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Monolayer coupling in phosphatidylserine bilayers: distinct phase transitions induced by magnesium interacting with one or both monolayers

N. Düzgüneş [a,c], C. Newton [a,*], K. Fisher [b,c], J. Fedor [a,**], and D. Papahadjopoulos [a,d]

[a] *Cancer Research Institute,* [b] *Cardiovascular Research Institute, and Departments of* [c] *Pharmaceutical Chemistry,* [d] *Pharmacology, and* [e] *Biochemistry and Biophysics, University of California, San Francisco, CA (U.S.A.)*

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We have investigated the thermotropic behavior of phospatidylserine bilayers interacting with Mg^{2+} either on one side or both sides, using differential scanning calorimetry. Large unilamellar vesicles (LUV) of phosphatidylserine exposed to Mg^{2+} on the external side only displayed an upward shift of the gel–liquid transition temperature (T_m) of about 6–8 C° relative to the T_m of LUV in Na^+. Mg^{2+} was shown not to enter the vesicle interior, by means of fluorescence measurements on encapsulated 8-hydroxyquinoline-5-sulfonate. Multilamellar vesicles prepared in the presence of Mg^{2+}, or vesicles prepared by Mg^{2+}-induced fusion of small unilamellar vesicles, had T_m values that were shifted upward by about 16–17 C°. When the latter preparation was treated with EDTA to produce vesicles with Mg^{2+} inside and Na^+ outside, the T_m was found to be shifted again by only 6–8 C°. These observations indicate that the monolayer interacting with Na^+ fluidizes the monolayer interacting with Mg^{2+}, and that the latter tends to solidify the former. The two monolayers thus appear to be coupled, possibly by hydrocarbon chain interdigitation.

Introduction

Studies on the interaction of divalent cations with phospholipid membranes are of fundamental importance in understanding the mechanism of action of these ions on biological membranes [1]. Calcium and magnesium induce drastic alterations in the structure of acidic phospholipid membranes as revealed by freeze-fracture electron microscopy [2–4], Raman spectroscopy [5], ^{31}P-NMR spectroscopy [6–8], Fourier transform infrared spectroscopy [9], X-ray diffraction and differential scanning calorimetry [10–14]. The changes in the gel–liquid crystalline transition temperature of these phospholipids is a sensitive indicator of the interaction of various ions with the membrane [15–18]. Most of these studies have been carried out with multilamellar vesicles, with the divalent cations on both sides of the membranes. Since many acidic phospholipid membranes undergo ex-

* Present address: Department of Biology, Kalamazoo College, Kalamazoo, MI 49007, U.S.A.
** Present address: Department of Pharmacy, University of California, San Francisco, CA 94143, U.S.A.

Abbreviations: LUV, large unilamellar vesicle; PS, phosphatidylserine; SUV, small unilamellar vesicles; MLV, multilamellar vesicles.

Correspondence: N. Düzgüneş, Cancer Research Institute, University of California, San Francisco, CA 94143-0128, U.S.A.

tensive aggregation and fusion in the presence of threshold concentrations of divalent cations [19–21], it is not possible to maintain a single-bilayer vesicle structure after addition of the cations.

In contrast to most cases studies so far, large (approx. 100 nm diameter) unilamellar vesicles composed of phosphatidylserine (LUV(PS)) are resistant to fusion by Mg^{2+}, which only aggregates the vesicles, but does not cause any intermixing of internal aqueous contents or their release into the external medium [22]. This property of LUV (PS) provides a unique experimental system for investigating the interaction of a divalent cation with one monolayer only of an acidic phospholipid bilayer. In the study reported here, we have investigated whether separate phase transition endotherms for the inner and outer monolayers of the LUV can be observed, or whether the monolayers are coupled to each other via hydrocarbon chain interaction and possibly interdigitation. Our results have been presented previously in preliminary form [23].

Materials and Methods

Lipids and other chemicals. Bovine brain PS was prepared as described by Papahadjopoulos and Miller [24] or obtained from Avanti Polar Lipids (Birmingham, AL) and stored in chloroform under argon in sealed ampoules at −70°C. 8-Hydroxyquinoline-5-sulfonate was obtained from ICN Pharmaceuticals (Plainview, NY). NaCl was obtained from Mallinckrodt, $MgCl_2$ and EDTA were from Fisher, and 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid (TES) was from Sigma.

Preparation of vesicles. LUV were made by reverse-phase evaporation followed by extrusion through polycarbonate membranes [25,26]. The lipid, dried from chloroform in a small screw-cap tube, was suspended in 1 ml diethyl ether (which was stored above water and washed with water immediately before use) at a concentration of 10 μmol/ml. An aqueous solution (0.34 ml) of 100 mM NaCl/5 mM TES (pH 7.4)/0.1 mM EDTA (NaCl buffer) was added and the mixture was sonicated in a bath-type sonicator (Laboratory Supplies Co., Hicksville, NY) under argon for 5 min. The resulting emulsion was transferred to a rotary evaporator, with the screw-cap tube resting in a larger glass tube connected to the evaporator. Water was placed in the larger tube to maintain thermal contact with a water bath maintained at 30C°. The ether was evaporated at a pressure of about 400 mmHg. The resulting gel was broken by brief vortexing and the evaporation was continued. This process was repeated two more times. At this stage, an aqueous dispersion of vesicles had formed. The vacuum was increased to about 60 mmHg, after which 0.66 ml of the buffer was added and the suspension was returned to the evaporator for an additional 20 min (approx. 10 mmHg) to eliminate any traces of diethyl ether. The vesicles were centrifuged for 20 min at 10000 × *g* to pellet any multilamellar vesicles, and the supernatant was extruded through a polycarbonate membrane of 0.1-μm pore diameter (Uni-Pore, Bio-Rad, Richmond, CA or Nucleopore, Pleasanton, CA) under approx. 80 psi of argon pressure, to achieve a uniform size distribution [25]. The yield was approx. 90% of the starting material. The vesicles were kept under argon throughout the experiments.

Small unilamellar vesicles (SUV) were prepared by sonication in NaCl buffer as described by Düzgüneş et al. [27]. Multilamellar vesicles (MLV) were obtained by first drying the lipid in high vacuum and then hydrating with NaCl buffer (+10 mM Mg^{2+}) at 25°C and vortexing under argon for 10 min.

Differential scanning calorimetry. $MgCl_2$ was added to a 5 ml vesicle suspension of 1 μmol lipid/ml at 25°C and the mixture was incubated for 30 min. The aggregated vesicles were harvested by centrifugation at 10000 × *g* for 20 min. Untreated LUV were concentrated by centrifugation at 150000 × *g* for 3 h at 25°C in a Beckman L3-50 ultracentrifuge. Multilamellar vesicles were pelleted at 10000 × *g* for 20 min. The pellets were transferred to aluminium calorimeter pans, which were then sealed by crimping.

Differential scanning thermograms were obtained with a Perkin-Elmer DSC-2, with a sensitivity of 1 mcal/s and a scan rate of 5 C°/min *.

* The differential scanning calorimetry curves were obtained at a relatively high scan rate and should, therefore, not be expected to represent the equilibrium excess heat capacity.

The temperature at the peak of the endotherm was designated as T_m.

Freeze-fracture electron microscopy. For electron microscopy, liposome suspensions were pipetted onto hat-shaped copper supports and rapidly frozen in liquid nitrogen-cooled Freon-22. Samples were transferred to a Balzers freeze-fracture apparatus, fractured at −115°C (non-etch) or −100°C (etch) and shadowed with Pt-C. Replicas were cleaned with 2.5% sodium dodecyl sulfate (w/v) for 60 min, washed 3× in glass-distilled water (1 min each) and examined with a Siemens 101 electron microscope. All micrographs are mounted with shadow direction from bottom to top. Contrast is not reversed photographically.

Measurement of magnesium ion influx. The fluorescent Mg^{2+} indicator 8-hydroxyquinoline-5-sulfonate [28] was encapsulated in LUV at a concentration of 10 mM together with 90 mM NaCl and 5 mM TES (pH 7.4) by the same procedure as that described above and were passed through a Sephadex G-75 (Pharmacia) column to eliminate unencapsulated material (elution buffer: 100 mM NaCl/5 mM TES (pH 7.4)/0.1 mM EDTA). Vesicles were suspended in 1 ml of NaCl buffer in a quartz cell stirred continuously with a magnetic stirrer and maintained at 25°C. Fluorescence was measured in an SLM-4000 fluorometer with the excitation wavelength at 360 nm and the emission at 490 nm (8 nm slit width). 100% fluorescence was determined by lysing the membranes in the presence of Mg^{2+} with 0.5% sodium cholate (pH 7.4).

Results and Discussion

Large unilamellar vesicles of phosphatidylserine exposed externally to magnesium

Binding of divalent cations to charged phospholipid membranes causes a reduction in membrane area [29,30] and a linear increase in T_m with an increasing number of occupied binding sites [31]. The increase in the T_m in the presence of divalent cations or protons has been attributed to a change in electrostatic free energy due to charge neutralization or reversal [32,33]. Other factors also thought to contribute to the increase in T_m include bridging of two PS molecules by a divalent cation and resulting alteration in the packing of the polar head groups, as well as changes in hydrogen bonding and the water of hydration [10,15].

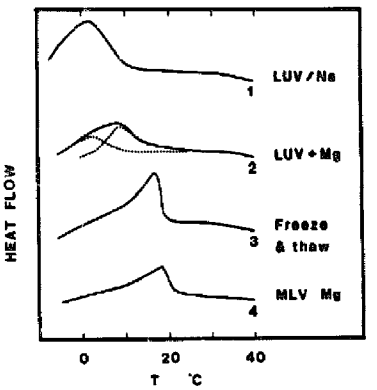
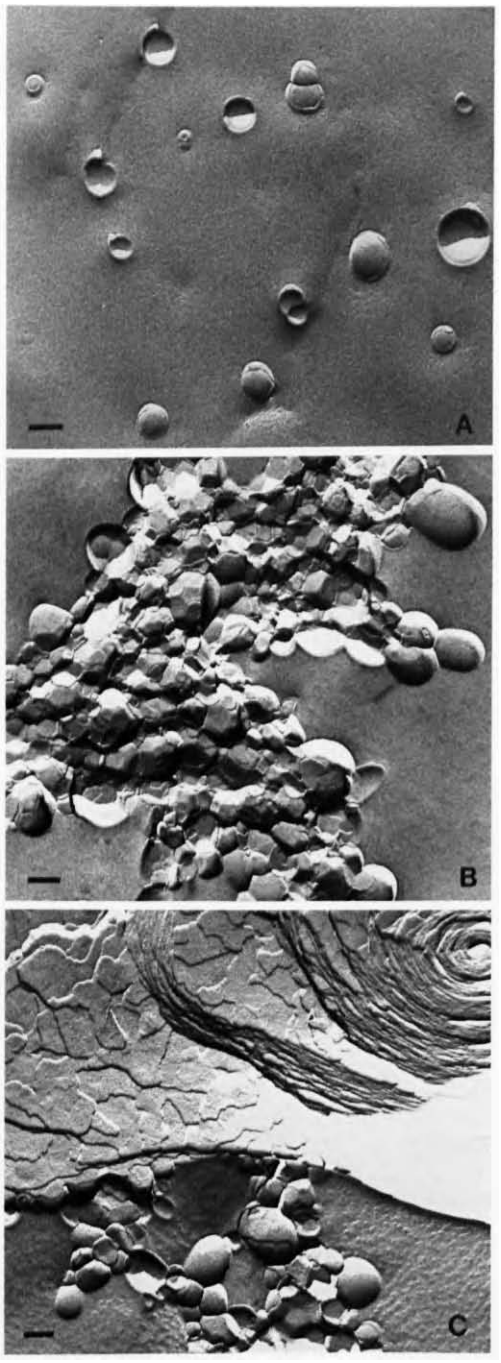


Fig. 1. Differential scanning calorimetry thermograms of large unilamellar vesicles (LUV) composed of phosphatidylserine (PS) in the presence of 100 mM NaCl/5 mM TES (pH 7.4) (curve 1) and LUV incubated with 10 mM $MgCl_2$ (curve 2). Hypothetical contributions from the transitions of the inner and outer monolayer are indicated by the dotted lines. LUV in the presence of Mg^{2+} on the outside were then frozen in the calorimeter and scanned while thawing (curve 3). Due to the broad ice-melting curve during the first heating scan, the second heating scan is shown. Curve 4 shows the endotherm of multilamellar vesicles (MLV) prepared in 100 mM NaCl/10 mM Mg^{2+}/5 mM TES (pH 7.4).

Large unilamellar vesicles of PS in 100 mM NaCl (pH 7.4) displayed a broad gel–liquid crystalline transition endotherm, ranging from approx. −8°C to 12°C with a peak at 2°C (Fig. 1, curve 1). When $MgCl_2$ was added externally to the vesicles at a concentration of 10 mM, the vesicles aggregated massively (Fig. 2B), and the endotherm was shifted upward with a broad peak between 7.5 and 9.5°C (Fig. 1, curve 2) *.

* It should be noted that different preparations of PS have different peak transition temperatures (T_m) in Na^+, and the corresponding upward shift in the presence of Mg^{2+} is somewhat different. For example, in one preparation, the T_m of PS/Na^+ was 6.5°C, and the addition of Mg^{2+} resulted in a broad endotherm with a peak between 11 and 15°C [23]. However, these differences do not affect our conclusions presented below regarding the interaction of Mg^{2+} with one or both monolayers of PS membranes.



Multilamellar phosphatidylserine bilayers exposed to magnesium on both sides

Multilamellar PS bilayers exposed to 10 mM Mg^{2+} on both sides displayed an endotherm with a peak at 18.5 °C (Fig. 1, curve 4). Similarly, if LUV in Mg^{2+} were frozen at −20 °C and scanned, the endotherm shifted upward to 17 °C (Fig. 1, curve 3). Freezing and thawing caused extensive fusion of the vesicles and the formation of multilamellar vesicles, as visualized by freeze-fracture electron microscopy (Fig. 2C). Under such conditions, Mg^{2+} is expected to equilibrate across the membranes, and the thermotropic properties of the membranes are expected to be similar to those of multilamellar vesicles *.

Magnesium does not enter the vesicle interior

Although Mg^{2+} does not cause release of the internal aqueous contents of LUV (PS), it may enter the interior of the vesicles and bind to the inner monolayer. Other divalent cations including Ni^{2+} and Mn^{2+} have been shown to translocate across PS membranes in the absence of extensive leakage of internal contents [34]. Thus, it is important to ascertain whether or not Mg^{2+} was translocated to the interior of the vesicle and bound to the inner monolayer.

When 1 mM Mg^{2+} was added externally to PS vesicles (LUV) encapsulating 8-hydroxyquinoline-5-sulfonate, no appreciable increase in fluorescence was detected, indicating that the Mg^{2+} was located exclusively on the outside of the vesicles. When the concentration of Mg^{2+} outside the vesicles was increased to 10 mM, the fluorescence increased slightly over a 30 min period, but to only 4% of the maximal fluorescence attainable (Fig. 3). The maximal fluorescence was established by lysing the vesicles with detergent in the presence of Mg^{2+}. These results indicate that the amount of Mg^{2+} able to enter the internal aque-

Fig. 2. Freeze-fracture electron micrographs of LUV composed of PS, in NaCl (A), in 10 mM $MgCl_2$ (B) and in 10 mM $MgCl_2$ after one freeze-thaw cycle (C). Each magnification marker represents 100 nm.

* The transition enthalpies of the Na^+ and Mg^{2+} complexes of PS have been found to be identical [14] and have, therefore, not been used in this study to compare the effects of the various Mg^{2+} distributions across the bilayer.

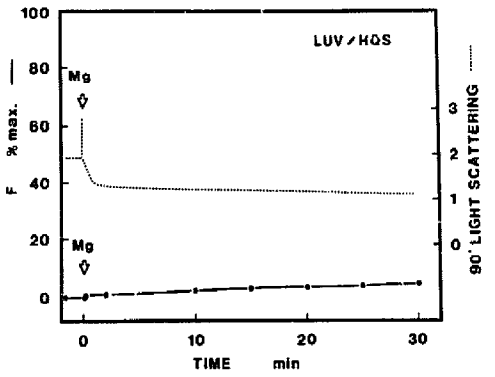


Fig. 3. Entry of Mg^{2+} into the internal aqueous space of LUV (PS), detected by the fluorescence of the Mg^{2+}-indicator 8-hydroxyquinoline-5-sulfonate (closed circles; left ordinate). Maximal fluorescence was obtained by lysing the vesicles with detergent in the presence of Mg^{2+}. Aggregation of the vesicles was monitored by 90° light scattering, given in arbitrary units on the right ordinate.

ous space was very limited. It is apparent, therefore, that the calorimetry scans obtained with LUV (PS) in the presence of 10 mM Mg^{2+} added externally represent the thermotropic behavior of PS bilayers exposed to Mg^{2+} primarily on one side.

Phosphatidylserine vesicles exposed to magnesium on both sides of the bilayer

The difference in thermotropic behavior shown above between membranes exposed to Mg^{2+} on one or both sides could also be due to the extensive stacking of large sheets of membrane against each other in the case of MLV or with frozen and thawed vesicles. Such stacking could contribute to the stabilization of the membrane and, hence, the higher shift in T_m compared to that observed for LUV. Thus, it was of interest to produce intact LUV with Mg^{2+} on both sides of the membrane. Furthermore, such vesicles would enable us to examine the effect of having Mg^{2+} on the inside and Na^+ on the outside, a condition that would be obtained by chelating the external Mg^{2+} with EDTA (vide infra).

We have attempted to place Mg^{2+} on both sides of LUV membranes by two methods: in the first, the ionophore A23187 was incorporated in the membrane at a ratio of 1 ionophore to 500 lipid molecules, yielding about 160 ionophores per vesicle. When the vesicles were incubated with 10 mM Mg^{2+}, the divalent ion entered the vesicle interior to a limited extent, less than 20% of maximal (data not shown). Calorimetric analysis indicated only a moderate (6 C°) upward shift of the T_m. This T_m was 4 C° lower than that obtained with MLV made in the presence of only 1 mM Mg^{2+}, again indicating that Mg^{2+} was not properly equilibrated on both sides of the bilayer of the LUV. The limited entry of Mg^{2+} into the vesicle interior in the presence of A23187 may have been the result of the creation of a diffusion potential. To overcome this potential, a reverse potential was created using K^+ inside the vesicles and valinomycin in the membrane. The fluorescence of 8-hydroxyquinoline-5-sulfonate did increase in the presence of Mg^{2+}. However, the fluorescence was determined to be present in the external medium by centrifuging the aggregated vesicles and measuring the supernatant. This result indicated that the vesicle contents had leaked under these conditions. The reasons for this phenomenon are not known at present. Because of the ambiguity introduced by the leakage, this preparation was not studied any further.

In the second method we utilized our earlier observation that SUV (PS) at 20–25 °C fuse up to limiting size of about 70 nm diameter in the presence of Mg^{2+} and that the fusion process is transiently leaky [22]. The Tb/dipicolinic acid assay for fusion also indicated that the vesicles did not collapse as a result of fusion, an observation which is corroborated by freeze-fracture electron micrographs of similar preparations [3]. Under these conditions, Mg^{2+} would be expected to equilibrate across the fusing membrane, and the fusion product would be in the form of LUV [22]. The differential scanning calorimetry thermogram of these vesicles is shown in Fig. 4, curve 2. The endotherm had a T_m of 18 °C, only 0.5 C° lower than the T_m of multilamellar vesicles prepared in the presence of Mg^{2+}.

Mg^{2+}-induced fusion of SUV at 12 °C has been shown to be extremely leaky [22] and to form large sheets of stacked membranes [3]. Such membranes containing Mg^{2+} on both sides displayed a T_m of 18.5 °C (Fig. 4, curve 1). Thus, the thermo-

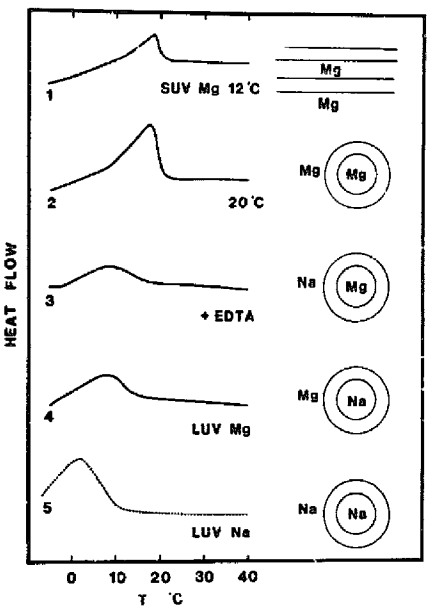


Fig. 4. Differential scanning calorimetry thermograms of PS membranes exposed to Mg^{2+} on one or both sides. The schematic diagrams of the membrane strutures and the distribution of Na^+ and Mg^{2+} across the membranes are shown on the right-hand side. Small unilamellar vesicles (SUV) were incubated with 10 mM Mg^{2+} at 12°C (curve 1) or at 20°C (curve 2). The former condition produces stacked sheets of membranes [3], and the latter treatment results in the formation of large, apparently unilamellar, vesicles [22]. Curve 3 shows the scan of SUV incubated at 20°C with Mg^{2+} and then with 20 mM EDTA to chelate the external Mg^{2+}. The phase-transition endotherm of LUV incubated with 10 mM Mg^{2+} is shown in curve 4. The dotted line indicates the endotherm of LUV in NaCl (curve 5).

tropic behavior of PS membranes in the presence of Mg^{2+} on both sides of the bilayer is independent of the macroscopic morphology of the membranes.

Phosphatidylserine vesicles exposed to magnesium inside and sodium outside

The formation of large unilamellar vesicles with Mg^{2+} on both sides enabled us to examine the calorimetric behavior of vesicles whose inner monolayer interacted with Mg^{2+} and outer monolayer with Na^+. To achieve this, Na-EDTA was added to the large vesicles formed by fusion of SUV at 20°C, in order to chelate the Mg^{2+} on the outside. Under these conditions (Fig. 4, curve 3), the T_m was lowered to the same temperature as the T_m of LUV interacting with Mg^{2+} on the outside (Fig. 4, curve 4). This observation indicates that the effect of Mg^{2+} on one monolayer of a vesicle membrane is independent of whether the monolayer is the inside monolayer, which is not in close apposition to any other membrane, or whether it is the outside monolayer, which is in apposition to a neighboring vesicle membrane.

Monolayer coupling in phosphatidylserine bilayers

The observation that the outer monolayer exposed to 10 mM Mg^{2+} does not exhibit a transition with a T_m as high as that of MLV exposed to Mg^{2+} on both sides, indicates that the inner monolayer fluidizes the outer monolayer, while the outer monolayer tends to solidify the inner monolayer *. Thus, the monolayers appear to be coupled. Our finding that the T_m is also shifted to a limited extent when the inner monolayer is exposed to Mg^{2+} and the outer monolayer to Na^+, lends further support to this interpretation. Thus, in the latter case the outer monolayer fluidizes the inner monolayer. The absence of two separate peaks, one for the inner monolayer corresponding to the Na^+/PS complex and the other for the outer monolayer corresponding to the Mg^{2+}/PS complex, also suggests that the two monolayers are sufficiently coupled. It is also possible, however, that the broad transition of LUV in the presence of external Mg^{2+} is a combination of two transition endotherms representing contributions from both the inner and outer monolayer transitions (Fig. 1, curve 2, dotted lines).

Coupling of monolayers has also been observed in small *N*-lignoceryl sphingomyelin vesicles when the outside monolayer transition is shifted by La^{3+}

* The shift in T_m is an indicator of the coupling between the monolayers. This should not be construed to suggest that the coupling exists only within the transition endotherm. It is because of the coupling that the transition occurs at an intermediate temperature. Although our results do not explicitly indicate a coupling at temperatures above the transition temperature, this is due to the nature of the method rather than because of the method rather than because of the absence of a coupling at these temperatures.

or Pr^{3+}, and has been attributed to the interdigitation across the bilayer of acyl chains of different hydrocarbon length [35]. In contrast, small dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine or *N*-stearoylsphingomyelin vesicles do not appear to exhibit any coupling between the monolayers [35,36]. Consistent with these observations, Georgallas et al. [37] have calculated that the interaction between two monolayers of dipalmitoylphosphatidylcholine is about 2% of the maximum interaction within the plane of the monolayer. Arnold [38] and Hunt and Tipping [39], however, have reported that the shift in transition of the outer monolayer of small dipalmitoylphosphatidylcholine vesicles can be transmitted to the inner monolayer. The reasons for this discrepancy are not apparent.

Conclusions

In summary, our observations indicate that the upward shift of the phase transition temperature of PS bilayers in the presence of Mg^{2+} depends on the distribution of Mg^{2+} across the bilayer. When both monolayers of a LUV (PS) are exposed to Mg^{2+} the T_m is very similar to that of MLV prepared in the presence of Mg^{2+} (Fig. 1) or of SUV fused to form large sheets (Fig. 4, curve 1). When Mg^{2+} is present on one side only, the shift is considerably less than when it is present on both sides of the bilayer.

Although other divalent cations such as Ba^{2+} and Sr^{2+} cause upward shifts of the T_m, it is not possible to ascertain the effect of their asymmetric distribution across the membrane, since they also induce fusion and an increase in the permeability of the membrane [18,40]. Other species of phosphatidylserine such as dimyristoylPS or dielaidylPS, which have well-defined phase transitions and considerably narrower widths at half-peak height than that of bovine brain PS, are not suitable for establishing the effect of Mg^{2+} asymmetry on the T_m, since they become leaky to encapsulated aqueous space markers in the presence of Mg^{2+} (Newton, C., and Düzgüneş, N., unpublished data). Such lipids of defined chain length are less likely to interdigitate between the inner and outer monolayers and thus to exhibit monolayer coupling. If so, then preservation of the integrity of brain PS vesicles in the presence of Mg^{2+} may be attributed partially to monolayer coupling.

Biological membranes have been proposed to act as bilayer couples, that is, the two monolayers of a membrane may respond differently to various perturbations while remaining coupled to one another [41]. Our results and those of Schmidt et al. [35] on monolayer coupling in phospholipid vesicles indicate, in addition, that the perturbation of one monolayer influences the properties of the other monolayer.

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