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# Is bupivacaine a decoupler, a protonophore or a proton-leak-inducer?

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This paper deals with the mechanism of bupivacaine uncoupling of oxidative phosphorylation in rat heart mitochondria. By comparison with the effects of QX 572, a permanently charged quarternary amine-type local anesthetic, it is concluded that the effects of bupivacaine and QX 572 may be explained by classical uncoupling behaviour. In the case of bupivacaine this uncoupling effect is mediated through a protonophore-like mechanism, whereas that of QX 572 is simply explained by an electrophoretic uptake.

Local anesthetic; Bupivacaine; Uncoupling; Mitochondria; Rat heart

## 1. INTRODUCTION

Bupivacaine, a local anesthetic of the tertiary-amine type, has been reported to affect the mitochondrial energy mechanism by uncoupling [1–7], but the mechanism is still under discussion. Different uncoupling modes have been proposed: bupivacaine may act as a protonophore [1,3,4] or as a decoupler [5,7]. The term protonophore, according to Rottenberg [8], is used to describe uncoupling agents that increase the proton permeability of membranes by a shuttling mechanism; this causes a decrease in the protonmotive force and simultaneously an increase in respiratory rate and a decrease in ATP synthesis. In contrast, the term decoupler describes agents which appear to uncouple oxidative phosphorylation without significant reduction of the protonmotive force [8].

Uncoupling by bupivacaine and other tertiary-amine local anesthetics is reinforced by lipophilic anions like tetraphenylboron ($TBP^-$), 1-anilinonaphtaleno-8-sulfonate (ANS) and picrate [3–5]. This observation has been taken by Terada et al. [5] as an indication for a third uncoupling mechanism, by the formation of ion-pairs within the membrane, where they increase proton conductance (proton-leak inducer).

Local anesthetics, which are tertiary amines, exist under physiological conditions in the protonated and deprotonated form. However, quarternary-amine analogs are always in the positively charged form, and therefore cannot act as protonophores. For this reason the latter could be useful reference compounds for the elucidation of the uncoupling mechanism of tertiary-amine local anesthetics. In the present paper the uncoupling action of bupivacaine and the quarternary-amine, QX 572 (Fig. 1), was studied under identical conditions with rat heart mitochondria. It is concluded that uncoupling by bupivacaine is due to a protonophore-like mechanism, whilst the uncoupling action of QX 572 (observed only in the presence of tetraphenylboron) seems to be best explained by dissipation of the electrochemical proton gradient caused by its electrophoretic uptake.

*Abbreviations:* CCCP, carbonylcyanide *m*-chlorophenylhydrazone; $TPB^-$, tetraphenylboron; VAL, valinomycin; BUPI, bupivacaine, 1-butyl-*N*-(2,6-dimethylphenyl)-2-piperidinecarboxamide; QX 572, dimethyldiphenyl-acetamido-ammonium; BCECF/AM, acetoxymethyl ester of 2,7-biscarboxy-ethyl-5(6)-carboxyfluorescein; $TPP^+$, tetraphenylphosphonium; RHM, rat-heart mitochondria

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## 2. MATERIALS AND METHODS

Male Wistar rats (250 g) were killed by cervical dislocation, and their hearts were rapidly put into ice-cold medium containing 70 mM sucrose, 210 mM mannitol, 10 mM EDTA and 50 mM Tris-HCl (pH 7.4). Mitochondria were isolated according to [9]. The mitochondrial pellet was resuspended in medium containing 75 mM sucrose, 225 mM mannitol, 0.1 mM EDTA and 10 mM Tris-HCl (pH 7.2). The protein content in the stock suspension was measured by the biuret method. Functional integrity was determined by measuring the respiratory control ratio with ADP and 5 mM glutamate plus 5 mM malate as substrates. Mitochondrial respiration was measured polarographically at 30°C using a Clarck-type electrode connected to a microcomputer giving an on-line display of rate-meter values. The incubation medium contained 25 mM sucrose, 75 mM mannitol, 100 mM KCl, 10 mM Tris-phosphate, 50 mM EDTA and 10 mM Tris-HCl (pH 7.4).

The transmembrane potential was estimated from the equilibration of tritiated tetraphenylphosphonium ($[^3H]TPP^+$) (0.5 μCi/ml) between mitochondria and medium. Mitochondria were separated from the medium by rapid centrifugation through a silicone oil layer. The radioactivity was counted in aliquots of the supernatant and the pellet, after washing and solubilization in a 2% SDS solution. Calculation of the transmembrane potential was based on a matrix volume of 1 μl/mg

protein. The data were corrected for non-specific binding of [$^3$H]TPP$^+$, estimated in separate experiments with de-energized mitochondria (incubated with 1 mM KCN plus 1 $\mu$M of the uncoupler, carbonylcyanide *m*-chlorophenylhydrazone (CCCP).

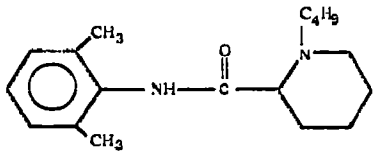
Passive proton permeability of the inner membrane in the presence and absence of local anesthetics was estimated by means of the swelling of non-respiring mitochondria in the potassium acetate/valinomycin system [10].

The effect of local anesthetics on the matrix pH was estimated using 2,7-biscarboxy-ethyl-5(6)-carboxyfluorescein (BCECF) as a pH-dependent fluorescent probe [11,12]. For this, mitochondria suspended in the isolation medium (25 mg/ml; 10 mM glutamate plus 10 mM malate) were loaded, 30 min at room temperature, with the membrane-permeable acetoxymethyl ester of the probe (BCECF/AM) (dissolved in dimethylsulfoxide) at a final concentration of 7 $\mu$M. After loading, mitochondria were centrifuged, washed, and resuspended in the isolation medium. The stock suspension was stored in ice. Fluorescence of BCECF-loaded mitochondria was recorded at 530 nm, using 509 nm as excitation wavelength.

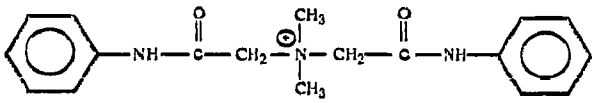
Bupivacaine was a gift from Laboratoire Roger Bellon (France); QX 572 was from Astra (Sweden); BCECF/AM was purchased from Molecular Probes Inc. (USA); [$^3$H]TPP$^+$ was from Amersham (Germany).

## 3. RESULTS AND DISCUSSION

Fig. 2 shows the dose-dependent effects of bupivacaine and QX 572 on the resting-state respiration of rat-heart mitochondria in the absence and presence of $TPB^-$. Bupivacaine stimulated respiration with and without $TPB^-$, a finding which is in line with recent results reported for rat-liver mitochondria [4–6]. In contrast, QX 572 did not stimulate respiration in the absence of $TPB^-$. However, with $TPB^-$ the respiration responded with a similar sensitivity to an addition of both local anesthetics. This synergistic effect of $TPB^-$ was attributed to the facilitated permeation of cationic local anesthetics across the inner membrane resulting from the formation of an electroneutral ion-pair [3]. Within this framework the missing effect of QX 572 on respiration (in the absence of $TPB^-$) may be explained by a lower lipophilicity of QX 572 (partition coefficient of 0.9 in the oleyl alcohol/phosphate buffer system;




BUPIVACAINE




QX 572

Fig. 1. Chemical structures of bupivacaine and QX 572.

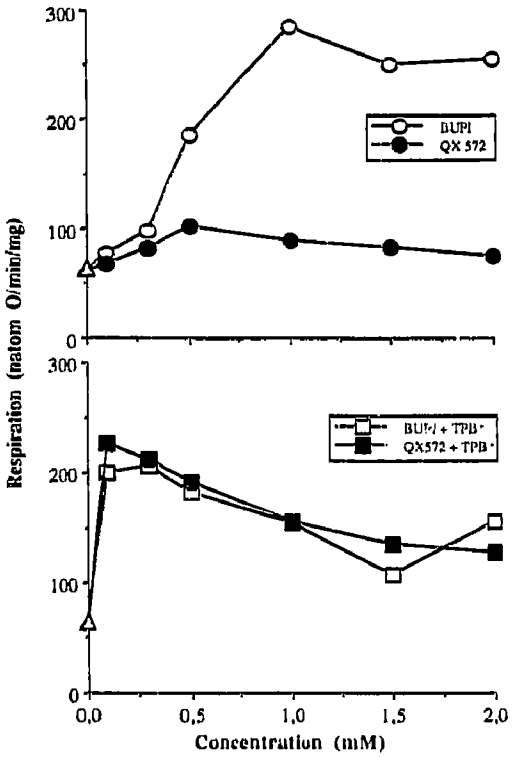



Fig. 2. Dose-response effect of bupivacaine and QX 572 on mitochondrial respiration in the resting state. Bupivacaine and QX 572 were added with and without 2 $\mu$M $TPB^-$ in the incubation medium containing rat-heart mitochondria (1 mg/ml) and 5 mM glutamate plus 5 mM malate as respiratory substrates. The data are the mean of three experiments.

Astra laboratory information) compared to bupivacaine (partition coefficient of 27.5 in *n*-heptane/phosphate buffer system [13]).

In order to see whether this respiration stimulation was due to a decoupling or an uncoupling mechanism we looked at the effect of both local anesthetics on the transmembrane potential. Since the maximal stimulatory effect of respiration by bupivacaine alone was obtained at a concentration of 1 mM (at higher concentrations inhibition occurs) we did not go beyond this concentration. Results are summarized in Fig. 3. Stimulation of respiration by bupivacaine (without $TPB^-$) was paralleled by a decrease in transmembrane potential (from 167 to 124 mV at 1 mM; see also [4,6]). A similar decrease was observed for both local anethetics in the presence of $TPB^-$ at the point of maximal stimulation of respiration (also from 165 to 135 mV at 0.2 mM). The further decrease in transmembrane potential can be explained by the observed inhibition of the respiratory chain at higher concentrations (see the lower panel of Fig. 2 and [4,5]).

All these results are in line with a classical uncoupling mechanism and clearly exclude a decoupling mechanism, contradicting the findings of Terada et al. [5] and van Dam et al. [7]. The fact that bupivacaine decreased

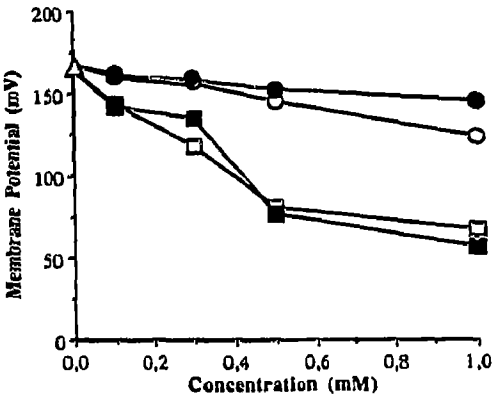



Fig. 3. Dose–response effect of bupivacaine and QX 572 on transmembrane potential. Experimental conditions and symbols are as for Fig. 2.

the transmembrane potential could be due to one of three modes of action. First, an increase in proton permeability of the inner membrane by a protonophoric mechanism. Second, an ion-leak could be formed as the results of incorporation of bupivacaine into the phospholipid membrane. Finally, the stimulation of respiration could be the consequence of the electrophoretic uptake of bupivacaine into the matrix space of the mitochondria when the membrane potential is negative inside.

To see whether the decrease in the membrane potential was due to an increased membrane permeability to protons the swelling of non-respiring mitochondria was studied with bupivacaine and QX 572 in the presence of potassium acetate and valinomycin. Acetate crosses the membrane in the neutral protonated form (AcH). Valinomycin allows the penetration of $K^+$. To maintain the electroneutrality of this process the $H^+$ accompanying acetate must go out again. This is obtained with an uncoupler such as CCCP. Conversely, any drug mediating mitochondrial swelling with the obligatory presence of valinomycin must permeabilize the membrane towards $H^+$ (and not towards $K^+$). Fig. 4 shows that bupivacaine was able to induce swelling of rat-heart mitochondria just like CCCP (this property of bupivacaine is reinforced by $TPB^-$), whereas the effect of QX 572 was negligible or very small (with $TPB^-$), a result which is simply explained by the inability of QX 572 to mediate a protonophoric effect.

The ability of bupivacaine to increase the proton permeability of the inner membrane can also be demonstrated with energized mitochondria. The expected effect was acidification of the matrix space which was followed by the fluorescence change of BCECF-loaded rat-heart mitochondria. It can be seen in Fig. 5 that bupivacaine induced a clear acidification of the matrix pH, whereas practically no effect was observed with QX 572. We had previously checked that there was no fluorescence change due to a direct interaction of all these drugs with BCECF.

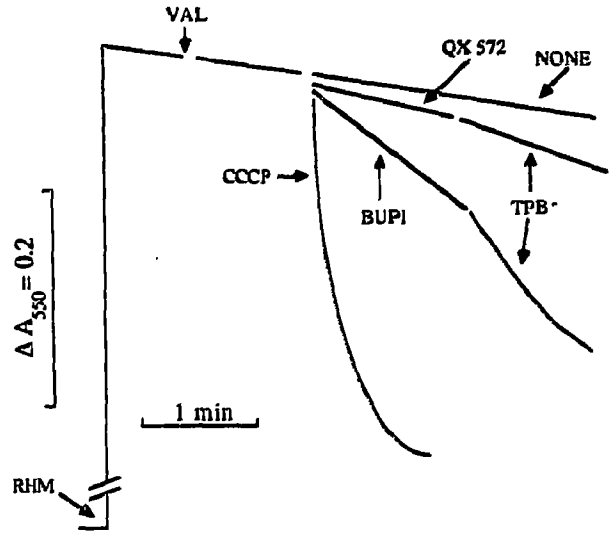



Fig. 4. Effect of CCCP, bupivacaine and QX 572 on passive swelling of mitochondria. CCCP (1 $\mu$M), bupivacaine (1 mM) and QX 572 (1 mM) were added to non-respiring rat-heart mitochondria suspended in 100 mM potassium acetate and 10 mM Tris-maleate buffer, pH 7.2, in the presence of 0.2 $\mu$g/ml antimycin and 25 $\mu$g/ml oligomycin. Valinomycin was added to an amount of 50 ng/mg protein, $TPB^-$ concentration was 2 $\mu$M. Swelling was measured as a decrease in optical absorption at 550 nm.

Comparing the effects of QX 572 (which cannot be protonated) and bupivacaine can lead to a better understanding of the uncoupling effect of the latter. The observed stimulation of respiration with both QX 572 and bupivacaine can be explained by a classical uncoupling mechanism, as evidenced by a decrease in the transmembrane potential and ATP synthesis (not shown). Terada et al [5] have proposed that bupivacaine forms intramembrane ion-pairs that create an electrophoretic conductance pathway for protons. In view of this it is

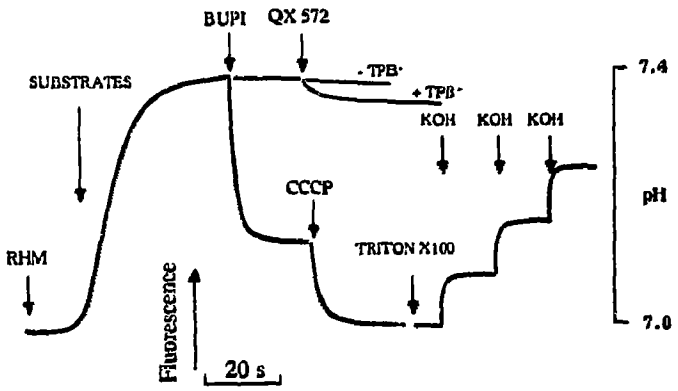



Fig. 5. Effect of bupivacaine and QX 572 on the matrix pH. BCECF-loaded mitochondria (0.5 mg/ml) were suspended in incubation medium (pH 7.0) containing 5 mM glutamate plus 5 mM malate, but without phosphate. The local anesthetics were added to a final concentration of 1 mM. At the end of each incubation CCCP (1 $\mu$M) was added to the cuvette. For calibration of fluorescence changes Triton X-100 (0.1% v/v) was added and the pH values were measured in the cuvette by a micro-electrode after addition of KOH aliquots.

not understandable why a leakage-type ion pathway would be not formed by the quarternary-amine, QX 572. An electrophoretic uptake of bupivacaine, facilitated by $TPB^-$ and similar to the case of QX 572, cannot be excluded. Nevertheless, under some experimental conditions we have proved that bupivacaine acts as a protonophore-like compound. This is the case in swelling experiments where proton movement is obligatory; in these experiments the action of bupivacaine exactly mimics thath of CCCP. This conclusion on the protonophoric action od bupivacaine is reinforced by the observation of acidification of the matrix space in the experiments in Fig. 5. In addition, there is no reason to assume a different uncoupling mechanism for bupivacaine with and without $TPB^-$. Taking all our results together, it seems more likely that the increased permeability of the inner membrane to protons by bupivacaine is the result of a protonophore-like action.

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