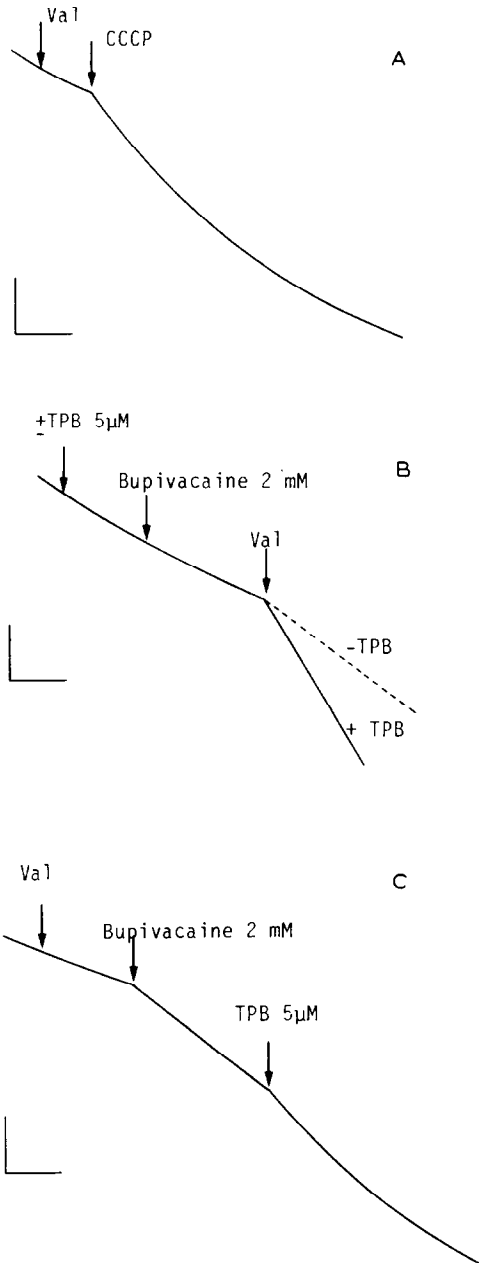

Fig.3. Iso-osmotic swelling in potassium acetate. Mitochondria (2 mg/ml) were incubated in a solution of 0.1 M potassium acetate and 10 mM Tris-maleate, pH 7.2, in the presence of 0.2 μg/mg antimycin and 25 μg/mg oligomycin. (A) Addition of valinomycin (50 ng/mg) and CCCP (1 μM) led to a swelling by K^+ influx and H^+ efflux. (B,C) Bupivacaine can replace CCCP, but not valinomycin. Bars indicate (vertically) 0.05 *A* and (horizontally) 1 min.

Table 1

[Bupivacaine] (mM)	$\Delta\psi$ (mV)	
	$-$ TPB$^-$	+ 5 μM TPB$^-$
0	166	160
0.5	144	110
3	80	20

4. DISCUSSION

The results presented in figs 1–3 and table 1 illustrate several features in the action of local anesthetics on mitochondrial metabolism.

(i) Contrary to the statement of Garlid and Nakashima [1], local anesthetics such as bupivacaine can modify state 4 respiration (fig.1A) or H^+ permeability (fig.3A) and $\Delta\psi$ (table 1), even in the absence of a lipophilic anion. When we compare the liposolubility of these local anesthetics with those which alone have no effect (lidocaine and those studied in [1]), it is reasonable to suggest that this effect on respiration depends on the liposolubility of the local anesthetic under study. This idea is reinforced by the observed role of TPB$^-$ which is known to facilitate the transport of cations across biological membranes.

(ii) The swelling experiments illustrated in fig.3 unambiguously show that bupivacaine can act alone as a protonophore. This property can be simply accounted for by the liposolubility of the drug, which can cross the membrane in either its neutral or protonated form, leading to a cycle of H^+ re-entry (fig.4B).

(iii) Swelling experiments are, however, rather different from those used for respiratory rate measurements because, in swelling experiments, the mitochondria are not energized. When a transmembrane potential is created (fig.1, table 1), it is expected that the main phenomenon should be an accumulation of protonated bupivacaine down its chemical potential gradient, collapsing the $\Delta\psi$ (fig.4A). Such accumulation of a local anesthetic has been demonstrated in large unilamellar vesicles in response to a membrane potential [8]. This electrophoretic movement (fig.4A), associated with the protonophoric mechanism of fig.4B, can ensure the stimulation of respiration (fig.1) and the $\Delta\psi$ decrease.

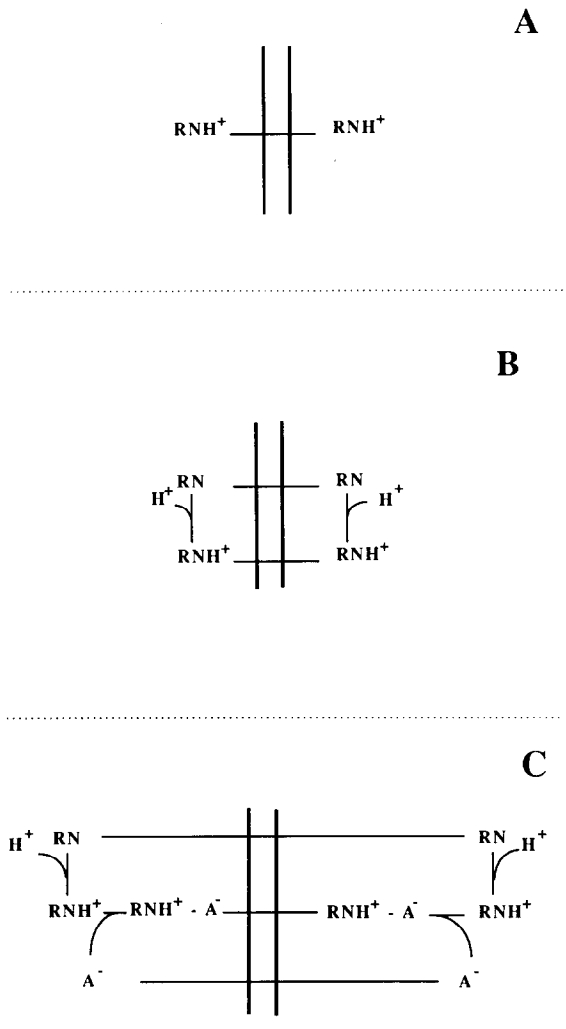

Fig.4. Models of uncoupling by local anesthetics. (C) Model proposed by Garlid and Nakashima [1]. A^-, lipophilic anion (TPB^- in our case); RN and RNH^+, neutral and protonated forms of the local anesthetics, respectively.

(iv) The inhibition of respiration can be explained by an inhibition of one or several constituents of the respiratory chain (fig.2). These results are in line with the observations of Chazotte and Vanderkooi [2] that demonstrate the existence of several sites or regions of the mitochondrial electron transport chain which are affected by tertiary amine local anesthetics.

(v) The necessity to add TPB^- in order to observe the same effects with lidocaine, or to decrease the efficient bupivacaine concentration, substantiates the model of Garlid and Nakashima [1] involving the formation of a neutral complex ($R-NH^+-TPB^-$) for transport across the mitochondrial membrane (fig.4C). The lipophilic anion and the neutral form of the local anesthetic (17% in the case of lidocaine at pH 7.2) can cross back through the membrane independently. However, as for bupivacaine, the respiratory stimulation is probably due mainly to electrophoretic accumulation of lidocaine mediated by the single TPB^- cycle (A^- cycle at the bottom of fig.4C), with the minor participation of the entire mechanism to explain the protonophoric effect in swelling experiments. The fact that the addition of TPB^- reinforces the action of bupivacaine indicates that a complex of bupivacaine with TPB^- can also be formed and that all mechanisms in fig.4 can operate simultaneously.

It emerges from this study that the anesthetic action of local anesthetics depends greatly on their liposolubility and on the natural presence of lipophilic anions. It involves different types of mechanisms: direct uncoupling by electrophoretic entry of the cationic form of the amine, protonophoric effects and the inhibition of mitochondrial membrane activities including respiratory chain components. The present findings could be of general pharmacological and toxicological significance. In particular, it can be thought that liposoluble local anesthetics can cross the cytoplasmic membrane and accumulate in mitochondria as a result of their high transmembrane potential (as has been reported for rhodamine 123 [9]), leading to the uncoupling of oxidative phosphorylation in vivo and a decrease in mitochondrial metabolism. This point is now under study in our laboratory.

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