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Differential effects of uncharged aminoamide local anesthetics on phospholipid bilayers, as monitored by ^{1}H-NMR measurements

Leonardo Fernandes Fraceto[a,b], Alberto Spisni[c,d], Shirley Schreier[e], Eneida de Paula[b,*]

[a]*Faculdade de Farmácia, Universidade de Sorocaba, SP, Brazil*
[b]*Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas, CP 6109, CEP 13083-970, Campinas, SP, Brazil*
[c]*Dipartimento di Medicina Sperimentale "Chimica e Strutturistica Biochimica", Universita di Parma, Italy*
[d]*Centro de Biologia Molecular e Estrutural, Lab. Nacional Luz Síncrotron, Campinas, SP, Brazil*
[e]*Departamento de Bioquímica, Instituto de Química/Universidade de São Paulo, SP, Brazil*

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Abstract

We have collected evidences of a "transient site" for the local anesthetics (LA) lidocaine, etidocaine, bupivacaine and mepivacaine in sonicated egg phosphatidylcholine (EPC) vesicles. The effects of the uncharged anesthetic species at a fixed LA/EPC ratio inside the bilayer were measured by chemical shifts (C.S.) and longitudinal relaxation times (T_1) of the lipid hydrogens. Two sort of changes were detected: (I) *decrease*, indicating specific orientation of the LA aromatic ring (measured as up-field C.S. changes by the short-range ring-current effect) and less rotational freedom (smaller T_1 values) for EPC hydrogens such as the two glycerol-CH_2 and the choline-CH_2 bound to the PO^{4-} group, probably due to the nearby presence of the LA; (II) *increase*, indicating the aromatic ring is now perpendicular to the orientation observed before (causing down-field changes in C.S.) and larger T_1 values for all the choline and glycerol hydrogens, as a result of LA insertion behind these well-organized bilayer regions. The less hydrophobic, linear and nonlinear (lidocaine and mepivacaine, respectively) aminoamide analogs provide similar effects—described in I; their hydrophobic counterparts (etidocaine and bupivacaine) also produced comparable effects (depicted in II). The preferential positioning and orientation of each LA inside the bilayer is then determined by its hydrophobic and steric properties. We propose that this "transient site" in the lipid milieu exists also in biological membranes, where it can modulates the access of the uncharged LA species to its site(s) of action in the voltage-gated sodium channel.

Keywords: Local anesthetic; Membrane; Liposome; Nuclear magnetic resonance; Longitudinal relaxation times

1. Introduction

The first Nuclear Magnetic Resonance (NMR) experiments concerning the interaction of local anesthetic (LA) with model membranes detected the immobilization of LA molecules when inserted in egg phosphatidylcholine (EPC) bilayers [1–5]. Similar effects were observed through the broadening of signals of paramagnetic analogs of LA inserted in model membranes, in Electron Paramagnetic Resonance spectra [6,7]. In the 1980s ^{2}H-NMR experiments revealed for the first time, preferential regions for the LA binding to these membranes [8–11]. The work of Boulanger et al. [9] became an obligatory quotation and continues to be one of the most conclusive works in the field, demonstrating different locations for the protonated –more superficial– and uncharged tetracaine species in large phosphatidylcholine vesicles. Other ^{2}H-NMR studies demonstrated the inter-

Abbreviations: C.S., chemical shifts; EPC, egg phosphatidylcholine; LA, local anesthetics; LDC, lidocaine; MVC, mepivacaine; BVC, bupivacaine; EDC, etidocaine; *P*, partition coefficient; NMR, nuclear magnetic resonance; SUV, small unilamellar vesicles; T_1, longitudinal relaxation time.

* Corresponding author. Tel.: +55 19 37886143; fax: +55 19 37886129.
E-mail address: depaula@unicamp.br (E. de Paula).

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action of ethanol, a general anesthetic, with many regions but mainly with the glycerol backbone of EPC unillamelar [12] and multillamelar vesicles [13]. Baber et al. [14] employed both ^{2}H-NMR and ^{1}H-NOE to show that halotane, isofurane and enfurane interact mainly with the hydrophobic core region of palmitoyloleyl phosphatidylcholine vesicles and have low preference for the membrane–water interface.

The development of high-resolution spectrometers and new pulse sequences, enhanced the power of ^{1}H-NMR for the study of the membranes, and novel information about nucleus proximity allowed for the mapping of the different bilayer regions. In relation to LA molecules some works have employed these techniques to describe the insertion of the charged species of tetracaine [15], procaine, dibucaine and tetracaine [16,17] in EPC vesicles. In those works, in agreement with ^{2}H-NMR results, the LA have altered the membrane dynamics to different extents, and the authors claimed that their ability to bind to the polar-head group of the membranes was directly related to the anesthetic potency. However, to our knowledge, there are no reports in the literature describing the effect of the uncharged LA species in membranes, using high resolution NMR.

There are a few works in the literature that made use of other spectroscopic techniques to study the uncharged LA species. We can highlight those of Smith et al. [8–11], Sikaris and Sawyer [18], Ueda et al. [19,20] and ours [21–23]. This lack of studies is inconceivable since the uncharged LA binds more strongly to the membranes than the charged species and also because LA potency and toxicity are directly related to the hydrophobic nature of the anesthetic molecule [24–26].

In the present work we have measured effects induced by the uncharged forms of aminoamide local anesthetics: lidocaine (LDC), mepivacaine (MVC), bupivacaine (BVC) and etidocaine (EDC) –Fig. 1– in EPC small unilamellar vesicles. Using ^{1}H-NMR we detected changes in the chemical shift (C.S.) and longitudinal relaxation times (T_1) of EPC hydrogens in the presence of these anesthetics. The short-range ring-current effect caused by the LA aromatic ring on the C.S. of EPC gave us direct information about its preferential location inside the membrane. In the same way, upon addition of LA, changes in longitudinal relaxation times of the lipid hydrogens disclosed details about the bilayer dynamics in presence of these molecules, as reported before [24].

At a molecular level, a new perspective for the understanding of the local anesthesia mechanism–considering both the specific binding to the voltage-gated sodium channel and the non-specific decrease in the lipid phase organization–takes into account that each LA presents a "transient site", i.e., a preferential bilayer insertion that is determined by its physicochemical properties such as hydrophobicity [22], steric hindrances [22,26] and uncharged/charged ratio at pH 7.4 [27].

2. Materials and methods

Egg phosphatidyl choline, deuterated water (D_2O, 99.9%) and Chelex resin were obtained from Sigma Chemical (St. Louis, MO). Lidocaine hydrochloride was a gift from Apsen Brasil, Ind. Quim. Farm. Ltda (São Paulo, SP). All other reagents were of analytical grade.

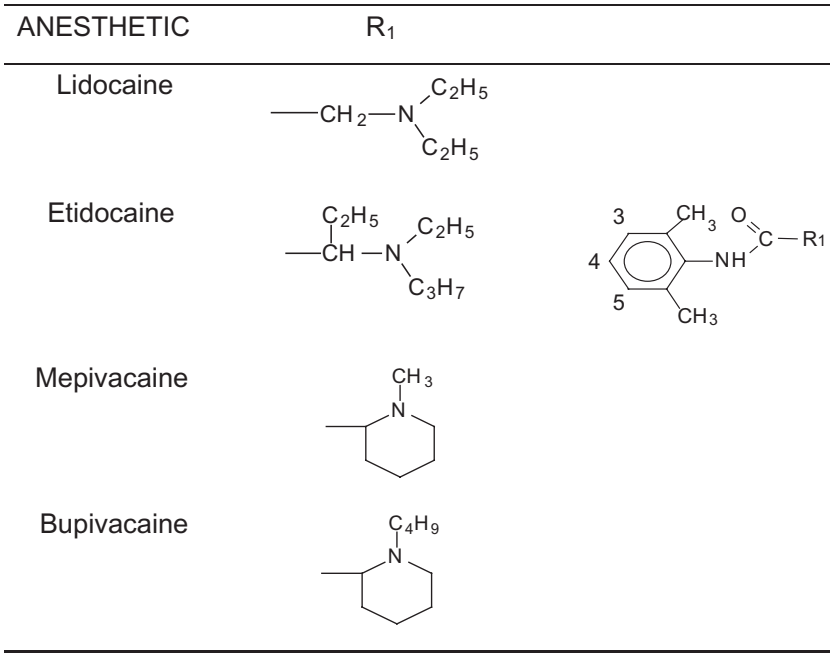


Fig. 1. Chemical structures of the local anesthetics.

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 were obtained after 3 min vortexing.

Small unilamellar vesicles (SUV) were obtained from freeze-dried large multilamellar vesicles, suspended in D_2O (pH 10.5). The samples were sonicated until clear (ca. 15 min) in a Sonics and Materials equipment (Newtown, CT). During sonication the temperature was kept at 0–4 °C by intermittent (1 min) agitation cycles, in an ice-water bath. The sonicated samples were centrifuged with Chelex resin at $1000\times g$ for 20 min to remove residual large particles and any titanium from the sonicator tip. Phospholipid concentration was determined according to Rouser et al. [28].

2.2. Nuclear magnetic resonance (NMR) experiments

The spectra were collected with a Bruker DRX 500 (Universidade de São Paulo, Brazil) equipment. The samples were degassed to avoid the interference of dissolved O_2 with the T_1 measurements. For ^{1}H-NMR, a 90° pulse was typically 10–15 μs and the recycling time was set to 5 times the largest T_1 (those of the aromatic protons of LA), typically 6 s. Longitudinal relaxation times were obtained by the conventional inversion-recovery technique, at 30 °C.

Using the partition coefficient values determined before [22], local anesthetics were added to the sonicated vesicles up to 1:3 (LDC, MVC, BVC) or 1:6 (EDC) LA:EPC molar ratio *within the membrane*. The low water solubility of the uncharged etidocaine species restrained incorporation of more EDC in the bilayer, as described before [22].

3. Results and discussion

Recently we have shown that uncharged lidocaine has a preferential location inside EPC bilayers using spectroscopic techniques such as electron paramagnetic resonance, fluorescence, infrared and nuclear magnetic resonance [24]. In that work T_1 results revealed consistent information about the dynamics of the LDC/EPC system, which encouraged us to employ the same approach to check the dynamics of EPC molecules in SUV, in the presence of three other aminoamide local anesthetics: mepivacaine, etidocaine and bupivacaine.

Table 1 shows the chemical shift (C.S.) and assignment for the NMR peaks of the uncharged LA hydrogens in D_2O, at pH 10.5.

Table 2 discloses changes in the C.S. of local anesthetic's hydrogens, when in D_2O or in EPC vesicles, at pH 10.5. As reported before for LDC [24] all the LA signals became broadened in the SUV (data not shown), indicating insertion of the anesthetic molecules into the lipid membrane. The major changes registered where: *up-field* for LDC and EDC (non-cyclic aminoamide LA), and *down-field* for MVC and BVC (cyclic aminoamides).

The C.S. and peak assignment of EPC hydrogen atoms, in accordance with previously reported results [16,24,29], is presented in Fig. 2. Notice that C.S. are referenced to the residual water peak (set at 3.9 ppm), in accordance to previous literature reports [15–17,29], avoiding the use of a reference such as TMS that could interfere in the lipid resonances [Fraceto, L.F., unpublished results].

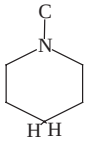
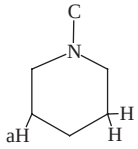
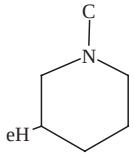
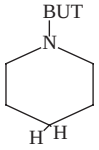
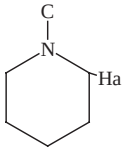
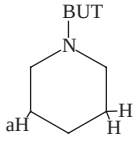
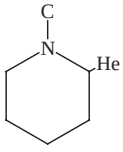
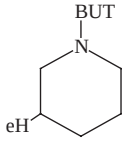
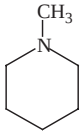
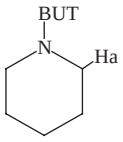
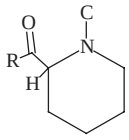
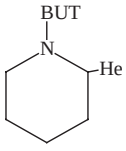
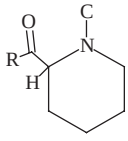
Fig. 3 shows the changes in the C.S. of lipid hydrogens in the presence of LA. Except for the up-field shifts on peaks G and H (CH_2 and CH_3 bound to the nitrogen of the choline $-\Delta$C.S.>0.08 ppm) and up and down-field shifts on peak J (glycerol CH_2 with the esther linkage to sn1$-\Delta$C.S.≥ 0.04 ppm) no important changes were registered in the EPC NMR spectra.

Literature reports that aromatic molecules in the bilayer can shift lipid resonances by the short-range ring current effect [30]. *Up-field* shifts in the choline hydrogens G and H were observed for LDC, while both LDC and MVC shifted the resonance of the glycerol peak J up-field. These results indicate that the aromatic groups of these two–less hydrophobic–analogs are equally oriented inside the bilayer, explaining the up-field shifts registered. However, the larger van der Waals volume of MVC relatively to LDC [24] and its piperidine ring input steric hindrances to its insertion among the phospholipids, resulting in a lower intermolecular proximity between its aromatic ring and the surrounding EPC molecules. This can counts for the lower short-range ring-current effect of MVC (ΔC.S. registered just for peak J), relatively to LDC.

EDC and BVC shifted the glycerol peak J *down-field* (Fig. 3), revealing an orientation of the aromatic group *perpendicular* to that observed for LDC and MVC inside the SUV. The higher hydrophobicity of EDC and BVC guides them to a deep insertion in the bilayer. The EPC groups surrounding their aromatic ring in this new situation or the need to bury their propyl and butyl groups (EDC and BVC, respectively) force the aromatic LA group to acquire a new orientation, explaining the *down-field* changes in C.S. registered.

Fig. 4 shows the T_1 profile of EPC hydrogens with and without LA. The EPC molecule is represented in its extended form, from hydrogens of the polar head-group (left) to those of the acyl chain region (right). One can notice that not all the EPC signals detected at 500 MHz resolution are displayed throughout panels a–d, since LA peaks can sometimes be juxtaposed to EPC signals, impeding accurate T_1 measurements. The T_1 values measured for EPC hydrogens on unilamellar vesicles (Fig. 4a–d, full symbol) are in close agreement with those reported by other authors [16,31]. Small T_1 values at the polar head-group hydrogens reflect the immobilization caused by the electrostatic

Table 1
Assignment and chemical shifts in the ^{1}H-NMR spectra of LDC, EDC, MVC and BVC in D_2O at pH 10.5 and 30 °C

Chemical shift (ppm)				Assignment			
LDC	EDC	MVC	BVC	LDC	EDC	MVC *	BVC *
0.16	0.17	0.40	−0.11	$N(C\text{-}CH_3)_2$	N-C-C-CH_3		CH_3-butyl group
1.25	0.34	0.59	0.29	ϕ-CH_3	CO-C-C-CH_3		N-C-C-CH_2-C
1.86	0.55	0.73	0.51	$N(CH_2\text{-}C)_2$	N-C-CH_3		N-C-CH_2-C-C, 
2.54	1.00	0.86	0.75	$COCH_2N$	N-C-CH_2-C		
6.34	1.33	1.05	1.00	ϕ-3,4,5	CO-C-CH_2-C		
	1.41	1.23	1.20		ϕ-CH_3	ϕ-CH_3	ϕ-CH_3, N-CH_2-C-C-C
	2.45	1.34	1.39		N-CH_2-C		
	2.57	1.94	1.65		N-CH_2-C-C		
	3.40	6.23	2.11		CO-CH-N	ϕ-3,4,5	
	6.43		2.50 6.20		ϕ-3,4,5		ϕ-NHCO- ϕ-3,4,5

The chemical shifts are referenced to the residual water signal, adjusted at 3.9 ppm, 500 MHz.
*aH/Ha and eH/He refer to the axial and equatorial hydrogen atoms of the piperidine ring. BUT=C_4H_9.

interaction between the amine and phosphate groups of adjacent EPC molecules in model membranes [32,33]. The profile of molecular dynamics through the acyl chain is also in accordance with data obtained through ^{13}C, ^{2}H and ^{1}H-NMR and EPR [34,35], indicating increasing mobility towards the terminal methyl group—peak A.

An analysis of the T_1 values after LA addition (Fig. 4a–d, open symbols) reveals specific bilayer regions that are affected by the presence of the uncharged species of LDC (panel a), EDC (panel b), MVC (panel c) and BVC (panel d).

For LDC and MVC the T_1 values changed mainly for the hydrogens in the polar head-group of EPC. The choline

Table 2
Changes in the chemical shifts of uncharged LA hydrogen NMR signals in the aqueous phase (D_2O) and inside EPC sonicated vesicles, at pH 10.5 and 30 °C

LA	Hydrogen	D_2O (ppm)	EPC (ppm)	Δ CS (ppm)
LDC	$N(C\text{-}\underline{H}_3)_2$	0.16	0.25	+0.09
	$CO\underline{H}_2N$	2.54	2.39	-0.15
	ϕ-3,4,5	6.34	6.20	-0.14
EDC	ϕ-$C\underline{H}_3$	1.41	1.30	-0.11
	ϕ-3,4,5	6.43	6.20	-0.23
MVC		0.73	0.80	+0.07
	ϕ-$C\underline{H}_3$	1.30	1.23	-0.07
		1.34	1.50	+0.16
BVC	ϕ-$C\underline{H}_3$	1.20	1.35	+0.15

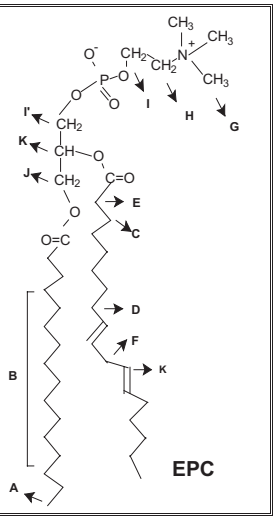
peaks G and H presented larger T_1 values, indicating an increase in the rotational freedom of ^1H in this region. In contrast, the choline (peak I) and glycerol (peaks I′) CH_2 adjacent to the PO_4^- group and the glycerol CH_2 (peak J) became more restricted (Fig. 4, panels a and c). T_1 values indicate that both LDC and MVC affect mainly the glycerol moiety in the vicinity of the PO_4^- group (peaks I, I′ and J), explaining the restricted motion of the glycerol hydrogens what is in agreement with ΔC.S. results (Fig. 3). The rotational freedom observed for hydrogens of peaks G and H (Fig. 4, panels a and c) could result from weaker electrostatic interactions between head groups belonging to adjacent EPC molecules, in the presence of LDC and MVC. So, the changes in T_1 can be related with the location of the LA inside the bilayer, as demonstrated with many other spectroscopic techniques for LDC [24], benzocaine [23] and other LA molecules (unpublished results).

MVC effect upon the T_1 values of choline and glycerol EPC hydrogens is stronger than that of LDC at the same LA/lipid (1:3) molar ratio in the membrane, in spite of the similar hydrophobicity between these LA in their uncharged form [22]. This is probably due to the disturbance caused by the bulky piperidine ring of MVC in-between the lipid molecules. The piperidine ring of MVC considerably reduces the number of possible conformations of the molecule, in comparison to LDC, as demonstrated by molecular dynamics calculations, in water [36].

In the presence of EDC (Fig. 4b) larger T_1 values were detected from the choline (peaks G, H, I) up to the glycerol (peaks I′, J) hydrogens of EPC. In contrast to the effects of LDC and MVC, the choline CH_2 adjacent to the PO_4^- group (peak I) and the glycerol CH_2 (peaks I′ and J) acquired more freedom, as shown in Fig. 4b,d. The effect of BVC is similar to that of EDC (Fig. 4d) but reaches up carbons 1 and 2 of the acyl chain. These results are in good agreement with ΔC.S. data (Fig. 3) since short-range ring current effects were detected for EDC and BVC at the glycerol backbone region, and denote an orientation of the aromatic rings perpendicular to that taken by LDC and MVC. The hydrophobic character of both analogs (EDC and BVC) probably force their aromatic ring in the glycerol moiety to assume an orientation that allows the LA to bury its less polar (propyl and butyl, respectively) groups in-between the first carbons of the acyl chain.

The deep insertion of EDC and BVC into phospholipid membranes relatively to their less hydrophobic counterparts LDC and MVC can also be compared to the differences in incorporation between uncharged and charged tetracaine species, described by Boulanger et al. using ^2H-NMR in large multilamellar vesicles [9].

Assignment		Chemical Shift (ppm)
A	CH_3	0.00
B	$(CH_2)n$	0.40
C	β-CH_2^*	0.70
D	$CH_2C{=}C$	1.15
E	α-CH_2^*	1.50
F	$=C\text{-}CH_2\text{-}C=$	1.90
G	$N^+(CH_3)_3$	2.39
H	CH_2N^+	2.80
I	O_3POCH_2C	3.20
I'	O_3POCH_2CHO	3.40
J	CH_2OCO	3.55
K	CH=CH,CHOCO	4.40



* α and β indicate positions 1 and 2 of the acyl chain.

Fig. 2. Assignment and C.S. of EPC hydrogens in SUV, assigned at a 500 MHz NMR spectrum; [EPC]=65 mM, pH 10.5, 30 °C.

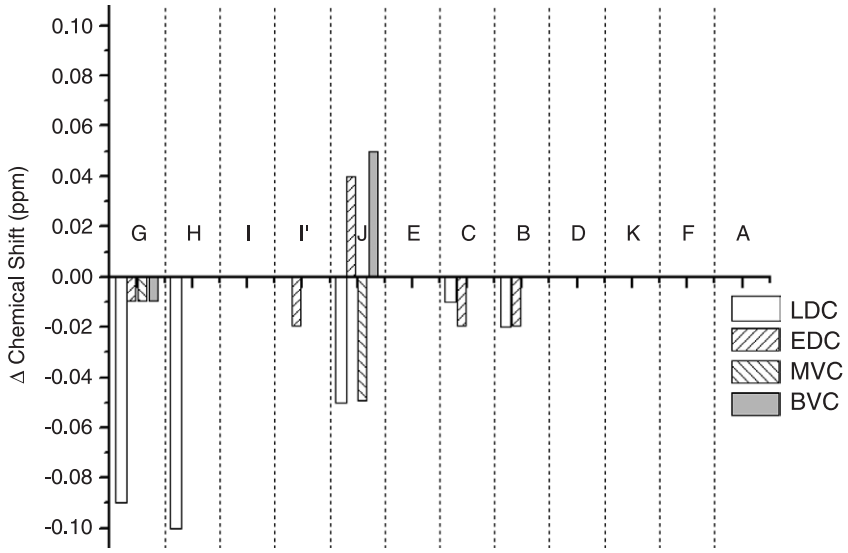


Fig. 3. Changes in the chemical shifts of EPC peaks induced by the local anesthetics. [EPC]=65 mM, pH 10.5, 30 °C, 500 MHz.

We propose that the physicochemical properties of each LA determine its preferential insertion into the membrane, what can be monitored by changes in the C.S. and T_1 of the lipids, as shown here. Nevertheless, we did not forget that LA molecules have fast equilibrium between adjacent membrane and water compartments [6,7], i.e., that they move quickly across the membranes. In fact we suggest that LA molecules, once in the membrane phase, occupy a

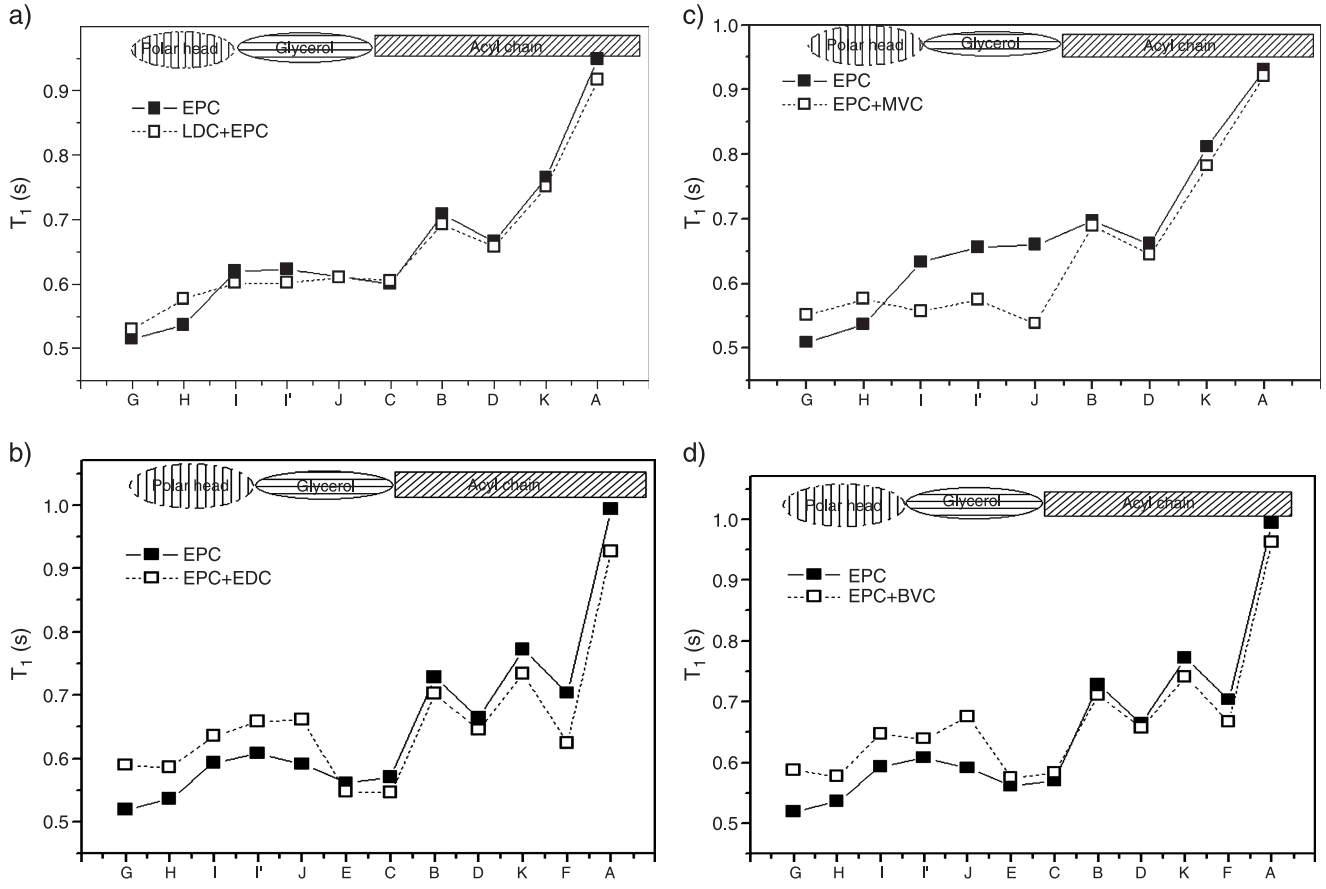


Fig. 4. Changes in the longitudinal relaxation times (T_1, s) of EPC hydrogens at SUV in the presence of LDC (a), EDC (b), MVC (c) and BVC (d). [EPC]=65 mM, pH 10.5, 30 °C, 500 MHz.

"transient site" where they can be found most of the time [12,13,24]. This transient location occurs at a specific bilayer depth–that is determined by hydrophobic, polarity and steric features of the LA [22,26,27,37]–and requires reorientation of the anesthetic molecule, as shown here through ^{1}H-NMR measurements.

4. Conclusion

The aminoamide local anesthetic family comprises some of the most important drugs in pain-relief treatment [38,39] because of their low toxicity, as compared to the esther family. LDC, for instance, is the most important local anesthetic in regional anesthesia, while BVC is worldwide the drug-of-choice for surgical procedures [38,39]. The binding to membranes is mainly determined by the physicochemical properties of each anesthetic molecule, modulating its specific binding to the voltage-gated Na^+ channels in the axons [37].

Stereo isomers of cyclic aminoamides such as S(-)-BVC and S(-)ropivacaine have been obtained and introduced in therapeutics in the last decade [38]. Biochemical and Biophysical studies have not followed this advancement and just a few works in these fields are devoted to elucidate the mode of action of aminoamide local anesthetics.

LDC and MVC are clinically used aminoamide local anesthetics with equivalent partition coefficients in membranes [22]. The same is true for EDC and BVC. Nevertheless MVC and BVC have a voluminous cyclic substituent: the piperidine ring that restricts the number of possible conformations of these molecules in comparison to LDC and EDC, as demonstrated by molecular calculations [36].

In the present work we followed the partition of the uncharged species of LDC and MVC, EDC and BVC into sonicated phosphatidylcholine membranes. Insertion was found to be quite different between the less (LDC and MVC) and more hydrophobic (EDC and BVC) aminoamide analogs.

LDC and MVC increased the mobility of the choline nuclei, releasing the amine-phosphate electrostatic interactions between adjacent EPC molecules. Their aromatic groups sit in the glycerol region, causing *up-field* shifts by the short-range ring current effect (ΔC.S. data) and decreasing the mobility of those hydrogens (T_1 data) [24]. The stronger effect of MVC over LDC in molecular dynamics probably reflects the disturbance caused by the insertion of its bulky piperidine ring into the well-organized glycerol region.

EDC and BVC effects on the polar head group of EPC vesicles in widespread, since their presence increase the mobility of all the choline and glycerol nuclei (T_1 data). Down-field, short-range ring current effects detected only in the C.S. of the glycerol hydrogens of EPC revealed that the aromatic ring of EDC and BVC have a deeper insertion in the bilayer, in relation to LDC and MVC, and it has a distinct orientation. Accordingly, the deeper insertion of EDC and BVC avoid the effective packing of EPC molecules, what is detected by the increased mobility of all choline and glycerol hydrogens.

Since the aromatic ring-current effect requires very close apposition of lipid hydrogens to the anesthetic aromatic ring [30,40], the induced ΔC.S. are a direct measure of LA location. However, at a resonance frequency of 500 MHz interpretation of changes in T_1 values in terms of anesthetic location *is rather indirect* since both an increase or a decrease in motional correlation-times, as well as changes in the amplitude of motions may alter the relaxation rates of hydrogens at this frequency [30,40]. Fortunately, as shown here, the changes in C.S. and T_1 values gave concordant information about the preferential positioning of the each LA studied into SUV.

LA effect on the lipid phase of the membrane can be viewed as the result of the preferential positioning and molecular orientation taken by the LA molecule inside the bilayers what could modulate the LA access [41] to the binding sites for the uncharged species in the Na^+ channels [42–45].

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References

[1] H. Hauser, R.M.C. Dawson, The displacement of calcium ions from phospholipid monolayers by pharmacologically active and other organic bases, Biochem. J. 109 (1968) 909–916.
[2] J. Cerbon, NMR evidence for the hydrophobic interaction of local anaesthetics. Possible relation to their potency, Biochim. Biophys. Acta 290 (1972) 51–57.
[3] A. Darke, E.G. Finer, A.G. Flook, M.C. Phillips, Nuclear magnetic resonance study of lecithin–cholesterol interactions, J. Mol. Biol. 63 (1972) 265–279.
[4] E.G. Finer, Nuclear magnetic resonance studies of lipid–water systems, Chem. Phys. Lipids 8 (1972) 327–332.
[5] A.G. Lee, J.M. Birdsall, Y.K. Levine, J.C. Metcalfe, High resolution proton relaxation studies of lecithins, Biochim. Biophys. Acta 255 (1972) 43–56.
[6] R.J. Gargiulo, G.J. Giotta, H.H. Wang, Spin-labeled analogs of local anesthetics, J. Med. Chem. 16 (1973) 707–708.
[7] G.J. Giotta, D.S. Chan, H.H. Wang, Binding of spin-labeled local anesthetics to phosphatidylcholine and phosphatidylserine liposomes, Arch. Biochem. Biophys. 163 (1974) 453–458.
[8] Y. Boulanger, S. Schreier, L.C. Leitch, I.C.P. Smith, Multiple binding sites for local anesthetics in membranes: characterization of the sites and their equilibria by deuterium NMR of specifically deuterated procaine and tetracaine, Can. J. Biochem. 58 (1980) 986–995.
[9] Y. Boulanger, S. Schreier, I.C.P. Smith, Molecular details of anesthetic–lipid interaction as seen by deuterium and phosphorous-31 nuclear magnetic resonance, Biochemistry 20 (1981) 6824–6830.

[10] J. Westman, Y. Boulanger, A. Ehrenberg, I.C.P. Smith, Charge and pH dependent drug binding to model membranes. A ^{2}H-NMR and light absorption study, Biochim. Biophys. Acta 685 (1982) 315–328.
[11] E.C. Kelusky, I.C.P. Smith, Anesthetic-membrane interaction: a ^{2}H nuclear magnetic resonance study of the binding of specifically deuterated tetracaine and procaine to phosphatidylcholine, Can. J. Biochem. 62 (1984) 178–184.
[12] J.A. Barry, K. Gawrisch, Direct NMR evidence for ethanol binding to the lipid–water interface of phospholipid bilayers, Biochemistry 33 (1994) 8082–8088.
[13] L.L. Holte, K. Gawrisch, Determining ethanol distribution in phospholipid multilayers with MAS-NOESY Spectra, Biochemistry 36 (1997) 4669–4674.
[14] J. Baber, J.F. Ellena, D.S. Cafiso, Distribution of general anesthetics in phospholipids-bilayers determined using ^{2}H NMR and ^{1}H-^{1}HNOE spectroscopy, Biochemistry 34 (1995) 6533–6539.
[15] S. Yokono, K. Ogli, S. Miura, I. Ueda, 400 MHz two-dimensional nuclear Overhauser spectroscopy on anesthetic interaction with lipid bilayer, Biochim. Biophys. Acta 982 (1989) 300–302.
[16] Y. Kuroda, Y. Fujiwara, Locations and dynamic perturbations for lipids of cationic forms of procaine, tetracaine and dibucaine in small unilamellar phosphatidylcholine vesicles as studied by nuclear Overhauser effects in ^{1}H nuclear magnetic resonance spectroscopy, Biochim. Biophys. Acta 903 (1987) 395–410.
[17] M. Wakita, Y. Kuroda, Y. Fujiwara, T. Nakagawa, Conformations of dibucaine and tetracaine in small unilamellar phosphatidylcholine vesicles as studied by nuclear Overhauser effects in ^{1}H nuclear magnetic resonance spectroscopy, Chem. Phys. Lipids 62 (1992) 45–54.
[18] K.A. Sikaris, W.H. Sawyer, The interaction of local-anesthetic with synthetic phospholipids-bilayers, Biochem. Pharmacol. 31 (1982) 2625–2631.
[19] I. Ueda, J.S. Chiou, P.R. Krishma, H. Kamaya, Local anesthetics destabilize lipid membranes by breaking hydration shell: infrared and calorimetry studies, Biochim. Biophys. Acta 1190 (1994) 421–429.
[20] H. Matsuki, K. Shimada, S. Kaneshima, H. Kamaya, I. Ueda, Differences in surface activities between uncharged and charged local anesthetics: correlation with their anesthetic potencies, Colloids Surf., B Biointerfaces 11 (1998) 287–295.
[21] W.A. Frezzatti Jr., W.R. Toselli, S. Schreier, Spin label study of local anesthetic–lipid membrane interactions. Phase separation of the uncharged form and bilayer micellization by the charged form of tetracaine, Biochim. Biophys. Acta 860 (1986) 531–538.
[22] E. de Paula, S. Schreier, Use of a novel method for determination of partition-coefficients to compare the effect of local-anesthetics on membrane-structure, Biochim. Biophys. Acta 1240 (1995) 25–33.
[23] L.M.A Pinto, D.K. Yokaichiya, L.F. Fraceto, E. de Paula, Interaction of benzocaine with model membranes, Biophys. Chem. 87 (2000) 213–223.
[24] L.F. Fraceto, L.M.A. Pinto, L. Franzoni, A.A.C. Braga, A. Spisni, S. Schreier, E. de Paula, Spectroscopic evidence for a preferential location of lidocaine inside phospholipid bilayers, Biophys. Chem. 99 (2002) 229–243.
[25] B.G. Covino, H.G. Vassalo, Local Aesthetics: Mechanisms of Action and Clinical Use, Grune and Stratton, New York, 1976.
[26] S.P. Gupta, Quantitative structure–activity relationship studies on local-anesthetics, Chem. Rev. 91 (1991) 1109–1119.
[27] S.V.P. Malheiros, L. Gottardo, L.M.A. Pinto, D.K. Yokaichiya, N.C. Meirelles, E. de Paula, A new look at the hemolytic effect of local anesthetics, considering their real membrane/water partitioning at pH 7.4, Biophys. Chem. 100 (2004) 213–221.
[28] G. Rouser, S. Fleicher, A. Yamamoto, Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots, Lipids 5 (1970) 494–496.
[29] Y. Kuroda, K. Kitamura, Intramolecular and intermolecular ^{1}H-^{1}H nuclear Overhauser effect studies on the interactions of chlorpromazine with lecithin vesicles, J. Am. Chem. Soc. 106 (1984) 1–6.
[30] W.M. Yau, W.C. Wimley, K. Gawrisch, S.H. White, The preference of tryptophan for membrane interfaces, Biochemistry 37 (1998) 14713–14718.
[31] P.E. Godici, F.R. Landsberger, Dynamics structure of lipid–membranes–^{13}C nuclear magnetic resonance study using spin labels, Biochemistry 13 (1974) 362–368.
[32] J. Seelig, ^{31}P nuclear magnetic resonance and the head group structure of phospholipids in membranes, Biochim. Biophys. Acta 515 (1978) 105–140.
[33] G. Büldt, R. Wohlgemuth, The head-group conformation of phospholipids in membranes, J. Membr. Biol. 58 (1981) 81–100.
[34] J.F. Ellena, S.J. Archer, R.N. Dominey, B.D. Hill, D.S. Cafiso, Localizing the nitroxide group of fatty-acid and voltage-sensitive spin-labels in phospholipid-bilayers, Biochim. Biophys. Acta 940 (1988) 63–70.
[35] B.J. Gaffney, H.M. McConnell, Paramagnetic-resonance spectra of spin labels in phospholipids membranes, J. Magn. Res. 16 (1974) 1–30.
[36] J.D.M. Neto, R.B. Alencastro, Theoretical studies on local anesthetics: procaine, lidocaine, tetracaine, bupivacaine and dibucaine-neutral and monoprotonated, Int. J. Quant. Chem. 61 (1997) 959–980.
[37] K.R. Courtney, Structure–activity relations for frequency-dependent sodium channel block in nerve by local anesthetics, J. Pharmacol. Exp. Ther. 213 (1980) 114–119.
[38] R.H. de Jong, Local anesthetics, Mosby, St. Louis, 1994.
[39] S.F. Malamed, Handbook of local anesthesia, Mosby-Year Book, St. Louis, 1997.
[40] A.E. Derome, Modern NMR Techniques for Chemistry Research, Pergamon Press, New York, 1987.
[41] E. de Paula, S. Schreier, Molecular and physicochemical aspects of local anesthetic–membrane interaction, Braz. J. Med. Biol. Res. 29 (1996) 877–894.
[42] D.S. Ragsdale, J.C. McPhee, T. Scheuer, W.A. Catterall, Molecular determinants of state-dependent block of Na^{+} channels by local anesthetics, Science 265 (1994) 1724–1728.
[43] D.S. Ragsdale, J.C. McPhee, T. Scheuer, W.A. Catterall, Common molecular determinants of local anesthetic, antiarrhythmic, and anticonvulsant block of voltage-gated Na^{+} channels, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 9270–9275.
[44] D.S. Ragsdale, M. Avoli, Brain sodium channels as molecular targets for antiepileptic drugs, Res. Brain Res. Rev. 26 (1998) 16–28.
[45] V. Yarov-Yarovoy, J.C. McPhee, D. Idsvoog, C. Pate, T. Scheuer, W.A. Catterall, Role of amino acid residues in transmembrane segments IS6 and IIS6 of the Na^{+} channel alpha subunit in voltage-dependent gating and drug block, J. Biol. Chem. 277 (2002) 35393–35401.