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The neuronal lipid membrane permeability was markedly increased by bupivacaine and mildly affected by lidocaine and ropivacaine

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Abstract

We investigated the local anesthetic action on ionic membrane conductance (membrane conductance) and selectivity in membranes formed with neuronal phospholipids in the absence and presence of cholesterol. In membranes without cholesterol, 1 mM bupivacaine and ropivacaine increased the membrane conductance ~ 4.5-fold; and 5 mM lidocaine, ropivacaine and bupivacaine increased the membrane conductance by 2.7-, 2.8- and 22.2-fold, respectively. In the presence of cholesterol, 5 mM ropivacaine had no effect, lidocaine decreased the membrane conductance by 2-fold, and bupivacaine increased the membrane conductance by 17-fold. Local anesthetics did not affect the ion selectivity in membranes without cholesterol, but they all decreased the Na^+ selectivity in membranes with cholesterol. Cholesterol reduced the lidocaine- and ropivacaine-induced membrane conductance increase by eliminating or reversing the Na^+ conductance increase and by lowering the Cl^- conductance increase. In the absence of cholesterol, 5 mM bupivacaine increased both Na^+ conductance (38-fold) and Cl^- conductance (19-fold), while in the presence of cholesterol it only increased Cl^- conductance (26-fold). Of the local anesthetics studied, ropivacaine was the least membrane toxic while bupivacaine was the most toxic.

Keywords: Lidocaine; Ropivacaine; Bupicaine; Cholesterol; Membrane ionic permeability

1. Introduction

During spinal and epidural anesthesia, the serum levels of local anesthetics are in the low μM range, while the levels at the site of application are high (20–80 mM) (Butterwordth, 2000). The local anesthetic concentration used for spinal nerve blocks for controlling hyperalgesic pain are lower than those used for surgery, however, the level of local anesthetics at the site of injection still remains relatively high (low mM range), and the duration of those high levels may last for hours or even days (Butterwordth, 2000; Van Dongen et al., 1999). In various experimental models, local anesthetics at mM levels have been shown to irreversibly affect neuronal function in a dose- and time-dependent manner (Bainton and Strichartz, 1994; Byers et al., 1973; Kanai et al., 1998; Lambert et al., 1994; Mateu et al., 1997).

Local anesthetics interactions with the cellular membranes may contribute to their toxicity. The membrane lipid composition contributes both directly and indirectly (e.g. modulating membrane protein function) in defining the biophysical properties of membranes, which in turn define the cell's electrical excitability and volume. Local anesthetics interact differently with different phospholipids (Kelusky et al., 1986; Singer and Jain, 1980) and in membranes formed with phosphotidylcholine the length and the level of saturation of the phosphatidyl chains (Kelusky et al., 1986; Singer and Jain, 1980) and the presence of cholesterol (Auger et al., 1988) have been shown to affect local anesthetic action on membrane ionic permeability.

Since the various cell membranes contain different cholesterol levels, we investigated whether three commonly used local anesthetics (lidocaine, bupivacaine and ropivacaine) altered ionic permeability and ionic selectivity of planar membranes formed with purified brain phospholipids

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in the absence and presence of cholesterol, and discuss whether such effects might be related to the neurotoxicity of local anesthetics.

2. Materials and methods

2.1. Lipid composition of planar lipid bilayers

Two lipid mixtures were used in proportions resembling those found in neuronal plasma membranes (Norton et al., 1975; Tamai et al., 1975). One mixture contained only purified rat brain phospholipids (Avanti Polar Lipids, Alabster, AL) in the following weight fraction 4PC:2PE:0.8PS:0.1PI (PC: L-(-Phosphatidylcholine) (MW ~ 760); PE: L-(-Phosphatidylethanolamine) (MW ~ 746); PS: L-(-Phosphatidylserine) (sodium salt) (MW ~ 812.); PI: Phosphatidylinositol-4-phosphate (di-ammonium salt) (MW ~ 1001). The second lipid mixture was the same as the first but also contained cholesterol (CH) in the following weight proportion 4PC:2PE:0.8PS:0.1PI:1CH, corresponding to ~ 13 wt.% or ~ 22 mol% of cholesterol. Cholesterol was from Sigma (St. Louis, MO; 5-Cholesten-3β-ol, Sigma Grade +99%, MW 386.7). Lipids were mixed and stored at −20 °C in chloroform and with N_2. On the day of the experiment chloroform was evaporated under a nitrogen flow and the lipids resuspended with decane (2.16 mg lipid/50 μl decane) (Andoh et al., 1997; Haydon et al., 1977). Decane was from Wiley Organics (99.9%, Coshocton, OH). The chamber contained two compartments (1.24 ml capacity each) separated by a partition with a hole diameter of 0.1 mm where the lipid bilayer was formed. Silver–Silver Chloride electrodes were placed in direct contact with the solution in each compartment, and the reference electrode was in the trans (back) compartment.

2.2. Membrane conductance and reversal potential measurements

Total ionic membrane conductance (membrane conductance), that is conductance caused by all ion species present in the solution, was measured using both symmetrical and asymmetrical solutions of NaCl to be able to estimate the individual contribution of the Na^+ conductance and the Cl^- conductance (see below). Initially, both compartments were filled with 1 ml of 20 mM NaCl. The concentration in the trans compartment was then increased stepwise to 80 mM and 200 mM NaCl. This was done by adding 30 and 60 μl of 2 M NaCl solution, respectively. The same volume of 20 mM NaCl solution was added to the cis (front) compartment to maintain equal the volume in both compartments. Solutions were stirred for 3–4 min after each increase in NaCl concentration. Membrane conductances were obtained from linear fits of the current–voltage relationships constructed by applying voltage ramps from −60 to +60 mV. For each current–voltage curve, the reversal potential (Vr, net ionic current is zero), was defined as the voltage-intercept of the linear fit. The holding potential (−60 mV) was maintained for 2–3 min to decrease the contribution of fast and slow capacitative currents. To determine more accurately the baseline current level at −60 mV, additional baselines were taken for each of the control and experimental conditions, by continuously applying −60 mV, while changing the ionic conditions. For a given membrane, for each ionic condition the voltage ramp was repeated four times. Data from the last three voltage ramps were used to obtain the average current–voltage relationship. For each condition, 5–8 membranes were used. All solutions contained 10 mM HEPES (Sigma) at pH 7.4, and the pH was maintained constant upon drug addition. When asymmetrical NaCl concentrations were used, the applied potential was corrected for the estimated electrode–liquid potential differences which were calculated by using the Clampex 8 software (Axon Instruments, Union City, CA). Experiments were done at room temperature (23–25 °C).

2.3. Estimation of Na^+ conductance and Cl^- conductance

The relative membrane ionic selectivity for Na^+ and Cl^- was estimated by using the Vr measurements obtained during the 200/20 NaCl gradient. Similar results were obtained when using Vr measurements during the 80/20 gradient (not shown). In our NaCl solutions, the Vr value is given by the following equation:

$$\mathrm{Vr} = (\mathrm{gNa} \times \mathrm{ENa}/\mathrm{gm}) + (\mathrm{gCl} \times \mathrm{ECl}/\mathrm{gm}) + (\mathrm{gH-OH} \times \mathrm{EH-OH}/\mathrm{gm}) + (\mathrm{gHEPES} \times \mathrm{EHEPES}/\mathrm{gm})$$

where gNa = Na^+ conductance; gCl = Cl^- conductance; gH–OH = proton-hydroxide conductance; gHEPES = HEPES conductance; ENa, ECl, EH–OH and EHEPES = equilibrium potentials (Nernst potentials) for each of the ions. As there was no pH or HEPES gradient, EH–OH and EHEPES are zero, and the equation becomes:

$$\mathrm{Vr} = (\mathrm{gNa} \times \mathrm{ENa}/\mathrm{gm}) + (\mathrm{gCl} \times \mathrm{ECl}/\mathrm{gm})$$

By assuming that only Na^+ and Cl^- ions carry the current (gNa/gm + gCl/gm = 1; gm = membrane conductance), the following equation was derived (Andoh et al., 1997):

$$\mathrm{Vr} = (1 - 2\mathrm{tNa}) \times (RT/F) \times \ln([\mathrm{NaCl}]_{\mathrm{trans}}/[\mathrm{NaCl}]_{\mathrm{cis}}) \quad (1)$$

where tNa = transference number of Na (tNa = gNa/gm); R = gas constant; T = temperature in Kelvin scale; F = Faraday constant. With this equation, we calculated the tNa that was then used to estimate the Na^+ conductance (gNa = gm × tNa) and Cl^- conductance (gCl = gm × (1 − tNa)) values.

2.4. Addition of local anaesthetics

Equal amounts of local anesthetics were added to both chamber compartments that contained 1.0 ml of 20 mM NaCl. Equilibrium of the local anesthetic with the membrane lipids was reached by first stirring the solution for 10 min and then reforming the bilayer at least three times before starting measurements. For lidocaine and bupivacaine (Sigma), the 1- and 5-mM final concentrations were obtained by adding 10 and 50 μl of a 0.1-M local anesthetic stock solution (ethanol), respectively. For ropivacaine (Astra USA), the 1 mM concentration was obtained by adding 55.5 μl of the original 18 mM solution; and for the 5 mM concentration, the following mix was prepared: 700 μl 20 mM NaCl, 3 μl 2 M NaCl and 277 μl 18 mM ropivacaine.

3. Results

3.1. Local anaesthetic effects on membrane conductance

We measured the effect of anaesthetics on membrane conductance and the selectivity between Na^+ and Cl^- ions. In membranes without cholesterol, 1 mM, lidocaine had no significant effect on the membrane conductance, while bupivacaine and ropivacaine increased the membrane conductance significantly and to a similar extent (~ 4.5-fold) (Fig. 1A). Lidocaine and bupivacaine (5 mM) produced a large membrane conductance increase (2.7- and 22.2-fold, respectively) (Fig. 1B). In contrast, 5 mM ropivacaine produced a slight but significantly lower ($P<0.01$) membrane conductance increase than 1 mM (4.3- and 2.8-fold at 1 and 5 mM, respectively). The membrane conductance increase induced by 5 mM bupivacaine (22.2-fold) was about 10-fold higher than that induced by either lidocaine or ropivacaine (Fig. 1B).

When cholesterol was added to the membranes (~ 22 mol%), the membrane conductance increased by about 2.5-fold as compared with membranes without cholesterol (Fig. 1A vs. C control groups). The presence of cholesterol in the phospholipid membrane also significantly affected the local anesthetic action. A 1 mM lidocaine tended to increase membrane conductance but the increase was significant only in the 20/20 NaCl group. A 5 mM lidocaine produced a small but significant membrane conductance decrease (Fig. 1D), this is in contrast to the membrane conductance increase observed in membranes without cholesterol (Fig.

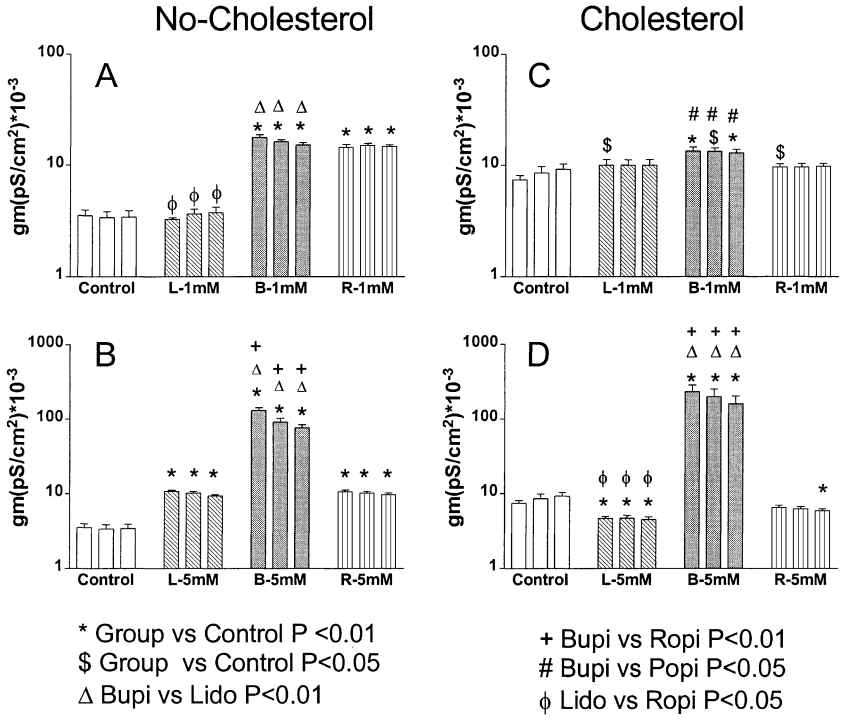


Fig. 1. Local anesthetic effects on membrane conductance. Total membrane conductance was measured in the absence (gray bars) and presence of lidocaine (L), bupivacaine (B) and ropivacaine (R) in phospholipid membranes without (left) and with (right) cholesterol. For each group there are three bars corresponding to measurements done in either symmetrical 20 mM NaCl (first bar), 80/20 mM NaCl gradient (middle bar) and 200/20 mM NaCl gradient (third bar). The effects of 1 mM local anesthetic are shown in A and C, that of 5 mM local anesthetic are shown in B and D. For phospholipid membranes without cholesterol, $N=7$ for control; $N=5$ for 1 and 5 mM lidocaine; $N=9$ and 6 for 1 and 5 mM bupivacaine, respectively; and $N=8$ and 7 for 1 and 5 mM ropivacaine, respectively. For phospholipid membranes with cholesterol, $N=6$ for control; $N=7$ and 8 for 1 and 5 mM lidocaine, respectively; $N=8$ and 5 for 1 and 5 mM bupivacaine; $N=7$ and 8 for 1 and 5 mM ropivacaine.

1B). A 1 mM ropivacaine produced a small membrane conductance increase in the 20/20 NaCl group (Fig. 1C) this is in contrast to the about 4-fold membrane conductance increase due to 1 mM ropivacaine in all the NaCl groups in membranes without cholesterol (Fig. 1A). A 5 mM ropivacaine tended to decrease the membrane conductance, but such effect was only significant in the 200/20 group (Fig. 1D). Again this is in contrast with the 2.8-fold membrane conductance increase observed in membranes without cholesterol (Fig. 1B). Most notable was the effect of bupivacaine in the presence of cholesterol. At 1 mM bupivacaine the membrane conductance increase was lower as compared to that observed in membranes without cholesterol (1.4- vs. 4.4-fold). However, the membrane conductance increase observed at 5 mM bupivacaine was similar in membranes with (17-fold) and without (22-fold) cholesterol, but the absolute membrane conductance value was higher in membranes with cholesterol. In experiments with 5 mM bupivacaine, membranes became very unstable, they broke very easily during experimental manipulations such as stirring of the solution or when adding the concentrated solution of NaCl. This membrane unsteadiness was higher in membranes containing cholesterol than in those without cholesterol. In contrast, the membrane stability in the presence of either 5 mM lidocaine or 5 mM ropivacaine was similar to that of membranes in the absence of local anesthetic.

3.2. Local anesthetic effects on membrane ionic selectivity

Fig. 2 shows current traces obtained in the absence (C) and presence of lidocaine (L), bupivacaine (B) and ropivacaine (R) at 5 mM. As described in the previous section under symmetrical 20 mM NaCl (Fig. 2A and C) and asymmetrical 200/20 mM NaCl (Fig. 2B and D), one can see the current increase (hence membrane conductance increase) induced by the various local anesthetics. In addition under asymmetrical 20/200 mM NaCl one can see that local anesthetics changed the membrane potential at which the ionic current is zero (Vr), and the magnitude of the change depended on both the local anesthetic and on whether the membrane contained cholesterol (Fig. 2B vs. D).

In Fig. 3, the data regarding changes in the Vr are summarized and for the two NaCl gradients the Nernst potential values for Na^+ and Cl^- are indicated. In the absence of anaesthetic, the phospholipid membranes containing no cholesterol had a Vr value that was close to that

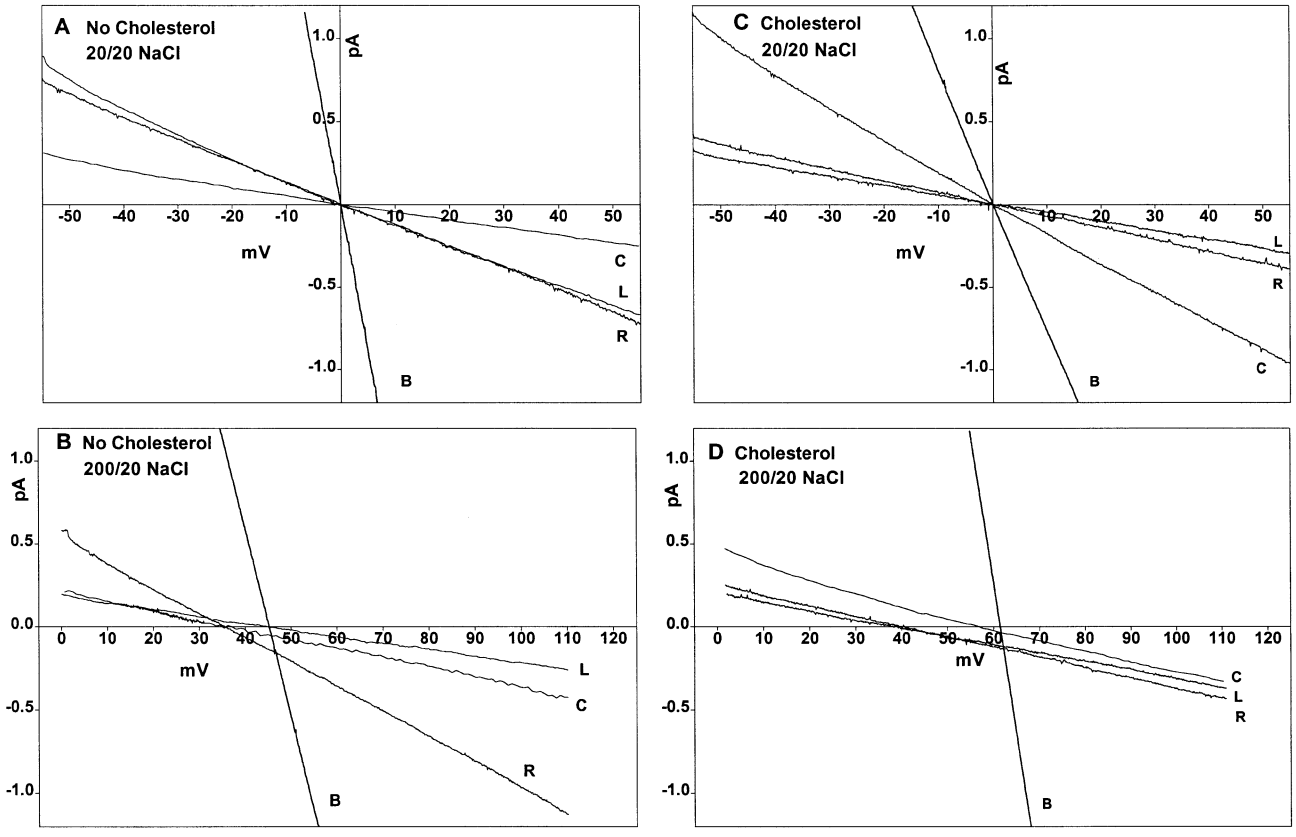


Fig. 2. Current–voltage relationships in the absence and presence of local anesthetics. Current traces obtained with voltage ramps under various conditions are shown. Current–voltage relationships obtained with phospholipid membranes without cholesterol (A,B) and with cholesterol (C,D) under symmetrical 20 mM NaCl (A,C) and under asymmetrical 200/20 NaCl (B,D). In the absence of control (labeled C), and presence of 5 mM local anesthetic: lidocaine (labeled L), bupivacaine (labeled B) and ropivacaine (labeled R).

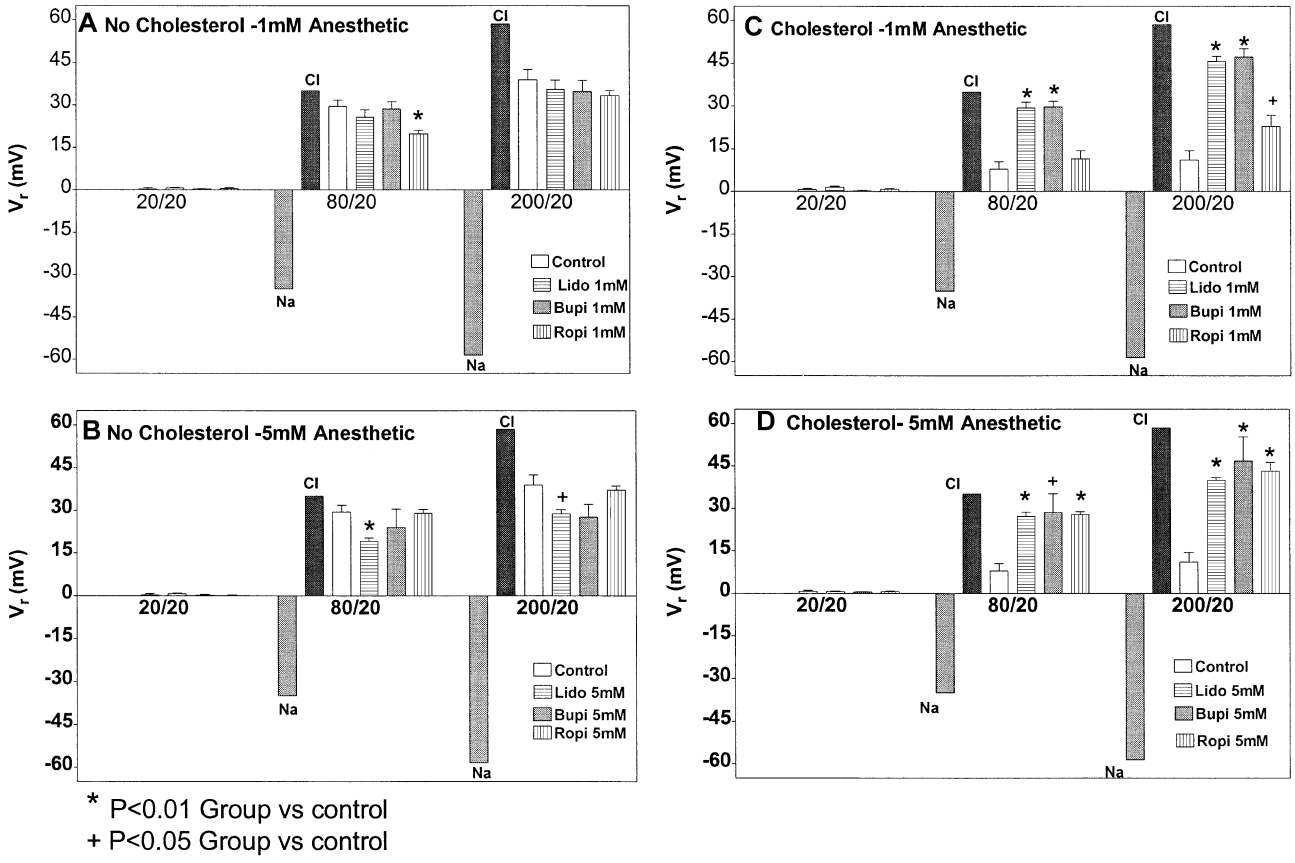


Fig. 3. Local anesthetic effects on reversal potential (Vr). The reversal potential was measured in the absence (open bars) and presence of lidocaine (horizontal lines-bars), bupivacaine (dotted-bars), and ropivacaine (vertical lines-bars) in phospholipid membranes without cholesterol (A,B) and with cholesterol (C,D). The effects of 1 mM local anesthetic are shown in A and C, and the effects of 5 mM local anesthetic are shown in B and D. The theoretical Vr values for a purely Cl^- selective membrane and purely Na^+ selective membrane in the two NaCl gradients (80/20 and 200/20) are indicated (bars labeled as Cl or Na). *N* are as in Fig. 1.

predicted for a purely Cl^- selective membrane (Fig. 3A and B, open bars). Only ropivacaine at 1 mM in the 80/20 NaCl group significantly changed Vr towards less Cl^- selective (Fig. 3A), but this effect disappeared at 5 mM ropivacaine. Only lidocaine at 5 mM significantly changed the Vr towards less Cl^- selective in both the 80/20 and 200/20 NaCl groups (Fig. 3B). Bupivacaine at 5 mM had a similar tendency but it did not reach statistically significance.

In the absence of anaesthetic, the phospholipid membranes containing cholesterol had a Vr value that indicated that the membranes were more selective for Cl^- than Na^+, but the level of Cl^- selectivity was significantly lower than that observed in membranes without cholesterol (Fig. 3C vs. A). Cholesterol enhanced the local anesthetic action on the Vr, at 1 mM all the local anesthetics changed the Vr to make it more Cl^- selective, the magnitude of the effect was comparable for lidocaine and bupivacaine. The effect was significantly lower for ropivacaine (Fig. 3C). At 5 mM, lidocaine and bupivacaine had the same effect as at 1 mM (Fig. 3C and D). The effect of ropivacaine was stronger at 5 than at 1 mM, and at 5 mM the magnitude of the effect was similar to that of lidocaine and bupivacaine (Fig. 3D).

3.3. Local anesthetic changes in Na^+ conductance and Cl^- conductance

The change in the membrane Na^+ selectivity was estimated by calculating the Na transference number (tNa = gNa/gm) using the reversal potential values as described in Section 2. In the absence of anaesthetics, the membranes without cholesterol had a lower Na^+ selectivity (tNa = 0.17) than membranes with cholesterol (tNa = 0.41) (Fig. 4A and E, respectively). In membranes without cholesterol, the various local anesthetics tended to increase the Na^+ selectivity. However, the Na^+ selectivity was only significantly increased by 5 mM lidocaine (~ 50% increase), a similar trend was observed for 5 mM bupivacaine but it did not reach statistical significance (Fig. 4A). In contrast, in membranes containing cholesterol, all the local anesthetics significantly decreased the Na^+ selectivity at both concentrations. The lowest effect was observed with 1 mM ropivacaine.

By using the tNa values, we further estimated the contribution of Na^+ conductance and Cl^- conductance to the total membrane conductance. In membranes without

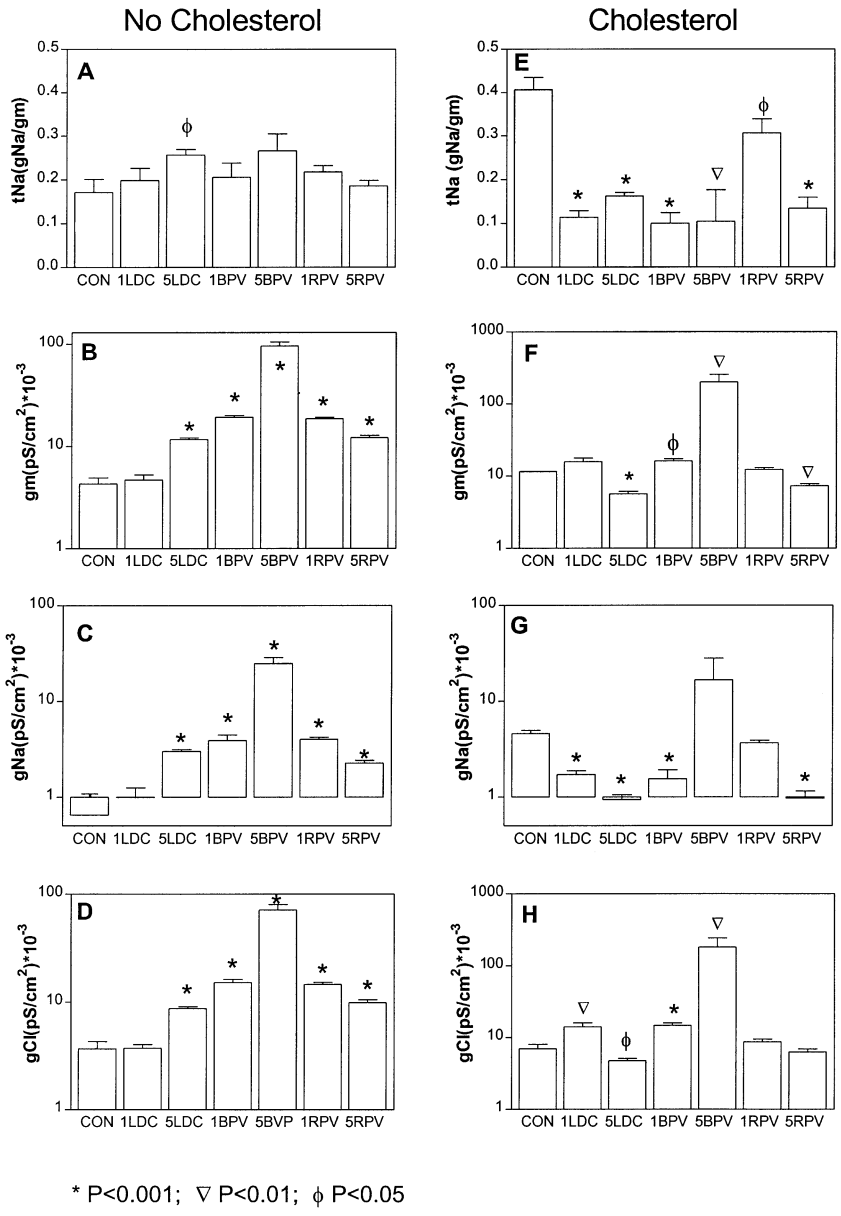


Fig. 4. Local anesthetic effect on tNa and the Na^+ conductance and Cl^- conductance contribution to membrane conductance. The Na^+ transference number (tNa), the Na^+ conductance and Cl^- conductance were estimated as described in Section 2. Data from membranes with cholesterol (A,B,C,D) and for membranes without cholesterol (E,F,G,H) are shown. *N* are as in Fig. 1.

cholesterol, in all conditions except for 1 mM lidocaine, the increase in membrane conductance resulted from an increase in the Na^+ conductance (Fig. 4C) and Cl^- conductance (Fig. 4D). In contrast, in membranes with cholesterol, in almost all conditions there was a decrease in Na^+ conductance, except in 5 mM bupivacaine and 1 mM ropivacaine where there was no significant change. In the presence of cholesterol, the effect on Cl^- conductance varied between local anesthetics. Bupivacaine increased Cl^- conductance at both concentrations, lidocaine at 1 mM increased but at 5 mM decreased the Cl^- conductance, while ropivacaine had no effect on Cl^- conductance at either concentration. The presence of cholesterol eliminated the lidocaine- and ropivacaine-induced membrane conductance increase, by reversing the action on Na^+ conductance (decreasing rather than increasing Na^+ conductance) and by eliminating or lowering the action on Cl^- conductance. In contrast, in the presence of cholesterol, bupivacaine at 1 mM induced a lower and at 5 mM produced a comparable membrane conductance increase as in membranes without cholesterol. Although 5 mM bupivacaine produced a comparable large membrane conductance increase in both membrane types, the effect on individual conductances was different. In the absence of cholesterol, 5 mM bupivacaine induced a large increase in both Na^+ conductance (38-fold) and Cl^- conductance (19-fold), while in the presence of

cholesterol it induced only a significantly large Cl^- conductance increase (26-fold).

4. Discussion

Previous studies found that in liposomes formed with only phosphatidylcholine, local anaesthetics at mM levels (1–10 mM) can increase, decrease or have no effect on the ^{22}Na permeability; the direction and magnitude of the effect depended on the local anesthetic structure, the length of the phosphatidylcholine fatty acyl chains and whether cholesterol was present or not (Auger et al., 1988; Singer and Jain, 1980). These reports suggest that the lipid composition is important in defining the local anesthetic action on lipid membrane permeability. Therefore, in order to investigate whether the known clinical local anesthetic toxicity in part results from the local anesthetic action on lipid membranes, we used membranes formed with phospholipids purified from neuronal tissue and in proportions resembling those found in neuronal tissues. Studies were done in the absence and presence of cholesterol at levels resembling those found in synaptosomal membranes.

We found that the magnitude of the local anesthetic-induced membrane conductance increase was higher in membranes without cholesterol than in membranes with cholesterol in all the groups except for the 5 mM bupivacaine group in which the membrane conductance increase was of similar magnitude. However, the effect of 5 mM bupivacaine on individual ion conductances was affected by cholesterol. In the absence of cholesterol, 5 mM bupivacaine induced a large increase in both Na^+ conductance and Cl^- conductance, while in the presence of cholesterol it induced only a large Cl^- conductance increase. The data indicates that local anesthetics will be more disruptive to membranes without cholesterol such as the mitochondria inner membrane or with very low cholesterol content such as the mitochondria outer membrane, endoplasmic reticulum and endocytic recycling compartments (Lange et al., 1999; Mukherjee et al., 1998; Schroeder et al., 1996) than in membranes with intermediate cholesterol content such as synaptosomal and somata membranes (22–25 mol% with phospholipids) (Breckenridge et al., 1972; Calderon et al., 1995) and with high cholesterol content such as myelinated and unmyelinated axonal membranes and myelin (~ 50 mol% with phospholipids) (DeVries et al., 1981, 1999). The data also indicates that regardless of whether cholesterol is present, of the local anesthetics studied the least disruptive one was ropivacaine followed by lidocaine, while the most disruptive one was bupivacaine.

In the bilayer system, cholesterol and phospholipids will be symmetrically distributed between the two leaflets of the lipid bilayer. In cells, however, there are mechanisms that lead to the nonsymmetrical distribution of the various membrane components including cholesterol (Liscum and Dahl, 1992). Plasma membranes, contain 80–90% of the total cellular cholesterol, of this about 72–84% is located in the inner leaflet (Schroeder et al., 1996). The asymmetrical distribution has also been reported in synaptosomal membranes, where 88% of synaptic plasma membrane cholesterol is located in the inner leaflet (Schroeder et al., 1996). Moreover, for a given membrane leaflet the cholesterol distribution is heterogeneous, since cholesterol preferentially interacts with a subset of membrane lipids in particular glycosphingolipids, and to a lesser extent phosphatidylcholine and also to certain proteins (e.g. some signalling molecules), giving rise to structurally and kinetically distinct cholesterol-rich (or rafts) and -poor domains (Lange et al., 1979; Schroeder et al., 1996). Our data indicate that when exposed to local anesthetics, one could expect significant Na^+ conductance and Cl^- conductance increases in the cholesterol-poor domains at the plasma and synaptic membranes. Increases in Na^+ conductance and Cl^- conductance will tend to depolarize the plasma membrane since both Na^+ and Cl^- ions have Nernst potential values that are more positive than that of the K^+ ion. This depolarizing action would add to other known depolarizing actions of local anesthetics, including inhibition of the Na,K-ATPase, which is required for the maintenance of the ion gradient across plasma membranes (Kutchai et al., 2000) and their block of a subpopulation of inward rectifier K^+ channels (Zhou et al., 2001). Then the observed local anesthetic-induced increase in Na^+ conductance and Cl^- conductance, especially when maintained for long periods, would contribute to the depolarization of the resting membrane potential and in turn contribute to the decrease in neuronal excitability by increasing the proportion of Ca^{2+} and Na^+ voltage-dependent channels that become inactivated.

Uncompensated increases in Na^+ conductance would also disturb the normal osmotic balance and hence allow for cell swelling. The latter effect could contribute to the reported observation that when toad sciatic nerves are exposed to 5 mM of various local anesthetics (lidocaine, tetracaine, dibucaine), there is a swelling of the cytoplasmic space in the myelin sheaths (Mateu et al., 1997). In the same study, it was also observed that the amplitude of the compound action potential was affected earlier than the structure of myelin, whereas conduction velocity closely followed the structural alterations (Mateu et al., 1997). The effect on the amplitude of the compound action potential is mostly due to the local anesthetic blocking action on the voltage-dependent Na^+ channels, while our data indicate that the local anesthetic effect on the myelin structure could result from disturbances in the osmotic balance.

Local anaesthetics have also been shown to increase the Ca^{2+} permeability in phospholipid vesicles containing no cholesterol (de Boland et al., 1975). Calcium containing intracellular membrane compartments such as mitochondria and ER have very little cholesterol, hence prolonged local anesthetic exposure could result in persistent increases in cytoplasmic Ca^{2+}. A combination of prolonged conductance changes at the plasma membrane and at intracellular

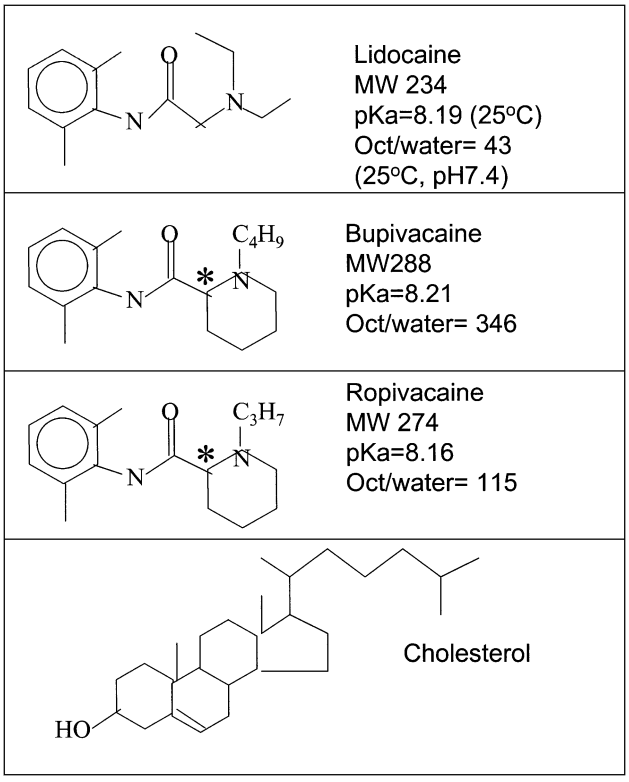
membrane compartments could explain the observation that in various experimental neuronal models structural and functional perturbations become irreversible after long exposure to mM levels of local anesthetics (Bainton and Strichartz, 1994; Byers et al., 1973; Kanai et al., 1998; Lambert et al., 1994; Mateu et al., 1997).

Unmyelinated axolemma contain higher lipid content (60 wt.%) (DeVries et al., 1999) than axolemma from myelinated sources (43 wt.%) (Detskey et al., 1988). A lower lipid content is found in soma (37 wt.%) and neuritic (15 wt.%) plasma membranes (Calderon et al., 1995). One would expect that the local anesthetic action on membrane conductance will be more dramatic in those membranes with higher lipid content for two reasons, first the local anesthetic can act on a wider surface area and second, in those membranes there will be a lower density of transporters that could oppose the local anesthetic membrane action. Therefore, we will predict that the membrane conductance effects of local anesthetics will be more damaging on unmyelinated axons than on myelinated axons and soma, and least damaging on neurites. Interestingly, most of sensory fibers are unmyelinated.

Local anesthetic molecules have been shown to reside at the polar headgroup region of lipid membranes, and depending on the local anesthetic their position may be affected by their charge. Charged tetracaine mostly locates at the lipid headgroup level, while the uncharged tetracaine is intercalated partly in the headgroup region and partly in the fatty acyl chains of lipid bilayers (Boulanger et al., 1981; Kelusky et al., 1986). In contrast, both charged and uncharged dibucaine have the same location, with their bulky quinoline ring located at the polar headgroup region and not between the hydrophobic acyl chains (Kuroda et al., 1996). The intercalation of local anesthetics in the lipid membranes results in a decrease of the lipid order parameters both in the plateau and in the tail regions of the acyl chains (Auger et al., 1988), and this action has been proposed to underlie the local anesthetic-induced membrane conductance increase (Singer and Jain, 1980).

With respect to increases in the membrane conductance, the most potent of the local anesthetic studied was bupivacaine, the one with the highest molecular size (MW 288) and the highest partition coefficient (~ 346) values (Table 1). The least potent was ropivacaine, which differs from bupivacaine by having one less methyl group and hence it has a similar molecular size (MW 274) but a significantly lower partition coefficient (~ 115). Lidocaine, the one with the lowest molecular size (MW 234) and lowest partition coefficient (~ 43), had an intermediate potency for increasing the membrane conductance. Therefore, the local anesthetic-induced membrane conductance increase does not correlate with the local anesthetic molecular size. This is consistent with previous observations in which it was found that although larger local anesthetic molecules partitioned preferentially into phosphatidylcholine bilayers, when present at equivalent molar concentrations in the bilayer

Table 1



*Asymmetric carbon atom, Values from Strichartz et al., 1990.

the relative perturbing effect on the membrane did not correlate consistently with molecular size (de Paula and Schreier, 1995). Of the local anesthetics studied, bupivacaine has the highest partition coefficient value, indicating a high level of intercalation in the fatty acyl chains and hence it could potentially have a strong disordering action, and in this way contribute to its high membrane conductance increase. However, the local anesthetic-induced membrane conductance increases does not consistently correlate with partition coefficient values (see above), and hence other molecular properties must contribute in defining the local anesthetic-induced membrane conductance increase.

Addition of cholesterol has been shown to reduce the partition coefficient of local anesthetics in the bilayers (Auger et al., 1988), and to change the location of the local anesthetic in the lipid bilayer such that the local anesthetic sits higher in the membrane, closer to the aqueous interface (Auger et al., 1988; Kuroda et al., 2000), presumably as a result of the increase in order of the lipid acyl chains in cholesterol-containing membranes (Auger et al., 1988; Kuroda et al., 2000). These effects could explain the cholesterol-mediated reduction on the lidocaine- and ropivacaine-evoked membrane conductance increase. The change in ionic selectivity with bupivacaine indicates that cholesterol probably has additional actions on the interactions between bupivacaine and the bilayer such that bupivacaine can still induce a high membrane conductance increase in the presence of cholesterol.

Based on results from studies measuring the ^{22}Na permeability of liposomes, changes in the electron spin resonance properties of lipids, and local anesthetic binding to lipids, it has been proposed that local anesthetics increase ionic permeability by preferentially binding to the boundary regions between gel and liquid crystal lipid, by increasing the number of boundary regions and increasing lipid fatty acyl chain motion (Singer and Jain, 1980). In our system we used biological glycerolipids that have transition temperatures that are lower than the experimental temperature, therefore we do not expect to have boundary regions between gel and liquid crystal lipid. However, the presence of different phospholipids may lead to phase-like separations due to preferential associations between some of them; this will be the case specially when cholesterol is present, since cholesterol prefers to interact with phosphatidylcholines than with phosphatidylethanolamines (Lange et al., 1979; Van Dijck et al., 1976). Moreover, since cholesterol increases the phospholipid order, then one would expect that the bilayer will have both a liquid ordered-like phase rich in cholesterol and phosphatidylcholines, and a liquid disordered-like phase rich in phosphatidylethanolamines. Then it would appear that the cholesterol-induced increase in phospholipid order cannot be overcome by lidocaine and ropivacaine but it can by bupivacaine. Another possibility is that bupivacaine has a strong effect at the boundary regions that appear in the presence of cholesterol, more so than lidocaine and ropivacaine.

As a local anesthetic, ropivacaine has a potency similar to that of bupivacaine, (Feldman and Covino, 1988) but ropivacaine is less cardiotoxic than bupivacaine (Rutten et al., 1989). Convulsive doses (serum levels) for bupivacaine and ropivacaine are similar and about four times lower than that for lidocaine (Rutten et al., 1989). In this study, we used local anesthetic at 1 and 5 mM, these concentrations are closer to those applied during prolonged spinal nerve block for controlling hyperalgesic pain but much lower than those used during spinal and epidural anaesthesia. Based on our results, at 5 mM, ropivacaine and lidocaine will be expected to produce lower local neurotoxicity than bupivacaine, and at 1 mM lidocaine will be the least disturbing local anesthetic. The higher incident of transient neurologic symptoms following spinal anaesthesia with lidocaine than with bupivacaine might reflect the much higher lidocaine (2% ~ 42 mM) than bupivacaine (0.5% ~ 17 mM) concentration normally used. The very high local anesthetic levels used during spinal and epidural anaesthesia (5–200 mM) have been shown to irreversibly affect neuronal function in various experimental models (Bainton and Strichartz, 1994; Byers et al., 1973; Kanai et al., 1998; Lambert et al., 1994; Mateu et al., 1997). Clinically, such high local anesthetic levels probably never reach the nerve, since the local anesthetic application is usually short allowing for local anesthetic redistribution and diffusion away from the site of application. When membranes containing no cholesterol were exposed to those high local anesthetic levels (>10 mM lidocaine), membrane stability was markedly reduced and measurements of membrane properties were not possible (unpublished observation). Therefore, as the techniques for local anesthetic application become more precise (closer to the nerve, less local anesthetic dilution), reduction of the local anesthetic concentration being applied should be considered.

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