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Effects of cations and polyamines on the aggregation and fusion of phosphatidylserine membranes

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Effects of various metal cations and polyamines on aggregation and fusion of phosphatidylserine vesicles and their associated physicochemical properties (such as surface tension and vesicle electrophoretic mobility) have been studied. It was found that metal polycations and hydrogen ion caused an increase in the surface tension of a phosphatidylserine monolayer, whereas the polyamines and other monovalent cations did not increase the surface tension of the membrane appreciably. All cations used affected the vesicle mobility roughly in the order of the number of their valencies and linearly with respect to the logarithm of their concentrations of ions; vesicle surface charge densities are reduced by adsorption and screening of the counter ions depending on their valencies and concentrations. The degree of aggregation of lipid vesicles parallels somewhat that of the reduction of vesicle electrophoretic mobilities. However, the degree of membrane fusion induced by these cations parallels that of the increase in surface tension of the membranes induced by these cations.

Introduction

A number of studies have demonstrated that various divalent cations induce fusion of acidic phospholipid membranes [1–4]. There have been several theories for such membrane fusion phenomena. Among them, it has been shown that the increase in surface tension of such acidic lipid membranes induced by divalent cation correlates well with the extent of the membrane vesicle fusion and it has been proposed that the surface tension increase is an important factor for the vesicle membrane fusion [3,5,6]. It has also been reported that some polyamines, which are polycationic molecules, enhance the rate of acidic lipid membrane fusion induced by divalent cations as well as the fusion capability of fusogenic agents, e.g., reducing Ca^{2+} or Mg^{2+} concentration required to induce a certain degree of membrane fusion [7]. Similarly to polyamines, certain polycations (polyamino acids) have been shown to induce fusion of acidic lipid membranes [8–10], especially those containing phosphatidylethanolamine [7], without using other fusogenic agents.

In this paper, we test the capabilities of various cationic molecules, including polyamines, having various valency numbers to induce aggregation and fusion of acidic phospholipid vesicles. We have studied the effect of polyamines as well as various metal cations on lipid vesicle aggregation and fusion, together with their effect on electro-

Abbreviation: Tes, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine.

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phoretic mobility of lipid vesicles and surface tension of lipid monolayers. From these studies, we would like to examine how the observed physical quantities of the membrane surface properties systematically correlate with the vesicle-membrane interaction such as aggregation and fusion for the various ionic substances tested.

Materials and Methods

Chemicals

Bovine brain phosphatidylserine was purchased from Avanti Biochemical Co., AL. The lipids showed a single spot on silica-gel thin-layer chromatographic plates. Spermine, spermidine and putrescine, all hydrochloride forms, were obtained from Sigma Chemical Co.. NaCl used was of ultrapure grade, purchased from Alfa Chemical Co. Before use, the NaCl was roasted at 400–500°C for 2 h to eliminate possible organic contaminants. Buffers used were a mixture of L-histidine (Ultrol grade, Calbiochem) and Tes (A grade, Calbiochem). The sodium citrate buffer was used for the solutions below pH 5.0. The pH of the solution was adjusted with HCl or NaOH. Hexane was used as the lipid solvent for spreading monolayers. It was obtained from Fluka (purum grade) and further purified through both alumina and silica-gel columns to remove possible contaminating surfactants. $LaCl_3$ (gold grade) was obtained from Aldrich Chemical Co. $CaCl_2$ and other chemicals used in the experiments were of reagent grade from Fisher Chemical Co. All experimental salt solutions except for La^{3+} contained a small amount of EDTA in order to remove any divalent and polyvalent ion contaminants in the experimental system. The water used was distilled three times, including a process involving alkaline permanganate.

Vesicle preparation

Small unilamellar vesicles were prepared in the NaCl buffer solution (0.1 M NaCl/2 mM histidine/2 mM Tes/0.02 mM EDTA (pH 7.0)). Phosphatidylserine was dispersed in the NaCl buffer solution at a concentration of 10 μmol lipid/ml, vortexed for 10 min and sonicated for 1 h in a bath-type sonicator under N_2 at 25°C. The samples were then centrifuged for 1 h at $100\,000 \times g$ to remove large vesicles and/or aggregates. The supernatants were kept as unilamellar lipid vesicle suspensions. The yield of unilamellar lipid vesicles was about 95% of the total lipids used for preparation [11]. Phosphatidylserine multilamellar vesicles were prepared by dispersing phosphatidylserine in the NaCl buffer solution at the same lipid concentration as that for the above small unilamellar vesicles and by vortexing the suspension for 15 min.

Surface tension measurements

Lipid monolayers were formed on an aqueous surface of a constant area (64 cm^2 in a glass dish) by placing an aliquot of the lipid spreading solution (approx. 1 mM lipid in hexane) by means of a microsyringe. Aqueous subphase solutions were 0.1 M NaCl/1 mM histidine/1 mM Tes/0.01 mM EDTA (pH 7.0). The surface tension of a monolayer-coated aqueous interface was measured with an electronic balance (Beckman), using either a microscopic cover glass ($18 \times 18 \times 0.2$ mm) or a thin Teflon plate ($11 \times 11 \times 1$ mm) as a Wilhelmy plate (accuracy of about 0.2 dyn/cm). The detailed procedures for measuring the surface tension are described in the literature [5,11]. The experiments were designed to measure the change in the surface tension of lipid monolayers as a function of various metal cation or polyamine concentrations in the subphase solution. The metal cation or polyamine concentrations in the subphase solution were altered successively by injecting a small amount of concentrated metal salt solutions or concentrated polyamine solutions.

Measurements of vesicle-monolayer fusion

Fusion between lipid vesicles and a lipid monolayer was monitored by measuring the surface tension of the monolayer in the presence of lipid vesicles in the subphase. The threshold of fusion of lipid vesicles to the lipid monolayer was determined as the point where a sudden, large and progressive decrease in the monolayer surface tension occurred. Such a surface tension decrease corresponds to the fusion of vesicles to the monolayer [5,11]. The detailed experimental procedures are published elsewhere [11]. The following is a general procedure: a lipid monolayer (phosphatidylserine) was formed at the air/water (0.1 M

NaCl buffer solution) interface, using a glass dish with a fixed area of 28.3 cm^2. The area per lipid molecule was either 70 Å or 85 $Å^2$. Then, the surface tension of the film was measured by use of an electrobalance with the Teflon plate as a Wilhelmy plate, where the monolayer surface tension decrease corresponded to the decrease in the upward force of the film exerted on the Wilhelmy plate. The depth of the dipped plate was kept constant, about 1.0 mm from the water surface. First, an aliquot of the stock lipid vesicle solution was injected into the subphase solution of the monolayer and the solution was stirred well with a magnetic stirrer. The addition of lipid vesicles to the subphase solution did not alter the surface tension of the monolayer. Then, the concentrated metal salt (La_3Cl_3, $CaCl_2$, NaCl) or polyamine solutions were injected into the subphase and the subphase solution was well stirred again. Each new addition of the above concentrated injection solutions into the subphase was made after the monolayer surface tension had stabilized. The concentrations of the injected substances were increased systematically: for example, in the case of $CaCl_2$, the $CaCl_2$ was injected by 0.1 or 0.2 mM increments, the solution was well stirred and the surface tension of the monolayer was measured at 2 min, this being followed by the next increment injection. However, near the 'critical concentration', at which the surface tension of the film started to decrease sharply, the concentration increment for the injecting substances was reduced. For example, in the case of $CaCl_2$ addition, the increment of the $CaCl_2$ concentration was reduced to 0.05–0.025 mM per injection (see Fig. 4) around the critical concentration. The point at which the monolayer surface tension started to decrease sharply, was called 'the threshold point' for vesicle-monolayer fusion. It was ascertained that this sharp decrease in surface tension was not due to the adhesion of vesicles to the monolayer, because polyvalent metal ion or polyamine adsorption causes an increase in surface tension of the monolayer which is opposite to the decrease in surface tension. The addition of equimolar EDTA to the Ca^{2+} concentration in the subphase during the fusion of vesicles to the monolayer showed no further change in surface tension of the monolayer. The 'threshold' point was influenced slightly by the manner of addition of the substances (e.g., adding amounts of the concentrated $CaCl_2$ solution, incubation (waiting) time until the next Ca^{2+} addition) as well as the concentration of vesicles in the subphase. However, the deviation of the experimental data points due to these factors was within the experimental error. In the pH-induced fusion of vesicles to the monolayer, the pH of the subphase solution was lowered from pH 6 stepwise by adding HCl into the subphase solution, and the pH of the solution was monitored continuously by a pH meter.

Turbidity measurements

The turbidity of phospholipid vesicle suspensions as a function of the concentration of various types of polyamine and metal cation was measured at 400 nm with a Hitachi (100-60) spectrophotometer. The vesicles were suspended at a concentration of 0.2 μmol lipid/ml of 0.1 M NaCl buffer solution. In the case of $LaCl_3$, the suspended lipid vesicle concentration was 0.02 μmol lipid/ml, which was a 10-times diluted concentration compared with the other cases. The metal cation or polyamine concentration was raised in increments. The absorbance was recorded 2 min after changing the cation or polyamine concentration. When the observed absorbance was plotted against cation or polyamine concentration, a maximum increase in the rate of absorbance change was obtained at a certain concentration of cation or polyamine. This was defined as the 'threshold' concentration of cation or polyamine to induce lipid vesicle aggregation.

Vesicle electrophoresis

The electrophoretic mobility of the multilamellar phosphatidylserine vesicle in 0.1 M NaCl buffer solution was measured with a microelectrophoresis apparatus (Mark II, Rank Bros., Bottisham, Cambridge) as a function of various metal cation or polyamine concentrations in the suspension solution. Great care was taken to focus on the stationary layer. The observed mobility, u, and ζ-potential are related by the following Helmholtz-Smoluchowski equation:

$$u = \zeta \epsilon_r \epsilon_0 / \eta$$

where η is the viscosity of the aqueous phase and

ϵ_r, ϵ_0 are the permititivities of the aqueous medium and a vacuum, respectively. All experiments were done at 23° ± 1°C.

Experimental results

Surface tension

Fig. 1 shows the surface tension increase in a phosphatidylserine monolayer (70 Å^2 per molecule) as a function of various cation and polyamine concentrations. A trivalent cation, La^{3+}, and divalent cations (Ca^{2+}, Mn^{2+}, Ba^{2+}, Sr^{2+} and Mg^{2+}) caused large changes in the surface tension of the phosphatidylserine monolayer in the 5–20 μM and the 0.5–10 mM ranges, respectively. Data for Mn^{2+}, Mg^{2+}, Ba^{2+} and Sr^{2+} are not shown in the figure. Also, H^+ resulted in a similar change in the surface tension of the monolayer in the range of pH 5 to pH 2 as seen in the case of Ca^{2+}. The surface tension increases showed saturation at about 10 dyn/cm for all the above cases. On the other hand, Na^+, Li^+ and K^+ did not cause such large changes in the surface tension of the phosphatidylserine monolayer up to 1.0 M.

Spermine up to 25 μM caused a monotonic increase in the surface tension of the phosphatidylserine monolayer. The surface tension increase was the greatest (2.5 dyn/cm) at about 25 μM of spermine, and at concentrations above 35 μM, the surface tension increase was slightly less than that at 25 μM spermine as the concentration increased. However, other polyamines (spermidine and putrescine) did not cause any significant change in surface tension of the phosphatidylserine monolayer at any concentration examined (Fig. 1). The data for putrescine are not shown.

For the case of spermidine, the change in surface tension of the monolayer with respect to the change in spermidine concentration was somewhat similar to those of spermine, but the magnitude of surface tension increase was much smaller and the concentrations of spermidine needed to cause such surface tension changes were much greater than those of spermine.

Putrescine did not cause any appreciable change in surface tension of the phosphatidylserine monolayer up to a concentration of 0.2 M.

Vesicle electrophoretic mobility

Fig. 2 shows the electrophoretic mobility of multilamellar phosphatidylserine vesicles (several μm in diameter) with respect to various cation and

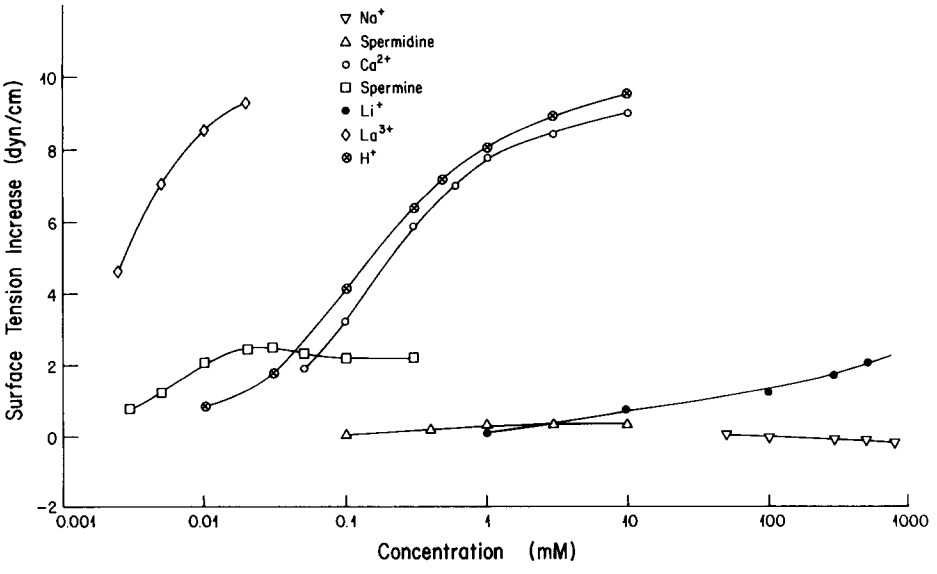

Fig. 1. The surface tension increase of a phosphatidylserine monolayer (70 Å^2 per molecule) formed at the air/water (0.1 M NaCl/1 mM histidine/1 mM Tes/0.01 mM EDTA (pH 7.0)) interface with respect to various metal cation and polyamine concentrations. ◇, La^{3+}; ○, Ca^{2+}; ⊗, H^+; ●, Li^+; ▽, Na^+; □, spermine^{4+}; △, spermidine^{3+}. Note: For La^{3+} the vesicle suspension solution did not contain EDTA.

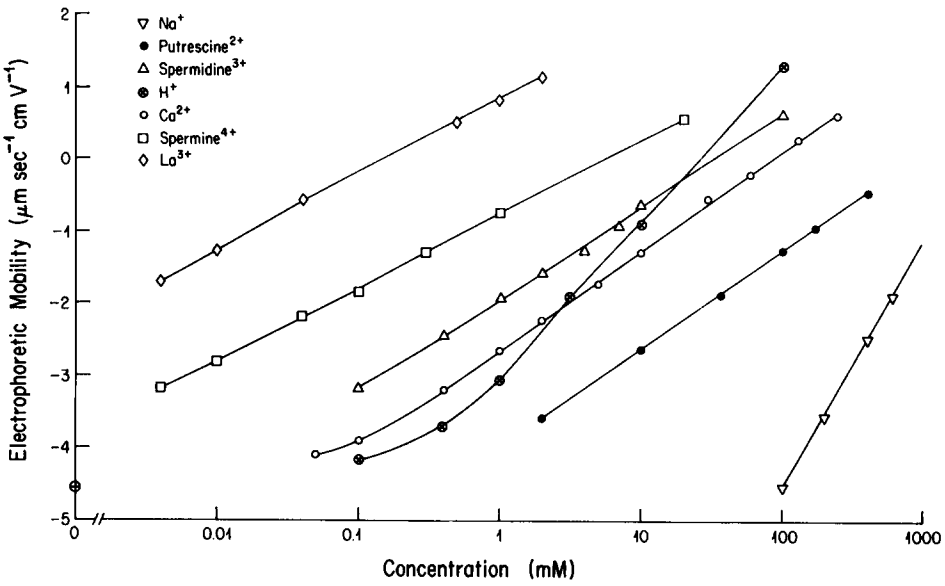

Fig. 2. The electrophoretic mobility of multilamellar phosphatidylserine vesicles suspended in 0.1 M NaCl/2 mM histidine/2 mM Tes/0.01 mM EDTA (pH 7.0) as a function of various polyamine and metal cation concentrations. For experiments involving variation in H^+ concentration the vesicle suspension solutions were 0.1 M NaCl/2 mM sodium citrate and the pH was adjusted with HCl or NaOH to the experimental pH value. ◇, La^{3+}; ○, Ca^{2+}; □, spermine^{4+}; △, spermidine^{3+}; ●, putrescine^{2+}; ⊗, H^+; ▽, Na^+. Note: For La^{3+} the vesicle suspension solution did not contain EDTA.

polyamine concentrations. Vesicle suspension solutions except for the La^{3+} case were all 0.1 M NaCl/2 mM histidine/2 mM Tes/0.01 mM EDTA (pH 7.0).

For La^{3+}, the slope of the vesicle mobility with respect to the logarithm of its concentration was straight and slightly smaller (1.2 $\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$ for a 10-fold change in La^{3+} concentration) than that in the case of Ca^{2+} (1.4 $\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$ per 10-fold change in Ca^{2+} concentration). In both cases, there were reversal points of the vesicle mobilities: 150 μM for La^{3+} and 90 mM for Ca^{2+}. At concentrations above these points, the vesicle mobility changed in sign. For Na^+ and Li^+, the relationships between the vesicle mobility and the logarithm of their concentrations (up to 1 M) were also linear and the slope of the relationship was larger (about 3.4 $\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$ per 10-fold change in ion concentration) than the others. The slope for the H^+ case lay between those of Ca^{2+} and Na^+.

For spermine, even at very low concentration, the ζ-potential of the vesicle was greatly reduced from that in its absence (Fig. 2). This suggests that the qaurternary valent positively charged spermine binds strongly to the phosphatidylserine membrane surface and greatly reduces its net negative charge. The spermine concentration causing the same reduction in the ζ-potential as that caused by the presence of 1 mM Ca^{2+} in the 0.1 M NaCl buffer solution was about 20 μM.

The relationship of the change in vesicle mobility with respect to the logarithm of the spermine concentration was almost linear up to 0.5 mM of the spermine concentration. The slope was about 1.0 $\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$ per 10-fold change in spermine concentration, which was the lowest slope among the cations tested. At a spermine concentration above 1 mM, the rate of reduction in the vesicle mobility decreased and the relationship between the change in vesicle mobility and the logarithm of spermine concentration became non-linear. At about 6 mM of spermine, the vesicle showed zero mobility and at a concentration above 6 mM the vesicle mobility became positive in sign. This shows that the positive charges due to adsorption of polyamines overcome the original negative surface charges of the phosphatidylserine membrane. Results similar to those mentioned above with spermine have been obtained by Chung

et al. [12]. The trend of vesicle mobility change with respect to spermidine and putrescine concentrations was similar to that for spermine. However, the concentrations of spermidine and putrescine needed to reduce the surface charges of the phosphatidylserine vesicle to the value in the case of 1 mM Ca^{2+} in 0.1 M NaCl were much greater than in the case of spermine: 0.3 mM for spermidine and 10 mM for putrescine, respectively. Also, the slopes of the vesicle mobility vs. polyamine concentration are slightly steeper as the valency becomes smaller (about 1.0 $\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$ for spermine, 1.2 $\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$ for spermidine and 1.4 $\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$ for putrescine).

Vesicle aggregation

Cation- or polyamine-dependent phosphatidylserine vesicle aggregation was followed by measuring the turbidity of lipid vesicle suspensions. Turbidities of small unilamellar phosphatidylserine vesicles with respect to various cation (La^{3+}, Ca^{2+}, Na^+, Li^+, H^+) or polyamine (spermine, spermidine and putrescine) concentrations are shown in Fig. 3. The sharp increase in turbidity of phosphatidylserine vesicles suspended in 0.1 M NaCl occurred at about 8 μM La^{2+}, 90 μM spermine, 1 mM Ca^{2+}, 3 mM spermidine, 4 mM H^+, 0.1 M putrescine, 0.7 M Na^+ and 0.8 M Li^+. Hong et al. [7] have obtained a similar result with spermine. The concentrations corresponding to these points were somewhat similar to those at which the vesicle mobility was reduced to about -1.5 to -2.0 $\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$, although there were slight deviations in these concentration values for the cases of spermidine, putrescine and Na^+.

Monolayer-vesicle fusion

In order to examine the vesicle fusion inducing ability of each cation, we have utilized a fusion assay method which we have developed in our laboratory [11] measuring the surface tension of a lipid monolayer interacting with lipid vesicles in the subphase solution. According to previous work, when the fusion of phosphatidylserine vesicles to a phosphatidylserine monolayer occurs at a certain threshold concentration of Ca^{2+}, a large and rapid decrease in surface tension of the monolayer, to about zero, was observed. The threshold concentrations of various divalent cations inducing such a decrease in the surface tension of the monolayer are the same as those of the corresponding divalent cations needed to induce lipid

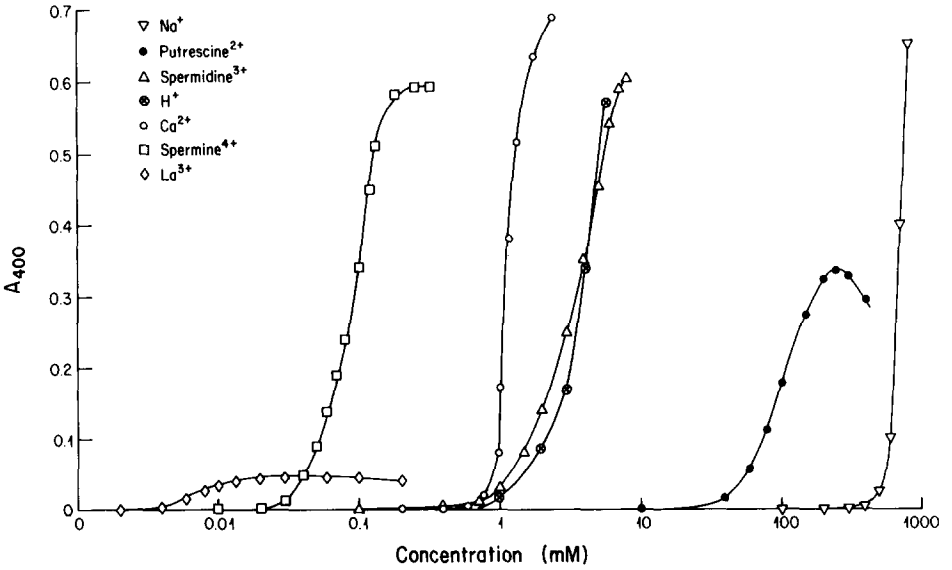

Fig. 3. Turbidity of small unilamellar phosphatidylserine vesicles (0.2 μmol lipid/ml of the NaCl buffer for all cases except for the La^{3+} case (0.02 μmole lipid/ml of the NaCl buffer)) suspended in 0.1 M NaCl buffer solution as a function of various metal cation and polyamine concentrations. ◇, La^{3+}; ○, Ca^{2+}; □, spermine^{4+}; △, spermidine^{3+}; ●, putrescine^{2+}; ⊗, H^+; ▽, Na^+.

vesicle membrane fusion measured by the method of the fluorescence assay [3].

As a control, the following experiment was carried out: the surface tension measurements of a phosphatidylserine monolayer in the presence of phosphatidylserine vesicles as a function of Ca^{2+} concentration in the subphase. The surface tension did not change appreciably until the Ca^{2+} concentration reached approx. 0.8 mM. At about 0.9 mM Ca^{2+}, the surface tension of the monolayer started to decrease drastically with time to the point of nearly zero surface tension (see Fig. 4). This did not occur in the presence of either Ca^{2+} or lipid vesicles alone in the subphase solution. We call this Ca^{2+} concentration, needed to induce such a decrease in surface tension of the monolayer, the 'threshold concentration' of the vesicle-monolayer fusion.

The reduced surface tension of the monolayer induced by Ca^{2+} was not restored or altered by the addition of EDTA, quantities of which were equivalent to divalent cation concentrations in the subphase solution. When EDTA was added while the surface tension of the monolayer was decreasing with time, no further change in surface tension was observed (see Fig. 4).

Spermine alone up to 500 μM in the subphase solution or spermine and lipid vesicles in the subphase solution did not exhibit any decrease in surface tension of the monolayers, but instead the surface tension increased slightly (Fig. 1). The presence of 10 μM spermine in the subphase solution increased the initial rate of decrease in surface tension at the threshold concentration of Ca^{2+} but did not change appreciably the threshold concentration of Ca^{2+} (about 0.9 mM). At 50 μM

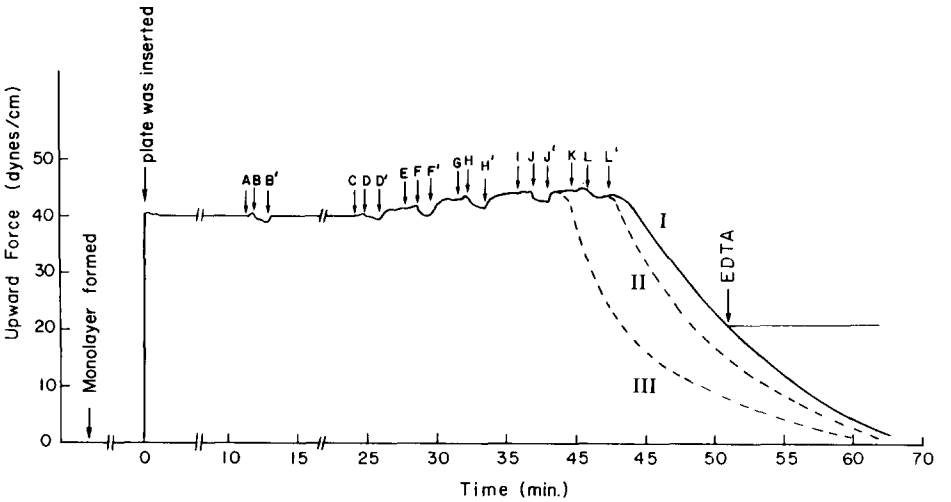

Fig. 4. A typical example of the time-course of the decrease in surface tension for the cases of a phosphatidylserine monolayer (70 Å^2 per molecule) in the presence of phosphatidylserine vesicles (0.1 μmol lipid/ml) and various concentrations of injection substances (polyamines or metal cations) in 100 mM NaCl buffer solution of the subphase. Here, instead of the film surface tension, the upward force exerted on the Wilhelmy plate by the monolayer is plotted against time. After a monolayer was completely formed at the air/water interface, the Teflon Wilhelmy plate was lowered from the air into the subphase to a constant depth. After the system stabilized, a certain amount of the concentrated vesicle suspension was injected into the subphase solution (about 0.1 μmol lipid/ml) (A), which was then stirred well for 1 min (started at B and stopped at B'), and the system was left for at least 10 min ('incubation time') to observe stability of the surface tension. Subsequently, successive aliquots of the injection solution were added to the subphase solution. For example, in the case of Ca^{2+}, $CaCl_2$ was added to raise the Ca^{2+} concentration of the subphase to 0.4 mM (C), and this was followed by stirring for 1 min (started at D and stopped at D'), and incubation for 2 min. Similar procedures were followed for each injection: 0.6 mM Ca^{2+} (E) and stirring (started at F and stopped at F'); 0.8 mM Ca^{2+} (G) and stirring (H to H'); 8.5 mM Ca^{2+} (I) and stirring (J to J'); 0.9 mM Ca^{2+} (K) and stirring (L to L'). In most cases, near the 'threshold concentration' the increment of calcium ion concentration was reduced to 0.05 mM for each injection. When an equivalent amount of EDTA to calcium ion concentration in the subphase was added to the subphase, the surface tension change was stopped at that level (shown with a thin solid line). This indicates that the fusion process was terminated and the reduction of the surface tension was not due to the adhesion of lipid vesicles. (I) no spermine in the subphase solution; (II) 10 μM spermine in the subphase solution; (III) 50 μM spermine in the subphase solution.

spermine, the threshold concentration of Ca^{2+} was reduced to a lower concentration of 0.85 mM, and the initial rate of decrease in surface tension was enhanced more than that in the case of 10 μM spermine. However, in both cases, the time taken for the monolayer surface tension to reach almost the zero value due to fusion of vesicles to the monolayer was the same as the control case (no spermine).

Similar experiments were done with spermidine as well as putrescine. At any concentration, each of these by itself did not produce any decrease in surface tension of the phosphatidylserine monolayer in the presence of the lipid vesicles. This seems to indicate that the polyamines used, by themselves, do not induce phosphatidylserine vesicle membrane fusion. These results compare well with the finding of the effects of polyamine on phosphatidylserine membrane fusion observed by use of the internal content mixing fluorescence fusion assay [13].

La^{3+} showed the similar decrease in surface tension of the phosphatidylserine monolayer in the range of 10–20 μM La^{3+} as for the Ca^{2+} case in the presence of the same lipid vesicle in the subphase. In this case, the threshold concentration depended more upon the area per lipid molecule than those observed in the case of Ca^{2+}: when the area per lipid molecule of the monolayer was 70 $Å^2$ the threshold concentration of La^{3+} needed to induce the monolayer-vesicle fusion was about 14 μM, and for 85 $Å^2$ per molecule, the threshold concentration was 20 μM.

Earlier workers showed that La^{3+} at low concentration (as low as 0.1 mM) destabilized small unilamellar phosphatidylserine vesicles to form large vesicular structures, which was implicated to be the result of vesicle fusion [14].

Unlike other monovalent cations, H^+ showed similar fusion phenomena as divalent and trivalent metal cations. The threshold concentration of H^+ required to induce such a vesicle-monolayer fusion was not clear-cut with respect to its concentration. The large change in the surface tension of the monolayer started in the range of pH 3.3–3.0, and rapid changes in surface tension were distinctly observed at or below pH 3.0. Restoration of the pH to 7.0 with NaOH stopped the change in surface tension, which indicates also that this decrease in surface tension associated with the pH change is due to the fusion of vesicles to the monolayer.

TABLE I

THRESHOLD CONCENTRATIONS

Threshold concentrations of various metal cations and polyamines at which the surface tension of phosphatidylserine monolayers formed on 0.1 M NaCl (pH 7.0) subphase solution decreased sharply and greatly with time.

	Area/molecule ($Å^2$)	
	70	85
La^{3+}	14±2 μM	20±2 μM
Ca^{2+}	0.9±0.1 mM	1.0±0.1 mM
$Spermine^{4+}$	none up to 500 μM; slight increase in surface tension	–
$Spermidine^{3+}$	none up to 100 mM; no change in surface tension	–
$Putrescine^{2+}$	none up to 1 M; no change in surface tension	–
Na^+	none up to 1 M; no change in surface tension	–
H^+	pH 3.1±0.2	–

Discussion

From the above experimental results, it is clear that there is a good correlation between the degree of membrane fusion and that of the incresed surface tension of the membrane. Those ions (La^{3+}, Ca^{2+} and H^+) which were found to induce membrane fusion of the phosphatidylserine membrane by our fusion assay also induced an increase in the surface tension of the membrane to a considerable degree, while those (polyamines, monovalent metal cations) which failed to induce membrane fusion also failed to increase the surface tension of the monolayer to any significant extent. A similar result was also obtained in the study of the divalent cation-induced fusion of acidic phospholipid membranes [3,5].

La^{3+}, Ca^{2+} and H^+ produced a large increase in the interfacial tension of the phosphatidylserine monolayer, but the polyamine and the other monovalent metal cations used showed little or no effect (Fig. 1). However, polyamines seemed to have a strong charge-neutralizing effect or charge-screening effect on a negatively charged membrane is seen from the experimental results of vesicle electrophoretic mobility shown in Fig. 2 (La^{3+} > spermine^{4+} > spermidine^{3+} > Ca^{2+} $\gtrsim$ H^+ > putrescine^{2+} > Na^+). The pH-dependent vesicle fusion studies were reported for membranes composed of different lipids other than phosphatidylserine using different fusion assay [15,16].

Although polyamine molecules have multivalent positively charged groups at neutral pH, since the shape of the molecule may be linear in a solution and the charged sites are separated by some distance, the molecules may not be able to interact with phospholipid charged polar groups in a manner of chelation binding as divalent or trivalent metal cations do [6]. Therefore, the affinity of the spermine molecule to approach and adsorb to the negatively charged membrane surface may be stronger than that of divalent metal cations (judging from the results shown in Fig. 2), but the spermine molecule may not be able to bind to the two close-neighboring charged polar groups of lipid molecules as strongly as do the divalent or trivalent metal cations, which are considered to have two or three closely situated point charges. The spermine molecule may not penetrate into the lipid polar group inter-shell upon its binding to the membrane surface. A similar explanation can be applied for the other polyamine cases.

It is to be noted is that the slope of the relation between the vesicle mobility and the logarithm of these cation concentrations is somewhat related to the inverse order of the valency of ions: the electrophoretic mobility changes per 10-fold ionic concentrations are 1.0 $\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$ for spermine^{4+}, 1.2 $\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$ for La^{3+} and spermidine^{3+}, 1.4 $\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$ for Ca^{2+} and putrescine^{2+} and 3.4 $\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$ for Na^+ and Li^+.

The trivalent cation, La^{3+}, was the most effective in causing the aggregation of the phosphatidylserine vesicles and the reduction of the vesicle electrophoretic mobility. The order of effectiveness causing the same extent of vesicle agregation among substances used was La^{3+} < spermine^{4+} < Ca^{2+} < spermidine^{3+} $\lesssim$ H^+ < putrescine^{2+} < Na^+ $\approx$ Li^+ in concentration. A fair correlation between the concentrations of various cations required to reduce vesicle electrophoretic mobility to a certain value (-1.5 to -2.0 $\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$) and the threshold concentrations of these cations needed to induce vesicle aggregation indicates that the charge density and aggregation of vesicle membranes are fairly well correlated.

Polyamines appear to be adsorbed strongly on the phosphatidylserine membrane surface, probably by electrostatic interaction, and reduce the ζ-potential or the surface charge density of the membrne. They induce the aggregation of phosphatidylserine vesicles but do not induce fusion of the same lipid vesicles. The latter compares well with the results found by others [13]. The large amounts of phosphatidylserine vesicle aggregation (caused by polyamines, especially spermine or spermidine) should help increase the overall rate of membrane fusion induced by other agents like Ca^{2+}, which is demonstrated by the experiments shown in Fig. 4. Since the vesicle fusion rate is expressed as the product of the number of aggregated vesicles and the reaction rate of an individual membrane fusion, the above interpretation is reasonable. The fact that the higher concentration of spermine (such as 50 μM) reduced slightly the 'threshold concentration' of Ca^{2+} for the fusion of lipid vesicles to a monolayer membrane suggests that spermine may also play a role in individual membrane fusion reactions. However, the role of the polyamine in membrane fusion seems limited, because up to a concentration of 500 μM spermine alone did not show any evidence of inducing membrane fusion (Table I). The surface tension of the monolayer interacting with the vesicles increased slightly (a few dyn/cm) with the increase in the spermine concentration rather than decreasing (see also Fig. 1). A greater degree of vesicle aggregation occurred at 100 μM spermine, at which point the ζ-potential of the phosphatidylserine membrane was about -25 mV (Fig. 3). The other polyamines tested gave results similar to those with spermine with respect to vesicle electrophoretic mobility and aggregation experiments,

although the concentrations producing the same effect as spermine were greater for those polyamines having a lower valency number.

According to our recent work [6], the increase in interfacial tension in the acidic lipid membrane caused by divalent cations seems to relate to the conformational change in lipid head group region due to divalent cation binding to lipids, as a result of which the membrane surface becomes more hydrophobic, and this change in hydrophobicity of the membrane surface seems to be essentially related to the induction of membrane fusion for the two opposed membranes [3,5]. It may be deduced from this that a polyamine molecule binds strongly with negatively charged membrane molecules at the membrane surface, but its bulky molecular shape would not allow it to penetrate into the inner charge sites of the polar groups of the lipids as much as divalent and trivalent metal cations do with acidic lipid membranes. This may be a possible explanation for spermine's ability to induce massive aggregation of the acidic phospholipid vesicles even at a low (40 μM) concentration, but not to induce the fusion of the membrane vesicles, even at higher (mM) concentration.

A similar argument can also be made for the other polyamines and monovalent metal cations. On the other hand, although H^+ is a monovalent cation, it can bind more easily to the negatively charged groups of the phospholipid not only in the outer shell (carboxyl group) but also in the inner shell (phosphate group), and these protonated forms may be more hydrophobic in nature than those of monovalent metal cations in the same situation.

Since we have studied the effect of polyamines on surface properties of only one type of lipid membrane, it may be too early to speculate any general statement regarding the effect of polyamines on membrane properties. However, it is likely that the role of polyamines in cells is to bind strongly with the negatively charged sites on the membrane or negatively charged molecules in the cells, which may strongly promote the aggregation of cell membranes or intracellular vesicle membranes as well as inhibit activity of certain macromolecules [17,18]. It has also been suggested that polyamines may enhance enzyme activity [19,20] – namely, stabilization of cell membranes [21,22] and regulation of some enzyme activation associated with membranes [23–26]. However, the polyamines seem to have little effect on membrane fusion.

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