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Divalent cation-induced phosphatidic acid membrane fusion. Effect of ion binding and membrane surface tension

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A study was made on the correlation between the degree of membrane fusion and surface tension increase of phosphatidic acid membranes caused by divalent cations. Membrane fusion was followed by the Tb^{3+}/dipicolinic acid assay, monitoring the fluorescent intensity for mixing of the internal aqueous contents of small unilamellar lipid vesicles. The surface tension and surface potential of monolayers made of the same lipids as used in the fusion experiments were measured as a function of divalent cation concentration. It was found that the 'threshold' concentration to induce massive vesicle membrane fusion was the same for Ca^{2+} and Mg^{2+}, and that the surface tension increase in the monolayer, induced by changing divalent cation concentration from zero to a concentration which corresponds to its threshold value, inducing vesicle membrane fusion, was approximately the same: 6.3 dyn/cm for both Ca^{2+} and Mg^{2+}. Both the divalent cation's threshold concentrations as well as the surface tension change corresponding to the threshold concentration for the phosphatidic acid membrane were smaller than those for the phosphatidylserine membrane. The different fusion capability of these divalent cations for phosphatidic acid and phosphatidylserine membranes is discussed in terms of the different ion binding capabilities of these ions to the membranes.

Introduction

Many exocytotic processes seen in biological systems seem to involve membrane fusion between the intracellular vesicle and plasma membranes, induced by the intracellular Ca^{2+} [1,2]. In order to gain insight into the mechanism for such membrane interaction processes, a number of membrane fusion studies using model membrane (lipid membrane) systems have recently been made [3,4]. It was demonstrated that acidic phospholipid membranes can fuse at a certain increased level of divalent cation concentration in a membrane bathing solution [3–5]. Out of the many acidic phospholipids, phosphatidylserine has often been used for such membrane fusion studies. So far, only a few workers have studied membrane fusion using pure phosphatidic acid membranes [6–8]. This may be due to the fact that phosphatidic acid is one of the least common lipids among those which are found in natural membranes [9]. However, it has a rather simple polar headgroup, which has only one dissociable polar group below pH 7.0 and has no other bulky polar groups as do phosphatidylserine, phosphatidylinositol or phosphatidylglycerol [10]. Therefore, the use of phosphatidic acid membrane may be more appropriate and may make it simpler to determine the mode of ion binding to the phospholipid membranes than the use of other

Abbreviation: Tes, 2-((2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino)-ethanesulfonic acid.

acidic phospholipid membranes. Since it is expected that the membrane fusion may depend on the nature of the surface polar group and the thickness of the polar phase, in this paper we examine the divalent-cation-induced fusion of phosphatidic acid membranes and its divalent-cation-dependent surface properties derived from surface-tension measurements and surface-potential measurements, in order to relate the ion binding properties to membrane fusion, and discuss the membrane fusion in relation to the phosphatidylserine membrane fusion.

Materials and Methods

Chemicals. Phosphatidic acid (derived from egg phosphatidylcholine) was purchased from Avanti Biochemical Co. (Birmingham, AL). The lipid sample showed a single spot on silica gel thin-layer chromatographic plate. NaCl used was of ultrapure grade purchased from Alfa Chemical Co. Before use, the NaCl was roasted at 400–500°C for 2 h to eliminate possible organic contaminants. The divalent cations used were all in the form of chloride salts obtained from Fisher Chemical Co. (reagent grade). $TbCl_3 \cdot 6\ H_2O$ (99.9% pure) and dipicolinic acid (pyridine 2,6-dicarboxylic acid) were obtained from Alfa and Sigma Chemical Companies, respectively, and used without further purification. Buffers used were a mixture of L-histidine (Calbiochem., Ultrol grade) and Tes (Calbiochem, A grade). Hexane was used as the solvent for the lipids. It was obtained from Fluka (purum grade) and further purified through activated alumina and silica gel (both from Fisher Chemical Co.) columns to remove possible contaminating surfactants. All experimental solutions contained a small amount of EDTA (Alfa Chemical Co.) in order to remove any divalent and polyvalent cation contaminants. The water used was distilled three times, including an alkaline permanganate process.

Vesicle preparation. Small unilamellar vesicles were prepared in either (a) 3 mM $TbCl_3$ and 100 mM sodium citrate or (b) 100 mM dipicolinic acid (sodium salt), both containing 2 mM L-histidine and 2 mM Tes. The pH of these salt solutions was adjusted to 7.0 with NaOH. The phospholipid (phosphatidic acid) was dispersed in either of the aqueous salt solutions, (a) or (b), at a concentration of 10 μmol/ml, vortexed for 10 min and sonicated for 1 h in a bath-type sonicator under N_2 at 25°C. The samples were then centrifuged for 1 h at 100 000 $\times$ g to remove large vesicles and/or aggregates. The supernatants were kept as unilamellar lipid vesicle suspensions. The yield of unilamellar lipid vesicles was about 90–95% of the total lipids used for preparation. Each lipid concentration was determined by the phosphate assay [11]. Vesicles were separated from non-encapsulated material using a Sephadex G-75 column according to the method of Wilschut et al. [12]. The elution buffer (0.1 M NaCl/2 mM histidine/2 mM Tes) contained 0.01 mM EDTA.

Assay of vesicle fusion. The fusion of vesicles induced by divalent cation was followed by a Tb^{3+}/dipicolinic acid assay, monitoring the fluorescent intensity (SLM-8000 spectrofluorimeter, SLM Instruments) due to mixing of the internal aqueous contents of unilamellar vesicles. The details are described in the earlier papers [5,12]. After $TbCl_3$ and dipicolinic acid encapsulated vesicles of an equimolar amount (0.1 μmol phospholipid each) were suspended in 2 ml of NaCl buffer (0.1 M NaCl/2 mM histidine/2 mM Tes/0.01 mM EDTA) solution in a quartz cuvette, divalent cations (40 mM $CaCl_2$ or $MgCl_2$) were injected into the vesicle suspension in small increments, and the solution was well shaken to give a homogeneous mixture. It took 10 s to change the divalent ion concentration in the experimental solution. The fluorescent intensity was measured about 15 s after each change in divalent ion concentration. The excitation wavelength was 275 nm and the emission fluorescence was measured at 542 nm, employing a Corning 3-68 cut-off filter to eliminate the contribution of light scattering to the signal. The value for 100% fusion was determined in the presence of 50 μM dipicolinic acid by release of the contents from Tb-containing vesicles (the same amounts as in the experiment, except that they were free from EDTA during the Sephadex G-75 column chromatography) with 0.5% (w/w) sodium cholate. However, since the leakage of internal contents from lipid vesicles during fusion is not compensated for percent fusion, the final results indicate only the relative quantity of vesicle fusion.

Threshold concentration of divalent ion-induced vesicle fusion. The fluorescence measured 15 s after each divalent ion concentration change was plotted as a function of the divalent ion concentration. The point at which the line extrapolated from the segment of the sharpest slope of the curve intercepted the concentration axis was determined. This was defined as the 'threshold concentration' of the divalent cation causing vesicle fusion.

Surface potential measurements. Surface potential of phospholipid monolayers was measured using an Americium air electrode and a calomel pH reference electrode [13]. The latter electrode was grounded and the ionizing air electrode was connected to the input of an electrometer (Keithley 610C). The output potential of the electrometer, which was related to 'surface potential', was monitored by a strip chart recorder. The entire apparatus was shielded by a Faraday cage.

A lipid (phosphatidic acid) monolayer was formed on the aqueous phase in a glass dish (9 cm in diameter) by spreading an aliquot of the hexane-lipid solution from a microsyringe (Hamilton). The subphase solution was 0.1 M NaCl containing 1 mM histidine/1 mM Tes (pH 7.0). Each experimental solution also contained 10 μM of EDTA to remove polyvalent cationic contaminants possibly present in trace amounts in the system. All the monolayers used in the experiment had the same area per molecule of 65 $Å^2$ (0.65 nm^2). This value was also determined to be correct within 2 $Å^2$ by surface pressure (π) measurements, the results of which corresponded to those determined for similar systems from a π vs. surface area curve.

Before the addition of salt, the aqueous subphase was stirred well and it was ascertained that the monolayer surface potential was not altered by further stirring of the subphase solution. Then a concentrated salt solution was injected into the subphase of the monolayer from a microsyringe. The subphase solution was again stirred until a stable surface potential was obtained. The standard mean error for the observed surface potentials were within ± 1 mV. Each data point represents the average of four or more experiments.

Surface tension measurements. The phosphatidic acid monolayers were prepared by placing an aliquot of the lipid spreading solution (approx. 1 mM lipid in hexane) by means of a microsyringe (Hamilton) on to an aqueous surface of constant area (64 cm^2 in a glass dish). The surface tension was measured after complete evaporation of hexane. The area per molecule of each monolayer was kept constant at 65 $Å^2$. Subphase solutions were 0.1 M NaCl containing a small amount of buffer (2 mM histidine and 2 mM Tes) and 0.01 mM EDTA. The pH of solutions was adjusted to 7.0 with NaOH. The surface tension * of the monolayers were measured with an electronic valance (Beckman), using a microscopic cover glass ($18 \times 18 \times 0.2$ mm) as a Wilhelmy plate (accuracy of ± 0.1 dyn/cm). The procedure for measuring the surface tension was almost the same as that published earlier [5]. The surface tension of a phosphatidic acid monolayer corresponding to the above area per molecule was reproducible within 3 dyn/cm for each monolayer. The experiments were designed to measure the change in the surface tension of such monolayers as a function of divalent cation concentration in the subphase solution. The salt concentrations were altered by injecting a small amount of a concentrated salt solution (1 M). After each injection of salts, the solutions were stirred well with a magnetic stirrer. All experiments were done at room temperature of 24 ± 1°C.

Experimental results

Fig. 1 shows the changes in fluorescence intensity of phosphatidic acid vesicle suspensions in 0.1 M NaCl with respect to various divalent cation concentrations where the two kinds of phosphatidic acid vesicles encapsulated with $TbCl_3$ or dipicolinic acid were suspended. All the experimental NaCl buffer solutions contained 2 mM histidine, 2 mM Tes and 0.01 mM EDTA. The pH of the solution was adjusted with NaOH to 7.0. The change in fluorescence intensity represents the degree of fusion of the two kinds of vesicles [12]. The divalent cation concentrations previously defined as the 'threshold concentration' to induce the vesicle membrane fusion were approx. 0.25 mM for both Ca^{2+} and Mg^{2+}. This result is

* To be exact, this should be called the surface tension of a lipid monolayer-coated air/water interface.

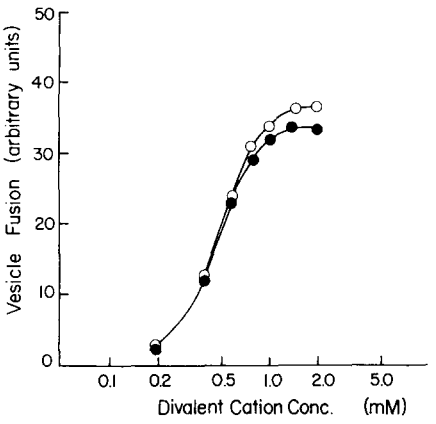

Fig. 1. Fusion of phosphatidic acid vesicles in 0.1 M NaCl with respect to various divalent cation (○, Ca^{2+}; ●, Mg^{2+}) concentrations. Two kinds of phospholipid vesicles (0.1 μmol lipid each) which are encapsulated with either 3 mM Tb/100 mM sodium citrate or 100 mM dipicolinic acid, respectively, were suspended in 2 ml of 0.1 M NaCl/2 mM histidine/2 mM Tes/0.01 mM EDTA (pH 7.0). The ordinate refers to the fluorescence intensity (see Materials and Methods) at 542 nm and the abscissa refers to divalent cation concentration. The fluorescent intensity is related to the relative quantity of vesicle fusion. The wavelength of excitation light was 275 nm (24°C).

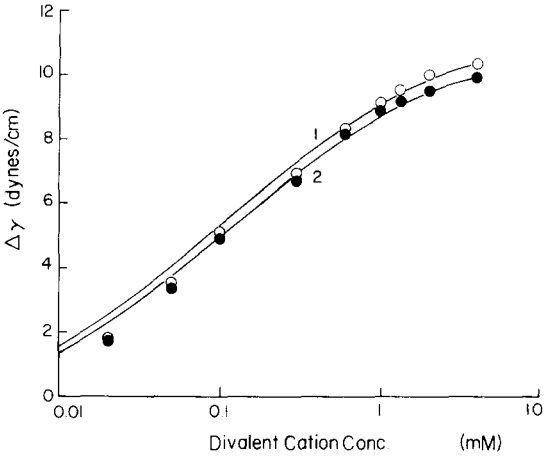

Fig. 2. Surface tension increase in the phosphatidic acid monolayer (approx. 65 Å per molecule) formed at the air/water interface with respect to various divalent cation concentrations. The subphase solution consisted of 0.1 M NaCl/1 mM histidine/1 mM Tes/0.01 mM EDTA (pH 7.0). The initial surface tension $\gamma(C_2 = 0)$ of a phosphatidic acid monolayer-coated (65 $Å^2$ per molecule) air/water interface was 24 ± 2 dyn/cm and $\Delta\gamma \equiv \gamma(n_2) - \gamma(n_2 = 0)$ where n_2 refers to the divalent cation concentration (○, Ca^{2+}; ●, Mg^{2+}). The solid lines in the figure refer to the calculated surface tension increase $\Delta\gamma$ as a function of divalent cation concentration with use of the following parameters: curve 1 refers to the Ca^{2+} case with $K_1 = 0.5$ M^{-1}, $K_2 = 10$ M^{-1}, $K_3 = 60$ M^{-1} and $u = 2.9$; curve 2 refers to the Mg^{2+} case with $K_1 = 0.5$ M^{-1}, $K_2 = 8$ M^{-1}, $K_3 = 50$ M^{-1} and $u = 2.8$.

comparable with those obtained in the earlier studies [6,8]. The threshold values obtained in those studies are slightly lower than ours and are in a wider range (0.05–0.2 mM). This may be due to (1) the use of different methods to detect membrane fusion [6] and (2) the use of large unilamellar vesicles of various size distributions [8]. The extent of fusion of the phosphatidic acid membranes was approximately the same for Ca^{2+} and Mg^{2+} in their overall concentration ranges examined.

In order to correlate the observed divalent cation-induced membrane fusion with the divalent cation-induced surface tension increase of the membrane, the surface tension measurements of phosphatidic acid monolayers were performed. Monolayers were formed on an aqueous solution of 0.1 M NaCl/1 mM histidine/1 mM Tes/0.01 mM EDTA (pH 7.0) at a constant area 65 $Å^2$ per molecule. The increase in surface tension of the monolayer is plotted as a function of divalent cation (Ca^{2+} and Mg^{2+}) concentration (Fig. 2). The surface tension increases in the phosphatidic acid monolayer with respect to divalent cation concentration were almost the same for both Ca^{2+} and Mg^{2+}. A similar result has been reported earlier [14] for the phosphatidic acid monolayer, at an equilibrium pressure different from that in this experiment. In the above two experiments with phosphatidic acid we have found at least two points which are different from those with the phosphatidylserine membranes: (1) the threshold concentration of Ca^{2+} for the phosphatidic acid vesicle fusion was slightly lower than that of the phosphatidylserine vesicle fusion, while the threshold concentration (0.25 mM) of Mg^{2+} was much lower than that (6 mM) for the phosphatidylserine membrane [5] and (2) the surface tension changes of the phosphatidic acid membrane were almost the same for both Ca^{2+} and Mg^{2+}, while in the case of the phosphatidylserine membrane there was a distinct difference between Ca^{2+} and Mg^{2+}: Ca^{2+} had a greater effect on surface tension as well as on membrane fusion of phosphati-

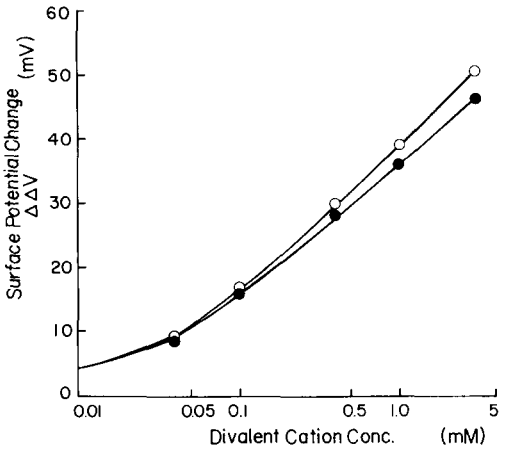

Fig. 3. Surface potential change ($\Delta\Delta V$) of the phosphatidic acid monolayer versus divalent cation (Ca^{2+}, Mg^{2+}) concentration in the subphase bulk solution (0.1 M NaCl/1 mM histidine/1 mM Tes/0.01 mM EDTA). The monolayers with an area 65 $Å^2$ per molecule were formed at the air/water interface. The surface potential change $\Delta\Delta V \equiv \Delta V(n_2) - \Delta V(n_2 = 0, n_1 = 0.1\ M)$. ○, Ca^{2+}; ●, Mg^{2+}. $t = 24°C$.

dylserine membranes than did Mg^{2+} [5]. The surface tension increase of the phosphatidic acid monolayer at the divalent cation concentration, which corresponds to the threshold concentration of the phosphatidic acid membrane fusion, is about 6.3 dyn/cm. This surface tension increase is smaller than that (8 dyn/cm) observed for the phosphatidylserine monolayer in the same situation (at the threshold concentration of divalent cation to induce the lipid vesicle membrane fusion). Similarly, in the vesicle fusion and surface tension experiments for phosphatidic acids, the surface potentials of the phosphatidic acid monolayer were more-or-less identical for Ca^{2+} and Mg^{2+} with respect to their concentrations in the subphase solution (Fig. 3), where the change in surface potential ($\Delta\Delta V = \Delta V(n_2, n_1 = 0.1\ M) - \Delta V(n_2 = 0, n_1 = 0.1\ M)$ is plotted as a function of divalent cation concentration (n_2 and n_1 refer to divalent and monovalent cation concentrations, respectively). This suggests that the binding strengths of both Ca^{2+} and Mg^{2+} for the phosphatidic acid membrane are approximately equivalent.

Analysis of Experimental Results and Discussion

Binding constants of Na^+, Ca^{2+} and Mg^{2+} for phosphatidic acid membranes with an area per molecule of 65 $Å^2$ can be calculated using of the method developed by Ohshima and Ohki [15]: we can solve two equations, surface tension equation (A1) and surface potential equation (A8) simultaneously (see Appendix), so as to fit the experimental data of both surface potential and surface tension measurements by adjusting the binding constants of each ionic species for the membrane. We assume the following binding mode for each ion: one Na^+ can bind to one phosphatidic acid molecule (1 : 1 binding), while one divalent cation can bind to either one lipid molecule (1 : 1 binding), or adjacent two lipid molecules in chelation binding (2 : 1 binding), and there is no anion binding to the membrane. We denote these ion binding constants by K_1 (1 : 1 binding for monovalent cation); K_2 (1 : 1 binding for divalent cation) and K_3 (2 : 1 binding for divalent cation). Thus, the calculated binding constants are $K_1 = 0.5\ M^{-1}$ for Na^+, $K_2 = 10\ M^{-1}$ for Ca^{2+} of the 1 : 1 mode binding and $K_3 = 60\ M^{-1}$ for Ca^{2+} of the 2 : 1 mode binding and $u = 2.9$. The corresponding values in the Mg^{2+} case are $K_1 = 0.5\ M^{-1}$, $K_2 = 8\ M^{-1}$ and $K_3 = 50\ M^{-1}$ and $u = 2.8$. Here u is defined as

$$u = (-1/2)\partial \ln K_3 / \partial \ln a$$

(a = area per lipid molecule), which corresponds to half the work necessary to form a bridged lipid pair from two unbridged lipids. Theoretical curves for $\Delta\gamma$ and ψ_s vs. divalent cation concentration, calculated with the above values of the binding parameters, are shown in Figs. 2 and 4, respectively, in comparison with the experimental results. As the experimental data for ψ_s gives only the change in ψ_s by addition of divalent cations, they are plotted in Fig. 4 so as to coincide with theoretical curves within the limit of zero divalent-cation concentration. The binding constant ($0.5\ M^{-1}$) of Na^+ for the phosphatidic acid membrane is smaller than that ($0.8\ M^{-1}$) for the phosphatidylserine membrane [16]. The similar binding constants of Na^+ for the phosphatidic acid and phosphatidylserine membranes are obtained from the electrophoretic mobility studies of these lipid vesicles (Ref. 17 and Ohki, S. unpublished data). The binding constants (1 : 1 binding and 2 : 1 binding) for Ca^{2+} are slightly greater than those ($K_2 = 6.5\ M^{-1}$, $K_3 = 50\ M^{-1}$) [15] for the phosphati-

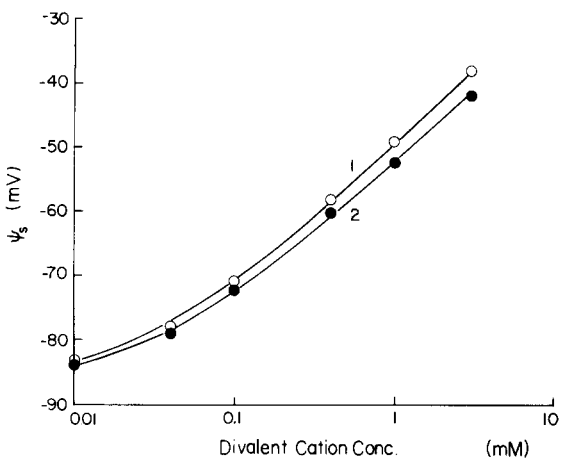

Fig. 4. Comparison of experimental and theoretical surface potentials, ψ_s, of the phosphatidic acid monolayer as a function of divalent cation (○, Ca^{2+} and ●, Mg^{2+}) concentration. The experimental values $\Delta\Delta V$ (in Fig. 3) are replotted as the surface potential ψ_s after knowing the calculated values of surface potential of the monolayer. The calculated values (solid lines; curve 1 for Ca^{2+} and 2 for Mg^{2+}) were obtained by using the following ion binding constants ($K_1 = 0.5\ M^{-1}$, $K_2 = 10\ M^{-1}$ and $K_3 = 60\ M^{-1}$ for the Ca^{2+} case; $K_1 = 0.5\ M^{-1}$, $K_2 = 8\ M^{-1}$ and $K_3 = 50\ M^{-1}$ for the Mg^{2+} case) which produced the best fit curves to the experimental points shown in Fig. 3 ($t = 24°C$).

dylserine membrane, while for Mg^{2+} they are much greater than those ($K_2 = 2.5\ M^{-1}$ and $K_3 = 15\ M^{-1}$) [15] of the phosphatidylserine membrane. It is interesting that the binding constants of Ca^{2+} and Mg^{2+} for the phosphatidic acid membrane are approximately the same for the 1 : 1 binding and the 2 : 1 binding modes, respectively. We also note that the parameter u takes similar values for different cations Ca^{2+} and Mg^{2+} and also for different lipid membranes : phosphatidic acid and phosphatidylserine (for the case of phosphatidylserine membrane, $u = 2.9$ for Ca^{2+} and 2.4 for Mg^{2+} [15]).

The reason why the phosphatidic acid membrane differs from the phosphatidylserine membrane in their ion binding behavior is probably due to the fact that in the phosphatidic acid membrane the negatively charged phosphate site is likely not to be hindered by the polar moiety for cation binding, and the charged site is directly and probably equally accessible to both Ca^{2+} and Mg^{2+}. For the phosphatidylserine membrane, on the other hand, the phosphate group is rather hindered for cation binding by the bulky serine headgroup, although another binding site (COO^-) for cations at the outer portion of the polar group may be equally accessible to both Ca^{2+} and Mg^{2+} for binding. Therefore, a smaller and less hydrated Ca^{2+} may be able to penetrate into the polar group region and bind to the charged phosphate group more easily than Mg^{2+}. These may be the reasons why there is a large difference between Ca^{2+} and Mg^{2+} binding constants for the phosphatidylserine membrane, while there is not so much difference between these two divalent cations for the phosphatidic acid membrane.

Each fraction of unbound lipid, Na^+-bound lipid, divalent cation bond (1 : 1 as well as 2 : 1 mode bindings) lipids to the total lipids ($X_i (i = 0–3)$ (see Appendix)) is shown in Figs. 5 and 6 as a function of divalent cation concentration. Similar to that for the phosphatidylserine membrane [15], the 2 : 1 binding mode (X_3) of divalent cations increases considerably in the range of 0.05 to 1.0 mM of divalent cations, and its fraction is about 2-times greater than that of the 1 : 1 binding mode of divalent cations in the same concentra-

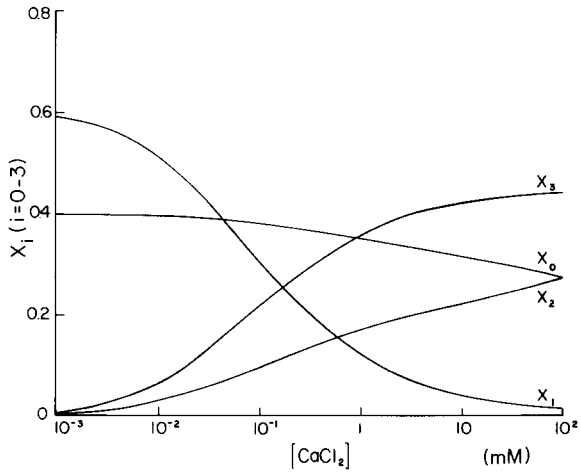

Fig. 5. The fraction of the lipids in four different forms, as a function of $CaCl_2$ concentration. X_0, unbound lipid; X_1, lipid bound to Na^+; X_2, lipid bound to Ca^{2+} in 1 : 1 binding. X_3, lipid bound to Ca^{2+} in 2 : 1 binding. Calculation was done with (K_1, K_2, K_3) = (0.5 M^{-1}, 10 M^{-1}, 60 M^{-1}). The definition of X_i is given in the Appendix.

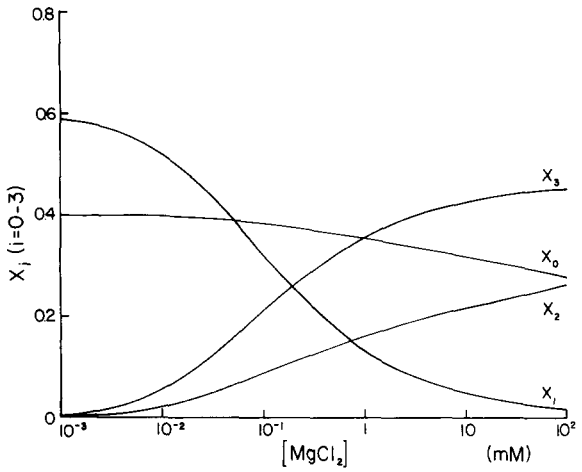

Fig. 6. The fraction $X_i (i = 0\text{–}3)$ as a function of $MgCl_2$ concentration. Calculation was done with $(K_1, K_2, K_3) = (0.5\ M^{-1}, 8\ M^{-1}, 50\ M^{-1})$.

tion range. From the previous work [18], we know that this 2 : 1 binding (bridge binding) of divalent cation is mainly responsible for the large increase in surface tension of acidic phospholipid membranes observed, and also we have hypothesized that this surface tension increase is related to the membrane fusion. From Figs. 1, 2, 5 and 6, we see that the membrane fusion for the phosphatidic acid membrane occurs at lower divalent cation concentration or at a smaller surface tension increase, or at a smaller degree of the 2 : 1 binding than those for the phosphatidylserine membrane fusion. This is reasonable given the following: membrane fusion may depend on the nature of the surface layer of the polar headgroups and its thickness. When the thickness of the surface layer is not great, the degree to which the surface hydrophilic layer has to become more hydrophobic in order to induce membrane fusion may be smaller than that for a membrane for which the surface hydrophilic layer is of greater thickness. Further studies investigating this situation are being undertaken in our laboratory.

Acknowledgements

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Appendix

The surface tension γ of a lipid-monolayer-coated air/electrolyte solution interface can be expressed [15] as

$$\gamma = \gamma_0 - \epsilon_r \epsilon_0 \kappa \left(\frac{kT}{e}\right)^2 \left[(2+e^{-y_s}) \left(\left(1-\frac{\eta}{3}\right) e^{y_s} + \frac{\eta}{3} \right)^{1/2} - 3 + \frac{1-\eta}{2} \left(\frac{3}{\eta}\right)^{1/2} \times \ln \frac{\left(1-(\eta/3)^{1/2}\right)\left[\left((1-\eta/3)\,e^{y_s}+\eta/3\right)^{1/2}+(\eta/3)^{1/2}\right]}{\left(1+(\eta/3)^{1/2}\right)\left[\left((1-\eta/3)\,e^{y_s}+\eta/3\right)^{1/2}-(\eta/3)^{1/2}\right]} \right] + \frac{4K_3 n_2 e^{-2y_s}}{\left[P+\left(P^2+4K_3 n_2 e^{-2y_s}\right)^{1/2}\right]^2} \frac{u}{a} kT \quad \text{(A1)}$$

with

$$\kappa = \left(\frac{2(n_1+3n_2)\,e^2}{\epsilon_r \epsilon_0 kT} \right)^{1/2} \quad \text{(A2)}$$

$$y_s = \frac{e\psi_s}{kT} \quad \text{(A3)}$$

$$\eta = \frac{3n_2}{n_1+3n_2} \quad \text{(A4)}$$

$$P = 1 + K_1 n_1 e^{-y_s} + K_2 n_2 e^{-2y_s} \quad \text{(A5)}$$

$$u = -\frac{1}{2} \frac{\partial \ln K_3}{\partial \ln a} \quad \text{(A6)}$$

where γ_0 is the surface tension when the monolayer is uncharged; ψ_s is the surface potential of the monolayer; n_1 and n_2 are respectively mono- and divalent cation concentrations; ϵ_r is the relative permittivity of the solution; ϵ_0 is the permittivity of a vacuum; k is the Boltzmann constant; T is the absolute temperature; e is the elementary electric charge; a is the area per lipid molecule; K_1 is the binding constant of monovalent cations; K_2 and K_3 are, respectively, the 1 : 1 and 2 : 1 binding constants of divalent cations. The surface tension increase by addition of divalent cations, which should be compared with experimental results, is then given by

$$\Delta\gamma = \gamma(n_2) - \gamma(n_2 = 0) \quad \text{(A7)}$$

where we assume that the dependence of γ_0 on n_2 can be neglected. The surface potential ψ_s (or y_s), appearing in Eqn. A1, can be obtained as the root of the following transcendental equation [15]:

$$(e^{-y_s}-1)\left[\left(1-\frac{\eta}{3}\right)e^{y_s}+\frac{\eta}{3}\right]^{1/2} - \frac{2e^2}{\epsilon_r\epsilon_0 kT\kappa a}\frac{1-K_2n_2e^{-2y_s}}{P+\left(P^2+4K_3n_2e^{-2y_s}\right)^{1/2}}=0 \quad \text{(A8)}$$

In order to calculate $\Delta\gamma$ and ψ_s as functions of n_2 for given values of a, T, n_1, K_i ($i=1$–3), and u, we first solve Eqn. A8 numerically to obtain ψ_s (which does not depend on u), and then substituting the result into Eqn. A1 and using Eqn. A7 we obtain $\Delta\gamma$. Putting $a=65$ Å^2, $T=297$ K, $\epsilon_r=79$ and $n_1=0.1$ M in accordance with the experimental conditions, we have calculated $\Delta\gamma$ and ψ_s for many sets of K_i and u to determine the values of these parameters that fit both the experimental data on $\Delta\gamma$ and ψ_s. The best-fit curves are shown in Figs. 2 and 4.

The fractions of unbound lipids, monovalent cation bound lipids, divalent cation bound lipids in 1:1 binding and in 2:1 binding, which we denote by X_i ($i=0$–3), respectively, are expressed [15] as

$$X_0=\frac{2}{P+\left(P^2+4K_3n_2e^{-2y_s}\right)^{1/2}} \quad \text{(A9)}$$

$$X_1=\frac{2K_1n_1e^{-y_s}}{P+\left(P^2+4K_3n_2e^{-2y_s}\right)^{1/2}} \quad \text{(A10)}$$

$$X_2=\frac{2K_2n_2e^{-2y_s}}{P+\left(P^2+4K_3n_2e^{-2y_s}\right)^{1/2}} \quad \text{(A11)}$$

$$X_3=\frac{4K_3n_2e^{-2y_s}}{\left[P+\left(P^2+4K_3n_2e^{-2y_s}\right)^{1/2}\right]^2} \quad \text{(A12)}$$

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