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SHORT COMMUNICATION

Mitochondrial Effects of L-Ropivacaine, a New Local Anesthetic

Guido Scutari,† Michela Marian,* Alberto Bindoli,‡ Maria Pia Rigobello,* Diana Deoni,* Ezio Vincenti§ and Marcantonio Bragadin¶*

*Department of Biological Chemistry, University of Padua, Padua; ‡CNR Center for the Study of Biomembranes, Padua; §Institute for Childhealth Burlo–Garofalo, Trieste; and ¶Department of Environmental Sciences, University of Venice, Venice, Italy

ABSTRACT. The effects of the local anesthetics ropivacaine and bupivacaine were investigated on isolated rat liver mitochondria. The efficiency of oxidative phosphorylation was evaluated by measuring the rates of respiration and ATP synthesis and the magnitude of the transmembrane electrical potential ($\Delta\psi$). Bupivacaine did not alter the ADP-stimulated respiration but strongly affected the resting respiration, which was more than doubled at 0.6 mM. In addition, it decreased the transmembrane electrical potential, and the ATP synthesis rate ($\Delta\psi$ was less than 100 mV at 0.6 mM). Ropivacaine did not alter the ADP-stimulated respiration, and the resting respiration seemed to be substantially unaffected up to 1.2 mM; a slight increase was observed at 1.8 and 2.4 mM. The transmembrane potential was decreased by anesthetic concentrations higher than 1.2 mM and ATP synthesis was consequently affected. The findings suggest that ropivacaine is less toxic than bupivacaine, in rat liver mitochondria. BIOCHEM PHARMACOL **56**;12:1633–1637, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. ropivacaine; bupivacaine; rat liver mitochondria; respiration; transmembrane electrical potential; ATP synthesis

Ropivacaine is a new local anesthetic now available for clinical use. It belongs to the local anesthetic pipecoloxylidide family, which also includes the well-known tertiary amines mepivacaine and bupivacaine with the latter widely used in recent decades. The three molecules differ in the aliphatic substituents on the piperidine nitrogen, since a methyl, propyl and butyl residue are present in mepivacaine, ropivacaine, and bupivacaine, respectively. Due to the presence of a chiral carbon, all three substances exist in both the D and L form, with only the latter being active as an anesthetic. However, while mepivacaine and bupivacaine are supplied as racemic solutions, only ropivacaine is available as a pure (99.5%) L enantiomer.

The pharmacokinetic profile of both ropivacaine and bupivacaine has been investigated after i.v. or epidural injection in dogs, *Rhesus* monkeys, and human volunteers [1–3]. In humans [3], the clearance of ropivacaine (0.82 L/min) is higher than that of bupivacaine (0.58 L/min), which is bound to plasma proteins to a greater extent. The theoretic distribution volume, at steady-state, is 73 L for bupivacaine and 59 L for ropivacaine. The half-life of ropivacaine is shorter than that of Bupivacaine [4], although a marked individual variability makes it difficult to establish an absolute value.

The effects of bupivacaine on mitochondrial energy-linked processes were reported some years ago by Schonfeld *et al.* [5, 6], Terada *et al.* [7] and by Sun and Garlid [8], and there is general agreement that it exerts an uncoupling effect on oxidative phosphorylation. However, the mechanism of this effect has been a matter of debate. In fact, different hypotheses have been proposed, such as that of the molecule acting as a proton carrier shuttle similar to CCCP‡ [5, 6], an action requiring a lipophilic anion able to form ion pairs within the membrane. This induces a proton leak [7] or the formation of intramembrane transient multimers of the free base, thus providing a sort of "flickering channel" for protons [8].

In this paper, we report the effects on rat liver mitochondrial energetic metabolism of L-ropivacaine, now used as a substitute for the racemic bupivacaine. The mitochondrial action of L-ropivacaine was compared to that of bupivacaine.

MATERIALS AND METHODS

Ropivacaine (hydrochloride monohydrate of 1-propyl-2,6-pipecoloxylidide) and bupivacaine (hydrochloride monohydrate of 1-butyl-2,6-pipecoloxylidide) were commercial preparations from Astra. All the reagents were of analytical grade; ADP, CCCP and rotenone were purchased from Sigma.

† Corresponding author: Prof. Guido Scutari, Dipartimento di Chimica Biologica, Viale G. Colombo 3; 35121 Padua (Padova), Italy.
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‡ *Abbreviations*: CCCP, carbonylcyanide-m-chlorophenylhydrazone; $\Delta\psi$, transmembrane electrical potential; RCI, respiratory control index.

TABLE 1. Effect of bupivacaine on respiration, steady-state transmembrane electrical potential and rate of ATP synthesis of isolated rat liver mitochondria

	Bupivacaine concentration					
	None	0.05 mM	0.1 mM	0.15 mM	0.3 mM	0.6 mM
St 3	96.6 ± 7.3	105.9 ± 10.4	98.7 ± 8.6	101.3 ± 8.8	101.6 ± 9.1	106.7 ± 9.8
St 4	27.8 ± 3.7	33.4 ± 5.4	**40.5 ± 5.6**	**40.9 ± 5.9**	**42.7 ± 6.2**	**68.5 ± 5.9**
RCI	4.1 ± 0.40	3.1 ± 0.38	2.6 ± 0.40	2.3 ± 0.39	2.1 ± 0.30	1.5 ± 0.39
CCCP	127.0 ± 6.1	125.5 ± 8.2	115.7 ± 8.7	117.6 ± 7.9	117.8 ± 11.3	**109.8 ± 8.1**
ΔΨ	188.4 ± 4.7	153.4 ± 6.7	**160.3 ± 5.4**	**151.6 ± 6.8**	**138.2 ± 4.9**	**92.0 ± 5.1**
ADP/O	1.58 ± 0.05	1.49 ± 0.04	1.36 ± 0.04	1.23 ± 0.05	1.16 ± 0.04	0.79 ± 0.07
ATP	168.4 ± 7.7	139.4 ± 5.4	**130.3 ± 4.9**	**128.0 ± 4.9**	74.0 ± 3.9	N.D.

State 3 (St 3) and state 4 (St 4) represent the oxygen consumption rates measured in the presence of 160 μM of exogenous ADP and in its absence, respectively. Oxygen consumption rates are expressed as ngatomO/min × mg of mitochondrial protein ± standard deviation. RCI is the ratio between the oxygen consumption rates measured in the presence (300 nmoles) and in the absence of exogenous ADP. CCCP indicates the oxygen consumption rate in the presence of the uncoupler carbonyl cyanide *m*-chlorophenyl-hydrazone (1.6 μM). ΔΨ is the transmembrane electrical potential expressed in mV. ATP indicates the ATP synthesis rate expressed as nmol ATP/minute × mg of mitochondrial protein. Values are the means (±SD) of 15 experiments in duplicate. The differences versus control, according to the Student's *t*-test, are significant when values are indicated in bold type ($P < 0.001$).

Mitochondria were isolated from liver of albino Wistar rats in 0.25 M of sucrose, 2.5 mM of Na-HEPES (pH 7.4), and 0.25 mM of EGTA by differential centrifugation as previously described [9], omitting EGTA in the final washing. Protein content of the mitochondrial suspension was assayed by the biuret method [10]. Oxygen consumption was followed using a Clark oxygen electrode (Yellow Springs Instruments Co.) in 2.5 mL of the basal medium supplemented with 5 mM K-succinate and 1.5 μM rotenone. Δψ was measured by monitoring the distribution of tetraphenylphosphonium cation across the inner mitochondrial membrane with a tetraphenylphosphonium-selective electrode in 5 mL of the basal medium supplemented with 5 mM K-succinate and 1.25 μM rotenone [11]. The rate of ATP synthesis was evaluated by using a semimicro combined pH electrode (Ingold Messtechnik AG) to monitor the rate of proton concentration decrease due to the shift of ATP, ADP and phosphate anion dissociation equilibria [12]. The rate of ATP synthesis was taken as the difference between the rates obtained in the absence and presence of 1μg oligomycin/mg of mitochondrial proteins. The experiments were performed in 2.5 mL of the basal medium (from which 3-*N*-morpholinopropanesulfonic acid was omitted) supplemented with 5 mM K-succinate, 1.25 μM rotenone and 150 μM ADP. For the ADP/O calculation, the actual concentration (nominal value 0.1 M) of ADP in the solution used for additions was assayed by an enzymatic method using phosphoenolpyruvate, lactate dehydrogenase, and NADH.

All experiments were carried out at 30° using 1 mg of mitochondrial protein/mL of a basal medium containing 0.25 M sucrose, 15 mM 3-*N*-morpholinopropanesulfonic acid, 1.6 mM $MgSO_4$, 2 mM KH_2PO_4, 6 mM NaCl, 1 mg/mL BSA, and 5 mM Na-succinate, pH 7.0. Each experimental value reported in the tables is the mean ± SD of 30 values obtained on 15 different mitochondrial preparations. Where indicated, the results were evaluated by the paired Student's *t*-test for comparison between anesthetic-tested and control mitochondria. Values of *p* less than 0.05 were considered statistically significant.

RESULTS

The effect of bupivacaine and ropivacaine on the respiration rates measured during (state 3) and after (state 4) a phosphorylation pulse induced by the addition of ADP and after the addition of the uncoupler CCCP are reported in the Tables 1 and 2 respectively. The ADP/O ratios and Δψ are also indicated in the same tables.

As shown in Table 1, the presence of bupivacaine in the incubation medium did not alter the rate of the ADP-stimulated respiration (state 3), whereas the resting respiration rate (state 4) was strongly stimulated by the anesthetic in a concentration-dependent manner. However, the increase in the rate of O_2 consumption in state 4 in the presence of bupivacaine was not linearly correlated to the concentration of the anesthetic since, after an initial increase at 0.1 mM, the rate remained approximately constant in the range 0.1–0.3 mM, with a rapid increase becoming apparent afterwards. In fact, when the concentration of the anesthetic approached 0.6 mM, the rate was more than doubled with respect to the control. On the other hand, the uncoupled respiration was not affected by bupivacaine up to 0.3 mM (differences were not significant) and was slightly inhibited at a concentration of 0.6 mM.

RCI indicates the ratio between the oxygen consumption rate in the presence of added ADP (state 3) and that obtained after the phosphorylation pulse is completed (state 4). Due to the ineffectiveness of the anesthetic on the state 3 respiration, RCI values showed a course opposite that of the resting respiration rate, reaching approximately 35% of the control value at 0.6 mM bupivacaine.

The ADP/O ratio, i.e. the ratio between the nmoles of ADP added and the ngatoms of oxygen consumed during the phosphorylation pulse (which reflects the efficiency of the oxidative phosphorylation system), gradually decreased, approaching approximately half of the control value at 0.6 mM. Accordingly, the Δψ, which supplies energy for ADP phosphorylation, and the ATP synthesis rate were gradually decreased by the scalar increase in bupivacaine concentra-

TABLE 2. Effect of ropivacaine on respiration, steady-state transmembrane electrical potential and rate of ATP synthesis of isolated rat liver mitochondria

	Ropivacaine concentration					
	None	0.3 mM	0.6 mM	1.2 mM	1.8 mM	2.4 mM
St 3	96.6 ± 7.3	104.2 ± 8.5	99.1 ± 11.7	102.9 ± 10.5	107.3 ± 14.8	111.0 ± 11.1
St 4	27.8 ± 3.7	30.2 ± 4.9	28.9 ± 4.1	31.5 ± 4.4	**34.8 ± 5.5**	**41.7 ± 4.7**
RCI	4.1 ± 0.40	3.9 ± 0.35	4.0 ± 0.39	3.7 ± 0.36	3.5 ± 0.27	2.8 ± 0.37
CCCP	127.0 ± 6.1	122.8 ± 6.9	128.0 ± 7.8	126.5 ± 6.6	124.6 ± 7.1	*120.6 ± 7.2*
ΔΨ	188.4 ± 4.7	175.0 ± 4.7	172.1 ± 5.5	*165.1 ± 4.7*	*135.3 ± 5.8*	*142.5 ± 6.5*
ADP/O	1.58 ± 0.05	1.50 ± 0.05	1.43 ± 0.04	1.40 ± 0.04	1.29 ± 0.03	1.20 ± 0.06
ATP	168.4 ± 7.7	**152.4 ± 5.1**	**148.3 ± 5.8**	**145.2 ± 4.7**	*119.7 ± 5.2*	*124.8 ± 5.7*

State 3 (St 30 and state 4 (St 4) represent the oxygen consumption rates as measured either in the presence of 160 μM of exogenous ADP or in its absence, respectively. Oxygen consumption rates are expressed as ngatom O/min × mg of mitochondrial protein ± standard deviation. RCI is the ratio between the oxygen consumption rates measured in the presence (300 nmoles) and in the absence of exogenous ADP. CCCP indicates the oxygen consumption rate in the presence of the uncoupler carbonyl cyanide m-chlorophenyl-hydrazone (1.6 μM). **ΔΨ** is the transmembrane electrical potential expressed in mV. ATP indicates the ATP synthesis rate expressed as nmol ATP/min × mg of mitochondrial protein. Values are the means (± SD) of 15 experiments in duplicate. The difference versus control, according to the Student's *t*-test, are significant when values are indicated in bold type ($P < 0.01$) or in italics ($P < 0.001$).

tion. A concentration of 0.6 mM of bupivacaine lowered the transmembrane potential to less than 100 mV.

In Table 2, the same parameters are reported for ropivacaine. ADP-stimulated respiration was not affected even at ropivacaine concentrations as high as 1.8 or 2.4 mM, i.e. two or three times the fully damaging bupivacaine concentration. The resting respiration rate, compared to that of control mitochondria, seemed to be substantially unaffected up to 1.2 mM of ropivacaine (differences versus control are not significant) and only the concentrations 1.8 and 2.4 mM produced a slight but significant increase. Consequently, also RCI values, i.e. the ratio between the ADP-stimulated and the resting respiration rate in the presence of 1.8 and 2.4 mM ropivacaine, were slightly decreased. Uncoupled respiration was unaffected, except in the presence of 2.4 mM of ropivacaine, where it exhibited a slight but significant difference versus control.

ADP/O ratios seemed to be significantly decreased, with respect to the control value, for concentrations of the anesthetic higher than 1.2 mM. Transmembrane electrical potential seemed to be slightly affected by 0.3 and 0.6 mM of ropivacaine, and seemed to be substantially decreased in the presence of anesthetic concentrations ranging from 1.2 to 2.4 mM. The ATP synthesis rate was consequently affected but, after a significant decrease in the presence of 0.3 mM of ropivacaine, it seemed to be maintained at a roughly constant level up to 1.2 mM, with a further decrease being observed at 1.8 and 2.4 mM.

DISCUSSION

Ropivacaine is a new local anesthetic of the tertiary–amine type similar to the well-known bupivacaine, whose aliphatic side-chain differs from that of ropivacaine in being longer by one carbon unit.

Bupivacaine has been reported to uncouple the mitochondrial energy-converting system; whether it acts as a classic uncoupler [5, 6] or as a transient multimer with proton-conducting properties within the membrane [8] has been a matter of debate. The first hypothesis implies that the anesthetic translocates protons by shuttling single protonated molecules through the mitochondrial inner membrane [5, 6], while the second hypothesis suggests the formation, through the membrane bulk phase, of transient linear bridges of molecules which provide sequences of weak binding sites for protons, thereby allowing their inward flux. [8].

Mitochondrial respiration rates, Δψ values, and ATP synthesis rates, measured in the presence of bupivacaine (Table 1), showed a more pronounced sensitivity to the anesthetic than that reported by other authors [6, 8].

Since the aim of this paper was to study the mitochondrial effects of ropivacaine, we used bupivacaine as a comparison to assess whether the minor change in molecular structure (a propyl instead of a butyl chain) may influence the mitochondrial energy production system. Bupivacaine was tested up to the concentrations which, in our preparations, completely impaired ATP synthesis and brought Δψ down to less than 100 mV; this break-point was found at 0.6 mM of bupivacaine in the presence of 1 mg of mitochondrial protein/mL. On the contrary, ropivacaine, even at a four-fold concentration (2.4 mM), maintained a Δψ of approximately 150 mV and a significant ATP synthesis rate.

According to the uncoupling mechanism based on the protonophoric properties of the tertiary-amine local anesthetics proposed by Dabadie *et al.* [5], Schonfeld *et al.* [6], and Sun and Garlid [8], it is apparent that even a small molecular change such as a 3C (ropivacaine) instead of a 4C (bupivacaine) side-chain, greatly modifies the protonophoric ability of the molecule. Indeed, 0.6 mM bupivacaine completely impaired the mitochondrial energetic metabolism, while 0.6 mM ropivacaine was substantially ineffective. As shown in Table 2, the protonophoric ability of ropivacaine, if compared to that of bupivacaine, is very limited. In fact, 0.6 mM ropivacaine decreased transmembrane electrical potential with respect to that of the control by approximately 10%, indicating that it neither transports

a relevant amount of H^+ nor induces an important increase in the passive membrane permeability to protons. The ATP synthesis rate seemed to be decreased by approximately 12%, i.e. the mitochondria were able to rephosphorylate ADP with an efficiency sufficient to preserve most of the energy of the gradient. Indeed, the ADP/O ratio, decreased by approximately 10%, reflects a very slight energy dissipation.

A gradual impairment of the energetic parameters appeared as the ropivacaine concentration increased. Between 1.8 and 2.4 mM (the highest concentration tested), the decrease in the $\Delta\psi$ showed a lag; consequently, the systems which utilize the energy gradient (e.g. ATP synthesis) or are related to it (e.g. ADP/O) were not further impaired. Our feeling is that a concentration interval was reached where a maximal mitochondrial effect seems to have been obtained. However, it is compatible with an appreciable maintenance of mitochondrial functionality.

As reported by Rosenberg *et al.* [13], the pKa for ropivacaine and bupivacaine are 8.0 and 8.1, respectively. Therefore, in the medium utilized (pH 7.4), 80% of ropivacaine and 84% of bupivacaine should be in the protonated, charged form, thereby allowing the molecules to behave like protonophores by entering energized mitochondria along the electrical gradient. However, as reported by Rosenberg *et al.* [13], the partitioning in an n-heptane/buffer (pH 7.4) system shows ratios of 20.5 ± 2.1 and 6.1 ± 0.6 for bupivacaine and ropivacaine respectively, thus emphasizing the more pronounced lipophilicity of bupivacaine. This might in part account for the strong mitochondrial toxicity of bupivacaine and for the milder action of ropivacaine. In addition, the mechanism proposed by Sun *et al.* [8] seems to fit well with the lipophilicity of bupivacaine, which favors the presence of transient sequences of the anesthetic molecules in the bulk phase of the membrane, allowing a quick proton crossing through the channel-like structures. On the contrary, the less hydrophobic structure of ropivacaine allows a shorter permanence of the anesthetic in the lipid membrane phase, with the shuttling system possibly being operant in this case.

It should be noted that the presence of hydrophobic molecules in the phospholipid bilayer of the inner mitochondrial membrane produces a perturbation of the membrane structure which might be much more marked as the lipophilicity of the molecule increases. In addition, it cannot be excluded that some type of interaction between the anesthetic molecule and specific target domains of the membrane proteins might occur.

Bupivacaine, the local anesthetic of first choice in many surgical fields, in some circumstances may give rise to potentially toxic plasmatic levels with persistent cardiac depression. Effective epidural blocks or peripheral nerve/plexus blocks may require 50–150 mg of 0.25–0.5% (w/v) bupivacaine in adults. If an accidental i.v. injection or a too rapid absorption occurs, the resulting high plasmatic levels of bupivacaine may cause systemic toxicity, resulting in convulsions at a mean dose of 5 mg/Kg [14]. In monkeys, bupivacaine produces convulsions at a blood concentration of approximately 4.5 μg/mL [15]. In isolated guinea pig-atria, 6 μg/mL of bupivacaine produces a 50% decrease in contractility [16].

Therefore, if 2–20 mM is the concentration useful for a clinically effective anesthetic action on peripheral nerves, a plasmatic concentration of approximately 15–20 μM induces symptoms of cardiovascular impairment and convulsions. Since an effective anesthetic block does not reduce bupivacaine concentration on nerves, it is very important to utilize local anesthetics having the same anesthetic potency but a lower systemic toxicity. L-ropivacaine clinically shows the same potency as bupivacaine but seems to be less toxic on organs and tissues.

Consequently, the pure L enantiomer of ropivacaine is now proposed as a substitute for the racemic bupivacaine in virtue of its higher efficacy/toxicity ratio. The results obtained, under the experimental conditions reported here, seem to corroborate the view of a more advantageous use of ropivacaine over bupivacaine.

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