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# Carboxyl groups at the membrane interface as molecular targets for local anesthetics

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**Abstract**

The interaction of the tertiary amine drugs chlorpromazine and dibucaine in their cationic form with carboxyl groups at the membrane surface is studied at concentrations relevant to anesthesia. Spin-labeled stearic acid is used both to provide the carboxyl groups and to monitor binding and ionization behavior in egg lecithin liposomes. Membrane anesthetic concentrations are spectrophotometrically obtained. They are shown to determine the drug influence on carboxyl groups at the membrane surface, independently of aqueous concentrations. The intramembrane association constants (related to the usual aqueous phase ones through the partition coefficient) of the drugs with fatty acids are determined. The same value ($10^2$ $M^{-1}$) is obtained for both drugs, suggesting that it is approximately the same for all tertiary amine local anesthetics. pH titrations of anesthetic-treated spin-labeled membranes are performed. The observed shifts in the fatty acid p$K$ are higher than can be produced assuming uniform distribution of the drug in the membrane surface, implying that there is an increased affinity of local anesthetics for superficial carboxyl. This affinity could account for the resting block of voltage-gated $Na^+$ channels. Under these considerations, local anesthetic binding sites at voltage-gated $Na^+$ channels and at sarcoplasmic reticulum $Ca^{2+}$-ATPase are proposed. 

*Keywords:* Local anesthetics; Biomembranes; Spin labels; Binding sites

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## 1. Introduction

The relevance of local anesthetics comes from their ability to relieve pain by blocking nerve transmission, besides promoting a variety of other physico-chemical changes on biological systems. A common feature of local anesthetics is their amphipathic character. A wide class of these drugs possesses aromatic rings in the hydrophobic region and a tertiary amine in the polar headgroup. Chlorpromazine and dibucaine belong to this class. It is well established that the anesthetic potency of local anesthetics is correlated to blockade of voltage-gated $Na^+$ channels of axonal membranes [1,2], which are essential for action potential generation. However, the molecular details of local anesthetic action have not yet been unveiled. The historical controversy on whether primary binding sites are lipidic or proteic still stands. More recently, specific segments of $Na^+$ channels have been implicated in the binding of local anesthetics [3,4]. On the other hand, the fact that the anesthetizing concentration of any narcotic is the same within a membrane cannot be ruled out. A hydrophobic protein site for anesthetic action agrees with this fact but the hydrophobicity of a protein portion compared to that of lipid chains would not provide the specificity required by the ion-channel site.

Tertiary amine local anesthetics occur in both positively-charged and uncharged forms. The ionization state of membrane-associated local anesthetics affects their physiological activity and transmembrane transport. The charged form is a more potent blocker of sodium conductance suggesting that electrostatic interactions play an important role in the molecular mechanism of anesthesia [5]. An interaction between the local anesthetic and negative groups of integral membrane proteins localized near the surface of the lipid layers, if strong enough, would provide specificity to a protein site. The carboxyl groups of the acidic amino acid residues aspartate and glutamate are negatively-charged sites in proteins. On the other hand, fatty acids intercalated in lipid bilayers have their carboxyl groups localized at the membrane surface. These acidic headgroups present ionization equilibrium with $pK_a$ values of approx. 7 in neutral phospholipid vesicles [6]. Thus, spin-labeled fatty acids are appropriate to probe the interactions between local anesthetics and anionic sites. Moreover, their ESR spectra are sensitive to the ionization state of the carboxyl groups [7,8].

Modification of the interfacial ionization equilibrium induced by changes in polarity and by electrostatic membrane potentials has been studied and analyzed according to equilibrium thermodynamics, using charged micelles and phospholipid mixtures [9–12]. In general, the results are consistent with the presence of a surface charge density. In the case of positively-charged drugs interacting with fatty acids in lipid bilayers, however, disagreement with theoretical expectations suggested localized charge effects [12,13].

In this work we evaluate the strength of the interaction of tertiary amine local anesthetics with carboxyl groups located at the level of the polar headgroup region of membranes, using spin-labeled stearic acid. We also study their influence on the carboxyl ionization equilibrium. We show that such carboxyl groups provide specific sites for anesthetic binding. This motivated us to look for negative residues in voltage-gated $Na^+$ channels and in $Ca^{2+}$-ATPase which would likely reside at the polar region of boundary lipids. In fact, such residues were found and proposed to be part of the anesthetic binding site.

## 2. Materials and methods

Chlorpromazine hydrochloride, dibucaine hydrochloride, egg lecithin and stearic acid spin-labeled at the C-5 position of the fatty acyl chain (5-SASL) were obtained from Sigma Chem. Co.

The stock solution for universal buffer (borate/citrate/phosphate) 0.067 M was prepared as described elsewhere [9]. The pH was adjusted by adding aliquots of 1 M HCl to diluted amounts of the stock solution. Spin-labeled liposomes were prepared by codissolving the spin-labeled stearic acid and egg phosphatidylcholine at a molar ratio of 1:100 in ethanol, drying by fluxing nitrogen and then under vacuum for 2 h. The dry lipid film was dispersed in a universal buffer of appropriate pH and 20 mM sodium ions

by vortex mixing, and washed two or three times. For drug treatment, appropriate quantities of concentrated ethanol solutions of chlorpromazine or dibucaine were evaporated in polypropylene tubes. Aliquots of spin-labeled liposomes containing 2 mg of lipids dispersed in buffer to a final volume of 1 ml were added. Samples were vortex mixed during 1 min and incubated for 2 h. After centrifugation, the supernatant was used for spectrophotometric measurements of anesthetic concentration, and the lipid containing pellet was transferred to glass capillaries and further centrifuged for ESR measurements.

X-band ESR spectra were obtained with a Bruker ESP300 spectrometer at room temperature. Apparent order parameter of spin labels, $S_{app}$, is obtained from ESR spectra according to [14]: $S_{app} = (A_{max} - A_{min})/[A_{zz} - (A_{xx} + A_{yy})/2]$, where the values of 3.36, 0.63 and 0.58 mT were used for $A_{zz}$, $A_{xx}$ and $A_{yy}$, respectively.

Aqueous anesthetic concentrations in the supernatant were measured spectrophotometrically using a HP 8452A diode array spectrophotometer. The amount of membrane-associated drug was calculated subtracting the amount of added drug from that in the aqueous phase. Membrane drug concentrations (moles of associated drug per volume occupied by the membranes) were then obtained considering a membrane density of 1 mg $ml^{-1}$. Partition coefficients, $K_p$, were obtained by the relation $K_p = (N_m/V_m)/(N_a/V_a)$, where $N$ is the number of moles of the drug and $V$ is the volume. The subscripts $m$ and $a$ refer to the membrane and to the aqueous phase, respectively.

## 3. Results

### *3.1. ESR spectra of the neutral and ionized stearic acid spin label and the effects of local anesthetics*

The ESR spectra of 5-SASL in egg PC dispersions are presented in Fig. 1A for bulk pH values of 5.2, 8.5 and 10.5. As observed previously [7,8], the ESR spectrum of membrane-associated fatty acids at low pH is characteristic of uncharged fatty acid molecules and has a smaller spectral anisotropy than that of ionized negatively-charged molecules occurring at high pH. This is explained considering that uncharged fatty acid molecules sink deeper into the bilayer so that the spin probe is affected by an environment of increased fluidity.

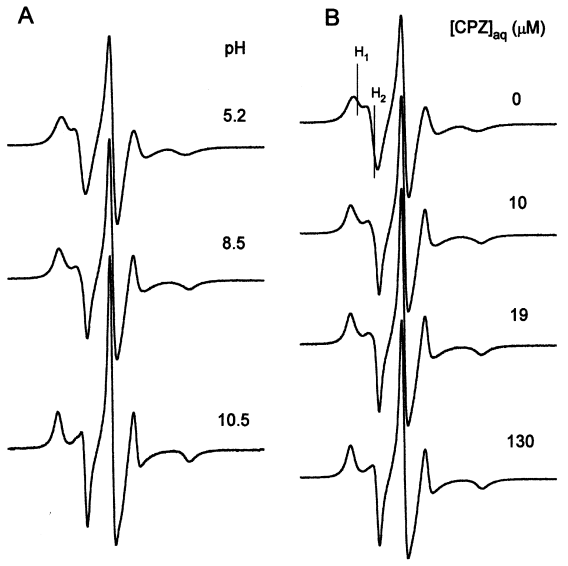


Fig. 1. ESR spectra of 5-SASL in egg lecithin liposomes: (A) at different pH values; (B) at different chlorpromazine concentrations, at pH 5.5.

Fig. 1B shows the effect of chlorpromazine treatment on the ESR spectrum of stearic-acid spin-labeled liposomes at pH 5.5. At low pH, increasing amounts of drug increase the spectral anisotropy, decreasing the motion of the fatty acid chains, up to saturation. Dibucaine produces the same kind of spectral changes, but higher concentrations of dibucaine are necessary to achieve the same spectral effect. Considering the total amount of drug added to the suspensions, chlorpromazine seems to be more effective in promoting the increase in spectral anisotropy.

Comparing the ESR spectral changes induced by local anesthetics (Fig. 1B) with those induced by increasing pH, it is noted that the major spectral features are the same. Anesthetics therefore induce deprotonation of stearic acid molecules causing the movement of the charged headgroup towards the membrane surface. Since the aque-

ous p$K_a$ of the drugs is approx. 9 (8.9 for dibucaine and 9.3 for chlorpromazine) [9,12], both chlorpromazine and dibucaine are positively charged at pH 5.5, even considering shifts of the ionization equilibrium due to membrane association. The positive electrical potential caused by the local anesthetic at the stearic acid headgroup shifts the ionization equilibrium favoring the charged form of the acid. This induces the observed increase in spectral anisotropy.

In order to present anisotropy changes as a function of membrane anesthetic concentrations, membrane:buffer partition coefficients of the drugs were obtained for all the samples used in the ESR measurements. Fig. 2 shows the results obtained from the spectrophotometric measurements. Fig. 2A is a plot of the drug concentration in the liposomes, $[drug]_{memb}$, as a function of the aqueous drug concentration, $[drug]_{aq}$, and Fig. 2B is the lipid:buffer partition coefficient, $K_p$, as a function of drug concentration in the liposomes. For both drugs, the partition coefficient decreases with increasing membrane-associated drug concentration, due to the electrostatic repulsion. For the same membrane-associated drug concentration, the membrane:buffer partition coefficient of chlorpromazine is eight times greater than that of dibucaine. This factor is very similar to the factor 7, obtained using the intrinsic dissociation constants calculated from binding curves to dipalmitoyl phosphatidylcholine [12].

The apparent order parameter $S_{app}$ of the spin-labeled stearic acid in anesthetic treated egg lecithin liposomes was obtained from the ESR spectrum of 5-SASL (see Section 2). Fig. 3 shows the plot of $S_{app}$ as a function of the membrane drug concentration, for dibucaine and chlorpromazine, at pH 5.5. The plot shows that, for the same membrane-associated drug concentration, both chlorpromazine and dibucaine have the same effect on stearic acid. The shape of the curve suggests a binding process.

It is worth noting that even low concentrations of drugs affect stearic acid. The value of $S_{app}$ almost reaches saturation at aqueous concentrations of 65 $\mu$M for dibucaine and 8 $\mu$M for chlorpromazine, which correspond to a membrane-associated drug concentration of 20 mM

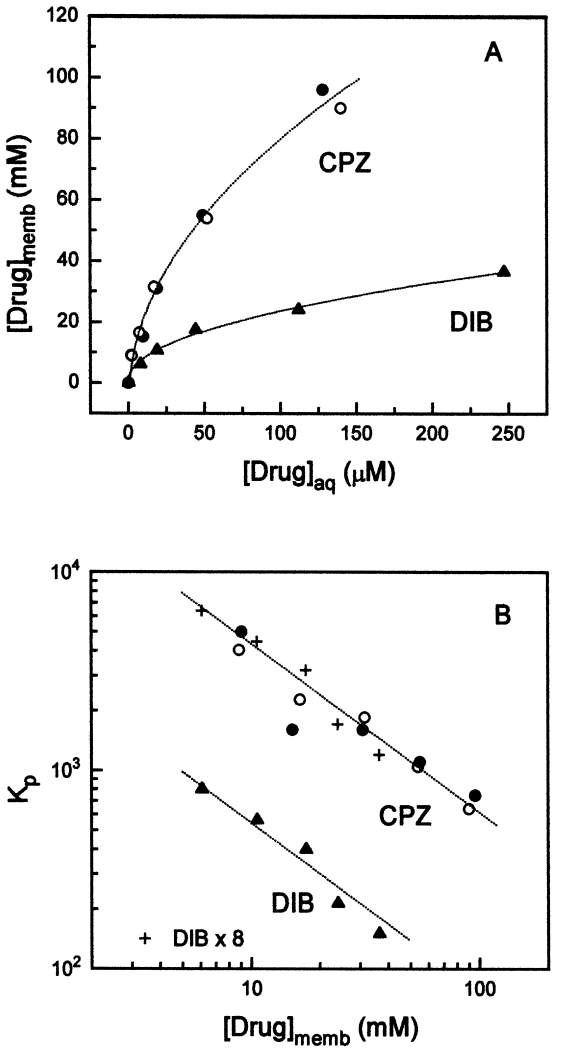


Fig. 2. Local anesthetics liposome partitioning: (A) membrane drug concentration in the spin labeled egg lecithin liposomes, $[drug]_{memb}$, as a function of aqueous drug concentration, $[drug]_{aq}$; (B) lipid:buffer partition coefficient, $K_p$, as a function of membrane drug concentration. Lipid concentration of all samples was 2 mg/ml.

(~ 20 mmol/kg). This is in the range of membrane concentrations relevant to anesthesia [15].

At pH 10.5 no changes in the ESR spectrum occur even at the highest drug concentration used in this work (aqueous chlorpromazine 0.13 mM). In fact, this is not surprising since at this pH anesthetic molecules are neutral and all stearic acid molecules are ionized.

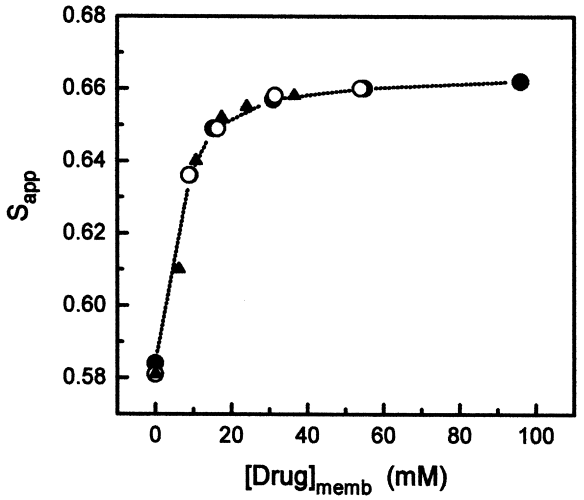


Fig. 3. Apparent order parameter, $S_{app}$, obtained from the ESR spectrum, as a function of membrane drug concentration, $[drug]_{memb}$, at pH 5.5, for chlorpromazine (open and solid circles) and dibucaine (triangles).

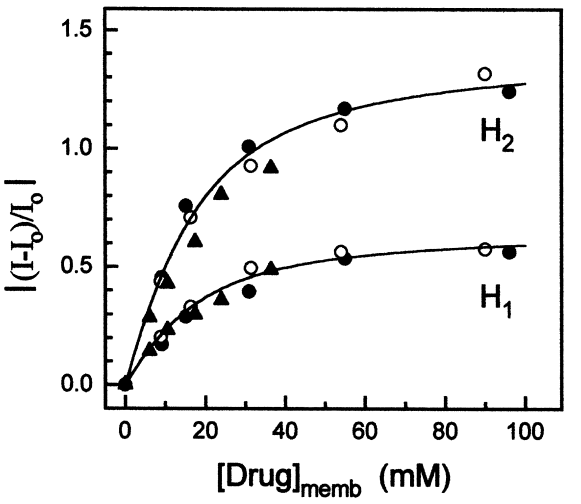


Fig. 4. Fractional intensity change of the ESR spectrum, $|(I_o - I)/I_o|$, at magnetic field values $H_1$ and $H_2$ indicated in Fig. 1, as a function of membrane drug concentration, for chlorpromazine (open and solid circles) and dibucaine (triangles), at pH 5.5. Solid curves were obtained using Eqs. (1),(2), with a drug/stearic acid intramembrane association constant $K_b^m = 100\ \mathrm{M}^{-1}$. Room temperature approx. 20°C.

### *3.2. Intramembrane anesthetic–stearic acid association constant*

The association of a drug to a probe can be monitored by any spectroscopic parameter of the probe which changes in the presence of the drug. The changes of the 5-SASL ESR spectrum upon addition of local anesthetics are used to obtain the association constants. All the spectra were normalized to the same double integral. Numerical spectral combinations showed that the ESR spectra consist of two components, one is that in the absence of drug and the other is assigned to anesthetic-associated stearic acid. The need to invoke anesthetic association to explain the second component is justified in the next section. The intensity of the spectrum at any magnetic field (or any $g$ value) is therefore the sum of these two components with different proportions depending on the degree of association. Fig. 4 shows the plot of the fractional intensity changes $|(I_o - I)/I_o|$ at magnetic fields $H_1$ and $H_2$, indicated in Fig. 1, as a function of the membrane concentration for both drugs. $I_o$ and $I$ are the intensities of the normalized ESR spectra in the absence of anesthetic and at a given anesthetic concentration, respectively.

To obtain the intramembrane association constant we assume that the association of a local anesthetic to stearic acid in the lipid phase can be represented by the equilibrium

local anesthetic (D) + stearic acid (SA)

$\leftrightarrow$ complex (DSA),

the lipid-phase association constant for the interaction is then given by:

$$K_b^m = \frac{[\mathrm{DSA}_m]}{[\mathrm{D}_m][\mathrm{SA}_m]} = \frac{\alpha[\mathrm{SA}_m^t]}{\left([\mathrm{D}_m^t] - \alpha[\mathrm{SA}_m^t]\right)\cdot(1-\alpha)[\mathrm{SA}_m^t]} \qquad (1)$$

where the subscript $m$ refers to the membrane, $[\mathrm{D}_m^t]$ and $[\mathrm{SA}_m^t]$ are the total membrane-bound concentrations of anesthetic and stearic acid, respectively, and $\alpha = [\mathrm{DSA}_m]/[\mathrm{SA}_m^t]$ is the molar fraction of anesthetic-bound stearic acid. The molar fraction $\alpha$ has the following expression, in

terms of the spectral intensity at a given magnetic field:

$$\alpha = \frac{[\mathrm{DSA}_m]}{[\mathrm{SA}^t_m]} = \frac{I_o - I}{I_o - I_\infty} = \frac{(I_o - I)/I_o}{(I_o - I_\infty)/I_o} \quad (2)$$

where $I_\infty$ is the intensity at infinite anesthetic concentration.

The experimental results in Fig. 4 are fitted using Eq. (1) with $\alpha$ given by Eq. (2). $K_b^m$ and $I_\infty$ are the fitting parameters. The theoretical curves in Fig. 4 were obtained with $K_b^m = 100\ \mathrm{M}^{-1}$. This is the average of $K_b^m$ values obtained from individual fittings (typical fitting errors are 20%). It is worth pointing out that this intramembrane association constant is the same for both chlorpromazine and dibucaine, and probably for all tertiary amine local anesthetics. The usual association constant $K_b$ of a local anesthetic to the membrane-bound stearic acid is obtained multiplying the intramembrane association constant by the anesthetic membrane:buffer partition coefficient: $K_b = K_p \cdot K_b^m$. It is therefore two orders of magnitude higher than the association constant to uncharged membrane lipids, which is approx. 0.75 $K_p$ $\mathrm{M}^{-1}$ [$K_b \approx (MW_{\mathrm{lip}}\ 10^{-3} \cdot K_p)\ \mathrm{M}^{-1}$, where $MW_{\mathrm{lip}}$ is the average molecular weight of lipids].

### 3.3. pH titration of stearic acid in the presence of local anesthetics

ESR spectra of 5-SASL were recorded for several pH values of the suspending medium, for untreated and CPZ-treated liposomes at several drug concentrations. The apparent order parameter $S_{\mathrm{app}}$ of the spin-labeled stearic acid is plotted against pH in Fig. 5. The data are fitted by the Henderson–Hasselbach equation giving the p$K$ values listed in Table 1. The p$K$ shifts due to the presence of CPZ molecules are plotted as a function of the membrane drug concentration in the insert of Fig. 5. These shifts mainly reflect the influence of the electrostatic potential due to charged anesthetic molecules at the stearic acid sites.

The electrostatic potential, $\Psi_o$, at a temperature $T$, due to a surface charge density, $\sigma$ , using the Poisson–Boltzmann approximation for symmetrical electrolytes with counter-ions and co-ions of valence $Z$ is given by:

$$\Psi_o = \frac{2kT}{Ze} \sinh^{-1}\left[\frac{Ze}{2kT}\frac{\sigma\lambda}{\varepsilon\varepsilon_o}\right] \quad (3)$$

where $\lambda = 0.304\ Z^{-1}\ c^{-1/2}$ (nm) is the Debye screening length, $c$ is the bulk ion concentration in mol/l, and parameters $e$, $\varepsilon$, $\varepsilon_o$ and $k$ are the elementary electronic charge, the dielectric constant of water, the permittivity of free space and the Boltzmann constant, respectively [16].

The electrostatic potential $\Psi_o$ changes the proton concentration in the solution adjacent to the membrane surface according to

$$[\mathrm{H}^+] = [\mathrm{H}^+]_{\mathrm{bulk}} \exp(-e\Psi_o/kT) \quad (4)$$

Under the assumption that the p$K$ shift, $\Delta$p$K$, is only due to the change in the local pH, it is expressed in terms of $\Psi_o$ by:

$$\Delta \mathrm{p}K = -e\Psi_o/(2.3kT) \quad (5)$$

On the other hand, a uniform surface charge density $\sigma$ is expressed in terms of the membrane drug concentration, $[drug]_{\mathrm{memb}}$, by

$$\sigma = \frac{e}{A}\frac{[drug]_{\mathrm{memb}}}{[PC]_{\mathrm{memb}}} \quad (6)$$

where the membrane concentration of lipids, $[PC]_{\mathrm{memb}}$, and the average area per lipid $A$ are

Table 1
p$K$ values and p$K$ shifts of stearic acid in egg-lecitin liposomes in the presence of CPZ at different concentrations

| [CPZ] (mM) | $[\mathrm{CPZ}]_{\mathrm{memb}}$ (mM) | p$K$ | $-\Delta$p$K$ |
|---|---|---|---|
| 0 | 0 | 7.7 | 0 |
| 0.02 | 9 | 5.6 | 2.1 |
| 0.04 | 16 | 5.4 | 2.3 |
| 0.08 | 31 | 5.1 | 2.6 |
| 0.16 | 54 | 4.7 | 3.0 |
| 0.32 | 93 | 4.4 | 3.3 |

*Note.* [CPZ] is the total concentration (mmol/l of the suspension); $[\mathrm{CPZ}]_{\mathrm{memb}}$ is the membrane concentration (mmol/l of the lipid phase).

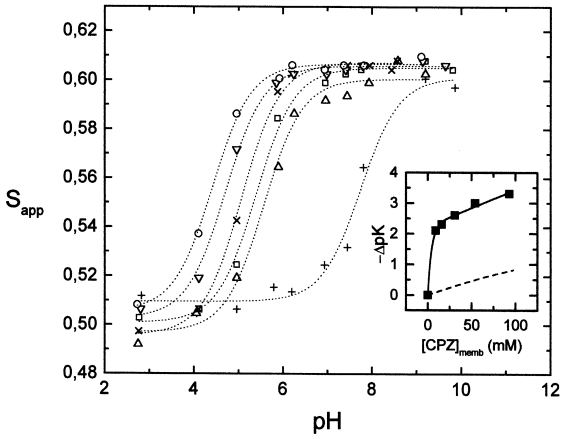


Fig. 5. Apparent order parameter, $S_{app}$, of the spin-labeled stearic acid 5-SASL obtained from the ESR measurements as a function of pH for different total CPZ concentrations: 0 mM (+); 1 mM (Δ); 2 mM (□); 4 mM (×); 8 mM (∇); 16 mM (○). Dashed lines were obtained with the Henderson–Hasselbach equation. Dispersion of $S_{app}$ values at low and high pH are due to different room temperatures (around 27°C) for each curve. Insert: p$K$ shift as a function of membrane CPZ concentration. The dashed line represents the theoretical calculation assuming uniform charge density according to Eq. (7).

taken as 1.3 M and 0.70 nm$^2$, respectively. After insertion of numerical values, $c = 20$ mM, $Z = 1$, $T = 300$ K, and substitution of Eq. (3) into Eq. (5), with $\sigma$ given by Eq. (6), one gets:

$$-\Delta \mathrm{p}K = 0.87 \sinh^{-1}(10.7[drug]_{\mathrm{memb}}) \qquad (7)$$

for $[drug]_{\mathrm{memb}}$, in mol/l. This expression is plotted in the insert of Fig. 5 (dashed line) for comparison with the experimental $\Delta$p$K$ values. Clearly a uniform distribution of anesthetic molecules cannot explain the p$K$ shifts. The initial slope of the experimental curve shows that there is a much higher increase in the electrostatic potential than that theoretically expected, indicating association of the drug with stearic acid molecules. On the other hand, the similar slopes observed in the insert of Fig. 5 for high concentration of the drug reveal that exceeding molecules tend to distribute uniformly.

The p$K$ shifts found in this work for low fractions of charged molecules in the membrane are of the same order of magnitude than those experimentally observed for membrane models with extremely high charge differences. For example, a p$K_a$ of 6.9 was found for myristic acid in DMPC bilayers [8] and of 8.0 in completely charged DMPG bilayers [17], resulting in a $\Delta \mathrm{p}K = 1.1$; dibucaine undergoes p$K$ shifts between 1.3 and 1.9 on going from neutral micelles to completely charged ones [9]. Thus, experimental findings also strengthen our proposal of preferential localization of the positively-charged drugs in the vicinity of stearic acid carboxyl groups at the membrane surface. The p$K$ of the stearic acid in the absence of the drug (Table 1) is somewhat higher than those found in the literature (6.9–7.3) [6–8]. Even taking the lower value (6.9), the resulting $\Delta$p$K$ would still be much higher than predicted by a uniform charge distribution.

## 4. Discussion

Membrane concentrations of local anesthetics relevant to anesthesia are of the order of 15 mmol/kg [15]. Anesthetic effects are frequently described in the literature for concentrations overcoming the physiologically significant value. In this work we report strong effects on carboxyl groups of stearic acids induced by membrane concentrations of anesthetics in the range of those inducing anesthesia.

The ionized form of the drugs causes changes in the ESR spectrum equivalent to increasing the pH. These changes are coincident for both drugs when plotted as a function of membrane drug concentration. The resulting intramembrane association constant to fatty acids is the same for both anesthetics. Its value 100 M$^{-1}$ indicates a preferential affinity with carboxyl groups at the polar headgroups level.

Anesthetics shift downwards the stearic acid ionization equilibrium. A uniform distribution of the drug in the membrane surface cannot account for the observed p$K$ shifts, implying in a specific interaction with the carboxyl groups.

Both anesthetics have substantially different apolar portions but show the same disturbing effect, implying that electrostatic interactions, and not hydrophobicity, control the binding to carboxyl groups. The hydrophobic character does

control the membrane partitioning which is related to anesthetic potency.

Local anesthetics block $Na^+$ channels. Hydrophobicity is, however, not enough to explain a higher affinity with channels than with the lipid portion of the membrane. The results of this work show that membrane-associated local anesthetics have a high affinity with the negative carboxyl groups at the membrane surface. This suggests that the anesthetic site of action involves a negatively-charged group accessible to drug molecules via the lipid phase of membranes. Carboxyl groups of negative residues located near the lipid headgroups at the lipid–protein interface of an ion channel can give the receptor site the required specificity.

Several biophysical results are consistent with a local anesthetic receptor site in the ion-conducting pore of the voltage-gated $Na^+$ channel [4,18,19]. The S6 membrane spanning segment in domain IV of rat brain $Na^+$ channels has been implicated in the binding of local anesthetics ([4] and refs. therein). The site proposed by Ragsdale et al. [4] is determined by residues Phe-1764 and Tyr-1771. Interestingly, there are conserved clusters of negatively-charged residues following the S6 segment. This region is assigned to the cytoplasmic side of the membrane and was proposed to be involved in the inactivation of the sodium channel [20]. The negative Glu at 1777 adjacent to the S6 segment is located at the level of lipid polar heads. This residue, which is adjacent to the site proposed by Ragsdale et al., provides an enhanced affinity site for charged anesthetic molecules.

The resting block of $Na^+$ channels by etidocaine has been described by a 1:1 binding relation with equilibrium association constant of $3.10^3$ $M^{-1}$ [4]. The affinity of resting $Na^+$ channel for lidocaine is approx. $2.10^3$ $M^{-1}$ [21]. Supposing that the intramembrane affinity of carboxyl is approximately the same for all tertiary amine local anesthetics and taking also $K_b^m = 10^2$ $M^{-1}$ for $Na^+$ channels, membrane-buffer partition coefficients ($K_b = K_p K_b^m$) of 30 and 20 are obtained for etidocaine and lidocaine, respectively. These are of the same order of magnitude as those estimated from the literature for etidocaine and dibucaine [22,23,12], showing that an interaction with carboxyl groups at the level of boundary lipid headgroups would be sufficiently strong to account for resting block. In contrast, this is not true for blocking of inactivated channels, described by an association constant two orders of magnitude greater [4].

More recently, Kuroda et al. [3] proposed that local anesthesia originates from the $\pi$-stacking interaction between the aromatic rings of the local anesthetic and of the Phe residue in the intracellular linker between domains III and IV of rat brain type IIA $Na^+$ channel, related to the inactivation gate. The presence of negative residues on both sides of the proposed sequence was suggested to facilitate the binding of local anesthetics. Our results suggest that if this linker penetrates the polar headgroup region of the membrane, then these negative residues could increase by two orders of magnitude the affinity with the site. This is, however, not enough to account for the association constant to inactivated channels. This point could be cleared up if energies involved in the proposed $\pi$-stacking interaction were known.

Local anesthetics also affect the function of $Ca^{2+}$-ATPase in sarcoplasmic reticulum, modifying the rate of $Ca^{2+}$ fluxes [24–26]. It has been shown that dibucaine molecules are at a shallow position in the membrane bilayer of sarcoplasmic reticulum vesicles [27]. The model for the $Ca^{2+}$-ATPase structure involves 10 transmembrane segments $M_1$–$M_{10}$ [28]. The short connection between segments $M_7$ and $M_8$ on the luminal face has seven negative and only two positive residues. This negatively-charged segment, adjacent to the transmembrane segments, is a good candidate for the local anesthetic binding site in this protein.

In all these cases, the candidate protein segments are surrounded by negative residues. The purely hydrophobic nature of a given segment will not be enough to explain a preferential association of the local anesthetic, which otherwise will equally prefer whatever hydrophobic region in the membrane. The enhanced affinity of carboxylic groups for local anesthetics shown in this work strongly supports domains of anionic

residues in the vicinity of hydrophobic regions as the action sites.

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