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DIVALENT CATION-INDUCED INTERACTION OF PHOSPHOLIPID VESICLE AND MONOLAYER MEMBRANES

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Summary

The effects of phospholipid vesicles and divalent cations in the subphase solution on the surface tension of phospholipid monolayer membranes were studied in order to elucidate the nature of the divalent cation-induced vesicle-membrane interaction. The monolayers were formed at the air/water interface. Various concentrations of unilamellar phospholipid (phosphatidylserine, phosphatidylcholine and their mixtures) vesicles and divalent cations (Mg^{2+}, Ca^{2+}, Mn^{2+}, etc.) were introduced into the subphase solution of the monolayers. The changes of surface tension of monolayers were measured by the Wilhelmy plate (Teflon) method with respect to divalent ion concentrations and time.

When a monolayer of phosphatidylserine and vesicles of phosphatidylserine/phosphatidylcholine (1 : 1) were used, there were critical concentrations of divalent cations to produce a large reduction in surface tension of the monolayer. These concentrations were 16 mM for Mg^{2+}, 7 mM for Sr^{2+}, 6 mM for Ca^{2+}, 3.5 mM for Ba^{2+} and 1.8 mM for Mn^{2+}. On the other hand, for a phosphatidylcholine monolayer and phosphatidylcholine vesicles, there was no change in surface tension of the monolayer up to 25 mM of any divalent ion used. When a phosphatidylserine monolayer and phosphatidylcholine vesicles were used, the order of divalent ions to effect the large reduction of surface tension was $Mn^{2+} > Ca^{2+} > Mg^{2+}$ and their critical concentrations were in between the former two cases. The threshold concentrations also depended upon vesicle concentrations as well as the area/molecule of monolayers. For phosphatidylserine monolayers and phosphatidylserine/phosphatidylcholine (1 : 1) vesicles, above the critical concentrations of Mn^{2+} and Ca^{2+}, the surface tension decreased to a value close to the equilibrium pressure of the monolayers within 0.5 h.

This decrease in surface tension of the monolayers is interpreted partly as the consequence of fusion of the vesicles with the monolayer membranes. The

order and magnitude of divalent cation concentrations at which phosphatidylserine/phosphatidylcholine (1 : 1) and phosphatidylserine vesicle suspensions showed a large increase in turbidity were similar to those obtained in the above mentioned experiments.

Introduction

Membrane fusion may be involved in many biological cellular processes, such as exocytosis, endocytosis, cell membrane assembly, etc. [1—4]. Interestingly enough, many of these phenomena are found to be strongly dependent on the Ca^{2+} concentration in the medium [5—18]. Morphological alterations of several membrane systems upon fusion reactions induced by divalent metal ions, have been studied by electronmicroscope [13,16,17], as well as optical microscope [10,18] methods. Although there are several theories on these membrane fusion reactions, stressing the important role of either lipids, proteins or complex subcellular structures [1,19—22], their molecular mechanisms are still not well understood.

Recently, many attempts have been made to elucidate the molecular mechanisms of these divalent ion-induced membrane fusion events by studying membrane incorporation reactions which occur in rather simplified model membrane systems, such as phospholipid vesicle-vesicle [23—26], phospholipid vesicle-cell membrane [27—29], phospholipid vesicle-bilayer membrane [30—33], and phospholipid bilayer-bilayer membrane systems [34—36]. Some molecular mechanisms of membrane fusion in the above systems have been proposed [24—26]. However, many studies with these systems have an ambiguity in identifying whether the membrane incorporation is due to fusion or molecular exchange. Here, we have investigated monolayer-vesicle membrane interactions induced by divalent metal ions by measuring the surface tension of the monolayer as a function of various divalent metal ion concentrations, to provide further insight into these phenomena. This system, together with the above-mentioned phospholipid vesicle-bilayer system, has a geometrical as well as functional resemblance to those observed in the exocytotic process at various secretory glands where the divalent ion-induced membrane interaction involves membranous vesicles and a relatively planar plasma membrane.

Materials and Methods

Chemicals. Bovine brain phosphatidylserine and egg phosphatidylcholine were purchased from Avanti Biochemical Co., AL. Both samples showed a single spot on silica gel thin-layer chromatographic plates.

Monolayer spreading solutions consisted of these phospholipids dissolved in hexane (purum, >99% GC, Fluka) (approx. $2 \cdot 10^{-4}$ M). The exact concentration of phospholipid in the spreading solution were determined by phosphate analysis. Subphase solutions were 100 mM NaCl containing 5 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, Calbiochem) buffer and 0.01 mM EDTA ('NaCl buffer solution') or this buffer solution diluted to 1/10 with water. The pH of the solution was titrated with NaOH to 7.4. The salts

(NaCl, Fisher Chemical) used were roasted at 500—600°C for 1 h. Divalent ion salts ($CaCl_2$, $MgCl_2$, $SrCl_2$, $BaCl_2$ and $MnCl_2$) used were Fisher reagent grade. Water was distilled three times, including the process of alkaline permanganate.

Methods. Vesicle preparation. Unilamellar phospholipid vesicles were prepared according to published methods [33]: phospholipids were suspended at a concentration of 10 μmol phospholipid/ml in the buffer solution, vortexed for 10 min and sonicated for 1 h in a bath type sonicator (Heat Systems, Ultrasonics) at 20 or 24°C and under an N_2 atmosphere, and then vesicle suspensions were centrifuged at 100 000 × *g* for 1 h and the supernatant was used as a vesicle suspension. In some experiments, vesicle suspensions without the centrifugation process were used and gave results identical to those with centrifugation.

Surface tension measurements. Monolayers were formed at the air-water interface of fixed area (33.2 cm^2) in a glass dish. Surface tensions were measured by use of an electronic microbalance (Beckman) with a teflon plate (11 × 11 × 1 mm) as a Wilhelmy plate. For each experiment, water surface tension was first measured to insure cleanness of the aqueous surface. Surface tension of water was also checked by used of a glass Wilhelmy plate. The depth of the dipped plate was kept constant at about 1.0 mm from the water surface, which was monitored by a microscope. For the monolayer surface tension measurement, the plate was redipped after a monolayer of a given area/molecule was formed on the water surface. Then certain amounts of concentrated lipid vesicle (10 μmol lipid/ml) and divalent ion (1 M) solutions were injected into the subphase solution of the monolayer by way of microsyringes (Hamilton), and the subphase solution was stirred well by a magnetic stirrer. Normally the vesicles were first introduced and stirred for 1 min, and then after waiting for 2 min, divalent ion concentrations were raised successively (injected with 1 or 2 mM increment, stirred for 1 min, incubated for 2 min before the following injection). However, near the 'critical concentration' at which the surface tension started to decrease sharply, the increment of divalent ion concentration was reduced to 1/3—1/4 mM for each injection. The manner of increasing the divalent ion concentration (e.g. injecting amounts of the concentrated solutions as well as incubation (waiting time)) influenced the experimental results slightly. However, the deviation of those results fell within the experimental error indicated in the tables.

Turbidity measurements. Turbidities of the vesicle suspensions as a function of divalent ion concentration were measured at 400 nm by use of Beckman DU-Gilford and Hitachi (100-60) spectrophotometers. The same NaCl buffer solution was used for the vesicle suspensions. The vesicles were suspended at 0.5 μmol phospholipid/ml in the solution and then divalent ion concentrations were raised step-by-step in a similar manner as described above, but the incubation time was 5 min at each divalent ion concentration.

All experiments were done at room temperature of 24 ± 1°C.

Results and Discussion

Fig. 1 shows the force-area curve for phosphatidylserine monolayers obtained by this Wilhelmy plate method. Since the contact angles between a

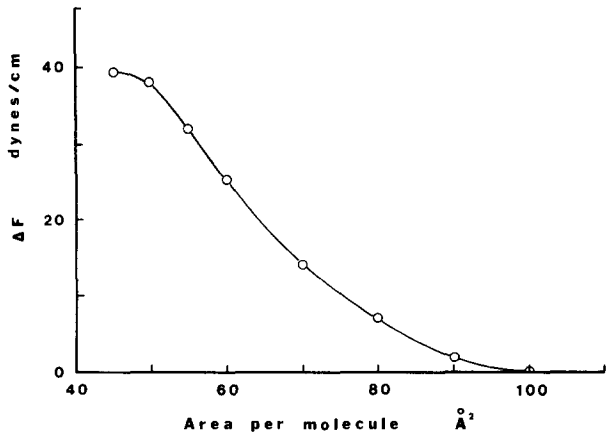

Fig. 1. The force vs. area curve for a phosphatidylserine monolayer at the air-water interface. The difference in the upward force on the Wilhelmy plate, between that at 100 Å^2 and that at a lower area/molecule, $\Delta F = F$(100 Å^2/molecule) $- F$(X Å^2/molecule), is plotted against the area/molecule.

teflon plate and the hydrocarbon phases of lipid monolayers may be different for monolayers at different areas/molecule, and were not known to us, the surface tension was not shown in the figure. When the area/molecule was 100 Å^2, the upward force acting on the plate was about 40 dynes/cm and when the area/molecule was below 50 Å^2, the force was nearly zero. The difference of about 40 dynes/cm between the forces at area/molecule of 100 Å^2 and 50 Å^2 is slightly smaller than the equilibrium pressure (43 dynes/cm) obtained by use of other surface tension methods for phosphatidylserine monolayers [37,54], but nevertheless compares rather well. The smaller value may be due to a small but finite contact angle of the hydrocarbon phase of the lipids and the Teflon plate [45].

When divalent ion concentrations were increased in the presence of a certain amount of phosphatidylserine/phosphatidylcholine (1 : 1) vesicles, the surface tension of the phosphatidylserine monolayer did not show any change until a certain concentration of the divalent ion was attained, which we call the 'threshold concentration'; but at or above this concentration the surface tension reduced sharply toward a value of zero for the upward force (Fig. 2). This sharp decrease in surface tension was not observed when only phospholipid vesicles or divalent ions were present in the subphase.

It must be emphasized that a Teflon Wilhelmy plate was essential for these measurements. A glass Wilhelmy plate resulted in abrupt and erratic behavior of the output of the microbalance, perhaps because the vesicles interfere with proper adhesion of the monolayer to the glass surface.

The threshold concentration for various divalent cations are given in Table I. Above their threshold concentrations Mn^{2+}, Ba^{2+} and Ca^{2+} caused sharp decreases in the surface tension of the monolayer, whereas Sr^{2+} and Mg^{2+} affected the decreases with rates several times slower. The value of the threshold concentration seemed to have a slight variation with different ways of increasing the divalent ion concentration. For example, the longer the incuba-

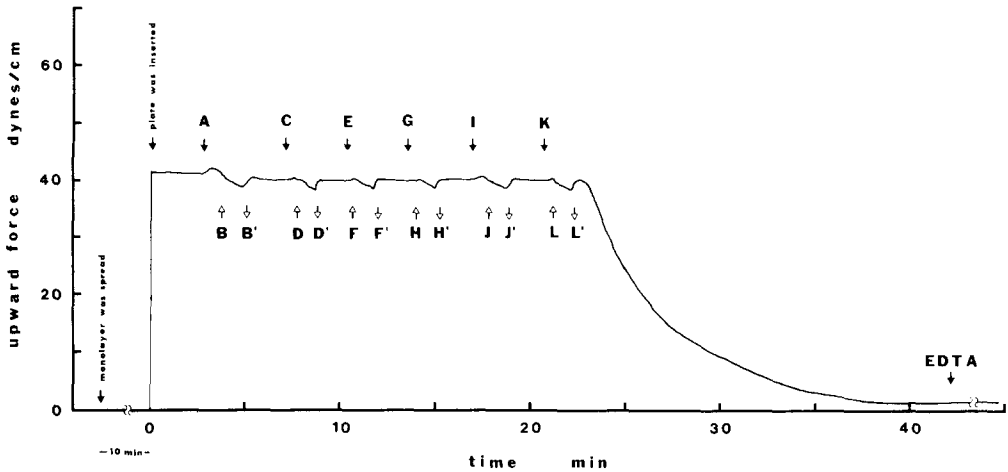

Fig. 2. A typical example of the time course of surface tension decrease for the case of a phosphatidylserine (PS) monolayer (100 $Å^2$/molecule) having PS/phosphatidylcholine (PC) (1 : 1) vesicles (0.067 μmol lipid/ml) and various concentrations of Ca^{2+} in 100 mM NaCl buffer solution in the subphase. Here, instead of film surface tension, the upward force exerted on the Wilhelmy plate by the monolayer is plotted with time. After a monolayer was completely formed at the air-water interface, a certain amount of the concentrated vesicle suspension (10 μmol lipid/ml) was injected into the subphase solution (A), which was then stirred well for 1 min (started at B and stopped at B'), and the system was left for 2 min ('incubation time') to observe any change in surface tension. Subsequently, an aliquot of $CaCl_2$ solution (1 M) was added so as to bring up the Ca^{2+} concentration of the subphase to 2 mM (C), and this was followed by stirring for 1 min (started at D and stopped at D'), and incubated for 2 min. Similar procedures were followed for each injection: 4 mM Ca^{2+} (E) and stirring (started at F and stopped at F'); 5 mM Ca^{2+} (G) and stirring (H to H'); 5.5 mM Ca^{2+} (I) and stirring (J to J'); 6.0 mM Ca^{2+} (K) and stirring (L to L'). In some cases, near the 'threshold concentration', the increment of divalent ion concentration was reduced to 1/3—1/4 mM for each injection. The added EDTA was equivalent to the divalent ion concentration in the subphase.

tion time, the lower the threshold concentration obtained. However, the deviation was not appreciably large, but fell within the experimental error obtained by a certain way of injection (see legend to Fig. 2). When divalent ions were injected first and then a certain amount of phospholipid vesicles was introduced into the subphase solution, no change in surface tension was observed unless the concentration of divalent ions exceeded the threshold concentration. The threshold concentration also depended upon the ionic strength of monovalent ions in the subphase. When the 1/10 diluted NaCl buffer solution was used, the threshold concentration was reduced to about one-half of that

TABLE I

Threshold concentrations (mM) of various divalent ions in cases of a phosphatidylserine monolayer (100 $Å^2$/molecule) and phosphatidylserine/phosphatidylcholine (1 : 1) vesicles of 0.067 μmol phospholipid/ml in 100 mM NaCl buffer solution, and its 1/10 diluted solution.

NaCl buffer solution	Mg^{2+}	Sr^{2+}	Ca^{2+}	Ba^{2+}	Mn^{2+}
100 mM NaCl buffer	16 ± 2	7 ± 1	6 ± 1	3.5 ± 0.7	1.75 ± 0.5
1/10 diluted buffer solution	6.5 ± 1	4 ± 1	3 ± 0.7	1.5 ± 0.5	0.75 ± 0.3

for the non-diluted NaCl buffer solution for each divalent ion (see Table I). It has been well demonstrated by both radioisotope tracer [37,38] and surface potential [39,40] studies that the amounts of divalent ions bound to phosphatidylserine membranes depend upon the concentration of monovalent ions in the subphase solutions.

With the same divalent ion, the values of the threshold concentration depended greatly upon the area/molecule for the monolayer; the smaller the area/molecule, the lower was the threshold concentration (Table II), indicating that this interaction is strongly related to the net negative charge density on the membrane surface.

The concentration of phospholipid vesicles in the solution greatly affected the rate of reduction of surface tension of the monolayer, and influenced slightly the magnitude of the threshold concentration; higher vesicle concentrations enhanced the rate of reduction of surface tension and lowered slightly the magnitude of the threshold concentration of divalent ions (Table III).

In all cases mentioned above, the order of the threshold concentrations of divalent cations was unchanged: $Mn^{2+} > Ba^{2+} > Ca^{2+} \geqslant Sr^{2+} > Mg^{2+}$. This order agrees well with those of the association constants of divalent cations on phosphatidylserine membranes obtained from electrophoretic measurements of phosphatidylserine vesicles [41], and surface potential measurements of phosphatidylserine monolayers [40]. The order also agrees well with those obtained from turbidity measurements for phospholipid vesicle suspensions (see below), and those from the direct measurements of divalent ion binding to phosphatidylserine molecules [42]. These agreements suggest that the present divalent ion-induced vesicle-monolayer interaction is strongly related to the degree of binding of divalent ions to the surface of the membrane.

It is important to mention that the reduced surface tension of the monolayer induced by divalent ions was not reversed or altered by the addition of EDTA, quantities of which were equivalent to divalent ion concentrations in the subphase solution. When EDTA was added while the surface tension of the monolayer was decreasing with time, no further reduction or increase in surface tension was observed.

Experiments similar to the above were done by varying phospholipid components of monolayer and vesicles, keeping other conditions the same. When a

TABLE II

Threshold concentrations (mM) of divalent ions at various areas/molecule of the phosphatidylserine monolayer in the presence of phosphatidylserine/phosphatidylcholine (1 : 1) vesicles of 0.067 μmol phospholipid/ml in the 100 mM NaCl buffer solution.

	Area/molecule ($Å^2$/molecule)					
	110	100	90	80	70	60
Mg^{2+}		16 ± 2			12 ± 2	
Sr^{2+}		7 ± 1			5 ± 1	
Ca^{2+}	6.5 ± 1	6 ± 1	5.5 ± 1	5 ± 0.8	4 ± 0.7	3.5 ± 0.6
Ba^{2+}		3.5 ± 0.7			2 ± 0.4	
Mn^{2+}		1.75 ± 0.5			1 ± 0.3	

TABLE III

Threshold concentrations (mM) of divalent ions when phosphatidylserine monolayers (100 $Å^2$/molecule) and various concentrations of phosphatidylserine/phosphatidylcholine (1 : 1) vesicles in the 100 mM NaCl buffer solution were used.

	Vesicle concentrations (μmol phospholipid/ml)			
	0.007	0.02	0.067	0.2
Mg^{2+}	18 ± 2	18 ± 2	16 ± 2	14 ± 1.6
Ca^{2+}	7.5 ± 1	7 ± 1	6 ± 1	5 ± 0.8
Mn^{2+}	2 ± 0.3	2 ± 0.3	1.75 ± 0.2	1.25 ± 0.2

phosphatidylserine/phosphatidylcholine (1 : 1) monolayer and vesicles of the same molecular components were used, higher values for the threshold concentrations of Mn^{2+} and Ca^{2+} (8 ± 1 and 20 ± 2 mM, respectively for a monolayer of 100 $Å^2$/molecule; 5 ± 0.5 and 13 ± 1.3 mM, respectively for 65 $Å^2$/molecule) were obtained than those with a phosphatidylserine monolayer (Table I); Mg^{2+} caused no change in surface tension up to 25 mM. The threshold concentrations were even higher in the case where a phosphatidylserine monolayer and phosphatidylcholine vesicles were used (Table IV). For a phosphatidylcholine monolayer and phosphatidylserine/phosphatidylcholine (1 : 1) vesicle system, similarly high threshold concentrations were observed for each divalent ion (Table IV). It is interesting to note that in this case the dependence of the threshold concentration on the area/molecule of the monolayer was reversed from that observed with phosphatidylserine monolayers: for Mn^{2+}, the threshold concentration was 9 ± 1 mM at 100 $Å^2$/molecule, 12 ± 1.3 mM at 80 $Å^2$ and 15 ± 1.5 mM at 65 $Å^2$; for Ca^{2+}, it was 20 ± 2 mM at 100 $Å^2$ and 25 ± 2.5 mM at 80 $Å^2$. At 65 $Å^2$/molecule, no change in surface tension was observed up to 25 mM Ca^{2+}. When a phosphatidylcholine monolayer and phosphatidylcholine vesicle were used, no surface tension change was observed up to 25 mM of any divalent ion used (Mn^{2+}, Ca^{2+} and Mg^{2+}).

In the case of a phosphatidylserine monolayer and phosphatidylserine vesicles, the critical concentrations of Ca^{2+} were about 1.5 mM for monolayers

TABLE IV

Threshold concentrations of divalent ions and the behavior of the surface tension when different combinations of phospholipids were used for monolayers (100 $Å^2$/molecule) and vesicles (0.067 μmol phospholipid/ml) in the 100 mM NaCl buffer solution.

	Phosphatidylserine monolayer + phosphatidylcholine vesicles	Phosphatidylcholine monolayer + phosphatidylserine/ phosphatidylcholine (1 : 1) vesicles	Phosphatidylcholine monolayer + phosphatidylcholine vesicles
Mg^{2+}	no change up to 25 mM	no change up to 25 mM	no change up to 25 mM
Ca^{2+}	slight change at 18 ± 2 mM	slight change at 20 ± 2 mM	no change up to 25 mM
Mn^{2+}	slight change at 8 ± 2 mM	slight change 10 ± 2 mM	no change up to 25 mM

of the area/molecule of 100 Å^2, and about 1 mM for monolayers of 70 Å^2/molecule, respectively, in the presence of a vesicle concentration of 0.067 μmol phospholipid/ml in the 100 mM NaCl buffer solution. The threshold values for Mg^{2+} were about 8 mM and 5 mM, respectively. 70 Å^2/molecule corresponds to that deduced from X-ray diffraction studies of lipid bilayer membranes [43], and it is interesting to note that the observed threshold concentration of Ca^{2+} is about the same magnitude at which fusion among phosphatidylserine vesicles was observed in an ionic environment similar to our experiments [26]. These experiments were particularly difficult because the surface tension, recorded as the output of the microbalance, occasionally showed erratic behavior, which was confirmed not to be due to the instrument. Above 1 mM Ca^{2+}, the subphase solution became turbid, which indicated the formation of large aggregates among phospholipid vesicles or conformation change of vesicles [26,44].

In order to make some correlation with the order of effectiveness of divalent ions on the monolayer-vesicle interaction, the turbidity of vesicle suspensions was measured as a function of divalent ion concentrations. Fig. 3 shows the

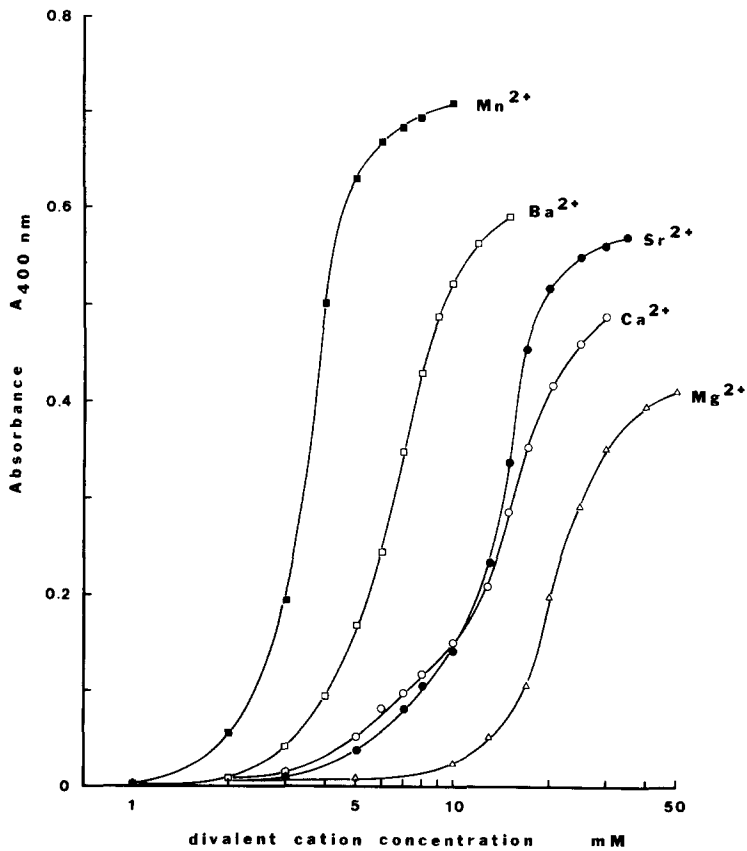

Fig. 3. The effect of divalent ions on the turbidity of phosphatidylserine/phosphatidylcholine (1 : 1) vesicle suspensions (0.5 μmol phospholipid/ml of 100 mM NaCl buffer solution). The absorbance (A_{400nm}) at 5 min after the addition of divalent ion concentration increments to a vesicle suspension is plotted against the final divalent ion concentration. ■, Mn^{2+}; □, Ba^{2+}; ○, Ca^{2+}; ●, Sr^{2+}; △, Mg^{2+}.

turbidity at 400 nm of phosphatidylserine/phosphatidylcholine (1 : 1) vesicles with respect to various divalent cation concentrations. The divalent ion concentrations at which the rate of increase in turbidity of vesicle suspensions were the sharpest (the 'critical concentrations for turbidity') were about 20 mM for Mg^{2+}, 16 mM for Sr^{2+}, 14 mM for Ca^{2+}, 7 mM for Ba^{2+} and 4 mM for Mn^{2+}. For similar experiments with the use of phosphatidylserine vesicles, the critical concentrations were about 2.5 mM for Mg^{2+}, 0.8 mM for Ca^{2+} and 0.6 mM for Mn^{2+}. The order as well as magnitudes of these critical concentrations of divalent ions were approximately the same as those threshold concentrations obtained for the vesicle-monolayer membrane systems of the same molecular components. The turbidity increase in the vesicle suspension is considered as the increase in possible aggregation and fusion among phospholipid vesicles. The sigmoid shape of the curve (turbidity versus divalent ion concentration) shown in Fig. 3 suggests that these processes are cooperative ones.

The observed sharp decrease in the surface tension of certain monolayers in the presence of both phospholipid vesicles and divalent ions is interpreted to be a consequence of fusion of vesicles with monolayer membranes. The reasons for this are the following:

(1) The presence of phospholipid vesicles either of phosphatidylserine or phosphatidylcholine or phosphatidylserine/phosphatidylcholine (1 : 1) only in the subphase solution without divalent ions, did not give any appreciable change (less than a few dynes/cm) in surface tension of the monolayer, at least withing a few hours, up to a vesicle concentration of 0.5 μmol phospholipid/ml.

(2) Concentrations up to 50 mM of divalent ions without the vesicles did not result in any large change in surface tension of the monolayer. It has been observed that divalent ions reduce the surface pressure of acidic phospholipid monolayers to a small extent (a few dynes/cm) [38,46,55].

(3) When a monolayer was left by itself, the surface tension was fairly stable, at least for a few hours. It has been found that temperature-dependent molecular dissolution occurs from the monolayer into the bulk phase [37]. However, the phosphatidylserine as well as phosphatidylcholine monolayers at the present experimental temperature (24°C) do not show significant molecular dissolution [37,47], and the effect of dissolution on surface tension is an increase in its magnitude, which would not account for the decrease in surface tension observed here.

(4) Adhesion of vesicles to a monolayer may contribute to the reduction of the film tension because the surface tension of phospholipid vesicles may be smaller than those of monolayers at relatively large area/molecule (100 $Å^2$/molecule). However, irreversibility of decreased surface tension by the addition of EDTA at the final (equilibrium) stage, as well as in the middle of the stage during which surface tension was decreasing, suggests that vesicle adhesion is not a predominant cause of the observed phenomenon. The incorporation of lipid molecules from the vesicles into the monolayer is most likely responsible for this event.

(5) There is a possibility that when the monolayer and vesicles are in close contact mediated by divalent ions, molecular [48,49] exchange occurs between vesicle and monolayer membranes, mainly from vesicle to monolayer. It is

clear, however, that the rate of reduction of the monolayer is relatively rapid (the change of 40 dynes/cm occurring within 10—30 min, depending on the concentration of phospholipid vesicles, the type of phospholipid vesicle, divalent ion concentration, etc.) so that molecular exchange, which is a rather slower process [48,50,51], especially at our experimental temperature, could not account for the observed large change in surface tension of the monolayers.

(6) Moreover, there are good correlations between the present experimental results and the results observed in the other membrane systems, regarding the possibility of membrane fusion. The threshold concentration for Ca^{2+} of 1 mM for a phosphatidylserine monolayer (at 70 $Å^2$/molecule) and phosphatidylserine vesicle system corresponds well to the concentration at which fusion among phosphatidylserine vesicles occurs in an ionic environment similar to our experiments [24,26]. This correspondence may perhaps indicate that the geometrical difference in membrane structures between planar phospholipid bilayer and vesicle membranes, does not appreciably contribute to the degree of divalent ion-induced interaction among these membranes. The possible presence of organic solvent in the monolayer may affect the fusion process, but probably to a small degree, judging from the good correlation between the two systems. Another correspondence is seen for the phosphatidylserine monolayer-phosphatidylserine/phosphatidylcholine (1 : 1) vesicle and the phosphatidylserine bilayer-phosphatidylserine/phosphatidylcholine (1 : 1) vesicle systems. The threshold concentration for Ca^{2+} of 4 mM for the phosphatidylserine monolayer at 70 $Å^2$/molecule in the presence of vesicles corresponds well to the Ca^{2+} concentration (about 4 mM) at which phosphatidylserine bilayers in a similar ionic environment showed a large increase and discrete fluctuations of membrane conductance, which was interpreted to be a consequence of vesicle fusion with the bilayer membrane [33].

Although explicit evidence for the molecular mechanism responsible for the surface tension decrease induced by divalent ions is not yet demonstrated with the present experiments and the work of others, we can speculate on the possible mechanism of divalent ion-induced membrane fusion in model membrane systems. At least two steps of reaction processes are necessary in order for two membranes to fuse. Firstly, the two membranes should be brought into close contact. Divalent metal ions would help make this process possible, especially for two acidic phospholipid membranes, by reducing surface charges by both screening and binding and/or bridging the two membrane surfaces [26,34,36,52]. Secondly, the surface properties of the membranes should be altered for the two membranes to be able to fuse. The interaction of divalent cations with phospholipid polar groups causes a water exclusion effect from the membrane surfaces, which renders the surface of the phospholipid-divalent ion complexes to be more hydrophobic in nature [53]. It has been suggested [36] that this increased hydrophobicity of the two membrane surfaces in close contact would be one of the main causes of membrane fusion in this type of membrane system. This change in surface properties seems to be intimately related to divalent ion binding with polar groups of lipids, but not to a simple charge screening effect. This proposal stresses the alteration of the membrane surface to be a more important factor for fusion, rather than the order-disorder phase transition associated with the interior hydrocarbon phase of the membrane.

Both of these processes could be involved in the membrane fusion. The phase transition can be induced by divalent ions [56,57] and has been implicated in the fusion of acidic phospholipid membranes [26]. However, under the conditions used in our experiments, the surface properties appear to be more important.

There are two observations which seem to support the above proposal: the observed threshold (or critical) concentrations of divalent ion exhibiting an accelerated interaction between two membranes do not depend appreciably upon the types of phospholipid membrane systems used (vesicle-vesicle, vesicle-bilayer and vesicle-monolayer). This suggests that the observed divalent ion-induced membrane interaction pertains primarily to the nature of the membrane surface. If the main rate-determining step of this interaction is at the level of hydrophobic phases of lipid membranes, since the physical state of hydrocation phase of the monolayer at the air-water interface is quite different from that of the bilayer membrane [54] at the same area/molecule corresponding to the bilayer membrane in the liquid crystalline state, different results for the threshold concentrations should be obtained for the cases of bilayer-vesicle, or vesicle-vesicle and vesicle-monolayer. The other observation is that in the cases of the phosphatidylserine monolayer, and phosphatidylserine or phosphatidylserine/phosphatidylcholine (1 : 1) vesicle systems, the threshold concentrations for divalent ions were lower for smaller area/molecule; but in the case of the phosphatidylcholine monolayer and phosphatidylserine/phosphatidylcholine (1 : 1) vesicle membrane system, the dependence of the threshold concentration on the area/molecule was opposite to that with the phosphatidylserine monolayer. The latter suggests the importance of the hydrophobicity of the membrane surface. The surface nature of phosphatidylcholine monolayers would not be appreciably affected by the presence of divalent ions of experimental concentrations, and the hydrophobicity of the surface should be increased by the increase in area/molecule.

In this study we have demonstrated a new approach to study divalent cation-induced membrane interaction (and possible fusion) in model membrane systems. Although the present system is a very simplified one, it bears a geometrical and functional resemblance to many biological systems where exocytosis is induced by Ca^{2+} [6,15,17].

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