Biochimica et Biophysica Acta 939 (1988) 267–276
Elsevier

BBA 73932

Partitioning of local anesthetics into membranes: surface charge effects monitored by the phospholipid head-group

Anna Seelig, Peter R. Allegrini * and Joachim Seelig

Department of Biophysical Chemistry, Biocenter of the University of Basel, Basel (Switzerland)

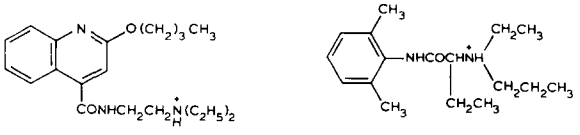
(Received 7 October 1987)

Key words: Dibucaine; Etidocaine; NMR, ^{2}H-; Anesthetic–membrane interaction

The binding of the charged form of two local anesthetics, dibucaine and etidocaine, to bilayers composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was measured simultaneously with ultraviolet spectroscopy and deuterium magnetic resonance. Because of their amphiphilic molecular structure, both drugs intercalate between the lipid molecules, increasing the surface area and imparting a positive electric charge onto the membrane. The ultraviolet (UV) binding isotherms were therefore analyzed in terms of a model which specifically took into account the bilayer expansion as well as the charge-induced concentration variations near the membrane surface. By formulating a quantitative expression for the change in surface area upon drug intercalation and combining it with the Gouy-Chapman theory, the binding of charged dibucaine and etidocaine to the lipid membrane was best described by a partition equilibrium, with surface partition coefficients of $660 \pm 80\ M^{-1}$ and $11 \pm 2\ M^{-1}$ for dibucaine and etidocaine, respectively (pH 5.5, 0.1 M NaCl/50 mM buffer). Deuterium magnetic resonance demonstrated further that the binding of drug changed the head-group conformation of the lipid molecules. Invoking the intercalation model, a linear variation of the deuterium quadrupole splittings of the choline segments with the surface charge density was observed, suggesting that the phosphocholine head-group may act as a 'molecular electrometer' with respect to surface charges.

Introduction

Most local anesthetics are tertiary amines which exist in both a neutral and a cationic form at physiological pH. Both forms are neurologically active, but their mode of action is still unclear [1–3]. Specific binding to membrane proteins as well as nonspecific adsorption to the lipid part of the membrane must be considered [4–7]. In the present work, we are concerned exclusively with the latter aspect. We have investigated the binding of the charged form of two commonly used local anesthetics, i.e., dibucaine (I) and etidocaine (II) (Scheme I), to phosphatidylcholine bilayers with UV spectroscopy and have monitored simultaneously the lipid head-group conformation with deuterium magnetic resonance.



I II

Scheme I.

* Present address: Ciba-Geigy AG, Basel, Switzerland.
Abbreviations: POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; UV, ultraviolet.

Correspondence: J. Seelig, Department of Biophysical Chemistry, Biocenter of the University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland.

Measurements of the electrophoretic mobility of phospholipid vesicles, which changes distinctly upon adsorption of charged local anesthetics, have led to different interpretations of the adsorption process. A partition equilibrium (unlimited incorporation of charged local anesthetics) [8] as well as a Langmuir adsorption isotherm (limited number of binding sites) [9] have been found to fit the experimental data. The latter model was also supported, at least qualitatively, by equilibrium dialysis [10]. In the present study, the binding isotherm could be measured over a larger concentration range than with either electrophoresis or equilibrium dialysis. The high concentration range was indeed essential to differentiate between the various modes of binding, since at low drug concentrations, all models eventually degenerate into a simple partition equilibrium.

The initial adsorption of local anesthetic to the membrane surface is followed by the penetration of drug into the membrane interior. The non-polar part of the local anesthetic will lie preferentially between the fatty acyl chains of the lipids, whereas the positive charge is in the vicinity of the lipid polar groups [4]. Hence, the adsorption of local anesthetics not only changes the surface charge but also causes a membrane expansion. This can be visualized most easily in monolayer experiments [11–14]. If a local anesthetic (either in the charged or the neutral form) is injected into the aqueous subphase of a lipid monolayer, an expansion of the monolayer to a new equilibrium value (at constant monolayer pressure) is observed. The intercalation of local anesthetics, therefore, pushes the lipid molecules apart and modifies the molecular details of lipid–lipid interaction. However, in spite of the generally accepted notion of membrane expansion, this model has not yet been incorporated into a quantitative interpretation of binding isotherms, presumably because the extent of drug incorporation was too low in previous studies. In contrast, quite high degrees of binding are reached in the present investigation, and a proper treatment of the binding isotherms is therefore required to quantitatively account for the increase in surface area.

A further point of interest is the change in lipid conformation upon drug binding. In particular, if the intercalation of local anesthetics leads to a reorientation of the lipid head-groups, strong electric dipole fields could result locally. Evidence for molecular changes in the lipid structure have indeed been obtained with ^{1}H- and ^{31}P-nuclear magnetic resonance [15–17], and, in particular, with deuterium NMR [18–20]. The observed variations in the NMR parameters were large for the lipid polar groups and small for the hydrocarbon chains, indicating that the local anesthetics sit closer to the head-groups than to the center of the hydrocarbon region. Furthermore, the charged form of the local anesthetic appeared to be more effective in changing the head-group conformation than the neutral form [18]. Likewise, the variation of the lipid head-group quadrupole splitting with pH was used to demonstrate that the pK of a local anesthetic at the membrane surface is identical with its value in solution [21].

In the present study, we investigate in more detail the molecular origin of the conformational change of the lipid head-group. By correlating the deuterium magnetic resonance spectra with the UV binding isotherms, it will be shown that the main driving force for the head-group reorientation is the electric charge density at the membrane surface, and that no specific chemical interactions need to be invoked.

Materials and Methods

Etidocaine · HCl was obtained from Astra Pharmaceutical Production AB (S-151 85 Södertälje, Sweden). Dibucaine · HCl was purchased from Sigma, (St. Louis, MO, U.S.A.). POPC was selectively deuterated at the α- and β-segments of the choline head-group, as described previously [22,23]. For simplification of the discussion, the two methylene segments of the choline head-group are denoted α and β:

$$-\mathrm{POCH_2CH_2\overset{+}{N}(CH_3)_3}.$$

$$\alpha \quad \beta$$

Determination of local anesthetic adsorption by UV absorption spectroscopy

In a typical experiment, about 20 mg (approx. 25 μmol) of deuterated POPC (the weight was

determined accurately) was suspended in 400 μl buffer (either 50 mM K_2HPO_4-KH_2PO_4 or borate-phosphate-citrate (BPC), (pH 5.5)) containing 0.1 M NaCl. A defined amount of local anesthetic was added from a stock solution prepared with the same buffer. In a second approach, the dry lipid was suspended in a defined amount of drug/buffer solution where the concentration of local anesthetic had been determined via UV spectroscopy. In order to achieve a homogeneous suspension, the sample was extensively vortexed at room temperature and subjected to several freeze-thaw cycles, with periodic vortexing at room temperature. The sample was then centrifuged at 25 °C and $30000 \times g$ for 30–60 min until a clear supernatant was obtained. A flat base-line at $\lambda \geqslant 380$ nm was used as a criterion for complete lipid removal. Lipid remaining in the supernatant led to light scattering which was detectable outside the local anesthetic absorption band. The clear supernatant was removed and was diluted to a concentration suitable for UV spectroscopy. Most measurements were performed in duplicate, and consistent results were obtained by this method of equilibration.

From the difference between the anesthetic content of the supernatant and that of the starting solution, it was possible to calculate the amount of anesthetic bound per mol of POPC, X_b (mol/mol). Only the excess amount of local anesthetic in the lipid pellet was measured by this approach. Drug trapped in the water phase of the lipid pellet did not affect the calculation as long as the drug concentration equalled that of the bulk phase. However, as will be discussed below, the drug concentration immediately adjacent to the lipid/water interface is always smaller than the bulk concentration. This results in a small underestimation of X_b, since the expulsion of local anesthetic from the interstitial water phase was not corrected for. The thickness of the boundary layer corresponds approximately to the Debye length (0.8 nm for 0.15 M NaCl), and from the concentration difference between the bulk solution and the membrane interface (cf. Tables I and II) it can be estimated that the error in X_b due to this effect is less than 1% for dibucaine and is 0.1% (10%) at the lowest (highest) concentration of etidocaine.

The concentration of etidocaine was determined by its absorption at 262 nm ($\varepsilon_{262} = 474$ $M^{-1} \cdot cm^{-1}$) and that of dibucaine by its absorption at 325 nm ($\varepsilon_{325} = 4144$ $M^{-1} \cdot cm^{-1}$). The absorption coefficients were determined in separate concentration series.

Deuterium NMR

All ^{2}H-NMR experiments were performed with coarse lipid dispersions. ^{2}H-NMR spectra were recorded at 46.1 MHz with the quadrupole echo technique [24]. The experimental conditions were the same as those described earlier [22]. The lipid pellets obtained from the UV binding assay were used without further manipulations. The observed quadrupole splitting could then be related to the amount of bound anesthetic, X_b, and to the equilibrium anesthetic concentration of the bulk solution, C_{eq}.

Results

The adsorption of dibucaine and etidocaine to multilamellar liposomes of POPC as measured with UV spectroscopy is summarized in Fig. 1. The degree of binding, X_b, which denotes the molar amount of drug adsorpbed per mol POPC, is plotted versus the equilibrium concentration, C_{eq}, of the local anesthetic. Note the different concentration scales for the two adsorption isotherms. Much lower concentrations of dibucaine than of etidocaine are needed to reach similar adsorption levels; obviously dibucaine (charged form) has a higher affinity towards POPC than etidocaine (charged form).

The cut-off of the adsorption isotherms has different origins for the two drugs. Etidocaine exhibits a rather limited solubility in water, even at pH 5.5; it was therefore not possible to measure at concentrations above 200 mM. For dibucaine, the limiting factor was the onset of 'micellization', which occurred for high loading of the membrane with dibucaine (at $X_b > 0.5$). Experimental evidence for micelle formation came from ^{2}H- and ^{31}P-NMR spectra of deuterated POPC. At low dibucaine concentrations ($C_{eq} < 10$ mM), the line shapes of both types of spectrum complied with theoretical bilayer spectra. Above $C_{eq} \simeq 10$ mM, a second, isotropic component was observed, which grew at the expense of the bilayer spectrum. The

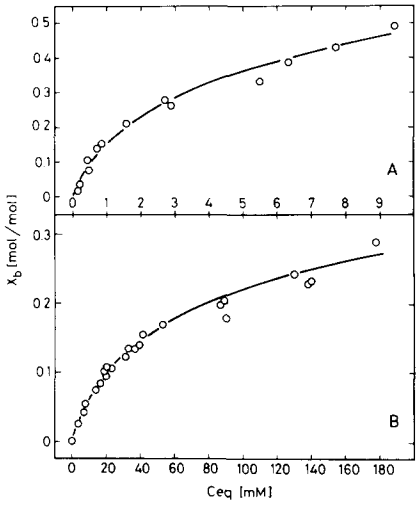


Fig. 1. Adsorption of charged local anesthetics to coarse liposomes composed of POPC. Adsorption isotherms measured with UV spectroscopy at 25 °C. Buffer composition: 0.1 M NaCl/50 mM phosphate buffer (pH 5.5). The degree of binding, X_b, denotes the molar amount of drug adsorbed per mol POPC. (A) Adsorption isotherm of dibucaine. (B) Adsorption isotherm of etidocaine.

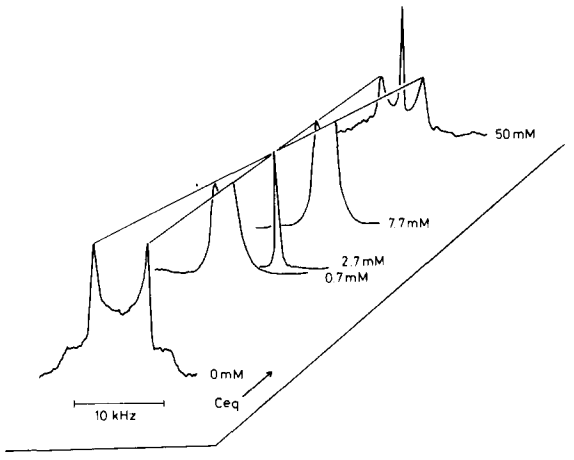


Fig. 2. Deuterium NMR spectra of aqueous dispersions of POPC as a function of dibucaine equilibrium concentration (25 °C, 0.1 M NaCl, BPC buffer (pH 5.5). The lipid was deuterated at the α-segment of the choline head-group.

non-bilayer phase was not further characterized. Hence, the term 'micellization' should be considered as simply indicating an isotropic motion of the lipids involved.

Selectively deuterated lipids were employed in the UV binding assay. It was therefore possible to measure simultaneously the ^{2}H-NMR spectra of the phosphocholine head-group and to correlate the observed spectral changes with the UV adsorption isotherm (for reviews on ^{2}H-NMR, cf. Refs. 25–28).

Fig. 2 summarizes a set of typical ^{2}H-NMR spectra obtained with coarse liposomes composed of POPC, deuterated at the α-choline segment ([α-C^{2}H$_2$]POPC). As the concentration of dibucaine is gradually increased, the ^{2}H-NMR spectra change in a systematic manner. First, we note that all ^{2}H-NMR spectra indicate a single, time-averaged head-group conformation at each dibucaine concentration. Separate signals for pure lipid and lipid interacting with dibucaine could not be observed, in agreement with previous ^{2}H-NMR studies on other local anesthetics [18,19]. These results suggest a rapid exchange of drug in and out of the bilayer as well as a fast in-plane translational diffusion. (Two quadrupole splittings were observed in mixtures of dibucaine and membranes composed of 1,2-dipalmitoyl-*sn*-glycerophosphocholine [20]; this result is probably due to imperfect mixing). Secondly, the deuterium quadrupole splitting, $\Delta\nu_\alpha$, which is defined as the separation of the two most intense peaks in the spectrum, is found to change dramatically. In the absence of dibucaine the residual quadrupole splitting was 6.0 kHz; upon addition of drug $\Delta\nu_\alpha$ decreased, collapsed to a single sharp resonance, and reappeared with reversed sign. (The assignment of a positive $\Delta\nu_\alpha$ to unperturbed POPC bilayers is arbitrary, since the sign of the quadrupole splitting cannot be determined from the usual ^{2}H-NMR spectra.) The numerical evaluation of the quadrupole splittings is summarized in Table I. A plot of $\Delta\nu_\alpha$ vs. C_{eq} parallels the UV adsorption isotherm (not shown). However, more interesting is the correlation between $\Delta\nu_\alpha$ and the amount of membrane-adsorbed dibucaine, X_b, as shown in Fig. 3A, since a linear relationship is observed up to $X_b \simeq 0.15$. The deviation from linearity (dashed line) at higher levels of dibucaine adsorption can be explained by a bilayer expansion due to drug intercalation, as discussed below.

Qualitatively similar ^{2}H-NMR spectra were obtained with etidocaine. For this molecule, the variations of both the α- and the β-choline head-group segment were measured. In parallel to dibucaine,

TABLE I

BINDING OF DIBUCAINE TO BILAYER MEMBRANES COMPOSED OF [α-C^2H_2]POPC (25 °C, 0.1 M NaCl, 50 mM PHOSPHATE OR BPC, pH 5.5)

C_{eq} (mM)	X_b [a,b] (mol/mol)	X_b^* [c] (mol/mol)	$\Delta\nu_\alpha$ [d] (kHz)	σ [e] (mC/m^2)	ψ_0 [f] (mV)	C_M [h] (μM)	X_b/C_M (M^{-1})
0.16	0.021	0.021	5.55	4.83	5.4	130	162
0.22	0.039	0.038	5.12	8.82	9.8	150	260
0.49	0.076	0.071	3.78	16.6	18.1	242	314
0.42	0.109	0.098	3.07	23.1	25.0	159	686
0.74	0.142	0.124	2.30	29.2	30.9 [g]	222	640
0.89	0.158	0.136	1.93	32.1	33.0 [g]	246	642
1.57	0.218	0.179	1.20	42.1	41.9	307	710
2.70	0.278	0.218	0	51.1	51.6 [g]	361	770
2.89	0.264	0.209	0	49.1	47.4	456	579
5.50	0.329	0.248	−1.37	58.2	56.5 [g]	608	541
6.34	0.389	0.280	−2.0	65.8	58.5	649	599
7.71	0.437	0.304	−2.19	71.5	65.0 [g]	612	714
9.39	0.494	0.331	−2.62	77.7	68.3 [g]	656	753

[a] Measured by UV absorption spectroscopy.
[b] Mol bound dibucaine per mol POPC.
[c] Calculated according to $X_b^* = X_b/(1+X_b)$.
[d] Measured by ^{2}H-NMR.
[e] Calculated according to $\sigma = (e_0/A_L)X_b^*$ with $A_L = 68$ Å^2.
[f] Calculated by means of Gouy-Chapman theory.
[g] 0.1 M NaCl, 30 mM Tris-HCl.
[h] Calculated according to $C_M = C_{eq}\exp(-\psi_0 F_0/RT)$.

TABLE II

BINDING OF ETIDOCAINE TO POPC BILAYER MEMBRANES (25 °C, 0.1 m NaCl, 50 mM PHOSPHATE OR BPC, pH 5.5)

C_{eq} (mM)	X_b (mol/mol)	X_b^* (mol/mol)	$\Delta\nu_\alpha$ (kHz)	σ (mC/m^2)	ψ_0 (mV)	C_M (mM)	X_b/C_M (M^{-1})
[α-C^2H_2]POPC							
3.3	0.026	0.025	5.26	5.96	6.4	2.57	10.1
6.6	0.044	0.042	4.47	9.9	10.6	4.4	10.1
7.5	0.055	0.052	4.05	12.3	13.1	4.5	12.1
14.9	0.075	0.070	3.08	16.4	17.3	7.6	9.9
16.3	0.085	0.078	2.96	18.4	19.2	7.7	11.0
18.8	0.098	0.089	2.50	21.0	21.4	8.2	12.0
22.3	0.108	0.098	2.41	22.9	23.2	9.0	12.0
30.3	0.124	0.110	1.71	25.9	25.3	11.3	11.0
32.9	0.138	0.121	1.41	28.5	27.7	11.2	12.3
37.1	0.135	0.119	1.37	28.0	26.9	13.0	10.4
39.2	0.142	0.124	1.20	29.2	27.8	13.3	10.7
53.3	0.170	0.145	0.20	34.1	30.9	16.0	10.6
87.0	0.167	0.143	0	33.6	28.5	28.6	5.8
91.4	0.177	0.150	0	35.3	29.6	29.0	6.0
131.5	0.240	0.193	−1.98	45.5	34.5	34.3	6.9
177.8	0.288	0.224	−2.16	52.5	36.6	42.8	6.7
[β-C^2H_2]POPC							
0			5.5				
19.8	0.112	0.101	7.28	23.7	24.0	7.8	14.4
18.4	0.104	0.094	7.18	22.1	22.5	7.7	13.6
41.1	0.156	0.135	7.86	31.7	29.7	12.9	12.1
88.2	0.204	0.169	8.74	39.8	33	24.4	8.4
87.0	0.198	0.165	8.79	38.8	32.3	24.7	8.01
138.0	0.230	0.187	9.28	43.9	33.1	38.0	6.05
138.7	0.234	0.190	9.23	44.6	36.6	33.3	7.03

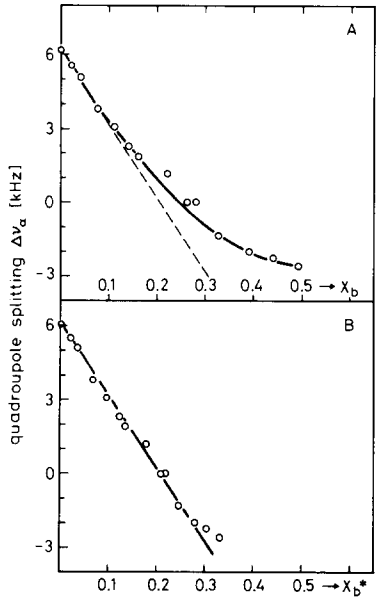


Fig. 3. Conformational change of the phosphocholine head group upon intercalation of dibucaine (charged form). POPC was deuterated at the α-segment of the choline moiety. (A) Variation of the deuterium quadrupole splitting with the mol fraction of adsorbed dibucaine, X_b (mol dibucaine/mol POPC). The dashed line indicates the initial slope. (B) Plot of the same set of quadrupole splittings as (A) against $X_b^* = X_b/(1 + X_b)$, which is proportional to the membrane surface charge density, σ. The slope is identical to the initial slope of (A).

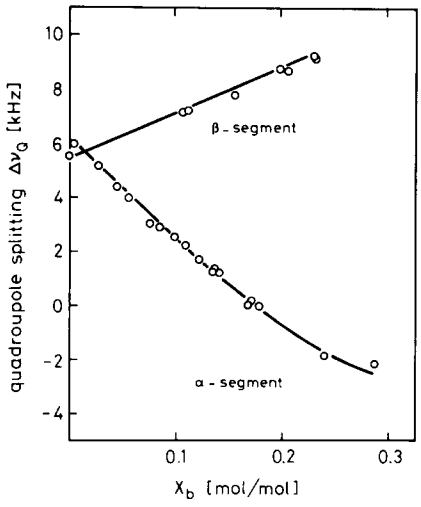


Fig. 4. Etidocaine adsorption to membranes composed of POPC. Variation of the quadrupole splittings of the α- and β-choline segment with X_b, the molar amount of bound dibucaine (pH 5.5).

the α-splitting of POPC decreased with increasing concentrations of etidocaine, whereas the β-splitting showed the opposite behavior and increased with increasing drug concentration. This counter-directional change of the α- and β-splitting has also been observed for the adsorption of charged tetracaine [18], for the binding of multivalent metal ions [29,30], and for the mixing of charged amphiphiles [31], providing evidence for a conformational change of the choline head-group conformation. An increase in the oscillatory fluctuations of the choline head-group can be excluded, since these would induce a simultaneous decrease of both the α- and the β-splitting. The ^{2}H-NMR quadrupole splittings for etidocaine adsorption are summarized in Table II, while the variations of the α- and β-quadrupole splittings with X_b are shown in Fig. 4. Again, an approximately linear relationship between $\Delta\nu_Q$ and X_b is observed. However, a closer inspection of the data reveals the same systematic deviations from linearity as noted for dibucaine.

Discussion

Intercalation of charged local anesthetics into lipid bilayers

In monolayer studies, dibucaine and etidocaine were found to penetrate into a POPC monolayer up to a surface pressure of 39 mN/m, leading to an area increase of the monolayer [14]. In the same series to experiments, a monolayer-bilayer equivalence pressure of 32 mN/m was established. The bilayer equivalence pressure is clearly below the critical cut-off pressure for drug penetration, and suggests that the binding of local anesthetics to POPC bilayers is accompanied by the intercalation of drug between the lipid molecules.

Additional evidence for an intercalation mechanism comes from ^{2}H-NMR, i.e., from the non-linearity of the $\Delta\nu_Q$ vs. X_b curves at large values of X_b. For a quantitative analysis, we first calculate the increase of the bilayer area as well as the surface charge density resulting from the intercalation of positively charged drug molecules. If n_D drug molecules of area A_D penetrate into a bilayer composed of n_L lipid molecules of area A_L,

the total surface area, A_T, is given by

$$A_T = n_D A_D + n_L A_L \quad (1)$$

At pH 5.5, each drug molecule carries a positive electric charge, e_0, while the lipid molecules are electrically neutral. The surface charge density of the POPC/dibucaine membrane is therefore given by

$$\sigma = n_D e_0 / A_T \quad (2)$$

$$\sigma = (e_0 X_b / A_L)/(1 + X_b(A_D/A_L)) \quad (3)$$

where $X_b = (n_D/n_L)$ is the degree of binding as measured with UV spectroscopy. The surface area of POPC in lipid bilayers is $A_L \approx 68$ Å^2 [30]. The surface area of amphiphilic drugs can only be approximated from inspection of molecular models. A_D is estimated as 45–68 Å^2, both for dibucaine and etidocaine. Numerical calculations show that, within this range of areas, the exact value of A_D is not critical in the present context. For simplification of the discussion, we hence choose $A_D = A_L$ which reduces Eqn. 3 to the simpler expression

$$\sigma = (e_0 X_b / A_L)/(1 + X_b) \quad (4)$$

We have recently summarized evidence that certain phospholipid head-groups may act as 'molecular electrometers', i.e., these head-groups undergo a conformational change dependent on the sign and the size of the membrane surface charge [31]. The variation of the head group conformation is reflected in quite dramatic changes of the head-group quadrupole splittings; in particular, the deuterium quadrupole splittings of the α- and β-choline segment were found to vary linearly with the surface charge density, σ, up to $\sigma \approx 0.3 e_0$/lipid. Applied to the problem of drug intercalation, this hypothesis predicts that a plot of $\Delta\nu_Q$ vs. σ should yield a better linearization than a plot of $\Delta\nu_Q$ vs. X_b. This is indeed borne out by the experimental results as demonstrated by Fig. 3B, where $\Delta\nu_\alpha$ was plotted vs. $X_b^* = X_b/(1 + X_b)$. Linear regression analysis yields

$$\Delta\nu_\alpha = 6.03 - 28.8 X_b^* \text{ (kHz)} \quad (5)$$

(correlation coefficient $R = 0.997$). For low values of X_b, the differences between X_b and X_b^* are small. Therefore, the influence of intercalation becomes most pronounced at high loading of the membrane with drug.

The effect of dibucaine intercalation is to expand the bilayer surface and to reduce the surface charge density. The maximum surface charge density which can be reached without destabilization of the POPC bilayer is about $0.3 e_0/68$ Å^2 ($\simeq 70$ mC/m^2) and, consistent with the results obtained for metal ions and other charged molecules [31], $\Delta\nu_\alpha$ of dibucaine varies linearly with the charge density in this interval.

At charge densities of $\sigma > 70$ mC/m^2, the lateral electrostatic repulsion forces between the dibucaine molecules become rather large. Therefore, the incremental area increase per inserted dibucaine molecule is no longer constant (as assumed in Eqn. 4) but increases gradually. This is indicated in Fig. 3B by the deviation from linearity at the largest X_b values. Eventually, this process leads to a micellization of the membrane.

The intercalation model has also been applied to etidocaine adsorption. Again, plots of $\Delta\nu_\alpha$ and $\Delta\nu_\beta$ vs. X_b^* (not shown) yield an improved linear fit to the data with

$$\Delta\nu_\alpha = 6.15 - 40.3 X_b^* \text{ (kHz)} \quad (6)$$

$$\Delta\nu_\beta = 5.4 + 19.8 X_b^* \text{ (kHz)} \quad (7)$$

(correlation coefficients of linear regression analysis better than 0.995 in both cases).

Elimination of X_b^* from eqns., 6 and 7 for etidocaine leads to

$$\Delta\nu_\beta = -0.49\,\Delta\nu_\alpha + 8.42 \text{ kHz} \quad (8)$$

This relationship demonstrates that (1) the quadrupole splittings of the α- and β-segment are linearly correlated over the total concentration range investigated, (2) the change of the α-segment (which is close to the negative phosphate group) induced by positively charged etidocaine is twice as large as that of the β-segment and (3) α- and β-quadrupole splittings vary in opposite directions.

Results analogous to Eqn. 8 have been obtained previously for the binding of Ca^{2+} to POPC bilayers ($\Delta\nu_\beta = -0.49\,\Delta\nu_\alpha + 7.6$ kHz [30]), for the

binding of various multivalent metal ions (Ca^{2+}, Mg^{2+}, Cd^{2+}, La^{3+}) to bilayers of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine ($\Delta\nu_\beta = -0.43\ \Delta\nu_\alpha + 6.7$ kHz [29]) and for the adsorption of dibucaine to the latter lipid ($\Delta\nu_\beta = -0.53\ \Delta\nu_\alpha + 7.3$ kHz; calculated from Fig. 3 of Ref. 20). Qualitatively, the same conformational change of the phosphocholine head-group is therefore caused by either the adsorption of etidocaine or dibucaine or by the binding of multivalent metal ions, even though the former intercalate between the lipid head-groups while the latter remain superficially adsorbed on the membrane surface. This finding lends additional support to the idea that the phosphocholine dipole responds essentially to the membrane surface charge and is not involved in specific chemical reactions.

The chemical nature of the membrane-bound ligand enters, however, indirectly, since it modulates the distance between the plane of phosphocholine dipoles and that of maximum charge density. Considering a hypothetical situation, where 1 mol of etidocaine, dibucaine or Ca^{2+} is adsorbed/bound to a POPC membrane, the quadrupole splittings of the α-segment would be reduced by 40.3 kHz (Eqn. 6), 28.8 kHz (Eqn. 5) or 20.5 kHz [30], respectively. Hence, etidocaine and dibucaine, which carry only one positive charge, bring about a larger conformational change than divalent Ca^{2+}. This may be explained by the hydrophobicity of dibucaine and etidocaine, which moves these molecules in closer vincinity to the phosphocholine dipoles than is possible for the ionic Ca^{2+}.

The orientation of the unperturbed choline dipoles is approximately parallel to the membrane surface [31]. The molecular details of the conformational changes induced upon anesthetic intercalation are not known. By comparison with related studies on metal ion binding, it can be concluded, however, that a change in the tilt angle by $\pm 15°$ would be sufficient to generate the observed variations of the quadrupole splitting [29].

Analysis of the adsorption isotherms.

Using Eqn. 3 (with $A_L = 68$ Å^2 for POPC), we can calculate the surface charge density σ from the experimentally determined X_b values. The surface charge density σ, in turn, generates a surface potential ψ_0, which may be approximated by means of the Gouy-Chapman theory [32]:

$$\sigma = \left(2000\epsilon_r\epsilon_0 RT\sum_i C_{i,eq}(e^{-z_i F_0\psi_0/(RT)} - 1)\right)^{1/2} \quad (9)$$

where $\epsilon_r = 78$ is the dielectric constant of water (at 25 °C), ϵ_0 the permittivity of free space, R the gas constant, F_0 the Faraday constant, $C_{i,eq}$ the concentration of the ith electrolyte in the bulk aqueous phase (in moles per liter), and z_i the signed charge of the ith species. The effect of the surface potential is to repel ions of like charge and to increase the concentration of ions of opposite charge at the membrane surface. The concentration of charged local anesthetic, C_M, at the plane of ion binding, i.e., in the solution immediately adjacent to the membrane surface, is related to its equilibrium concentration, C_{eq}, in bulk solution, according to the Boltzmann equation:

$$C_M = C_{eq}\exp(-\psi_0 F_0/RT) \quad (10)$$

Numerical data for σ, ψ_0, and C_M are summarized in Tables I and II for dibucaine and etidocaine, respectively. Having determined the amount of bound local anesthetic as well as its concentration immediately adjacent to the membrane interface, various models of drug adsorption may be tested, such as a partition equilibrium

$$X_b/C_M = K_p \quad (11)$$

or a Langmuir adsorption isotherm

$$X_b/C_M = K_L(1 - X_b) \quad (12)$$

In the former, the ratio X_b/C_M is independent of the degree of binding, X_b, in the latter it decreases with X_b, exhibiting a slope of $m = -1$. Employing a more general formulation of the Langmuir adsorption isotherm where one drug binds to n lipids, i.e., $(X_b/C_M) = K_L(1 - nX_b)$, the variation of (X_b/C_M) with X_b should be even more pronounced.

In the last columns of Tables I and II, we have calculated, therefore, the ratio X_b/C_M for dibucaine and etidocaine, respectively. Considering the etidocaine data first, we note an almost constant ratio $X_b/C_M \simeq 11$ M^{-1} in the concentration range $0 \leqslant C_{eq} \leqslant 60$ mM. This implies that, for

physiologically relevant concentrations of etidocaine, the binding to POPC membranes can best be described by a simple partition equilibrium with K_p (etidocaine) = 11 ± 2 M^{-1}. At higher concentrations, etidocaine adsorption follows a different mechanism which was not further investigated due to the limited solubility of etidocaine in water.

A similar conclusion holds true for dibucaine adsorption. Except at very low concentrations (where the experimental error is large), the X_b/C_M ratios are constant within $\pm$ 20%. Hence, dibucaine adsorption to POPC membranes can also be described by a partition equilibrium with K_p (dibucaine) = 660 ± 80 M^{-1}. The partition coefficient as defined by Eqn. 11 must be considered as a surface partition coefficient. If C_M is assumed to be 1 M, then K_p denotes the molar amount of drug adsorbed per mole POPC surface.

The adsorption of dibucaine at pH 6.0 to vesicles composed of egg phosphatidylcholine has been investigated by electrophoretic measurements [8]. A numerical analysis by means of the Gouy-Chapman theory was reported for four different concentrations in the range of 0.1–3 mM dibucaine. A surface partition equilibrium with a partition coefficient of $K_{pc} = 9.4 \pm 0.5$ $(M \cdot Å^2)^{-1}$ (Table I of Ref. 8) was found to yield the best fit to the experimental data. Multiplication by 68 $Å^2$, the average area per lipid molecule, converts K_{pc} into the molar partition coefficient, K_p, as defined by Eqn. 11, yielding $K_p = 640 \pm 34$ M^{-1}. This result is in excellent agreement with the present values derived by UV and ^{2}H-NMR spectroscopy.

In contrast, equilibrium dialysis data of dibucaine with vesicles of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine were analysed in terms of the Langmuir adsorption isotherm, since apparently saturating binding profiles were found. At pH 5.0, the association constant was 10^3 M^{-1} and the number of phospholipids comprising a binding site was about 3.5, taking into account surface potential effects by the Gouy-Chapman theory [10]. Compared to the present data, the extent of dibucaine binding to 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine vesicles was almost a factor of 2 smaller at large dibucaine concentrations. In addition, the divergence between the theoretical model and the experimental data was considerable in Ref. 10.

The application of a Langmuir-type adsorption model appears to be justified for ligands which bind exclusively to the membrane surface. Under these conditions, the constant surface area limits the maximum amount of bound ligands. In fact, the surface binding of multivalent metal ions to zwitterionic and negatively charged membrane surfaces is best described by the Langmuir adsorption isotherm assuming a 1 metal ion: 1 lipid stoichiometry. In contrast, dibucaine and etidocaine are accommodated between the lipid molecules. Since the drugs are an integral part of the bilayer, almost unlimited swelling is conceivable until the bilayer converts into a non-bilayer phase. Hence, the thermodynamic result of a partition equilibrium is consistent with the molecular details of lipid–anesthetic interaction. However, a constant partition coefficient is only obtained via the application of the Gouy-Chapman theory. If, instead of the interfacial concentration C_M, the bulk equilibrium concentration C_{eq} is entered into Eqn. 11, the partition coefficients for etidocaine and dibucaine vary by a factor of 4 in the same concentration interval.

It has been argued that the intrinsic pK values of drug molecules when bound to lipid are considerably different from pK values measured in solution [9]. On the other hand, using ^{2}H-NMR, it has been possible to determine directly the ionization state of a local anesthetic at the membrane surface, and no significant pK shift was observed [21]. A consistent interpretation of the present data is possible only if a full ionization of the drug molecules in the lipid/water interface is assumed, in agreement with conductance [7] and electrophoretic [8] measurements.

Conclusions

Local anesthesia is a multifaceted problem, and only one physicochemical aspect, the interaction of the cationic form of local anesthetics with the lipid part of the membrane, has been addressed in the present study. Since the pK values of the two drugs investigated are about p$K \approx 8$ (etodicaine 7.7 (Ref. 1, p. 46); dibucaine 8.0 [8] or 8.85 [10] the cationic form predominates (over 80%) at physiological pH. Leaving aside specific effects on the membrane proteins, the adsorption of even moderate amounts of local anesthetic has three

immediate physicochemical and probably functional consequences for the membrane surface. First, the membrane expansion may alter the membrane permeability [35]. Secondly, drug adsorption increases the membrane surface potential by some 20 mV, which, in turn, will reduce the concentration of Ca^{2+} at the membrane surface by about a factor of 5. Only by drastically increasing the Ca^{2+} concentration in the medium would it be possible to return to the original Ca^{2+} interfacial concentration. This would explain the observed antagonistic action of high Ca^{2+} levels in local anesthesia. The actual site of Ca^{2+} action is, however, at the protein part of the membrane. Thirdly, drug intercalation changes the phospholipid head-group conformation and necessarily modifies the electric dipole field in the immediate vicinity of the phosphocholine dipoles. The variations of the local dipole fields are of the order of 50–100 mV [31], which would be sufficient to trigger conformational transitions in proteins. In summary, we conclude that (1) adsorption of dibucaine and etidocaine (charged form) to electrically neutral POPC bilayers in the experimentally accessible concentration range is best described by a surface partition equilibrium; (2) the partion equilibrium constant for dibucaine (660 M^{-1}) is larger than for etidocaine (11 M^{-1}); (3) membrane expansion and surface potential effect must be taken into account for a proper analysis of the adsorption isotherm; (4) the intercalation of drug changes the conformation of the phosphocholine group; and (5) the deuterium quadrupole splittings vary linearly with the electric charge density at the membrane surface.

Acknowledgement

This work was supported by the Swiss National Science Foundation Grant 3.521.86. We are indebted to P. Ganz and H. Stücheli for the competent synthesis of deuterated lipids.

References

1 Seeman, P. (1972) Pharmacol. Rev. 24, 583–655.
2 Covino, B.G. and Vassallo, H.G. (1976) Local Anesthetics, Mechanism of Action and Clinical Use, Grune & Stratton, New York.
3 Hille, B. (1984) Ionic Channels of Excitable Membranes, Sinauer Ass., Sunderland, MA.
4 Blaustein, M.P. and Goldman, D.E. (1966) Science 153, 429–432.
5 Seeman, P. (1975) Molecular Mechanisms of Anesthesia (Fink, B.R., ed.), Progress in Anesthesiology, Vol. 1, 243–251, Raven Press, New York.
6 Franks, N.P. and Lieb, W.R. (1984) Nature 310, 599–601.
7 McLaughlin, S. (1975) Molecular Mechanisms of Anesthesia (Fink, B.R., ed.) Progress in Anesthesiology, Vol. 1, 193–221, Raven Press, New York.
8 Ohki, S. (1984) Biochim. Biophys. Acta 777, 56–66.
9 Rooney, E.K. and Lee, A.G. (1983) Biochim. Biophys. Acta 732, 428–440.
10 Eftink, M.R., Puri, R.K. and Ghahramani, M.D. (1985) Biochim. Biophys. Acta 813, 137–140.
11 Skou, J.C. (1954) Acta Pharmacol. Toxicol. 10, 325–337.
12 Skou, J.C. (1958) Biochim. Biophys. Acta 30, 625–629.
13 Clements, J.A. and Wilson, K.M. (1962) Proc. Natl. Acad. Sci. USA 48, 1008–1014.
14 Seelig, A. (1987) Biochim. Biophys. Acta 899, 196–204.
15 Westman, J., Eriksson, L.E.G. and Ehrenberg, A. (1984) Biophys. Chem. 19, 57–68.
16 Siminovitch, D.J., Brown, M.F. and Jeffrey, K.R. (1984) Biochemistry 23, 2412–2420.
17 Eriksson, G.L.E. (1987) Biophys. Chem. 26, 9–18.
18 Boulanger, Y., Schreier, S. and Smith, I.C.P. (1981) Biochemistry 20, 6824–6830.
19 Kelusky, E.C. and Smith, I.C.P. (1984) Mol. Pharmacol. 26, 314–321.
20 Browning, J.L. and Akutsu, H. (1982) Biochim. Biophys. Acta 684, 172–178.
21 Watts, A. and Poile, T.W. (1986) Biochim. Biophys. Acta 861, 368–372.
22 Tamm, L.K. and Seelig, J. (1983) Biochemistry 22, 1474–1483.
23 Harbison, G.S. and Griffin, R.G. (1984) J. Lipid Res. 25, 1140–1142.
24 Davis, J.H., Jeffrey, K.R., Bloom, M., Valic, M.I. and Higgs, T.P. (1976) Chem. Phys. Lett. 42, 390–394.
25 Seelig, J. (1977) Q. Rev. Biophys. 10, 353–418.
26 Griffin, R.G. (1981) Methods Enzymol. 72, 108–174.
27 Davis, J.H. (1983) Biochim. Biophys. Acta 737, 117–172.
28 Seelig, J.and Macdonald, P.M. (1987) Acc. Chem. Res. 20, 221–228.
29 Akutsu, H. and Seelig, J. (1981) Biochemistry 20, 7366–7373.
30 Altenbach, Ch. and Seelig. J. (1984) Biochemistry 23, 3913–3920.
31 Seelig, J., Macdonald, P.M. and Scherer, P.G. (1987) Biochemistry 26, 7535–7541.
32 Aveyard, R. and Haydon, D.A. (1973) An Introduction to the Principles of Surface Chemistry, University Press, Cambridge.
33 Macdonald, P.M. and Seelig, J. (1987) Biochemistry 26, 1231–1240.
34 Macdonald, P.M. and Seelig, J. (1987) Biochemistry 26, 6292–6298.
35 Escudero, B. and Gutiérrez-Merino, C. (1987) Biochim. Biophys. Acta 902, 374–384.