Ca^{2+} Binding to Phosphatidylcholine Bilayers As Studied by Deuterium Magnetic Resonance. Evidence for the Formation of a Ca^{2+} Complex with Two Phospholipid Molecules†

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ABSTRACT: The binding of Ca^{2+} to bilayer membranes composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was investigated with atomic absorption spectroscopy and deuterium magnetic resonance, leading to the following conclusions. (1) Atomic absorption spectroscopy allowed the determination of the amount of Ca^{2+} bound to the membrane surface (C_b) at low Ca^{2+} concentrations (3–100 mM). (2) Simultaneous measurements of the deuterium magnetic resonance spectra of POPC with specifically deuterated choline head groups revealed a linear relationship between the quadrupole splitting and the amount of bound Ca^{2+}. (3) With this calibration, the amount of bound Ca^{2+} could be determined from the deuterium spectra under conditions where atomic absorption spectroscopy was technically not feasible, i.e., in the concentration range of 0.1–5 M $CaCl_2$. (4) The Ca^{2+} binding isotherm exhibited saturation behavior. The quadrupole splitting at the saturation limit corresponded to a binding stoichiometry of one Ca^{2+} per two POPC molecules. (5) The surface charge density (σ) could be evaluated from the amount of bound Ca^{2+} and the surface area per POPC molecule. By employing the Gouy–Chapman theory, it was then possible to determine the surface potential (ψ_0) and the Ca^{2+} concentration immediately at the lipid–water interface (C_I). (6) With this set of experimental parameters, various models for the mode of Ca^{2+} binding were tested. A simple partition equilibrium or a Langmuir absorption model could be ruled out. However, a very good fit to the experimental data was obtained by applying the law of mass action in the form $C_b/(1 - 2C_b)^2 = KC_I$ in which K is the only adjustable parameter. This model independently supports the above conclusion of a Ca^{2+} to phospholipid stoichiometry of 1:2. (7) For POPC in the liquid-crystalline state, this model predicts Ca^{2+} binding constants of K = 13.8 M^{-1} (0.1 M NaCl, 25 °C) and 7.0 M^{-1} (no NaCl, 40 °C).

The adsorption of calcium to the surface of biological membranes has important physiological consequences [for reviews, see McLaughlin (1977) and Scarpa & Carafoli (1978)]. Studies on model membranes have demonstrated that these physiological effects may be due not only to a binding of Ca^{2+} but also to a screening effect in the aqueous diffuse double layer at the lipid–water interface (McLaughlin et al., 1971). Different physicochemical techniques have been employed in order to elucidate in more detail the mechanism of metal ion binding, the most prominent among these being electrophoretic measurements (Bangham & Dawson, 1962; McLaughlin, A., et al., 1978, 1983; McLaughlin, S., et al., 1971), nuclear magnetic resonance (NMR)[1] [cf. Hauser et al. (1977), Hutton et al. (1977), Grasdalen et al. (1977), McLaughlin et al. (1978), Westman & Eriksson (1979) Hauser & Phillips (1979), and Chrzeszczyk et al. (1981)], and X-ray diffraction (Inoko et al., 1975; Oshima & Mitsui, 1978; Lis et al., 1981a,b; Rand, 1981; Oshima et al., 1982). As a result of these studies, it is generally agreed that the binding of Ca^{2+} to a neutral lipid bilayer such as a phosphatidylcholine membrane is rather weak, and the reported binding constants are typically on the order of 1–100 M^{-1}.

However, the exact mechanism of Ca^{2+} binding is still not well understood for a number of reasons. First, the amount of Ca^{2+} bound to the membrane surface has not been measured directly but was inferred from other parameters, such as the electrophoretic mobility (McLaughlin et al., 1978), the competition of Ca^{2+} with lanthanide shift reagents (Grasdalen et al., 1977), or the electrostatic interbilayer repulsive force (Lis et al., 1981a,b; Oshima et al., 1982). As far as the data were interpreted in terms of the Gouy–Chapman theory, two adjustable parameters, the binding constant and the surface potential (ψ_0), had to be evaluated from the same set of experimental parameters [cf. McLaugthlin et al. (1983) and references cited therein]. In experiments involving shift reagents, the hyperfine shift constant had to be fitted as a third independent parameter. The complexity and ambiguity of such multiparameter fits make a critical comparison of various binding models difficult. Second, metal binding studies have been performed with either single-shelled vesicles (NMR shift reagents, electrophoretic mobility) or coarse lipid dispersions (X-ray diffraction). In the latter experiments, the swelling properties of lipid bilayers as a function of metal ion concentration and osmotic pressure were measured with X-ray small-angle scattering. The binding of metal ions imparts a net positive charge to the membrane surface, causing an electrostatic repulsion between adjacent bilayers to which the system reacts by imbibing more water. As a net effect, the binding constant becomes dependent on the interlamellar distance, i.e., the thickness of the water layer. It is therefore important to determine the binding constant of metal ions under conditions where the electrostatic repulsion is sufficiently screened by counterions. Finally, most previous studies have been limited to low concentrations of metal ions ($C_{Me^{2+}} \leq 30$ mM in NMR studies and $\leq$100 mM in X-ray diffraction studies). An extension of the concentration range would be desirable in order to allow a better differentiation between the various modes of ion binding.

In the present study, we have used ^2H NMR to study the binding of Ca^{2+} to unsaturated phosphatidylcholine bilayers. As has been reported previously, the binding of Ca^{2+} and other multivalent metal ions causes a small conformational change of the phosphocholine polar group which is reflected in distinct changes of the quadrupole splittings of the two choline

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[1] Abbreviations: NMR, nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine.

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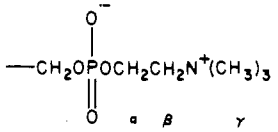
methylene segments (Brown & Seelig, 1978; Akutsu & Seelig, 1981). The strength of the interaction increases with increasing charge and concentration of the metal ion, and the residual quadrupole splitting may change by as much as 10 kHz. This effect is limited to the phosphocholine head group since deuterated segments in the hydrocarbon chains or the glycerol backbone are not affected under the same conditions.

We have previously reported the binding isotherms of Ca^{2+} and La^{2+} for membranes composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) in the concentration range of 5 mM to 2 M (Akutsu & Seelig, 1981). The present work is characterized by the following new features. (1) The Ca^{2+} binding isotherm was determined for an unsaturated lipid. Naturally occurring lipids contain a cis double bond in the *sn*-2 chain which increases the surface area per lipid by about 10–20 $Å^2$ compared to the saturated analogues. The larger spacing between the polar groups should be reflected in a different binding affinity for metal ions. As a lipid, we have chosen 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) which is one of the most common naturally occurring lipid species. (2) By means of atomic absorption spectroscopy, we have measured the amount of Ca^{2+} bound to the membrane surface, which, in turn, allowed a calibration of the quadrupole splitting. The Ca^{2+} binding to the POPC membrane could thus be evaluated over the whole concentration range investigated (3 mM–5 M $CaCl_2$). (3) Due to this information, the membrane surface charge density (σ) and the surface potential (ψ_0) could be determined prior to and independent of the evaluation of the binding constant. The assumption made was the applicability of the Gouy–Chapman theory in order to relate the surface potential to the surface charge density. It was further assumed that no anions bind to the membrane. (4) The knowledge of the amount of bound Ca^{2+} and of the Ca^{2+} concentrations at the lipid–water interface allowed a detailed comparison of various binding models.

Materials and Methods

For simplification of discussion, the following nomenclature is employed for the phosphocholine head-group segments:



1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was selectively deuterated at the α- and β-segments as described by Tamm & Seelig (1983).

Measurement of Ca^{2+} Binding by Atomic Absorption Spectroscopy. In a typical experiment, 20 mg (26.28 μmol) of α-CD_2-POPC was suspended in 400 μL of $CaCl_2$ solution containing in addition 0.1 M NaCl. Equilibrium was attained by several freeze–thaw cycles, leaving the suspension at rest for 48 h at 4 °C, and vortexing from time to time at 25 °C for a period of 6 h. During all manipulations, the lipid was kept in the dark. The suspension was then centrifuged in a desk centrifuge for 10 min and the clear supernatant removed with a pipet.[2] The supernatant was diluted to a concentration suitable for atomic absorption spectroscopy and the Ca^{2+} concentration determined with a Perkin-Elmer 5000 atomic absorption spectrophotometer. As a control, the Ca^{2+} concentrations of the starting solutions were also determined, yielding deviations of less than ±1.5% from the nominal concentration. In contrast, incubation with POPC reduced the Ca^{2+} concentration of the supernatant by 6–17%, indicating a considerable binding of Ca^{2+} to the POPC bilayers. From the difference between the Ca^{2+} content of the supernatant and that of the starting solution, it was possible to calculate the amount of Ca^{2+} bound per molecule of POPC. This parameter is denoted C_b (moles of Ca^{2+} per mole of POPC) in the following. The Ca^{2+} concentration of the supernatant is identical with $C_{CaCl_2}^{eq}$, which is the thermodynamic equilibrium concentration of free Ca^{2+} in bulk solution. Atomic absorption spectroscopy measurements were performed in the concentration range of 3–100 mM $CaCl_2$ (plus 0.1 or 1.0 M NaCl). At Ca^{2+} concentrations larger than 100 mM, the change in the Ca^{2+} content due to binding to POPC was too small to allow an accurate enough measurement.

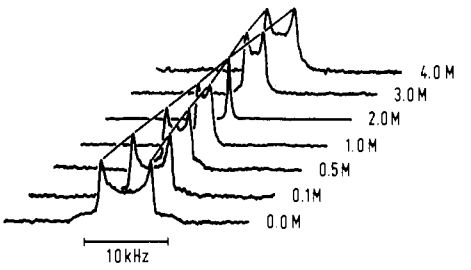


FIGURE 1: ^2H NMR spectra of coarse dispersions of POPC bilayers at various $CaCl_2$ concentrations (no NaCl). The lipid was deuterated at the α-segment ($-NCH_2CD_2OP-$). Measuring temperature, 40 °C.

Deuterium NMR. All ^2H NMR studies were performed with coarse lipid dispersions since no quadrupole splitting can be observed with sonicated bilayer vesicles due to rapid vesicle tumbling (Stockton et al., 1976). ^2H NMR spectra were recorded at 46.1 MHz with a quadrupole echo technique (Davis et al., 1976). The experimental conditions were the same as those described earlier (Seelig et al., 1981; Tamm & Seelig, 1983). For the ^2H NMR measurements, two different sets of samples were prepared. In the first set, the lipid pellets from the atomic absorption spectroscopy studies were used without further manipulations. The observed quadrupole splitting could then be related to the concentration of bound Ca^{2+}, C_b, and to the equilibrium concentration of the bulk solution, $C_{CaCl_2}^{eq}$. In the second set of experiments, the lipid (~20 mg) was dispersed in a vast excess of buffer (~10 mL) and centrifuged to form a pellet. Since the relative change in Ca^{2+} concentration was small under these conditions, the Ca^{2+} concentration of the starting solution was identical with the thermodynamic equilibrium concentration $C_{CaCl_2}^{eq}$. In this type of experiment, the experimental quadrupole splitting could only be related to $C_{CaCl_2}^{eq}$ whereas C_b was not determined.

The gel to liquid-crystal phase transition of pure α- and β-CD_2-POPC in water was −5 °C as determined from the broadening and the loss of the ^2H NMR signal at and below this temperature, respectively. This transition temperature is in agreement with differential scanning calorimetry studies on nondeuterated POPC (de Kruijff et al., 1973). At small and medium Ca^{2+} concentrations, the phase transition of POPC was not altered. However, at the highest Ca^{2+} concentrations measured (C_{CaCl_2} = 5 M), the transition temperature was raised by about 30 °C.

Results

Figure 1 shows a sequence of ^2H NMR spectra of α-CD_2-POPC dispersions recorded at increasing concentrations

[2] A clear supernatant is obtained only if the suspension also contains NaCl at concentrations >0.1 M. POPC suspended in pure $CaCl_2$ solutions (3–100 mM) exhibited a quite pronounced swelling [cf. Inoko et al. (1975) and Lis et al. (1981a,b)]. Even after high-speed centrifugation for an extended period, the supernatant still retained a slightly milky appearance.

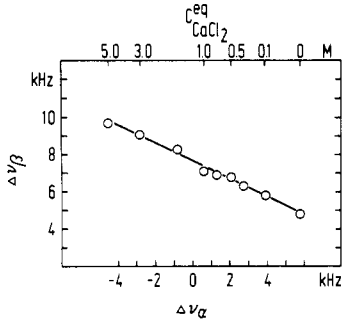


FIGURE 2: Comparison of the quadrupole splittings of the α- and β-segments of POPC bilayers at various $CaCl_2$ concentrations (plus 0.1 M NaCl). Each data point corresponds to two separate measurements with α- and β-deuterated POPC dispersed in the same buffer. The observed quadrupole splitting at the β-segment is plotted vs. that of the α-segment. Measuring temperature, 40 °C.

of $CaCl_2$. In the absence of Ca^{2+}, the residual quadrupole splitting amounts to 6.1 kHz. As the Ca^{2+} concentration is raised, the quadrupole splitting decreases, collapses to a single resonance around 2 M $CaCl_2$, and reverses its sign at even higher Ca^{2+} concentrations. It should be noted that only the absolute value of the quadrupole splitting is accessible by ^2H NMR. The assignment of a positive quadrupole splitting to α-CD_2-POPC in the absence of Ca^{2+} is therefore arbitrary.

Monovalent ions alone have little effect on the ^2H NMR spectra. At 1 M NaCl (no Ca^{2+} present), the quadrupole splitting of α-CD_2-POPC is 5.5 kHz, which is only a few hundred hertz smaller than that for α-CD_2-POPC in pure water.

However, the addition of NaCl enhances the binding of $CaCl_2$. α-CD_2-POPC in the presence of about 7 mM $CaCl_2$ exhibits a splitting of 5.2 kHz; if the same measurement is repeated with 1 M NaCl, the quadrupole splitting is reduced to 4.3 kHz. A similar effect has been noted before for DPPC bilayers (Akutsu & Seelig, 1981).

Addition of Ca^{2+} and other metal ions to bilayers of β-CD_2-POPC *increases* the quadrupole splitting of this segment. In particular, if the α-segment is reduced to $\Delta\nu_\alpha = 0$ kHz, the β-segment exhibits a splitting of $\Delta\nu_\beta = 7.6$ kHz, excluding the possibility of a completely random motion of the phosphocholine head group. Empirically, a simple linear relationship between the two quadrupole splittings $\Delta\nu_\alpha$ and $\Delta\nu_\beta$ has been observed for DPPC (Akutsu & Seelig, 1981). A quantitatively similar result was obtained in the present study of POPC. Figure 2 shows a plot of $\Delta\nu_\beta$ vs. $\Delta\nu_\alpha$ at corresponding $CaCl_2$ concentrations at 40 °C, yielding a straight line with

$$\Delta\nu_\beta = -0.49\Delta\nu_\alpha + 7.6 \text{ kHz} \quad (1)$$

In principle, both segments may hence be used to investigate the binding of metal ions. However, eq 1 demonstrates that the spectral changes at the α-segment are twice as large as those at the β-segment, which makes the former a more sensitive probe.

The α- and β-segments can be further differentiated by their temperature dependence. The α-splitting is practically independent of temperature whereas the β-splitting decreases linearly with temperature with a temperature coefficient of 65 Hz/°C. A similar behavior has also been observed for DPPC (Gally et al., 1975; Akutsu & Seelig, 1981). The binding of metal ions has no influence on the individual temperature characteristics. The insensitivity toward temperature is an additional advantage for using the α-segment in studying metal binding.

^2H NMR spectra were recorded for α- and β-CD_2-POPC over a wide range of concentrations, and the numerical data

Table I: Binding of Ca^{2+} to Bilayers of α-CD_2-POPC Dispersed in $CaCl_2$ Solutions of Varying Concentration[a]

$C_{CaCl_2}^{eq}$ (M)	$\Delta\nu_\alpha$ (kHz)	C_b[b] (M/M)	σ[c] (mC/m²)	ψ_0[d] (mV)	C_I[e] (M)
0	6.1				
0.005	5.76	0.017	7.8		
0.01	5.52	0.028	13.3		
0.02	5.32	0.038	17.9		
0.05	5.03	0.052	24.6		
0.1	4.54	0.076	35.9		
0.2	4.00	0.103	48.3	30	0.022
0.4	3.13	0.145	68.3	30	0.043
0.5	2.73	0.165	77.5	30.4	0.052
0.6	2.39	0.180	85.3	30.5	0.062
0.8	1.95	0.202	95.4	29.6	0.089
1.0	1.32	0.234	109.9	30.5	0.104
2.0	0	0.298	140.3	27.7	0.255
3.0	−2.0	0.396	186		
4.0	−3.32	0.460	216		
5.0	−3.86	0.486	229		

[a] Measuring temperature, 40 °C; no NaCl added. [b] Calculated with $\Delta\nu_\alpha = 6.1 - 20.48C_b$. [c] $\sigma = 0.471C_b$ (assuming a surface area of $S = 68$ Å² per POPC molecule). [d] Calculated with the Gouy–Chapman theory. [e] Concentration of Ca^{2+} ions in solution at the membrane–water interface; $C_I = C_{CaCl_2}^{eq} \exp[-2F\psi_0/(RT)]$.

Table II: Binding of Ca^{2+} to Bilayers of POPC in the Presence of 0.1 or 1 M NaCl

	$\Delta\nu_\alpha$ (kHz) at		$\Delta\nu_\beta$ (kHz) at		C_b[a] (mmol/
$C_{CaCl_2}^{eq}$	298 K	313 K	298 K	313 K	mol)
		$CaCl_2$ + 0.1 M NaCl[b]			
0	5.75	5.75	5.5	4.8	
4.15	5.52	5.37			13.3[d]
5.0	5.45[c]				
8.56	5.27	5.03			25.5[d]
10.0	5.22[c]				
20.0	4.74[c]				
22.65	4.98	4.69			40.7[d]
41.75	4.63				57.1[d]
47.75	4.49	4.30			
50.0	4.10[c]				
93.0	4.0	4.00			87.5[d]
		$CaCl_2$ + 0.1 M NaCl[e]			
0.1	3.91	3.86	6.54	5.81	
0.3	2.83	2.73	7.18	6.30	
0.5	1.85	2.05	7.61	6.73	
0.7	1.20	1.32	7.86	6.88	
1.0	~0.6	~0.6	8.10	7.08	
2.0	~−1.0	~−0.8	9.18	8.25	
3.0	~−3.0	~−2.8	10.25	9.08	
5.0	gel	~−4.5	gel	9.7	
		$CaCl_2$ + 1 M NaCl[f]			
0	5.5				
30.5	3.91				126.0[d]

[a] C_b denotes the amount of Ca^{2+} (in millimoles) which is bound to 1 mol of POPC. [b] $C_{CaCl_2}^{eq}$ values are in millimolar. [c] Solution contained 0.1 M NaCl plus 10 mM Tris buffer, pH 7.5. [d] Measured by atomic absorption spectroscopy. [e] $C_{CaCl_2}^{eq}$ values are in molar. [f] $C_{CaCl_2}^{eq}$ values are in millimolar.

are summarized in Tables I and II (Table I, POPC in pure $CaCl_2$ solutions; Table II, POPC in $CaCl_2$ solutions plus 0.1 or 1 M NaCl). The adsorption isotherm for α-CD_2-POPC dispersions in $CaCl_2$ solutions plus 0.1 M NaCl (at 25 °C) is reproduced in Figure 3. The solid line was calculated with the ternary complex model to be discussed below.

Atomic absorption spectroscopy was applied to POPC dispersions in the range of 3–100 mM $CaCl_2$ containing in addition 0.1 or 1.0 M NaCl. The outcome of these studies is summarized in the last column of Table II where C_b denotes the amount of Ca^{2+} (in millimoles) bound per 1 mol of POPC. Next, Figure 4 shows a plot of the quadrupole splitting, $\Delta\nu_\alpha$,

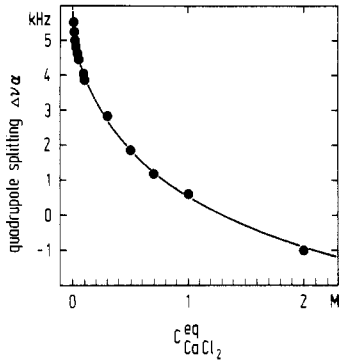


FIGURE 3: Binding of Ca^{2+} to bilayers of POPC. The figure represents the binding isotherm as measured by the variation of the quadrupole splitting of the α-segment with the equilibrium $CaCl_2$ concentration, $C_{CaCl_2}^{eq}$ (plus 0.1 M NaCl, 25 °C). The solid line was calculated by assuming a binding model with $M + 2L \rightleftarrows ML_2$ and $K = 13.8\ M^{-1}$ (cf. text).

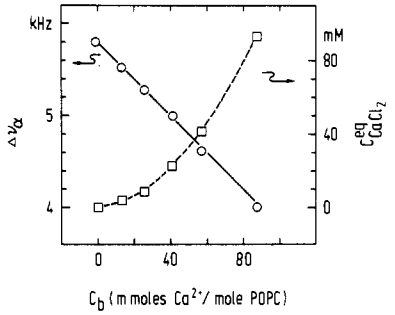


FIGURE 4: Quadrupole splitting of the α-segment of POPC bilayers as a function of the bound Ca^{2+}, C_b (open circles). Also shown is the relationship between C_b and the thermodynamic equilibrium concentration $C_{CaCl_2}^{eq}$ of the supernatant (open squares). Measuring temperature, 25 °C. All data were obtained in the presence of 0.1 M NaCl.

vs. C_b (0.1 M NaCl, 25 °C), demonstrating a linear relationship between the two parameters. Linear regression analysis yields

$$\Delta\nu_\alpha = 5.8 - 20.48C_b \quad (2)$$

(conditions: $\Delta\nu_\alpha$ in kilohertz, C_b in moles of Ca^{2+} per mole of POPC, 25 °C, 0.1 M NaCl). Figure 4 also shows the variation of the Ca^{2+} equilibrium concentration, $C_{CaCl_2}^{eq}$, for the same set of data points. The correlation between $C_{CaCl_2}^{eq}$, and C_b, and therefore also that between $C_{CaCl_2}^{eq}$ and $\Delta\nu_\alpha$, is clearly nonlinear.

The atomic absorption studies in the presence of 1 M NaCl exhibit a larger scattering for reasons which are not clear at present. However, there is no systematic deviation from eq 2. In the following, the validity of eq 2 is also assumed for POPC–$CaCl_2$ dispersions in the absence of NaCl (with the modification that $\Delta\nu_\alpha = 6.1$ kHz for $C_b = 0$). As has been mentioned above, atomic absorption spectroscopy cannot be applied to the latter system because it is not possible to obtain clear supernatants for small Ca^{2+} concentrations.

Discussion

One of the key results of the present study is the observation that a linear relationship exists between the quadrupole splitting and the amount of Ca^{2+} bound per lipid head group, C_b. This calibration of the quadrupole splitting by means of eq 2 allows the evaluation of C_b at Ca^{2+} concentrations where atomic absorption spectroscopy is no longer helpful, i.e., at high Ca^{2+} concentrations or for POPC dispersed in pure $CaCl_2$ solutions. Underlying this extrapolation is of course the assumption that the mode of Ca^{2+} binding remains constant. Within experimental error, this is certainly true for the binding of Ca^{2+} in the presence of 1 M NaCl where an experimental comparison can be made with the 0.1 M NaCl Ca^{2+} adsorption isotherm. Any further experimental justification is, however, difficult at present. The best support for the validity of the above assumption comes from the rather consistent molecular picture which can be derived by analyzing the various consequences.

Stoichiometry of the Ca^{2+}–POPC Interaction. The binding of metal ions to phosphatidylcholine bilayers is a dynamic equilibrium [cf. Akutsu & Seelig (1981)]. The ^2H NMR spectrum is characterized by a single quadrupole splitting and arises as the weighted average of ion-free and ion-complexed phospholipid head groups. Since the quadrupole splittings of the two forms differ by at least 10 kHz, it follows from NMR chemical exchange theory that the residence time of Ca^{2+} at the individual phosphocholine head group is less than 10^{-5} s [cf. Carrington & McLachlan (1967)].

Experimentally, the minimum quadrupole splitting is observed for α-CD_2-POPC dispersed in 5 M $CaCl_2$ (0.1 M NaCl, 40 °C) with $\Delta\nu_\alpha = -4.5$ kHz. From eq 2, the concentration of bound Ca^{2+} is calculated as $C_b = 0.5$ mol of Ca^{2+} mol of POPC, predicting a stoichiometry of one Ca^{2+} bound to two phospholipid head groups at the saturation limit. This straightforward conclusion is further corroborated by the more detailed analysis of the binding process to be discussed below.

Surface Charge Density (σ). If C_b is the surface concentration of bound Ca^{2+}, S the surface area per POPC molecule, and e_0 the charge of an electron, then the surface charge density (σ) is given by

$$\sigma = 2e_0C_b/S \quad (3)$$

In the following, it is assumed that the surface charge is distributed homogeneously in the plane of the membrane, even though the correct molecular picture involves the distribution of discrete charges.

In order to apply eq 3 to the present problem, the surface area of a POPC molecule in the liquid-crystalline membrane must be known. By comparison, the surface area of DPPC has been determined quite accurately with neutron diffraction methods as $S = 57$ Å^2 (Büldt et al., 1979). The introduction of a cis double bond into the *sn*-2 chain is accompanied by a considerable increase in the surface area as is evidenced most clearly by monolayer studies (Demel et al., 1967; Stoffel & Pruss, 1969). Unfortunately, no X-ray or neutron diffraction studies are available for synthetic POPC. The closest comparison is egg lecithin, which contains about 70% POPC (Tattrie et al., 1968) and which has been studies extensively by X-ray diffraction. At 25 °C and 50 wt % water, the average surface area per lipid molecule amounts to $S = 68$–75 Å^2 [cf. Shipley (1973) and Rand (1981)]. For the following calculations, we assume the lower value of $S = 68$ Å^2 since the heterogeneity of the lipid composition of egg lecithin will probably increase the surface area compared to synthetic POPC. With $S = 68$ Å^2, eq 3 takes the numerical form

$$\sigma = 0.471C_b \quad (4)$$

or by means of eq 2

$$\sigma = 0.133 - 0.023\Delta\nu_\alpha \quad (5)$$

(with σ in Coulombs per square meter, C_b in moles per mole, and $\Delta\nu_\alpha$ in kilohertz). Equation 5 demonstrates that the surface charge density σ may be inferred directly from the quadrupole splitting $\Delta\nu_\alpha$. This was not possible in our earlier study on DPPC (Akutsu & Seelig, 1981).

Grasdalen et al. (1977) have argued that chloride ions also bind to phosphatidylcholine membranes. If correct, this effect would reduce the surface charge density as determined from the ^2H NMR spectra. On the other hand, McLaughlin et al. (1978) know of no evidence that either sodium or chloride can bind to phosphatidylcholine vesicles.

Surface Potential (ψ_0). The surface charge density σ creates a surface potential ψ_0. For a homogeneously charged surface immersed in an electrolyte solution, the surface potential is usually calculated by the Gouy–Chapman theory [cf. Aveyard & Haydon (1973), Overbeek (1952), and Adamson (1976)]. In spite of its simplifying assumptions, the Gouy–Chapman theory has been remarkably successful in explaining many membrane phenomena [cf. McLaughlin (1977)]. However in the application of this approach to coarse, multilamellar dispersions of phospholipids, an additional complication arises because of the electrostatic interaction of juxtaposed bilayers. The Gouy–Chapman theory as originally conceived describes the behavior of an isolated surface. In contrast, the onionlike structures of coarse liposomes constitute an array of interacting surfaces. Modifications of the Gouy–Chapman theory have been developed which specifically taken into account the electrostatic bilayer–bilayer repulsion [cf. Overbeek (1952) and Lis et al. (1981a,b)]. These treatments show that an additional parameter, namely, the interbilayer distance, must be measured in order to establish an unambiguous quantitative relationships between the surface charge density and the surface potential.

Since the bilayer–bilayer separation was not measured in the present study, the calculation of the surface potential had to be confined to bilayer dispersions where the surface potential was effectively screened by the counterions. This is generally the case for solutions of high ionic strength. For example, at 0.1 M NaCl, the Debye length (κ^{-1}) of the electrolyte solution is 9.7 Å (at 25 °C) which means that at this distance from the bilayer surface the electrostatic potential is reduced to 37% of its surface value ψ_0. For a solution composed of 0.1 M NaCl and 0.1 M $CaCl_2$ κ^{-1} is already reduced to 4.9 Å.

The equilibrium separation of fully hydrated DPPC and POPC bilayers in the absence of metal ions is about 30 Å and is determined mainly by the balance of van der Waals attraction forces and hydration forces [for a review, see Rand (1981)]. Hence, if the electrostatic surface potential is effectively screened within half of this distance, the mutual electrostatic repulsion of juxtaposed bilayers can be neglected and the application of the conventional version of the Gouy–Chapman theory appears to be justified. This assumption was made here for pure $CaCl_2$ solutions with $C_{CaCl_2} \geq 0.2$ M and for $CaCl_2$ solutions containing 0.1 M NaCl with $C_{CaCl_2} \geq 0.05$ M.

The surface potential was calculated according to [cf. Aveyard & Haydon (1973)]

$$\sigma = [2000\epsilon_r\epsilon_0 RT\sum_i C_i^{eq}(e^{-z_iF_0\psi_0/(RT)} - 1)]^{1/2} \qquad (6)$$

where $\epsilon_r = 78$ is the dielectric constant of water (at 25 °C), ϵ_0 the permittivity of free space, R the gas constant, F_0 the Faraday constant, C_i^{eq} the concentration of the ith electrolyte in the bulk aqueous phase (in moles per liter), and z_i the signed charge of the ith species.[3] The summation is taken over all ions, i, in solution. The surface potentials of pure $CaCl_2$ solutions and for $CaCl_2$ plus 0.1 M NaCl are summarized in Tables I and III, respectively. The application of the Gouy–Chapman theory in the present context raises a number of problems: (1) The positive surface charge is not distributed smoothly on the surface but localized in discrete charges; (2) the theory may not work at high salt concentrations; and (3) the dielectric constant may vary with the distance from the membrane surface. Nevertheless, we are not aware of any better alternative to relate the surface charge density with the surface potential.

Table III: Binding of Ca^{2+} to Bilayers of α-CD_2-POPC in the Presence of 0.1 M NaCl[a]

$C_{CaCl_2}^{eq}$ (M)	$\Delta\nu_\alpha$ (kHz)	C_b[b] (M/M)	σ[c] (mC/m²)	ψ_0[d] (mV)	C_I[e] (M)
0.00415	5.52	0.0137	6.4	8.0	0.00222
0.00856	5.27	0.0259	12.2	15.3	0.00261
0.0227	4.98	0.0400	18.9	20.5	0.00460
0.0260	4.83	0.0474	22.3	23.3	0.00424
0.0418	4.63	0.0571	26.9	25.3	0.00583
0.0478	4.49	0.0640	30.1	27.3	0.00570
0.0930	4.00	0.0879	41.4	30.5	0.00865
0.1	3.91	0.0923	43.5	31.3	0.00874
0.3	2.83	0.145	68.3	31.8	0.0253
0.5	1.85	0.193	90.9	33.5	0.0369
0.7	1.20	0.225	105.8	33.3	0.0522
1.0	0.60	0.254	119.6	31.9	0.0835
2.0	−1.00	0.332	156.4	29.9	0.195

[a] Binding data were analyzed according to the Gouy–Chapman theory; the temperature was 25 °C. [b] Calculated with $\Delta\nu_\alpha = 5.8 - 20.48C_b$. [c] $\sigma = 0.471C_b$ (assuming a surface area of $S = 68$ Å² per POPC molecule). [d] Calculated by means of the Gouy–Chapman theory. [e] Concentration of Ca^{2+} ions in solution at the membrane–water interface; $C_I = C_{CaCl_2}^{eq}\exp[-2F_0\psi_0/(RT)]$. In the range of $0 \leq C_{CaCl_2}^{eq} \leq 50$ mM, the application of the Gouy–Chapman theory must be considered as an approximation (for details, cf. the text).

Knowledge of the surface potential allows calculation of the concentration of free Ca^{2+} ions, C_I, at the plane of ion binding via the Boltzmann equation:

$$C_I = C_{CaCl_2}^{eq}\exp[-2F_0\psi_0/(RT)] \qquad (7)$$

These interfacial concentrations are also listed in Tables I and III.

Models for Ca^{2+} Binding. The present study yields the concentration of bound Ca^{2+}, C_b, as well as the interfacial concentration of Ca^{2+} ions in solution, C_I. Hence, it is possible to test various models of cation binding to lipid bilayers.

(1) Henry–Dalton Partition Equilibrium. The simplest approach is to assume a nonspecific partitioning of Ca^{2+} between the interface and the bilayer surface which in the presence notation can be written as

$$K = C_b/C_i \qquad (8)$$

Using the numerical values listed in Tables I and III, it can easily be verified that K is not constant but changes in the relevant concentration range (0.05–2 M $CaCl_2$) by at least a factor of 10.

(2) Langmuir Adsorption Isotherm. This model is usually written as

$$\theta/(1 - \theta) = KC \qquad (9)$$

where θ is the mole fraction of complex formed. If we denote with C_b^{max} the maximum amount of Ca^{2+} (in moles) which can be bound to 1 mol of POPC in the liquid-crystalline bilayer, then θ is defined as

$$\theta = C_b/C_b^{max} \qquad 0 \leq \theta \leq 1 \qquad (10)$$

With this notation, eq 9 may be rewritten as

$$C_b/(C_b^{max} - C_b) = KC_I$$

[3] The factor 1000 in eq 6 accounts for the fact that the concentrations are given in moles per liter. However, all other factors should be expressed in SI units.

or

$$C_b/C_I = K(C_b^{max} - C_b) \quad (11)$$

A plot of C_b/C_I vs. C_b should thus yield a straight line. A rather poor fit of the experimental data is obtained if the results of Tables I and III are plotted according to eq 11. The scatter of the data points is considerable, and the intersection with x axis is C_b^{max} = 0.3–0.4 mol/mol, suggesting a maximum loading of 1 Ca^{2+} per 3.3–2.5 POPC molecules. This is not consistent with the limiting quadrupole splitting of −4.5 kHz at saturating Ca^{2+} concentrations which indicates a higher loading of the membrane with Ca^{2+}.

(3) Formation of a Ternary Complex. A much better fit is obtained by assuming a ternary complex involving one Ca^{2+} (M) and two lipids (L) according to

$$M + 2L \rightleftarrows ML + L \rightleftarrows ML_2 \quad (12)$$

Let us denote with C_{ML_2}, C_{ML}, and C_L the surface concentrations of the metal complexes and of the free lipid, respectively. Mass conservation requires

$$C_L^0 = C_L + C_{ML} + 2C_{ML_2} \quad (13)$$

where C_L^0 is the surface concentration of the total lipid. Next we assume that only one complex, ML_2, is formed and that the concentration of the intermediate, C_{ML}, can be neglected. This is suggested first by the observation that the binding of monovalent metal ions to phospholipids is very weak. Second, the binding constants of metal ions to adenosine di- and triphosphate are 2–3 orders of magnitude larger than those of the corresponding metal ion–adenosine monophosphate complexes [Sigel et al., 1967; cf. Table IV of Martin & Mariam (1979)]. Even though the latter complexes are not exactly comparable to the complex formed at the membrane surface, they at least suggest an increased complex stability if two phosphate moieties are involved. By neglecting C_{ML}, eq 13 simplifies to

$$C_L^0 = C_L + 2C_{ML_2} \quad (14)$$

The surface concentration C_L^0 is constant and independent of the total amount of lipid. Since a single POPC molecule occupies a surface area of S = 68 Å^2, the surface concentration is equal to $C_L^0 = (2.45)10^{-6}$ mol/m^2. The mole fraction of the ion–lipid complex is given by C_{ML_2}/C_L^0 and corresponds to the experimental parameter C_b defined above:

$$C_b = C_{ML_2}/C_L^0 \quad (15)$$

The law of mass action for the overall reaction (eq 12) may then be written as

$$K^* = C_{ML_2}/(C_L^2 C_I) \quad (16)$$

and can be transformed by means of eq 14 and 15 into

$$K = K^* C_L^0 = C_b/[(1 - 2C_b)^2 C_I] \quad (17)$$

[This equation is identical with eq 5 of Grasdalen et al. (1977) for the special case of n = 2.] For convenience in testing data, eq 17 may be put in linear form:

$$(C_b/C_I)^{1/2} = K^{1/2}(1 - 2C_b) \quad (18)$$

This model predicts that a plot of $(C_b/C_I)^{1/2}$ vs. C_b should yield a straight line intercepting the x and y axes at C_b = 0.5 mol/mol (which is the maximum loading for a 1M:2L stoichiometry) and $K^{1/2}$, respectively.

Figure 5 shows corresponding plots for the data of Tables I and III, demonstrating an excellent fit for both binding isotherms. In particular, both straight lines intersect the x axis at C_b = 0.5 mol/mol, providing a second and independent support for a binding stoichiometry of one Ca^{2+} per two lipids. The binding constants which can be evaluated from the intercepts with the y axis are K = 13.8 M^{-1} (0.1 M NaCl, 25 °C) and 7.0 M^{-1} (no NaCl, 40 °C).

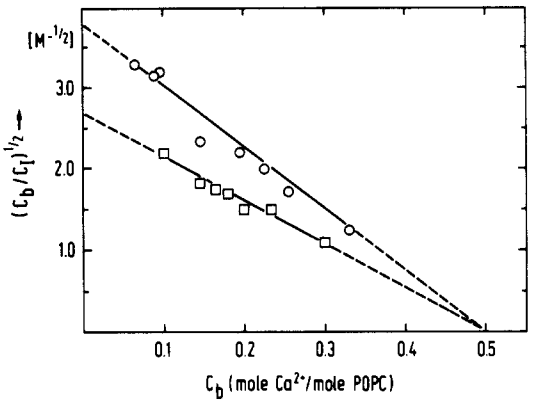


FIGURE 5: Analysis of the Ca^{2+} binding data in terms of a surface equilibrium involving two lipids and one metal ion according to M + 2L ⇄ ML_2. (□) Ca^{2+} binding in the absence of NaCl, 40 °C. (○) Ca^{2+} binding in the presence of 0.1 M NaCl, 25 °C.

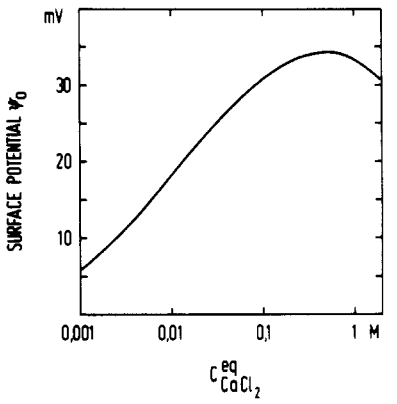


FIGURE 6: Variation of the surface potential of POPC dispersions as a function of the $CaCl_2$ concentration in 0.1 M NaCl. The curve was calculated with a binding constant of 13.8 M^{-1} and a binding stoichiometry of 1 Ca^{2+} per 2 POPCs. The calculation refers to the case of noninteracting bilayers.

A second possibility of analysis is a least-squares fit program based on the combination of the law of mass action, the Boltzmann equation, and the Gouy–Chapman theory. The solid line in Figure 3 represents the result of such an analysis, assuming a binding stoichiometry of one Ca^{2+} per two POPC. The binding constant is found to be 13.8 M^{-1}, and the average deviation of all data points, including the low concentration range $0 \leq C_{CaCl_2}^{eq} \leq 50$ mM, is about ±75 Hz. This is well within the experimental accuracy of the measured quadrupole splittings. For comparison, if a 1:1 or 1:3 stoichiometry is assumed, the average deviations are distinctly larger and amount to ±425 and ±575 Hz, respectively.

Finally, the above results may be used to calculate the surface potential ψ_0 for arbitrary Ca^{2+} concentrations. Figure 6 displays the variation of the surface potential, ψ_0, as a function of the equilibrium Ca^{2+} concentration, $C_{CaCl_2}^{eq}$ (at 25 °C, 0.1 M NaCl). The surface potential increases almost linearly with the logarithm of the Ca^{2+} concentration in the range of 1–100 mM Ca^{2+}. Physiological Ca^{2+} concentrations generally do not exceed 10 mM, and the surface potential of a POPC dispersion would amount to 5–15 mV under these conditions. It should further be noted that in the same concentration range (1–100 mM $CaCl_2$) eq 6 can be approximated by

$$\sigma = \epsilon_0 \epsilon_r \kappa \psi_0 \quad (19)$$

Therefore, the initial part of Figure 6 also represents the behavior of the surface charge density, since κ remains approximately constant for low Ca^{2+} concentrations due to the dominant influence of 0.1 M NaCl.

Comparison with Earlier Investigations. In comparing the present results with previous investigations, it should first be noted that at low metal ion concentrations (where $C_b \ll 1$) both the Langmuir adsorption isotherm and the ternary complex model degenerate into the Henry–Dalton partition equilibrium. Thus, a clear distinction between the various models and stoichiometries can only be made by measuring at high enough concentrations. However, for technical reasons, most previous studies were confined to rather low concentrations. Second, earlier interpretations of binding isotherms required the simultaneous adjustment of at least two, sometimes three, independent parameters, introducing a considerable ambiguity into the analysis. Finally, again for technical reasons, most previous studies were concerned with the binding of lanthanide ions rather than Ca^{2+}.

The most definitive earlier result on the problem of ion–lipid stoichiometry is that of Chrzeszczyk et al. (1981), who studied the binding of Pr^{3+} ions to DPPC bilayers at 52 °C, i.e., in the liquid-crystalline state. By comparing the extrapolated limiting hyperfine shift of DPPC with that of the dimethyl phosphate anion, they obtained a binding stoichiometry of two DPPCs per Pr^{3+}. No data of comparable quality are available for Ca^{2+}. Most authors in recent publications assume a 1:1 stoichiometry for Ca^{2+}–lipid binding (Grasdalen et al., 1977; McLaughlin et al., 1978; Lis et al., 1981). Hauser et al. (1977) suggest a 1:2 stoichiometry, but their interpretation has been criticized by Chrzeszczyk et al. (1981).

As far as the binding constants of Ca^{2+} are concerned, numerical values of 1–2 M^{-1} have been reported for egg yolk lecithin vesicles based on a 1:1 stoichiometry (Grasdalen et al., 1977; McLaughlin et al., 1978). Using a modified Langmuir adsorption isotherm with interaction potential, we estimated a binding constant of 19 M^{-1} for the interaction of Ca^{2+} with DPPC at 59 °C (Akutsu & Seelig, 1981). However, in view of the present interpretation which is based on a larger data set and characterized by a well-defined molecular picture, the binding of Ca^{2+} to DPPC bilayers must be reinvestigated. Binding constants of Ca^{2+}–DPPC complexes *below* the phase transition temperature have also been reported and are of the order of 8–50 M^{-1}, assuming again a 1:1 stoichiometry (Lis et al., 1981; Ohshima et al., 1982). The latter studies have taken advantage of the electrostatic repulsion between bilayers, and the binding constants therefore vary with the intermembrane distance. Lanthanide ions exhibit a much stronger binding affinity toward phospholipid bilayers [cf. Grasdalen et al. (1977), Akutsu & Seelig (1981), and Chrzeszczyk et al. (1982)] and are therefore not relevant analogues for Ca^{2+} as far as the binding constants are concerned.

Concluding Remarks. The data presented here suggest a simple molecular picture for the Ca^{2+}–POPC interaction. The binding of Ca^{2+} to the membrane surface is a highly dynamic process with a Ca^{2+} residence time at an individual lipid head group of less than 10^{-5} s. In contrast to the nonspecific adsorption of hydrophobic ions, the binding of Ca^{2+} appears to involve the formation of a well-defined chemical complex in which one Ca^{2+} coordinates with two POPC molecules. This stoichiometry is evidenced (1) by the limiting quadrupole splitting at high Ca^{2+} concentrations and (2) by the correct prediction of the variation of the quadrupole splitting with Ca^{2+} concentration. However, the limitations of the Gouy–Chapman theory should also be borne in mind, and additional experiments to test the theory, e.g., by diluting the membrane with a neutral lipid to which Ca^{2+} does not absorb, are necessary.

The quadrupole splittings of the choline methylene segments vary linearly with the amount of Ca^{2+} bound. An analogous linear relationship has been observed for the phosphocholine head group upon binding of a local anesthetic (Boulanger et al., 1981) and tetraphenylphosphonium (C. Altenbach and J. Seelig, unpublished results). The quantitative relationship appears to be similar for Ca^{2+} and the local anesthetic but different for tetraphenylphosphonium. Since Ca^{2+} has been found to reverse the physiological effects of local anesthetic drugs [cf. Covino & Vassallo (1976)], ^2H NMR appears to be a promising tool to examine the interrelationship of the two agents at the membrane surface. It will also be interesting to study in more detail the nonspecific adsorption of monovalent hydrophobic ions such as tetraphenylphosphonium and tetraphenylborate as well as to extend these investigations to other phospholipid head groups.

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Registry No. POPC, 26853-31-6; Ca, 7440-70-2; Na, 7440-23-5.

References

Adamson, A. W. (1976) *Physical Chemistry of Surfaces*, 3rd ed., Wiley, New York.

Akutsu, H., & Seelig, J. (1981) *Biochemistry 20*, 7366–7373.

Aveyard, R., & Haydon, D. A. (1973) *An Introduction to the Principles of Surface Chemistry*, Cambridge University Press, Cambridge, England.

Bangham, A. D., & Dawson, R. M. (1962) *Biochim. Biophys. Acta 59*, 103–115.

Boulanger, Y., Schreier, S., & Smith, I. C. P. (1981) *Biochemistry 20*, 6824–6830.

Brown, M. F., & Seelig, J. (1977) *Nature (London) 269*, 721–723.

Büldt, G., Gally, H. U., Seelig, J., & Zaccai, G. (1979) *J. Mol. Biol. 134*, 673–691.

Carrington, A., & McLachlan, A. D. (1967) *Introduction to Magnetic Resonance*, Harper and Row, New York.

Chrzeszczyk, A., Wishnia, A., & Springer, C. S. (1981) *Biochim. Biophys. Acta 648*, 28–48.

Covino, B. G., & Vassallo, H. G. (1976) *Local Anesthetics. Mechanism of Action and Clinical Use*, Grune & Stratton, New York.

Davis, J. H., Jeffrey, K. R., Bloom, M., Valic, M. I., & Higgs, T. P. (1976) *Chem. Phys. Lett. 42*, 390–394.

de Kruijff, B., Demel, R. A., Slotboom, A. J., van Deenen, L. L. M., & Rosenthal, R. F. (1973) *Biochim. Biophys. Acta 307*, 1–19.

Demel, R. A., van Deenen, L. L. M., & Pethica, B. A. (1967) *Biochim. Biophys. Acta 135*, 11–19.

Gally, H. U., Niederberger, W., & Seelig, J. (1975) *Biochemistry 14*, 3647–3652.

Grasdalen, H., Eriksson, L. E. G., Westman, J., & Ehrenberg, A. (1977) *Biochim. Biophys. Acta 469*, 151–162.

Hauser, H., & Phillips, M. C. (1979) *Progr. Surf. Membr. Sci. 13*, 297–413.
Hauser, H., Hinckley, C. C., Krebs, J., Levine, B. A., Phillips, M. C., & Williams, R. J. P. (1977) *Biochim. Biophys. Acta 468*, 364–377.
Hutton, W. C., Yeagle, P. L., & Martin, R. B. (1977) *Chem. Phys. Lipids 19*, 255–265.
Inoko, Y., Yamaguchi, T., Furuya, F., & Mitsui, T. (1975) *Biochim. Biophys. Acta 413*, 24–32.
Lis, L. J., Parsegian, V. A., & Rand, R. P. (1981a) *Biochemistry 20*, 1761–1770.
Lis, L. J., Lis, W. T., Parsegian, V. A., & Rand, R. P. (1981b) *Biochemistry 20*, 1771–1777.
Martin, R. B., & Mariam, Y. H. (1979) *Met. Ions Biol. Syst. 8*, 57–124.
McLaughlin, A., Grathwohl, C., & McLaughlin, S. (1978) *Biochim. Biophys. Acta 513*, 338–357.
McLaughlin, A., Eng, W. K., Vaio, G., Wilson, T., & McLaughlin, S. (1983) *J. Membr. Biol. 76*, 183–193.
McLaughlin, S. A. (1977) *Curr. Top. Membr. Transp. 9*, 71–144.
McLaughlin, S., Szabo, G., & Eiseman, G. (1971) *J. Gen. Physiol. 58*, 667–687.
Oshima, H., & Mitsui, T. (1978) *J. Colloid Interface Sci. 63*, 525–537.
Oshima, H., Inoko, Y., & Mitsui, T. (1982) *J. Colloid Interface Sci. 86*, 57–72.
Overbeek, J. Th. G. (1952) in *Colloid Science* (Kruyt, H. R., Ed.) Vol. I, pp 245–276, Elsevier, Amsterdam.
Rand, R. P. (1981) *Annu. Rev. Biophys. Bioeng. 10*, 277–314.
Scarpa, A., & Carafoli, E. (1978) *Ann. N.Y. Acad. Sci. 307*, 1–655.
Seelig, J., Tamm, L., Hymel, L., & Fleischer, S. (1981) *Biochemistry 20*, 3922–3933.
Shipley, G. (1973) in *Biological Membranes* (Chapman, D., & Wallach, D. F. H., Eds.) Vol. 2, pp 1–89, Academic Press, New York.
Sigel, H., Becker, K., & McCormick, D. B. (1967) *Biochim. Biophys. Acta 148*, 655–664.
Stockton, G. W., Polnaszek, C. F., Tulloch, A. P., Hasan, F., & Smith, I. C. P. (1976) *Biochemistry 15*, 954–966.
Stoffel, W., & Pruss, H. D. (1969) *Hoppe-Seyler's Z. Physiol. Chem. 350*, 1385–1393.
Tamm, L. K., & Seelig, J. (1983) *Biochemistry 22*, 1474–1483.
Tattrie, N. H., Bennett, J. R., & Cyr, R. (1968) *Can. J. Biochem. 46*, 819–824.
Westman, J., & Eriksson, L. E. G. (1979) *Biochim. Biophys. Acta 557*, 62–68.

Thermotropic and Dynamic Characterization of Interactions of Acylated α-Bungarotoxin with Phospholipid Bilayer Membranes†

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ABSTRACT: The interactions of palmitoyl-α-bungarotoxin (PBGT) with dipalmitoylphosphatidylcholine (DPPC) bilayers have been studied by using high-sensitivity differential scanning calorimetry together with steady-state and time-resolved phosphorescence and fluorescence spectroscopy. The incorporation of PBGT into large single lamellar vesicles causes a decrease in the phospholipid phase transition temperature (T_m), a broadening of the heat capacity function, and a decrease in the enthalpy change associated with the phospholipid gel to liquid-crystalline transition. Analysis of the dependence of this decreased enthalpy change on the protein/lipid molar ratio indicates that each PBGT molecule exhibits a localized effect upon the bilayer, preventing approximately six lipid molecules from participating in the lipid phase transition. Additional calorimetric experiments indicate that binding to acetylcholine receptor enriched membranes causes a small increase in the T_m of the PBGT/DPPC vesicles. Steady-state fluorescence depolarization measurements employing 1,6-diphenyl-1,3,5-hexatriene (DPH) indicate that the association of PBGT with the phospholipid bilayer decreases the apparent order of the bulk lipid below T_m while increasing the order above T_m. These results have been further supported by rotational mobility measurements of erythrosin-labeled PBGT associated with giant (about 2-μm) unilamellar vesicles composed of dielaidoylphosphatidylcholine or dioleoylphosphatidylcholine using the time-dependent decay of delayed fluorescence/phosphorescence emission anisotropy. Rotational correlation times in the submillisecond time scale (about 30 μs) indicate that the protein is highly mobile in the fluid phase and that below T_m the rotational mobility is only slightly restricted. Lateral diffusion measurements of fluorescein-labeled PBGT ($PBGT^F$) associated with multilamellar liposomes composed of egg PC or DPPC measured by fluorescence recovery after photobleaching technique indicate that the lateral mobility of $PBGT^F$ is dependent upon the bilayer fluidity. Lateral diffusion coefficients at 25 °C of $(1.7 \pm 1.2) \times 10^{-8}$ cm^2/s (egg PC) and 1×10^{-11} cm^2/s (DPPC) were obtained. These results are consistent with a model in which PBGT interacts with the phospholipid bilayer principally via the fatty acid chain while the polypeptide region of the molecule does not interact strongly with the bilayer.

Specific interactions between membranes are of fundamental importance in many biological processes, including cellular recognition, immune responses, cellular adhesion and sorting, cell fusion, fertilization, and neuronal recognition. Presently, however, very little is known regarding the molecular details of these processes as well as the effects of membrane–membrane interactions mediated by specific ligand–receptor pairs on the physical and functional properties of the two adjacent membranes.

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