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PHYSICAL STUDIES OF PHOSPHOLIPIDS

XI. Ca^{2+} BINDING TO MONOLAYERS OF PHOSPHATIDYLSERINE AND PHOSPHATIDYLINOSITOL

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SUMMARY

Measurements have been made of the binding of radioactive Ca^{2+} to unimolecular films of purified phosphatidylserine and phosphatidylinositol at various film pressures. Three theoretical treatments based on the mass equation have been applied to the experimental data to evaluate the binding of Ca^{2+} in terms of K_A, the apparent association constant, and n, the fraction of phospholipid molecules acting as binding sites for a Ca^{2+}.

At low film pressures (or low charge densities) the binding of Ca^{2+} follows the mass equation. Deviations from the mass equation are observed with increasing film pressure (charge density) and are attributed to electrostatic interaction between adjacent binding sites. The ratio of Ca^{2+} bound/lipid reaches a maximum at about 30 dynes/cm when the spacing between phospholipid molecules is most favourable for two-point electrostatic attachment of Ca^{2+}. Ca^{2+} bound to phosphatidylserine and phosphatidylinositol form a Stern layer and do not penetrate into the plane of the Gouy potential.

INTRODUCTION

Phospholipids are important constituents of cell membranes, and one of their characteristic properties is their ability to interact with metal ions. It is well known that phospholipids such as cephalin[1], phosphoinositides[2-5], gangliosides[2,6,7], phosphatidic acid[2,8,9] and phosphatidylserine[2,5,10,11] have a high affinity for both Ca^{2+} and Mg^{2+}. The binding abilities of phosphatidylserine and phosphatidylethanolamine for Ca^{2+} have been related by ROJAS AND TOBIAS[12] to the ionization of their polar head groups. Previous work by HAUSER AND DAWSON[2] on the adsorption of Ca^{2+} to phospholipid monolayers at high film pressures has shown that the binding of Ca^{2+} depends on the surface-charge characteristics of the phospholipid monolayer and the chemical structure of the phospholipid molecule matters only to the extent to which it determines the number of net charges per molecule. The surface charge of phospholipids is significant in relation to cell adhesiveness[13,14], to the action of phospholipases[1, ,16], to the mechanism of blood coagulation[17] and many other important properties of biological membranes. These physiological activities are known to be remarkably influenced by the presence of metal ions interacting with the phospholipid surface.

In this paper we study the adsorption of Ca^{2+} on phospholipid monolayers of pure phosphatidylserine and phosphatidylinositol and its variation with surface-charge density or film pressure assuming full ionization of the phospholipid at the pH used[2,32]. With the monolayer technique the packing of the molecules can be readily controlled and thus the surface-charge density is known accurately.

METHODS

A polytetrafluoroethylene trough (Fluon, I.C.I.) of dimensions 20 cm × 4.5 cm × 0.8 cm was used. It was stirred by a glass-sheathed steel stirrer which was moved by a reciprocating magnet underneath the trough. Phosphatidylinositol dissolved in chloroform–methanol (1:1, v/v) and phosphatidylserine dissolved in chloroform–light petroleum (1:1, v/v) were spread on the surface from a micrometer syringe (Agla, Burroughs Wellcome). The surface pressure was adjusted by moving a teflon barrier and measured by means of a Wilhelmy dipping plate made from half a microscope cover slip suspended from the arm of a torsion balance. The molecular areas were reproducible to ± 5% at 10 dynes/cm, at higher pressures the experimental error decreased to ± 2%. The molecular areas were lower by up to 3% when determined on the Fluon trough described above as compared with areas determined on a conventional Langmuir trough (area = 300 cm^2).

The Ca^{2+} adsorption was measured by using $^{45}CaCl_2$ (Radiochemical Centre, Amersham, England). Aliquots of $^{45}CaCl_2$ (0.154 mg $^{45}Ca^{2+}$/mC) were added to the trough which had been filled to the brim with distilled water. The water was deionized before double distillation, the average pH of which was 5.5. The increase in radioactivity due to the adsorption of Ca^{2+} from the bulk phase to the monolayer was detected by a gas-flow counter with a large window (6.5 cm × 1.2 cm) covered with a sheet of 6-μ-thick polyethyleneterephthalate plastic (Melinex I.C.I.)[18]. The pulses were integrated in an Ekco ratemeter (over 20 sec) and continuously recorded with a pen recorder.

In order to relate the surface radioactivity to the number of Ca^{2+} per cm^2 two 1-ml samples of the bulk phase were evaporated on planchettes and counted with a gas-flow counter, the first one before spreading the phospholipid and the second one after spreading the phospholipid on the surface of the trough. If we define the ratio

$$f = \frac{\text{counts/min from 1 ml without film}}{\text{counts/min from 1 ml with film}}$$

then

$$[Ca^{2+}]_s = [Ca^{2+}]_{total} - \frac{[Ca^{2+}]_{total}}{f} = [Ca^{2+}]_{total}\left(1 - \frac{1}{f}\right)$$

where $[Ca^{2+}]_s$ = total amount of Ca^{2+} adsorbed to the monolayer; $[Ca^{2+}]_{total}$ = amount of Ca^{2+} added to the trough. The increase in surface radioactivity above the background was directly proportional to the amount of $[Ca^{2+}]_s$, or the amount of

Ca^{2+} bound to 1 cm^2 of monolayer (Fig. 1). The reduction of the original background caused by the adsorption of Ca^{2+} on the film was considered to be too small to make a correction necessary. The characteristics of the curve preclude any possibility of contaminating Ca^{2+} or other divalent ions affecting the results appreciably.

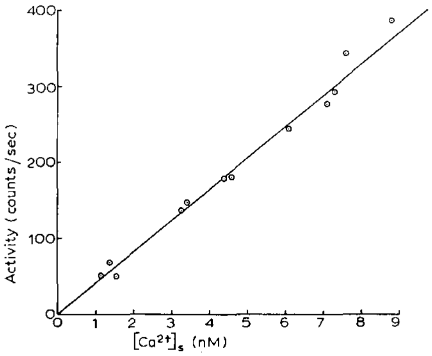


Fig. 1. Increase in surface radioactivity above the background as a function of $[Ca^{2+}]_s$, the total amount of Ca^{2+} adsorbed to phosphatidylserine and phosphatidylinositol monolayers of 32.4 and 31.5 dynes/cm, respectively.

MATERIALS

Phosphatidylinositol was prepared from frozen peas by a method developed in R.M.C. Dawson's laboratory and based on methods described by ROUSER *et al.*[19] and ANSELL AND HAWTHORNE[20]. Phosphatidylserine was a gift from Mr. N. Miller. Both phospholipids were converted to the sodium salts. Thin-layer chromatography and alkaline degradation indicated that both were pure.

RESULTS

The experimental data are analysed in terms of apparent association constants, K_A, for the reaction $Ca^{2+} + Lip \rightleftharpoons Ca^{2+}{}_s$ ($Ca^{2+}{}_s$ referring to Ca^{2+} adsorbed on the surface) and n, the fraction of phospholipid molecules acting as binding sites for Ca^{2+} at a given film pressure. The various methods used for calculating K_A and n based on the application of the mass equation in the form

$$K_A = \frac{[Ca^{2+}]_s}{[Ca^{2+}]_f[Lip]_f} \tag{1}$$

where $[Ca^{2+}]_f$ = Ca^{2+} concentration in the bulk phase; $[Lip]_f$ = concentration of free binding sites in the surface; and since $[Ca^{2+}]_f = [Ca^{2+}]_{total} - [Ca^{2+}]_s$

$$[Lip]_f = n[Lip]_{total} - [Ca^{2+}]_s$$

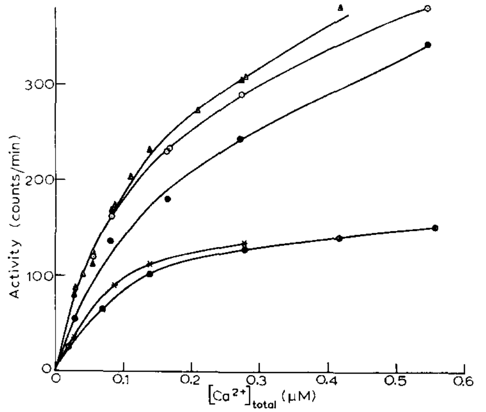
where $[Lip]_{total}$ = total number of lipid molecules spread on the surface, hence

$$K_A = \frac{[Ca^{2+}]_s}{([Ca^{2+}]_{total} - [Ca^{2+}]_s)\,(n[Lip]_{total} - [Ca^{2+}]_s)} \tag{2}$$

Three theoretical methods are used for analysing the experimental data in terms of K_A and n and we shall number them I, II and III.

Method I

The adsorption of Ca^{2+} expressed in counts/sec onto monolayers of phosphatidylserine and phosphatidylinositol of various film pressures is shown in Fig. 2. For comparison the pressures of corresponding monolayers of phosphatidylserine and phosphatidylinositol are chosen from the respective force–area curves so that they possess approximately equal spacing between the phospholipid molecules. With all films increasing amounts of Ca^{2+} are adsorbed with increasing Ca^{2+} concentration in the bulk phase, the relation, however, is not linear, but resembles rather a Langmuir adsorption isotherm.



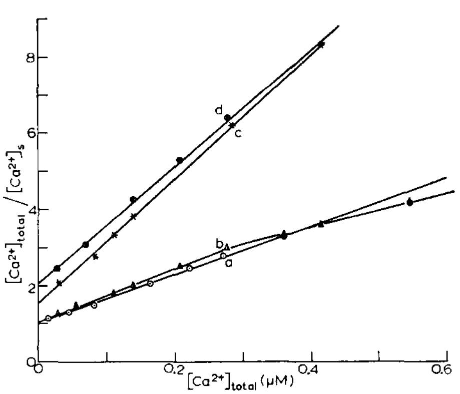


Fig. 2. Binding of Ca^{2+} onto monolayers of phosphatidylserine and phosphatidylinositol of various film pressures as a function of the total amount of Ca^{2+}. The Ca^{2+} bound is expressed in counts/sec, the total concn. of Ca^{2+} in μM. ⊗—⊗, phosphatidylserine at 7 dynes/cm; ●—●, phosphatidylserine at 32.4 dynes/cm; ▲—▲, phosphatidylserine at collapse pressure; ✱—✱, phosphatidylinositol at 10 dynes/cm; ⊙—⊙, phosphatidylinositol at 31.5 dynes/cm; △—△, phosphatidylinositol at collapse pressure.

Fig. 3. Langmuir-type plot. The ratio of the total amount of Ca^{2+} to the amount of Ca^{2+} bound at various film pressures is plotted as a function of the total amount of Ca^{2+} present in μM. ⊗—⊗, phosphatidylserine at 7 dynes/cm; ✱—✱, phosphatidylinositol at 10 dynes/cm; ⊙—⊙, phosphatidylinositol at 31.5 dynes/cm; △—△, phosphatidylinositol at collapse pressure.

Rearranging Eqn. 2 leads to

$$[Ca^{2+}]_s = \frac{n[Lip]_{total}[Ca^{2+}]_{total}}{1/K_A + n[Lip]_{total} + [Ca^{2+}]_{total}} \qquad (3)$$

since, as a first approximation, $[Ca^{2+}]_s^2$ is small compared with the other terms. Since K_A, n and $[Lip]_{total}$ are constant Eqn. 3 is equivalent to a Langmuir adsorption isotherm as derived originally for the adsorption of gas on solid surfaces. If the experimental data conform with a Langmuir-type adsorption isotherm as derived in Eqn. 3 a straight line should be obtained plotting $[Ca^{2+}]_{total}/[Ca^{2+}]_s$ against $[Ca^{2+}]_{total}$. Straight lines result for the adsorption of Ca^{2+} on phosphatidylserine and phosphatidylinositol monolayers of various film pressures (Fig. 3), although some deviations occur at high film pressures and higher values of $[Ca^{2+}]_{total}$ (Fig. 3, Curves a and b). Under these circumstances the assumption that $[Ca^{2+}]_s^2$ is small is no longer valid.

Values for K_A and n can be obtained from the slope and intercept of the straight lines, respectively. For a phosphatidylserine monolayer of 7 dynes/cm, $K_A = 1.46 \cdot 10^7$

and $n = 0.297$; for a phosphatidylinositol monolayer of 10 dynes/cm, $K_A = 2.97 \cdot 10^7$ and $n = 0.278$.

At higher film pressures greater deviations in the K_A and n values are observed as compared with the values obtained with either of the following methods. For instance n from the slope of Curves b and a (Fig. 3) amounts to 0.724 and 0.62 for the adsorption of Ca^{2+} on phosphatidylinositol monolayers of collapse pressure and 31.5 dynes/cm, respectively. The n values are considerably higher than the corresponding values obtained from the other methods used.

Method II

The direct application of the mass equation in the form of Eqn. 2 to the experimental data is not possible even if $[Ca^{2+}]_{total}$, $[Ca^{2+}]_s$ and $[Lip]_{total}$ are known from the experiment because Eqn. 2 contains two unknown quantities, K_A and n. Using a similar mathematical treatment to that of MILDVAN AND COHN[21] who examined the interaction of Mn^{2+} with bovine serum albumin, and solving Eqn. 2 for $[Ca^{2+}]_s$ and dividing by $[Ca^{2+}]_{total}$, we get Eqn. 4.

$$\frac{[Ca^{2+}]_s}{[Ca^{2+}]_{total}} = \frac{(1/K_A + [Ca^{2+}]_{total} + n[Lip]_{total}) \pm}{2[Ca^{2+}]_{total}} \frac{\pm \sqrt{(1/K_A + [Ca^{2+}]_{total} + n[Lip]_{total})^2 - 4[Ca^{2+}]_{total} n[Lip]_{total}}}{2[Ca^{2+}]_{total}} \quad (4)$$

As $[Ca^{2+}]_{total} \rightarrow$ zero the expression $[Ca^{2+}]_s/[Ca^{2+}]_{total}$ becomes indeterminate.

Del'Hospital's rule can be applied to Eqn. 4. By differentiation of both numerator and denominator of Eqn. 4 with respect to $[Ca^{2+}]_{total}$ we obtain Eqn. 5

$$\lim_{[Ca^{2+}]_{total} \rightarrow 0} \frac{[Ca^{2+}]_s}{[Ca^{2+}]_{total}} = \frac{n[Lip]_{total}}{1/K_A + n[Lip]_{total}} \quad (5)$$

Generally the amount of Ca^{2+} bound on a phospholipid film in the limit of $[Ca^{2+}]_{total} \rightarrow 0$ varies from none to all, depending on $1/K_A = K_D$ (dissociation constant) and

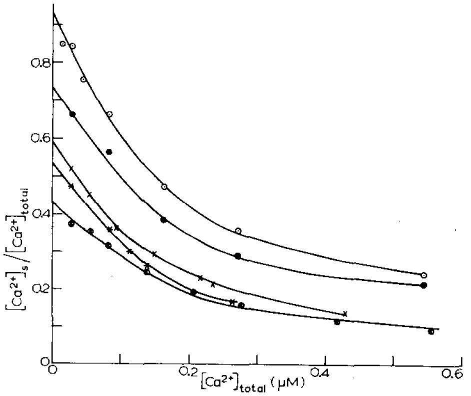


Fig. 4. Variation of the ratio of Ca^{2+} bound/total amount of Ca^{2+} with the total Ca^{2+} concn. expressed in μM. The curves are extrapolated to $[Ca^{2+}]/_{total} \rightarrow 0$. ⊗—⊗, phosphatidylserine at 7 dynes/cm; ●—●, phosphatidylserine at 32.4 dynes/cm; ✱—✱, phosphatidylinositol at 10 dynes/cm; ×—×, phosphatidylinositol at 12.5 dynes/cm; ⊙—⊙, phosphatidylinositol at 31.5 dynes/cm.

$n\ [\mathrm{Lip}]_{\mathrm{total}}$. From Eqn. 5 we see that all the Ca^{2+} is bound as $[Ca^{2+}]_{\mathrm{total}} \rightarrow 0$ if the number of binding sites $n[\mathrm{Lip}]_{\mathrm{total}}$ is large compared with K_D. In Fig. 4 $[Ca^{2+}]_S/[Ca^{2+}]_{\mathrm{total}}$ is plotted as a function of $[Ca^{2+}]_{\mathrm{total}}$. The $\lim_{[Ca^{2+}]_{\mathrm{total}} \rightarrow 0} [Ca^{2+}]_S/[Ca^{2+}]_{\mathrm{total}}$ is obtained by extrapolation of the curve to $[Ca^{2+}]_{\mathrm{total}} \rightarrow 0$.

TABLE I

Film	*Pressure (dynes/cm)*	*n*	$K_A \times 10^{-7}$
Phosphatidylserine	7	0.25	1.46
Phosphatidylinositol	10	0.25	2.4
Phosphatidylinositol	12.5	0.27	2.51
Phosphatidylserine	32.4	0.46	1.89

Both Eqns. 2 and 5 can be solved for K_A and n. Values of K_A and n for phosphatidylserine and phosphatidylinositol monolayers of various pressures are listed in Table I.

It can be seen from Fig. 5 that the K_A values are concentration dependent. The values in Table I have been obtained by extrapolation to $[Ca^{2+}]_S/[\mathrm{Lip}]_{\mathrm{total}} \rightarrow 0$. Because at collapse pressure $\lim_{[Ca^{2+}]_{\mathrm{total}} \rightarrow 0} [Ca^{2+}]_S/[Ca^{2+}]_{\mathrm{total}}$ cannot be determined unambiguously, K_A values for collapse pressure are not included in Table I.

Eqn. 2 can be rearranged to give Eqn. 6 (ref. 22).

$$\frac{\theta}{1-\theta} = [Ca^{2+}]_f K_A \qquad (6)$$

where $\theta = [Ca^{2+}]_S/n[\mathrm{Lip}]_{\mathrm{total}}$ = (number of ions bound)/(number of binding sites).

The association constant is given by $K_A = e^{-\Delta G_0/kT}$, where ΔG_0 is the standard free energy change of the reaction. ΔG_0 can be resolved into an electrostatic, polarization and Van der Waals term[23].

$$-\Delta G_0 = -Ze\psi_S + \lambda_p + W \qquad (7)$$

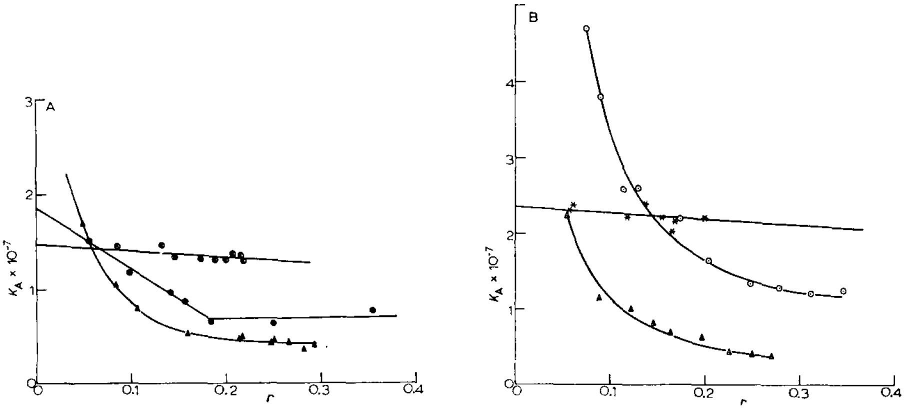


Fig. 5. Variation of the apparent association constant K_A with r = Ca^{2+} bound (ions/cm^2)/total amount of phospholipid in the surface (molecules/cm^2). K_A values for monolayers of phosphatidylserine of various film pressures: ⊗—⊗, 7 dynes/cm; ●—●, 32.4 dynes/cm; ▲—▲, collapse pressure. K_A values for monolayers of phosphatidylinositol of various film pressures: ∗—∗, 10 dynes/cm; ⊙—⊙, 31.5 dynes/cm; △—△, collapse pressure.

where Z = valency of the metal ion; ε = electronic charge; ψ_S = potential in the plane of the first layer of counterions (Stern potential); λ_p = energy of polarization; W = Van der Waals energy.

Introducing $K_A = e^{-\Delta G_0/kT}$ and using Eqns. 7 and 6 one obtains Eqn. 8. This resembles the Stern equation and enables us to calculate ψ_S.

$$\log \frac{\theta}{1-\theta} - \log\,[\mathrm{Ca}^{2+}]_f - \frac{\lambda_p + W}{2.3kT} = \frac{-Z\varepsilon\psi_S}{2.3kT} \qquad (8)$$

k = Boltzmann constant and T = absolute temperature. The value[24] for $(\lambda_p + W)/kT$ is taken as 9.36 (see later) and so ψ_S can be obtained. The Stern potential ψ_S is plotted in Fig. 6 as a function of $[\mathrm{Ca}^{2+}]_s/[\mathrm{Lip}]_{\mathrm{total}} = r$.

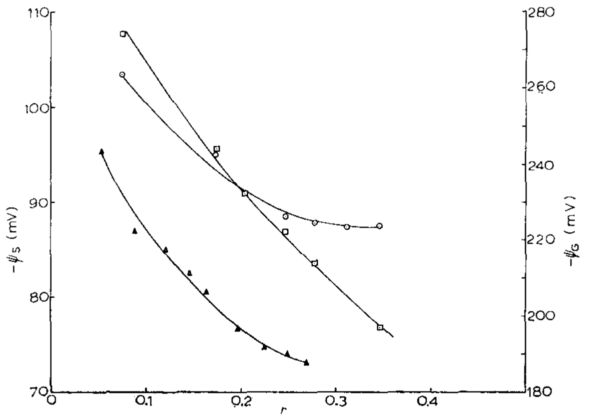


Fig. 6. Variation of the Stern potential ψ_S calculated from Eqn. 8 with the amount of Ca^{2+} bound. The amount of Ca^{2+} bound is expressed as $r = \mathrm{Ca}^{2+}$ bound/total amount of phospholipid in the surface. ⊙—⊙, phosphatidylinositol at 31.5 dynes/cm; ▲—▲, phosphatidylinositol at collapse pressure; ⊡—⊡, variation of the Gouy potential ψ_S with the amount of Ca^{2+} bound if Ca^{2+} penetrated into the plane of the negative charges of the phospholipid molecules. The curve is calculated from Eqn. 11 for a monolayer of phosphatidylinositol at 31.5 dynes/cm.

Method III (Hughes–Klotz plots)

In this method we apply the mass equation to the interaction of Ca^{2+} with phospholipids in a similar way as to that carried out by HUGHES AND KLOTZ[25] for the binding of metal ions to proteins.

$$\mathrm{Lip} + \mathrm{Ca}^{2+} \rightleftharpoons [\mathrm{Lip{\cdot}Ca}]; \qquad K_{A_1} = \frac{[\mathrm{Lip{\cdot}Ca}]}{[\mathrm{Lip}]a_{\mathrm{Ca}^{2+}}}$$

$$[\mathrm{Lip{\cdot}Ca}] + \mathrm{Ca}^{2+} \rightleftharpoons [\mathrm{Lip{\cdot}Ca_2}]; \qquad K_{A_2} = \frac{[\mathrm{Lip{\cdot}Ca_2}]}{[\mathrm{Lip{\cdot}Ca}]a_{\mathrm{Ca}^{2+}}}$$

and so on, where K_{A_1}, K_{A_2} are the equilibrium constants for the consecutive reactions, [Lip], [Lip·Ca], [Lip·Ca$_2$] are the concentrations of phospholipid and phospholipid–metal complexes, respectively, $a_{\mathrm{Ca}^{2+}}$ is the Ca^{2+} activity. When each binding site of the phospholipid is uninfluenced by its neighbours and each one has the same affinity for Ca^{2+}, then a definite statistical relation exists between the diffierent equilibrium constants K_{A_1}, K_{A_2} .... and to a single association constant K_A.

$$K_{A_i} = [(n - i + 1)/i]K_A; \quad i = 1, 2, 3 \ldots n$$

Using this relation and the general mass equation the quantity r defined above is given by the following equation

$$\frac{r}{[Ca^{2+}]_f} = nK_A - rK_A \tag{9}$$

Provided that there is no electrostatic interaction between neighbouring binding sites, a plot of $r/[Ca^{2+}]_f$ as a function of r should give a straight-line relation. This plot is shown in Fig. 7 for phosphatidylserine and phosphatidylinositol monolayers of various film pressures. With low pressure films, as predicted by Eqn. 9 straight lines are obtained. As the pressure increases a greater deviation from a straight-line relationship occurs.

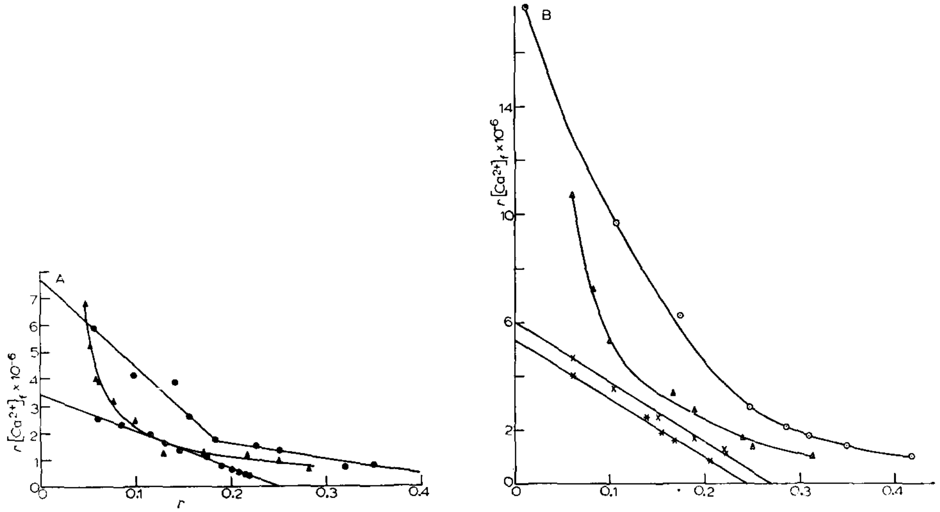


Fig. 7. Hughes–Klotz plots. The ratio $r/[Ca^{2+}]_f$ is plotted as a function of r = Ca^{2+} bound/total amount of phospholipid in the surface. $[Ca^{2+}]_f$ = concn. of Ca^{2+} in the bulk phase in M. ⊗—⊗, phosphatidylserine at 7 dynes/cm; ●—●, phosphatidylserine at 32.4 dynes/cm; ▲—▲, phosphatidylserine at collapse pressure; ✱—✱, phosphatidylinositol at 10 dynes/cm; ×—×, phosphatidylinositol at 12.5 dynes/cm; ⊙—⊙, phosphatidylinositol at 31.5 dynes/cm; △—△, phosphatidylinositol at collapse pressure.

TABLE II

Monolayer	*Pressure (dynes/cm)*	*n*	$K_A \times 10^{-7}$
Phosphatidylserine	7	0.25	1.38
Phosphatidylserine*	7	$n^1 = 25 \cdot 10^{14}$	1.36
Phosphatidylserine	32.4	0.46	1.7
Phosphatidylinositol	10	0.24	2.23
Phosphatidylinositol	12.5	0.27	2.21
Phosphatidylinositol	31.5	0.50	3.93

* The whole phospholipid film is regarded as the reacting entity. Knowing the total number of phosphatidylserine molecules in the surface ($97 \cdot 10^{14}$) the n^1 value can be related to n (n = 0.26).

Table II contains the K_A and n values obtained from such plots. As $r \rightarrow 0$: then $r/[Ca^{2+}]_f = nK_A$; also as $r/[Ca^{2+}]_f \rightarrow 0$: then $r = n$.

The K_A and n values of low-pressure films are readily obtained by extrapolation of the straight lines. Since the extrapolation is not unambiguous for monolayers of higher film pressures, the K_A and n values must be regarded as approximate. For this reason K_A and n values for collapse pressure are not included in Table II.

The shape of Curves b, c, f and g in Fig. 7 could also be interpreted in terms of two different binding sites with different K_A values. The extrapolation of the curves, however, was carried out on the assumption that there is only one kind of binding site per phospholipid molecule. This seems reasonable since there is no indication of a second binding site at low film pressures.

DISCUSSION

The surface-radioactivity technique precisely measures net movements of Ca^{2+} into the entire surface phase adjacent to the film, as distinct from surface-potential (ΔV) measurements which are presumed to register changes in the various dipole contributions to ΔV brought about by the interaction of Ca^{2+} with the phospholipid monolayer[31]. However, since the $^{45}Ca^{2+}$ adsorbed at the phospholipid/water interface are completely and rapidly removed when nonactive Ca^{2+} is added to the subphase[2], it is perhaps artificial to assume that there can be a clear distinction between a 'fixed' and diffuse electrical double layer of Ca^{2+}. It is probable that on moving out from the interface the bonding forces are reduced by the usual inverse square relationship for electrostatic adhesion so there is a constant gradation of binding.

The observation that predictions from the mass equations are borne out by the experimental results is good evidence that it is legitimate to use the surface-radioactivity measurement of bound Ca^{2+} in the derived expressions.

Binding of Ca^{2+} on phosphatidylserine and phosphatidylinositol monolayers at collapse pressure

It is clear that the adsorption of Ca^{2+} is dependent on the film pressure or the number of negative charges per cm^2. This is shown in Figs. 2 and 8. The adsorption isotherm for phosphatidylserine at collapse pressure is equivalent to that of phosphatidylinositol (Fig. 2). This indicates that the adsorption of Ca^{2+} on phosphatidylserine and phosphatidylinositol monolayers of collapse pressure is determined by the number of net negative charges per phospholipid molecule[2] and differences in the chemical structure have no influence on the metal binding. At the bulk pH used (5.5) the phosphate group on phosphatidylinositol is presumed to be fully ionized[2]. With phosphatidylserine it is also known that the carboxyl group is fully ionized at this pH (ref. 11) so that this phospholipid like phosphatidylinositol would have a single net negative charge. From the fact that (a) the negatively charged group in phosphatidylinositol is the phosphate group, (b) equal adsorption of Ca^{2+} on phosphatidylserine and phosphatidylinositol monolayers at collapse pressure takes place, and (c) that the adsorption of Ca^{2+} on the carboxylic groups of gangliosides appears to be less than that for a phosphate group of phospholipids[2], it seems reasonable to conclude that it is the negatively charged phosphate group in phosphatidylserine which is responsible for the attraction of Ca^{2+} and that the carboxyl group is compensated by the positively charged amino group.

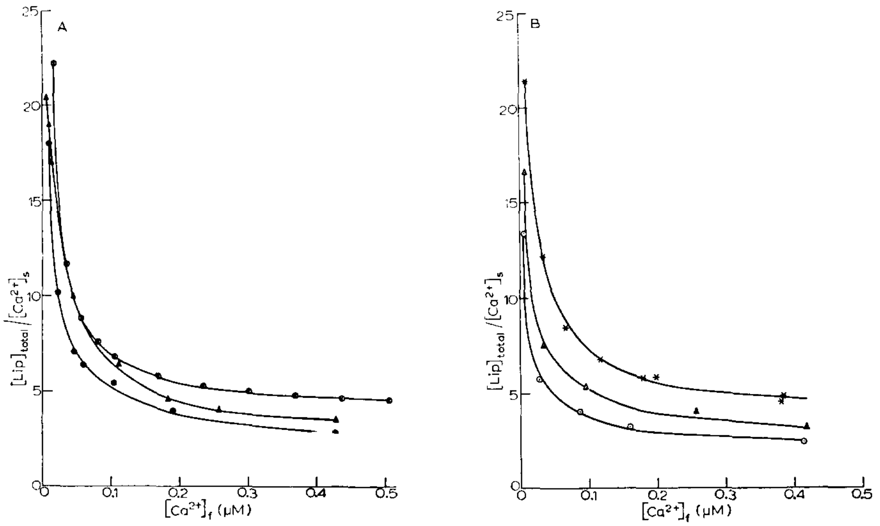


Fig. 8. Variation of the phospholipid to Ca^{2+} bound ratio $[Lip]_{total}/[Ca^{2+}]_s$ with the Ca^{2+} concn. $[Ca^{2+}]_f$ in the bulk phase. $(Ca^{2+}]_f$ in μM. a. For binding of Ca^{2+} to monolayers of phosphatidylserine: ⊗—⊗, 7 dynes/cm; ●—●, 32.4 dynes/cm; ▲—▲, collapse pressure. b. For the binding of Ca^{2+} to monolayers of phosphatidylinositol ✱—✱, 10 dynes/cm; ⊙—⊙, 31.5 dynes/cm; △—△, collapse pressure.

Binding of Ca^{2+} on phosphatidylserine and phosphatidylinositol monolayers at film pressures lower than collapse pressure

At film pressures lower than collapse pressures phosphatidylinositol molecules exhibit a stronger adsorption for Ca^{2+} than phosphatidylserine; the difference in K_A, however, is less than an order of magnitude. This difference may be due to the different chemical structure of the polar groups of these molecules. The serine moiety of phosphatidylserine and the inositide ring of phosphatidylinositol may contribute differently to the metal binding by chelating effects. These effects appear to become negligible at collapse pressure. Fig. 8 shows that the $[Lip]/[Ca^{2+}]_s$ ratio at any Ca^{2+} concentration used in the bulk phase is smaller at 30 dynes/cm than at collapse pressure, indicating that the binding of Ca^{2+} goes through a maximum both for phosphatidylserine and phosphatidylinositol[26]. This is clearly demonstrated in Fig. 9 where the $[Ca^{2+}]_s/[Lip]_{total}$ ratio is plotted as a function of the charge density, σ, where $\sigma = 10^{16}\varepsilon/A$; A = area/molecule and ε = electronic charge. This maximum of adsorption at a pressure of approx. 26–28 dynes/cm may be due to the spacing between the phospholipid molecules at that pressure becoming particularly favourable for two-point electrostatic attachment of Ca^{2+} (refs. 2 and 26).

At low pressures the wider spacing may impede the two-point electrostatic attachment of Ca^{2+}, while the closest spacing at collapse pressure may inhibit the penetration of the hydrated Ca^{2+} to the most suitable position. HAUSER AND DAWSON[26] point out that the distance between the negative charges of a phosphatidylinositol monolayer approaches the diameter of hydrated Ca^{2+} (9.6 Å) at the pressure at which Ca^{2+} binding reaches a maximum.

The K_A and n values obtained by the three different methods compare reasonably with each other. Examination of the n values listed in Tables I and II shows that as the film pressure is increased to 30 dynes/cm the value approaches 0.5. The approach of n to a value of 0.5 means by definition that there are two phospholipid molecules for every Ca^{2+} bound, or in other words two-point electrostatic attachment. At higher Ca^{2+} concentration it seems possible that n could become larger and approach one, implying a single-point electrostatic attraction[12].

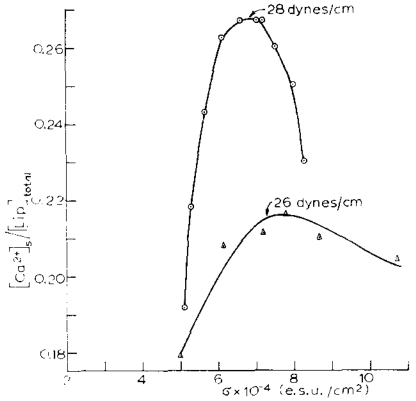


Fig. 9. Relation between the ratio of Ca^{2+} bound to phospholipid ($[Ca^{2+}]_s/[Lip]_{total}$) and the charge density σ of monolayers of phosphatidylserine and phosphatidylinositol. σ is expressed in e.s.u./cm². The total Ca^{2+} concn. $[Ca^{2+}]_{total}$ = 0.21 μM. △—△, phosphatidylserine; ⊙—⊙, phosphatidylinositol.

Electrostatic effects account for deviations from the mass equation

Figs. 5A and 5B show that only with low-pressure films do the K_A values remain constant or decrease slightly with increasing r ($r = [Ca^{2+}]_s/[Lip]_{total}$). At higher film pressures the K_A values are dependent on the amount of Ca^{2+} bound and decrease considerably with increasing r. The K_A values decrease more rapidly at low r values and appear to level out at higher r values. The Hughes-Klotz plots in Fig. 7 confirm the results shown in Fig. 5. Only with low-pressure films is an ideal behaviour observed. A straight-line relationship is obtained, the experimental data following Eqn. 9. This equation is derived for a single set of binding sites with no electrostatic interaction between them. At higher film pressures this assumption is no longer valid, and electrostatic interaction causes the deviations from a straight-line relationship.

Comparing the distance between the negative charges in a phosphatidylinositol monolayer of 10 dynes/cm (area/molecule = 94 Å², distance 11 Å) with the distance at 30 dynes/cm (area/molecule = 68 Å², distance 9.3 Å) it is surprising that with the former a straight line is obtained consistent with negligible electrostatic forces between adjacent binding sites. However, for a given concentration of Ca^{2+} added, the Ca^{2+} adsorbed on the monolayer will be much higher with the film at 30 dynes/cm (Fig. 2). Calculation shows that at a film pressure of 10 dynes/cm the electrostatic force of atrraction between one Ca^{2+} and a phosphate group is at least 30 times larger than the electrostatic force of repulsion between two Ca^{2+} in the surface. Fig. 5 shows that even at 7 dynes for phosphatidylserine and 10 dynes for phosphatidylinositol, the K_A values slightly decrease with increasing r, indicating that also at lower pressures small electrostatic interaction occurs between neighbouring binding sites. These electrostatic forces between adjacent binding sites, however, are not big enough to cause an observable deviation from a straight line in the Hughes–Klotz plot.

Comparison of the K_A values with apparent association constants determined by other techniques

BARTON[24] determined the apparent association constant for the interaction of Ca^{2+} with aqueous dispersion of phosphatidylserine and phosphatidic acid by measuring the charge reversal concentration. At the charge reversal point $\psi_S = 0$ and the standard free energy change of the Ca^{2+} adsorption reduces to $-\Delta G_0 = \lambda_p + W$. BARTON determined ΔG_0 and was able to calculate the apparent association constant. His values of $K_A = 1.17 \cdot 10^4$ for phosphatidylserine and $K_A = 1.05 \cdot 10^4$ for phosphatidic acid are consistent with values obtained for the reaction of Ca^{2+} with aqueous dispersions of phosphatidylserine and phosphatidic acid by titration[5,9] and turbidity methods[27]. These K_A values are, however, three orders of magnitude lower than the values that we obtain for the binding of Ca^{2+} onto monolayers of low film pressures ($K_A = 1.4 \cdot 10^7$ for a phosphatidylserine monolayer at a pressure of 7 dynes). This discrepancy can be explained in two ways. (1) Since with the charge reversal method $\psi_S = 0$, the difference between the apparent association constant of BARTON and our values might be accounted for by the electrostatic term $-Z\varepsilon\psi_S$ of the standard free energy $-\Delta G_0$ of Eqn. 7. Whereas in our experiments using distilled water as a bulk phase the electrostatic term is considerable, the contribution of this term is either zero (with the method of BARTON) or probably small (with the titration and turbidity methods) owing to the use of an ionic strength of at least 0.1. (2) Although both HENDRICKSON AND FULLINGTON[5] and ABRAMSON and co-workers[8,28] found that all acidic groups in the aqueous phospholipid dispersions prepared by ultrasonic irradiation were available for titration this need not necessarily apply for the interaction with Ca^{2+}. From $K_A = e^{-\Delta G_0/kT}$ and Eqn. 7 Eqn. 10 is derived[29]

$$K_A = K_I \, e^{-Z\varepsilon\psi_S/kT} \tag{10}$$

where K_I is the intrinsic association constant[30] describing the specific binding of Ca^{2+} by the charged phospholipid. K_I* is identical with $e^{(\lambda_p + W)/kT}$, the association constant of BARTON determined from the charge reversal concentration when $\sigma \rightarrow 0$ ($\psi_S = 0$). The association constants of BARTON are identical with the values of HENDRICKSON AND FULLINGTON, although these authors claim that their association constants include an electrostatic term due to the field of the phosphatidylserine particles. This is not in agreement with Eqn. 10.

Ca^{2+} bound to phosphatidylserine and phosphatidylinositol monolayers form a Stern layer

In Fig. 6 the Stern potential ψ_S for phosphatidylinositol monolayers at 31.5 dynes/cm and collapse pressure calculated from Eqn. 8 is plotted as a function of r. If, however, Ca^{2+} did not form a Stern layer, but penetrated into the plane of the anionic binding sites of the phospholipid molecule (Fig. 10B), then the charge density of the monolayer would be reduced and the Gouy potential would change steeply. Such a penetration cannot be interpreted by the Stern equation, since (see Fig. 10A),

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* $e^{(\lambda_p + W)/kT} = K_I$ would only be justified if $\lambda_p + W$ were independent of the film pressure.

the distance between the Gouy and the Stern potential becomes zero (Fig. 10B). The Gouy potential can be calculated from Eqn. 11.

$$\psi_G = \left(\frac{2kT}{\varepsilon v}\right) \sin h^{-1} \left[\frac{\sigma}{c^{1/2}} \left(\frac{500\pi}{DkT}\right)^{1/2}\right] \tag{11}$$

where c = concentration of $[Ca^{2+}]_f$ in M; v = valency of Ca^{2+}; k = Boltzmann constant; T = absolute temperature; ε = electronic charge; σ = charge density; D = bulk dielectric constant; R = gas constant.

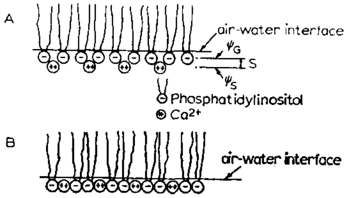

Fig. 10. Possible positions of Ca^{2+} beneath a negatively charged monolayer. A. Ca^{2+} forming a Stern layer. B. Ca^{2+} penetrating into the plane of the negative charges of the monolayer. The plane of the Gouy potential and the plane of the Stern potential are marked by ψ_G and ψ_S, respectively.

With the penetration of Ca^{2+} into the plane of the anionic binding sites σ of Eqn. 11 is given by $\sigma = \varepsilon(10^{16}/A - 2[Ca^{2+}]_s)$ where $[Ca^{2+}]_s$ is the concentration of Ca^{2+} per cm^2.

Eqn. 10 describes the variation of the apparent association constant with the surface potential ψ. In the case of Ca^{2+} penetrating into the plane of the anionic binding sites the Gouy potential ψ_G has to be inserted into Eqn. 10.

Fig. 6 demonstrates how the Gouy potential of a phosphatidylinositol monolayer (31.5 dynes/cm) would change if Ca^{2+} penetrated into the plane of the charged phosphate group. Using the Gouy potential in Eqn. 10 leads to values not consistent with the experimental K_A values of Fig. 5. We, therefore, conclude that the Ca^{2+} adsorbed to the monolayer forms a Stern layer as shown in Fig. 10A. This can be compared with the view of SHAH AND SCHULMAN[31] who concluded from measurements of the surface potential that the Ca^{2+} penetrated beyond the Gouy layer in lecithin films.

CONCLUSIONS

1. The amount of Ca^{2+} adsorbed on monolayers of pure phosphatidylserine and phosphatidylinositol is mainly a function of the charge density.

2. At collapse pressure the binding of Ca^{2+} is directly related to the net excess negative charge on the phospholipid molecule and largely independent of the chemical nature of the phospholipid molecule. Both with phosphatidylserine and phosphatidylinositol the Ca^{2+} is bound to the phosphate group.

3. At film pressures lower than collapse pressure, small differences in the binding of Ca^{2+} to phosphatidylserine and phosphatidylinositol are observed. These differences are accounted for by the different chemical structure of the polar groups of these molecules.

4. At low film pressures (or low charge densities) the binding of Ca^{2+} follows the mass equation. Deviations from the mass equation are observed with increasing film

pressure (charge density) and are attributed to electrostatic interaction between adjacent binding sites.

5. Ca^{2+} is bound to the phospholipid molecules by two-point electrostatic attachment.

6. The Ca^{2+} bound/lipid ratio reaches a maximum at about 26–28 dynes/cm when the spacing between phospholipid molecules approaches the diameter of the hydrated Ca^{2+}.

7. Ca^{2+} bound to phosphatidylserine and phosphatidylinositol form a Stern layer and do not penetrate into the plane of the Gouy potential.

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