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Local anesthetic-phospholipid interactions. The pH dependence of the binding of dibucaine to dimyristoylphosphatidylcholine vesicles

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The interaction of the local anesthetic dibucaine with unilamellar vesicles of dimyristoylphosphatidylcholine was studied by equilibrium dialysis. Saturating binding profiles (as a function of dibucaine) were found, with apparent association constant ranging from $1.26 \cdot 10^3$ M^{-1} to $2.57 \cdot 10^3$ M^{-1} as pH is increased from 5.0 to 7.5. The number of phospholipid molecules comprising a binding site was found to be about 5 at each pH. Analysis of the data was also achieved using the Stern model, which takes into account the electrostatic effect on binding of the cationic drug due to the build up of a surface potential.

A variety of amphipathic drug molecules, such as the tertiary amine local anesthetics, interact with phospholipid bilayer membranes [1]. Such drug-membrane interactions have been subjected to numerous investigations, with particular attention being focused on the orientation and degree of penetration of the bound drug and the extent to which the fluidity or phase transition temperature of the phospholipid bilayer is perturbed by the drug [2–6]. The thermodynamics of the drug-phospholipid bilayer interaction is most often described in terms of a partition coefficient [7,8]. For example, the local anesthetic dibucaine has been found to have a partition coefficient of 235 for phosphatidylcholine vesicles (at a particular dibucaine and phospholipid concentration [5]). In studies of the interaction of certain drugs with model membranes, however, the binding profiles have shown a saturating effect. In such cases a more quantitative description of a drug-membrane binding equilibrium is possible in terms of a Langmuir binding isotherm for the drug [8–10]. In this treatment one can describe the binding process in terms of an apparent binding constant and a saturation level. Even more sophisticated treatments of drug-membrane interactions include changes in the membrane surface potential due to drug binding [6,9,11,14,15].

Here we report an equilibrium dialysis procedure for obtaining binding profiles for the interaction of drugs with phospholipid vesicles. We present data for the association of dibucaine with sonicated, unilamellar vesicles of dimyristoylphosphatidylcholine. These molecules were selected because dibucaine is known to perturb the phase transition of phosphatidylcholine bilayers [5,11] and thus must interact with the bilayers. Also dibucaine is fluorescent, which allows for handy determination of its relative concentrations. Dimyristoylphosphatidylcholine (DMPC) vesicles were selected because their structure and preparation are well characterized and because they have a zwitterionic, electrically neutral surface.

The materials L-α-DMPC, dibucaine-HCl, and

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Fiske-SubbaRow reducing agent were obtained from Sigma Chemical Co.. DMPC was found to migrate as a single spot on silica gel plates with the solvent system chloroform/methanol (80 : 20, v/v). Single lamellar phospholipid vesicles were prepared using the procedure of Barenholz et al. [13]. All studies were performed within two days of preparation of a vesicle sample. The procedure of Bartlett [12] was used for the determination of lipid phosphate. The DMPC vesicles prepared were typically $1 \cdot 10^{-2}$ M in phospholipid.

An 8-cell equilibrium dialysis chamber, together with Spectra-por 3 dialysis sheets, was used for the binding studies. To one side was added 0.5 ml of DMPC vesicles and to the other was added 0.5 ml of dibucaine solutions of varying concentrations (i.e., $16 \cdot 10^{-3}$ M to $1.25 \cdot 10^{-4}$ M). The buffers used were 0.05 M sodium acetate at pH 5.0, and 0.05 M Tris-HCl at pH 7.0 and 7.5. The dialysis chamber was incubated in a shaking water bath at 35°C for at least four hours. Aliquots (25 μl) from each side of the dialysis cells were then added to 1.0 ml of 0.05 M acetate buffer (pH 5.0) and the fluorescence intensity at 390 nm was measured with excitation at 340 nm, using a Perkin-Elmer MPF 44A spectrophotofluorometer. From comparison of the fluorescence from both sides to that of a series of dibucaine standards, the molar concentration of dibucaine, [D], in both cell compartments can be obtained (i.e., $[D]_{free}$ in one compartment and $[D]_{bound} + [D]_{free}$ in the vesicles containing compartment). From the difference, the concentration of vesicle bound dibucaine, $[D]_{bound}$, can be determined, from which one can determine the moles of dibucaine bound per mole of phospholipid, ν, as $\nu = [D]_{bound}/[DMPC]_o$, where $[DMPC]_o$ is the total molar concentration of phospholipid in the lipid containing compartment.

The emission wavelength mentioned above was selected after scanning the emission spectrum of dibucaine in the absence and presence of increasing concentrations of DMPC. As shown in Fig. 1, there is an isoemissive point at approx. 390 nm. Thus, at this wavelength, the fluorescence intensity will be proportional to dibucaine concentration, regardless of whether a small amount of DMPC is present or not in the fluorescence cell. After equilibration of the dialysis cells for 40 h at 35°C, a phosphate assay of both compartments revealed less than 1% of the phospholipid had diffused to the opposite side.

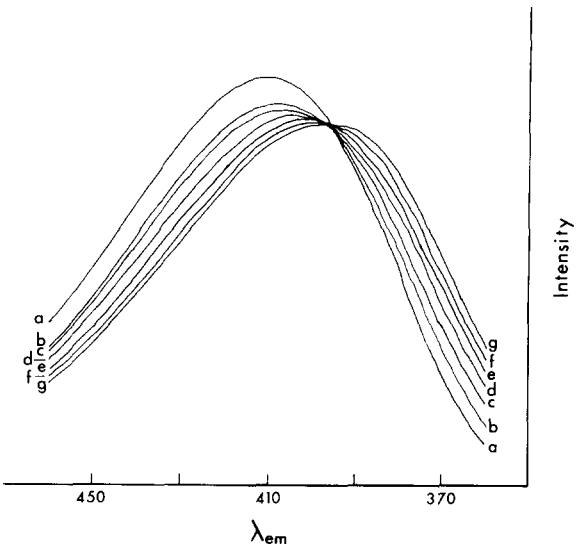

Fig. 1. The emission spectrum of dibucaine in the absence (a) and presence of the following concentrations of vesicular DMPC: (b), $0.250 \cdot 10^{-3}$ M; (c), $0.48 \cdot 10^{-3}$ M; (d), $0.75 \cdot 10^{-3}$ M; (e), $0.91 \cdot 10^{-3}$ M; (f), $1.11 \cdot 10^{-3}$ M; and (g) $1.30 \cdot 10^{-3}$ M;. Conditions: room temperature, pH 5.0, 0.5 M acetate buffer, excitation at 310 nm, $2.5 \cdot 10^{-5}$ M dibucaine.

The tertiary amino group of dibucaine has a pK_a of 8.85. This we have determined by a fluorescence pH titration of a $1 \cdot 10^{-5}$ M solution of dibucaine. Thus in the physiological pH range, this local anesthetic will exist mostly in its protonated form. However, the unprotonated form will be populated to a minor extent. Because both states of protonation will exist, we have studied the interaction at pH 5.0, where the protonated form dominates, and at pH 7.0 and 7.5, where both states of protonation will be found. Due to the low solubility of the unprotonated form of dibucaine, it was not feasible to work at a higher pH.

The binding of dibucaine, at pH 5.0, 7.0 and 7.5, to DMPC vesicles at 35°C, $I = 0.05$, is shown in Fig. 2A as plots of ν, the moles of bound dibucaine per mole of DMPC, versus the concentration of free dibucaine, $[D]_{free}$. At each pH value the binding profiles show a tendency to approach saturation. Analysis of the data was therefore attempted by use of a Langmuir adsorption isotherm, which describes the binding of a drug molecule to fixed adsorption sites on the

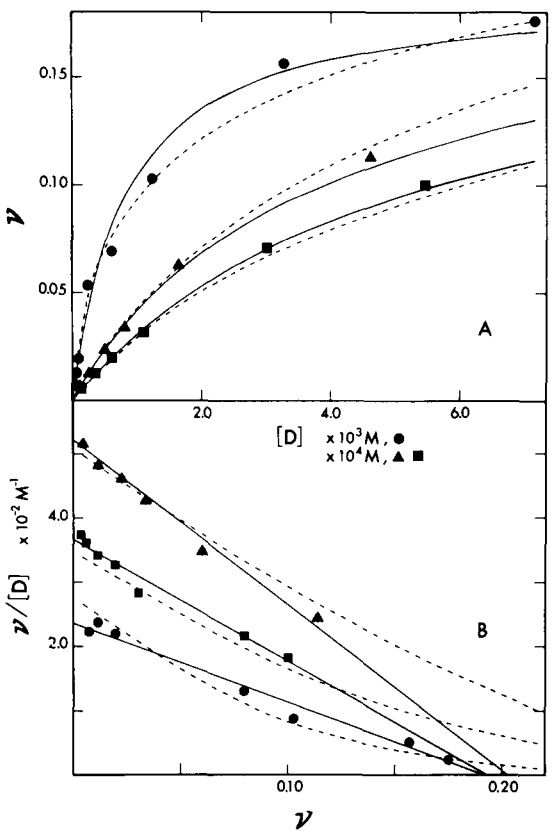

Fig. 2. (A) Binding profile for the association of dibucaine to DMPC unilamellar vesicles at pH = 7.5 (▲), pH = 7.0 (■), and pH = 5.0 (●). Conditions: 35°C, ionic strength of 0.05, approximately $1 \cdot 10^{-2}$ M DMPC in each case. Note the different concentration axis for free dibucaine for the experiments at different pH. (B) Scathchard plots of the above data. See Table I for fitting parameters for the solid lines. The dashed lines are a fit to the Stern model as described in the text with $K^+ = 1.0 \cdot 10^3$ M^{-1}, $\sigma_m = 1/270$ $Å^{-2}$, and $\Delta pK = -1.35$ (and using $pK = 8.85$ for free dibucaine and a molecular surface area of 70 $Å^2$ for DMPC in the liquid-crystalline phase).

phospholipid vesicle

$$\nu = \frac{nK[D]_{free}}{1 + K[D]_{free}} \quad (1a)$$

$$\nu/[D]_{free} = nK - \nu K \quad (1b)$$

where K is an association constant and n is the number of dibucaine molecules bound per DMPC molecule at saturation. The plots of $\nu/[D]_{free}$ versus ν in Fig. 2B are approximately linear, from which the n and K values given in Table I are obtained.

TABLE I

APPARENT BINDING PARAMETERS FOR THE INTERACTIONS OF DIBUCAINE WITH DMPC VESICLES

pH	n	K (M^{-1})	nK (M^{-1})
5.0	0.190	$1.26 \cdot 10^3$	240
7.0	0.192	$1.93 \cdot 10^3$	370
7.5	0.202	$2.57 \cdot 10^3$	520

The magnitude of n is 0.19–0.20 at each pH. This indicate that a dibucaine binding site comprises approx. 5 DMPC molecules (if the internal phospholipid molecules of the vesicles are inaccessible to dibucaine, the latter number will be smaller). The magnitude of K increases with increasing pH, indicating that the neutral form of the drug binds better than the cationic form. Previous studies have shown the uncharged form of the tertiary amine anesthetics procaine and tetracaine to bind better than their cationic form [14].

To this point we have neglected effects due to a change in the electrostatic surface potential of the vesicles due to drug binding [6,9,15]. As the cationic drug binds to the initially neutral phosphorylcholine vesicle surface, a surface potential will arise which will resist the binding of subsequent cationic drug molecules. As a result there will be a negative cooperativity in the binding of the drug to the vesicle surface. To take into account this electrostatic effect, we have also analyzed the dibucaine binding data in terms of the Stern relationship [6,9,14,15]. Briefly, this analysis assumes that both the protonated and unprotonated forms of the drug bind (possibly with different intrinsic association constant) to the vesicle surface as described by a Langmuir adsorption isotherm, that the concentration of cationic species (i.e., the protonated drug and hydrogen ions) close to the vesicle surface is related to their bulk concentration and the electrostatic surface potential by the Boltzman relationship, and that the surface potential is related to the surface charge density (i.e. due to bound cationic drug molecules) via the Grahamme equation. By using equations 1–14 in the development by Rooney and Lee [15], we have fitted this Stern model to our dibucaine binding

data at the three pH values with three fitting constants: the intrinsic association constant for the cationic drug, K^+; the maximum possible number of drug molecules (charged or uncharged) adsorbed per unit surface area of the vesicle, σ_m; and the shift in the pK of the drug upon binding to the vesicle, ΔpK (also the independently determined pK = 8.85 of the free drug and the surface area per molecule of the phospholipid, $\gamma_L = 70$ Å^2 [16], were used). To achieve a fit we first assumed a value for the surface charge density, σ^+, then calculated the corresponding surface potential and hence the pK of the vesicle-bound drug and the ratio of unprotonated to protonated forms of the bound drug (see Rooney and Lee [15]). From the Boltzman relationship, the concentration of free, protonated drug corresponding to the assumed charge density was calculated, and the concentration of total free drug was calculated from its pK. Lastly the concentration of total bound drug (protonated and unprotonated), and hence ν_{calc}, were calculated from the assumed surface charge density and the ratio of unprotonated to protonated forms of the bound drug.

The dashed lines in Figs. 2A and 2B are fits (not necessarily unique) to the data with $K^+ = 1.0 \cdot 10^3$ M^{-1}, $\sigma_m = 1/270$ Å^{-2}, and $\Delta pK = -1.35$. An adequate fit is found for all three pH with the same set of constants, thus supporting the Stern model. The above σ_m, together with the phospholipid surface area value of 70 Å^2, indicates that, at saturation with drug, one dibucaine molecule is adsorbed per approx. 3.9 phospholipid molecules (again if the internal phospholipids are inaccessible to dibucaine the latter number is smaller and the value of σ_m is larger). The $\Delta pK = -1.35$ indicates that the neutral dibucaine molecule binds with an association constant approximately 22-times larger than that for the cationic form. Possibly the enhanced binding of the neutral drug is due to its ability to penetrate more deeply into the bilayer structure.

Further studies (i.e., ionic strength, temperature dependencies) are necessary to more completely characterize the nature of the forces involved in dibucaine binding. This equilibrium dialysis system, with unilamellar vesicles, allows for great flexibility in experiment design. It should be possible to use different phospholipids, or mixed component vesicles. We have also used a gel filtration method [17] to study the binding of dibucaine to phosphatidylcholine unilamellar vesicles (data not shown) and have obtained data qualitatively similar to that reported in Fig. 2. Our experience is that the equilibrium dialysis method is much more convenient to use. Separation of lipid-drug complexes from free drug molecules by centrifugation is the most frequently used method of determining partition coefficients [7,8]. However, this employs multilamellar vesicles (unilamellar vesicles do not easily sediment). Also the equilibration of charged drug molecules across the layers of multilamellar liposomes can be very slow [14]. The equilibrium dialysis method with unilamellar vesicles described here is both convenient and flexible for the study of drug-membrane interactions.

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