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Calcium Binding to Mixed Cardiolipin–Phosphatidylcholine Bilayers As Studied by Deuterium Nuclear Magnetic Resonance†

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ABSTRACT: Calcium binding to bilayer membranes containing cardiolipin (CDL) mixed with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was investigated by using phosphorus-31 and deuterium nuclear magnetic resonance (NMR) spectroscopy. The destabilizing effect of Ca^{2+} on CDL bilayers, including the formation of hexagonal H_{II} and isotropic phases, was eliminated when CDL was mixed with sufficiently large proportion of POPC. Thus, for the mixture CDL–POPC (1:9 M/M), ^{31}P NMR spectra retained a line shape typical of fluid bilayer lipids even in the presence of 1.0 M Ca^{2+}. Specifically head-group-deuteriated CDL or POPC showed in this mixture ^2H NMR spectra indicating that both lipids remained in a fluidlike bilayer at Ca^{2+} concentrations up to 1.0 M. Any phase separation of Ca^2–CDL clusters could be excluded. The residence time of Ca^{2+} at an individual head group binding site was shorter than 10^{-6} s. The deuterium quadrupole splitting, $\Delta\nu_Q$, of POPC deuteriated at the α-methylene segment of the choline head group was found to be linearly related to the number of bound calcium ions, X_2, for the CDL–POPC (1:9 M/M) mixture. The effective surface charge density, σ, could be determined from the measured amount of bound Ca^{2+}. Subsequently, the surface potential, ψ_0, and the concentration of free Ca^{2+} ions at the plane of ion binding were calculated by employing the Gouy–Chapman theory. Various possible models of the equilibrium binding of Ca^{2+} could then be tested. The Langmuir adsorption isotherm with a Ca^{2+} binding constant of 15.5 M^{-1} gave the best fit to the experimental data. Sodium binding was comparatively weak with a binding constant of 0.75 M^{-1}. A comparison of Ca^{2+} binding constants for different membrane lipid compositions revealed that the increase in Ca^{2+} binding observed in the presence of negatively charged lipids was predominantly an electrostatic effect rather than being due to differences in the intrinsic Ca^{2+} affinity. Ca^{2+} was able to reduce the surface potential by binding and neutralizing negative surface charges in addition to having a screening effect.

Cardiolipin, by virtue of its negative charge, should enhance the membrane surface binding of cationic ligands. The binding to the membrane surface of metal ions such as calcium can alter the physical properties of membrane lipids (Tilcock, 1986) and influence the electrical and functional properties of the membrane itself (McLaughlin, 1977). For other ligands, such as anaesthetics and particular signal peptides and proteins, binding to the membrane surface is intrinsic to their mode of functioning. Clearly, given the biological relevance of these various surface binding events, it is desirable to obtain a quantitative understanding of the properties of the lipid membrane surface, how these properties are influenced by the lipid composition, and how their alteration can affect ligand binding to the membrane surface.

While numerous techniques have been employed to study the binding of metal ions (and other ligands) to membrane lipids, ^2H nuclear magnetic resonance (NMR)[1] of specifically deuteriated lipid polar head groups offers certain advantages. The measurements are performed on large multilamellar vesicles so that the lipid configuration is not strained as it would be in the case of small sonicated vesicles. The binding data can be obtained even at quite high ion concentrations so that the complete ion binding isotherm can be described. This allows a distinction to be made between various possible modes of binding. ^2H NMR is sensitive to the charge at the surface of the membrane rather than at an idealized plane of shear some distance from the surface as with measurements of ζ potentials via electrophoretic mobility. Finally, ^2H NMR not only provides a quantitative measure of the thermodynamics of ion binding but also simultaneously monitors the molecular conformation of the membrane lipids.

Using ^2H NMR, we have been able to describe quantitatively the binding of calcium to neutral phosphatidylcholine membranes (Altenbach & Seelig, 1984) and to negatively

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[1] Abbreviations: NMR, nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycerol 3-phosphate; CDL, cardiolipin; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

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charged membranes consisting of mixtures of phosphatidylcholine and phosphatidylglycerol (Macdonald & Seelig, 1987), and to differentiate the various effective modes of ion binding. In this paper, we report our investigations of calcium–lipid interactions in mixtures of cardiolipin and phosphatidylcholine. Cardiolipin is a negatively charged lipid found in the inner mitochondrial membrane of eukaryotes and in the cytoplasmic membranes of Gram-positive and Gram-negative bacteria. Structurally, cardiolipin consists of two phosphatidic acid units (i.e., 1,2-di-fatty acyl-*sn*-glycerol 3-phosphate) bridged by a glycerol moiety (Ioannou & Golding, 1979). In addition to apparently being essential for the function of some membrane-bound enzymes (Robinson, 1982; Cheneval et al., 1983), cardiolipin is known to readily form nonbilayer lipid phases in the presence of various agents including divalent metal ions (Cullis et al., 1978; de Kruijff & Cullis, 1980). In this paper, we will demonstrate first that, when sufficiently diluted by mixing with neutral phosphatidylcholine, cardiolipin retains a fluid bilayer configuration even at abnormally high calcium concentrations. Second, we will describe the determination of the calcium binding isotherm using the ^2H NMR technique for membranes containing cardiolipin and phosphatidylcholine mixed in a biologically relevant proportion. Finally, we present the analysis of the calcium binding isotherm for this cardiolipin–phosphatidylcholine mixture. This involves considering both an electrostatic contribution as encompassed within the Gouy–Chapman theory and a chemical binding equilibrium as represented by particular models of ion binding.

Theory

The binding of charged species to a membrane surface involves electrostatic effects in addition to the binding equilibrium. The simplest available model of surface electrostatics is the Gouy–Chapman theory (Aveyard & Haydon, 1973). When combined with the Langmuir adsorption isotherm representation of the binding equilibrium, one obtains the so-called Gouy–Chapman–Stern equation (McLaughlin, 1977) which accounts for both the electrostatics and the binding equilibrium. However, we wish to consider possible modes of calcium binding other than a Langmuir adsorption, and, therefore, we have employed binding equations describing more complex equilibria and tested these in conjunction with the Gouy–Chapman theory.

Since we have described earlier our approach in detail (Macdonald & Seelig, 1987), we will highlight here only the most salient features. A charged surface will attract (repel) ions of opposite (like) charge so that, at equilibrium, their concentration in the solution immediately adjacent to the surface is greater (less) than their concentration in the bulk solution far from the surface. The charge on the surface may arise either from the presence of charged lipids in the membrane or from the binding of charged species to the surface or both. The density of charge at the surface for a binary mixture of negative and neutral lipids is then

$$\sigma = \left[\frac{e_0}{S(1 + PX_N - X_N)}\right][z_N X_N + \sum_i z_i X_i] \qquad (1)$$

where e_0 is the electrical charge, S is the surface area per neutral lipid molecule (assumed to be 68 Å^2 for POPC), P is the ratio of the surface areas of negative to neutral lipid (assumed to be 2 for CDL–POPC), X_N is the mole fraction of negatively charged lipid with the signed charge z_N, and X_i is the mole fraction of bound ion i with valence z_i (X_i equals the moles of bound ion i per mole of total lipid). As will be shown later, $X_{Ca^{2+}}$ can be obtained from ^2H NMR data.

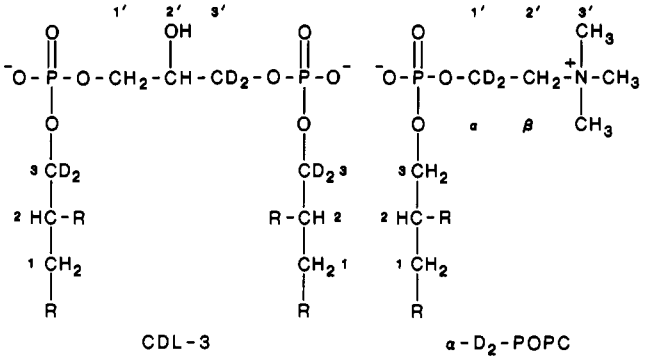
Having determined the surface charge density, σ, we calculate a membrane surface potential, ψ_0, from the Gouy–Chapman theory and the calcium concentration at the membrane surface, $C_{2,M}$, using the Boltzmann relation. With values for the amount of bound calcium (X_2) and the calcium concentration at the plane of ion binding ($C_{2,M}$), we can obtain the calcium binding constant, K_2, using

$$\frac{K_2 C_{2,M}}{(1 + K_1 C_{1,M})^n} = \frac{X_2}{(1 - nX_2)^n} \qquad (2)$$

where K_1 and $C_{1,M}$ are the association constant and surface concentration, respectively, for sodium ions (Macdonald & Seelig, 1987). We will test eq 2 for different values of n, the lipid/calcium stoichiometry. Note that for $n = 1$, eq 2 corresponds to the Langmuir adsorption isotherm.

Materials and Methods

Materials. The following nomenclature is employed for phosphatidylcholine and cardiolipin containing deuterons at the indicated positions:



Nondeuteriated 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycerol 3-phosphate (POPA), and bovine heart cardiolipin (CDL) were purchased from Avanti Polar Lipids (Birmingham, AL). POPC was selectively deuteriated at the α-segment starting from POPA as described by Tamm and Seelig (1983). Deuteriated cardiolipin (CDL-3) was obtained by incorporating *sn*-[3,3-$^2H_2$]glycerol into the phospholipids of *Escherichia coli* B131GP, a strain defective in both the biosynthesis and degradation of glycerol, and subsequently isolating and purifying the labeled cardiolipin as described by Allegrini et al. (1984). This protocol leads to deuteriation of the cardiolipin glycerol backbone(s) as well as the head-group segment.

Measurement of Calcium Binding by Atomic Absorption Spectroscopy. Calcium binding could be assayed in the concentration range up to 20 mM by using the procedure described by Altenbach and Seelig (1984) and Macdonald and Seelig (1987). A volume of dichloromethane–methanol (1:1 v/v) containing 15.3 μmol of POPC plus CDL in the correct proportion was dried under a stream of nitrogen, and any residual solvent was removed overnight under high vacuum. The lipids were dispersed in 400 μL of aqueous solution containing 100 mM NaCl–10 mM Tris-HCl, pH 7.4, plus the desired concentration of $CaCl_2$, via repeated warming to 45 °C and vortexing until a homogeneous suspension was obtained. Equilibrium was achieved through repeated cycles of freeze–thaw, maintenance for 48 h at 4 °C, and finally intermittent vortexing over a 6-h period at 25 °C. The suspension was centrifuged for 30 min at 20000g in a Sorvall RC-2B centrifuge and the clear supernatant removed. The calcium concentration in the supernatant was determined by using a Perkin-Elmer 5000 atomic absorption spectrophotom-

eter. The quantity denoted as X_2, corresponding to the moles of bound calcium per mole of total phospholipid, equalled the difference in the amount of calcium present before and after the addition of lipids divided by the molar quantity of lipids. The value of $C_{2,eq}$, which is the equilibrium concentration of free calcium in the bulk solution, corresponded to the calcium concentration of the supernatant after lipid binding.

Deuterium and ^{31}P NMR. ^2H NMR spectra were recorded on a Bruker CXP-300 spectrometer operating at 46.1 MHz, employing a quadrupole echo technique (Davis et al., 1976) and using the experimental conditions described previously (Seelig et al., 1981; Tamm & Seelig, 1983). The lipid pellets resulting from the calcium binding/atomic absorption procedure were transferred into NMR sample tubes and measured immediately.

^{31}P NMR spectra were recorded at 121.48 MHz as described previously (Seelig et al., 1981). A Hahn echo sequence with proton decoupling and cycling of the pulses was employed as described by Rance and Byrd (1983). Particulars regarding 90° pulse lengths (3.5 μs), interpulse delays (50 μs), recycling delays (1 s), spectral widths (50 kHz), number of acquisitions (1000), and data size (2K) are given in the figure captions when these differ from the typical values given in parentheses.

Computer Simulations. Experimental calcium binding isotherms (corresponding to $\Delta\nu_Q$ vs. $C_{2,eq}$) were simulated by using the equations outlined under Theory in conjuction with a nonlinear least-squares fit program involving a Marquardt routine as described by Bevington (1969). The quality of the fit of simulated binding isotherms to experimental isotherms was expressed as a χ value (hertz) representing the mean deviation of experimental points from the simulated binding curve.

Results

Macroscopic Phase of CDL and CDL–POPC Mixtures plus Calcium. Calcium can have various physical effects on negatively charged lipids including increasing the temperature of the thermotropic gel to liquid-crystal phase transition (e.g., phosphatidylglycerol; Van Dijck et al., 1978; Fleming & Keough, 1983), inducing transitions to nonbilayer structures (e.g., cardiolipin; Deamer et al., 1970; Rand & Sengupta, 1972; Cullis et al., 1978; de Kruijff & Cullis, 1980; Seddon et al., 1983) and causing lateral phase separation in mixed lipid systems (e.g., phosphatidylserine; Tilcock & Cullis, 1981).

As evident in the ^{31}P NMR spectra shown in Figure 1A,B, a lamellar bilayer organization is present for CDL/POPC (1:9 M/M) mixtures both in the absence of Ca^{2+} and in the presence of 1.0 M Ca^{2+}. These broad, asymmetric lipid phosphorus spectra are the result of the fast but anisotropic lipid rotation in a fluid bilayer which leads to incomplete averaging of the phosphorus chemical shift anisotropy (Seelig, 1978). This spectral line shape is diagnostic of fluid lipids arranged in a lamellar bilayer organization [e.g., see Seelig (1978) and Cullis and de Kruijff (1979)]. Control experiments showed that only at such high dilutions of CDL with POPC was a lamellar structure stable to the addition of high Ca^{2+} concentrations. CDL–POPC (1:2 M/M) and pure CDL bilayers, for example, underwent transistions to, respectively, isotropically averaging and hexagonal H_{II} phases upon the addition of even relatively low Ca^{2+} concentrations (^{31}P NMR spectra not shown).

The ^2H NMR spectra in Figure 1C,D were provided by CDL–POPC (1:9 M/M) mixtures prepared with CDL-3 isolated from *E. coli* B131GP grown on *sn*-[3,3-$^2H_2$]glycerol. In the absence of Ca^{2+}, the spectrum was typical of a random dispersion of lipids in a fluid bilayer (Seelig, 1977). Two

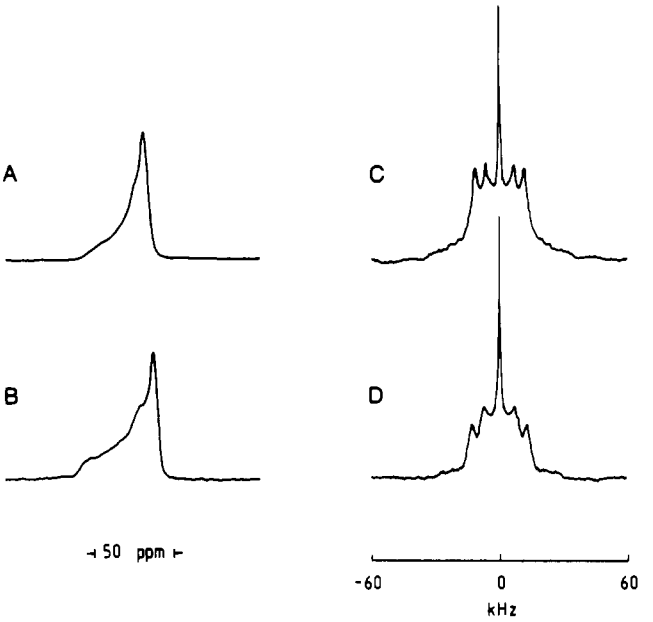


FIGURE 1: NMR evidence for the stability of a fluid, lamellar bilayer organization for CDL–POPC (1:9 M/M) mixtures in the presence of Ca^{2+}. (A, B) ^{31}P NMR spectra of CDL–POPC (1:9 M/M) with 0 and 1.0 M Ca^{2+}, respectively. (C, D) ^2H NMR spectra of CDL-3–POPC (1:9 M/M) with 0 and 1.0 M Ca^{2+}, respectively. All mixtures contained in addition 0.1 M NaCl and 0.01 M Tris-HCl, pH 7.4. All spectra were obtained at 25 °C as described under Materials and Methods except that the ^2H NMR spectra were obtained with a sweep width of 120 kHz and averaging 300 000 and 125 000 scans for (C) and (D), respectively.

quadrupole splittings were observed, one corresponding to deuterons located on glycerols incorporated into the CDL backbone ($\Delta\nu_Q$ = 22 kHz) and the other to deuterons located on glycerol incorporated in the CDL head group ($\Delta\nu_Q$ = 13 kHz) (Allegrini et al., 1984). Upon the addition of Ca^{2+} concentrations as high as 1.0 M, the ^2H NMR spectrum was still readily observable while the two quadrupole splittings decreased.

The ^{31}P and ^2H NMR spectra of CDL–POPC (1:9 M/M) mixtures therefore demonstrate unambiguously that a fluid, lamellar bilayer structure is maintained at this CDL/POPC stoichiometry over a wide range of Ca^{2+} concentrations.

Measurement of the Calcium Binding Isotherm for CDL–POPC (1:9 M/M). We base our measurements of calcium binding on the response of the ^2H NMR quadrupole splitting of head-group-deuteriated phosphatidylcholines to changes in the membrane surface charge which occur upon ion binding (Akutsu & Seelig, 1981; Altenbach & Seelig, 1984, 1985; Macdonald & Seelig, 1987). Apparently, the choline head group acts as a "molecular electrometer", reflecting the charge density at the membrane surface via the measured quadrupole splitting.

For example, all negatively charged species which associate with membranes increase the quadrupole splitting for α-deuteriated POPC-containing membranes, whether these be lipids such as phosphatidylglycerol, phosphatidylserine, or phosphatidic acid [Borle & Seelig, 1985; Macdonald & Seelig, 1987; P. Scherer and J. Seelig, unpublished results; cf. Sixl and Watts (1983)] or hydrophobic ions such as tetraphenylborate (Altenbach, 1985). Figure 2 compares the deuterium quadrupole splittings of α-D_2-POPC bilayers as a function of the content of cardiolipin (two negative charges per molecule) or POPG (one negative charge per molecule). The mole fraction of negative lipid has been expressed here in terms of the formal surface charge density (see eq 1) assuming that

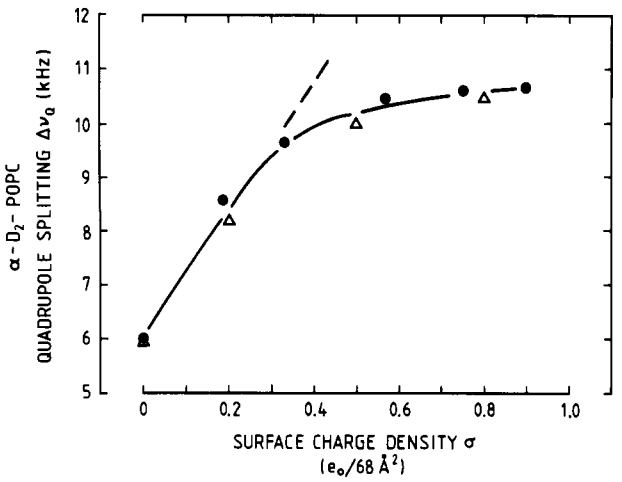


FIGURE 2: Deuterium quadrupole splitting, $\Delta\nu_Q$, of α-D_2-POPC in mixtures with CDL (circles) or POPG (triangles) as a function of the surface charge density, σ (25 °C, 0.1 M NaCl/0.01 M Tris-HCl, pH 7.4). The surface charge density was calculated from the mole fraction of negative lipid by using eq 1 and assuming cross-sectional areas of 68 Å^2 for POPC and POPG and 136 Å^2 for CDL. The POPG data were adapted from Macdonald and Seelig (1987).

CDL (four fatty acyl chains) occupies a cross-sectional area twice that of POPG (two fatty acyl chains). Both lipids caused the quadrupole splitting to increase from 6 kHz (pure POPC) to a saturating value of about 11 kHz. When expressed in this fashion (as opposed to merely plotting $\Delta\nu_Q$ directly as a function of the mole fraction of negative lipid) the effects of CDL and of POPG are seen to be identical. The nonlinear variation of $\Delta\nu_Q$ with σ toward higher formal surface charge densities suggests that the true surface charge density might be smaller at high mole fractions of negative lipid than that calculated formally.

All positively charged species which associate with membranes decrease the quadrupole splitting for α-deuteriated POPC-containing membranes whether these be water-soluble ions such as Ca^{2+} or La^{3+} (Akutsu & Seelig, 1981; Altenbach & Seelig, 1984; Macdonald & Seelig, 1987), hydrophobic ions such as tetraphenylphosphonium (Altenbach & Seelig, 1985), or local anaesthetics (P. Allegrini, A. Seelig, and J. Seelig, unpublished results), or even synthetic positively charged lipids (P. Scherer and J. Seelig, unpublished results). As a further example, Figure 3 shows a series of ^{2}H NMR spectra from membranes consisting of a (1:9 M/M) mixture of CDL with α-D_2-POPC to which have been added various concentrations of $CaCl_2$. The values of the quadrupole splittings decreased progressively as the calcium concentration increased. Regardless of the calcium concentration, a single quadrupole splitting was observed, indicating that the lipids were exchanging between a calcium-"bound" and a -"free" state at a rate which was fast on the ^{2}H NMR time scale of 10^{-5}–10^{-6} s.

In order to calibrate the change in quadrupole splitting with the amount of bound calcium ions, we employed an independent assay of calcium binding involving atomic absorption spectroscopy (Altenbach & Seelig, 1984; Macdonald & Seelig, 1987). The results from these determinations are listed in Table I. For the interdependence of X_2, the moles of Ca^{2+} bound per mole of total lipid as determined by using atomic absorption, and the quadrupole splitting $\Delta\nu_Q$, we obtain a linear relationship of the form

$$\Delta\nu_Q = 8.6 - 14.5X_2 \quad (3)$$

Similar results have been obtained previously for membranes consisting of POPC (Altenbach & Seelig, 1984) or mixtures of POPG plus POPC (Macdonald & Seelig, 1987). Extrapolation of eq 3 to include Ca^{2+} concentrations at which X_2 could not be measured independently assumes that the mode of Ca^{2+} binding remains the same irrespective of Ca^{2+} concentration. The useful range of the atomic absorption/calcium binding assay is limited by several factors. Primarily, since we are measuring the difference in calcium concentration before and after binding to lipids, above 100 mM Ca^{2+} this difference becomes small relative to the accuracy of the in-

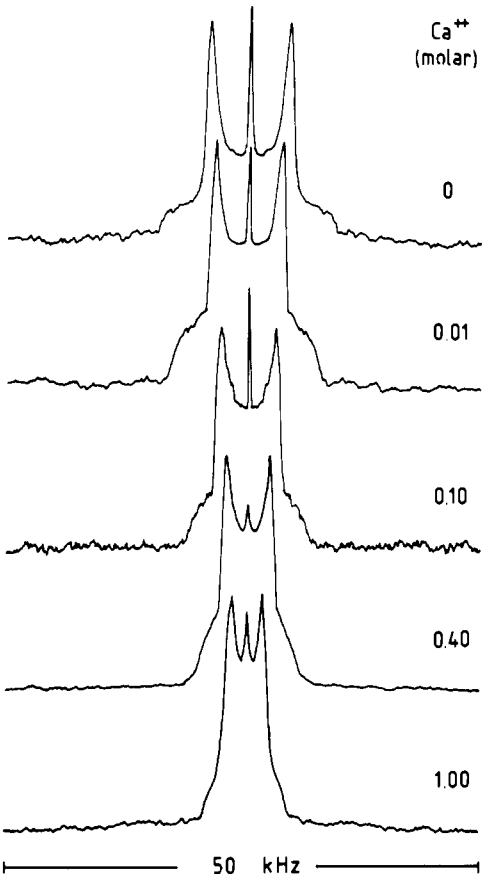


FIGURE 3: ^{2}H NMR spectra of α-D_2-POPC mixed with CDL (9:1 M/M) for various Ca^{2+} concentrations. All spectra were obtained at 25 °C using the conditions described under Materials and Methods. All mixtures contained 0.1 M NaCl/0.01 M Tris-HCl, pH 7.4, plus the indicated Ca^{2+} concentration.

Table I: Binding of Ca^{2+} to Mixtures of 10 mol % CDL plus 90 mol % α-D_2-POPC (0.1 M NaCl and 0.01 M Tris-HCl, pH 7.4, 25 °C)

$C_{2,eq}$[a] (M)	X_2[a] (M/M)	$\Delta\nu_Q$[b] (kHz)	X_2[c] (M/M)	ψ_0[d] (mV)	$C_{2,M}$[d] (mM)
0	0	0.60	0	−53.4	0
0.003	0.063 ± 0.004	7.75 ± 0.05	0.062	−5.6	4.6
0.010	0.082 ± 0.001	7.20	0.096	4.7	6.9
0.014	0.113 ± 0.002	7.00	0.110	7.8	7.7
0.045		6.55 ± 0.05	0.138	18.5	10.7
0.085		6.00	0.179	24.0	13.2
0.175		5.45 ± 0.05	0.214	29.6	17.5
0.340		4.70	0.269	33.9	24.2
0.485		4.10	0.310	36.0	30.0
0.497		4.00	0.317	36.0	30.1
0.645		3.50	0.352	37.3	35.4
0.657		3.60	0.345	37.4	35.9
0.792		3.30	0.365	38.1	40.7
0.802		3.30	0.365	38.2	41.0

[a] Measured by using atomic absorption spectroscopy. [b] Measured in duplicate. The range of values is shown for cases in which $C_{2,eq}$ but not $\Delta\nu_Q$ values were identical. When both $C_{2,eq}$ and $\Delta\nu_Q$ were different in the duplicates, both are shown individually. [c] Calculated by using $\Delta\nu_Q = 8.6 - 14.5X_2$. [d] Calculated as described in the text using $K_1 = 0.75$ M^{-1}, $K_2 = 15.5$ M^{-1}, and $n = 1$.

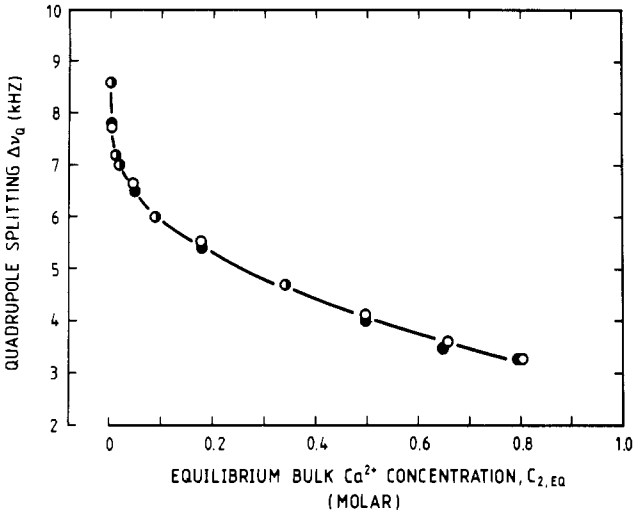


FIGURE 4: Deuterium quadrupole splitting, $\Delta\nu_Q$, vs. the equilibrium bulk Ca^{2+} concentration, $C_{2,eq}$, for the mixture α-D_2-POPC–CDL (9:1 M/M). The solid line represents the result of a computer simulation combining the Gouy–Chapman equations, the Langmuir adsorption isotherm, and the calibrated dependence of $\Delta\nu_Q$ on X_2. The binding constants for Na^+ and Ca^{2+} were $K_1 = 0.75\ M^{-1}$ and $K_2 = 15.5\ M^{-1}$, respectively, with $n = 1$. Closed and open circles represent duplicates at 25 °C with 0.1 M NaCl–0.01 M Tris-HCl, pH 7.4, plus the indicated Ca^{2+} concentration. $C_{2,eq}$ was measured by atomic absorption. See also Table I.

dividual atomic absorption determinations. Further limitations arise from considerations intrinsic to the manner in which these assays are performed, such as the fact that, at Ca^{2+} concentrations high relative to Na^+ concentrations, excess free ions in the diffuse double layer contribute significantly to the measured X_2 values [e.g., see Macdonald and Seelig (1987) and Ohki and Sauve (1978)]. Therefore, for the calibration of the change in $\Delta\nu_Q$ with X_2, we rely on values of X_2 determined at calcium concentrations up to 20 mM (sodium concentration at 0.1 M).

Figure 4 shows the calcium binding isotherm ($\Delta\nu_Q$ vs. $C_{2,eq}$) determined for the mixture CDL–α-D_2-POPC (1:9 M/M) over the range 0–0.8 M Ca^{2+}. The solid curve represents the best computer fit to the binding data determined as will be discussed below. The numerical values of $\Delta\nu_Q$ for duplicate determinations made at each concentration of calcium are listed in Table I.

Discussion

The results described here indicate that CDL can be stabilized into a fluid bilayer configuration, even at high Ca^{2+} concentrations, when mixed with sufficient POPC. Furthermore, calcium binds to these mixtures in a fashion such that the lipids equilibrate rapidly on the ^2H NMR time scale between a "calcium-bound" and a "free" state. Both types of head groups are involved in the ion binding with no separation of CDL–Ca^{2+} clusters.

The macroscopic phase behavior of the lipids and the details of calcium binding will be interrelated. We will first consider the bilayer-stabilizing effect of mixing CDL with POPC within the context of present concepts regarding the macroscopic phase preferences of lipids and lipid mixtures. Second, in order to determine the mode of calcium binding and to obtain a value for the calcium association constant, we will analyze the calcium binding isotherm obtained for the CDL–POPC (1:9 M/M) membranes on the basis outlined under Theory.

Phase Behavior of CDL–POPC Mixtures plus Ca^{2+}. Both POPC and CDL, alone or in mixtures together, assume lamellar macroscopic organizations. Within the context of the shape concept of the macroscopic phase behavior of lipids [for a recent review, see Tilcock (1986)], the geometric area occupied by the polar head groups of these two lipids is postulated to balance the cross-sectional area occupied by their fatty acyl chains.

When Ca^{2+} ions bind to pure CDL bilayers, the charge repulsion between head groups is neutralized, and the effective size of the CDL head group is reduced. The resulting lipid geometry is more conelike than cylindrical and so promotes the formation of nonlamellar macroscopic phases.

By diluting CDL into POPC mixtures, we alleviate the bilayer-destablizing effects of Ca^{2+} in two ways. First, since the membrane surface as a whole becomes less negatively charged upon diluting CDL with POPC, fewer calcium ions are attracted to the surface and fewer are available to bind. Second, it becomes increasingly more likely that CDL molecules neutralized by calcium binding will encounter the larger POPC head group rather than the smaller CDL head group.

In short, the bilayer-stabilizing influence of mixing CDL with POPC as observed here is readily understood within the context of present explanations for the macroscopic phase preferences of lipids. This effect of POPC is not unique to CDL but has also been observed with another negatively charged lipid, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) (Borle & Seelig, 1985; Macdonald & Seelig, 1987). Here, mixing POPG with as little as 20 mol % POPC prevented the Ca^{2+}-induced lipid phase transition temperature increase and the formation of quasi-crystalline lipid clusters seen with pure POPG. It is interesting that the extent to which CDL must be diluted with POPC in order to prevent these complicating effects of Ca^{2+} binding is far greater than that necessary to achieve a similar prevention with POPG but corresponds to that in effect in biological membranes commonly containing CDL (McMurray, 1978).

Calcium Association Constant and Lipid/Ion Stoichiometry. The computer simulation shown in Figure 4 represents the best fit to an experimental data set involving ^2H NMR quadrupole splittings ($\Delta\nu_Q$) and equilibrium bulk calcium concentrations ($C_{2,eq}$). For a more readily appreciable picture, we have plotted directly in Figure 5 the moles of calcium bound per mole of lipid, X_2, vs. $C_{2,eq}$ for the CDL–POPC (1:9) membranes. For a comparison, the results obtained previously for pure POPC (Altenbach & Seelig, 1984) and for POPG–POPC (1:4) membranes (Macdonald & Seelig, 1987) are plotted as well. Clearly, the membranes containing negative lipids have bound far greater levels of Ca^{2+} at a given value of $C_{2,eq}$ than have neutral POPC membranes. Furthermore, membranes containing 10 mol % CDL bind amounts of Ca^{2+} nearly identical with membranes containing 20 mol % POPG. To a good approximation, the two negative lipids appear remarkably similar in their Ca^{2+} binding capabilities.

The binding isotherms in Figure 5 represent a combination of effects due to the electrical double layer's influence on interfacial Ca^{2+} concentrations and the equilibrium binding of Ca^{2+} from the interfacial solution onto the lipid surface. By modeling the electrostatic contribution in terms of the Gouy–Chapman theory, we isolate the equilibrium binding contribution. The success of the modeling was judged from the quality of the fit (expressed as a mean deviation χ in hertz) between the simulated and the experimental values of the quadrupole splittings.

Choosing the initial surface charge density in the absence of calcium to be equal to that expected for membranes consisting of 10 mol % CDL plus 90 mol % POPC, we obtained excellent fits of the simulations to the experimental data for

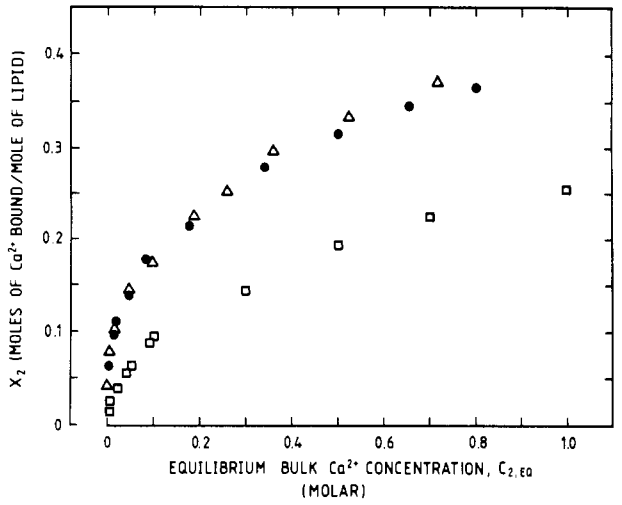


FIGURE 5: Calcium binding isotherms (X_2 vs. $C_{2,eq}$) for three membrane lipid compositions. Ca^{2+} binding data are shown for the mixtures CDL–POPC (1:9 M/M) (circles) and POPG–POPC (1:4 M/M) (triangles) as well as for pure POPC (squares). The values of X_2 were obtained from the calibrated dependence of $\Delta\nu_Q$ on X_2 as measured for POPC by Altenbach and Seelig (1984), for POPG–POPC (1:4 M/M) by Macdonald and Seelig (1987), and for CDL–POPC (1:9 M/M) in the present study. This step allows a direct comparison of the Ca^{2+} binding to these three membranes by eliminating the complications due to the dependence of $\Delta\nu_Q$ on the mole fraction of negatively charged lipid and the somewhat different slopes in the relationship $\Delta\nu_Q = \Delta\nu_0 - mX_2$ observed for the various mixtures. Note as well that these data still reflect both electrostatic and equilibrium binding differences between these three membranes.

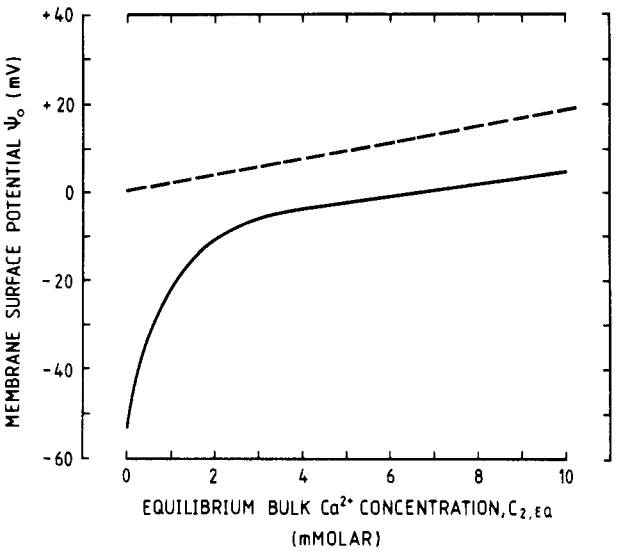


FIGURE 6: Dependence of the membrane surface potential, ψ_0, on the equilibrium bulk Ca^{2+} concentration, $C_{2,eq}$. The value of ψ_0 was calculated from the effects of Ca^{2+} binding on the surface charge density, σ, and thereafter invoking the Gouy–Chapman equations as outlined under Theory. The solid line is for CDL–POPC (1:9 M/M) while the dashed line is for pure POPC (Altenbach & Seelig, 1984).

a 1:1 lipid/ion binding model (χ = 100 Hz) with a calcium binding constant K_2 = 15.5 M^{-1} and a sodium binding constant K_1 = 0.75 M^{-1}. Any increase in the presumed value of n (the lipid/ion stoichiometry) lead to clearly inferior simulations (i.e., χ = 250 Hz for n = 2). If the range of Ca^{2+} concentrations to be fit was restricted to less than 100 mM, all binding models provided equally low χ values. Thus, any differences in the expected binding behavior for different binding models vanish below 100 mM Ca^{2+} (Macdonald & Seelig, 1987).

Regardless of the particular binding model chosen, by including a sodium competition term we substantially improved the fit of the simulated to the experimental data. Several studies indicate that Na^+ ions bind to membrane lipids, particularly negative lipids [e.g., see Eisenberg et al. (1979)]. Our optimal Na^+ binding constant was K_1 = 0.75 M^{-1} which agrees well with the value reported by these authors and with our values reported previously for POPG–POPC mixtures (Macdonald & Seelig, 1987).

We have applied our ^2H NMR approach now to study Ca^{2+} binding to three differently composed lipid membranes. In the present study, mixtures of CDL–POPC (1:9 M/M) bound Ca^{2+} according to n = 1 with K_2 = 15.5 M^{-1}. Membranes consisting of POPG–POPC (1:4 M/M) were shown to bind Ca^{2+} according to n = 1 with K_2 = 19.5 M^{-1} (Macdonald & Seelig, 1987). Altenbach and Seelig (1984) concluded that calcium binding to pure POPC membranes was best described in terms of a specific binding with n = 2 and K_2 = 13.8 M^{-1}. Thus, the calcium binding affinity of these various lipids and lipid mixtures appears to be very similar. We may conclude that the much higher levels of calcium binding which are observed in the presence of negatively charged lipids are primarily the result of the electrostatic attraction of calcium ions toward the negatively charged membrane surface and the consequently greater opportunity for ion binding to occur.

Among other techniques, electrophoretic mobility measurements of lipid vesicle ζ potentials have been used extensively to study Ca^{2+} binding to neutral and negative lipids and mixtures of the two (McLaughlin et al., 1981; Lau et al., 1981). Such investigations generally have been limited to Ca^{2+} concentrations below approximately 100 mM and quite reasonably, therefore, have considered the calcium binding equilibria only in terms of a Langmuir adsorption isotherm (while electrostatic considerations were accounted for by using the Gouy–Chapman theory). These authors have reported intrinsic 1:1 calcium association constants (M^{-1}) of 8.5 for phosphatidylglycerol, 12 for phosphatidylserine, and 20 for cardiolipin (S. McLaughlin, personal communication). The electrophoretic mobility results and the ^1H NMR results agree remarkably with respect to the calcium association constants for membranes containing negatively charged lipids, despite the different assumptions inherent to the two techniques. Moreover, the ^2H NMR results with POPG (Macdonald & Seelig, 1987) and CDL (present study) confirm the validity of employing the Langmuir adsorption isotherm to analyze the calcium binding equilibrium, at least for membranes containing these negative lipids.

Membrane Surface Potential. The many possible biological implications of the membrane surface potential and its alteration by various agents have been discussed by others (McLaughlin, 1977; Hille, 1984). When Ca^{2+} binds to the membrane surface, it alters the surface charge and thereby the surface potential. The effect of calcium on the membrane surface potential, ψ_0, of POPC–CDL (9:1 M/M) membranes is shown in Figure 6. In the absence of calcium, ψ_0 is initially approximately −55 mV. Upon the addition of even very low concentrations of calcium (<2 mM), the surface potential rises rapidly toward zero and soon assumes positive values. This very sensitive response occurs for membranes containing a biologically relevant amount of CDL within a physiologically significant range of Ca^{2+} concentrations.

Finally, we would point out that in the presence of a negative surface potential there is an excess of calcium ions present at the membrane surface due to attraction and due to binding. Since these calcium ions appear to be trapped by but floating freely in a "trough" of negative potential, this "excess" Ca^{2+} represents a reserve which may be activated by altering the membrane surface potential.

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

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