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Spectroscopic evidence for a preferential location of lidocaine inside phospholipid bilayers

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Abstract

We examined the effect of uncharged lidocaine on the structure and dynamics of egg phosphatidylcholine (EPC) membranes at pH 10.5 in order to assess the location of this local anesthetic in the bilayer. Changes in the organization of small unilamellar vesicles were monitored either by electron paramagnetic resonance (EPR)—in the spectra of doxyl derivatives of stearic acid methyl esters labeled at different positions in the acyl chain (5-, 7-, 12- and 16-MeSL)—or by fluorescence, with pyrene fatty-acid (4-, 6-, 10- and 16-Py) probes. The largest effects were observed with labels located at the upper positions of the fatty-acid acyl-chain. Dynamic information was obtained by ^{1}H-NMR. Lidocaine protons presented shorter longitudinal relaxation times (T_1) values due to their binding, and consequent immobilization to the membrane. In the presence of lidocaine the mobility of all glycerol protons of EPC decreased, while the choline protons revealed a higher degree of mobility, indicating a reduced participation in lipid–lipid interactions. Two-dimensional Nuclear Overhauser Effect experiments detected contacts between aromatic lidocaine protons and the phospholipid-choline methyl group. Fourier-transform infrared spectroscopy spectra revealed that lidocaine changes the access of water to the glycerol region of the bilayer. A 'transient site' model for lidocaine preferential location in EPC bilayers is proposed. The model is based on the consideration that insertion of the bulky aromatic ring of the anesthetic into the glycerol backbone region causes a decrease in the mobility of that EPC region (T_1 data) and an increased mobility of the acyl chains (EPR and fluorescence data).

Keywords: Lidocaine; Membrane; Nuclear magnetic resonance

Abbreviations: EPC, egg phosphatidylcholine; EPR, electron paramagnetic resonance; FTIR, Fourier-transform infrared spectroscopy; LA, Local anesthetics; LDC, lidocaine; MeSL, methyl ester of doxyl-stearic acid spin label; LMV, large multilamellar vesicles; *P*, partition coefficient; Py, pyrene-derivative fluorescent probe; NMR, nuclear magnetic resonance; T_1, longitudinal relaxation times; ROESY, Rotational nuclear Overhauser Effect SpectroscopY; SUV, small unilamellar vesicles

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1. Introduction

Local anesthetics (LA) are known to exert their anesthetic effect by blocking inward sodium transport and therefore, the action potential of axons [1]. In addition to binding to the voltage-gated sodium channel, these molecules are known to bind to other membrane proteins affecting their function [2]. Local anesthetic also interact with and alter the organizational properties of lipid membranes [3–13]. In biological membranes such changes could interfere with lipid–protein interactions, leading to protein conformational changes with a reflection on their activity [1,2,14,15]. Moreover, according to the so called Meyer–Overton rule—that the more hydrophobic, the more potent is the anesthetic [16–18]—partitioning into the lipid bilayer can modulate the access of local anesthetic to their binding sites in Na^+ channels [16,19–21].

In spite of their different structures, the majority of local anesthetic share common chemical features that are relevant for their biological function: an aromatic ring, a polar group and an ionizable amine with pK around physiological pH [2,18,22].

Although evidence for a specific binding of charged local anesthetic to Na^+ channels has been reported [19,22–24] and studies conducted at physiological pH have indicated that local anesthetic activity is related to the protonated form, the importance of the uncharged species has been increasingly recognized in view of: (i) the postulated existence, in voltage-gated Na^+ channels, of a binding site for the uncharged local anesthetic form located deep inside the hydrophobic membrane core [20,21]; (ii) more pronounced partitioning and perturbation of lipid bilayer organization for uncharged local anesthetic species [5,12]; and (iii) a recognized correlation between local anesthetic hydrophobicity, potency and toxicity [16–18,25,26].

The advantages of the uncharged species might be related to their stronger binding to the membrane. In fact, their dispersion into the bilayer could provide a way to protect the molecule from metabolic processes that would produce their elimination [2]. As a result, one would expect delayed clearance in time, justifying long-lasting anesthesia. Indeed, good evidence for this hypothesis is given by the observation that hydrophobic anesthetics show longer half-lives than hydrophilic ones [17,24].

However, we have shown that while the uncharged forms of nine local anesthetic bind to egg phosphatidylcholine (EPC) model membranes, decreasing membrane organization as detected by EPR [12], their perturbing effect does not correlate with local anesthetic hydrophobicity or class (esters vs. amides). In fact, lidocaine (Fig. 1), one of the less hydrophobic anesthetics [26] studied, has been found to decrease membrane organization to a greater extent than the more hydrophobic local anesthetic such as etidocaine, bupivacaine, dibucaine or tetracaine. These results therefore suggest that binding to the membrane is not regulated only by hydrophobicity. Indeed, polar interactions or other steric parameters might determine a specific or preferential location for each local anesthetic molecule inside the membrane.

Here, we present a study on lidocaine (LDC) binding to EPC liposomes where, by measuring the anesthetic's effect on lipid organization, we collected strong evidence for the existence of a preferential location for lidocaine insertion into the bilayer. The detection of a preferential location for each different local anesthetic molecule in the bilayer—determined by its own physicochemical properties—opens a new perspective for the understanding of local anesthetic activity at the molecular level. Even considering the fast movement of local anesthetic molecules across the membrane, this 'transient site' in the bilayer could modulate the access of these molecules to their site(s) in the voltage-gated sodium channel.

2. Materials and methods

Egg phosphatidyl choline, deuterated water (D_2O, 99.9%), Chelex resin and spin labels, methyl esters of doxyl stearic acid (MeSL) labeled at carbons 5, 7, 12 and 16 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 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, USA). Lidocaine

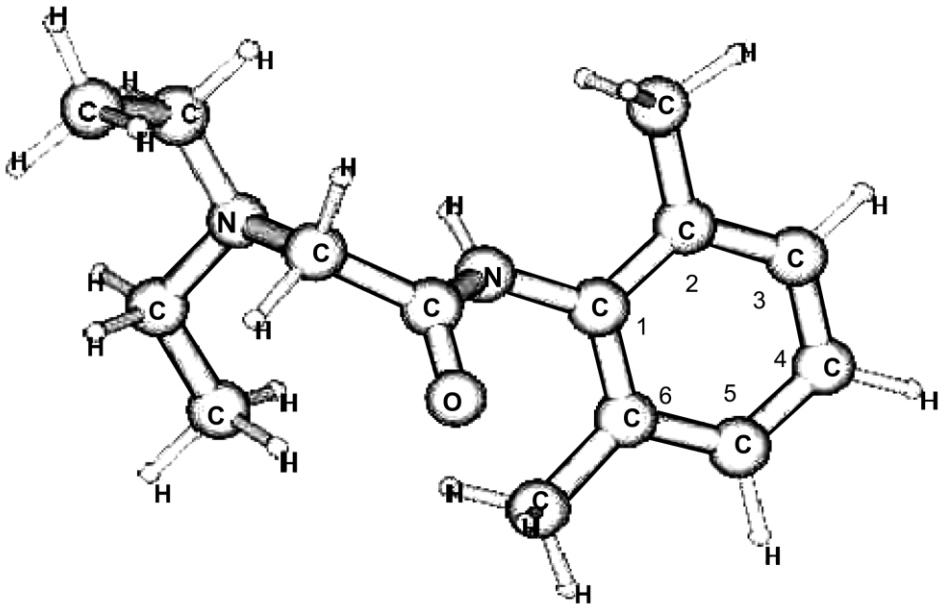

Fig. 1. Lidocaine molecular modeling, in vacuum.

hydrochloride was a gift from Apsen Brasil, Ind. Quim. Farm. Ltda, São Paulo, Brazil. All other reagents were of analytical grade.

2.1. Membrane preparation

Liposomes were obtained by evaporating stock chloroform solutions of EPC under a stream of nitrogen. The samples were left under vacuum for no less than 2 h to remove residual solvent. The lipids were then suspended in 0.02 M carbonate/bicarbonate buffer solution, pH 10.5. Large multilamellar vesicles (LMV) were obtained by vortexing for 3 min. For EPR and fluorescence experiments the labels were dissolved in chloroform and added at a concentration of 1 mole% with respect to lipid.

Small unilamellar vesicles (SUV), were obtained from freeze-dried LMV suspended in D_2O (pD 10.5). The samples were sonicated until clear (approx. 15 min) using a Sonics and Materials (Newtown, CT, USA) equipment. During sonication, the temperature was kept at 0–4 °C by intermittent (1 min) apparatus agitation cycles in an ice water bath. The sonicated samples were centrifuged with Chelex resin at $1000\times g$ for approximately 20 min to remove residual large particles and any titanium from the sonicator tip.

2.2. Partition coefficient determination

The LDC/EPC molar ratios inside the membrane were calculated by the membrane–water partition coefficient, P, of lidocaine determined by phase-separation between SUV/water at pH 10.5, according to the Eq. (1):

$$P=\frac{n_m/V_m}{n_w/V_w} \quad (1)$$

where n denotes the number of moles of lidocaine, V is the volume, and the subscripts m and w refer to the membrane and aqueous phase, respectively. The amount of lidocaine bound to the lipid phase was optically determined at 262 nm ($\varepsilon_M=420$) after ultra-centrifugation at $120\,000\times g$ for 2 h, by subtracting the supernatant concentration from the total drug concentration measured before phase mixing.

Phospholipid concentration was determined according to [27].

2.3. Electron paramagnetic resonance (EPR) experiments

The spectra were obtained with a Bruker ER-200 SRC spectrometer operating at 9 GHz (3.4 kG). 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 calculated the h_{+1}/h_0 parameter, the height ratio of low-field to mid-field resonance, after lidocaine addition to the membranes. h_{+1}/h_0 is an empirical parameter that measures changes in overall membrane organization and is expressed as a percent 'effect' relative to the control [12], according to Eq. (2):

$$\%\ \text{Effect} = \frac{(h_{+1}/h_0)\ \text{sample} - (h_{+1}/h_0)\ \text{control}}{(h_{+1}/h_0)\ \text{control}} \times 100 \quad (2)$$

This empirical parameter comprises the effect of both molecular mobility and order in the bilayer: the slower and the more anisotropic the motion of the probe, the greater the difference between the low-field (h_{+1}) and the mid-field (h_0) peak heights. As membrane organization decreases h_{+1}/h_0 approaches 1, as for the probe in an isotropic environment [6,28].

2.4. Fluorescence experiments

The experiments were conducted using a Hitachi F4500 fluorimeter (Tokyo, Japan) at room temperature (22 °C). Pyrene probes were excited at 345 nm and the emission spectra collected in the 350–500 nm range. Quenching titrations were carried out by adding aliquots of lidocaine stock solution directly to the fluorescence cuvette containing the labeled SUV. Quenching (I_0/I, %) was obtained by the ratio between the emission intensity with (I) and without (I_0) lidocaine.

2.5. Nuclear magnetic resonance (NMR) experiments

The spectra were collected either with a Bruker AMX 400 (University of Parma, Parma, Italy) or Bruker DRX 500 (Universidade de São Paulo, Brazil) apparatus. The samples were degassed to avoid interference of dissolved O_2 with the T_1 and/or NOE measurements. For ^{1}H-NMR, a 90° pulse was typically 10–15 μs and the recycling time was set to five times the largest T_1 (those of the aromatic protons), typically 6 s. Longitudinal relaxation times (T_1) were obtained by the conventional inversion recovery method at temperature between 20 and 40 °C. The Rotational nuclear Overhauser Effect SpectroscopY (ROESY) experiments [29] were carried out using 50 ms mixing times, for the detection of build-up NOEs [30,31]. ^{31}P-NMR decoupled spectra were recorded at 202 MHz using a single pulse sequence [d_1-π/6-τ_1(acquisition)]; pulse length was 7 μs and the recycling time was 3 s. Eighty-five percent H_3PO_4 was used as standard.

2.6. Fourier transform infrared (FTIR) determinations

The experiments were carried out using the CaF_2 cell of a BOMEM-MB Series FTIR spectrometer, at Instituto de Química/Unicamp. Spectra were deconvoluted using different Gaussians/Lorentzians rates. Each spectrum of 64 scans was averaged with the detector at 2 cm^{-1} resolution. The samples were prepared in D_2O buffered solution to avoid the strong water absorption band at 1650 cm^{-1}.

2.7. Molecular modeling

The molecules were built with the Molden software and processed/visualized with the GAMESS (General Atomic and Molecular Electronic Structure System) program that uses a semi-empirical AM1 approach and MNDO approximation. The programs were licensed to Centro Nac. Processamento de Alto Desempenho em São Paulo (CENAPAD) that allowed us to calculate the molecules in a parallel process.

3. Results and discussion

In a previous study we demonstrated that lidocaine, an amino–amide local anesthetic (Fig. 1), was the most efficient among the nine local anes-

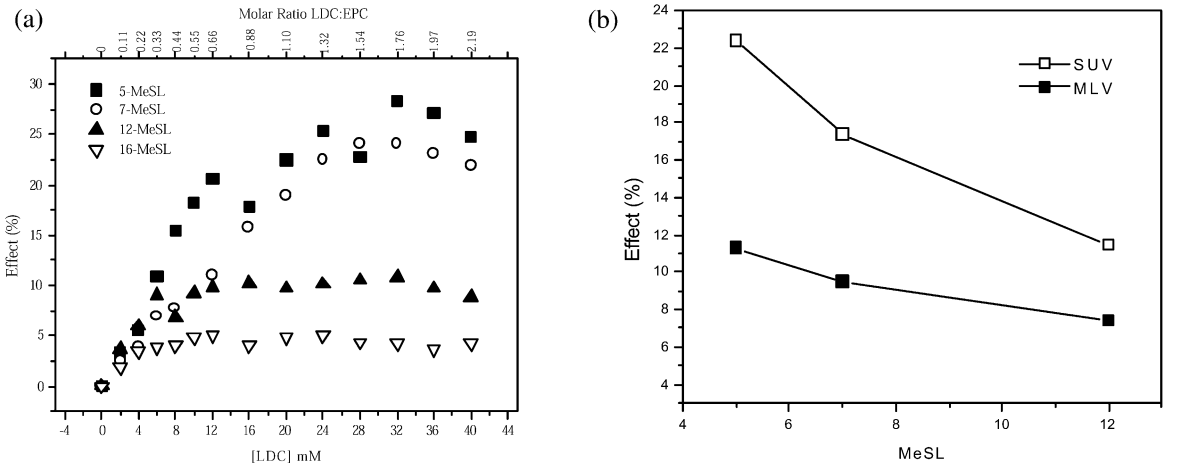

Fig. 2. (a) Effect of lidocaine on membrane organization detected by the EPR spectra of spin probes at different depths (5-,7-,12- and 16-MeSL) in large multilamellar EPC (8 mM) vesicles in 0.02 M carbonate buffer, pH 10.5, at 22 °C. (b) Comparative effect for a 0.33:1, LDC/EPC molar ratio in the membrane on small unilamellar (□) and large multilamellar (■) vesicles.

thetic tested in decreasing the membrane organization of EPC multilamellar vesicles at pH 10.5 [12]. This finding was quite intriguing if we consider the Meyer–Overton rule and the relatively low hydrophobicity of lidocaine.

3.1. Electron paramagnetic resonance experiments

The effect of lidocaine on egg phosphatidylcholine membrane organization was followed by methyl ester derivatives of stearic acid (MeSL) spin labels. These probes carry paramagnetic doxyl groups at carbons 5, 7, 12 or 16, monitoring different depths of the bilayer [32]. In LMV (Fig. 2a) the decrease in membrane organization induced by lidocaine was observed with all the spin-labels used. The hyperbolic curves were indicative of membrane saturation, in agreement with our previous observation using the 5-MeSL probe [12]. The maximum decrease in membrane organization reached 26% with 5-MeSL. Using the partition coefficient of lidocaine between LMV/water ($P=144$ at pH 10.5 [12]), we calculated that membrane saturation in the curves illustrated in Fig. 2a occurred when the anesthetic reached a 0.4:1 molar ratio (LDC/lipid, in the membrane).

According to the curves in Fig. 2a, the sensitivity of the MeSL probes to the effect of lidocaine decreased as the nitroxide assumed deeper positions in the acyl chain ($5>7>12\geq16$ MeSL), as predicted by the profile of the acyl chain order [33,34] and dynamics [30,32,35] inside the phospholipid bilayers.

We determined the partition coefficient for Lidocaine between SUV/water ($P=74\pm24$ at pH 10.5) and carried out experiments like those in Fig. 2a using unilamellar vesicles. While membrane saturation was reached at the same molar ratio (approx. 0.4:1, LDC/lipid) in the membrane as for LMV, the maximum effect reached 36.5% in the curve for 5-MeSL. Knowledge of P values allowed us to carry out the analysis of EPR data at a fixed lidocaine/lipid molar ratio in the membrane. Fig. 2b compares the effect of lidocaine, at a 0.33:1 LDC/lipid molar ratio in the membrane—in the different membrane regions monitored by the MeSL probes—both for LMV and SUV. Data from 16-MeSL were not included because changes were quite small, within the error of the experiment. The profile for LMV and SUV was similar: the effect of lidocaine on membrane organization was stronger at positions near the membrane/water interface (5 and 7 MeSL), although the decrease in membrane organization was much more evident in the unilamellar vesicles, probably due to the

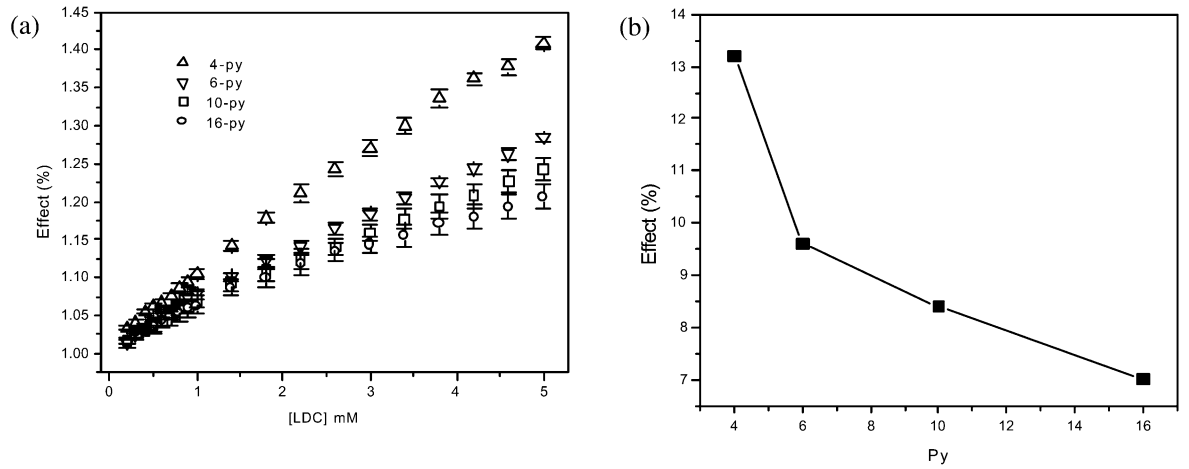

Fig. 3. Effect of lidocaine on membrane organization detected by the spectra of fluorescence probes at different depths (4-,6-,10- and 16-Py) on small unilamellar EPC (0.7 mM) vesicles, pH 10.5, 22 °C. (b) Comparative effect for a 0.07:1 (LDC/EPC, molar ratio in the membrane).

looser packing of the lipids in these vesicles [36,37].

Differences in the acyl chain order and dynamics could not explain the profiles observed in Fig. 2b. Moreover, the effect of lidocaine on membrane organization detected by EPR ($5>7>12$ MeSL) differs from that of tetracaine ($5\approx7>12$ MeSL) and benzocaine ($5<7>12$), as we have shown before [13].

These data suggest that lidocaine penetrates the lipid bilayer in the region comprised between the glycerol backbone and the 2–5 carbons of the acyl chain, acting as a spacer between lipid molecules. Lidocaine is rather spherical, and the presence of two *o*-methyl groups in its aromatic ring creates a significant steric hindrance for its insertion between the lipids. This extra volume in the aromatic ring could explain the higher effect of lidocaine on membrane organization in comparison to tetracaine [12], an amino-ester local anesthetic that does not have *o*-methyl groups. Tetracaine is essentially cylindrical in shape and has a long amino-butyl tail in the *para* position of the benzoic acid ring that is expected to favor the orientation of the molecule parallel to the acyl chains of the phospholipids [2]. Previous results from our laboratory have shown that in LMV monitored by 5-MeSL probes, tetracaine was not able to decrease membrane organization by more than 15% [12].

Local anesthetic disturb the packing of the lipids since they are shorter (lidocaine length = 9.4 Å) than the lipid molecules (approx. 25 Å). Local anesthetic is believed to create inter-lipid spaces that would permit an increase in the probability of Gauche–CH_2 bonds, decreasing the acyl chain order [2]. This disturbing effect can be more pronounced if the local anesthetic occupies more restricted portions of the bilayer such as the glycerol backbone and first carbons of the acyl chain [32–35]. In fact, etidocaine and bupivacaine—hydrophobic analogs of lidocaine—did not have the pronounced effect of lidocaine on membrane organization [12] and we have evidence for a deeper insertion of both local anesthetics inside EPC bilayers (Fraceto and de Paula, in preparation).

3.2. Fluorescence experiments

The effect of local anesthetic on membrane organization has been studied also by fluorescence using fatty-acid pyrene derivatives as probes; like the spin labels, these fluorescent molecules were able to monitor different depths of the acyl chain [38], but light scattering limited the studies in SUV up to 1 mM concentration.

In Fig. 3 the quenching of the pyrene probes is located at different membrane depths and was

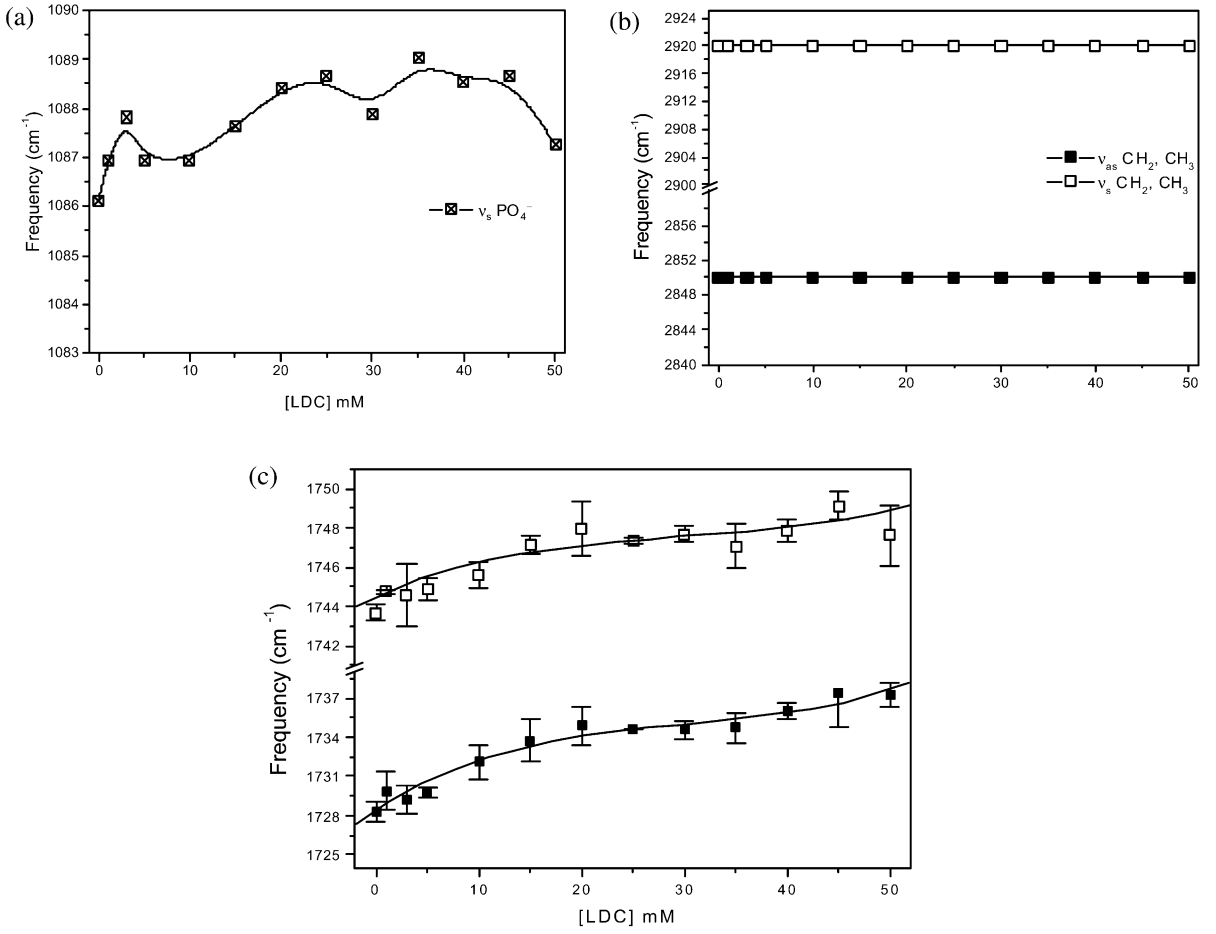

Fig. 4. Changes in the FTIR absorption of the phosphate stretching band, ν_s (a); acyl chain stretching bands $-CH_2-CH_3$ ν_s, and ν_{as} (b), and carbonyl stretching bands (c) of EPC small unilamellar vesicles in the presence of increasing lidocaine concentration. [EPC] = 65 mM, CaF_2 cell, 22 °C, pH 10.5.

considered to be the *effect* resulting from decreased order or increased molecular mobility inside the bilayer [39] caused by lidocaine. Partitioning of the anesthetic into the membrane decreased its organization in a concentration-dependent manner, in agreement with the EPR results. Since lipid concentration was limited to 1 mM to avoid scattering, lidocaine could not reach an adequate concentration (0.4:1, mole%) in the membrane to induce saturation (as in Fig. 2b).

As evidenced by the pyrene probes in Fig. 3b, for a fixed molar ratio (0.07:1, LDC/EPC) in the membrane, the *effect* of Lidocaine 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 profile similar to that observed for spin-label probes.

3.3. ^{31}P-NMR and FTIR experiments

Since EPR and fluorescence data indicated that Lidocaine affected mainly the more superficial portions of the bilayer, we decided to verify the degree of perturbation that could be induced at the polar head-group of phosphatidylcholine.

Boulanger et al. reported an 8% change in the ^{31}P-NMR chemical shift anisotropy of EPC mul-

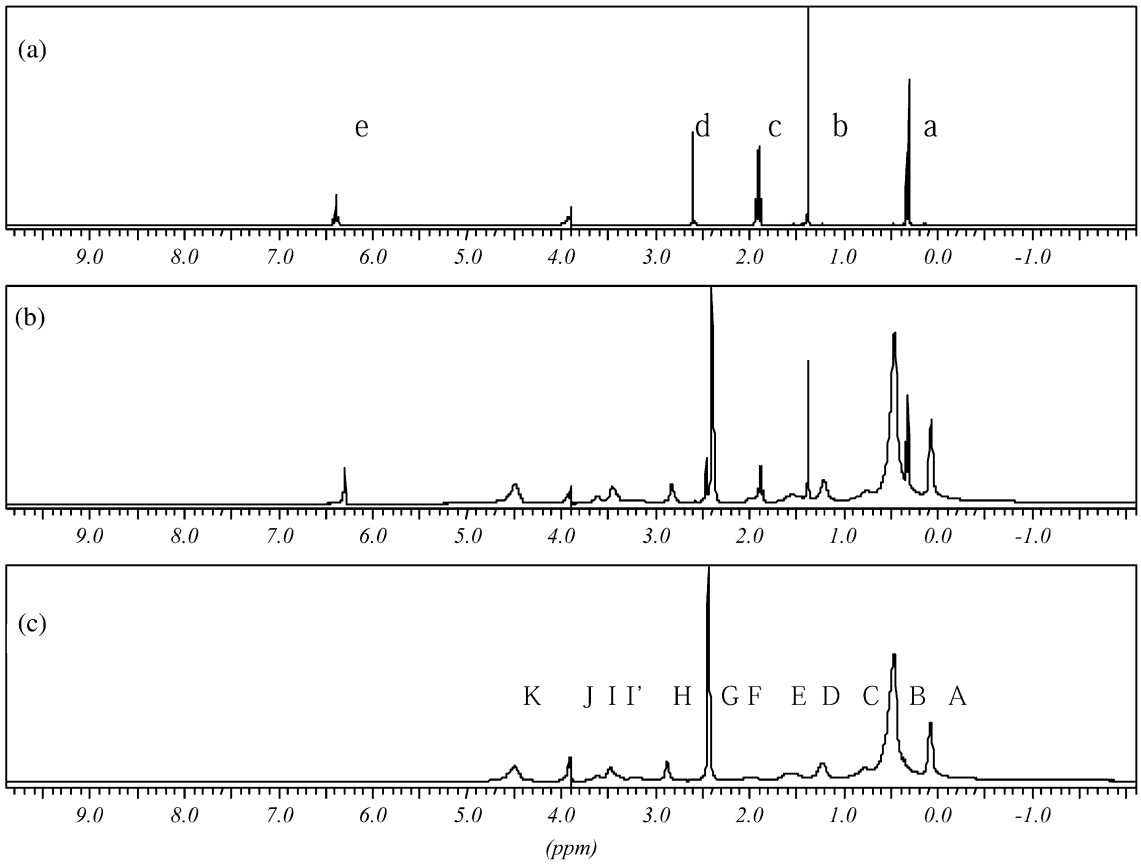

Fig. 5. Four hundred megaHertz ^{1}H-NMR spectra of lidocaine (a), lidocaine/EPC 0.33:1, molar ratio in the membrane (b), and 65 mM small unilamellar EPC vesicles (c); pH 10.5, 30 °C. Assignment as in Table 1.

tilamellar vesicles in the presence of charged tetracaine and no changes with the uncharged form, which penetrates deeper into the membrane [2]. Indeed, we could not detect any significant effect in the phosphate NMR spectra of EPC multilamellar vesicles (de Paula et al., in preparation) after the addition of lidocaine or other local anesthetic, at pH 10.5.

In sonicated vesicles Kuroda and Fujiwara reported discrete shifts in the ^{31}P resonance of EPC induced by the charged tetracaine, dibucaine and procaine [40]. Here, we detected no significant changes in the ^{31}P-NMR spectra of SUV after the addition of uncharged lidocaine.

Analysis of the phosphate stretching band in FTIR experiments (Fig. 4a) with sonicated EPC vesicles did not demonstrate any specific effect of lidocaine in the neighborhood of the PO_4^- group. The PO_4^- stretching band (ν_{as}) and acyl chain-CH_2 and CH_3-stretching bands (ν_{as} and ν_s) were equally insensitive to the presence of lidocaine (Fig. 4b).

Interestingly, the carbonyl stretching bands were changed in the presence of lidocaine (Fig. 4c). Lidocaine shifted the band position of both ester groups of EPC, i.e. the more (1728 cm^{-1}) and the less hydrated (1741 cm^{-1}), to higher wave numbers—1738 and 1748, respectively. This effect was interpreted in terms of a decrease in hydrogen bonding for both ester C=O. Data from the literature have shown that anesthetics disturb membrane organization in such a way that they improve the access of water molecules to the carbonyl bands [41]. However, this is not the case

Table 1
Chemical Shifts in the ^{1}H-NMR spectra of lidocaine, EPC and lidocaine/EPC (0.33:1 mole% in the membrane)

Peak	Assignment[a]	Chemical shift (ppm)		
		LDC in D_2O	EPC	LDC/EPC
A	ω-CH_3		0.00	0.00
a	**Ethyl–CH_3**	**0.16**		**0.25**
B	$(CH_2)_n$		0.40	0.38
C	β–CH_2		0.70	0.69
D	$CH_2C{=}C$		1.15	1.15
b	**Aromatic–CH_3**	**1.25**		**1.30**
E	α–CH_2		1.50	1.50
c	**Ethyl–CH_2**	**1.86**		**1.80**
F	$=C–CH_2–C=$		1.90	1.90
G	$N^+(CH_3)_3$		2.39	2.30
d	**CO–CH_2–N:**	**2.54**		**2.39**
H	$CH_2–N^+$		2.80	2.70
I	$O_3PO–CH_2$ (choline)		3.20	3.20
I'	$O_3PO–CH_2$ (glycerol)		3.40	3.40
J	CH_2–OCO		3.55	3.50
	HDO	3.90	3.90	3.90
K	CH=CH, CH–OCO		4.40	4.40
e	**Aromatic -3,4,5**	**6.34**		**6.20**

[a] Capital letters refer to EPC; lower-case letters refer to lidocaine peaks.

for lidocaine. The upshift in the C=O stretching band frequencies supports the hypothesis that the presence of the lidocaine molecule in the glycerol neighborhood creates a more hydrophobic environment in which lipids are less hydrogen-bonded to water. No changes in the proportion of the bands were detected after lidocaine addition, excluding the possibility of conformational changes in the polar head-group bending angle relatively to the acyl chain [42,43].

These FTIR data suggest that lipid molecules directly adjacent to a hydrophobic group of lidocaine (probably the aromatic ring) may experience a transition from a stronger to a weaker hydrogen-bonding environment caused by the displacement of water molecules from the carbonyl region.

3.4. ^{1}H magnetic resonance experiments

Fig. 5 shows the ^{1}H-NMR spectra of lidocaine in D_2O (a), EPC unilamellar vesicles (c) and EPC plus Lidocaine, at a 0.33:1 molar ratio (LDC/lipid) in the membrane (b). The assignment of EPC and lidocaine protons is reported in Table 1, where capital letters refer to the phospholipid [40], and lower-case letters identify the local anesthetic resonances. Upon mixing, all lidocaine protons were broadened, confirming their insertion into the lipid membrane; and their chemical shifts were slightly changed to up-field (peaks c, d and e) or to low-field (methyl protons, peaks a and b) frequencies. No significant changes in the line-width or chemical shifts of EPC resonance peaks were observed, except for the up-field shifts of peaks (g) and (h) (0.1 ppm choline protons) and of peak (j) (0.05 ppm), in the glycerol backbone. Overall, these results suggest that just a few protons, at the polar head-group of EPC unilamellar vesicles, are affected by the presence of lidocaine. Since the literature reports that aromatic molecules in the bilayer can shift resonances by the short-range ring current effect [44] this result indicates a superficial insertion of lidocaine into this bilayer.

3.5. Nuclear Overhauser experiments

ROESY experiments were carried out in SUV of egg phosphatidylcholine with the addition of Lidocaine (0.33:1, LDC/EPC, molar ratio in the membrane) in order to identify lidocaine/EPC contacts. To reduce artifacts due to spin diffusion the ROESY spectra were run at short mixing times, i.e. up to 50 ms [30,40,45–48]. Only two intermolecular dipolar interactions were detected between protons from lidocaine and the lipid molecules (Fig. 6) and we have checked that these cross-peaks were not due to magnetization transfer between lidocaine protons [i.e. (b)–(d); (d)–(e)]. The cross-peaks were assigned to protons e-G and b-G, indicating interactions between the local anesthetic aromatic ring and the choline methyl protons of EPC.

3.6. T_1 measurements

Longitudinal relaxation time (T_1) measurements provided complementary information about the dynamics of the LDC/EPC system. Fig. 7 reports the T_1 values for lidocaine protons in water and when incorporated into the anisotropic membrane

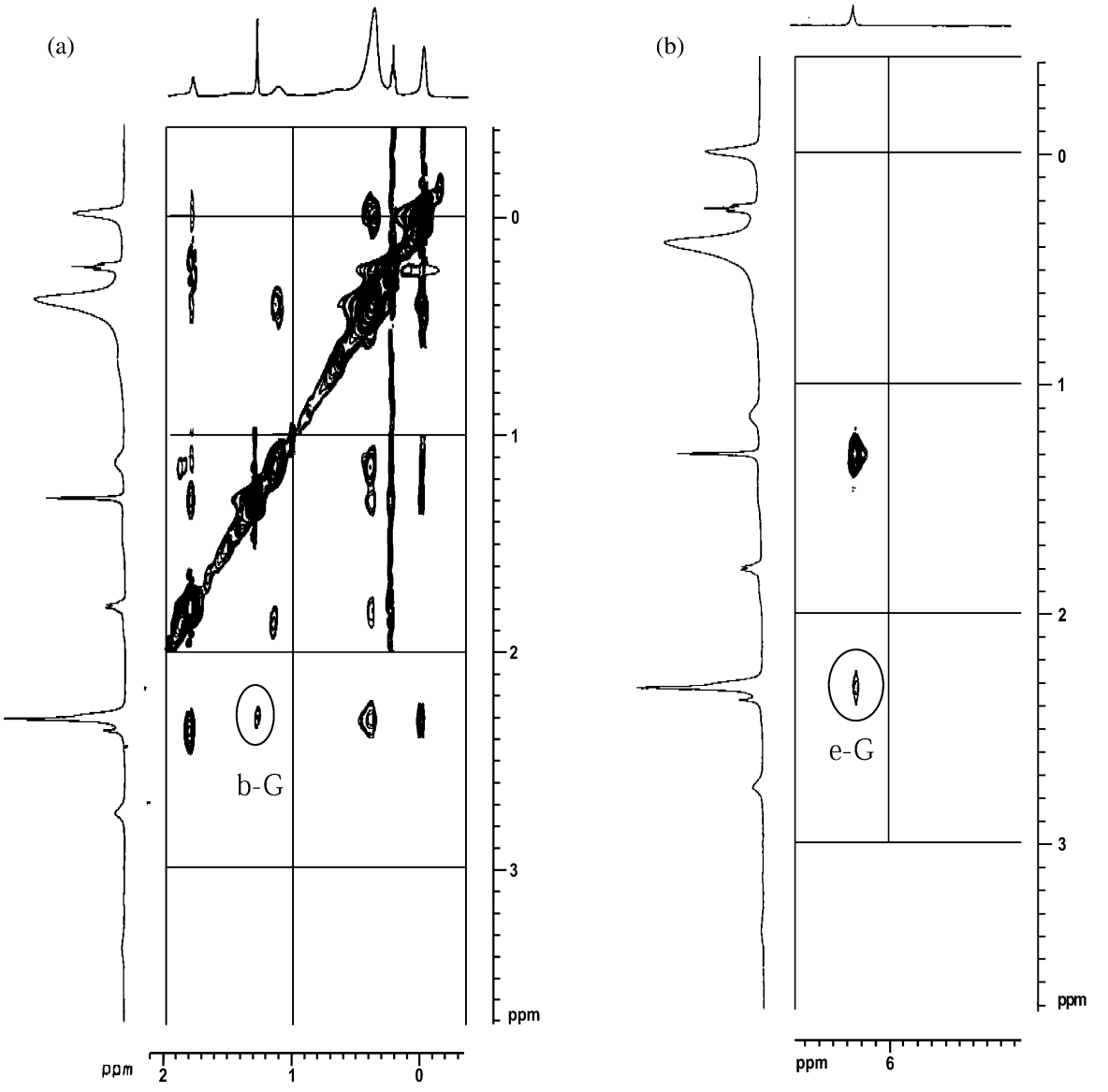

Fig. 6. Detail of a ROESY spectrum of lidocaine/EPC (0.33:1, molar ratio in the membrane) system, revealing intramolecular (lidocaine-EPC) cross-peaks. 65 mM EPC, pH adjusted to 10.5, spectra run at 500 MHz, 30 °C.

environment. In the presence of EPC vesicles we observed a clear reduction of the T_1 values of all lidocaine protons. This observation, in agreement with the observed broadening of their resonance peaks (Fig. 5), is indicative of a restriction in the mobility of the lidocaine protons, confirming that the molecule is fully inserted into the membrane.

Analysis of the T_1 values for the EPC protons (Fig. 8a) revealed the bilayer regions that were more affected by the presence of lidocaine. The

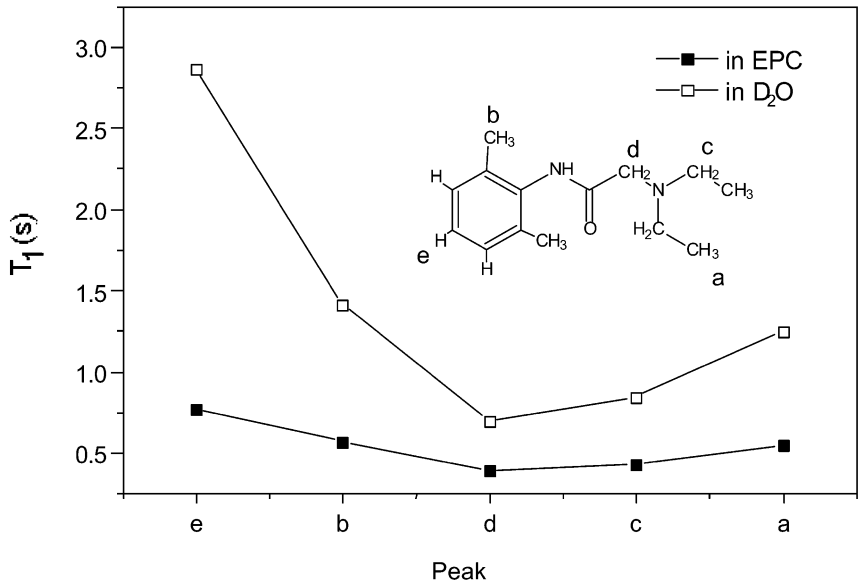

Fig. 7. Longitudinal relaxation times (T_1) of Lidocaine protons in D_2O and in EPC small unilamellar vesicles (0.33:1, LDC/lipid, molar ratio in the membrane). Peak assignment as in Table 1; experimental condition as in Fig. 6.

T_1 values for EPC protons are in good agreement with those reported in the literature [30,49]; the polar head group protons show shorter T_1 values, reflecting the increased relaxation caused by the electrostatic interaction between the choline $-N^+(CH_3)_3$ and phosphate groups of adjacent lipids [50]. The small acyl chain dynamics in the first 2–8 carbons-peak C in Fig. 8a—increases towards the hydrophobic core-peaks B, D, K and A—in agreement with the profile of phospholipid dynamics in lipid membranes reported in ^{13}C, ^{2}H, ^{1}H-NMR [30,32,35] and EPR [51] studies.

Upon addition of Lidocaine the T_1 values changed mainly for the protons in the polar headgroup region of EPC. Protons bound to the choline amine group—the methyl (G) and methylene (H) peaks—presented longer T_1 values, indicating that they lacked lipid–lipid inter-molecular interaction in the presence of lidocaine. In contrast, peaks I—corresponding to the choline CH_2 adjacent to the phosphate group—I′ and J, at the glycerol backbone, became more restricted allowing us to conclude that the lidocaine molecule stays there, increasing the relaxation of these protons. The effect of lidocaine on the choline and phosphate protons of EPC is highlighted in Fig. 8b, with experiments run between 20 and 40 °C.

4. Conclusion

Clinically used local anesthetics are small amphiphiles containing an ionizable amino group with a pK between 7 and 9. It is believed that the stronger binding of the uncharged species to the membrane would protect local anesthetic from blood clearance, thus justifying long lasting anesthesia. If this hypothesis is accepted, uncharged local anesthetic clearly acquire a crucial role in anesthesia.

The interaction of uncharged lidocaine with small unilamellar and multilamellar EPC vesicles resulted in a decrease of the overall membrane organization, as revealed by EPR and fluorescence data. Lidocaine induced a less tightly packing of the lipids in the vesicles, possibly by creating inter-lipid spaces. This effect was more evident in the glycerol region and also in the small unilamellar vesicles.

To describe the lidocaine/lipid interaction we employed a variety of spectroscopic techniques.

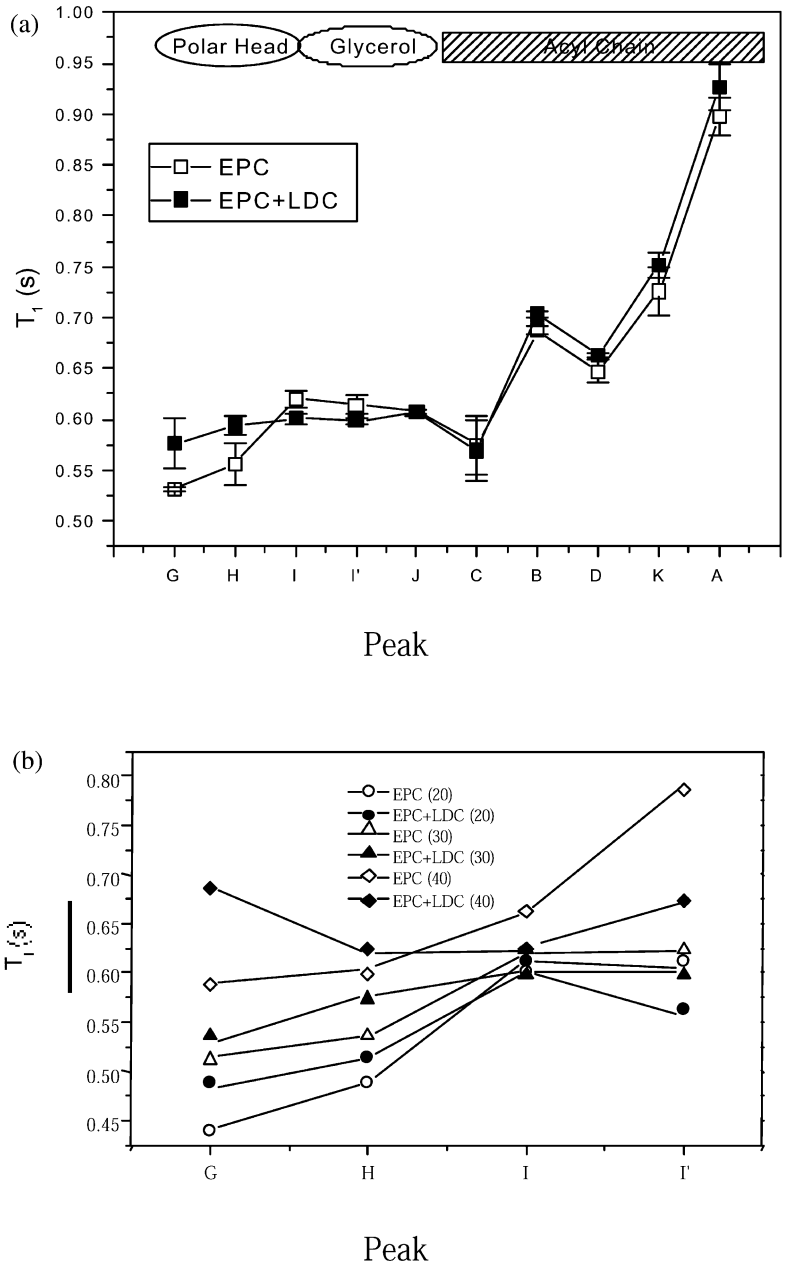

Fig. 8. Effect of Lidocaine (0.33:1 LDC/lipid, molar ratio in the membrane) on the relaxation times of EPC protons at 30 °C (a). Detail of the effect on the polar head group and glycerol region at variable temperatures of 20, 30 and 40 °C (b). T_1 values were determined by the inversion-recovery technique. Assignment as in Table 1.

For the unilamellar EPC vesicles in particular, our data suggest that lidocaine possesses a preferential location inside the EPC vesicle, with its aromatic ring (the bulky part of the molecule) in the vicinity of the glycerol backbone. This positioning affects atoms at the choline, glycerol (NMR), carbonyl

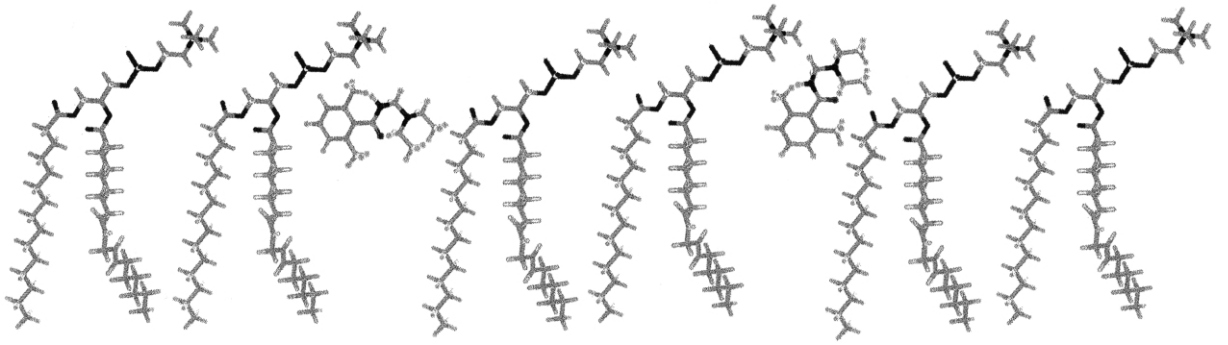

Fig. 9. Proposed model for lidocaine insertion into EPC small unilamellar vesicles.

(FTIR) and at the first portion of the acyl chain (EPR and fluorescence data). Although up to now no realistic models are available for phospholipid bilayer [52] representation at atomic resolution, the cartoon in Fig. 9 is useful to outline the depth of insertion of lidocaine, while its conformation and orientation can probably change. In fact, it is quite probable that lidocaine diffuses laterally at the level of the glycerol backbone.

It is important to point out that the existence of a preferential membrane location for the local anesthetic does not mean that these molecules remain steadily at that site. In fact, anesthesia requires a rapid equilibrium of local anesthetic between adjacent membrane and water compartments, implying that they have to move quickly across 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 local anesthetic [53,54] upon local anesthetic addition to liposomes. Therefore, we suggest that while local anesthetic are able to cross the membrane, they occupy a 'transient site' at a specific bilayer depth, determined by their hydrophobicity, polarity and steric features.

In a previous report [12] we demonstrated the importance of steric properties and location in determining the degree of membrane perturbation caused by local anesthetic. 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) [19,20].

Lidocaine provides good evidence for this hypothesis because even though it is not a highly hydrophobic anesthetic, it decreases membrane organization to a greater extent than other anesthetics with a higher hydrophobic character, such as tetracaine. The van der Waals volume of lidocaine (228.5 $Å^3$) is smaller than that of tetracaine (251.4 $Å^3$) [18]; nevertheless, because of its spherical shape, the spacing created by lidocaine inserted into the bilayer is greater than that of tetracaine [2].

The features of the specific bilayer region (polarity, fluidity, etc.) where the anesthetic stays most of the time could then determine its orientation and conformation, directing the molecule to the hydrophobic site(s) of action [20,21] of the voltage-gated sodium channel.

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