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Interfacial ionization and partitioning of membrane-bound local anaesthetics

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Consideration of the interfacial protonation equilibria of membrane-associated amphiphiles indicates that the partition coefficients of the protonated and unprotonated species will differ considerably. The partition coefficients of the charged and uncharged forms of spin-labelled myristic acid in dimyristoylphosphatidylcholine bilayer dispersions have been measured by EPR spectroscopy and found to be approximately 140-fold higher for the protonated acid than for the dissociated salt form. This ratio of partition coefficients is found to be in good agreement with that predicted from the interfacial shift in pK_a of the fatty acid on its partitioning into the membrane. The latter was determined from the changes in the EPR spectra of the membrane-associated fatty acid with pH and was found to be +2.1 pH units. The interfacial shifts in pK_a for a series of spin-labelled analogues of tertiary amine local anaesthetics have been determined from the pH dependence of the partition coefficients in dimyristoylphosphatidylcholine bilayer dispersions and are found mostly to be in the range of approx. −1.0 to −1.5 pH units, corresponding to a 10- to 30-fold higher partition coefficient of the uncharged base compared with that of the charged ammonium form.

Introduction

The ionization state of membrane-associated local anaesthetics will affect both their transmembrane transport [1] and their physiological activity [2,3]. The membrane concentrations of local anaesthetic, which also determine activity, are additionally specified by the partition coefficients of the protonated and dissociated forms [4,5]. Since the differences in partitioning equilibria for the charged and uncharged species are controlled by the same thermodynamic factors as those for the interfacial ionization equilibria, the two are expected to be directly related. It is the purpose of this paper to demonstrate unambiguously that such a relation does hold for one particular class of local anaesthetic molecules and hence to demonstrate the generality of these principles for local anaesthetic–membrane interactions.

Interfacial ionization equilibria have been analysed in detail by Fernández and Fromherz [6]. The pK_a of any protonatable group at a membrane interface, pK_a^i, will differ from the intrinsic value, pK_a^0, for the amphiphile in water because of thermodynamic differences in the ionization equilibria at the two locations. For charged membranes, an additional shift in pK_a arises because the interfacial proton concentration is different from that in the bulk aqueous phase. In general, the interfacial pK_a is given by [7]:

$$pK_a^i = pK_a^0 + \Delta pK_a^{el} \pm |\Delta pK_a^{pol}| \quad (1)$$

where $\Delta pK_a^{el} = -e\Phi/(\ln 10 \cdot kT)$ is the electrostatic shift which is determined by the surface potential, Φ,

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Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; *n*-MASL, *n*-(4,4-dimethyloxazolidine-*N*-oxyl)myristic acid; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance.

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of the membrane. The polarity-induced shift, ΔpK_a^{pol}, accounts for the intrinsic difference in ionization equilibria and takes the positive sign for dissociation of a molecular acid ($HA \rightleftharpoons H^+ + A^-$) and the negative sign for the dissociation of a cationic acid ($HB^+ \rightleftharpoons H^+ + B$), since the polarity at the interface is lower than that in bulk water. This shift has been related theoretically to the hydration of the amphiphile at the interface [7]. Interfacial shifts in pK_a also have been demonstrated experimentally by direct determination of the pK_a for tetracaine bound to micelles [8]. In general, the pK_a of the membrane-associated local anaesthetic will differ by one pH unit or more from that in bulk water.

The connection between the difference in partitioning of the charged and uncharged species and the shift in pK_a on association with the membrane has been taken into account in the analysis of several studies on the pH dependence of the membrane partitioning of local anaesthetic molecules [9–11]. The effects of such shifts on the *apparent* pK_a as deduced solely from partitioning data, with the consequence that this quantity is dependent on the membrane concentration, have also been emphasized [12]. In certain cases, the results of such studies have been equivocal in that shifts in pK_a have been detected for certain local anaesthetics but not for others [10]. This result is unexpected in view of the quite general nature of the considerations concerning interfacial ionization equilibria that are given above. A direct, critical demonstration of the connection between the difference in partition coefficients and the interfacial shift in pK_a has so far been lacking.

Fatty acids are known to have a local anaesthetic function as non-competitive blockers of the acetylcholine receptor ion channel [13,14]. A spin-labelled fatty acid derivative has also been demonstrated to have local anaesthetic activity [15]. In the present work we have determined the partition coefficients of the charged and uncharged forms of a spin-labelled fatty acid at low concentrations in neutral bilayer model membranes. The interfacial pK_a of the membrane-associated form of this molecule has also been determined directly from the electron paramagnetic resonance (EPR) spectra. The 140-fold greater partition coefficient found for the uncharged form of the fatty acid compared with the dissociated form correlates quantitatively with the large upward shift observed in the pK_a of the fatty acid on association with the membrane. Additionally, the interfacial shifts in pK_a of spin-labelled analogues of tertiary amine local anaesthetics, which are known to associate with acetylcholine receptor-rich membranes [16] and to have local anaesthetic potency [17], have been determined from the pH dependence of the partitioning. The shifts in pK_a are all found to be similar to those expected from the lower polarity at the membrane surface.

Materials and Methods

Dimyristoylphosphatidylcholine (DMPC) was obtained from Fluka (Buchs, Switzerland). Spin-labelled myristic acids (*n*-MASL) were synthesized according to the methods of Hubbell and McConnell [18]. The spin-labelled local anaesthetic analogues (see Fig. 1) were synthesized as described in Hideg et al. [19] or by analogous methods.

For sample preparation, 0.5 mol% of the spin-labelled analogue was codissolved with the required amount of DMPC in dichloromethane. The solution was dried in a water bath at 45 °C for 15 min and then under vacuum overnight. The dry lipid film was hydrated in 50 μl of 10 mM buffer, dispersed at 45 °C by vortex mixing and then bath sonicated for 10 min. Buffers used were acetate (pH < 5), phosphate (5 ≤ pH ≤ 8) and borate (pH > 8), and all contained 0.1 mM EDTA. The pH of the dispersion was then measured and a 10 μl aliquot transferred to a 1 mm o.d. glass capillary for EPR measurement. Where required, the lipid concentrations were checked by phosphate analysis [26].

EPR spectra were recorded on a Varian E-Line 9 GHz spectrometer equipped with nitrogen gas flow temperature regulation. Sample capillaries were accommodated in standard 4 mm quartz EPR tubes which contained light silicone oil for thermal stability. Temperature was measured with a fine-wire thermocouple positioned within the silicone oil at the top of

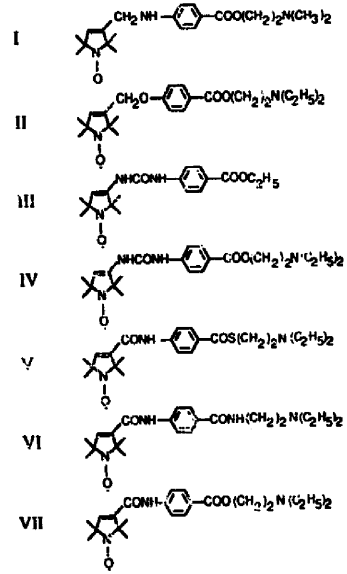

Fig. 1. Structures of the spin-labelled local anaesthetic analogues used in this study.

the microwave cavity. Data were collected on an IBM PC computer interfaced to the spectrometer. Spectral subtractions were performed digitally as described in Ref. 20.

Theoretical background

The protonation equilibria of an amphiphile which partitions between the aqueous and membrane phases are indicated schematically in Fig. 2. The intrinsic acid-base dissociation constant of the amphiphile in water is defined by:

$$K_a^w = [L][H^+]/[LH] \quad (2)$$

where L and LH are the unprotonated and protonated forms, respectively, of the amphiphile in water, and $[H^+]$ is the bulk hydrogen ion concentration in water. The interfacial acid-base dissociation constant for the amphiphile bound to the membrane is defined by:

$$K_a^i = [L_i][H^+]/[L_iH] \quad (3)$$

where L_i and L_iH are the unprotonated and protonated forms, respectively, of the bound amphiphile. At low concentrations of amphiphile, the partition coefficients of the unprotonated and protonated forms (K_L and K_{LH}, respectively) are given by:

$$K_L = [L_i]/[L] \quad (4)$$

and

$$K_{LH} = [L_iH]/[LH] \quad (5)$$

Because of the cyclical nature of the equilibria (Fig. 2), the acid-base dissociation constants are related to the partition coefficients by (cf. Eqns. 2–5):

$$K_a^w/K_a^i = K_{LH}/K_L \quad (6)$$

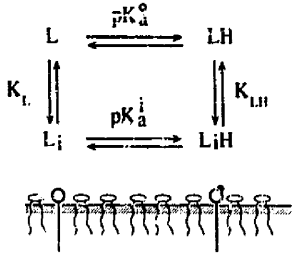

Fig. 2. Proton ionization equilibria (dissociation constants: K_a^w, K_a^i) of a local anaesthetic, L, partitioning between the aqueous phase and a membrane with partition coefficients: K_L and K_{LH}. The index i indicates an interfacial localization of the protonatable group of the local anaesthetic.

Therefore the interfacial shift (ΔpK_a^i) in pK_a is given by:

$$pK_a^i - pK_a^w = \log_{10}(K_{LH}/K_L) \quad (7)$$

This latter equation illustrates the fact that the thermodynamic factors giving rise to a pK_a shift at the interface inevitably result also in a difference in partitioning between the protonated and unprotonated forms of the amphiphile. For a typical value of the interfacial polarity shift: $|\Delta pK_a^{pol}| \approx 1.1$ [6,7], it is predicted that the partition coefficient of the uncharged form of the amphiphile will be approximately 13-times greater than that of the charged form, in neutral membranes.

The experimentally measured partition coefficient, in the case where both protonated and unprotonated species are present, is given by:

$$K_{exp} = ([L_i]+[L_iH])/([L]+[LH]) \quad (8)$$

Combining Eqn. 8 with Eqns. 2, 3 and 5 then yields:

$$K_{exp} = K_{LH}([H^+]+K_a^i)/([H^+]+K_a^w) \quad (9)$$

which describes the pH dependence of the experimentally measured partition coefficient.

Results and Discussion

Membrane partitioning of spin-labelled fatty acids

The EPR spectra of the spin-labelled myristic acid, 10-MASL, in dispersions of DMPC bilayer model membranes are given as a function of the membrane (DMPC) concentration in Fig. 3. The spectra all consist of two components: a sharp three-line component from the spin labels tumbling rapidly in water and a broad anisotropic component from the spin labels intercalated in the membrane. As can be seen from the figure, the proportion of the membrane-associated component increases progressively with the membrane concentration.

The relative proportions of the free and membrane-bound fatty acid in Fig. 3 can be obtained by digital subtraction of a spectrum of the spin-labelled fatty acid alone in water, followed by double integration of the spectral components to determine relative spin concentrations. If f_L and f_W are the fractions of the total spectral intensity in the lipid-bound and free components, respectively, then:

$$f_L/f_W = K_{exp}\bar{V}_l c_l \quad (10)$$

where c_l is the lipid concentration (in g/ml) and $\bar{V}_l$ is the partial specific volume of the lipid. The dependence of the ratio f_L/f_W on the lipid concentration is given for dispersions at two different pH values in Fig.

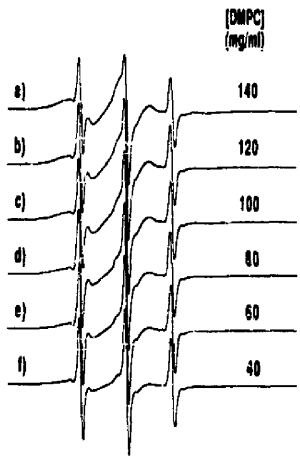

Fig. 3. EPR spectra of the 10-MASL myristic acid spin label in aqueous dispersions of DMPC bilayers at pH 9.0 and with different DMPC concentrations. (a) 140 mg/ml DMPC; (b) 120 mg/ml DMPC; (c) 100 mg/ml DMPC; (d) 80 mg/ml DMPC; (e) 60 mg/ml DMPC; (f) 40 mg/ml DMPC. $T = 15\,^{\circ}C$, total scan width = 100 gauss.

4. The linearity of the concentration dependence as predicted by Eqn. 10 demonstrates that the fatty acid establishes a partition equilibrium between the membrane and aqueous phases. At the low relative concentrations of fatty acid used in the present experiments, electrostatic effects of the bound fatty acid may be neglected.

Since the fatty acid will be fully protonated at pH 4.0 and fully dissociated at pH 9.0, the partition coefficients of the two protonation states can be determined. From the linear regressions in Fig. 4 the (scaled) partition coefficients are: $K_{LH}\bar{v}_l = 2570$ ml/g (pH 4.0) and $K_L\bar{v}_l = 17.9$ ml/g (pH 9.0), i.e. the partition coefficient

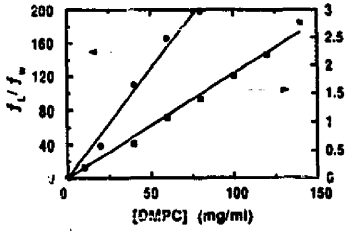

Fig. 4. Dependence on DMPC concentration of the ratio, f_L/f_W, of 10-MASL myristic acid partitioning into DMPC to that in water, at 15 °C. Data are deduced from spectral subtractions of ESR spectra of the type shown in Fig. 3 and are given for two pH values of the suspending buffer: (●) pH 4.0, (■) pH 9.0. The left-hand ordinate corresponds to the data for pH 4.0 and the right-hand ordinate to the data for pH 9.0. The solid lines represent linear regressions of the data yielding the (scaled) partition coefficients: $K_{LH}\bar{v}_l = 2570$ ml/g (pH 4.0) and $K_L\bar{v}_l = 17.9$ ml/g (pH 9.0) where $\bar{v}_l$ is the partial specific volume of DMPC, cf. Eqn. 10.

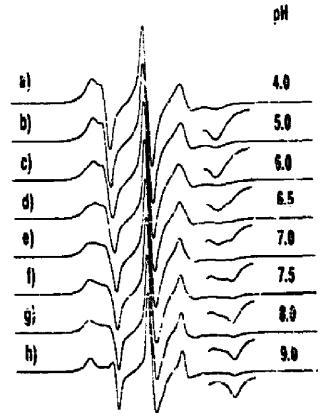

Fig. 5. ESR spectra of the 6-MASL myristic acid spin label in DMPC bilayers dispersed in buffers of different pH values. (a) pH 4.0; (b) pH 5.0; (c) pH 6.0; (d) pH 6.5; (e) pH 7.0; (f) pH 7.5; (g) pH 8.0; (h) pH 9.0. $T = 41\,^{\circ}C$, total scan width = 100 gauss.

of the uncharged fatty acid is approximately 145-times greater than that of the charged species. Measurements on the 6-MASL derivative with the spin label located at a different position in the fatty acid chain yielded comparable data with a 130-fold difference in the partition coefficients of the uncharged and charged forms.

Interfacial ionization of membrane-bound fatty acid

The ESR spectra of the spin-labelled myristic acid, 6-MASL, in DMPC bilayer model membranes are given as a function of the pH of the suspending medium in Fig. 5. Under the conditions of this experiment, the spin-label essentially is wholely bound to the membrane. As found previously for spin-labelled stearic acid in a different membrane system [21], the EPR spectra in the titration region of the membrane-bound fatty acid consist of two components. One component has a smaller spectral anisotropy characteristic of the protonated (uncharged) form of the fatty acid observed at pH 4.0 (Fig. 5a), and the other component has a larger spectral anisotropy characteristic of the ionized (charged) form of the fatty acid at pH 9.0 (Fig. 5h).

The relative proportions of the protonated and unprotonated membrane-bound fatty acid in Fig. 5 can be obtained by digital subtraction of the spectra at the low and high pH extremes from those in the titration region. If f_H is the fraction of the total spectral intensity in the protonated component, then a conventional pH titration gives:

$$f_H = 1/(1 + K_a^l/[H^+]) \qquad (11)$$

The data for the pH dependence of f_H are given in

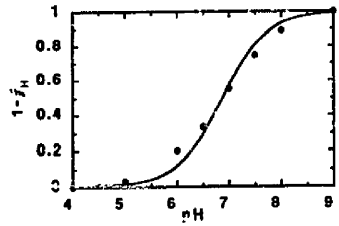

Fig. 6. pH dependence of the fraction, $1-f_H$, of unprotonated 6-MASL myristic acid deduced from spectral subtractions of the ESR spectra shown in Fig. 5. The solid line represents a non-linear least-squares fit of the data to Eqn. 11 yielding a $pK_a^i = 6.9$.

Fig. 6 and a non-linear least-squares fit to Eqn. 11 yields a value of $pK_a^i = 6.9$ for the interfacial pK_a of the 6-MASL myristic acid. Since the intrinsic pK_a of a fatty acid in water is approximately $pK_a^w = 4.8–4.9$ [22], the interfacial pK_a shift is: $pK_a^i - pK_a^o = 2.0–2.1$. The positive sign of the shift is that expected for the dissociation of a molecular acid binding to neutral membranes [6]. The magnitude of the shift is greater than the polarity-induced shifts in interfacial pK_a that are normally observed, $|\Delta pK_a^{pol}| \approx 1.1$ [6,7] because the protonated fatty acid sinks deeper into the bilayer on protonation, as evidenced by the considerably smaller anisotropy in the EPR spectrum at low pH (see Fig. 5 and the discussion in Ref. 29).

The value of the interfacial shift in pK_a predicted from the measured partition coefficients of the protonated and unprotonated species by using Eqn. 7 is: $pK_a^i - pK^o = 2.11$ for 6-MASL and 2.16 for 10-MASL. The agreement with the value that was measured directly demonstrates the validity of Eqn. 7 in describing the partitioning and ionization equilibria in amphiphile-membrane interactions. Clearly, the interfacial shifts in pK_a must be taken into account when interpreting data on the binding of local anaesthetics to membranes. The data for fatty acids indicate that these effects can be very appreciable and it is of considerable interest to determine the magnitude of the shifts for other local anaesthetic molecules, particularly those of the tertiary amine class.

pH dependence of the partitioning of spin-labelled local anaesthetics

The spin-labelled analogues of local anaesthetics whose structures are given in Fig. 1 are known to bind to synaptic membranes [16]. The EPR spectra of these spin-labelled analogues in DMPC bilayers were found not to be sensitive to pH (data not shown) therefore precluding direct determination of the interfacial pK_a as was possible in the case of the spin-labelled fatty acid. However, since the principles are well established by these latter measurements, the pH dependence of the partitioning obtained with the above methods can be used to estimate the shifts in pK_a.

The EPR spectra of the different spin-labelled local anaesthetic analogues in DMPC bilayer dispersions are given in Fig. 7. As for the spin-labelled fatty acid, the spectra are clearly resolved into two components corresponding to the free and membrane-associated species. The ratios f_L/f_W of the membrane-bound to free spin-labelled local anaesthetic analogues were determined from the EPR spectra as described above. These are given in Fig. 8 as a function of the pH of the suspending medium for the different local anaesthetic analogues. The nonprotonatable benzocaine analogue, III, showed very little pH dependence of partitioning over the ranges for which a titration is found in Fig. 8, confirming that the pH dependences in Fig. 8 correspond to titration of the tertiary amine group of these analogues. For the benzocaine analogue the (scaled) partition coefficient was $K_L\bar{v}_l \approx 4700$ ml/g. The non-linear least-squares fits of the partitioning data to Eqn. 9 (and 10) are given for the other analogues in Fig. 8. The pH dependences are all reasonably well described by this equation and yield the fitting parameters given in Table I.

The intrinsic $pK_a^o = 8.3$ obtained for the tetracaine analogue, I, agrees very well with values of $pK_a^o = 8.26$

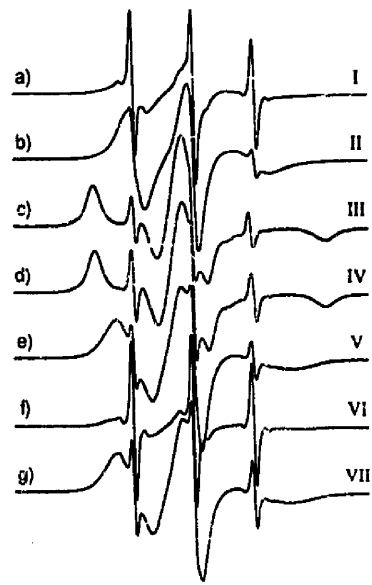

Fig. 7. ESR spectra of the different spin-labelled local anaesthetic analogues (cf. Fig. 1) in aqueous dispersions of DMPC bilayers at pH 10.0 and $T = 15\,^{\circ}C$. (a) analogue I, [DMPC] = 150 mg/ml; (b) analogue II, [DMPC] = 150 mg/ml; (c) analogue III, [DMPC] = 15 mg/ml; (d) analogue IV, [DMPC] = 30 mg/ml; (e) analogue V, [DMPC] = 15 mg/ml; (f) analogue VI, [DMPC] = 100 mg/ml; (g) analogue VII, [DMPC] = 100 mg/ml. Total scan width = 100 gauss.

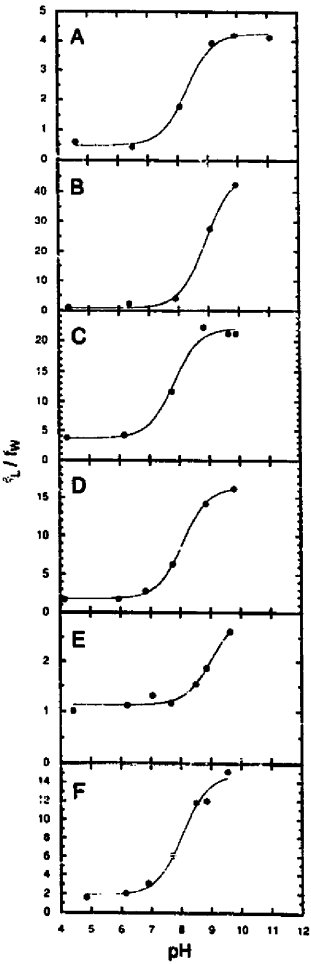

Fig. 8. pH dependence of the ratios, f_L/f_W, of the different spin-labelled local anaesthetic analogues (cf. Fig. 1) partitioning into DMPC to those in water, at 15 °C. (A) Analogue I, [DMPC] = 150 mg/ml; (B) analogue II, [DMPC] = 150 mg/ml; (C) analogue IV, [DMPC] = 30 mg/ml; (D) analogue V, [DMPC] = 15 mg/ml; (E) analogue VI, [DMPC] = 100 mg/ml, (F) analogue VII, [DMPC] = 100 mg/ml. Data are deduced from spectral subtractions of ESR spectra such as those in Fig. 7. The solid lines represent non-linear least-squares fits of the data to Eqn. 9 yielding the intrinsic and interfacial pK_a values and the partition coefficients given in Table I.

[8] and 8.24 [23] that have been obtained directly for tetracaine itself in aqueous solution. The intrinsic $pK_a^o = 8.9$ obtained for the procaine-like analogue, II, agrees well with the value of $pK_a^o = 8.9$ for procaine itself [24], but not with the values for the other procaine analogues, IV and VII. Interestingly, however, the values for pK_a^i for the different procaine analogues are all rather similar.

TABLE I

Intrinsic and interfacial pK_a values (pK_a^o and pK_a^i, respectively) and (scaled) partition coefficients ($K_{L/H}\bar{r}_1$) of spin-labelled local anaesthetic analogues, L, partitioning into DMPC bilayers at 15 °C

Data are deduced from the non-linear least-squares fits to Eqn. 9 that are presented in Fig. 8.

L	[DMPC] (mg/ml)	pK_a^o	pK_a^i	$K_{L/H}\bar{r}_1$ (ml/g)
I	150	8.3	7.4	3.2
II	150	8.9	7.2	6.5
IV	30	7.8	7.0	120
V	15	8.1	7.1	120
VI	100	9.0	8.6	11
VII	100	8.0	7.1	19

The interfacial pK_a shifts of the different tertiary amine local anaesthetic analogues are all negative, as expected for the dissociation of a cationic acid binding to neutral membranes [6]. The values for the different analogues vary somewhat, but have a mean value taken over all derivatives of $\Delta pK_a^i = -0.95$. This is of the size expected for a polarity-induced shift where the molecule, unlike the fatty acid, does not move in the membrane on protonation. The latter supposition is consistent with the insensitivity to pH of the ESR spectra of the membrane-associated local anaesthetic analogues *. The interfacial pK_a shift for the tetracaine analogue, I, ($\Delta pK_a^i = -0.9$) is comparable to the value of $\Delta pK_a^i = -0.68$ measured directly for tetracaine in neutral detergent micelles [8]. Some small difference between the two values might be expected in view of the somewhat different interfaces in the two cases. Using the calibrations of Ref. 8, the value of pK_a^i obtained here for analogue I corresponds to an effective interfacial dielectric constant at the site of the amine group of $\varepsilon_{int} \approx 40$–44 for DMPC bilayers, as opposed to $\varepsilon_{int} \approx 51$ for the neutral micelles (cf. footnote below).

Other workers have determined the pH dependence of the partitioning of unlabelled tertiary amine local

* Based on indirect evidence from ^{2}H-labelled phosphatidylcholine, it was suggested in Ref. 27 that unlabelled tetracaine changes its location in the bilayer on deprotonation. However, NMR results from ^{2}H-labelled tetracaine [28] have indicated further that such effects are dependent on the particular lipid host, which is different in the present case. The EPR spectra from the spin-labelled local anaesthetic analogues used here provide no positive evidence for a change in their bilayer location on deprotonation, although a limited change cannot be excluded. The variation in size of ΔpK_a^{pi} between the different spin-labelled analogues might be taken to indicate such a change for some of the analogues, but clearly this is considerably smaller than the large shift observed for the fatty acid. In cases where the amphiphile does move on deprotonation, the effective interfacial polarity or dielectric constant deduced from the shift in pK_a corresponds to some average for the two locations.

anaesthetics from which the interfacial shift in pK_a can be extracted. Westman et al. [9] have derived values of $\Delta pK_a^{pol} = -0.77$ and -1.64 for tetracaine and procaine, respectively, in bilayer membranes. These values are in good accord with those obtained here for analogues I and II, respectively. Correcting for an error in sign (cf. Ref. 9), polarity-induced shifts of $\Delta pK_a^{pol} = 0$, -1.0 and -1.0 were found for tetracaine, procaine and lidocaine, respectively, in Ref. 10. The vanishing shift in pK_a for tetracaine is inconsistent with the results quoted here and with the above theoretical considerations regarding interfacial ionization. Eftink et al. [25] have also obtained a polarity-induced shift of $\Delta pK_a^{pol} = -1.35$ for dibucaine, which again is consistent with the general expectations for tertiary amine local anaesthetics.

Conclusions

It has been demonstrated directly that the partitioning of the charged and uncharged forms of a fatty acid local anaesthetic is correlated with the shift in pK_a on membrane association, according to the predictions of equilibrium thermodynamics. Hence, the membrane partitioning of protonated and unprotonated amphiphiles will differ in all cases, since this difference is determined by the same interfacial energetic factors that govern the shift in pK_a (cf. Eqn. 7). In general, the pK_a shift for the tertiary amine local anaesthetics due to the lower polarity of the interfacial site is in the region of $\Delta pK_a^{pol} \approx -1.0$ to -1.5 and therefore the uncharged anaesthetic is expected to have a 10- to 30-fold greater partition coefficient for neutral membranes than that of the charged form. Other local anaesthetics, such as the fatty acids, which have a different interfacial location in the charged and uncharged forms may give rise to larger differences in partition coefficient (cf. footnote on p. 67). In the case of negatively charged membranes, the partition coefficient of the protonated species will be increased by an amount determined by the surface potential, as indicated by Eqns. 1 and 7. Additional shifts will also arise from the electrostatic gathering or depletion of the charged species at the charged membrane surface. At high levels of partitioning, the modification of the surface charge by the bound anaesthetic must also be taken into account, as was done in Refs. 9 and 10.

Acknowledgements

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