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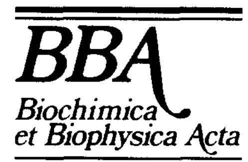

Use of a novel method for determination of partition coefficients to compare the effect of local anesthetics on membrane structure

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Abstract

A new, simple procedure for the determination of partition coefficients (P) was developed based on spectral effects caused upon addition of solutes to spin labeled model lipid membranes, and on the knowledge of their water solubility. Values of P were determined for nine local anesthetics (LA), amino-esters and amino-amides. The results were in good agreement with those found by phase separation and by a more complex, previously reported, methodology (Lissi et al. (1990) Biochim. Biophys. Acta 1021, 46–50) applied to either EPR or fluorescence spectra of probes incorporated in the bilayers. Both the present and the previously reported procedures make use of effects on membrane structure evaluated by spectroscopic techniques and offer the advantage of not requiring phase separation. The spectral effects, indicative of a decrease in bilayer organization increased with LA concentration, reaching a maximum at the drug water solubility, indicating that partitioning in the membrane is limited by saturation of the aqueous phase. A thermodynamic analysis of the partition data according to Hill (Hill, M.W. (1974) Biochim. Biophys. Acta 356, 117–124) showed that the LAs did not display ideal behavior. Knowledge of the partition coefficients allowed a comparison between effects at the same drug concentration *in the membrane*. Within a given family (esters, acyclic amides, cyclic amides) no clear proportionality was observed between effect and LA hydrophobicity, as reflected in the partition coefficient. Rather, the membrane perturbing ability is a result of steric effects originating in the mismatch between anesthetic and phospholipid shapes.

Keywords: Local anesthetic; Partition coefficient; Membrane; Steric property; Lipid bilayer; Spin label; EPR; Fluorescence

1. Introduction

Local anesthetics (LAs) act by binding to the sodium channel protein, inhibiting Na^+ uptake and blocking the nervous impulse. Most local anesthetics are ionizable amines and both the charged and uncharged forms are thought to be relevant for the mechanism of anesthesia. It has been proposed that while the uncharged form is the main species for transporting the anesthetic across the cell membrane, the protonated form binds to a specific site on the channel, triggering the anesthetic effect [1–3]. Nevertheless, some local anesthetics are not ionizable compounds (e.g., benzocaine, benzyl alcohol) and different, as yet unidentified, mechanisms for blocking the sodium channel have been suggested for these compounds as well as for barbiturates, some volatile general anesthetics and the unprotonated form of the amines [4].

Structure–activity relationship studies have tried to elucidate the molecular features relevant for the action of LAs [5–8]. Hydrophobicity, pK_a, and molecular size [5–7], and polar interactions [8] have been invoked as factors influencing biological effects. Amino-esters were found to be more potent than amino-amides.

The lipid bilayer could be involved in the mechanism of anesthesia in different ways: as the medium from which the LA reaches the sodium channel and/or by undergoing drug-induced organizational changes that would modulate functionally related conformational changes at the protein level. In addition, alterations of lipid structure are very

Abbreviations: BVC, bupivacaine; CLP, chloroprocaine; DBC, dibucaine; EDC, etidocaine; LDC, lidocaine; MVC, mepivacaine; PLC, prilocaine; PRC, procaine; TTC, tetracaine; DPPC, dipalmitoylphosphatidylcholine; EPC, egg phosphatidylcholine; EPR, electron paramagnetic resonance; LA, local anesthetics; 9-PyNA, 9-(1-pyrene)-n-nonanoic acid; P, partition coefficient; 5-SASL, 5-doxylstearic acid; 5-MeSL, methyl ester of 5-SASL.

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likely related to toxicity effects [9]. Therefore, it is important to understand the effects of anesthetics on lipid membranes. Studies designed to examine the action of LA upon model lipid membranes have made use of various techniques such as monolayer measurements [10,11], calorimetry [12,13], and spectroscopic methods, like UV absorption [14], fluorescence [15–18], infra-red [19,20], X-ray diffraction [21,22], electron paramagnetic resonance [9,23–31], and nuclear magnetic resonance [32–41].

Under physiological conditions the amino-ester and amino-amide LAs are present in both the protonated and unprotonated forms. The understanding at a molecular level, of the events related to each species requires that studies be performed in systems containing only one species.

In the present work, we have investigated the role of physicochemical and structural properties of the uncharged form of a series of ester (procaine, chloroprocaine and tetracaine) and amide (lidocaine, prilocaine, etidocaine, mepivacaine, bupivacaine and dibucaine) LAs (Fig. 1) on membrane binding and perturbation.

Partition coefficients were determined employing EPR and fluorescence spectra of lipid incorporated probes. A novel method making use of spectral alterations due to anesthetic-induced bilayer disorganization, and on measured water solubilities is presented, and the results are compared to those obtained by other methodologies, based on spectroscopic measurements and on phase separation. The knowledge of the partition coefficients allowed the comparison of the effects on bilayer structure at the same anesthetic concentration in the membrane.

2. Materials and methods

Egg phosphatidylcholine (EPC) was extracted and purified according to [42], as modified by Kamp et al. [43]. The fluorescent probe 9-PyNA was obtained from Molecular Probes, Eugene, OR. Spin labels: 5-SASL and 5-MeSL came from Sigma Chemical Co., St. Louis, MO. The sources of LAs (in the hydrochloride form) were: TTC: Sigma Chemical Co., St. Louis, MO; CLP and EDC: Astra

ANESTHETIC		R_1	R_2	R_3	X
Procaine	PRC	-H	$-C_2H_5$	$-C_2H_5$	-H
Chloroprocaine	CLP	-H	$-C_2H_5$	$-C_2H_5$	-Cl
Tetracaine	TTC	$-C_4H_9$	$-CH_3$	$-CH_3$	-H
Lidocaine	LDC	$-CH_3$	$-CH_2-N(C_2H_5)_2$		
Prilocaine	PLC	-H	$-CH(CH_3)-N(H)(C_3H_7)$		
Etidocaine	EDC	$-CH_3$	$-CH(C_2H_5)-N(C_2H_5)(C_3H_7)$		
Mepivacaine	MVC	$-CH_3$	N-CH_3 piperidyl		
Bupivacaine	BVC	$-CH_3$	N-C_4H_9 piperidyl		
Dibucaine	DBC				

$R_1-NH-C_6H_3(X)-C(=O)-O-(CH_2)_2-N(R_2)(R_3)$

$C_6H_3(R_1)(CH_3)-NH-C(=O)-R_2$

H_9C_4O – quinoline – $C(=O)-NH-(CH_2)_2-N(C_2H_5)_2$

Fig. 1. Chemical structure of the local anesthetics studied.

Pharmaceutical Products Inc., Westborough, MA; MVC: Sterling Drug Inc., Rensselaer, NY; BVC and LDC: Apsen Brasil, Indústria Química e Farmacêutica Ltda, São Paulo, SP; PLC: Cristália Produtos Químicos e Farmacêuticos Ltda, Itapira, SP; DBC: Berlimed Produtos Químicos, Farmacêuticos e Biológicos Ltda, São Paulo, SP; PRC was synthesized according to [44] by Dr. A.T. do Amaral, from the Institute of Chemistry, University of São Paulo. All other compounds were reagent grade.

All experiments were done in 0.2 M carbonate-bicarbonate buffer (pH 10.5) except for the determination of water solubility of the charged forms of the LAs. In this case, 0.2 M sodium acetate buffer (pH 5.5) was used. Doubly distilled water was used throughout.

Solubility determination. Aqueous LA solutions of increasing concentrations were centrifuged at $10\,000 \times g$ for 5 min at room temperature. The concentration in the supernatant was determined making use of the extinction coefficients in the UV region (Table 1).

Membrane preparation. EPC bilayers were obtained by evaporating stock chloroform solutions of EPC under a stream of wet nitrogen. The samples were left under vacuum for no less than 2 h. Multilamellar liposomes were obtained by addition of buffer and vortexing for 5 min. Phospholipid concentration was determined according to [45].

Spectroscopic measurements. EPC multibilayers containing 5-SASL or 5-MeSL (2% of total lipid, in moles), or 9-PyNA (2.3–3.3%, in moles), were prepared by adding the proper amount of probe to the chloroform solution.

Membrane organization was monitored by measuring the ratio of heights of the low-field (h_{+1}) to the center-field (h_0) resonances in the EPR spectra of 5-MeSL. It has been previously discussed that this parameter contains the contribution of both order and mobility and is taken as expressing changes in the overall membrane organization [9,26], increasing h_{+1}/h_0 values being ascribed to a less organized bilayer. The order parameter (S) was measured in the spectra of membrane-bound 5-SASL. For 5-SASL, whose long molecular axis is approximately parallel to the bilayer normal, the order parameter, S, can be obtained experimentally from Eq. (1):

$$S = \frac{A_{\parallel} - A_{\perp}}{A_{zz} - (A_{yy} + A_{xx})/2} \quad (1)$$

where $A_{\parallel}(A_{\perp})$ is the hyperfine splitting corresponding to the spin label long molecular axis oriented preferentially parallel (perpendicular) to the external magnetic field. $A_{\parallel}$ and $A_{\perp}$ are measured as half the separation between the outer and the inner extrema, respectively [46]. A_{xx}, A_{yy} and A_{zz} are the principal components of the hyperfine tensor and were taken as 6.0, 6.0 and 32.0 gauss [47], respectively. No corrections were made to account for differences in polarity.

For the fluorescent probe, the intensity was monitored before (I_0) and after (I) addition of the anesthetic. The excitation and emission wavelengths were 337 and 376 nm, respectively. Addition of LAs decreases membrane organization, leading to an increase in static and dynamic fluorescence quenching [18,30].

EPR spectra were obtained either in a Varian E-4 or in a Bruker ER-200D-SRC spectrometer at room temperature. Flat quartz cells came from James Scanlon, Costa Mesa, CA. Fluorescence spectra were obtained with a Perkin Elmer LS-5 spectrofluorimeter at room temperature (22 ± 2°C).

Partition coefficient determination by phase separation (P_{ps}). Lipid multibilayers were prepared as described above. A known amount of local anesthetic was added to EPC multibilayers, and the drug concentration remaining in the supernatant after centrifugation at $105\,000 \times g$ for 2 h was determined as in the solubility experiments. The drug concentration in water was subtracted from the total drug concentration, to give the amount bound to the lipid phase. The partition coefficient, P, of a solute (s) between two phases is given by Eq. (2):

$$P(\mathrm{s}) = \frac{n_{\mathrm{m}}(\mathrm{s})/V_{\mathrm{m}}}{n_{\mathrm{w}}(\mathrm{s})/V_{\mathrm{w}}} \quad (2)$$

where: n = number of moles of solute; V = volume; the subscripts m and w refer to the membrane and aqueous phase, respectively.

P determination by spectroscopic methods. P was determined from EPR (P_{epr}) and fluorescence (P_{fluor}) experiments, making use of the methodology described by Lissi et al. [30]. Briefly, the method consists in assuming that a given effect on membrane structure is proportional to the amount of solute bound to the membrane. Experiments are done by adding increasing amounts of solute to a given membrane concentration. Then, a family of plots of effect vs. total number of moles of solute, n_{t} ($n_{\mathrm{t}} = n_{\mathrm{w}} + n_{\mathrm{m}}$), is obtained for various membrane concentrations. The same effect in each plot represents the same solute:lipid molar ratio, in the membrane. Plots of n_{t} vs. membrane volume for the same effect yield a straight line described by Eq. (3):

$$n_{\mathrm{t}}(\mathrm{s}) = \frac{C_{\mathrm{m}}(\mathrm{s}) \cdot V_{\mathrm{w}}}{P(\mathrm{s})} + C_{\mathrm{m}}(\mathrm{s}) \cdot V_{\mathrm{m}} \quad (3)$$

where $C_{\mathrm{m}}(\mathrm{s})$ is the solute concentration in the membrane, and corresponds to $n_{\mathrm{m}}/V_{\mathrm{m}}$ in Eq. (2). The partition coefficient can be obtained from Eq. (3a):

$$P(\mathrm{s}) = \frac{\text{slope}}{\text{intercept}} \cdot V_{\mathrm{w}} \quad (3a)$$

P was also determined by a novel procedure, based on the measurement of the concentration of LA necessary to cause the maximum membrane perturbation ($E_{\max}$, as monitored by spin label EPR spectra) and on its water

solubility (see Results). P values calculated by this procedure are represented by P_{sol}.

3. Results

The pK_a values of the LAs in aqueous medium vary between 7.6 and 9.2 [7,48,49]. It has been shown that the apparent pK of positively charged compounds decreases in the presence of zwitterionic membranes [26,50–52]. Therefore, in the present experiments, performed at pH 10.5, the drugs are essentially in the uncharged form.

Table 1 shows the optical absorption properties of the LAs in the UV region and the water solubility of the charged and uncharged forms. Clearly, the protonated form is more soluble by two or three orders of magnitude.

Addition of the uncharged LA to EPC membranes led to a change in bilayer organization. An increase in the empirical parameter h_{+1}/h_0 was calculated from the EPR spectra of 5-MeSL, as well as a decrease in the order parameter from the spectra of 5-SASL. Previous work has shown that uncharged PRC and TTC have a larger effect on bilayer structure than their protonated counterparts [26,32].

Spectra of 5-MeSL indicated that the decrease in bilayer organization is a function of LA concentration, a maximum effect (E_{max}) being reached at different concentrations for the different LAs. Beyond E_{max}, the ester and amide anesthetics displayed different behavior: while the amides led to constant h_{+1}/h_0 (Fig. 2), ester anesthetics yielded decreasing h_{+1}/h_0 values (Fig. 3).

Since the neutral forms of the LAs have a limited water solubility (Table 1), under the conditions of the experiment, eventually an aqueous concentration is reached that corresponds to this solubility. At this point, *since the aqueous phase becomes saturated with the anesthetic, no more drug partitions into the membrane*. This explains the constant maximum effect observed in the case of the amide anesthetics. The more complex behavior of h_{+1}/h_0 for the esters was previously reported for TTC and was ascribed to the appearance of a second, anesthetic-enriched, lipid phase [9]. Partitioning of the probe into this phase leads to the appearance of an additional spectral component, causing the decrease of h_{+1}/h_0 at the higher drug concentrations (Fig. 3). Fig. 4 illustrates the spectral effects for CLP. Initially, the drug causes an increase in h_{+1}/h_0 values (Fig. 4B). At higher CLP concentration a more immobilized spectral component is observed (Fig. 4C). Also in these cases E_{max} occurs at the anesthetic solubility in water.

The breaks in the plots of spectral parameters vs. LA concentration such as those in Figs. 2 and 3 can be used to calculate the drug membrane-water partition coefficient. As mentioned above, the breakpoints occur at the drug solubility in water, S_w. This means that n_w in Eq. (2) is known. Since the total number of drug molecules (n_t) is

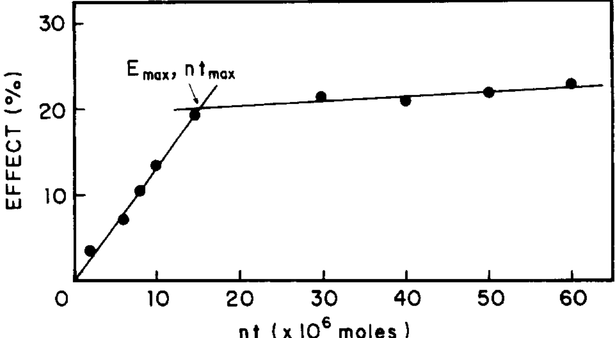

Fig. 2. Effect of LDC concentration on the h_{+1}/h_0 ratio for spectra of 5-MeSL in EPC multilamellar vesicles. n_t represents the number of moles of LDC in 1.0 ml of aqueous phase (V_w). [EPC] = 2 mM, 0.2 M carbonate/bicarbonate buffer (pH 10.5), 22°C. Effect (%) = $100((h_{+1}/h_0)_{sample} - (h_{+1}/h_0)_{control})/((h_{+1}/h_0)_{control})$.

also known, it is possible to derive n_m and, therefore, P (P_{sol}). In spite of the more complex behavior of the ester anesthetics, the procedure is also applicable to these compounds.

The values of P_{sol} calculated from the above analysis are presented in Table 2. These values are compared to those determined by phase separation (P_{ps}) and by the procedure described by Lissi et al. [30], making use of both EPR (P_{epr}) and fluorescence measurements (P_{fluor}). It is seen that the agreement between the values obtained by the various procedures is very good. In particular, the values of P_{sol} are in close agreement with those found by phase separation. Thus, the knowledge of the drug aqueous solubility enables one to use a very simple procedure to evaluate P without requiring phase separation. It is also

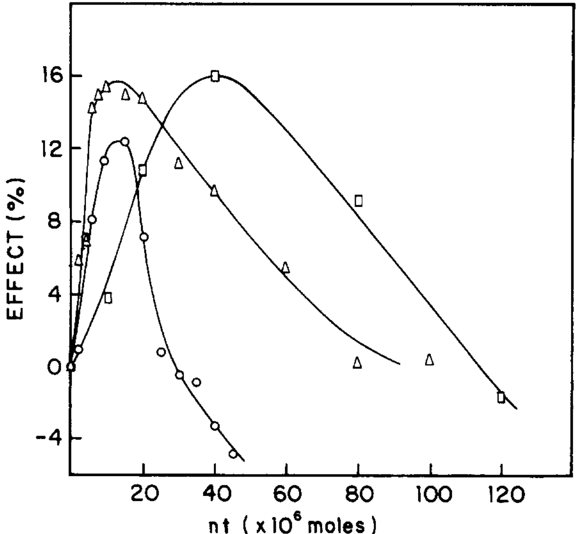

Fig. 3. Effect of the ester LAs concentration on the h_{+1}/h_0 ratio for spectra of 5-MeSL in EPC multilamellar vesicles: PRC (□), TTC (△) and CLP (○). [EPC] = 20 mM, n_t represents the number of moles of LA in 1.0 ml of aqueous phase (V_w), 0.2 M carbonate/bicarbonate buffer (pH 10.5), 22°C.

Table 1
Wavelength of maximum absorption (λ_{max}), molar extinction coefficient (ε), and aqueous solubility (S_w) of the local anesthetics at 22°C

LA	Charged LA [a]			Uncharged LA [b]		
	λ_{max} (nm)	ε (M^{-1} cm^{-1})	S_w (μM)	λ_{max} (nm)	ε (M^{-1} cm^{-1})	S_w (mM)
PRC	290	16500	2.95	287	15000	16.3
CLP	290	25000	0.20	287	23000	1.98
TTC	310	21800	1.34	307	24900	0.76
LDC	263	480	2.30	262	420	13.1
PLC	265	280	0.83	270	850	23.1
EDC	263	480	0.20	262	420	0.16
MVC	263	550	1.30	263	440	8.82
BVC	263	470	0.07	263	600	0.58
DBC	327	43000	1.90	327	38000	0.03

[a] 0.2 M sodium acetate buffer (pH 5.5). [b] 0.2 M carbonate/bicarbonate buffer (pH 10.5).

more advantageous over the methodology previously described [30], since it does not require working with different membrane concentrations.

Studies were also performed with the spin label 5-SASL. The results for LDC and PLC are in agreement with those found with 5-MeSL: a decrease in order parameter upon addition of LA is observed, followed by a plateau for higher concentrations (Fig. 5). The breakpoints in these plots yield P_{sol} values for LDC and PLC (Table 2) in good agreement with the values found with 5-MeSL.

High LA/phospholipid molar ratios (Table 3, see below) can be reached at the maximum effect. One might expect that such ratios would cause changes in partition coefficient and, eventually, micellization of the bilayer. In fact, Abuin and Lissi [53] have found changes in the partitioning of alcohols into micelles as the alcohol concentration increased. Bilayer micellization has been found in the presence of high concentration of cationic TTC [9,54] and of uncharged analogs of TTC [55]. Nevertheless, within the accuracy of our experiments, no significant deviation of linearity was detected in plots of spectral parameters vs. LA concentration, which would be suggestive of changes in P. Moreover, under no circumstances did the spin probe spectra provide any indication of anesthetic-induced micellization.

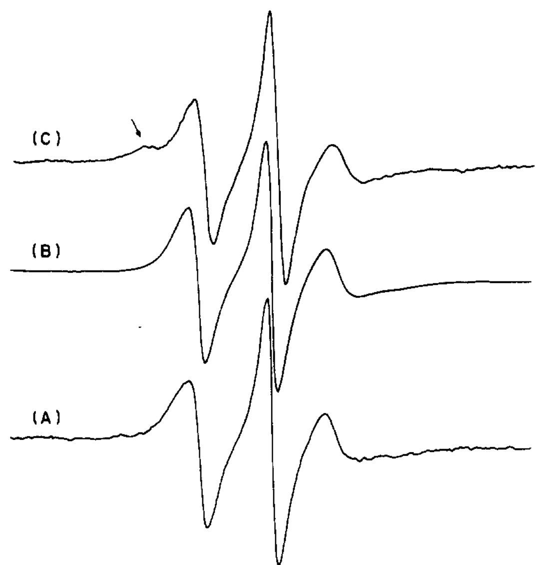

Fig. 4. EPR spectra of 5-MeSL in EPC multibilayers in the absence (A) and in the presence of 10 mM (B) and 40 mM (C) CLP. The arrow indicates the low field resonance of the less mobile spectral component that appears at high CLP concentration. [EPC] = 20 mM, 0.2 M carbonate/bicarbonate buffer (pH 10.5), 22°C.

A quantitative evaluation of the LA–bilayer interaction was done making use of the LA water solubility and of spectral effects. Table 3 displays several parameters calculated by this approach. Values of E_{max} for the various anesthetics are shown, together with the values of n_t needed to reach E_{max} for a given membrane concentration (16.7 mM EPC). The experimentally obtained values of

Table 2
Partition coefficients for the uncharged forms of the LAs in EPC multibilayers at pH 10.5 [a,b]

LA	P_{ps}	P_{epr}	P_{fluor}	P_{sol} [c]	$P_{ps} \cdot S_w$ [e]
PRC	84 ± 32	96	228	72	1.05
CLP	250 ± 46	134	396	258	0.38
TTC	868 ± 23	780	980	697	0.51
LDC	144 ± 54	127	223	92; 130 [d]	1.45
PLC	110 ± 61	134	305	96; 86 [d]	1.96
EDC	1202 ± 480	–	1251	1077	0.15
MVC	98 ± 12	246	245	75	0.67
BVC	798 ± 147	–	1168	870	0.36
DBC	2240 ± 1050	–	–	8280	0.051; 0.191 [f]

[a] Definitions of P_{ps}, P_{epr}, P_{fluor} and P_{sol} and experimental conditions are given in Materials and methods. [b] P_{ps} values are the average of four experiments; P_{epr}, P_{fluor} and P_{sol} are the average of two experiments. [c] From 5-MeSL spectra (see text). [d] From 5-SASL spectra. [e] Calculated taking the concentrations as mole solute/mole solvent. [f] Calculated using P_{sol}.

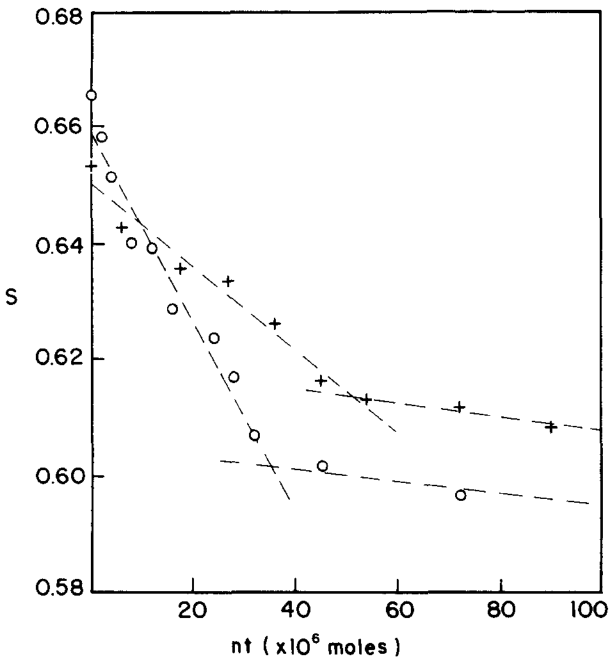

Fig. 5. Effect of LDC (O) and PLC (+) concentration on the order parameter of 5-SASL in EPC multilamellar vesicles. [EPC] = 18 mM, n_t represents the number of moles of LA in 1.0 ml of aqueous phase (V_w), 0.2 M carbonate/bicarbonate buffer (pH 10.5), 22°C.

$n_{t\,max}$ were compared to values calculated from the partition coefficients measured by phase separation, and from the drug solubility in water, according to Eq. (4):

$$n_{t\,max}(s) = V_m \cdot P_{ps}(s) + S_w(s) \quad (4)$$

Except for DBC, whose water solubility is very low, the agreement between experimental and calculated values is very good. The table also shows $n_{m\,max}$, i.e., the number of moles of LA in the membrane, at E_{max}, obtained from Eq. (5):

$$n_m(s) = \frac{n_t(s)}{1 + S_w \cdot \frac{1}{V_m}} \quad (5)$$

The values of n_m are shown as LA/EPC molar ratios at the maximum effect (fifth column) in Table 3.

As shown in Table 3, both E_{max} and the LA:lipid molar ratio at E_{max} are different for the different anesthetics. Clearly, it is desirable to compare the structural effects caused by the different LAs at the same concentration *in the membrane*. This was accomplished making use of Eq. (5) and plots of effect vs. n_t. The effects at LA/EPC (1:3, mole/mole) are given in Table 3. For EDC and DBC, the limiting membrane concentration is reached at LA/EPC molar ratios smaller than 1:3. For the sake of comparison, these values were obtained by extrapolation. Except for the smaller effects found for PRC (3.8%) and for the cyclic amides MVC (6.6%) and BVC (5.4%) all other anesthetics displayed effects ranging from 9.2 to 15.9%.

Table 3
Quantitative comparison of the effect [a] of the LAs upon EPC multibilayers [b], derived from EPR data

LA	E_{max} [a]	$n_{t\,max}$ [c] (expt.) (μmoles)	$n_{t\,max}$ [d] (calc.) (μmoles)	LA/EPC at E_{max}	Effect for LA/EPC 1:3 (mole/mole)
PRC	17.0	31.5	34.0	2.7:3	3.76
CLP	15.0	8.60	8.35	1.2:3	12.1
TTC	15.0	9.38	9.28	1.5:3	13.9
LDC	26.5	28.6	37.4	2.8:3	10.4
PLC	13.0	51.7	56.0	5.1:3	9.22
EDC	7.5	2.21	2.67	0.4:3	15.9 [e]
MVC	10.5	16.5	20.0	1.4:3	6.64
BVC	7.8	7.75	6.56	1.3:3	5.38
DBC	8.2	3.56	0.90	0.6:3	13.3 [e]

[a] Effect is defined in the caption of Fig. 2, E_{max} is the maximum effect achieved. [b] In this experiment [EPC] = 16 mM; experimental conditions are described in Materials and methods. [c] Total number of moles of LA in 1.0 ml aqueous phase necessary for the observation of E_{max}. [d] Total number of moles of LA in 1.0 ml aqueous phase necessary to obtain E_{max}, calculated from Eq. (4). [e] The maximum concentration of LA in the membrane occurs at an LA/EPC ratio smaller than 1:3 (see text).

4. Discussion

The present results report on qualitative and quantitative aspects of local anesthetics–lipid bilayer interaction. The effects of the uncharged forms of three ester and six amide anesthetics upon the organizational properties of egg phosphatidylcholine multibilayers were examined making use of EPR spin label spectroscopy. EPR and fluorescence data were utilized to calculate partition coefficients, in the line of previous work from this laboratory [30,31].

4.1. Determination and thermodynamic analysis of partition coefficients

We have previously presented a methodology making use of spectroscopic measurements for the determination of membrane-water partition coefficients without the requirement of phase-separation [30]. The formalism developed can be applied to any measurement providing information about concentration-dependent effects of partitioning solutes upon membrane properties. The elimination of the phase separation step avoids errors due to eventual solute co-sedimentation [56] as well as membrane remaining in the supernatant. Moreover, problems due to time-dependent processes, such as chemical reactions involving the solute (or the membrane) are also largely circumvented. The methodology described by Lissi et al. [30] is based on the addition of increasing solute concentrations to a family of fixed membrane concentrations.

In this paper we show that an even simpler procedure can be used to evaluate P, as long as the drug water solubility is known, and is sufficiently low to reach water

saturation under the conditions of the experiment. Thus, we found that the disorganizing effect of the LAs upon the bilayer structure increases with LA concentration, reaching a maximum (E_{max}). While E_{max} remains constant for the amide series upon further drug addition (Figs. 2 and 5), a decrease is observed for the ester series (Fig. 3). E_{max} was found to occur at the drug water solubility, S_w. Since P is a thermodynamic constant; once the aqueous phase becomes saturated, no more solute can partition into the membrane. A similar effect was found by Hill for n-alcohols and general anesthetics, in dipalmitoylphosphatidylcholine (DPPC) bilayers [57]. In addition, Pringle et al. observed that the anesthetic potency of n-alcohols upon murine neurons increased with the acyl chain length, up to 12 carbons. Then, a cut-off effect occurred, longer n-alcohols (whose $S_w < 10^{-6}$ M) having either the same effect as shorter ones, or none [58].

In a study of the binding of hexachlorocyclohexane insecticides to dioleoylphosphatidylcholine bilayers, Jones and Lee also found that the binding was limited by saturation of the aqueous rather than the lipid phase, and that the maximum concentration reached in the lipid does not correspond to saturation of available binding sites [59]. Holland and Schreier found a similar behavior for the antichagasic drug nifurtimox in DPPC bilayers (unpublished results).

As shown in Results, the knowledge of S_w and the total drug concentration ($n_{t\ max}$) to reach E_{max} for a given membrane concentration allows the calculation of the partition coefficient (P_{sol}). In Table 2 the values of P calculated by this procedure are compared to those found by phase separation and by the Lissi et al. methodology [30] from EPR and fluorescence measurements (P_{ps}, P_{epr}, P_{fluor}, respectively). It is seen that all values are in reasonable agreement, and that the values of P_{sol} are the closest to P_{ps}.

Hill [57] has described a thermodynamic analysis of the partition coefficient and derived the equation:

$$P \cdot S_w = 2 \tag{6}$$

(concentrations in mole solute/mole solvent), for the ideal case. While the author found that this product was observed for even-numbered, but not for odd-numbered chain length alcohols, Lee [60] found that the product decreased as a function of chain length.

Table 2 shows that in the case of the LAs, the $P \cdot S_w$ product is far from 2 in most cases, indicating that partitioning is non-ideal. Values of $P \cdot S_w < 2$ were also obtained for inhalation anesthetics [57] and hexachlorocyclohexanes [59]. In the present study, values closer to 2 were obtained for the more water-soluble anesthetics (LDC, 1.45, and PLC, 1.96). A larger deviation for the less soluble compounds was also found in the n-alcohol series ([60] and [61], as pointed out in ref. [60]).

The lack of ideal behavior has been ascribed to incomplete exclusion of solute from the gel phase and non-ideal mixing between solute and phospholipid [14,62–64].

4.2. Structure–activity analysis of the LAs effects on membrane organization

The spin label spectra indicated that addition of the LA causes a decrease of overall organization (h_{+1}/h_0, 5-MeSL) and of the degree of bilayer order (S, 5-SASL). The results are in agreement with those obtained from deuterium NMR data that revealed both structural (decrease of quadrupole splittings) and dynamical (increase of relaxation times) effects of the LAs on phospholipid membranes (de Paula, E., Jarrell, H.C. and Schreier, S., in preparation).

A quantitative analysis was made by calculating the amount of membrane bound drug from P values. Table 3 shows that the LA/EPC molar ratio at E_{max} varies by a factor of ca. 10, being smaller for the less water soluble compounds. This is one more indication that the amount of drug bound to the membrane is restricted by its water solubility, and is a consequence of the fact that P increases less rapidly than the solubility decreases.

The effects of local anesthetics have been analysed in terms of structure–activity relationships. Several pharmacological effects have been interpreted as being the result of various factors: drug hydrophobicity, as measured by the partition coefficient, pK_a (extent of ionization in aqueous medium), and molecular size [5–7], as well as polar interactions [8]. The ionization degree is not dealt with in the present work, since it focuses only on the uncharged form of the LAs. In a previous study [26], we have analysed the effect of membrane binding on the extent of drug ionization, taking into account that it is affected by the differences in partition coefficient between the protonated and unprotonated forms, i.e., $\Delta pK = pK_{membrane} - pK_{water}$ is proportional to ΔP ($P_{uncharged} - P_{charged}$) [26,50–52]. In summary, both forms bind to the lipid bilayer, and their molar ratio is not the same as that in water at a given pH. As for the role of hydrophobicity, it has been found that several pharmacological effects are proportional to the partition coefficient, within a family of related structure [5–7,49]. Thus, the ester anesthetics were seen to be more active than the amides. In some analysis, the cyclic amides were found to be less active than the acyclic analogues [5,7]. The differences between families have been ascribed to molecular size. The latter contribution has been analysed in detail in terms of different parts of the molecule: the bulkiness of the aromatic methyl groups, the orientation of the ester vs. the amide bond, and the branched vs. cyclic amide portion in the amino-amides [6].

The present results do not reveal a clear proportionality between P and effect on membrane organization within a given family of LAs, when compared at the same concen-

tration in the bilayer (Table 3). This is not unexpected, since membrane perturbation should be related to steric effects.

Balgavý and co-workers [65] have proposed that the differences in free volume generated by the mismatch between the shapes of the anesthetic and the phospholipid could be responsible for the different structural effects. In fact, a comparison between PRC (from the amino-ester series) and LDC and PLC (from the amino-amide series), whose partition coefficients are quite similar, shows that the latter exert a much larger membrane perturbing effect. PLC and LDC posses one to two -CH_3 groups in the aromatic ring, respectively, and very branched aliphatic portions. The hydrophobic volume of a methyl group is quite large, and would give rise to a large free volume. This might be a prevalent factor in the PLC, LDC, EDC series, leading to a smaller range of effects (9–16%) than in the ester series (3–13%), where the chlorine atom of CLP and the straight *N*-butyl chain of TTC are responsible for the differences with respect to PRC. The smaller effects observed for MVC and BVC might be due to replacement of the branched side chains by the cyclic amine moiety, leading to a decrease in the free volume. Here, again, essentially no difference is seen between MVC ($P \cong 100$) and BVC ($P \cong 800$). We are tempted to speculate that the greater hydrophobic character of the cyclic amine portion might facilitate its insertion (upside-down) in the bilayer, thereby decreasing the steric perturbation.

It is also conceivable that the different effects might be, in part, associated with different membrane location of the LAs. Indeed, NMR data has indicated that PRC is located more superficially than TTC [32] and that charged TTC is located closer to the aqueous interface than its uncharged counterpart [33]. The interactions between the LAs and phospholipids can be of hydrophobic, as well as polar nature. The balance between them will determine the preferential membrane location. Work is under way to examine this possibility.

It has been proposed that the lesser biological activity of the amide anesthetics is due to the more difficult fitting in a specific site at the sodium channel due to steric hindrance [6]. Interestingly, the differences found in the present work at the lipid bilayer level seem to be of the same nature.

In conclusion, we have shown that spin label spectra in conjunction with the knowledge of water solubility allows the determination of membrane-water partition coefficients through a very simple procedure that does not require phase separation. The values thus obtained are in very good agreement with those obtained by classical procedures, and by other methodologies employing spectroscopic measurements. The work has also allowed the comparison of the effects of different LAs on membrane organization at the same concentration in the membrane. It was found that differences between the LA are a function of steric effects due to the mismatch between anesthetic and phospholipid shapes. The possibility of different membrane location will be further investigated.

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