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BINDING OF HYDROPHOBIC DRUGS TO LIPID BILAYERS AND TO THE (Ca^{2+} + Mg^{2+})-ATPase

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Microelectrophoretic studies of the binding of a number of commonly used hydrophobic amine drugs to liposomes demonstrated the existence of relatively large surface potentials associated with binding of the protonated forms of the drugs. A theoretical treatment based on Langmuir adsorption isotherms and the Gouy-Chapman theory of the diffuse double layer allows estimation of drug-binding constants from electrophoretic mobility data. Such constants allow calculation of the charge effects arising from drug binding in more complex membrane systems, and it is shown that shifts in the apparent Ca^{2+} affinity of the (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum in the presence of hydrophobic amine drugs are readily explicable in terms of the electrostatic effects of drug binding.

Introduction

Many drug molecules are hydrophobic and so would be expected to show extensive binding to biological membranes, either to the lipid component of the membrane or to hydrophobic sites on membrane proteins. Binding to the lipid component of the membrane could affect the function of membrane proteins in two ways. Binding could change the fluidity of the lipid (either the bulk lipid or the annular lipid around the membrane protein), and it is known that the activities of membrane proteins are sensitive to the fluidity of the surrounding lipid (see Ref. 1). However, although some drugs increase the fluidity of lipid bilayers in the liquid crystalline phase and some decrease it, all effects are rather small [2,3]. Alternatively, if the drug is charged, then binding of drug to the lipid component of the membrane will alter the charge on the membrane. Since most enzyme substrates, transmitters, etc. are also charged, their concentrations close to the membrane surface will be altered by charge interactions, with consequent alterations in enzyme activity or receptor activation, and so forth. A formalism is therefore necessary to describe the binding of charged drugs to lipid bilayers, to allow the calculation of charge effects that could follow from binding.

As well as any binding to the lipid component of the membrane, direct interaction is possible with proteins in the membrane. As we show in the following paper [4], such direct interaction can be demonstrated using fluorescent analogues of drugs. In analysing binding to membrane proteins, however, it is necessary to take into account the significant fraction of the drug that is bound to the lipid component of the membrane. Again, this requires a formalism to describe drug binding to lipid.

Here we show that drug-binding constants for lipid bilayers can be obtained from measurements of electrophoretic mobility, and that charge

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

effects that follow from drug binding are sufficiently large to explain observed effects of drugs on the Ca^{2+} affinity of the $(Ca^{2+} + Mg^{2+})$-ATPase from sarcoplasmic reticulum.

Materials and Methods

Lipids were obtained from Lipid Products. The antihistamines were obtained as follows: antazoline-HCl (CIBA), Methdilazine-HCl (Duncan, Flockhart), diphenhydramine-HCl (Parke Davis), cyproheptadine-HCl (Merck, Sharp & Dohme). Chlorpromazine-HCl was obtained from Sigma, and its quarternary methyl derivative was synthesised according to Huang et al. [5]. Dansyl propranolol was obtained from Calbiochem.

Measurements of electrophoretic mobility were made on a Rank Bros. Mark I microelectrophoresis apparatus with a cylindrical sample cell. Care was taken to focus on the stationary layer.

$(Ca^{2+} + Mg^{2+})$-ATPase was prepared from female rabbit (New Zealand White) hind leg muscle as described previously [6]. Polyacrylamide gels showed the presence of essentially pure ATPase (over 95%). The molar ratio of protein/phospholipid was found to be 1 : 30. ATPase activity was determined using a coupled enzyme assay [7], in a medium containing 40 mM Hepes (pH 7.2 at 37°C), 5 mM $MgSO_4$, 2 mM ATP, 0.42 mM phospho*enol*pyruvate, 0.15 mM NADH, pyruvate kinase (7.5 IU) and lactate dehydrogenase (18 IU) in a total volume of 2.5 ml at 37°C, with $CaCl_2$ and EGTA added to buffer Ca^{2+} at the required concentration.

Free calcium concentrations were calculated using the binding constants for Mg^{2+} and Ca^{2+} to EGTA and ATP, and for H^+ to ATP given in Fabiato and Fabiato [8], and for H^+ to phospho*enol*pyruvate given by Vianna [9]. The constants listed for proton equilibria in Fabiato and Fabiato [8] are true thermodynamic constants, involving H^+ concentration rather than the H^+ activity that is measured by a pH electrode. Accordingly, we have used the pK values for EGTA given by Boyd et al. [10] which are mixed constants, incorporating H^+ activity and EGTA concentration as is more usual in the biochemical literature. Drug pK values taken from the literature are also assumed to be mixed constants, and pH values reported in this paper are activity values. Curves expressing the dependence of ATPase activity on calcium concentration were fitted to the Hill equation by use of a derivative-free non-linear least-squares technique [11].

Analysis of lipid-binding data

Our analysis of the binding of positively charged drugs to lipid bilayers is analogous to that adopted for describing the binding of fatty acids to lipid bilayers [12]. The drugs studied here are derivatives of amines, and so will exist as mixtures of charged and uncharged species at physiological pH, the relative proportions of the two forms being given by the Henderson-Hasselbach equation:

$$\mathrm{pH} = \mathrm{p}K + \log([\mathrm{A}]/[\mathrm{AH}^+]) \quad (1)$$

where the pK of the drug determines the relative amounts of the neutral (A) and positively charged (AH^+) forms. Both forms can bind to the membrane, and we have shown elsewhere [12–14] that binding can be described by Langmuir adsorption isotherms:

$$\sigma^{\mathrm{A}} = (\sigma^{\max} - \sigma^{\mathrm{A}} - \sigma^{\mathrm{AH}})[\mathrm{A}]_{x=0}/K^{\mathrm{A}} \quad (2)$$

$$\sigma^{\mathrm{AH}} = (\sigma^{\max} - \sigma^{\mathrm{A}} - \sigma^{\mathrm{AH}})[\mathrm{AH}^+]_{x=0}/K^{\mathrm{AH}} \quad (3)$$

where σ^{A} and σ^{AH} are, respectively, the number of molecules of A and of AH^+ adsorbed to the membrane per unit area, $\sigma^{\max}$ is the maximum possible number of molecules adsorbed per unit area, K^{A} and K^{AH} are dissociation constants for binding of A and AH^+, respectively, and $[\mathrm{A}]_{x=0}$ and $[\mathrm{AH}^+]_{x=0}$ are the aqueous concentrations of A and AH^+, respectively, at the membrane/solution interface. The two dissociation constants are related by [13]:

$$K^{\mathrm{A}}/K^{\mathrm{AH}} = \exp(2.303\,\Delta\mathrm{p}K) \quad (4)$$

where ΔpK is the shift in pK on binding to the membrane. Although the concentration of A close to the surface will be equal to the bulk concentration, the concentration of AH^+ close to the surface will be less than the bulk concentration of AH^+ because of charge repulsion by the positive charges on the membrane surface, which are the result of the binding of AH^+. The charge effect can be

described by the Boltzmann relationship:

$$[AH^+]_{x=0} = [AH^+]_{bulk} \exp(-F\psi_0/RT) \quad (5)$$

where ψ_0 is the surface potential, the electrostatic potential in the aqueous phase immediately adjacent to the membrane surface, which is related to the surface charge density σ^+, by the Grahame equation [12]. With an initially electroneutral membrane, and in the absence of any other adsorbed charges, the surface charge density σ^+ will be equal to the surface concentration of AH^+, σ^{AH}.

The concentration of bound drug can be expressed in units of bulk concentration ($mol \cdot l^{-1}$) by:

$$[D]_{bnd} = \gamma_m(\sigma^A + \sigma^{AH}) \quad (6)$$

where γ_m represents the area of the membrane expressed on a molar basis, in units of $Å^2 \cdot mol \cdot l^{-1}$. The total area of membrane is dependent on the concentration of bound drug according to

$$\gamma_m = [lipid]\gamma_{lipid} + [D]_{bnd} \cdot \gamma_D \quad (7)$$

where [lipid] is the total concentration of lipid ($mol \cdot l^{-1}$) and γ_{lipid} and γ_D are, respectively, the area per molecule of a lipid and of a drug molecule in the membrane. From Eqns. 6 and 7,

$$[D]_{bnd} = \gamma_{lipid}(\sigma^A + \sigma^{AH})[lipid]/(1 - \gamma_D(\sigma^A + \sigma^{AH})) \quad (8)$$

For the drug free in solution, we obtain the ratio of the concentrations of A and AH^+ from Eqn. 1 as:

$$[A]_{free}/[AH^+]_{free} = 10^{(pH-pK)} = C \quad (9)$$

where the subscripts denote unbound drug. Accordingly, the unbound concentration of the neutral form of the drug is given by:

$$[A]_{free} = [D]_{total} - [D]_{bnd}/(1 + 1/C) \quad (10)$$

and the isotherm (Eqn. 2) can be rewritten as:

$$\sigma^A = (\sigma^{max} - \sigma^{AH})/(1 + K^A/[A]_{free}) \quad (11)$$

Turning now to the membrane-bound drug, the pK of the drug in the membrane can be conveniently written as:

$$pK_{bnd} = pK_{apparent} + \Delta pK \quad (12)$$

where $pK_{apparent}$ is the apparent pK in the membrane, altered because of the increase in local pH in the interfacial region due to the build-up of positive charge on the membrane. The proton activity at the surface ($x = 0$) is given by an equation analogous to Eqn. 5 so that

$$pK_{apparent} = pK_{bulk} - (F\psi_0/2.303\,RT) \quad (13)$$

The relative concentrations of A and AH^+ bound to the membrane are given by:

$$[A]_{bnd}/[AH^+]_{bnd} = \sigma^A/\sigma^{AH} = 10^{(pH-pK_{bnd})} \quad (14)$$

The concentrations of A and AH^+ bound to the membrane may be calculated for any given set of binding parameters (K^A, ΔpK, σ^{max}) by numerical solution of the above equations, using the Bolzano method [15].

Briefly, an initial estimate of the surface potential, ψ_0, allows calculation of the corresponding surface charge density, σ^+, via the Grahame equation [12]. This is equated with σ^{AH}. From the relationship between ψ_0 and $pK_{apparent}$ (Eqn. 13) and Eqn. 14, σ^A is obtained. Knowing σ^A and σ^{AH}, the concentration of the unbound, uncharged form of the drug can be calculated (Eqn. 10). Substitution of this value in the binding isotherm (Eqn. 11) gives σ^A, and the ratio of σ^A/σ^{AH} defines pK_{bnd} (Eqn. 14), from which a new estimate of ψ_0 is made, using Eqns. 12 and 13. The cycle is repeated until there is no significant difference between successive estimates of ψ_0.

To fit data obtained at relatively high salt (0.1 M NaCl) concentrations, it was found to be necessary to introduce specific binding of Cl^- to the ionized form of the bound drug, AH^+. This was accommodated in the following way: the presence of a positive surface potential increases the Cl^- concentration close to the membrane surface:

$$[Cl^-]_{x=0} = [Cl^-]_{bulk} \cdot \exp(F\psi_0/RT) \quad (15)$$

When $[Cl^-]_{bulk}$ is in large excess over the concentrations of AH^+ bound to the membrane, the surface concentration of Cl^- bound to AH^+ is

given by:

$$\sigma^{Cl} = (\sigma^{AH}[Cl^-]_{x=0})/(K^{Cl} + [Cl^-]_{x=0}) \quad (16)$$

where K^{Cl} is the dissociation constant for the interaction between bound AH^+ and Cl^-. The surface charge density, σ^+, will now be given by:

$$\sigma^+ = \sigma^{AH} - \sigma^{Cl} \quad (17)$$

and the surface concentration of AH^+ will be greater than the net surface charge density:

$$\sigma^{AH} = \sigma^+ + \sigma^{Cl} \quad (18)$$

The zeta potential, ξ, the potential at the hydrodynamic plane of shear, was calculated from the measured value of the electrophoretic mobility, $\bar{u}$, using the Helmholtz-Smoluchowski equation:

$$\xi = \bar{u}\eta/\epsilon_r\epsilon_0 \quad (19)$$

where η is the viscosity of the aqueous phase, ϵ_r is the relative permittivity of water and ϵ_0 is the permittivity of free space. In previous studies [12,16,17], it has been concluded that ξ corresponds to the potential 2 Å from the surface of the membrane. This can be calculated from the surface potential, ψ_0, using the equations of Bentz and Nir [12,18].

In previous publications [13,14], we have shown that it is possible to estimate the concentration of drug bound to a lipid bilayer from the effect of drug binding on the temperature of the gel-to-liquid-crystalline phase transition for a lipid such as dipalmitoylphosphatidylcholine. The relationship between the depression of the phase transition temperature, ΔT_c, and the concentration of bound drug is:

$$\Delta T_c = \left(-RT_c^2/\Delta H_c\right)\cdot\ln(1-x) \quad (20)$$

where T_c is the temperature of the mid-point of the phase transition, ΔH_c is the enthalpy change of the transition and x is the mole fraction of drug in the membrane. The mole fraction of bound drug is related to the surface concentration by:

$$x = \gamma_{lipid}(\sigma^A + \sigma^{AH})/\{1 + (\gamma_{lipid} - \gamma_D)(\sigma^A + \sigma^{AH})\} \quad (21)$$

Results

Binding to lipid bilayers

The results of measurements of electrophoretic mobility of egg-yolk phosphatidylcholine in the presence of the antihistamine, methdilazine, are shown in Figs. 1 and 2 as a function of concentration and pH. Zeta potentials were calculated from mean electrophoretic mobilities by application of the Helmholtz-Smoluchowski equation (Eqn. 19).

Binding parameters were estimated from zeta potentials by fitting to the equations derived above. Zeta potentials were related to surface potentials by taking the distance between the plane of shear, at which the zeta potential is measured, and the plane of the surface charges on the liposomes to be

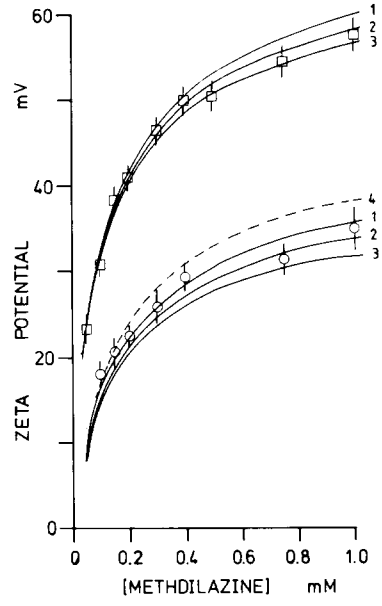

Fig. 1. Zeta potentials of liposomes of egg phosphatidylcholine (0.36 mM) in the presence of the given concentrations of methdilazine at 25°C. Buffer was 10 mM Tris-HCl (pH 7.3) containing either 10 mM NaCl (□) or 100 mM NaCl (○). Symbols represent potentials (mean ± S.D.) calculated from at least ten mobility measurements. The curves represent the predicted variation of zeta potential with drug concentration, calculated using the following parameters: $pK = 9.9$, $\Delta pK = -1.5$ and (1) $K^A = 1\cdot10^{-5}$, $\sigma^{max} = 1/60$; (2) $K^A = 6.7\cdot10^{-6}$, $\sigma^{max} = 1/90$; (3) $K^A = 5\cdot10^{-6}$, $\sigma^{max} = 1/120$. Chloride ions are assumed to bind to the protonated form of the adsorbed drug with a dissociation constant of 0.5: the dashed line (4) was calculated using the same parameters as in (2) in 100 mM NaCl, but neglecting Cl^- binding to the adsorbed drug.

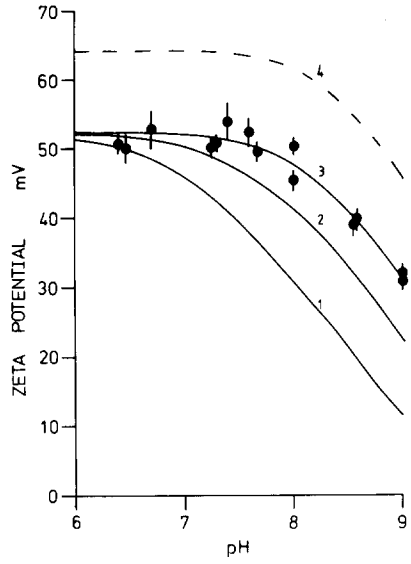

Fig. 2. Variation of zeta potential of liposomes of egg phosphatidylcholine (0.67 mM) with pH in the presence of 0.5 mM methdilazine. Buffer was 10 mM Tris-HCl/10 mM NaCl/0.1 mM EDTA, at 25°C. Symbols represent the means ± S.D. of at least ten measurements. Solid lines represent theoretical fits to the data calculated assuming $K^A = 1 \cdot 10^{-5}$, $\sigma^{max} = 1/60$, $\Delta pK = -1.5$ and (1) $pK = 9.0$, (2) $pK = 9.5$, (3) $pK = 9.9$. The dashed line (4) was calculated for $pK = 9.9$, $K^A = 1 \cdot 10^{-5}$, $\sigma^{max} = 1/60$ and $\Delta pK = -1.0$. Binding of Cl^- to the adsorbed cations was assumed, with a dissociation constant of 0.5 M.

2 Å [12,16,17]. In the analysis, the bulk pK of the drug is required. For methdilazine, this is not available in the literature but can be estimated to be between 9 and 10 by comparison with molecules of similar structure [19]. The area occupied by a lipid molecule in the surface of the membrane has been taken to be 60 Å^2 [20], and that occupied by a drug molecule has been taken to be between 30 and 60 Å^2; as is shown below, the analysis is not particularly sensitive to this figure. The variables remaining in the fitting procedure were ΔpK, the shift in pK on binding, K^A, the dissociation constant for binding of the neutral form of the drug, and σ^{max}, the maximum number of drug molecules that can be bound per unit area. As found in other studies [12,16,21], it was not possible to determine independently both K^A and σ^{max}, since within the experimentally accessible range of saturation of binding sites equally good fits to the data can be obtained over a range of values of σ^{max} and K^A provided that the quotient σ^{max}/K^A is maintained at a constant value. We have therefore fitted the data with values of σ^{max} of 1/60, 1/90 and 1/120 Å^{-2}, corresponding to unlimited binding of a drug with an area of 60 Å^2, and to binding of drugs of areas 30 and 60 Å^2, with a lipid:drug stoichiometry of 1:1, respectively.

Ignoring chloride binding, it was not possible to fit satisfactorily the data at both 0.1 M NaCl and 0.01 M NaCl (Fig. 1). Introduction of chloride binding to bound AH^+, however, allows a good fit of the data at both high and low NaCl concentrations as a function of drug concentration (Fig. 1) and as a function of pH (Fig. 2).

Chloride binding to electroneutral phospholipid membranes is normally insignificant [22], as shown by the measured zeta potential of less than −2 mV for liposomes of phosphatidylcholine in 0.1 M NaCl. The dissociation constant for binding of chloride to phosphatidylcholine cannot therefore be smaller than about 15 (M). Binding of other halides is, however, more favourable, since liposomes suspended in 0.1 M solutions of NaBr and NaI show significant negative zeta potentials (unpublished observations). Whilst the presence of a positive surface potential will concentrate Cl^- in the solution immediately adjacent to the membrane surface, the consequent reduction in potential due to chloride binding to lipid will still be insignificant. Westman and Eriksson [23] have, however, suggested that chloride binding to lipid bilayers is enhanced in the presence of adsorbed cations. We find a good fit to our data assuming a dissociation constant, K_d, of 0.5 (M) for binding of chloride to methdilazine cations adsorbed in the membrane. The effect is not significant at low concentrations of NaCl, but produces a measurable effect at 0.1 M (curve 4, Fig. 1).

As shown in Fig. 1, equally good fits can be obtained over a range of values of σ^{max} and K^A, as long as the quotient, K^A/σ^{max} is maintained approximately constant. As shown in Fig. 2 (curves 1–3), the fit is sensitive to the assumed pK value, with the best fit being obtained at 9.9. Fig. 2 also illustrates the sensitivity of the fit to the value of ΔpK. The binding parameters giving the best fit are listed in Table I.

We have shown previously that it is also possible to obtain estimates of drug-binding constants

TABLE I

BINDING CONSTANTS OF DRUGS TO LIPID BILAYERS

In all cases, binding of Cl^- to the protonated form of the adsorbed drug was accounted for with a K_d of 0.5 M. Lipid was egg-yolk phosphatidylcholine for the electrophoresis experiments, and dipalmitoylphosphatidylcholine for the transition temperature experiments. pK values were taken from (1) Ref. 19; (2) Ref. 26; (3) Ref. 27 and (4) Ref. 28. All others were determined indirectly by fitting the data.

Drug	σ^{max} (Å^{-2})	pK	ΔpK	K^A
A. From both electrophoresis and transition temperature data:				
Methdilazine	1/60	9.9	−1.5	$1.0 \cdot 10^{-5}$
Methdilazine	1/90	9.9	−1.5	$6.7 \cdot 10^{-6}$
Methdilazine	1/120	9.9	−1.5	$4.0 \cdot 10^{-6}$
Antazoline	1/60	10.1[1]	−1.5	$3.0 \cdot 10^{-4}$
Diphenhydramine	1/60	9.2	−1.5	$2.5 \cdot 10^{-4}$
Cyproheptadine	1/60	10.0	−1.5	$1.0 \cdot 10^{-5}$
Chlorpromazine	1/60	9.3[2]	−1.5	$3.0 \cdot 10^{-6}$
B. From electrophoresis data alone:				
Methylchlorpromazine	1/60	–	–	$1.0 \cdot 10^{-3}$
C. From transition temperature data alone[a]:				
Trimeprazine	1/60	9.15[2]	(−1.5)	$6.0 \cdot 10^{-6}$
Dimethothiazine	1/60	9.15[2]	(−1.5)	$8.0 \cdot 10^{-6}$
Diphenylpyraline	1/60	9.5	(−1.5)	$8.0 \cdot 10^{-5}$
Mepyramine	1/60	8.9[1]	(−1.5)	$8.0 \cdot 10^{-4}$
Pheniramine	1/60	9.0	(−1.5)	$1.0 \cdot 10^{-3}$
Triprolidine	1/60	8.77[3]	(−1.5)	$2.0 \cdot 10^{-3}$
Dibucaine	1/60	8.5[4]	(−1.5)	$5.5 \cdot 10^{-5}$

[a] Fitted assuming $\Delta pK = -1.5$.

from measurements of the effects of drugs on the phase transition temperatures of lipids [14]. The binding constants derived from the electrophoresis data for methdilazine differ from those previously estimated, and zeta potentials calculated from the previous binding constants with a ΔpK of zero [14] are considerably higher than those observed experimentally. However, the present set of binding constants (Table I) also provide a good fit to the data on the depression of the phase transition temperature in dipalmitoylphosphatidylcholine (Figs. 3 and 4). We have refined our previous calculations [14] in four ways. Firstly, instead of a value of 9.67 kcal/mol for the transition enthalpy of dipalmitoylphosphatidylcholine [24], we have used a value of 8.7 kcal/mol as determined by Wilkinson and Nagle [25]. Secondly, we now take account of the temperature-dependence of the pH of Tris buffer, which can cause significant alterations in the relative concentrations of the charged and uncharged forms of the drug. Thirdly, our analysis now allows for the binding of a significant fraction of the drug to the bilayer and takes account of the contribution of the drug to the membrane surface area. Fourthly, we now take account of the possibility of Cl^- binding to the adsorbed drug. Calculations of the depression of transition temperature (Eqns. 20 and 21) using the parameters derived from the electrophoresis experiments gives very good agreement with the experimental data. Compared with our previous estimations, the net result of these changes is to keep the total amount of drug bound to the membrane approximately the same, but to decrease the proportion of drug that is bound in the charged form.

Similarly good agreement is obtained for other drugs, including some for which the pK is known (antazoline, chlorpromazine and diphenhydramine) (Table I). Despite the very different structures of these compounds, all can be fitted with a ΔpK value of −1.5. We have therefore refitted our previous data for effects of drugs on transition

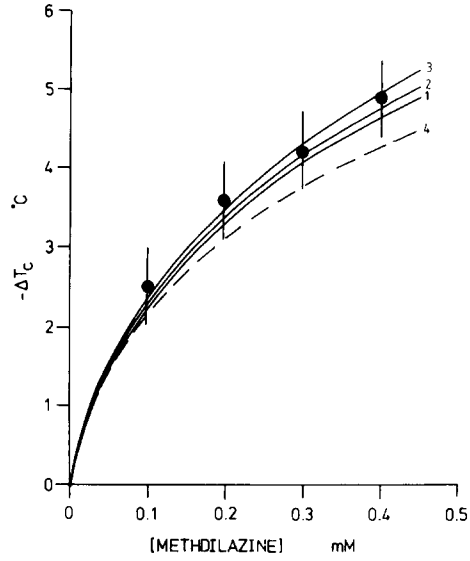

Fig. 3. Variation of the depression of the mid-point temperature (T_c) of the gel-liquid crystalline phase transition in dipalmitoylphosphatidylcholine as a function of methdilazine concentration. Lipid concentration was 0.15 mM. Buffer was 10 mM Tris-HCl/0.1 M NaCl, pH 7.2 at 20°C. Solid lines show theoretical fits to the data calculated assuming $pK = 9.9$, $\Delta pK = -1.5$ and (1) $K^A = 1 \cdot 10^{-5}$, $\sigma^{max} = 1/60$, (2) $K^A = 6.7 \cdot 10^{-6}$, $\sigma^{max} = 1/90$, (3) $K^A = 5 \cdot 10^{-6}$, $\sigma^{max} = 1/120$, and assuming that Cl^- binds to the adsorbed cation with a dissociation constant of 0.5. The dashed line (4) was calculated as for (2), but neglecting Cl^- binding.

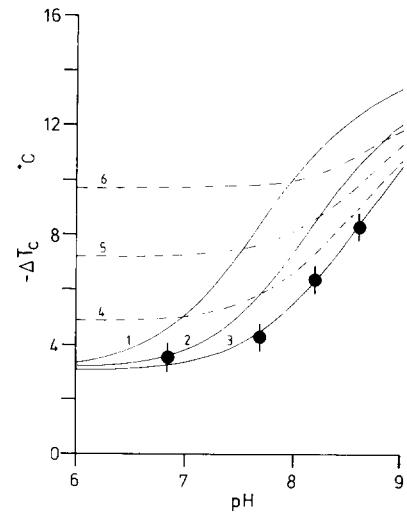

Fig. 4. Variation of the depression of the mid-point temperature (T_c) of the gel-liquid crystalline phase transition in dipalmitoylphosphatidylcholine (0.15 mM) as a function of pH at the transition temperature in the presence of 0.5 mM methdilazine. Buffer was 10 mM Tris HCl/0.1 M NaCl. Solid lines represent theoretical fits to the data calculated for $K^A = 1 \cdot 10^{-5}$, $\sigma^{max} = 1/60$, $\Delta pK = -1.5$ and (1) $pK = 9.0$, (2) $pK = 9.5$, (3) $pK = 9.9$. Dashed lines were calculated for $pK = 9.9$, $K^A = 1 \cdot 10^{-5}$, $\sigma^{max} = 1/60$ and (4) $\Delta pK = -1.0$, (5) $\Delta pK = -0.5$, (6) $\Delta pK = 0$. Binding of chloride ions to the adsorbed cations with a dissociation constant of 0.5 was assumed.

temperatures [14] to conform to this value of ΔpK with the results given in Table I.

Effects on the (Ca^{2+} + Mg^{2+})-ATPase

The data in Figs. 1 and 2 show that the binding of charged hydrophobic drugs to phospholipid bilayers can result in high surface potentials. In the same way, binding to the lipid component of biological membranes could be expected to produce significant changes in the surface potential of the membrane. For the (Ca^{2+} + Mg^{2+})-ATPase/lipid system, the introduction of positive charge into the membrane by binding to the lipid component will decrease the concentration of Ca^{2+} close to the surface of the membrane according to the Boltzmann relationship, and so reduce the apparent affinity of the ATPase for Ca^{2+}. If K_m^0 and K_m^1 are the affinities of the ATPase for Ca^{2+} in the absence and presence of drug respectively, then:

$$K_m^1 = K_m^0 \cdot \exp(2F\psi/RT) \quad (22)$$

where ψ is the potential at the Ca^{2+}-binding site on the ATPase and the factor of 2 represents the valency of Ca^{2+}. The apparent affinity of the ATPase for Ca^{2+} can be estimated from plots of ATPase activity as a function of Ca^{2+} concentration at saturating concentrations of ATP. Such plots cannot be fitted to a simple binding curve, and show positive co-operativity. Non-linear least-squares fits to the Hill equation give values for the Hill coefficient, n, between 1.2 and 1.9, the value of n varying between preparations, with storage conditions and with buffer. Highsmith [29] has also reported large changes in n with sample treatment.

Fig. 5 shows the effect of methdilazine on ATPase activity as a function of free Ca^{2+} con-

TABLE II

RESULTS OF FITS OF THE Ca^{2+}-DEPENDENCE OF THE ($Ca^{2+} + Mg^{2+}$)-ATPase TO THE HILL EQUATION

The Hill equation is: $v_0/V_{max} = S^n/(K_H + S^n)$, where v_0 and V_{max} are the initial and the maximum rates observed under conditions of substrate excess, S is the substrate concentration and n the Hill coefficient. K_H is a constant related to K_m in the Michaelis-Menten equation, and has units of $(mol/l)^n$. The substrate concentration for half-maximal activity ($S_{0.5}$) is therefore the nth root of K_H. One international unit is 1 μmol phosphate formed per min per mg ATPase protein. K_i was obtained as the drug concentration necessary for 50% inhibition of ATPase activity at high [Ca^{2+}] ($pCa = 5.4$).

Drug	Concentration (mM)	V_{max} (I.U.)	K_H	n	$S_{0.5}$ (pCa)	K_i (mM)
Control		10.8 ± 0.2	0.22 ± 0.03	1.23 ± 0.05	6.53	
Methdilazine	0.2	3.6 ± 0.3	0.76 ± 0.16	1.23 ± 0.16	6.10	0.065
Control		7.5 ± 0.1	0.084 ± 0.005	1.6 ± 0.03	6.67	
Chlorpromazine	0.2	2.8 ± 0.9	0.887 ± 0.65	1.46 ± 0.28	6.04	0.200
Control		15.3 ± 0.2	0.212 ± 0.02	1.53 ± 0.05	6.44	
Methylchlorpromazine	0.5	8.4 ± 2.6	0.323 ± 0.51	2.24 ± 1.03	6.22	0.600
Control		21.5 ± 1.4	0.131 ± 0.06	1.95 ± 0.25	6.45	
Diphenhydramine	3.2	18.1 ± 1.5	0.190 ± 0.09	2.96 ± 0.50	6.24	> 6.0 [a]
Control		22.3 ± 1.2	0.363 ± 0.09	1.51 ± 0.15	6.29	
Dansyl propranolol	0.012	6.3 ± 0.4	0.895 ± 0.12	1.5 ± 0.2	6.03	0.005

[a] Plots of ATPase activity at high [Ca^{2+}] against drug concentration were biphasic for diphenhydramine. Enzyme activity decreased abruptly at drug concentrations over 6 mM. Possibly this was due to a detergent effect of the drug at such high concentrations.

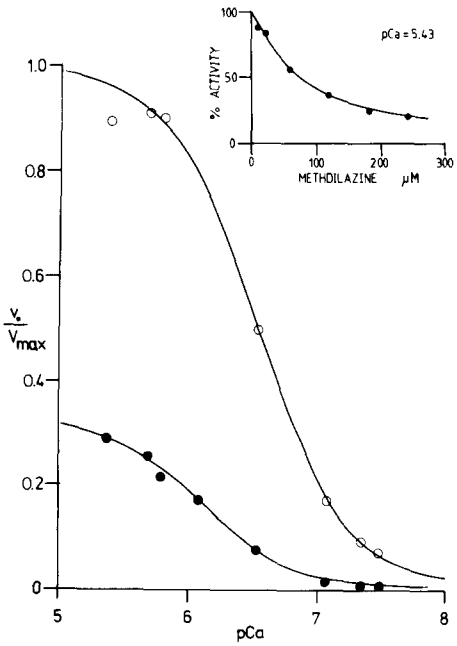

Fig. 5. ($Ca^{2+} + Mg^{2+}$)-ATPase activity as a function of free Ca^{2+} concentration in the presence (●) and absence (○) of 0.2 mM methdilazine. The lines are the results of non-linear least-squares fits of the experimental data to the Hill equation, using the parameters listed in Table II. Insert shows the effect of increasing drug concentration on ATPase activity at a high ($pCa = 5.43$) Ca^{2+} concentration. The line is a fit of the data to a simple binding model where the drug binds with a K_i (concentration for half-maximum inhibition) of 65 μM.

centration. There are clearly two effects: a reduction in V_{max} of the ATPase and a decrease in the apparent affinity for Ca^{2+}. Non-linear least-squares fits to the Hill equation suggests no effect on the Hill coefficient, n. The inset to Fig. 5 shows the effect of increasing concentration of methdilazine on the ATPase at a Ca^{2+} concentration sufficient for maximal activation of the enzyme; a simple (hyperbolic) inhibition curve calculated with an inhibition constant, K_i, of 65 μM agrees well with the experimental data.

Similar effects are observed with chlorpromazine (and its quaternary amine methyl derivative), diphenhydramine and the fluorescent probe dansyl propranolol [4] and the results are summarised in Table II. Non-linear regression to the Hill equation was used in order to establish V_{max} and to detect any changes in co-operativity due to the presence of drug. Estimated values of both the Hill coefficient (n) and the free Ca^{2+} concentra-

tion necessary to obtain half-maximal activity ($S_{0.5}$) varied between batches of ATPase, and therefore a control experiment was performed for each drug tested. Effects of drugs on n were generally small, the only large effect being observed for diphenhydramine with an ATPase preparation exhibiting an unusually high initial n value (Table II).

Discussion

Firstly, the detection of large, positive zeta potentials for liposomes in the presence of a variety of drugs containing an amine group show that the charged form of the drug is able to bind to bilayers of phosphatidylcholines. It has sometimes been suggested that the charged forms of local anaesthetics are unable to bind to bilayers of zwitterionic lipids [30,31]. We have shown that it is possible to describe the charge effects that follow from drug binding with a formalism based on the Langmuir adsorption isotherm. The Langmuir isotherm was originally developed on the assumption of spatially fixed adsorption sites (localised adsorption) and it might be more appropriate to use an isotherm such as the Volmer isotherm [32]. However, this isotherm is less convenient mathematically and we find, as reported by McLaughlin and Harary [21], that in the limit $\sigma \ll \sigma^{max}$ (which corresponds to the experiments reported here) the two are indistinguishable provided that σ_v^{max} is equated with $2\,\sigma_l^{max}$ and that K_v is equated to $2K_l$ where the subscripts v and l correspond to parameters used in the Volmer and Langmuir isotherms, respectively.

As in previous studies [12,16], it was not possible to obtain values for K^A and σ^{max} separately. The quotient K^A/σ^{max} was, however, well defined so that it is possible to use the parameters given in Table I to calculate amounts of drug bound to the membrane in the region $\sigma \ll \sigma^{max}$, which corresponds to the experimentally useful range. For all the drugs tested, it was necessary to assume a large decrease in pK on binding, favouring binding of the neutral form of the drug. Fromherz [33] has reported a similar shift in pK (+0.6 to +1.7) for binding of the anionic pH indicator 4-heptadecylumbelliferone, and Westman et al. [34] have reported shifts in pK of −0.8 to −1.6 on binding of tetracaine and procaine, respectively.

In making previous estimates of binding constants for drugs to lipid bilayers based on effects on transition temperatures, data were fitted assuming no shift in pK on binding [14]. Since the shift in transition temperature depends on the total concentration of bound drug, whereas the electrophoretic measurements detect only the concentration of the bound charged form of the drug, the latter is more sensitive to shifts in pK value. Good fits to the observed shifts in transition temperature can be obtained using the binding parameters derived from electrophoresis experiments, for methdilazine, antazoline, chlorpromazine, diphenhydramine and cyproheptadine, all with a ΔpK value of −1.5. Since this shift in pK on binding seems a very general phenomenon, we have refitted some of our other data with this value of ΔpK, to give the binding constants listed in Table I.

We have also studied the effects of some of these drugs on the activity of the $(Ca^{2+} + Mg^{2+})$-ATPase that can be purified from sarcoplasmic reticulum. We believe that the effects of drugs on V_{max} of the ATPase are due to direct binding of the drug to the ATPase [4]. Effects of drugs on the apparent Ca^{2+} affinity could, however, be an indirect effect of changing the charge on the membrane. Although calculations of such effects must be very model-dependent, we can readily show that charge effects are large enough to explain the observed shifts.

As shown in the following paper [4], binding constants for drugs to the lipid component of the $(Ca^{2+} + Mg^{2+})$-ATPase/lipid system appear to be comparable to those for simple lipid bilayers. It should therefore be possible to use the binding constants listed in Table I to describe binding to the lipid component of the ATPase system, and to calculate the change in surface potential that results from such binding. Introduction of positive charge into the membrane will reduce the apparent affinity of the ATPase for Ca^{2+} by reducing the Ca^{2+} concentration close to the membrane surface. Taking methdilazine as an example, we see from Fig. 5 and Table II that the apparent K_m for Ca^{2+} ($S_{0.5}$, the concentration of Ca^{2+} necessary for half-maximal activity) is shifted from pCa 6.53 to pCa 6.10 in the presence of 0.2 mM methdilazine. Application of Eqn. 22 shows that this shift would

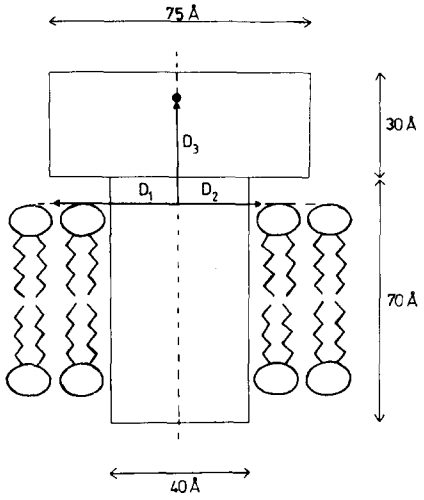

Fig. 6. Cross-sectional view of $(Ca^{2+} + Mg^{2+})$-ATPase in a membrane, based on the data of Le Maire et al. [36]. Adjacent proteins are omitted for clarity. The cytoplasmic face of the protein is at the top. Distance D_1 represents the distance from the axis of the protein to drug molecules bound in the lipid phase. Distance D_2 represents the distance from the axis of the protein to drug molecules bound at the protein-lipid interface (annular sites). Distance D_3 represents the distance of the Ca^{2+}-binding site from the plane of the lipid headgroups.

arise from an increase of 13.3 mV in the potential at the Ca^{2+}-binding site on the ATPase.

Using the lipid binding constants for methdilazine (Table I), and knowing the ATPase concentration (0.05 μM) and the lipid/protein ratio (30:1), we can calculate the surface potential (ψ_0) on the lipid portion of the membrane due to the binding of the drug. Under the conditions of the experiment illustrated in Fig. 5, 0.2 mM methdilazine creates a surface potential of 49 mV. In this calculation we have ignored the contribution of negatively charged lipid (acidic phospholipids make up approx. 10% of the total phospholipid) to the potential at the Ca^{2+}-binding site, since this contribution will not be affected by addition of the drug.

The potential some distance, x, from the surface can be calculated using the equations derived by Bentz and Nir [12,18] to describe the dependence of potential on distance in solutions containing multiple ionic species. For the potential at the Ca^{2+}-binding site to be 13.3 mV, the site must be approx. 20 Å from the lipid bilayer surface. This distance is not unreasonable in view of structural studies [35–37] which depict the ATPase (monomer) as being about 40 Å in diameter and extending some 40 Å into the aqueous phase from the cytoplasmic face of the sarcoplasmic reticulum membrane (Fig. 6).

In the above calculation, the charge was averaged only over the lipid component of the membrane. However, at the high protein/lipid ratio of the ATPase preparation, a large fraction of the membrane surface will be occupied by protein (approx. 60%) and it may be that the surface charge density due to drug binding to the lipid bilayer component of the membrane should be averaged over the entire membrane surface; the result would be to reduce the surface potential to approx. 22 mV, and the corresponding distance between the charged surface and the Ca^{2+}-binding site to 8 Å.

The calculation should be further refined to take account of drug binding at the lipid-protein interface, which can be detected using fluorescent probes [4,38,39]. For dansyl propranolol it has been shown that the binding constant obtained from fluorescence titrations is equal to the inhibition constant, K_i, obtained from plots of ATPase activity as a function of the concentration of dansyl propranolol at high Ca^{2+} concentrations [4]. We can therefore tentatively equate inhibition constants for the drugs studied here with binding constants at the lipid/protein interface. For methdilazine, the inhibition constant is 65 μM (Fig. 5) so that in the presence of 0.2 mM drug, the annular sites will be 75% occupied. The pK of drug bound at the lipid/protein interface is not known. For simplicity, we will assume it to be equal to the bulk pK. With 30 annular sites (see Ref. 4), then under the conditions of Fig. 5, each ATPase molecule will have associated with it an average of 25.4 additional positive charges, comprised of 22.5 protonated drug molecules bound at the annular sites and 2.8 bound to the surrounding lipid. If we consider only one face of the membrane, as under the assay conditions the Debye length $1/k$ is only 16 Å, then we have a surface charge density of 12.7 charges per 2157 Å^2 (area of protein plus 15 lipids), which corresponds to a uniformly charged surface with $\psi_0 = 114$ mV. To

obtain the observed potential of 13.3 mV at the Ca^{2+}-binding site the site would be some 29 Å from the surface.

It is arguable whether or not the uniform-field approach used above is appropriate to describe the microenvironment of a site on a membrane protein. For example, binding of a small number of positively charged drug molecules in very close proximity to the Ca^{2+}-binding site would grossly distort the local field. If we possessed detailed structural information on the ATPase and its organisation within the membrane, then a discrete-charge approach might well be preferable. In the absence of such detailed structural information we can only guess at the location of the Ca^{2+}-site in relation to bound drug molecules. We might, for example, take the structural model of Le Maire et al. [36] illustrated in Fig. 6, and suggest, for mathematical convenience, that the Ca^{2+}-binding site is located somewhere on the cytoplasmic projection of the molecule, on the axis of symmetry and at some undetermined height above the plane of the lipid headgroups. Under these constraints we can apply a discrete-charge treatment such as that of Brown [40] in which the electrostatic potential at any point, x, depends on the sum of the fields due to n point charges arranged on the membrane surface:

$$\psi_x = (q(1+k_2)/4\pi\epsilon_0\epsilon_w) \sum_{n}^{i=1} [\exp(-kr_i)/r_i] \qquad (23)$$

where q represents a quantity of charge (in this case, one formal positive charge, $1.6021 \cdot 10^{-19}$ abs. coulombs), ϵ_0 is the permittivity of free space and ϵ_w is the relative permittivity (dielectric constant) of the aqueous medium, k_2 is a constant reflecting the difference between the dielectric constants of water and membrane (estimated as 0.882 in Ref. 40), k is the Debye reciprocal length constant, approx. 1/16 Å under the assay conditions, and r_i is the distance between x and the ith of the

TABLE III

CHARGE CALCULATIONS FOR DRUG EFFECTS ON THE $(Ca^{2+} + Mg^{2+})$-ATPase

ψ_x is the apparent increase in potential at the Ca^{2+}-binding site, calculated from the data in Table II using Eqn. 22. For (A), the surface potential (ψ_0) is calculated for drug binding to the lipid component of the ATPase under the assay conditions, using the binding constants listed in Table I. The distance, x, is the required separation between the Ca^{2+}-binding site and a uniformly charged surface of surface potential, ψ_0, to give potential ψ_x. In (B), the surface charge density σ^+ used to calculate ψ_0 in (A) has been averaged over the entire membrane area to give a new, lower, ψ_0. The surface area occupied by a lipid has been taken as 60 $Å^2$, and that occupied by a protein molecule as 1257 $Å^2$. Protein/lipid ratio is 1:30. In (C), the number of annular sites on the ATPase molecule has been taken as 30 [4], of which 15 are on the same side of the membane as the Ca^{2+}-binding site. If the pK of drug bound at the annular sites is equal to the bulk pK (Table I), then all the protein-bound drug will be ionized. The dissociation constant for drug binding to the annular sites is taken to be the same as that listed in Table II as K_i (not obtainable for diphenhydramine).

Drug:	Methdilazine	Chlorpromazine	Methylchlorpromazine	Diphenhydramine	Dansyl propranolol
Concn. (mM)	0.2	0.2	0.5	3.2	0.012
ψ_x (mV)	13.3	19.6	13.6	6.4	8.0
A. Surface potentials (ψ_0) calculated over area of lipid alone					
ψ_0 (mV)	48.6	62.1	46.4	40.4	33.9
x (Å)	19.7	16.9	18.7	28.4	22.5
B. Surface potentials averaged over entire membrane area					
ψ_0 (mV)	22.3	30.5	21.4	18.7	15.3
x (Å)	8.1	6.8	7.1	17.0	8.3
C. As in (B), but allowing for binding of drug to annular sites with pK = pK_{bulk}					
ψ_0 (mV)	114.0	98.4	90.7	–	107.8
x (Å)	29.4	22.1	27.0	–	37.0
D. As in (C), but using a discrete charge treatment [40]					
D_3 (Å) [a]	19.7	6.0	11.4	–	8.4

[a] See Fig. 6.

n positive charges. We can use Eqn. 23 to calculate the potential at any height, D_3, in Fig. 6, due to the presence of 11.3 positive charges located at the annular sites (distance D_2 from the mid-line in Fig. 6) and 1.4 positive charges bound to the lipid (distance D_1). A potential of 13.3 mV is obtained at a distance $D_3 = 19.7$ Å, well within the physical limits set by the size of the protein.

The results of these calculations for methdilazine and for the other drugs tested on the $(Ca^{2+} + Mg^{2+})$-ATPase are set out in Table III.

Our main conclusion is that the predictable consequences of binding of charged drug are sufficient to explain the observed effect on the Ca^{2+}-dependence of the $(Ca^{2+} + Mg^{2+})$-ATPase, whether calculated from uniform-field or symmetrically-arranged discrete-charge models. As relatively little is known about the structure of the ATPase, the uniform-charge approach may be preferred on grounds of simplicity. It is interesting that the calculations involving only lipid-bound drug give quite a consistent estimate of the position of the Ca^{2+}-binding site, whereas when the possible annular binding is taken into account, the results are more variable. This could be a reflection of effects of surface roughness, with the charge arising from drug molecules bound at the lipid/protein interface being shielded to some extent by the 'cross-piece' of the ATPase molecule. Unfortunately, surface potential effects due to drug binding to membranes containing protein cannot readily be quantitated by electrophoresis. Thus we find that binding of chlorpromazine (0.1–0.4 mM) produces only a small increase (5.7 mV) in the zeta potential of vesicles of sarcoplasmic reticulum (protein/lipid molar ratio, 1:90), because the hydrodynamic plane of shear, at which the zeta potential is measured, is relatively distant from the lipid surface (see Fig. 6). Since hydrodynamic calculations are not possible for such molecularly rough surfaces, surface potentials cannot be obtained from measurements of zeta potentials for protein-containing membranes.

Acknowledgements

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