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Effect of Local Anesthetic Ropivacaine on Isolated Rat Liver Mitochondria

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ABSTRACT. Ropivacaine is a new long-acting aminoamide local anesthetic with a reduced systemic and cardiac toxicity. Since the latter seems to be related, at least partially, to an interference with mitochondrial energy transduction, the effect of ropivacaine on the metabolism of rat liver mitochondria was studied. Ropivacaine alone exhibited little effect on mitochondrial metabolism, whereas effects were strongly enhanced by tetraphenylboron (TPB^-) anion. At low drug concentrations, state 4 respiration was stimulated and mitochondrial membrane potential collapsed. At higher concentrations, state 4 and uncoupled respiration were inhibited by impairment of electron transfer from NAD- and flavine adenine dinucleotide-linked substrates to the respiratory chain. The fact that TPB^- increased drug effects indicated that stimulation of respiration was due to dissipation of the electrochemical proton gradient caused by its electrophoretic uptake, although a classical uncoupling mechanism cannot be excluded. The mechanism for the lower toxicity of ropivacaine *in vivo* was ascribed to low liposolubility leading to reduced access to the mitochondrial membrane, resulting in a minimal perturbation of mitochondrial metabolism. BIOCHEM PHARMACOL **58**;6:1009–1016, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. local anesthetics; ropivacaine; mitochondria; uncoupler; electron transport chain; rat liver

Although the most relevant physiological effect of local anesthetics is their ability to block the action potential responsible for nerve conduction thus causing both sensory and motor paralysis, they can modify a large variety of non-neuronal processes [1]. Among these, it has been shown that local anesthetics interfere with several mitochondrial functions. In particular, they stimulate and inhibit electron transport [2–5], uncouple oxidative phosphorylation [3, 6–8], stimulate and inhibit cation transport [9–12], and inhibit F_1ATPase [13, 14]. Nevertheless, some local anesthetics, such as bupivacaine, show a severe cardiotoxicity associated with their clinical use. Several mechanisms, e.g. the inhibition of adenosine 3′,5′-cyclic monophosphate production [15], and L-type Ca^{2+} current [16], have been proposed to explain this toxic effect. However, the marked depression of myocardial contractile force induced by bupivacaine cannot be explained on the basis of its electrophysiological properties alone. Indeed, it has been demonstrated that the cardiodepressant effect of bupivacaine is almost counteracted by ATP [17, 18], thus suggesting that its cardiotoxicity might be at least partially due to an interference with mitochondrial energy transduction [4, 19]. Local anesthetics are mostly tertiary amines with pKa values ranging from 7 to 9, so that both cationic and neutral forms could have several biological effects. The overall effects should depend on the relative amount of these molecular species and their hydrophobicities [6].

The cardiac toxicity of bupivacaine stimulated research to develop a less toxic local anesthetic. The result was ropivacaine, the (*S*)-enantiomer of 1-propyl-2′,6′ pipecoloxylidide, a new aminoamide long-acting, injectable local anesthetic which produces relatively less blockade of motor fibers than does bupivacaine, but with similar sensory blockade [20]. Even though structurally very similar to bupivacaine, ropivacaine causes fewer central nervous system symptoms and possesses a higher threshold for systemic and cardiac toxicity [21, 22]. Ropivacaine is slightly liposoluble, as demonstrated by a partition coefficient lower than that of bupivacaine, i.e. 6.1 versus 27.5 in *n*-heptane, suggesting that its lower toxicity could involve reduced impairment of mitochondrial metabolism.

Employing a widely used model to investigate the effect of several local anesthetics on mitochondria [3–5, 23–25], we present data on the effect of ropivacaine on state 4 and FCCP‖-uncoupled respiration, the electron flow through specific segments of the respiratory chain, the redox state of endogenous $NAD(P)^+$ and cytochrome *b*, mitochondrial potential, and membrane permeability in isolated rat liver

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‖ *Abbreviations:* FCCP, carbonyl cyanide *p*-trifluormethoxyphenylhydrazone; RP, ropivacaine; and TPB^-, tetraphenylboron.

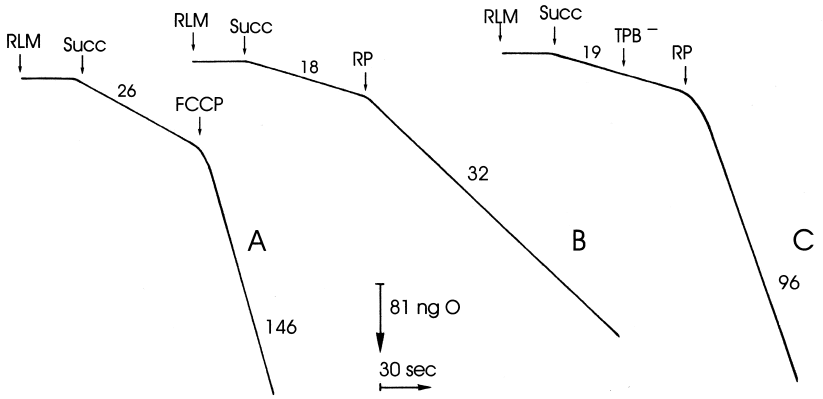

FIG. 1. Typical polarographic traces showing the effect of ropivacaine on state 4 respiration of rat liver mitochondria (RLM). Mitochondria (3 mg protein), succinate (Succ; 5 mM), ropivacaine (2 mM), FCCP (0.5 μM), and TPB^- (5 μM) were added as indicated by arrows. The numbers along the traces represent the rate of oxygen consumption (ng atoms O min^{-1} mg^{-1}). The experiments were repeated four times and yielded reproducible results (±5%). $P < 0.01$ versus unstimulated respiration.

mitochondria. The experiments were essentially designed to obtain information on the site(s) as well as on the mechanism of action of ropivacaine.

MATERIALS AND METHODS

Chemicals

The following chemicals were purchased from the indicated sources: fatty acid-free BSA, EGTA, FCCP, HEPES, and safranine O from Sigma Italia; glutamate, malate, succinate, nigericin, oligomycin, valinomycin, and antimycin A from Boehringer Italia; and rotenone and duroquinol from K&K Laboratories. All other reagents were of analytical grade and were purchased from BDH Italia. Ropivacaine–HCl was a gift from Astra Farmaceutici.

Preparation of Mitochondria

Rat liver mitochondria were isolated from adult male Sprague–Dawley rats, fasted overnight according to Pedersen *et al.* [26]. The mitochondria were resuspended in a minimal volume of H-medium (70 mM sucrose, 210 mM mannitol, 2.1 mM Li-HEPES, pH 7.20) without BSA at a concentration of 50 mg/mL. Protein content was determined by the biuret method in the presence of 0.2% deoxycholate, using BSA as the standard [27]. Only mitochondrial preparations with a respiratory control ratio ranging between 5 and 8 were used.

Assay of Oxygen Consumption

The rates of oxygen consumption were determined with a Clark oxygen electrode (Yellow Spring Instruments Co.) equipped with an ultrathin Teflon membrane. The electrode was inserted horizontally in a thermostatted closed chamber of 2.0 mL capacity (Gilson Medical Electronics) and contained final concentrations of 180 mM sucrose, 40 mM KCl, 3 mM Li-HEPES (pH 7.20), 1 mM EGTA, and 3.0 mg of mitochondrial protein. Other additions were described in the figure legends. Experiments were performed at 25°, and the solubility of dissolved oxygen was 442 ng atoms mL^{-1} when the medium was air-equilibrated at 760 torr (≈10,180 Pa) [28].

Spectrophotometric Determinations

The effect of ropivacaine on the oxidoreduction state of $NAD(P)^+$ and cytochrome *b* was evaluated by dual wavelength spectrophotometry (Aminco DW-2a) at 340–370 nm and 430–410 nm, respectively. The cuvette, thermostatted at 25°, was provided with magnetic stirring. The reaction medium was the same as that used for oxygen consumption, with a final volume of 2.5 mL. The addition of substrates and other compounds, as indicated in the figure legends, was performed by rapid injection from microsyringes in such a way as to achieve the shortest possible mixing time.

Estimation of Mitochondrial Membrane Potential

Variations in membrane potential were measured spectrophotometrically using the dye safranine O at 25°. Absorbance changes were monitored with an Aminco DW-2a spectrophotometer at the wavelength pair 511–533 nm [29]. $\Delta\psi$ was estimated by Nernst's equation as described previously [29]. Each experiment was repeated with at least five different mitochondrial preparations and the traces shown were from a representative experiment.

Swelling

Osmotic swelling was recorded at 550 nm and 25° using an Aminco DW-2a spectrophotometer. Mitochondria (0.8 mg/mL) were incubated in 0.1 M potassium acetate or nitrate with 10 mM Li-HEPES, pH 7.2, in the presence of rotenone, antimycin A (0.2 μg/mg), and oligomycin (25 μg/mg). Other additions are indicated in the figure legends.

RESULTS

Effect of Ropivacaine on Mitochondrial Respiration

In Fig. 1, representative polarographic traces of oxygen consumption by rat liver mitochondria as affected by ropivacaine are shown. Ropivacaine at 2 mM increased state 4 respiration of rat liver mitochondria respiring on succinate from 18 ng O min^{-1} mg^{-1} to 32 ng O min^{-1} mg^{-1} (trace B), whereas with a low concentration of

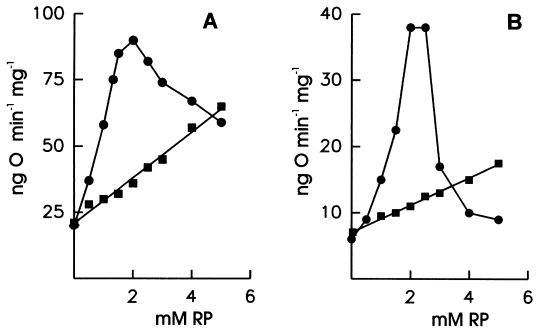

FIG. 2. (A) Effect of ropivacaine concentration on succinate oxidation in the presence (●) and in the absence (■) of 5 μM TPB^-. Mitochondria (3 mg protein) were added to air-equilibrated buffered medium, 5 mM succinate was then added at 1 min, and the rate of oxygen consumption recorded. When about 20% of dissolved oxygen had been utilized, an established concentration of ropivacaine was injected and the rate of subsequent oxygen uptake compared to that prior to drug addition. Each point represents the mean of twelve different mitochondrial preparations. Error bars within the symbols. (B) Effect of ropivacaine concentration on glutamate plus malate oxidation in the presence (●) and in the absence (■) of 5 μM TPB^-. Each point represents the mean of five mitochondrial preparations. Error bars within the symbols.

lipophilic anion TPB^- basal respiration rose to 96 ng O min^{-1} mg^{-1} (trace C). Ropivacaine partially released oligomycin-inhibited state 3 respiration, whereas in the presence of hydrophobic anion TPB^- the rate of oxygen consumption was similar to that stimulated by ADP (not shown).

Figure 2A shows the concentration-dependent effect of ropivacaine on the resting-state respiration of rat liver mitochondria oxidizing succinate in the presence or absence of TPB^-. The mitochondria were added to air-equilibrated medium, 5 mM succinate added at 1 min, and the rate of state 4 oxygen consumption was recorded. When approximately 20% of the available oxygen had been utilized, the drug was injected into the glass chamber and the rate of subsequent oxygen consumption determined. In the absence of the hydrophobic anion TPB^-, respiration increased linearly with drug concentration, but did not reach the maximum even at the highest concentration tested, i.e. 5 mM. Far more conspicuous was the effect with TPB^-: the curve was biphasic with respiration stimulated by low and inhibited by high drug concentration. Maximum oxygen uptake was reached at 1.5–2 mM ropivacaine. Figure 2B shows the effect of ropivacaine concentration on the oxidation of glutamate plus malate by rat liver mitochondria. The effect of the drug, both with and without TPB^-, was qualitatively similar to that observed with mitochondria respiring on succinate, but a remarkably greater sensitivity of complex I to inhibition by the drug was found. Indeed, at 3 mM ropivacaine the respiration rate was lowered from 38 ng O min^{-1} mg^{-1} to 17 ng O min^{-1} mg^{-1} (Δ%: −55), whereas with succinate the rate of oxygen uptake was decreased from 90 ng O min^{-1} mg^{-1} to 74 ng O min^{-1} mg^{-1} (Δ%: −18). At 5 mM ropivacaine, the rate of oxygen consumption was similar to that of the control, i.e. 9 ng O min^{-1} mg^{-1} versus 7 ng O min^{-1} mg^{-1}. The biphasic behavior was indicative of at least two different effects, and the inhibition of oxygen consumption was probably due to an impairment of component(s) of the respiratory chain. This hypothesis can be conveniently tested by evaluating the drug effect on uncoupled respiration. In this case, inhibition of the respiratory chain is not influenced by variations in membrane permeability, since it is always maximum.

Figure 3A shows the effect of increasing concentrations of ropivacaine (with and without TPB^-) on the respiration uncoupled by the addition of 0.5 μM FCCP. The rate of respiration decreased as drug concentration increased, and the inhibition of oxygen uptake was enhanced by TPB^-. The ability of ropivacaine to inhibit succinate-supported uncoupled respiration strongly suggested that its site of interaction should be localized in the Q → oxygen segment of the respiratory chain or between succinate and Q. To discriminate between these two possibilities, two different approaches were employed. The first experimental approach involved duroquinol as electron donor to site 2 of the respiratory chain. Figure 3B shows the rate of oxygen consumption when increasing concentrations of ropivacaine were added to a system containing 0.5 mM duroquinol in the presence of rotenone to inhibit electron flow from site 1 and FCCP so as to yield the maximal rate of duroquinol oxidation. It was observed that, with or without

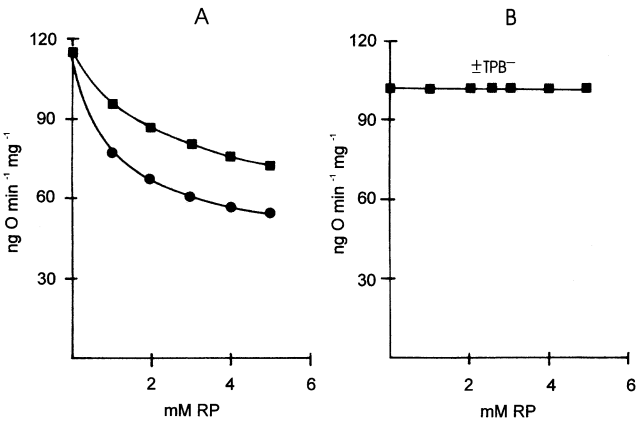

FIG. 3. (A) Concentration-dependent effect of ropivacaine on uncoupled respiration of rat liver mitochondria in the presence (●) and the absence (■) of 5 μM TPB^-. Mitochondria (3 mg protein) were preincubated for 1 min with the indicated concentration of ropivacaine in buffered medium (see Methods), 5 mM succinate was then added, and the rate of oxygen consumption recorded. After 20% of the dissolved oxygen was utilized, FCCP was added at a final concentration of 0.5 μM and the rate of oxygen uptake compared to that prior to the addition of FCCP. Each point represents the mean of seven different mitochondrial preparations. Error bars within the symbols. (B) Effect of ropivacaine concentration on duroquinol oxidation in the presence and absence of 5 μM TPB^-. The final concentration of duroquinol was 0.5 mM. Other experimental conditions were as in Fig. 3A. Each point represents the mean of seven different mitochondrial preparations. Error bars within the symbols.

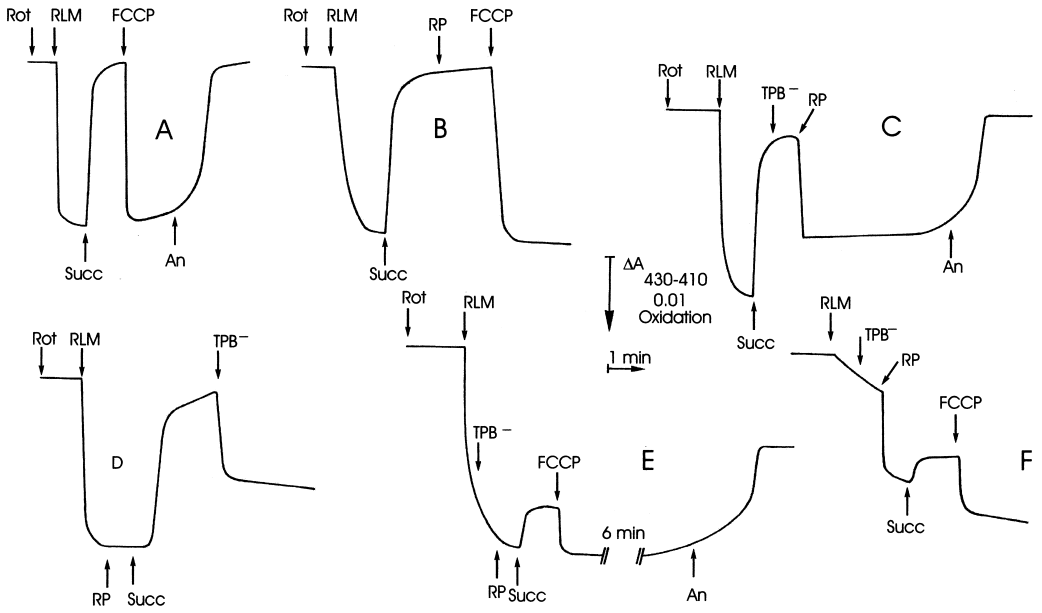

FIG. 4. Representative spectrophotometric trace showing the effect of ropivacaine on the oxidoreduction state of cytochrome *b* of rat liver mitochondria (RLM). Mitochondria (3 mg protein) were incubated in 2.5 mL of buffered medium at 25°. Two μM rotenone (Rot), 0.5 μM FCCP, 5 mM succinate (SUCC), 3 mM ropivacaine (RP), and 5 μM TPB⁻ were added as indicated by the arrows. An: anaerobiosis. Experiments were repeated five times and yielded reproducible results (±4%).

TPB^-, ropivacaine did not inhibit the high rate of duroquinol oxidation, thus clearly demonstrating that the Q → oxygen span of the respiratory chain was not the site of ropivacaine action.

Effect of Ropivacaine on the Oxidoreduction State of Mitochondrial Electron Carriers

The second type of experimental approach also excluded the segment Q → oxygen as the site of ropivacaine action. Figure 4 shows the effect of ropivacaine on the oxidoreduction level of cytochrome *b*. Preincubation of mitochondria in medium supplemented with rotenone, which inhibits electron flow from site 1 endogenous substrates, induced a large and rapid oxidation of cytochrome *b* (Fig. 4A). The addition of succinate, at the point shown, promptly reduced cytochrome *b*. The addition of FCCP caused a rapid oxidation of cytochrome *b*, as respiration was stimulated by the uncoupler. Then, 2 min later, dissolved oxygen was exhausted and cytochrome *b* became reduced, as indicated by the upward deflection of the 430–410 nm trace. In the absence of TPB^-, the addition of ropivacaine to succinate-reduced cytochrome *b* (Fig. 4B) did not modify the reduced cytochrome *b* which, on the contrary, was promptly and extensively reoxidized by the addition of the uncoupler. In contrast, ropivacaine, in presence of TPB^-, induced a partial (62%) and rapid oxidation of reduced cytochrome *b* (Fig. 4C). Nevertheless, because the rate of the oxygen consumption in the presence of ropivacaine (Fig. 1C) was lower than that of FCCP, the time taken to reach anaerobiosis was twice that of controls. A similar oxidoreduction pattern was observed when ropivacaine was added to state 1 mitochondria (no substrate, no ADP) with cytochrome *b* oxidized by rotenone (Fig. 4D). When TPB^- was added prior to ropivacaine (Fig. 4E), reduction by succinate of rotenone-oxidized cytochrome *b* was inhibited by approximately 80%. The reduced cytochrome *b* was then oxidized by FCCP. However, the time needed to reach anaerobiosis was far greater than controls, i.e. 10 min versus 2 min.

In state 1 mitochondria preincubated without rotenone, the slow, spontaneous oxidation of cytochrome *b* was greatly enhanced upon the addition of ropivacaine (Fig. 5F), implicating a second site between site 1-entering substrates and Q, at which ropivacaine may have inhibited electron transfer. To localize the site of action of ropivacaine, its effect on the oxidoreduction of $NAD(P)^+$ was, therefore, evaluated. Figure 5A shows the oxidoreduction pattern of NAD(P)H in state 4 (with substrate, no ADP). Preincubation of mitochondria resulted in oxidation of NAD(P)H. When the trace became stable, addition of glutamate plus malate induced a rapid reduction. NAD(P)H was rapidly and extensively reoxidized by FCCP and almost completely reduced by rotenone. Ropivacaine, in the absence of TPB^-, reversed substrate-induced reduction, but was unable to oxidize NAD(P)H further (Fig. 5B). Instead, in the presence of TPB^-, the extent of drug-induced NAD(P)H oxidation was similar to that found with FCCP (Fig. 5C). The addition of ropivacaine plus TPB^- to state 1 mitochondria induced a rapid and extensive oxidation of NAD(P)H, one only partially reverted by rotenone (Fig. 5D). Similarly, the extent of $NAD(P)^+$ reduction by substrates was about 50% lower (Fig. 5E) than in controls (Fig. 5A).

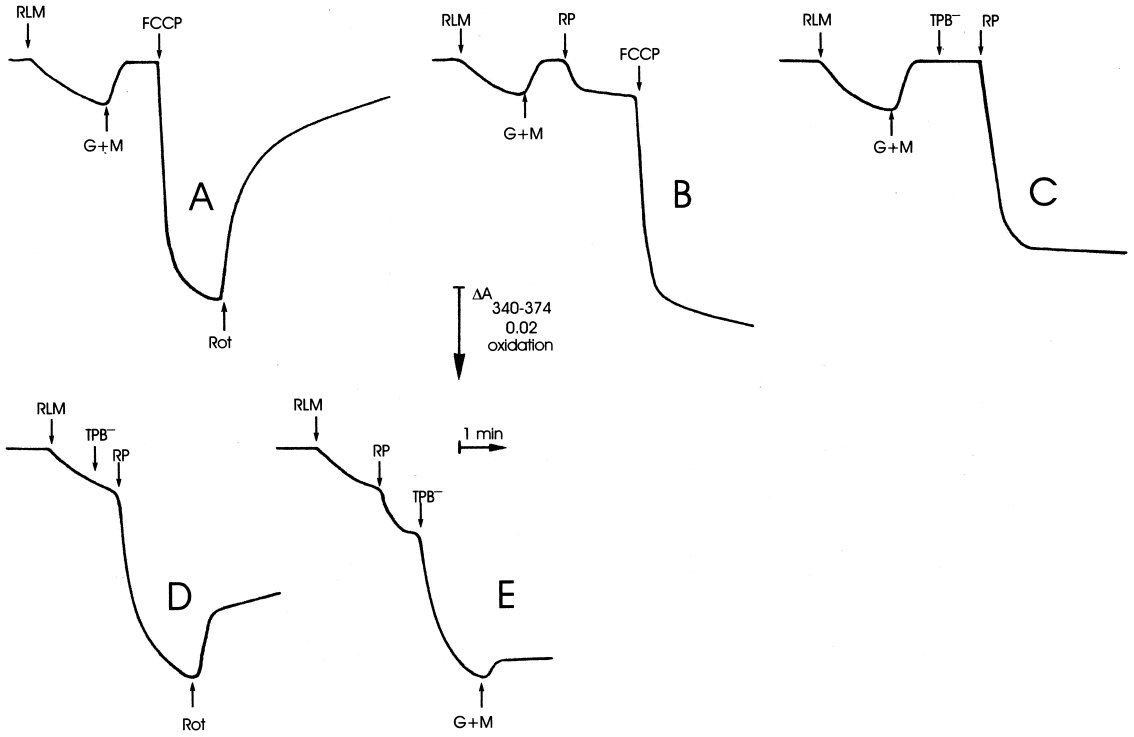

FIG. 5. Representative spectrophotometric trace showing the effect of ropivacaine on the oxidoreduction state of NAD(P)$^+$ of rat liver mitochondria (RLM) oxidizing glutamate plus malate (G + M). Other experimental conditions and abbreviations as in Fig. 4. Experiments were repeated five times and yielded reproducible results (±3%).

Effect of Ropivacaine on Mitochondrial Potential and Membrane Permeability

To ascertain whether the stimulation of respiration was due to an uncoupling mechanism, the effect of ropivacaine on transmembrane potential was investigated. Since the maximal stimulation of oxygen consumption by ropivacaine, in the presence of the hydrophobic anion TPB^-, was achieved by a concentration of 1.5–2 mM, with inhibition occurring at higher concentrations, we did not go beyond this concentration. The addition of succinate to rat liver mitochondria incubated with 10 μM safranine induced a membrane potential of 175 ± 5 mV (Fig. 6A) on the basis of the spectral shift and the comparison to the calibration curve with the same mitochondrial preparation (not shown). The addition of 1.5 mM ropivacaine, i.e. the concentration at which the maximal stimulation of state 4 respiration occurred (Fig. 2A), did not affect membrane potential at all, but the injection of the hydrophobic anion TPB^- completely dissipated membrane potential, as did the classical uncoupler FCCP (Fig. 6B).

To address the question of whether the decrease in membrane potential might be ascribed to an increased membrane permeability to protons, the swelling of nonrespiring mitochondria was studied with ropivacaine in potassium acetate as well as in potassium nitrate medium. When mitochondria were incubated in acetate buffer with rotenone and antimycin A to block electron flow from NADH-dehydrogenase and QH_2-cytochrome *c* oxidoreductase in the presence of valinomicyn, the addition of ropivacaine decreased absorbance at 55 nm (Fig. 7B), indicating that partial permeabilization of membrane towards H^+ had occurred. This effect was reinforced by the lipophilic anion TPB^-. Moreover, ropivacaine induced swelling, enhanced by TPB^-, in the absence of valinomycin (Fig. 7C).

A passive swelling of mitochondria was also observed if the suspending medium was KNO_3. In this case, valinomy-

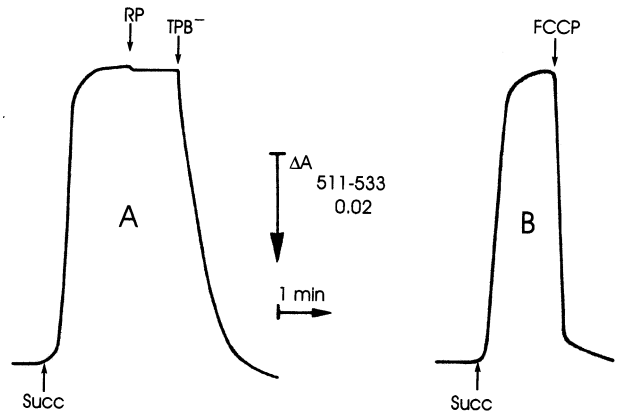

FIG. 6. Effect of ropivacaine on rat liver mitochondria membrane potential. Mitochondria (3 mg protein) were added to 2.5 mL of buffered medium containing 10 μM safranine. The final concentrations of FCCP, ropivacaine, succinate, and TPB^- were 0.5 μM, 1.5 mM, 5 mM, and 5 μM, respectively. Experiments were repeated five times and yielded reproducible results (±4%). Abbreviations as in Fig. 4.

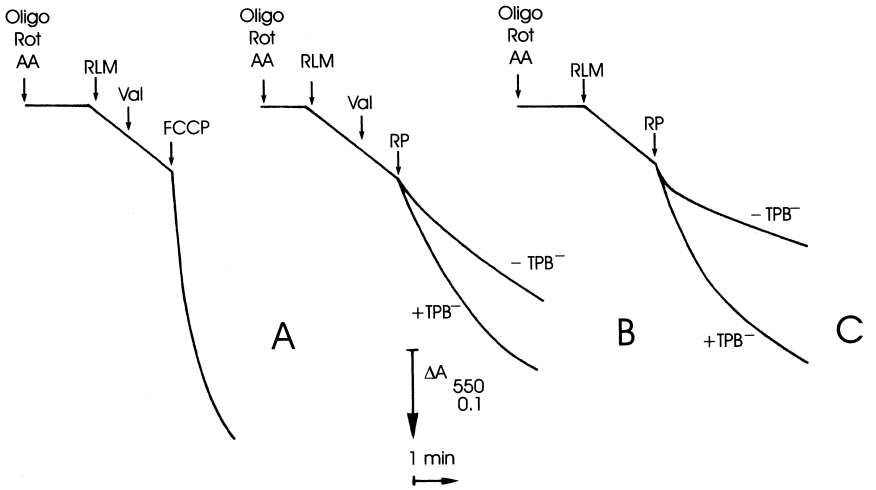

FIG. 7. Effect of FCCP and ropivacaine on passive swelling of rat liver mitochondria (RLM). At the points indicated, FCCP (0.5 μM), 2 mM ropivacaine (RP), and 5 μM TPB^- were added to non-respiring mitochondria (0.8 mg/mL) suspended in 2.5 mL of 100 mM potassium acetate and 10 mM HEPES buffer, pH 7.2, in the presence of 0.2 μg/mL antimicyn A (AA), 25 μg/mg protein oligomycin (Oligo), and 2 μM rotenone (Rot). Valinomycin (Val) was added to an amount of 50 ng/mg protein. Swelling was measured as a decrease in optical absorbance at 550 nm. Experiments were repeated five times and yielded reproducible results (±6%).

cin promoted the swelling, as NO_3^- was permeable across the inner mitochondrial membrane, thus maintaining electroneutrality (Fig. 8A). The addition of ropivacaine to mitochondria suspended in KNO_3 medium induced a decrease in optical density after a lag period of about 2 min (Fig. 8B), which was enhanced by TPB^- anion (Fig. 8B). Nigericin, in the absence of an uncoupler, did not induce mitochondrial swelling in KNO_3 in contrast to ropivacaine (Fig. 8C).

DISCUSSION

The results presented in this paper illustrate several features concerning the action of the local anesthetic ropivacaine on mitochondrial metabolism. Its mode of action was not unique, but involved a modification of inner mitochondrial membrane permeability and inhibition of membrane-bound enzyme activities. Ropivacaine had two main effects on liver mitochondria, as already observed for heart mitochondria [19]. Respiration was enhanced by low and inhibited by high drug concentrations. The stimulation of oxygen uptake was due to a partial uncoupling of oxidative phosphorylation, whereas its decrease must be ascribed to an inhibition of the respiratory chain at the level of complex I and II, with a remarkably higher effect on the former (Fig. 2B). The effect of local anesthetics on the energy-transducing processes in mitochondria is roughly proportional to their liposolubility [2, 4, 25], suggesting that the limited stimulatory effect of ropivacaine on state 4 respiration, both with site 1- and 2-entering substrates (Fig. 2), was due mainly to its low liposolubility. Lidocaine, which exhibits a lower partition coefficient, i.e. 2.9 in *n*-heptane [1], is unable to stimulate the basal rate of oxygen consumption even at high concentrations, i.e. 5 mM [4], whereas bupivacaine,

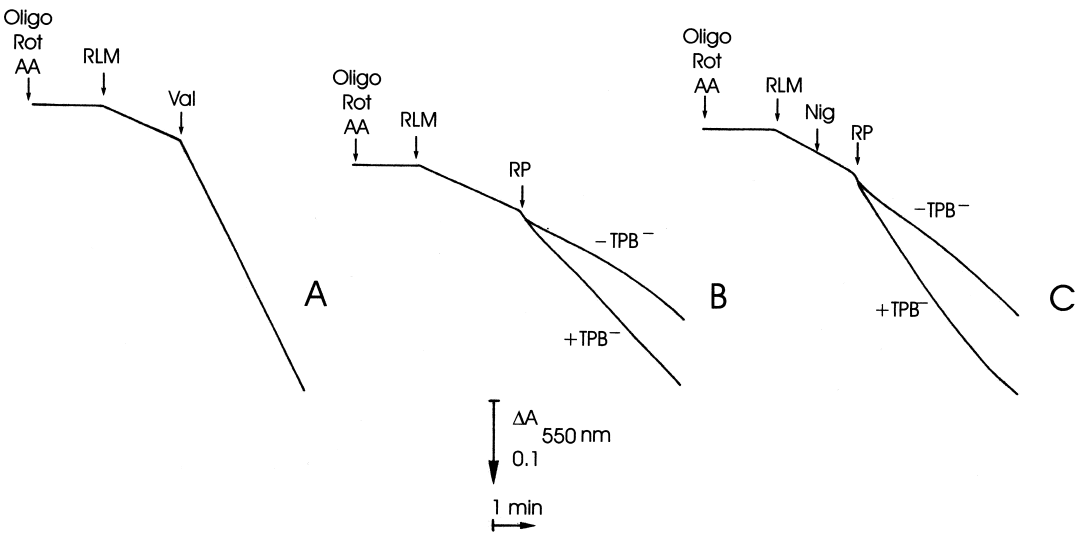

FIG. 8. Effect of FCCP and ropivacaine on passive swelling of rat liver mitochondria (RLM). At the points indicated, FCCP (0.5 μM), ropivacaine (2 mM), and TPB^- (5 μM) were added to non-respiring mitochondria (0.8 mg/mL) suspended in 2.5 mL of 100 mM potassium nitrate and 10 mM HEPES buffer, pH 7.2. Nigericin (Nig) was added to an amount of 50 ng/mg protein. Other experimental conditions and abbreviations as in Fig. 7. Experiments were repeated five times and yielded reproducible results (±5%).

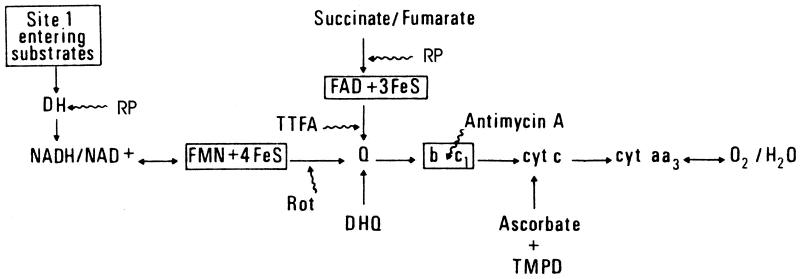

FIG. 9. Simplified scheme of the electron transport chain illustrating the suggested inhibition sites of ropivacaine (RP). DH, dehydrogenase; FMN, flavin mononucleotide; TTFA, 2-thenoyltrifluoroacetone; FAD, flavin adenine dinucleotide; Rot, rotenone; DHQ, duroquinol; cyt c, cytochrome c; TMPD, tetramethyl-*p*-phenylene-diamine.

with a high partition coefficient [1], is much more active both in stimulating and in inhibiting mitochondrial respiration [4, 5, 19]. The enhancing effect of the hydrophobic anion TPB^- (Fig. 1) can be explained on the basis of the model proposed by Garild and Nakashima [2], which implicates the formation of a neutral complex, RP-NH-TPB^-, in order to cross the inner mitochondrial membrane. Clearly, the lipophilic anion TPB^- and the neutral form of ropivacaine (12% at pH 7.20) can cross the mitochondrial membrane independently.

Swelling experiments performed in acetate medium and valinomycin, where proton movement was obligatory, suggested that ropivacaine may behave as a mild uncoupler. The neutral form of acetate (AcH) crosses the mitochondrial membrane, whereas valinomycin induces an electrical uniport for K^+. Since an overall charge balance across the membrane is required for swelling to occur, the H^+ accompanying the acetate must be extruded by an uncoupler such as FCCP (Fig. 7A). A similar effect, reinforced by TPB^-, was obtained by ropivacaine, whose action exactly mimicked that of FCCP (Fig. 7B). Furthermore, the RP-NH-TPB^- complex, but not ropivacaine alone, was able to replace valinomycin (Fig. 7C), demonstrating that the inner mitochondrial membrane was made permeable to K^+ as well. This was confirmed by mitochondrial swelling in KNO_3 medium and nigericin. Indeed, the capacity of nigericin to exchange K^+ and H^+ supports the uptake of cation, but an uncoupler must be added to recycle the protons extruded as a result of the obligatory K^+ exchange (Fig. 8C). Taken together, these results indicate a slight increase in inner mitochondrial membrane permeability to protons. However, the necessity of TPB^- to increase the drug effect suggested that stimulation of respiration in addition to the decrease and/or collapse of mitochondrial transmembrane potential was mainly due to an electrophoretic uptake of ropivacaine. However, an uncoupling mechanism by a futile cycle of H^+, similar to that described for the classical uncouplers [30] or bupivacaine and etidocaine [4, 5, 7], cannot be excluded. The inhibition of state 4 (Fig. 2, A and B) and uncoupled respiration (Fig. 3A) by ropivacaine was due to the inhibition of one or more constituents of the respiratory chain. The failure of ropivacaine, either in the presence or absence of lipophilic anion TPB^- (Fig. 3B), to inhibit the oxidation of duroquinol, which feeds electron directly to Q (Fig. 9), clearly demonstrated that the drug did not affect electron transport in the Q → oxygen span of the respiratory chain.

More detailed studies of the site(s) of action of ropivacaine were restricted to an examination of the point(s) at which the drug inhibited mitochondrial respiration. Spectrophotometric experiments demonstrated that the inhibition of succinate oxidation by the RP-NH- TPB^- complex was due to a block of electron transfer from substrate to Q, because of the inability of succinate to reduce the cytochrome *b* oxidized by the addition of rotenone (Fig. 4D). The oxidation of cytochrome *b* in state 1 mitochondria induced by the RP-NH- TPB^- complex (Fig. 4F) indicated that there must be a second point at which electron transfer was affected by the drug. The measurements of the redox state of $NAD(P)^+$ clearly demonstrated that ropivacaine also inhibited electron transfer from site 1-entering substrates to respiratory carriers (Fig. 9). Indeed, in mitochondria oxidizing endogenous substrates, ropivacaine induced NAD(P)H oxidation, which was only partially reverted by rotenone (Fig. 5D). Furthermore, NAD(P)H, reduced by the addition of glutamate plus malate, was oxidized by the RP-NH-TPB^- complex (Fig. 5C), confirming impairment of electron transport at the dehydrogenase–coenzyme level and suggesting two mechanisms of action: the first, a direct interaction of drug with enzymes, leading to partial protein denaturation and loss of enzyme activity; and the second, and more likely, that enzyme activities were affected by modified membrane fluidity following drug incorporation into membrane lipids. Protein hydrophobic sites are usually buried within the membrane so that small changes in fluid state may result in large changes in membrane-bound protein activity [25]. Indeed, the effect of local anesthetics on mitochondrial energy-transducing processes in mitochondria has been mainly ascribed to perturbation of membrane structure by decreasing bilayer order and/or to increasing membrane fluidity, which is an important factor in regulating membrane activity and biological functions [23, 24].

The results reported in this study show that ropivacaine, by itself, exhibits little effect on mitochondrial metabolism, but that in the presence of a hydrophobic anion, such as TPB^-, its effects are strongly enhanced. Therefore, a relevant question concerns whether the concentration of such anions *in vivo* is sufficiently high to influence mitochondrial energy-transducing processes in a decisive manner. Since the toxicity of ropivacaine *in vivo* is half that of bupivacaine [22, 31], it is reasonable to suppose that the role of lipophilic anions *in vivo* is negligible. This is supported by the observation *in vitro* that the lipophilic

anion TPB^- lowers the effective concentration of ropivacaine to values similar to those found for bupivacaine [4, 5]. Therefore, the lower toxicity of ropivacaine may be mainly ascribed to its low liposolubility, with reduced access to the mitochondrial membrane resulting in a limited perturbation of mitochondrial metabolism [32].

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