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SPECIFICITY OF Ca^{2+} AND Mg^{2+} BINDING TO PHOSPHATIDYLSERINE VESICLES AND RESULTANT PHASE CHANGES OF BILAYER MEMBRANE STRUCTURE

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Summary

Differences in the interaction of Ca^{2+} and Mg^{2+} with phosphatidylserine vesicles were revealed by binding studies, differential scanning calorimetry and X-ray diffraction. The two cations produced structurally different complexes with phosphatidylserine as evidenced by phase transition characteristics, lamellar spacings, and hydrocarbon packing. Ca^{2+} effectively competes with Mg^{2+} for phosphatidylserine binding sites. Analysis of the binding data showed that Ca^{2+} had a ten-fold greater intrinsic binding constant for phosphatidylserine.

Ca^{2+} is a key regulatory factor in many biological processes. Some of this control may very well be expressed at the membrane level. Therefore, we have been interested in studying the interactions of Ca^{2+} with model membranes of phosphatidylserine and have found Ca^{2+} to increase permeability [1] and vesicle fusion [1, 2]. It also induces phase separations in vesicles composed of acidic and neutral lipids [2, 3]. Mg^{2+}, on the other hand, is much less effective in causing phase separations [2, 3] and inducing vesicle fusion [1]. Here we study the association of Ca^{2+} and Mg^{2+}, both separately and together, with phosphatidylserine vesicles. We present results and analyses of binding, differential scanning calorimetry, and X-ray diffraction experiments.

The lipids used in these experiments were chromatographically pure. Phosphatidylserine was isolated from beef brain as previously described [1, 4, 5]. Dispersions of multilamellar vesicles and sonicated unilamellar vesicles of phosphatidylserine were prepared as described before [1] in a buffer containing 100 mM NaCl, 2 mM L-histidine, and 2 mM *N*-tris-(hydroxymethyl)methyl-2-amino ethanesulfonic acid (TES), adjusted to pH 7.4. Binding studies were carried out by equilibrium dialysis. Endothermic phase transitions were detected with the Perkin-Elmer DSC-2 differential scanning calorimeter. Details of the experimental methods are in the captions to the figures. X-ray diffraction was used to determine both the structural organization of the phospholipid phase and the nature of the hydrocarbon chain packing [6, 7]. Samples were examined using CuK$_\alpha$ radiation in either a quartz monochromatized Guinier camera or a Ni filtered Franks camera.

Results of the binding studies are shown in Fig. 1. Qualitatively, it appears that Ca^{2+} has a stronger affinity for phosphatidylserine than does Mg^{2+} with both cations eventually reaching a saturation ratio slightly lower than 0.5. When both metals are present, Ca^{2+} is able to compete with Mg^{2+} even when the Ca^{2+} concentration is much lower. This implies that the phosphatidylserine·cation complex involves specific 'chemical' binding. Binding studies with multilamellar vesicles where Ca^{2+} or Mg^{2+} was added to phosphatidylserine during initial dispersion gave substantially similar values, except that at high Ca^{2+} concentrations the Ca^{2+} to phosphatidylserine ratio reached a plateau at a lower value, 0.35. A pronounced preference for Ca^{2+} over Mg^{2+} was again observed (data not shown).

Ca^{2+} and Mg^{2+} not only showed differences in the extent of binding to the head group of phosphatidylserine but also in their effects on the phase transition and acyl chain packing. Thermal scans of the cation·lipid complex are shown in Fig. 2. Ca^{2+} causes the disappearance of the phosphatidylserine ·Na$^+$ phase transition within the temperature range 0—70°C as previously

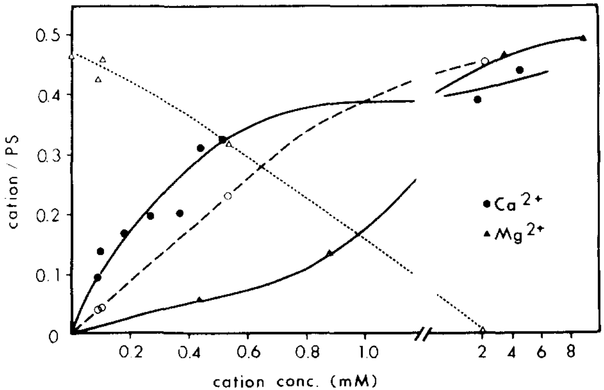

Fig. 1. The sonicated lipid dispersion was centrifuged at $1 \cdot 10^5 \times g$ for 30 min to remove any large particles. The supernatant was removed and diluted to give a lipid concentration of approximately 2 mM. 1-ml aliquots of the lipid suspension were placed in dialysis bags and dialyzed for 6 h at 37°C against bulk solutions containing 100 mM NaCl, 2 mM L-histidine, 2 mM TES, and varying amounts of CaCl$_2$ or MgCl$_2$. The contents of the dialysis bags were analyzed for Ca^{2+}, Mg^{2+}, and PO$_4^{3-}$. Ca^{2+} was determined by using ^{45}Ca as a radioactive tracer. Mg^{2+} content was measured with a Model 290 B Perkin Elmer atomic adsorption spectrophotometer (Perkin Elmer Corp., Norwalk, Conn.). A modified Fiske-Subba Row [8] method was used to determine the amount of phosphate in the samples. The molar ratios of cation to lipid were then calculated for various bulk concentrations. The ratio for the bound cation in the presence of both 0.5 Ca^{2+} and 3 mM Mg^{2+} and the ratio for Mg^{2+} alone at 9 mM are estimates based on the probable amount of lipid exposed to the bulk solution as determined by permeability studies [1]. The complete range of values is shown in Table I. The points shown in the figure refer to: Ca^{2+} alone (●), Mg^{2+} alone (▲), Ca^{2+} in the presence of 3 mM Mg^{2+} (○), Mg^{2+} at constant concentration (3 mM) and varying concentrations of Ca^{2+} (△).

reported [3, 9]. In contrast, Mg^{2+} causes an increase of about 12°C in the temperature of the midpoint of the transition [3, 10] with no significant decrease in ΔH (Fig. 2). The curve shown was obtained with vesicles prepared in 3 mM Mg^{2+} but the same results are given by Mg^{2+} concentrations of 30 mM. Fig. 2 also shows that Ca^{2+} induces the disappearance of the transition of the phosphatidylserine$\cdot Mg^{2+}$ complex when Mg^{2+} is present at 30-fold higher concentrations. Other data not shown indicate that the Ca^{2+} effect predominates even when the Mg^{2+} concentration is 100-fold higher.

Samples prepared in the same manner as above were also examined by X-ray diffraction. The data indicates that the structure of cation$\cdot$phosphatidylserine complex is distinctly different for each cation. The phosphatidylserine$\cdot Ca^{2+}$ complex (formed at Ca^{2+} concns. 2—20 mM) is lamellar with a spacing of 53 Å (Fig. 3). The hydrocarbon chain packing is characterized by two sharp high angle reflections at 4.5 and 4.1 Å as previously noted [3]. While these are not typical of one of the common methylene subcell packings, they do indicate a two-dimensional ordering of the chains. The diffraction pattern remains unchanged over a temperature range 0—60°C. Similarly, there is no change in either the spacing or, to a visual approximation, the relative intensities of the diffraction maxima of the phosphatidylserine$\cdot$Ca precipitate after drying in high vacuum. This indicates there is little, if any, removable water in the phosphatidylserine$\cdot$Ca complex. A displacement of the water of hydration upon binding of Ca^{2+} was detected earlier with NMR by Hauser et al. [11].

The diffraction pattern obtained with the phosphatidylserine$\cdot Mg^{2+}$ complex (formed at 20 mM Mg^{2+}) at low temperature (5°C) shows a 67 Å lamellar structure with a hydrocarbon packing characterized by the 4.2 Å spacing

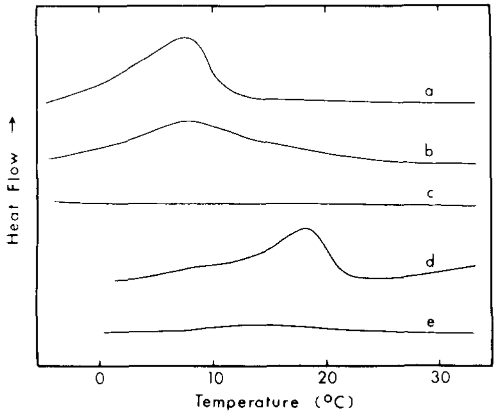

Fig. 2. Multilamellar vesicles were prepared by dispersing 5 μmol phosphatidylserine in 10 ml of buffer containing 100 mM NaCl, 2 mM histidine, 2 mM TES, and the indicated amount of divalent cations. The samples were given three brief (15 s) bursts of sonication [1] to aid in equilibration. The samples were incubated for 30 min at 37°C and several hours at room temperature and then spun at $1 \cdot 10^5 \times g$ for 30 min. The wet pellets were examined with the Perkin-Elmer DSC-2 (Perkin Elmer Corp., Norwalk, Conn.). The heating rate was 5°C/min. a. Phosphatidylserine prepared in NaCl buffer alone. ΔH=4.5 kcal/mol (± 0.5). b. Phosphatidylserine prepared in NaCl buffer with 0.10 mM $CaCl_2$ initially present. c. Phosphatidylserine prepared in NaCl buffer with 0.15 mM $CaCl_2$ initially present. d. Phosphatidylserine prepared in NaCl buffer containing 3 mM $MgCl_2$. ΔH=4.0 kcal/mol (± 0.5). e. Phosphatidylserine prepared in NaCl buffer containing 3 mM $MgCl_2$ with 0.10 mM $CaCl_2$ initially present. Each sample contained 1—2 μmol lipid.

typical of hexagonally-packed chains (Fig. 3). This is the most disordered of the solid chain packing arrangements. At higher temperature (25°C), the lamellar spacing is 53 Å with high angle diffraction characteristic of fluid chains (4.6 Å, diffuse). The crystalline phosphatidylserine·Mg complex shrinks to 60 Å after drying in high vacuum, indicating the loss of considerable water. An indication of the stronger binding of Ca^{2+} to phosphatidylserine, as opposed to that of Mg^{2+}, is gained by an examination of the complex formed by phosphatidylserine in the presence of 3 mM Mg^{2+} and 0.1 mM Ca^{2+}. In this system, despite the presence of a thirty-fold greater concentration of Mg^{2+}, the only structure observed by X-ray diffraction is that of the phosphatidylserine·Ca complex.

All of the above experimental results indicate that there are striking differences in the interaction of Ca^{2+} and Mg^{2+} to phospholipids. This would not be the case if the cations simply resided in the double layer region. In order to further analyze the binding data we have distinguished between the amount of cations tightly or chemically bound and the amount of cations in the double layer region. The latter amount is given by calculating n_{si}, the excess number of cations of type i per unit area of membrane above the solution value. This quantity is given by the equation

$$n_{si} = \int_0^{x_0} (n_i(x) - n_i(\infty))\, dx. \tag{1}$$

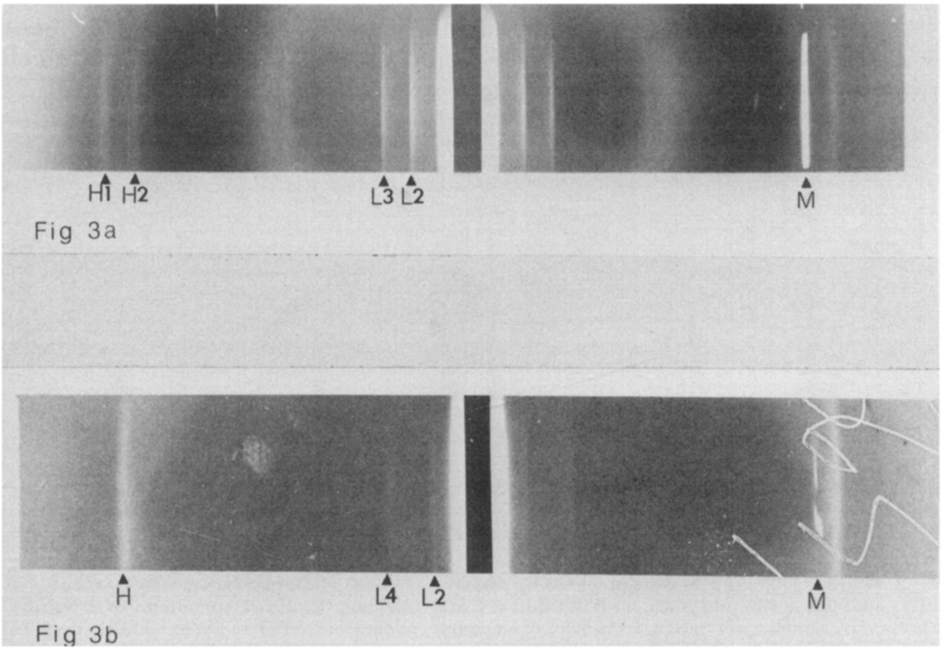

Fig. 3. a. X-ray diffraction pattern of the phosphatidylserine·Ca^{2+} complex at room temperature. Reflections H1 and H2 are the high angle reflections of 4.1 Å and 4.5 Å arising from the hydrocarbon chain packing. Reflections L2 and L3 are the 2nd and 3rd order reflections of a 53 Å lamellar structure. b. X-ray diffraction pattern of the phosphatidylserine·Mg^{2+} complex at 5°C. Reflection H is the characteristic 4.2 Å reflection of hexagonally packed hydrocarbon chains. Reflections L2 and L4 are the 2nd and 4th order reflections of a 67 Å lamellar structure. Reflection M is from the mica window of the sample holder.

The number of cations i per unit volume at distance x, $n_i(x)$, and the number far away from the surface, $n_i(\infty)$, are related by

$$n_i(x) = n_i(\infty) \exp\left(-\frac{ez_i\psi(x)}{kT}\right) \tag{2}$$

where $\psi(x)$ is the electrical potential, ez_i is the charge of a cation of valency z_i, k is Boltzmann's constant, and T is the absolute temperature.

An analytical expression for n_{si} was derived by integrating the solution given by Abraham-Shrauner [12] for the case of a system containing both monovalent and divalent cations. The calculation of n_{si} was independently performed by R. Sauve and S. Ohki (personal communication) who employed numerical integration. Our results showed only a slight increase of n_{si} with x_0 for x_0 values above 20 Å. (For more details see ref. 22.)

In calculating the amount of tightly-bound cations, we followed the approach of McLaughlin et al. [13] and extended the treatment for the case of several bound cations. The binding constants were determined by fitting the calculated values of total bound Ca^{2+} and bound Mg^{2+} to the experimental values. Inspection of Table I illustrates that the present formalism can quantitatively predict binding results by employing the following intrinsic binding constants: Ca^{2+} (35.0), Mg^{2+} (4.0), and Na^+ (0.8) all in units of

TABLE I

EXPERIMENTAL AND CALCULATED BINDING DATA FOR Ca^{2+} AND Mg^{2+} TO PHOSPHATIDYLSERINE VESICLES

The experimental binding data were obtained with unilamellar (sonicated and centrifuged) vesicles. At low cation concentrations where the vesicles are relatively impermeable [1], only the outer half of the bilayer is exposed to the bulk solution. In calculating the experimental ratios of cation/phosphatidylserine for low concentration it was assumed that 60% of the total lipid was available for interaction with the cations. At high cation concentrations, the vesicles appear to be fully permeable [1] and so 100% of the total lipid was used to calculate cation/phosphatidylserine ratios. In cases where the vesicles are partially permeable, a range is given for the ratio. The calculated ratios of cation/phosphatidylserine assume these values of the intrinsic binding constants in units of liter/mol: Ca^{2+} (35.0), Mg^{2+} (4.0), Na^+ (0.8).

Ca^{2+} concentration (mM), with 0.1 M Na^+	0.10	0.44	1.80	4.69
Experimental ratio Ca^{2+}/phosphatidylserine	0.14	0.31	0.39	0.44
Calculated total Ca^{2+}/phosphatidylserine	0.15	0.26	0.35	0.39
Calculated tightly-bound Ca^{2+}/phosphatidylserine	0.14	0.25	0.34	0.38
Calculated double layer Ca^{2+}/phosphatidylserine	0.002	0.01	0.01	0.02
Ca^{2+} concentration (mM), with 10 mM Na^+	0.08	0.43	1.79	
Experimental ratio Ca^{2+}/phosphatidylserine	0.44	(0.34—0.57)	0.44	
Calculated total Ca^{2+}/phosphatidylserine	0.40	0.44	0.47	
Ca^{2+} concentration (mM), with 0.1 M Na^+ and 3 mM Mg^{2+}	0.10	0.53	2.12	
Experimental ratio Ca^{2+}/phosphatidylserine	0.05	0.23(0.16—0.27)	0.46	
Calculated total Ca^{2+}/phosphatidylserine	0.06	0.18	0.31	
Experimental ratio Mg^{2+}/phosphatidylserine	0.47	0.32(0.20—0.36)	0.01	
Calculated total Mg^{2+}/phosphatidylserine	0.21	0.14	0.06	
Mg^{2+} concentration (mM), with 0.1 M Na^+	0.43	0.88	3.50	9.18
Experimental ratio Mg^{2+}/phosphatidylserine	0.06	0.14	0.47	0.49(0.32—0.54)
Calculated total Mg^{2+}/phosphatidylserine	0.10	0.15	0.27	0.33
Calculated tightly bound Mg^{2+}/phosphatidylserine	0.09	0.14	0.23	0.28

M^{-1}. The intrinsic binding constant of Ca^{2+} to phosphatidylserine is about ten times larger than that of Mg^{2+}. Thus, Ca^{2+} is very effective in competing with Mg^{2+} for phosphatidylserine binding sites as illustrated in Table I and Fig. 1. The binding of Na^{+} cannot be ignored, although most of the Na^{+} adsorbed resides in the double-layer region. NMR results (Kurland, R., personal communication) also indicate that some sodium is tightly bound.

We find much lower binding constants for Ca^{2+} and Mg^{2+} than the experimental constants given by others [14—16]. It should be noted that these constants [14—16] are apparent binding constants and, therefore, subject to the particular ionic environment present during the experiment. Our approach is closer to that of McLaughlin et al. [13] although our findings disagree with those presented by this group. McLaughlin et al. saw little difference between Ca^{2+} and Mg^{2+} binding to phosphatidylserine and estimated the binding constant for both to be $K\sim$ 0.1 M^{-1}. With their low value for the binding constants, they concluded that the predominant effect of Ca^{2+} and Mg^{2+} on the electrical potential is through screening (double-layer effects), whereas our results emphasize the role of specific binding. This disagreement stems mainly from the fact that our binding data were directly determined, whereas McLaughlin et al. inferred binding from their conductance data.

The difference between the interaction of Ca^{2+} and Mg^{2+} with phosphatidylserine, as shown by the X-ray diffraction studies, demonstrates that Ca^{2+} forms a more tightly packed, highly ordered, and less hydrated structure than does Mg^{2+} with these phospholipids. Together with the differential scanning calorimetry and binding results, these findings indicate differences in the interaction of Ca^{2+} and Mg^{2+} with phosphatidylserine which cannot be explained if the cation association to the membrane is simply due to the electrical double layer. Instead, it is necessary to invoke a 10-fold stronger specific 'chemical' binding of Ca^{2+} to phosphatidylserine. The electrostatic repulsion between phosphatidylserine vesicles exposed to Ca^{2+} is, therefore, expected to be smaller than in the presence of Mg^{2+}.

It is well documented that Ca^{2+} plays a vital role in natural membrane fusion phenomena [17, 18] while Mg^{2+} is ineffective in most such systems. In view of the same specificity in the induction of phase separations and fusion in pure phospholipid vesicles, it is tempting to speculate that Ca^{2+}-induced phase separations of acidic phospholipids may be responsible for initiating fusion events in natural membranes [19]. The data presented in this paper indicate that Ca^{2+} is effective in competing with Mg^{2+} and inducing phase changes in phosphatidylserine vesicles even in the presence of much higher concentrations of Mg^{2+}. It is therefore reasonable to propose that the increased Ca^{2+} influx intracellularly prior to a secretory event [20, 21] displaces intracellular Mg^{2+} and produces crystallization of phosphatidylserine domains in the inner monolayer of the plasma membrane. Fusion of secretory vesicles then may occur at domain boundaries as suggested earlier [1].

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