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Preferential location of lidocaine and etidocaine in lecithin bilayers as determined by EPR, fluorescence and ^{2}H NMR

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Abstract

We have examined the effect of the uncharged species of lidocaine (LDC) and etidocaine (EDC) on the acyl chain moiety of egg phosphatidylcholine liposomes. Changes in membrane organization caused by both anesthetics were detected through the use of EPR spin labels (5, 7 and 12 doxyl stearic acid methyl ester) or fluorescence probes (4, 6, 10, 16 pyrene-fatty acids). The disturbance caused by the LA was greater when the probes were inserted in more external positions of the acyl chain and decreased towards the hydrophobic core of the membrane. The results indicate a preferential insertion of LDC at the polar interface of the bilayer and in the first half of the acyl chain, for EDC. Additionally, ^{2}H NMR spectra of multilamellar liposomes composed by acyl chain-perdeutero DMPC and EPC (1:4 mol%) allowed the determination of the segmental order (S_{mol}) and dynamics (T_1) of the acyl chain region. In accordance to the fluorescence and EPR results, changes in molecular orientation and dynamics are more prominent if the LA preferential location is more superficial, as for LDC while EDC seems to organize the acyl chain region between carbons 2–8, which is indicative of its positioning. We propose that the preferential location of LDC and EDC inside the bilayers creates a "transient site", which is related to the anesthetic potency since it could modulate the access of these molecules to their binding site(s) in the voltage-gated sodium channel.

Keywords: Local anesthetic; Membrane; Fluorescence; Magnetic resonance; Liposomes

1. Introduction

Local anesthetics (LA) act by binding to the voltage-gated Na^+ channel, inhibiting Na^+ uptake and blocking the nervous impulse propagation. Binding of drug molecules to these channels depends on their conformation, with LA generally having a higher affinity for the open and inactivated channel states, induced by membrane depolarization [1]. Equally important for the anesthesia mechanism is the proposed role of the membrane lipid milieu in controlling the rate-limiting step for LA/protein association via the so-called "hydrophobic pathway" from the voltage-gated Na^+ channels [2] Many LA are ionizable amines and both the charged and uncharged forms are considered relevant for their pharmacological action.

There are only a few works in the literature focusing on the effect of the uncharged LA species upon model membranes, using spectroscopic techniques. It is possible to highlight the works of Smith et al., using NMR [3–6], others with FTIR [7], fluorescence [8] and molecular dynamics [9], beside some previous works from our group employing NMR, EPR and fluorescence [10–14]. The lack of works in this issue is perplexing since the uncharged LA species bind stronger to the membranes than do the charged species and both LA's potency and toxicity are directly related to the hydrophobicity of the LA molecule [15–17]. These features point out the uncharged LA species as good candidates for modulating the access of local anesthetic

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molecules to their binding sites at the voltage-gated sodium channel.

FTIR studies have shown that uncharged local anesthetic molecules can be found in the membrane–water interface region, where they compete with the phospholipid head groups for binding to water molecules [7,18]. ^{1}H NMR studies with four uncharged amino-amide local anesthetics in phosphatidylcholine membrane [14] suggested different preferential location of the LA inside the membrane, determined by the hydrophobic and steric features of each compound.

In the present work, we have investigated the insertion of the uncharged forms of two amino-amide local anesthetics (Fig. 1): lidocaine (LDC) and etidocaine (EDC) [15,19] inside the hydrophobic core of EPC bilayers. EPR, fluorescence and ^{2}H NMR probes positioned at different depths in the acyl chain core provided information about the local organization of the bilayer and detected changes caused by the presence of the LA at each specific spot monitored by the probes. The detection of a preferential location for each different LA molecule in the bilayer, determined by its particular physicochemical features, opens a new perspective for the understanding of LA action mechanism, in which this "transient site" could modulate the access of these molecules to their site(s) in the voltage-gated sodium channel [20].

2. Materials and methods

Egg phosphatidylcholine(EPC), 1,2-dimyristoyl-*d*54-*sn*-glycero-3-phosphocholine, local anesthetics (lidocaine and etidocaine), Chelex resin and methyl esters of doxyl stearic acid (MeSL spin probes) labeled at carbons 5, 7 and 12, were obtained from Sigma Chemical Co (St. Louis, MO). The fluorescent fatty-acid probes *n*-pyrene butanoic, hexanoic, decanoic and hexadecanoic acid (4-, 6-, 10- and 16-Py, respectively) were purchased from Molecular Probes Inc (Eugene, OR). All other reagents were of analytical grade.

2.1. Membrane preparation

Large multilamellar vesicles (MLV) were prepared as described before [14]. For EPR and fluorescence experiments, the labels were added at a concentration of 1 mol% with respect to lipid (EPC), prior to membrane suspension in 0.02 M carbonate/bicarbonate buffer solution, pH 10.5. For the ^{2}H NMR experiments, mixed liposomes containing EPC and DMPC perdeuterated in the acyl chains (DMPC-*d*54), in a 4:1 molar ratio were prepared in 0.2 M carbonate/bicarbonate buffer, pH 10.5.

The small unilamellar vesicles (SUV) used in the EPR and fluorescence experiments were prepared by sonication of MLV [14] using a Sonics and Materials (Newtown, CT) equipment. During sonication, the temperature was kept between 0 °C and 4 °C by intermittent (1 min) apparatus agitation cycles in an ice water bath. The sonicated samples were centrifuged with Chelex resin at 1000 ×*g* for about 20 min to remove large residual particles and any titanium from the sonicator tip.

2.2. EPR experiments

The spectra were obtained with a Bruker ER-200 SRC spectrometer operating at 9 GHz (3.4 kG). EPR Flat cells for aqueous solutions (Wilmad Co., USA) were used and the experiments were conducted at room temperature (22 °C). From the spin label spectra we have calculated the h_{+1}/h_0 parameter, given by the height ratio between low-field and mid-field resonances, before and after LA addition to the membranes. h_{+1}/h_0 is an empirical parameter that measures changes in overall membrane organization and was expressed as a percent "effect" relatively to the control [21,22] according to Eq. (1):

$$\%\text{Effect} = \frac{(h_{+1}/h_0)\text{sample} - (h_{+1}/h_0)\text{control}}{(h_{+1}/h_0)\text{control}} \times 100 \qquad (1)$$

This empirical parameter comprises the effect of both molecular mobility and order in the bilayer: The slower and the more anisotropic is the motion of the probe, greater is the difference between the low-field (h_{+1}) and the mid-field (h_0) peak heights. As membrane organization decreases, h_{+1}/h_0 gets closer to 1, as for the probe in an isotropic environment [21,23].

2.3. Fluorescence experiments

The experiments were conducted in a Hitachi F4500 fluorimeter (Tokyo, Japan), at room temperature (22 °C). Pyrene probes were excited at 345 nm and the emission spectra were collected in the 350–500 nm range. Quenching titrations were carried out by adding aliquots of LA stock solution directly to the fluorescence cuvette containing the labeled SUV.

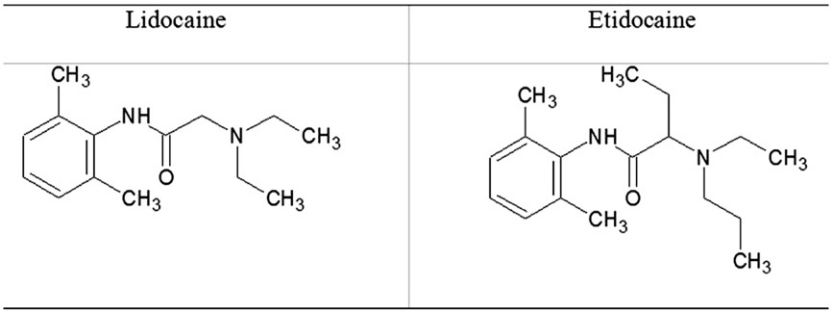

Fig. 1. Chemical structure of the LA.

Quenching (I_0/I %) was obtained by the ratio between the emission intensity, with (I) and without (I_0) LA.

2.4. Deuterium NMR experiments

The experiments were conducted in a Bruker MSL 300. ^{2}H quadrupolar splittings were determined at 30.7 MHz. The modified quadrupolar echo sequence [24] with quadrature detection was used to record echo signals. Pulse spacing was typically 60 μs and $\pi/2$ pulse length was 5.5 μs (10 mm coil); the recycle time was greater than $5T_1$ (800 ms). Longitudinal relaxation times (T_1) were obtained by Hahn-echo and inversion recovery technique, coupled with the quadrupolar echo sequence [25]. The frequency of the spectrometer was carefully set at the center of the quadrupolar powder patterns. Samples were enclosed in a glass jacket, in which the temperature was regulated to 30 °C. The spectra were "dePaked" according to Bloom et al. [26] to obtain the 90° oriented sample spectra from which the quadrupolar splittings were estimated.

2.5. ^2H quadrupolar splittings and calculation of the S_{mol} order parameter

For lipids in lamellar liquid-crystalline phases, in which the bilayer normal is the axis of motional averaging, the ^{2}H NMR spectra of C–^{2}H fragments present the shape corresponding to axially symmetric motion. In this case, the quadrupolar splitting, $\Delta\upsilon$, between the powder spectrum peaks is related to the orientational order parameter, S_{C-^2H}, according to Eq. (2) [27]

$$\Delta\upsilon = \left(\frac{3}{4}\frac{e^2qQ}{h}\right)\cdot S_{C-^2H} \quad (2)$$

where the static deuterium quadrupolar coupling constant, QC (QC=e^2qQ/h) is 170 kHz for the aliphatic C–^{2}H bonds [28]. Assuming a symmetric rotation around the membrane perpendicular axis (normal), it is possible to determine the local order parameter at this segment, S_{mol}, related to the order parameter of the C–^{2}H bound vector, according to Eq. (3) [27].

$$S_{mol} = -2S_{C-^2H} \quad (3)$$

3. Results

In a previous study, we have demonstrated that LDC was the most efficient among nine local anesthetics tested in decreasing the bilayer organization of EPC multilamellar liposomes, as measured by the EPR probe 5-methyl doxyl stearate, at pH 10.5 [11]. This finding was quite intriguing if the relatively low hydrophobic character of LDC is considered in comparison to other analyzed anesthetics such as EDC, bupivacaine and tetracaine.

In a different work, the interaction of the uncharged species of LDC, EDC, mepivacaine and bupivacaine with small unilamellar EPC vesicles was investigated through the measurement of changes in EPC dynamics, determined by ^{1}H NMR [14]. That approach revealed changes mainly in the polar head group and glycerol moiety of the bilayer, suggesting different depths of insertion for each LA that seemed to be determined by the hydrophobicity of the molecule and volume of its amine group region.

Herein, we employed three different spectroscopic techniques, using probes inserted at different depths along the acyl chain of unilamellar and multilamellar phosphatidylcholine liposomes in order to investigate the effect of LDC and EDC, at pH 10.5, on the hydrophobic core packing.

3.1. Electron paramagnetic resonance experiments

The effect of LA on egg phosphatidylcholine membrane organization was monitored with methyl ester derivatives of stearic acid (MeSL) spin labels. The probes carried paramagnetic nitroxide groups covalently attached to carbons 5, 7 or 12 and they are known to sense different depths of the hydrocarbon acyl chain region [29].

The hyperbolic curves obtained with increasing LA concentrations were exemplified in Fig. 2, for EDC, which is indicative of membrane saturation, in agreement with our previous observation [12,29]. Fig. 2 also reveals that the sensitivity of the MeSL probes to the effect of LA upon membrane organization decreased as the nitroxide assumed deeper positions in the acyl chain (5>7>12 MeSL), as predicted by the profile of the acyl chain order [30,31] and dynamics [29,32,33] inside the phospholipid bilayers.

Since we have previously determined the partition coefficient of these LA between EPC multilamellar vesicles and carbonate buffer, pH 10.5 [11], the EPR experiments were designed, so that the same LA:lipid molar ratio was found at the membrane. The quantitative results for 1:6 LA:lipid are shown in Fig. 3 for LDC and EDC.

LDC (EDC) has decreased the membrane organization monitored by 5-, 7- and 12-MeSL to different extents: 14.2, 10.4 and 5.3% (10.6, 9.0 and 7.7%), respectively. Local

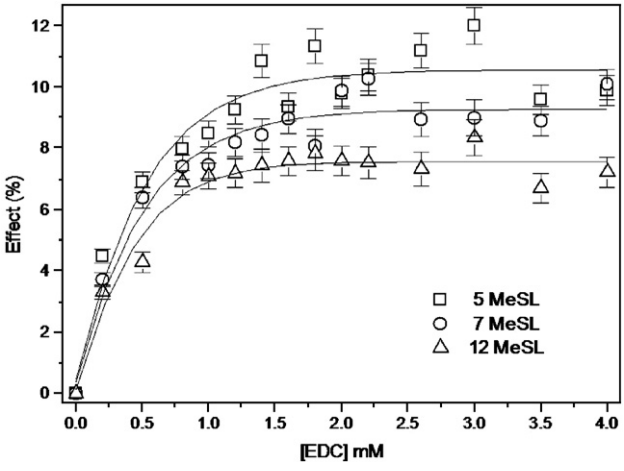

Fig. 2. Effect of EDC upon membrane organization, measured with spin probes at different positions inside the bilayer. EPC unilamellar vesicles (8 mM), 0.02 M carbonate buffer, pH 10.5, 22 °C.

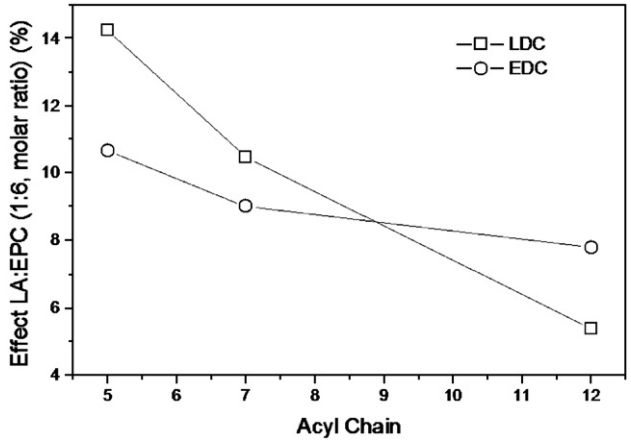

Fig. 3. LA effect at a 1:6 LA:EPC molar ratio in membrane organization, as monitored by EPR MeSL probes at different positions inside the bilayer. Experimental conditions as in Fig. 2.

anesthetics disturb the lipid packing since they are shorter (length ≅ 9 Å) than the lipid molecules (≅ 25 Å). LA are believed to create inter-lipid spaces that would permit an increase in the probability of trans-gauche conformations, decreasing the acyl chain order [13]. This disturbing effect is more pronounced if the LA occupies more restricted portions of the bilayer such as the glycerol backbone and first carbons of the acyl chain [29–31,33], as evinced for LDC, both in multilamellar [11] and unilamellar EPC vesicles [13,14].

The h_{+1}/h_0 parameter comprises information of both molecular mobility and orientation. Analysis of Fig. 3 shows that, although the major changes in the h_{+1}/h_0 parameter were detected by the 5-MeSL spin label (as expected), each LA elicited different effects on membrane organization: LDC produced a more evident decrease in membrane organization than EDC at the position monitored by 5-MeSL probe, which can be explained by the more hydrophilic character of LDC, as compared with EDC (Table 1) and its more superficial preferential location in the membrane [14].

In fact, EDC did not elicit the pronounced effect of LDC on the membrane organization measured by 5-MeSL [11] and ^{1}H NMR experiments detected no intermolecular NOE cross-peaks for EDC such as the one found between hydrogens belonging to the aromatic ring of LDC and the choline group of EPC vesicles [13]. Instead, ^{1}H NMR provided evidence for a deeper insertion of EDC inside unilamellar EPC vesicles [14].

3.2. Fluorescence experiments

The effect of LA on membrane organization has also been studied by fluorescence, using fatty-acid pyrene derivatives as membrane probes. As well as the spin labels, these fluorescent molecules were able to monitor different depths of the acyl chain [8].

Fig. 4 shows changes in the fluorescence of the pyrene probes located at different membrane depths, in the presence of increasing EDC concentrations. The quenching *effect* is favored by the decrease in the order or increase in molecular mobility

Table 1
Physical–chemical and pharmacological properties of the anesthetics studied: ionization constant (pKa), van der Waals volume and area, partition coefficient (*P*), water solubility (Sw), potency and half-life

Property	LDC	EDC
pKa [a]	7.8	7.7
Volume (angstron^3) [b]	274.04	327.43
Area (angstron^2) [b]	296.80	339.93
P_{SUV} (uncharged form) [c]	74±24	1.207±202
P_{MLV} (uncharged form) [d]	144±54	1.202±480
Sw (uncharged form) [e]	131.1 mM	0.16 mM
Potency [f]	4	16
Half-life (min) [a]	96	162

[a] According to [15].
[b] Calculated in vacuum, according to [16].
[c] Partition coefficient between EPC unilamellar vesicles and water, determined at pH 10.5 [Fraceto L. F., unpublished results].
[d] Partition coefficients between EPC multilamellar vesicles and water, determined at pH 10.5 by phase-separation, according to [11].
[e] Water solubility, determined at pH 10.5, according to [11].
[f] According to [19] in comparison to procaine (potency = 1).

[10] caused by the presence of LA inside the bilayer. As for EDC, partitioning of LDC into the membrane has decreased its organization in a concentration-dependent manner up to membrane saturation, in agreement with the EPR results reported above.

Since lipid concentration was limited to 1 mM to avoid light scattering, we could not reach the same LA:lipid molar ratios used in the EPR experiments; nevertheless, Fig. 5 shows that, at equivalent concentrations (1:14 LA:EPC, mol% in the membrane), the *effect* of LA on membrane organization was stronger for the probes near the membrane surface and weakened towards the bilayer core (4 > 6 > 10 > 16 Py-fatty acid), in a similar profile to that observed with the spin labels. LDC decreased the membrane organization monitored by 4-, 6-, 10 and 16-Py-fatty acids by 13.2, 9.6, 8.4 and 7.0%, while EDC had a stronger effect: 28.4, 24.2, 21.9 and 16.1%, respectively.

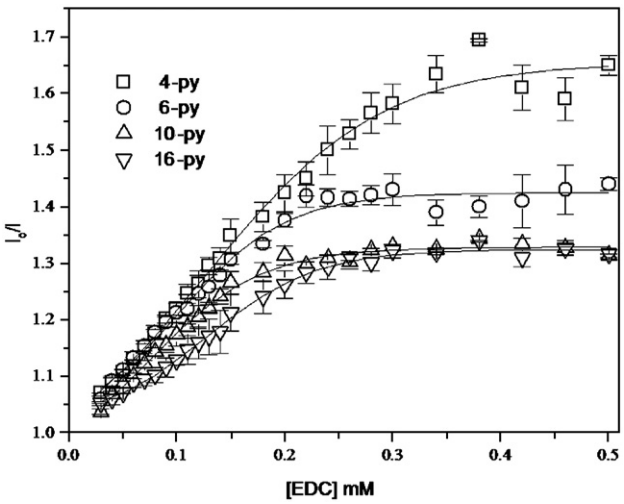

Fig. 4. Quenching of pyrene-fat acids fluorescence by EDC. Probes incorporated to EPC unilamellar vesicles, at different depths of the bilayer. [EPC] = 0.7 mM, pH 10.5, 22 °C.

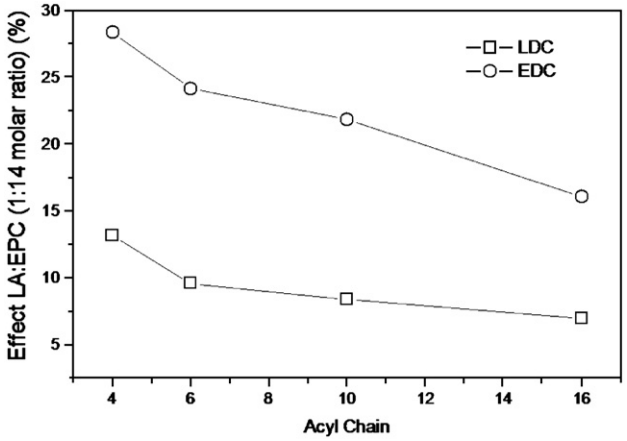

Fig. 5. LA effect upon EPC organization at a 1:14 LA:EPC molar ratio inside the membrane (as seen by fluorescence labels at different position inside the membrane). Experimental conditions as in Fig. 4.

Because EDC presented an outstanding quenching effect on the four Pyrene probes used (Fig. 5), we decided to check its quenching efficiency over the fluorescent probe in an isotropic medium (water:ethanol 95:5 v:v). A quantitative assessment of the quenching efficiency of LDC and EDC was obtained by plotting the I_0/I values vs. anesthetic concentration (Fig. 6) and assuming a simple Stern–Volmer kinetics [34].

The determined Stern–Volmer quenching constants (K_{SV}) for the 4-Py probe were 76.7 M^{-1} and 247.5 M^{-1} for LDC and EDC, respectively. The values of K_{SV} confirmed the high quenching efficiency of EDC over LDC [34], justifying the differences observed with the use of EPR (LDC>EDC) and fluorescence (EDC>LDC) probes as monitors of membrane organization.

Considering the possibility that the regions monitored by the MeSL and Pyrene probes could not be strictly related to the positions of the EPC acyl chain carbons, we have measured the ^{2}H NMR signals of DMPC-*d*54 loaded EPC multilamellar vesicles.

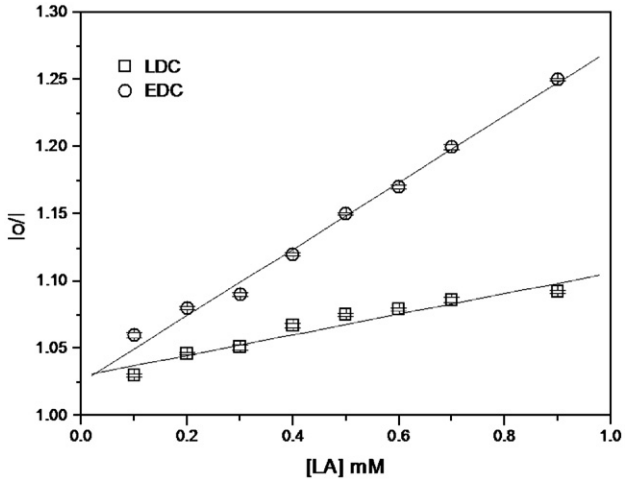

Fig. 6. Stern–Volmer plots for quenching in 4-Py butyric acid fluorescence dissolved in water:ethanol (95:5 v:v) solution, induced by etidocaine and lidocaine, pH 10.5, 22 °C.

3.3. Deuterium nuclear magnetic resonance experiments

^{2}H NMR of chain perdeuterated PC was used to evaluate the interaction of the LA (1:3 LA:EPC molar ratio inside the membrane) with the hydrophobic membrane core and its effect upon both membrane order and dynamics. Fig. 7 shows the spectra of DMPC-*d*54 loaded EPC liposomes, registered in absence and in the presence of LDC. LA effect (Fig. 7B) is evident in external shoulders, an "envelope" of deuteron peaks nearby 14 MHz that corresponds to the first carbon positions of the acyl chain [35]. The ^{2}H NMR "dePaked" spectra – revealing the 90° oriented sample [26] – were used for peak assignment, quadrupolar splittings and T_1 measurements.

The variation of the deuterium quadrupolar splitting ($\Delta\nu/2$) for the liposomes gives a pure structural measurement of the rotational freedom (orientation) of carbon–deuterium bonds relatively to the external magnetic field, and it is related to the segmental order parameter, S_{mol}, according to Eqs. (2) and (3). The profile of S_{mol} values obtained in the presence of LA (Fig. 8) is in accordance with the literature and resembles those

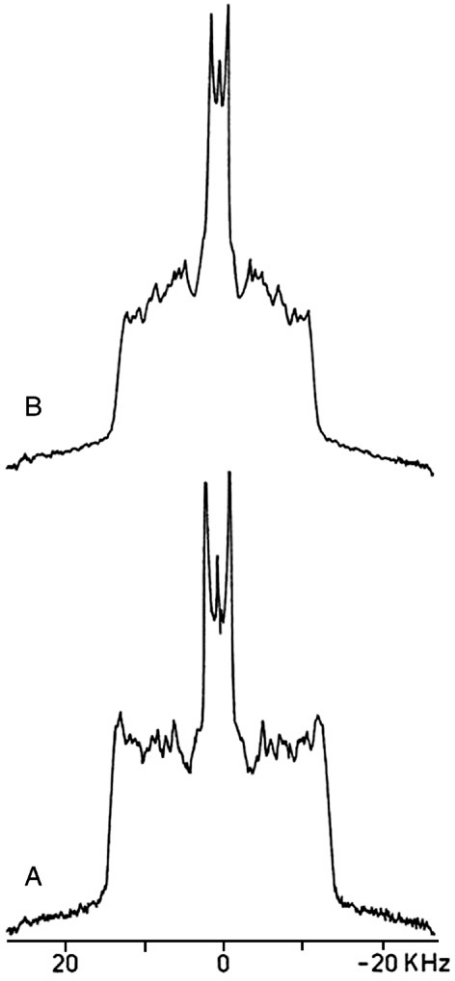

Fig. 7. ^{2}H NMR spectrum of acyl chain-perdeutero DMPC incorporated in EPC (1:4 mol%) multilamellar vesicles (A), in the presence of lidocaine (1:3, LA: lipid, mol%) (B). [lipid]=143 mM., pH 10.5, 22 °C, 30.7 MHz, echo-quadrupolar pulse sequence ($(\pi/2_x - t_1 - \pi/2_y - t_2 -$ acquisition), $\pi/2$=5.5 μs.

found with perdeutero POPC vesicles, with the general anesthetics halothane, enflurane and isoflurane [36].

Fig. 8 shows that LA caused changes in the segmental order parameter of the DMPC-*d*54 acyl chain but, as discussed above (Fig. 7), mainly at the "envelope" of deuteron peaks corresponding to the first carbon positions of the acyl chain (C_2–C_8 in control), so that the signals belonging to the C_7 and C_8 deuterons become identifiable when the LA is present (Fig. 8), in agreement with results previously reported for neutral tetracaine species [4]. LDC decreased the segmental order parameter of the acyl chain as a whole, probably due to its preferential positioning towards the polar head-group region of the bilayer [13], increasing the rotational freedom of the acyl chain. EDC effect was more discrete than that of lidocaine and distinctive, since it increased the acyl chain molecular orientation of deuterons at the carbons 2–7, which is a strong evidence of its insertion at that spot.

The dynamics of the EPC acyl chain core were accessed through longitudinal relaxation (T_1) measurements of deuterons from DMPC-*d*54 loaded EPC liposomes. Fig. 9 gives the T_1 values for the acyl chain deuterons, measuring the rates of rotation of their C–^{2}H bonds, in the presence of LA. As expected, the dynamics inside the acyl chain increase towards the terminal-CH_3 carbon [36,37].

The capacity of different LA to affect the mobility (T_1) of the acyl chain lipids resembles that of the order parameter, i.e., LDC was more effective than EDC. In the presence of LDC the dynamics of the acyl chain deuterons was increased as a whole, probably because of the inter-lipid spaces created by lidocaine preferential insertion nearly to the polar head-group region of the bilayer. T_1 values were slightly decreased up to C_6 in the presence of EDC, reflecting its site of insertion, while it has increased at deeper positions.

The inflection observed for the deuteron belonging to carbon 9 of DMPC-*d*54 in the presence of EDC reflects the presence of double bonds between C_9–C_{10} of EPC, as previously observed for perdeutero DOPC inside biological membranes [37]. The reason why the inflection got so evident in the presence of EDC

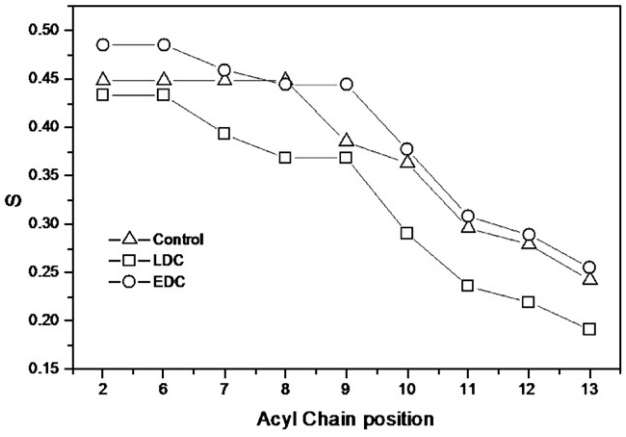

Fig. 8. Changes in the segmental order parameter S_{mol}, taken from the ^{2}H quadrupolar splittings of DMPC-*d*54:EPC multilamellar vesicles in the presence of local anesthetics (1:3 LA:lipid, molar ratio inside the membrane), pH 10.5, 23 °C.

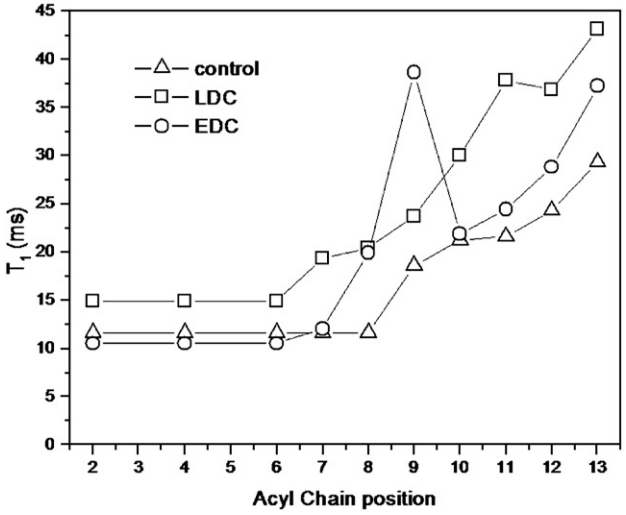

Fig. 9. Changes in the spin-lattice relaxation time (T_1) in DMPC-*d*54:EPC multilamellar vesicles in the presence of local anesthetics (LA:lipid 1:3, molar ratio inside the membrane), pH 10.5, 23 °C.

is probably related to the shorter length of the LA in comparison to the lipid (as discussed above) and also to the positioning of EDC around the first carbons of the acyl chain, so that its voluminous side chain fulfills spaces in between the lipids up to carbon 7.

4. Discussion

Amphiphilic molecules such as local anesthetics are able to quickly cross membranes, reestablishing their concentration among lipid bilayers and intracellular fluids. These molecules bind to membranes, exhibiting a preferential position inside the bilayer, where they can be found most of the time, determined by their physicochemical properties, [14,20].

The existence of a preferential membrane location for the local anesthetics does not mean that these molecules remain steadily at that site. In fact, anesthesia requires a fast equilibrium of the LA between the adjacent membrane and water compartments, implying in fast movement across the membrane and water. EPR experiments have demonstrated the existence of such a fast steady-state equilibrium since it is not possible to detect distinct populations of spin-labeled LA [38,39] upon their addition to liposomes. Nevertheless, the preferential location of the LA molecules inside the bilayer can create a "hydrophobic pathway" that could modulate the access of these molecules to their site(s) in the voltage-gated sodium channel [20,40].

Indeed, the location and orientation of anesthetics in the membrane could play a crucial role in the mechanism of anesthesia since they can modulate LA–Na^+ channel binding by directing the molecule to access the proper site at the channel and/or by enhancing LA concentration in the surroundings of the binding site(s) [41,42]. The polarity of the bilayer region in which the anesthetic resides most of the time could then determine the orientation of the molecule, addressing its binding to the site(s) of action, inside the voltage-gated sodium channel [20].

In the present work, we demonstrate that the uncharged form of LDC and EDC interacts with PC liposomes. EPR and fluorescence experiments have measured changes in the acyl chain packing sensed by probes inserted at different spots. ^{2}H NMR experiments gave a more realistic picture of the membrane interior, allowing us to distinguish between the effect of EDC and LDC, mainly in the upper portion of the acyl chains. ^{2}H NMR shows that etidocaine is preferentially located in the first half of the acyl chain (i.e., up to carbons 7–8 of perdeutero DMPC). As for LDC, it induced a less tightly packing (lower order, Fig. 8) and increased lipid dynamics (higher T_1 values, Fig. 9) in the acyl chain of multilamellar vesicles, possibly due to the inter-lipid spaces created by the preferential insertion of its aromatic group in the glycerol moiety [13,14]. The findings obtained through EPR, fluorescence and ^{2}H NMR experiments support the existence of a "transient site" both for LDC and EDC, determined mainly by their hydrophobic and steric features, where the LA can be found, most of the time.

The "transient site" description gives a good picture of the complex relationship between LA effects upon lipids and proteins of the excitable membranes, which are profoundly dependent on the insertion of the LA molecule (amount, depth, orientation) in the bilayer. As stated by Yun et al. [43], "the fluidization of membrane lipids may provide an ideal microenvironment for optimum local anesthetic effects and interaction with sodium channels".

The insertion of EDC resembles that reported for the uncharged species of tetracaine, a more hydrophobic and cylindrical-shape LA that penetrates more deeply into the membrane [4,40]. Similar results were observed in literature for chlorpromazine (CPZ, an antipsychotic agent) with erythrocytes membranes [44] and phospholipids monolayers [45,46], where CPZ induces ordering of the lipid being preferentially located in the lipid tail region.

EDC's "transient site" comprises the first half of the lipids acyl chain, as confirmed by changes in the order and mobility of the lipids, seen by ^{2}H NMR. This result is quite compatible with the hydrophobic binding site proposed for EDC inside the S6/IV segment of the voltage-gated Na^+ channel, revealed by site-direct mutagenesis experiments [41].

In conclusion, we did not detect a direct correlation between LA effect (decrease in membrane organization) and anesthetic potency, as claimed by other authors [47] using experimental and theoretical approaches, respectively, in model membranes at pH 7.4. Nevertheless, the results presented here show that a deeper insertion into the membrane guarantees an increased LA potency, as pointed out by ours and other works in the literature [3,13,14].

As a possible connection to explain the mechanism of action of local anesthetics we propose that if the "transient site" of a given LA is at the hydrophobic core region, the potency of this LA will be higher, probably because it will gain free access to the hydrophobic LA sites in the voltage-gated Na^+ channel, such as those reported for EDC by Ragsdale et al. [41,48]. By the other hand, changes in membrane fluidity (decrease in membrane organization/increase in membrane dynamics) are more pronounced for LA with "transient sites" at the polar headgroup region which is not related with its potency, as for LDC.

The effect of LA on the lipid phase organization probably involves changes in membrane hydration [47] being determined by steric parameters such as van der Waals' volume and molecular orientation [14]. These points deserve deeper analysis and forthcoming molecular dynamic simulations (Pickholz, M., Fraceto, L.F., de Paula, E. in preparation) should help the understanding, at a molecular level, of the features that determine LA effect on the bilayer and its significance on the anesthesia mechanism.

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