Biochimica et Biophysica Acta, 769 (1984) 231–237
Elsevier

BBA 71936

EFFECT OF LIPID MEMBRANES ON THE APPARENT p*K* OF THE LOCAL ANESTHETIC TETRACAINE
SPIN LABEL AND TITRATION STUDIES

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(Received June 8th, 1983)

Key words: Phosphatidylcholine membrane; Anesthetic membrane interaction; Tetracaine; Spin label; Partition coefficient; ESR

Electrometric titrations and spin label data demonstrate changes in the experimentally determined apparent p*K* of an ionizable drug in the presence of membranes. This effect is attributed to the difference in partition coefficients for the charged and uncharged forms of the drug. Investigation of the binding of a local anesthetic, tetracaine, to egg phosphatidylcholine membranes indicates that the drug apparent p*K* decreases in the presence of membranes, the decrease being a function of membrane concentration. The agreement between titration and spin label studies is very good and could be simulated by calculating membrane-bound and free populations of charged and uncharged tetracaine from the independently-measured partition coefficients for the two forms.

Introduction

A large amount of work has been done on the effect of drugs on membranes. Most of these drugs undergo partitionining between the aqueous and membrane phases, and this process depends, among other variables, on the nature of the membrane, temperature and pH. The influence of pH is observed when the drug and/or membrane carry ionizable groups. Nevertheless, the values of partition coefficients (K_p) that are used by some authors have often been obtained in unrelated systems.

Boulanger et al. [1] have determined the K_p values for both the protonated and unprotonated forms of the local anesthetic tetracaine in egg PC membranes and have shown that these values differ from those obtained for the drug in other systems.

McLaughlin and Harary [2] analysed the binding of charged species to lipid membranes. Lee [3] applied their formalism to compounds that undergo ionization equilibria. The formalism shows that when both the charged and uncharged forms bind to the membrane with different binding constants, the p*K* for the drug in the membrane should differ from that in the aqueous phase. In this case, $\Delta pK = pK_m - pK_a$ (adapted from Eqn. 8, Ref. 3) where pK_m and pK_a correspond to values of pH in the bulk aqueous phase at which the drug is half ionized in the membrane and in the bulk aqueous phase, respectively. Lee found $\Delta pK = 1$ for procaine, but ΔpK was zero for tetracaine [3]. This latter effect would occur if the binding constants were the same for the charged and uncharged species.

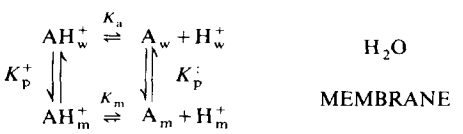
Using a formalism similar to that developed by Lee, Westman et al. [4] simulated binding curves

Abbreviations: 5-MeSL, stearic acid methyl ester containing the 2,2-dimethyl-*N*-oxyl oxazolidine moiety at position 5.

for charged and uncharged tetracaine from the distribution of the anesthetic between membrane and aqueous phase as a function of pH. By introducing different binding constants for protonated and unprotonated tetracaine, and $pK_a = 8$, 0, pK_m was found to be 7.23.

In studies of partitioning of ionizable compounds into micelles, Chaimovich et al. [5] have defined an apparent pK, pK_{app}, which corresponds to the pH in the aqueous phase where the population of the charged species equals the population of the uncharged species, both populations calculated as the sum of fractions in the membrane and in the aqueous phase. This treatment has also been applied to ionizable compounds binding to bilayer vesicles of synthetic amphiphiles [6].

Thus, for the ionization equilibria of compounds that partition between two phases, the following scheme must be considered:



Scheme I.

where K_a and K_m are the ionization constants in the aqueous and membranes phases, respectively, and K^+_p and $K^:_p$, the partition coefficients for the charged (AH^+) and uncharged (A) forms of the compound, respectively. The subscripts w and m represent the aqueous and membrane phases, respectively.

We can define three pK values for this system:

(1) pK_a, the pH of the bulk aqueous phase at which the populations of charged and uncharged forms in the aqueous phase are equal

$$\left(K_a = [H^+_w] \times \frac{[A_w]}{[AH^+_w]}\right);$$

(2) pK_m, the pH of the bulk aqueous phase at which the populations of charged and uncharged forms in the membrane are equal

$$\left(K_m = [H^+_w] \times \frac{[A_m]}{[AH^+_m]}\right);$$

(3) pK_{app}, the pH of the bulk aqueous phase at which the total charged population equals the total uncharged population

$$\left(K_{app} = [H^+_w] \times \frac{[A_w + A_m]}{[AH^+_w + AH^+_m]}\right).$$

The latter can be seen to depend on membrane concentration. This behavior is relevant for the determination of proper doses of drugs whose active species is one of the (ionized or non-ionized) forms.

In this paper we determine pK_{app} values for tetracaine as a function of concentration of egg PC multilamellar bilayers, both by electrometric titration and from spin label data. Making use of independently determined values of partition coefficients (K^+_p and $K^:_p$) for tetracaine, we calculate the populations of membrane-bound and free, charged and uncharged, forms of the drug as a function of membrane concentration and estimate pK_{app} values in good agreement with the experimentally determined ones. In addition, the formalism also allows the calculation of pK_m.

A preliminary account of the spin label results has been published elsewhere [7].

Materials and Methods

Egg phosphatidylcholine was obtained by the method of Nielsen [8], tetracaine hydrochloride was from Sigma Chemical Co., St. Louis, MO. The spin probe 5-MeSL was obtained from Syva, Palo Alto, CA. All other reagents were analytical grade.

Sample preparation. EPC membranes were prepared by evaporating a chloroform solution of the lipid under wet N_2. For EPR experiments the spin probe 5-MeSL, also in chloroform solution, was added to yield systems containing 1–2 mol% probe. The samples were left under vacuum for no less than 2 h. Multilamellar dispersions were prepared by adding 0.12 M phosphate/borate/citrate buffer (for EPR samples) or 0.12 NaCl (for the electrometric titrations). For EPR experiments, tetracaine was added to samples either from a concentrated solution or as a solid, pH adjustments were done by addition of concentrated NaOH or HCl. For electrometric titrations, the solution used to disperse the lipid already contained the anesthetic.

Electrometric titrations. Titrations were per-

formed by adding aliquots of 0.1–0.2 M NaOH solutions to tetracaine in the absence or presence of membranes starting at pH 3. A Metrohm E-300 pH meter was used for the titrations. The sample volume was 2.0 ml and the NaOH concentration was chosen so that the final volume would not change by more than 5%. Titrations were done at 30°C under an atmosphere of nitrogen. The pK was determined from plots of pH vs. added NaOH volume.

EPR experiments. Spectra were run in a Varian E-4 spectrometer, at room temperature (22 ± 2°C). The samples were placed in flat quartz cells for aqueous solutions from James Scanlon, Costa Mesa, CA. pH measurements of EPR samples were done with a Metrohm E-512 pH meter.

Results

Electrometric titrations

The titration of tetracaine in the concentration range 0.5–1.4 mM in the absence of membrane yielded for the aliphatic amino group pK_a = 8.5, in good agreement with data from Ritchie and Greengard [9]. Titrations of tetracaine were performed as a function of membrane concentration. Fig. 1 shows the variation of apparent pK.

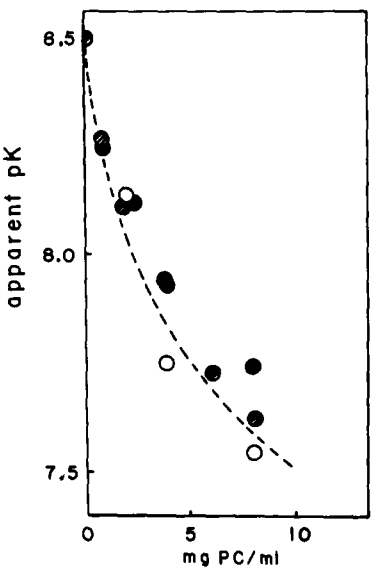


Fig. 1. Tetracaine apparent pK as a function of membrane concentration. (⊘) electrometric titration, (○) EPR data, (●) calculated from data in Table I and similar ones for other membrane concentrations. The concentrations of anesthetic were: 0.8 mM, 0.6 mM, 1.2 mM, 2.5 mM, 3.7 mM and 5.0 mM for 0, 1, 2, 4, 6, and 8 mg egg PC/ml, respectively.

It has been reported that the pK_a for tetracaine is concentration dependent [10]. It is likely that this variation was due to precipitation of the uncharged form of the anesthetic. We believe that the decrease in pK_{app} observed in the presence of EPC membranes cannot be ascribed to tetracaine precipitation since the concentrations used for the experiments in Fig. 1 were much lower than those given by Butler et al. [10]. The experiments in Fig. 1 (titrations and EPR) were performed at a constant molar ratio of total anesthetic to lipid (ca 1 : 2). In addition, in the presence of increasing egg PC concentrations, 0.8 mM tetracaine (when no precipitation occurs) gave pK_{app} values similar to those in Fig. 1.

EPR experiments

EPR spectra were obtained for 5-MeSL in EPC multilamellar dispersions as a function of pH in the absence and presence of tetracaine. A typical spectrum is presented in Fig. 2. The probe is an ester, therefore is not strongly anchored at the membrane-water interface. As a result, 5-MeSL exhibits a high degree of motional freedom. Nevertheless, the probe still undergoes some degree of ordering which does not allow the calculation of rotational correlation times from line heights and widths [11]. However, we have taken the ratio of

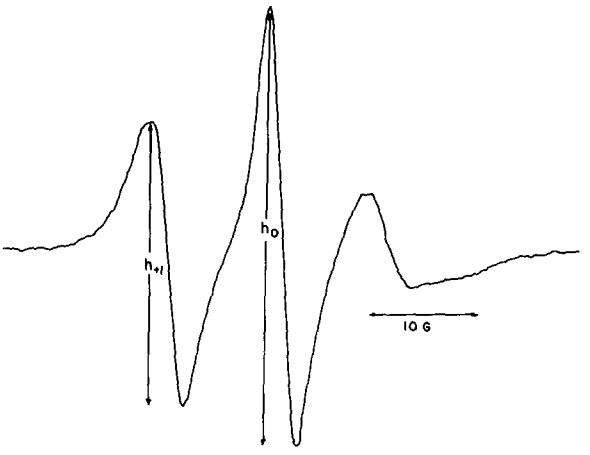


Fig. 2. EPR spectrum of 5-MeSL in egg PC membranes, pH 8.0. The figure shows how the heights of the low field (h_{+1}) and center (h_0) lines were measured to calculate h_{+1}/h_0.

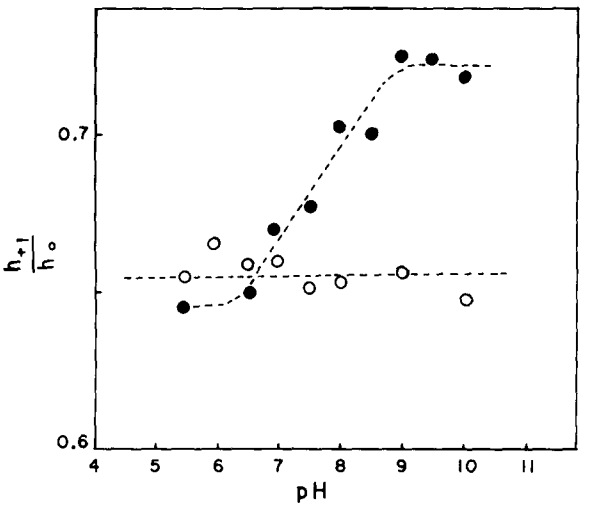


Fig. 3. h_{+1}/h_0 ratio as a function of pH for the spectra of 5-MeSL in egg PC membranes (4 mg/ml) in the absence (○) and in the presence (●) of 2.3 mM tetracaine.

the heights of the low field line (h_{+1}) and of the center line (h_0) as an empirical measurement of the effect of the drug on the organization of the membrane lipids.

Fig. 3 shows the variation of the h_{+1}/h_0 ratio as a function of pH for one membrane concentration (4 mg EPC/ml). In the presence of tetracaine, the values of h_{+1}/h_0 increase with increasing pH and then level off. The increase in h_{+1}/h_0 as a function of pH is probably due to changes in the organization of membrane components caused by uncharged tetracaine. Increasing amounts of drug bind to the membrane as a result of the increase in the average partition coefficient, $K_{p_{av}}$, as a function of pH (see Discussion and Table I). That the uncharged form of the anesthetic is capable of decreasing the degree of order of lipid bilayers has already been shown by spin label [10] and by deuterium NMR [12] studies.

The mid-point of the inflection in the h_{+1}/h_0 vs. pH curve occurs at a pH value lower than the pK_a of tetracaine in the absence of membranes. The pH values for mid-points of similar curves obtained at variable membrane concentration are given in Fig. 1. The inaccuracy involved in the EPR experiment is larger than that for the titration data. Nevertheless, the agreement between the two sets of results is very good.

Discussion

Scheme 1 shows that when an ionizable compound partitions between aqueous and membrane phases, there are four species present: AH^+_w, AH^+_m, A_w and A_m. The relative amounts of these species

TABLE I

CALCULATED AVERAGE K_p VALUES AND PERCENTAGES OF MEMBRANE-BOUND AND FREE CHARGED AND UNCHARGED TETRACAINE AS A FUNCTION OF pH AND MEMBRANE CONCENTRATION

pH	$K_{p_{av}}$	4 mg egg PC/ml				10 mg egg PC/ml			
		Charged		Uncharged		Charged		Uncharged	
		aqueous	membrane	aqueous	membrane	aqueous	membrane	aqueous	membrane
5.5	25.7	90.6	9.06	0.09	0.26	79.5	19.9	0.08	0.56
6.0	27.2	89.9	8.99	0.29	0.81	78.4	19.6	0.25	1.76
6.5	31.8	87.8	8.78	0.90	2.51	75.2	18.8	0.75	5.31
7.0	46.0	81.9	8.19	2.58	7.35	66.4	16.6	2.10	14.9
7.5	87.3	67.4	6.74	6.74	19.1	48.6	12.1	4.85	34.5
8.0	190	43.2	4.32	13.7	38.8	26.2	6.57	8.22	59.0
8.5	368	20.3	2.03	20.3	57.4	10.7	2.66	10.7	76.0
9.0	545	7.55	0.76	23.9	67.8	3.72	0.92	11.8	83.6
9.5	648	2.53	0.25	25.3	71.9	1.21	0.31	12.1	86.4
10.0	689	0.82	0.08	25.8	73.3	0.38	0.09	12.3	87.3
10.5	703	0.26	0.03	26.0	73.8	0.12	0.03	12.2	87.7
11.0	708	0.08	0.01	26.0	73.9	0.04	0.01	12.3	87.8

depend on pH and on membrane concentration, and their determination is a problem of theoretical and practical relevance. The analysis of pK_{app} is intrinsically related to this question.

Fig. 1 shows that pK_{app} for tetracaine, determined by electrometric titration depends on membrane concentration. We also found that the mid-points in the h_{+1}/h_0 vs. pH curves (e.g., Fig. 3) are sensitive to membrane concentration, as shown in Fig. 1.

The experimental results can be described by a model that allows the calculation of pK_{app} from the estimation of the populations of charged and uncharged anesthetic both membrane-bound and free in the aqueous phase. These populations can be estimated making use of pK_a, of the experimentally determined partition coefficients, K_p^+ and $K_p^:$, for the two forms of tetracaine, and of average partition coefficients, $K_{p_{av}}$. $K_{p_{av}}$ measures the total amount of drug that partitions into the membrane and its values as a function of pH can be calculated from weighted averages of K_p^+ and $K_p^:$ using the Henderson-Hasselbalch equation.

Boulanger et al. [1] have determined partition coefficients of 22 and 660 for tetracaine at pH 5.5 and 9.5, respectively. Using $pK_a = 8.5$, the actual partition coefficient for uncharged tetracaine, $K_p^:$, can be calculated by the Henderson-Hasselbalch equation as being 710. Using $K_p^+ = 25$ and $K_p^: = 710$ we calculated the values of $K_{p_{av}}$, given in Table I.

By combining pK_a, $K_{p_{av}}$, K_p^+ and $K_p^:$, it is possible to calculate the percentages of charged and uncharged membrane-bound and free anesthetic as a function of membrane concentration and pH. Table I presents the results for 4 and 10 mg membrane/ml. Fig. 4 displays the effect of pH on the sum of (membrane-bound + free in aqueous phase) charged and uncharged tetracaine populations. The intersections give pK_{app} at 4 mg egg PC/ml (7.96, Fig. 4A) and at 10 mg egg PC/ml (7.69, Fig. 4B). The values of pK_{app} determined from the above procedure are also in Fig. 1. The agreement between experimental and calculated pK_{app} values is satisfactory. For higher membrane concentrations, both electrometric titration and spin label experiments gave lower pK_{app} values than the calculated ones. We have no explanation for these results at present.

The analysis of the amounts of membrane-bound anesthetic allows the determination of pK_m. Fig. 5 shows the variation of the populations of membrane-bound charged and uncharged tetracaine at membrane concentrations of 1, 4 and 10 mg egg PC/ml (Fig. 5A, B and C, respectively).

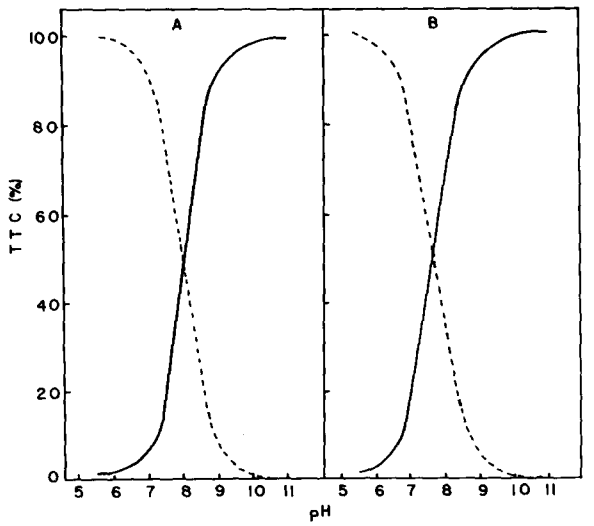


Fig. 4. Calculated sums of membrane-bound + free, charged (- - - - - -) and uncharged (———) populations of tetracaine (TTC) as a function of pH. A. 4 mg egg PC/ml; B. 10 mg egg PC/ml. The intersections give the apparent pK values.

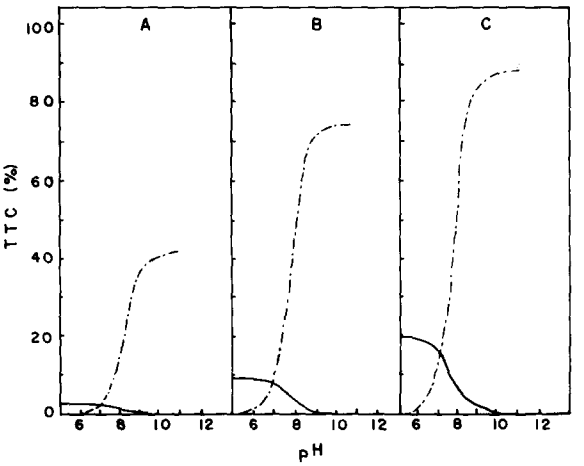


Fig. 5. Calculated percentages of membrane-bound charged (———) and uncharged (- · -) populations of tetracaine (TTC) as a function of pH. A. 1 mg egg PC/ml; B. 4 mg egg PC/ml; C. 10 mg egg PC/ml. The intersections give the anesthetic pK in the membrane (pK_m).

While the amounts of bound charged and uncharged anesthetic increase with increasing membrane concentration, the intersections always occur at pH 7.05, which is the value of pK_m.

The large decrease in the dissociation constant for membrane-bound tetracaine can be ascribed mainly to a smaller dielectric constant in the membrane. Boulanger et al. [12] have shown that the average membrane location of charged tetracaine differs from that of uncharged tetracaine, the former being closer to the membrane-water interface. Since the dielectric constant is thought to decrease when going from the interface to the bilayer center [13,14], the dielectric constant experienced in the ionization process should be thought of as an average between those characterizing the binding regions of charged and uncharged anesthetic.

Westman et al. [4] found $pK_m = 7.23$ from computer simulations and 7.0 from experimental data obtained for 50 mg egg PC/ml. The difference was ascribed to the positive charge conferred to the membrane by protonated tetracaine. It should be noticed that the present pK_m value contains the contribution from charge effects, since the calculation of the population of each species was based on experimental values of partition coefficients. This means that $K_p +$ contains the contribution of charge to the binding of protonated tetracaine. However, the high ionic strength used in this work should minimize the surface charge effects, rendering the estimated pK_m value very close to the actual one. Furthermore, we used $pK_a = 8.5$ and values of K_p^+ and $K_p^{\pm}$ that do not correspond exactly to the binding constants given in Ref. 4.

Lee [3] did not find a difference between pK_m and pK_a values for tetracaine. We believe that the lack of Lee's observation of changes in the anesthetic pK could be due to the use of a very low membrane concentration (ca. 0.055 mg/ml) which would not cause a detectable decrease in pK_{app}.

In a recent paper, Rooney and coworkers [15] were able to detect pK changes for fatty acids bound to low membrane concentrations. In this case, partitioning into the membrane is highly favoured. Increased partitioning was also observed as a function of membrane concentration.

Our results show that, when working with ionizable drugs, especially those with pharmacological activity, one should take into account that the ionized and unionized forms will probably display different partition coefficients. This leads to a variety of consequences that should not be neglected:

(1) When variable pH studies are being effected at constant total drug and membrane concentration, different amounts of drug will partition into the membrane at different pH values. This is the case in Fig. 3. In order to distinguish which form is responsible for an observed effect, it is necessary to work at concentrations that will yield the same amount of bound drug at different pH values.

(2) Increasing membrane concentration results in an increase of the percentage of both charged and uncharged forms in the membrane (Fig. 5 and Table I).

(3) Finally, the pH where the total drug is half ionized (pK_{app}) also depends on membrane concentration (Figs. 1 and 4) while the pH where the drug is half ionized in the membrane (pK_m) does not (Fig. 5).

These phenomena are particularly relevant in the case of ionizable drugs when only one of the forms is active, and should be taken into account when studying the mechanism of drug action.

Acknowledgements

We are indebted to Mr. Wilson R. Toselli for excellent technical work. W.A.F. Jr. was recipient of a fellowship from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). S.S., H.C., and I.M.C. are recipients of research fellowships from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The work was supported by a grant from Financiadora de Projetos (FINEP).

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