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# A MECHANISM OF DIVALENT ION-INDUCED PHOSPHATIDYLSERINE MEMBRANE FUSION

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**A mechanism for the divalent cation-induced membrane fusion of phosphatidylserine membranes is proposed. Fusion was followed by the Tb/DPA (dipicolinic acid) assay, monitoring the fluorescent intensity for mixing of the internal aqueous contents of unilamellar lipid vesicles, and the threshold concentrations required for various divalent cations to induce membrane fusion were determined from the fluorescence spectrum of the lipid vesicle suspension with respect to various concentrations of divalent ions. Also, the surface tension of monolayers made of the same lipids as used in the fusion experiments was measured with respect to the variation of divalent cation concentrations. The surface tension increase in the monolayer, induced by changing divalent ion concentrations from zero to a concentration which corresponded to its threshold concentration to induce vesicle membrane fusion, was the same (approx. 8 dyn/cm) for all divalent ions used. From these experimental data and the theory concerning ion binding to the membrane, it is deduced that the main cause of divalent cation-induced membrane fusion of phosphatidylserine membranes is the degree of increased hydrophobicity (surface tension increase) of the membrane surface, which results from the binding of cations to acidic phospholipid membrane surfaces. Some discussion on the molecular mechanism of phospholipid membrane fusion is given.**

## Introduction

Membrane fusion is one of the essential events involved in many biological cellular processes, such as exocytosis and endocytosis, cell membrane assembly, development, fertilization, etc.

A number of membrane fusion events have been observed and several proposals concerning the mechanisms of biological membrane fusion processes have been described in the literature [1,2]. However, none of these proposals has, as yet, adequate and clear experimental evidence to ascertain the molecular mechanism of membrane fusion.

Abbreviations: Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]amino}ethanesulfonic acid; Tb-DPA, terbium-dipicolinic acid complex.

Recently, a number of studies on membrane fusion dealing with artificial phospholipid membrane systems have been made in order to gain insight into the mechanism of biological membrane fusion.

It has been shown that acidic phospholipid vesicles will fuse upon the introduction of certain polyvalent metal ions [3–13]. These fusion events are directly related to the strong binding of such polyvalent metal ions to charged sites on the membranes. Polyvalent ion-induced membrane fusion has also been studied with the use of other types of model membrane system such as vesicle-monolayer [14], vesicle-bilayer [15–19] and bilayer-bilayer [21,22].

On the other hand, spherical phospholipid bilayer membranes fuse when the temperature is

increased to a value characteristic for each phospholipid [21,22]. An osmotic gradient across the cell seems to induce membrane fusion in secretory gland systems [23]. This was also shown with the use of model membranes [24]. In these cases, the expansion of the membrane seems to be responsible for the fusion reaction, which appears to be different from the divalent ion-induced membrane fusion.

There have been several molecular mechanisms proposed for the above-mentioned membrane fusion reactions. For divalent ion-induced acidic phospholipid membrane fusion, Papahadjopoulos and co-workers [7,11] have stressed the importance of lateral segregation of acidic lipids in the region of contact. Fusion is then thought to occur at high-energy boundaries between the areas of acidic lipid-$Ca^{2+}$ complex and the remaining liquid-crystalline state membrane lipids.

Alternatively, Lucy and co-workers [25,26] have suggested that a lipid micelle configuration in the membrane may be responsible for membrane fusion when two membranes are in close contact. A similar view has been presented by Breisblatt and Ohki [21] for the temperature-induced spherical membrane fusion. The formation of some nonlamellar lipid structures (e.g., hexagonal lipid structures) for phospholipid membranes containing phosphatidylethanolamine, in the presence of $Ca^{2+}$ has been observed [27,28]. These authors proposed the hypothesis that an intermediate state in membrane fusion is the appearance of such an inverted micelle at the region of contact of the apposing membranes, while a recent study on membrane fusion with use of a similar lipid membrane system suggests that the inverted micelle is not a necessary intermediate state for membrane fusion [29].

For temperature-induced membrane fusion, we have recently attempted to correlate temperature-dependent membrane expansion with the fusion temperature for each phospholipid spherical membrane. We have concluded that membrane expansion, e.g., the increase in surface free energy, is responsible for the temperature-induced membrane fusion [30].

In the case of divalent ion-induced membrane fusion, we originally proposed [14,22,30] that the alteration of membrane surfaces to a more hydrophobic nature (higher surface free energy) by divalent ion binding is the important factor in membrane fusion, rather than a high-energy boundary between the acidic phospholipid-$Ca^{2+}$ complex and liquid-crystalline-state membrane lipids [7,11].

Recently, Portis et al. [12] reported the importance of a transmembrane $Ca^{2+}$ complex as the site of direct interaction between the two apposed membranes. The evidence in support of their hypothesis, however, is not entirely clear.

Although the processes of membrane fusion induced by divalent cations (or polyvalent cations) and temperature-induced membrane fusion appear to be different in their molecular pathways, both seem to have a common feature of an increase in the surface free energy of the membrane under the environmental conditions necessary for membrane fusion.

In order to examine this possibility, we have established experimentally in this paper a correlation between the degree of membrane fusion and the surface tension increase in phosphatidylserine membranes as a function of divalent cation concentration.

## Materials and Methods

### *Chemicals*

Phosphatidylserine was extracted and purified from bovine brain by a slightly modified method of Rouser et al. [31]. The purified lipid showed a single spot on silica gel thin-layer chromatographic plates. NaCl 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. $TbCl_3 \cdot 6\,H_2O$ (99.9% pure) and dipicolinic acid (pyridine-2,6-dicarboxylic acid) were obtained from Alfa and Sigma Chemical Companies, respectively. Buffers used were a mixture of L-histidine (Calbiochem, Ultrol Grade) and Tes (Calbiochem, A grade). Hexane was used as the lipid solvent. It was obtained from Fluka (purum grade), and further washed with distilled water several times to remove possible contaminating surfactants. Other chemicals used were of reagent grade, obtained from Fisher Chemical Co.

In addition, some solutions contained a small amount of EDTA (Alfa Chemical Co.) in order to remove any divalent and polyvalent ion contaminants in the experimental solutions. The divalent

cations used were all in the form of chloride salts obtained from Fisher Chemical Co. (reagent grade). The water used was distilled three times, including the process of alkaline permanganate.

*Vesicle preparation*

Small unilamellar vesicles were prepared in either (a) 10 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 7.4 with NaOH. The phospholipid (phosphatidylserine) was dispersed in either of the aqueous salt solutions, (a) or (b), at a concentration of 10 $\mu$mol/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 100000 $\times$ $g$ 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% of the total lipid used for preparation. Vesicles were separated from non-encapsulated material using a Sephadex G-75 column according to the method of Wilschut et al. [32]. The elution buffer (0.1 M NaCl/2 mM histidine/2 mM Tes) contained 0.05 mM EDTA.

*Assay of vesicle fusion*

Fluorescence intensity was measured by an SLM-8000 spectrofluorimeter (SLM Instruments) in a manner similar to that described in earlier papers [13,32]. The output signal of the fluorescence was monitored with a strip chart recorder. Equimolar amounts (0.2 $\mu$mol phospholipid) of $TbCl_3$- or dipicolinic acid-encapsulated vesicles were suspended in 2ml of NaCl buffer solution in a quartz cuvette. The temperature of the sample holder was maintained at 23°C. Divalent ions were injected into the vesicle suspension in small increments, and the solution was well shaken to give a homogeneous mixture. It took 10 s for changing the divalent ion concentration in the cuvette. The fluorescence intensity was then measured about 15 s after each change in divalent ion concentration.

The Tb-DPA complex was excited at 272 nm and the emission fluorescence was measured at 492 nm. The value for 100% Tb-DPA complex fluorescence was determined in the presence of 20 $\mu$M dipicolinic acid by releasing the contents from Tb-containing vesicles (total 0.2 $\mu$mol phospholipid, freed from EDTA by Sephadex G-75 column chromatography) with 0.5% (w/v) sodium cholate.

*Threshold concentration of divalent ion-induced vesicle fusion*

The fluoresence measured 15 s after each change divalent ion concentration was plotted as a function of the divalent ion concentration. The concentration 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 ion in question causing vesicle fusion. The threshold concentration may also be defined in other ways; e.g. the time course of vesicle fusion was monitored at a given concentration of divalent ion, and the initial slopes were plotted with respect to the divalent ion concentrations. The concentration corresponding to the sharpest point of the initial slope-divalent ion concentration curve can be taken as a 'threshold' concentration. The threshold concentrations obtained by this method were essentially the same (within the experimental error) as those obtained by the above method, used in the present work.

The light intensity contributed by the scattered light due to vesicle aggregation was also measured for suspensions of the same phospholipid vesicles containing no fluorescence materials. The net fluorescence intensity due to vesicle fusion was obtained by subtracting the intensity contributed by the scattered light from the total light intensity at 492 nm. A reduction in fluorescence intensity with time was observed at divalent ion concentrations above the threshold value, but this was not observed below the threshold concentration of divalent ions.

*Surface tension measurements*

The phosphatidylserine monolayers were prepared by placing an aliquot of the lipid spreading solution (approx. 1 mM lipid in hexane) by means of a micro-syringe (Hamilton) on 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 67 $Å^2$. Sub-

phase solutions were NaCl mixtures containing a small amount of buffer (2 mM histidine and 2 mM Tes) and EDTA. The pH of solutions was adjusted to 7.4 with NaOH. The surface tension of the monolayers were measured with an electronic balance (Beckman), using a microscopic cover glass ($18 \times 18 \times 0.2$ mm) as a Wilhelmy plate (accuracy of $\pm 0.1$ dyn/cm). The procedure for measuring the surface tension was almost the same as that published earlier [14]. The surface tension of a phosphatidylserine 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 phosphatidylserine monolayers as a function of divalent or monovalent cation concentration in the subphase solution. The salt concentrations were altered by injecting a small amount of a concentrated salt solution (3 M or more). After each injection of salts, the solutions were stirred well with a magnetic stirrer.

In order to clarify whether the surface tension change induced by divalent cations in the subphase was due to the effective surface lipid concentration change or not, the force-area curve measurements were done with phosphatidylserine monolayers formed on a 0.1 M NaCl buffer solution as well as on the same buffer solution containing divalent ions at concentrations corresponding to the respective observed threshold concentrations for phosphatidylserine vesicle fusion. A Teflon Langmuir trough with surface area dimension of $5 \times 30$ cm, was used. The surface tension of monolayer was measured by the method described above, and the monolayer was gradually compressed by a movable Teflon barrier from 120 $Å^2$ per molecule to 40 $Å^2$ per molecule.

All experiments were done at room temperature (23°C).

## Experimental results

Fig. 1 shows the changes in fluorescence intensity of phosphatidylserine vesicle suspensions in 0.1 M NaCl with respect to various divalent ion concentrations where two kinds of phosphatidylserine vesicle encapsulated with $TbCl_3$ or dipicolinic acid were suspended. All the experimental NaCl solutions contained 2 mM histidine, 2 mM Tes and 0.01 mM EDTA. The pH of the solution was adjusted with NaOH to 7.4. The change in fluorescence intensity represents the degree of fusion of the two kinds of vesicles [32]. The divalent ion concentrations previously defined as the 'threshold concentration' of the divalent ion which

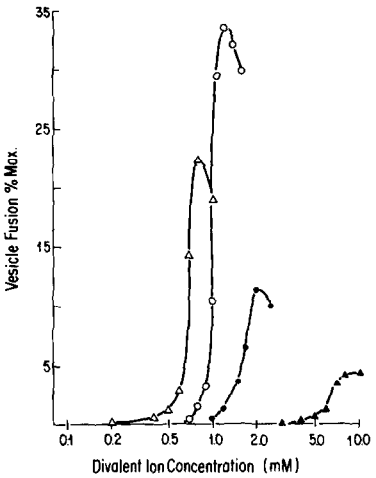


Fig. 1. Fusion of phosphatidylserine vesicles in 0.1 M NaCl with respect to various divalent ion concentrations. Two kinds of phospholipid vesicle (0.1 μmol lipid each) which are encapsulated with 10 mM Tb/100 mM sodium citrate and 100 mM dipicolinic acid, respectively, were suspended in 2 ml 0.1 M NaCl/2 mM histidine/2 mM Tes/ 0.01 mM EDTA, pH 7.4. The ordinate refers to the fluorescence intensity at 492 nm and the abscissa refers to divalent ion concentration. The fluorescence intensity was corrected for the background scattered light by subtracting the scattered light intensity from the total light intensity at 492 nm. The intensity is expressed in percentage of the maximum fusion. The wavelength of excitation light was 272 nm. △, $Ba^{2+}$; ○, $Ca^{2+}$; ●, $Sr^{2+}$; and ▲, $Mg^{2+}$.

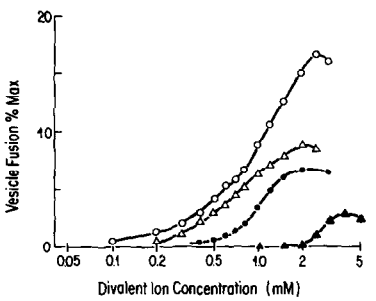


Fig. 2. Similar plots of the experimental results as described in Fig. 1, except that the vesicle suspension solution was 0.01 M NaCl instead of 0.1 M NaCl. △, $Ba^{2+}$; ○, $Ca^{2+}$; ●, $Sr^{2+}$; and ▲, $Mg^{2+}$.

induced vesicle membrane fusion were 0.7 mM for $Ba^{2+}$, 1.0 mM for $Ca^{2+}$, 1.4 mM for $Sr^{2+}$ and 6 mM for $Mg^{2+}$. The threshold values obtained for $Ca^{2+}$ and $Mg^{2+}$ in this experiment correspond to those reported earlier (Refs. 32, 43, respectively).

Fig. 2 shows the same experiments as described above, except for the use of vesicle suspension solutions of 0.01 M NaCl. In this case, the threshold concentrations were lower than those obtained in 0.1 M NaCl. They were 0.2 mM for $Ba^{2+}$, 0.37 mM for $Ca^{2+}$, 0.6 mM for $Sr^{2+}$ and 2.2 mM for $Mg^{2+}$. A similar observation was made for $Ca^{2+}$-induced phosphatidylserine vesicle fusion when the medium salt concentration was changed from 100 mM to 3 mM NaCl [33].

Fig. 3 also shows experimental results similar to those mentioned above, but for $Ca^{2+}$-induced membrane fusion in a suspension of various monovalent ionic strengths (0.01, 0.1, 0.3, 0.55, 0.8 M). The figure indicates that the threshold concentration for fusion increased as the monovalent ionic strength in the suspension was increased. In 0.55 M and 0.8 M NaCl solutions, no appreciable fusion was observed up to 10 mM $Ca^{2+}$, although the degree of vesicle aggregation in such suspensions increased with an increase in $Ca^{2+}$ concentration. In 0.8 M NaCl, a great degree of aggregation of phosphatidylserine vesicle was already observed in the absence of $Ca^{2+}$.

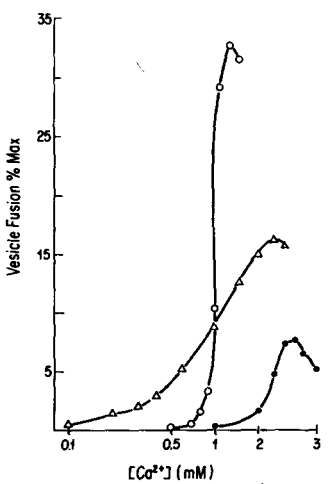


Fig. 3. Fusion of phosphatidylserine vesicles suspended in various NaCl concentration solutions with respect to $Ca^{2+}$ concentrations. Other experimental conditions were the same as in Fig. 1. △, 0.01 M NaCl; ○, 0.1 M NaCl; ●, 0.3 M NaCl.

Fig. 4 shows the increase in surface tension of a phosphatidylserine monolayer (67 Å$^2$/molecule) as a function of divalent cation concentration. The monolayer was formed on an aqueous solution of 0.1 M NaCl/2 mM histidine/2 mM Tes/0.01 mM EDTA, pH 7.4.

As seen in Fig. 4, at the threshold concenration (0.7 mM $Ba^{2+}$, 1.0 mM $Ca^{2+}$, 1.4 mM $Sr^{2+}$, 6 mM $Mg^{2+}$) for each divalent ion which induced vesicle fusion, the surface tension increase in the monolayer was of the same magnitude (8 dyn/cm for all divalent cation cases (also see Table IA)), although for $Mg^{2+}$ the surface tension increase approached, but was of slightly lower value than, 8 dyn/cm at 10 mM $Mg^{2+}$. For $Ba^{2+}$, $Ca^{2+}$ and $Sr^{2+}$, the surface tension increase exceeded 8 dyn/cm when the divalent ion concentration was increased above the threshold concentration for membrane fusion, and tended to saturate at about 9–10 dyn/cm. However, with $Mg^{2+}$ the increase was not as great as those obtained for the other ions, appearing instead to saturate at about 8 dyn/cm. This result corresponds to the observation that for $Mg^{2+}$ the degree of membrane fusion was very small in comparison with the other divalent ions. The surface tension of a phosphatidylserine monolayer on a 130 mM NaCl subphase has been measured previously as a function of divalent ion concentration [34]. However, the

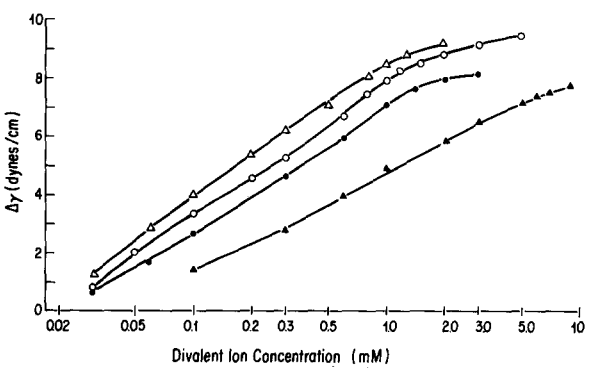


Fig. 4. Surface tension increase in the phosphatidylserine monolayer (67 Å$^2$ per molecule) formed at the air/water interface with respect to various divalent ion concentrations. the subphase solution consisted of 0.1 M NaCl/2 mM histidine/2 mM Tes/0.01 mM EDTA, pH 7.4. The initial surface tension $\gamma(C_2=0)$ of a phosphatidylserine monolayer of 67 Å$^2$ per molecule was $27\pm2$ dyn/cm and $\Delta\gamma \equiv \gamma(C_2)-\gamma(C_2=0)$ where $C_2$ refers to the divalent ion concentration. △, $Ba^{2+}$; ○, $Ca^{2+}$; ●, $Sr^{2+}$; ▲, $Mg^{2+}$.

TABLE I

THRESHOLD CONCENRATION ($C_{Th}$) OF DIVALENT CATIONS

Threshold concentration required to induce phosphatidylserine vesicle fusion; observed surface tension change, $\Delta\gamma$, of phosphatidylserine monolayer (67 Å$^2$/molecule); the calculated fraction of bound divalent cations per phosphatidylserine molecule and the surface charge ($f$) of the phosphatidylserine membrane (67 Å$^2$/molecule) at divalent ion concentrations corresponding to the threshold concentrations for membrane fusion. The values ($K_1$ and $K_2$) of binding constants for monovalent and divalent ions were those obtained in the surface potential study [40] and the vesicle aggregation studies [37]. A, 0.1 M NaCl; B, 0.01 M NaCl.

| $M^{2+}$ | $C_{Th}$ (mM) | $\Delta\gamma$(dyn/cm) | Bound divalent cation/lipid ratio | $f$ | $K_2$ ($M^{2+}$) ($M^{-1}$) | $K_1$ ($Na^+$) ($M^{-1}$) |
|---|---|---|---|---|---|---|
| A: 0.1 M NaCl | | | | | | |
| $Ba^{2+}$ | 0.7 | 8.0 | 0.32 | 0.22 | 37 | 0.6 |
| $Ca^{2+}$ | 1.0 | 8.0 | 0.32 | 0.22 | 30 | 0.6 |
| $Sr^{2+}$ | 1.4 | 8.0 | 0.33 | 0.22 | 25 | 0.6 |
| $Mg^{2+}$ | 6.0 | 7.8 | 0.34 | 0.22 | 10 | 0.6 |
| B: 0.01 M NaCl | | | | | | |
| $Ba^{2+}$ | 0.2 | 8.1 | 0.41 | 0.15 | 37 | 0.6 |
| $Ca^{2+}$ | 0.4 | 8.1 | 0.42 | 0.15 | 30 | 0.6 |
| $Sr^{2+}$ | 0.6 | 8.0 | 0.42 | 0.15 | 25 | 0.6 |
| $Mg^{2+}$ | 2.2 | 7.9 | 0.4 | 0.17 | 10 | 0.6 |

surface tension values obtained were smaller than those found in the present study, and the change in surface tension did not increase monotonically as a function of divalent ion concentration. It should be noted that the previous experiment was done using a monolayer at the equilibrium surface pressure (approx. 44 dyn/cm) which corresponds to 28 dyn/cm monolayer surface tension, indicating a fairly condensed state for the lipid monolayer (area per molecule approx. 50 Å$^2$), while the present study was done at a monolayer surface pressure of 27 ± 3 dyn/cm, which corresponds to an area per molecule of approx. 67 ± 2 Å$^2$. The latter area per molecule corresponds to that ob-

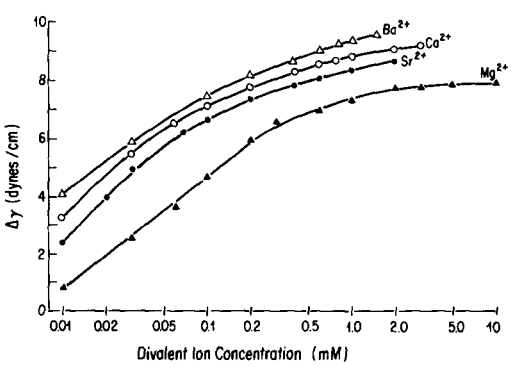


Fig. 5. The experimental results similar to those indicated in Fig. 4 except that the subphase solution was 0.01 M NaCl instead of 0.1 M NaCl. △, $Ba^{2+}$; ○, $Ca^{2+}$; ●, $Sr^{2+}$; ▲, $Mg^{2+}$.

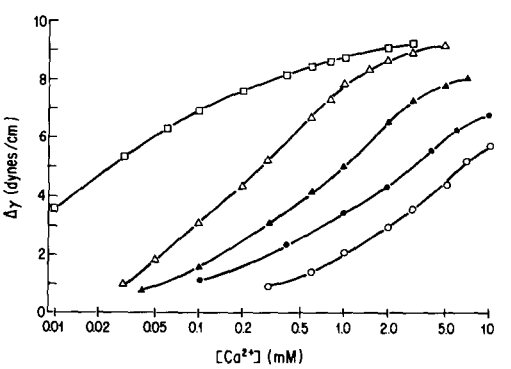


Fig. 6. Surface tension increase of the phosphatidylserine monolayer (67 Å$^2$ per molecule) formed on various NaCl concentration solutions with respect to $Ca^{2+}$ concentrations. Each subphase solution contained 2 mM histidine, 2 mM Tes and 0.01 mM EDTA. The pH was 7.4. [NaCl](M): □, 0.01; △, 0.1; ▲, 0.3; ●, 0.55; ○, 0.8.

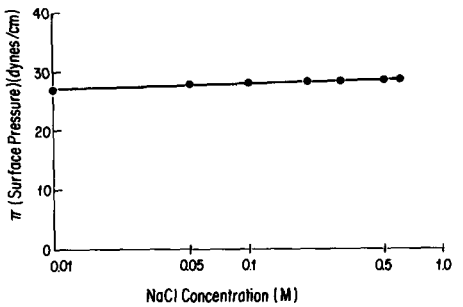


Fig.7. Surface pressure, $\pi$, of the phosphatidylserine monolayer (67 Å$^2$ per molecule) with respect to various NaCl concentrations in the subphase solution. The subphase solution contained 2 mM histidine, 2 mM Tes and 0.01 mM EDTA. The pH was 7.4. A concentrated NaCl solution was injected ino the subphase solution in order to alter the concentrations of the subphase salt concentration. Each experimental value was corrected for the change in surface tension due to the volunme change resulting from the solution injection. The surface pressure is expressed: $\pi = \gamma_0 - \gamma$ where $\gamma_0$ is the surface tension of water and $\gamma$ is the surface tension of a monolayer spread on the water surface.

tained for phospholipid bilayers composed of naturally occurring phospholipids [35].

Fig. 5 shows experimental results similar to those above except in that 0.01 M NaCl was used as the subphase solution. The results also correlate well with the vesicle fusion results obtained with these experimental solutions (see Table IB). The increase in the surface tension of phosphatidylserine monolayers, in various NaCl solutions, as a function of $Ca^{2+}$ concentration is shown in Fig. 6. When the NaCl concentration was 0.3 M, the increase in surface tension as a function of the subphase $Ca^{2+}$ concentration was suppressed, and at 7 mM $Ca^{2+}$ the increase in surface tension was below 8 dyn/cm. With higher NaCl concentrations in subphase solutions (0.5–0.8 M), the increases in surface tension with increasing $Ca^{2+}$ concentrations were more suppressed, being below 7 dyn/cm even at 10 mM $Ca^{2+}$. Correspondingly, the degree of vesicle membrane fusion induced by $Ca^{2+}$ in these high monovalent salt concentration solutions was also drastically reduced (almost zero), although 0.8 M NaCl alone caused a great degree of phosphatidylserine vesicle aggregation, as also observed by others [36,37].

Fig. 7 shows the surface tension change in a phosphatidylserine monolayer with respect to increasing the NaCl concentration of the subphase solution. In contrast to the cases of divalent ions, increasing the NaCl concentration resulted in a slight decrease in monolayer surface tension. It has previously been observed that phosphatidylserine vesicles aggregate in NaCl suspensions greater than 0.6 M, and the massive aggregation occurs at around 0.7–0.8 M [30,37]. Each data point in the above figures represents the average of four or more experiments.

Fig. 8 shows the surface pressure-area curves for phosphatidylserine monolayers formed on 0.1 M NaCl/2 mM histidine/2 mM Tes/0.05 mM

TABLE II

THRESHOLD CONCENTRATION ($C_{Th}$) OF $Ca^{2+}$ REQUIRED TO INDUCE PHOSPHATIDYLSERINE VESICLE FUSION IN VARIOUS NaCl CONCENTRATION SOLUTIONS

Threshold concentration; the observed surface tension change of a phosphatidylserine monolayer (67 Å$^2$/molecule); the calculated fraction of bound $Ca^{2+}$ per phosphatidylserine molecule and the surface charge ($f$) of the phosphatidylserine membrane (67 Å$^2$/molecule) at $Ca^{2+}$ concentrations corresponding to the threshold concentrations for membrane fusion. The values indicated with * symbols are not the 'threshold concentration' values. The magnitudes in the parentheses are those corresponding to the case of 10 mM $Ca^{2+}$.

| NaCl (M) | $C_{Th}$ (mM) | $\Delta\gamma$(dyn/cm) | Bound $Ca^{2+}$/ lipid ratio | $f$ | $K_2$ ($Ca^{2+}$) ($M^{-1}$) | $K_1$ ($Na^+$) ($M^{-1}$) |
|---|---|---|---|---|---|---|
| 0.01 | 0.4 | 8.1 | 0.42 | 0.15 | 30 | 0.6 |
| 0.1 | 1.0 | 8.0 | 0.32 | 0.22 | | |
| 0.3 | 1.8 | 7.8 | 0.25 | 0.25 | | |
| 0.55 | (10)* | (6.7) | (0.29) | (0.21) | | |
| 0.8 | (10)* | (5.6) | (0.24) | (0.22) | | |

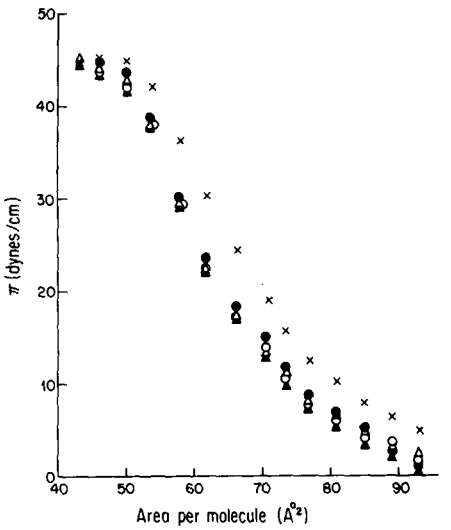


Fig. 8. Force-area curves of phosphatidylserine monolayers formed at the air/water interface. Subphase solutions composed of 0.1 M NaCl, 2 mM histidine and 2 mM Tes with and without divalent cation, the concentration of which was the same as its threshold concentration for phosphatidylserine vesicle fusion. pH of the solution was 7.4. ×, no divalent cation; △, $Ba^{2+}$ (0.7 mM); ○, $Ca^{2+}$ (1.0 mM); ●, $Sr^{2+}$ (1.4 mM); ▲, $Mg^{2+}$ (6.0 mM).

EDTA, pH 7.4, and on the same salt solutions containing divalent ions corresponding to its threshold concentrations ($Ba^{2+}$, 0.7 mM; $Ca^{2+}$, 1.0 mM; $Sr^{2+}$, 1.4 mM; and $Mg^{2+}$, 6 mM) for inducing the fusion of phosphatidylserine vesicles. The condensation of the phosphatidylserine monolayer by divalent cation of its threshold concentration at the constant surface pressure of 27 dyn/cm was approx. 5 Å$^2$ for all divalent cation cases and the surface tension change was about 7.7 dyn/cm at the constant surface area (67 Å$^2$) per molecule. All four cases showed approximately the same condensation effect and the surface tension change in the range of area per molecule from 55 Å$^2$ to 80 Å$^2$. The result in the case of $Ca^{2+}$ was approximately the same as that observed earlier [34].

## Discussion

From the above vesicles fusion and monolayer surface tension studies, it is proposed that an increase in the suface tension of the membrane beyond a certain threshold value would be a major cause of phospholipid fusion. In order for membrane fusion to occur, it is presumed that the close adhesion of two membranes has occurred. The adhesion of two membranes is, however, necessary but not sufficient for membrane fusion. For example, it was shown that phosphatidylserine vesicles aggregated to a great degree in a 0.8 M NaCl solution at pH 7.4, but did not fuse under this environmental condition. Thus in addition to membrane adhesion, a further change in the nature of the membrane surface is necessary for membrane fusion to occur, as proposed previously [14,22,30]. This further change would be accomplished by the adsorption of divalent cations to phosphatidylserine headgroups on the membrane surface. Because of the strong binding affinity of divalent or polyvalent metal ions to the negatively charges sites, at least two processes would occur: (1) structural water would be removed from the membrane surface [38]; and (2) the chelation of metal ions with polar groups would cause bridging of the polar groups close together and hence the more hydrocarbon phase of the membrane would tend to be revealed to the water phase. Consequently and significantly, the membrane surface would thus become more hydrophobic in nature, resulting in an increase in the surface tension of the membrane. This is clearly shown by the progressive increase in the surface tension of phosphatidylserine monolayers with increasing divalent ion concentrations. This idea is consistent with the fact that a high concentration of NaCl (0.5–0.8 M in the subphase solution resulted neither in as large an increase in the surface tension of the phosphatidylserine monolayers as with the divalent ions (see Fig. 7), nor in membrane fusion in the above concentration range (in this study).

It has been shown that membrane fusion can be induced by increasing the environmental temperature [21,22,26], or by applying an osmotic pressure gradient across the membrane [24]. These actions would cause the expansion of the membrane which should result in an increase in surface tension or surface free energy with respect to an unexpanded state of the membrane [30].

An explanation for the fact that $Na^+$ and most other monovalent cations induce aggregation of phosphatidylserine vesicles [36,37], but not vesicle

fusion, is that, in general, monovalent cation bindings [37,39,40] are not as strong as those of divalent or polyvalent ions to the negatively charged sites of lipids – nor do they bridge two polar groups. Indeed, such monovalent ion binding did not increase the surface tension of the monolayer (see Fig. 7). We propose that the affinity of divalent ion binding to the polar groups relates directly to the degree of increase in surface free energy of the membrane surface. This surface tension increase is not due to electrosatic effects, but rather to molecular effects. The study correlating the number (fraction) of tightly bound divalent ions to a lipid polar group with vesicle membrane fusion [33], coincides with the molecular mechanisms for membrane fusion presented here. However, this author believes that it is the degree of surface free energy or surface tension of the membrane which is the appropriate parameter for examining the mechanism of divalent ion-induced phosphatidylserine membrane fusion. It is indeed seen from Tables I and II that the fraction of divalent ions bound to a phospholipid molecule and the threshold concentrations of divalent ions which induce vesicle fusion are well correlated for the experiments done under the conditions of the same ionic strength of monovalent salt; but they are not correlated among the experiments under different ionic strengths of monovalent salt (see Tables I and II). In contrast, the correlation between the increased surface tension and the degree of membrane fusion is good regardless of the different ionic strengths of monovalent salts used in this experiment (see Tables I and II). The values for ion binding and the surface change of a lipid molecule were calculated according to the method described in the Appendix.

It is known that $Ca^{2+}$ condenses acidic lipid monolayers, probably by chelating polar groups of membrane molecules [34,41]. The good correspondence (Fig. 8) between the surface tension increase, caused by the effective surface lipid concentration changes due to condensation, and the degree of fusion at the corresponding divalent ion concentration indicates that membrane fusion was due to the increase in surface tension of the membrane caused by the divalent ion binding of phospholipid polar groups. In the case of divalent cation-induced fusion of sonicated lipid vesicles, since divalent cations were added only to the outer bilayer surface, the degree of condensation of the outer surface by divalent ions would be much reduced by the constraint of the highly condensed monolayer of the internal surface. Therefore, the situation of a constant area per molecule for the outer monolayer of the vesicle was maintained, which was similar to that of the monolayer experiments in the present study. This coincides with the observation that the larger the vesicle size, the less susceptible the vesicles became to fusion by divalent ions [10].

There seem to be at least two common factors for two apparently opposing actions (expansion and condensation) for membrane fusion: (1) creating a high-energy surface state, and (2) creating an unstable configuration (nonbilayer type structure) in two stable closely apposed membrane systems.

The value of 8 dyn/cm observed for the increased monolayer surface tension relative to the reference state (surface pressure 27 dyn/cm) may not correspond directly to the increase in surface tension of a lipid bilayer membrane required for its membrane fusion, since the air/water interface monolayer is not exactly a half of a bilayer in terms of its physical state [42]. Also, the area of the outer surface of a vesicle membrane may not be kept constant, although the inner monolayer of vesicle membrane should impose the constraint on the outer layer not to be condensed. In addition, another factor should be considered in determining the surface free energy of a vesicle membrane, namely the shape of the membrane surface (e.g., curvature). However, for this factor, as long as the relative magnitude of the surface tension increases is compared between the two cases (monolayer and bilayer), since the curvature of the vesicle is assumed to be the same for all cases in the present experiments (therefore, the curvature factor contributing the surface energy is a constant), the correlation between the surface tension increase and the degree of membrane fusion discussed in this paper, should still be applicable.

It should be noted that the fusion of phospholipid membranes containing phosphatidylethanolamine [27,28] may proceed via a pathway different from that for the divalent cation-induced lipid membrane fusion. The latter is due to an elevation to a high free energy state of the lipid

polar group region, whereas the former may be due to the high energy state of the lipid hydrocarbon (hydrocarbon interior) region relative to the polar group region.

It should also be mentioned that the present proposal for a mechanism of membrane fusion is applicable only to simple phospholipid membrane systems, and may not be, in its present form, for those observed in biological membrane systems. In the latter cases, more complicated and various molecular mechanisms probably involving membrane macromolecules may be necessary to explain the membrane fusion processes [44].

## Appendix

The Gouy-Chapman double layer potential (which we term here 'surface potential') at the memrane-electrolyte interface is expressed by

$$\sigma = \frac{1}{271}\left[\sum_i C_i \left(\exp\left(-\frac{ez_i\phi(0)}{kT}\right)-1\right)\right]^{1/2} \quad (1)$$

where $\sigma$ is the surface charge density (charge per Å$^2$); $C_i$ the molar concentration of the $i$th ionic species in the bulk phase; $z_i$ the valency of the $i$th ion; $\phi(0)$ the surface potential at the membrane interface; $k$ the Boltzmann constant, and $T$ the temperature 23°C.

We assume that one divalent ion would bind with two phospholipids simultaneously, and one monovalent ion with one phospholipid, independently. Then, we have the following reactions:

$$C^+ + A^- \overset{K_1}{\rightleftharpoons} C^+A^-$$

$$C^{2+} + A^{2-} \overset{K_2}{\rightleftharpoons} C^{2+}A^{2-} \quad (2)$$

where $K_1$ and $K_2$ are the association constants for the above reactions:

$$K_1 = \frac{[C^+A^-]}{[C^+][A^-]}$$

$$K_2 = \frac{[C^{2+}A^{2-}]}{[C^{2+}][A^{2-}]} \quad (3)$$

Here, $[A^{2-}] \equiv [A^-]/2$, where $[A^-]$ is the surface concentration of phospholipid molecules.

Then, the surface charge density, $\sigma$, is expressed by

$$\sigma = \frac{\sigma^{int}}{1 + K_1C^+ \exp\left(\frac{-e\phi(0)}{kT}\right) + K_2C^{2+} \exp\left(\frac{-2e\phi(0)}{kT}\right)} \quad (4)$$

where $\sigma^{int}$ is the surface charge density with no ion binding.

With Eqns. 1 and 4, and knowing all ionic concentrations in the bulk phase, and the initial surface charge density of the phosphatidylserine bilayer ($\sigma^{int} \equiv -e/67$ Å$^2$), the surface potential is calculated by a computer (CDC 4000 at SUNY/Buffalo) giving $K_1$ the association constant for monovalent ions and $K_2$ the association constant for divalent ions.

The fraction of the surface charge density, $f$, is defined by

$$f \equiv \frac{\sigma}{\sigma^{int}} \quad (5)$$

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