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Sodium-induced aggregation of phosphatidic acid and mixed phospholipid vesicles

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Sodium-induced aggregations of sonicated vesicles prepared from synthetic phosphatidic acid and from its 1 : 1 mixtures with synthetic phosphatidylethanolamine and phosphatidylcholine were studied by turbidimetric measurements. The aggregation reactions were almost completely reversible on change in the Na^+ concentration, pH or temperature. The threshold concentrations of Na^+ for aggregations of pure dipalmitoylphosphatidic acid vesicles and mixed dipalmitoylphosphatidylethanolamine- and dimyristoylphosphatidylcholine-dipalmitoylphosphatidic acid vesicles were found to be 200, 310 and 550 mM, respectively, at 25°C and pH 7.2. The hydrocarbon chain lengths of phosphatidic acid and phosphatidylethanolamine had little effect on the threshold concentrations. The threshold concentrations for phospholipid vesicles composed of phosphatidic acid alone or its 1 : 1 mixture with phosphatidylethanolamine were changed by varying either the pH or temperature, while that for phosphatidylcholine-phosphatidic acid vesicles was almost independent of the pH and temperature, implying that aggregation of the latter vesicles is induced by a somewhat different mechanism.

Introduction

Aggregation of phospholipid vesicles is an interesting phenomenon in relation to not only the initial step of membrane fusion but also intermembrane adhesion and communication. There have been extensive studies on this phenomenon, which may be classified into three categories: studies on cation-induced aggregation of phospholipid vesicles [1–7] and aqueous phospholipid dispersions [8,9], studies on lectin-induced aggregation (agglutination) of phospholipid vesicles containing glycolipid [10–13] and studies on polysaccharide-induced aggregation of phospholipid vesicles [14–17].

The first information on cation-induced aggregation was obtained by Abramson et al. [8,9], who observed that aqueous dispersions of phosphatidic acid and phosphatidylserine aggregate in the presence of monovalent or divalent cations. Subsequently, Lansman and Haynes [3] reported that vesicles composed of phosphatidic acid or phosphatidylserine aggregate in the presence of divalent cations, but not in the presence of monovalent cations at physiological concentrations. Recently, Day et al. [6] found that high concentrations of Na^+ induce reversible aggregation of phosphatidylserine vesicles. Therefore, phosphatidic acid vesicles may also aggregate reversibly in the presence of high concentrations of Na^+. In this paper

Abbreviations: DMPA, β,γ-dimyristoyl-L-α-phosphatidic acid; DPPA, β,γ-dipalmitoyl-L-α-phosphatidic acid; DSPA, β,γ-distearoyl-L-α-phosphatidic acid; DLPE, β,γ-dilauroyl-L-α-phosphatidylethanolamine; DMPE, β,γ-dimyristoyl-L-α-phosphatidylethanolamine; DPPE, β,γ-dipalmitoyl-L-α-phosphatidylethanolamine; DMPC, β,γ-dimyristoyl-L-α-phosphatidylcholine, Mes, 4-morpholineethanesulfonic acid.

we demonstrate reversible aggregation of sonicated phosphatidic acid vesicles by Na^+. We also describe the effects of the phospholipid head group and hydrocarbon chain length on vesicle aggregation.

Materials and Methods

The phospholipids DMPA, DPPA, DSPA, DLPE, DMPE, DPPE and DMPC were purchased from Sigma Chemicals Co., or Calbiochem-Behring Corp. Mes was obtained from Nakarai Chemicals, Ltd. Other chemicals were commercial products of reagent grade.

Phospholipid vesicles were prepared as follows. Weighed amounts of phospholipid were mixed in chloroform/methanol (2 : 1, v/v). The solvent was removed under reduced pressure and after continuous evacuation (10^{-4} Torr) for 15 h, the dried lipid film was dispersed in an appropriate buffer solution by vortex-mixing at 70°C. The suspension was sonicated at 60–65°C several times for 2-min periods with 1-min intervals in a Branson sonifier Model W-185, equipped with a microtip. The resulting optically clear suspension was filtered through a 0.45-μm membrane filter.

The turbidity of the vesicle suspension was measured at 500 nm in a Hitachi 624 digital double-beam spectrophotometer, equipped with a constant temperature cell holder. The trace was started 10 s after mixing the sample with salt, and the turbidity change in a 1-min trace was denoted as ΔA_{500}.

The threshold concentration for vesicle aggregation was obtained by plotting the turbidity change at 500 nm (ΔA_{500}) as a function of the cation or salt concentration, and then extending the line from the linearly increasing portion of the plot to the abscissa. This cation concentration on the abscissa was defined as the threshold concentration of the cation for inducing vesicle aggregation.

Results

Reversible aggregation of phospholipid vesicles

When DPPA vesicles were mixed with various concentrations of NaCl, turbidity increase was observed at above a certain concentration of the salt. Fig. 1 shows the dependence of the turbidity change on the NaCl concentration. At 25°C in 10 mM Tris-HCl (pH 7.2), no vesicle aggregation occurred at salt concentrations of less than 190 mM, but steep increase in turbidity was observed at higher concentrations. The threshold concentration of Na^+ for the aggregation, determined as described in the Materials and Methods, was 195 mM. Aggregation of DPPA vesicles was also observed in the presence of KCl, but the threshold concentration of K^+ was appreciably higher, being 450 mM at 20°C in 10 mM Tris-HCl (pH 7.2), whereas that of Na^+ was 170 mM under the same conditions.

Fig. 1 also shows the turbidity changes of DPPA-DPPE (1 : 1) and DPPA-DMPC (1 : 1) vesicles with NaCl concentration. At 25°C and pH 7.2, the threshold Na^+ concentrations for their aggregation were 310 and 550 mM, respectively. The effects of the phospholipid head group and hydrocarbon chain length on vesicle aggregation are summarized in Table I. These results show that the phospholipid head group affected the threshold concentration for vesicle aggregation, but that the hydrocarbon chain length had little effect.

The threshold concentrations in Fig. 1 and Table I were determined from plots of ΔA_{500}, the turbidity change in a 1-min trace, against the concentration of NaCl. Almost identical values for the threshold concentrations were obtained, when

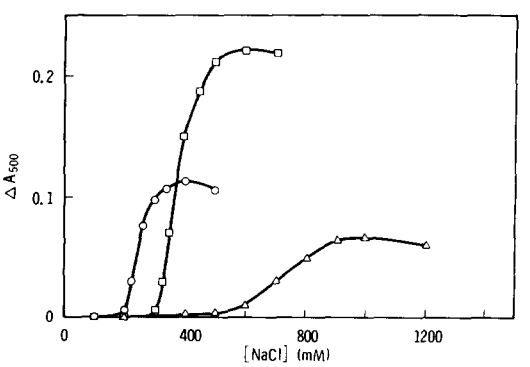

Fig. 1. Turbidity change of phospholipid vesicle suspensions as a function of NaCl concentration. Increase in turbidity at 25°C was traced 10 s after mixing the sample with NaCl in 10 mM Tris-HCl (pH 7.2). Turbidity changes in 1-min traces (ΔA_{500}) of DPPA (○), DPPA-DPPE (1 : 1) (□) and DPPA-DMPC (1 : 1) (△) vesicles are plotted against the final salt concentration. The phospholipid concentrations of these vesicles were 260, 310 and 340 μM, respectively.

TABLE I

THRESHOLD CONCENTRATIONS OF SODIUM ION FOR AGGREGATION OF SONICATED PHOSPHOLIPID VESICLES

Experimental conditions were as described in Fig. 1.

Vesicles	Vesicle concn. (μM phospholipid)	Threshold concn. of Na^+ (mM)
DMPA	280	200
DPPA	260	195
DSPA	280	215
DPPA-DLPE (1 : 1)	260	310
DPPA-DMPE (1 : 1)	260	320
DPPA-DPPE (1 : 1)	310	310
DPPA-DMPC (1 : 1)	340	550

turbidity changes in 30 s or 2 min were plotted against the salt concentration.

The threshold concentration was apparently independent of the vesicle concentration. For example, DPPA vesicles with phospholipid concentrations of 50, 190 and 330 μM gave threshold concentrations of Na^+ of 170, 165 and 165 mM, respectively, at 20°C and pH 7.2, and the threshold concentrations of Na^+ for aggregation of DPPA-DPPE (1 : 1) vesicles at phospholipid concentrations of 60, 310 and 620 μM were 310, 305 and 300 mM, respectively, at 25°C and pH 7.2.

The reversibility of the aggregation reaction was examined by varying the pH, temperature and cation concentration. As described later, the threshold concentration for aggregation of DPPA vesicles increased on either decrease in pH or increase in temperature. When the pH or temperature of the solution was changed, the aggregation was reversed: at pH 8.4 the turbidity increased in the first 5 min in the presence of 200 mM NaCl, and on change to pH 5.8, the turbidity immediately returned to its initial level (Fig. 2A); DPPA vesicles aggregated when incubated at 20°C for 25 min in the presence of 200 mM NaCl, and when the temperature of the circulating bath was changed to 40°C, the turbidity decreased to about a quarter of its maximum value (Fig. 2B). Moreover, decrease of the temperature from 40°C to 20°C again led to rapid aggregation of the vesicles. Results on the reversibility of sodium-induced aggregation of various phospholipid vesicles upon

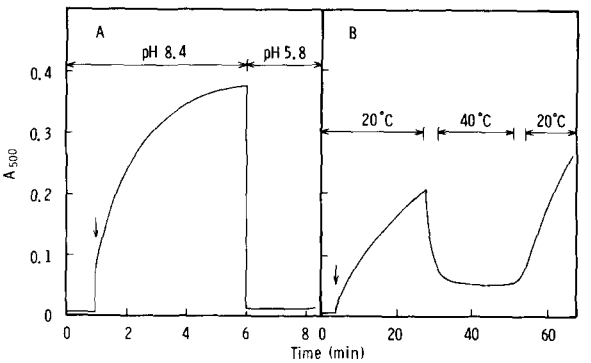

Fig. 2. Reversibility of Na^+-induced aggregation of sonicated DPPA vesicles on change in the pH (A) and temperature (B). For A, the aggregation reaction was carried out at 25°C for 5 min in 10 mM Tris-Mes (pH 8.4) in the presence of 200 mM NaCl, and then 0.2 ml of 500 mM Tris-Mes (pH 5.5) was added quickly to the suspension (total volume 2 ml). For B, vesicles were allowed to aggregate at 20°C for 25 min in 10 mM Tris-Mes (pH 7.2) in the presence of 200 mM NaCl, and then the temperature of the circulating bath was changed rapidly to 40°C; the mixture reached 40°C in about 4 min. After tracing turbidity at 40°C for 20 min, the temperature was rapidly changed back to 20°C; the mixture cooled to 20°C in about 3 min. The arrows show the starting points of traces at 10 s after mixing. The phospholipid concentrations of the vesicles used in experiments A and B were 260 and 130 μM, respectively.

dilution of the salt are shown in Table II. For aggregation, vesicles were incubated at 25°C for 1 h, which is sufficient time to allow completion of reactions in the presence of higher concentrations of NaCl than the threshold concentration. Then, the resulting aggregates were collected by centrifugation and incubated in the buffer solution for 3 h. After this incubation, more than 90% decrease in turbidity of all the kinds of vesicles examined was observed, irrespective of the vesicle concentration. Thus, the sodium-induced aggregations of phospholipid vesicles were almost completely reversed by change in the pH, temperature and cation concentration, indicating that there was little fusion of vesicles.

pH-dependency of vesicle aggregation

The pH dependencies of aggregation of DPPA, DPPA-DPPE (1 : 1) and DPPA-DMPC (1 : 1) vesicles were examined at 25°C. In Fig. 3, the threshold concentrations for aggregation of these

TABLE II

REVERSIBILITY BY CATION CONCENTRATION OF SODIUM-INDUCED AGGREGATION OF SONICATED PHOSPHOLIPID VESICLES

Phospholipid vesicles were kept at 25°C in 10 mM Tris-HCl (pH 7.2) in the presence of NaCl. After 1 h, the turbidity of the suspension (+NaCl) was measured at 500 nm. The suspensions were centrifuged at 3500×*g* for 10 min, and then the resulting precipitates were suspended in 10 mM Tris-HCl (pH 7.2) and kept at 25°C for 3 h. Then the residual turbidity (−NaCl) was measured at 500 nm.

Vesicles	NaCl concn. (mM)	Vesicle concn. (μM phospholipid)	Turbidity (A_{500})	
			+NaCl	−NaCl
DPPA	300	520	0.940	0.069
	400	260	0.540	0.050
	400	520	1.001	0.064
DMPA	300	280	0.302 [a]	0.050
	400	280	0.283 [a]	0.031
DPPA-DPPE (1 : 1)	600	310	0.913	0.056
DPPA-DMPC (1 : 1)	1500	850	0.958	0.062

[a] These low values were due to formation of large flocculates.

vesicles are plotted against the pH of the solution. The threshold concentrations were obtained from plots of ΔA_{500} against NaCl concentration at different hydrogen ion concentrations, in which the linearly increasing portions of the plots were almost parallel to each other in the aggregation reactions of DPPA and DPPA-DPPE vesicles, but not in the reaction of DPPA-DMPC vesicles. The threshold concentration of Na^+ for aggregation of DPPA vesicles decreased with increase in pH. Since the second pK of phosphatidic acid is in the range of 8–8.5 in 0.1 M NaCl and of 8.5–9 at lower ionic strength [9,18,19], ionization of the phosphate group of phosphatidic acid seems to be responsible for the pH dependency of the threshold concentration. The pH dependency of the threshold concentration for aggregation of DPPA-DPPE vesicles was quite similar to that of DPPA vesicles, except that its value was always about 100 mM greater. In contrast, with DPPA-DMPC vesicles, the threshold concentration to induce vesicle aggregation was essentially independent of the pH.

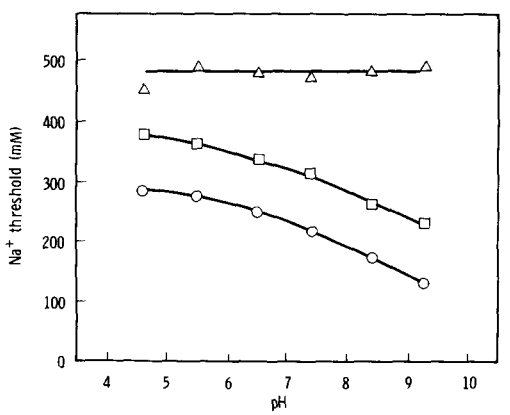

Fig. 3. Threshold concentrations of Na^+ for aggregation of sonicated phospholipid vesicles as a function of pH. One ml of 100 mM Tris-Mes buffer of a given pH was added to 0.1 ml of phospholipid vesicles in 5 mM Tris-Mes (pH 7.2). The total volume of the sample was adjusted to 2 ml. The threshold concentrations for aggregations of DPPA (○), DPPA-DPPE (1 : 1) □) and DPPA-DMPC (1 : 1) (△) vesicles were determined from plots of turbidity change at 25°C against NaCl concentration. The phospholipid concentrations of the two former types of vesicles were 180 μM and that of the latter was 360 μM.

Temperature dependence of vesicle aggregation

Fig. 4 shows the effect of temperature on aggregation of DPPA, DPPA-DPPE (1 : 1) and DPPA-DMPC (1 : 1) vesicles. The results were obtained from plots of ΔA_{500} at different temperatures against NaCl concentration, in which the linearly increasing portions of the plots for aggregation reactions of DPPA and DPPA-DPPE vesicles, but not DPPA-DMPC vesicles, were almost parallel to each other. The threshold concentrations for aggregation of DPPA and DPPA-DPPE vesicles increased with increase in temperature, while that of DPPA-DMPC vesicles was almost constant, as with decrease in pH.

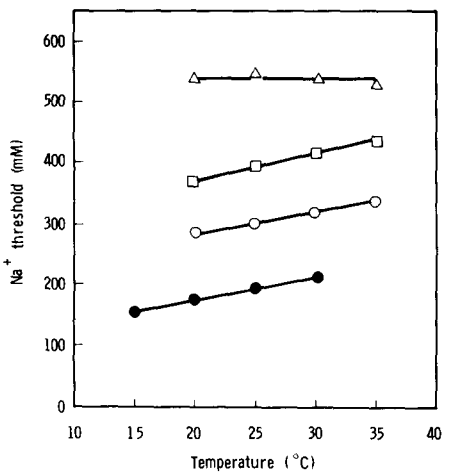

Fig. 4. Temperature dependence of the threshold concentrations of Na^+ for aggregation of sonicated phospholipid vesicles. The aggregation reactions were carried out in 20 mM Tris-Mes (pH 5.5) for DPPA (○) and DPPA-DPPE (1 : 1) (□) vesicles and in 20 mM Tris-HCl (pH 7.2) for DPPA (●) and DPPA-DMPC (1 : 1) (△) vesicles. The threshold concentrations were determined from plots of turbidity change at 500 nm against NaCl concentration. The phospholipid concentrations of the two former preparations of vesicles were 190 μM and those of the latter were 260 and 340 μM, respectively.

The threshold concentrations of Na^+ in Fig. 4 were somewhat higher than those in Fig. 3 even at similar temperatures and hydrogen ion concentrations. This is possibly due to differences in the buffer concentrations used in the two experiments, since increase in the ionic strength of the solution results in the reduction of the membrane surface potential, and since the threshold concentration for Na^+ was shifted slightly by change in buffer concentration.

Discussion

Divalent cations such as Ca^{2+} and Mg^{2+} induce both aggregation and fusion of phosphatidic acid [20,21] and phosphatidylserine [22, 23] vesicles and vesicles composed of their mixtures with neutral phospholipids [4,24–27], although Mg^{2+}-induced fusion of phosphatidylserine vesicles depends on the bilayer curvature [28]. Ca^{2+}-induced aggregation of phosphatidic acid vesicles occurs at a lower ratio of Ca^{2+} to phosphatidic acid [4]. On the contrary, sodium ion, a monovalent cation, merely induces aggregation of phosphatidylserine vesicles [6]. The present study demonstrated that sodium ion also induced aggregation of sonicated vesicles composed of phosphatidic acid and its 1 : 1 mixtures with phosphatidylethanolamine and phosphatidylcholine. It did not induce fusion of these vesicles, because the aggregation reactions were almost completely reversible on change in the cation concentration, pH or temperature.

The mechanism of cation-induced aggregation of anionic vesicles remains to be elucidated, but it has been postulated that the surface potential of the vesicles is reduced by the direct binding and/or the screening effect of Na^+, resulting in decrease in charge repulsion between vesicles and consequently leading to vesicle aggregation. Abramson et al. [29] and Barton [30] observed binding of Na^+ to phosphatidic acid and mixed phosphatidylcholine-phosphatidic acid dispersions, respectively. Moreover, Hauser and Dawson [31] and Puskin [32] showed that divalent cations bound to phosphatidic acid vesicles are displaced by Na^+. These results suggest that aggregation of phosphatidic acid vesicles is induced by the direct binding of Na^+. This suggestion is supported by the present finding that the threshold concentration of Na^+ for aggregation of DPPA vesicles was lower than that of K^+, since the binding constant for Na^+ is higher than that for K^+ [29,30]. However, there is an observation that substantially no sodium ion is bound to phosphatidic acid vesicles [33]. Eisenberg et al. [34] found that the electrostatic potentials at the hydrodynamic plane of shear (ζ potentials) for phosphatidic acid vesicles in solutions containing alkali metal cations, such as Li^+, Na^+, K^+, Rb^+, and Ca^+, differ from those of phosphatidylserine vesicles. They interpreted their data for the latter vesicles in terms of the Stern equation, which assumed ion binding to the membrane surface in addition to formation of a diffuse layer of ions depending on the potential. This means that their results for the former vesicles might not be explained simply in terms of the Stern equation. Thus, the possibility that the screening effect of Na^+ is also responsible for aggregation of phosphatidic acid vesicles cannot be ruled out. Further studies are required to elucidate the mechanism of monovalent cation-induced aggregation of phosphatidic acid vesicles.

When DPPA vesicles contained 50 mol% DPPE, DMPE, DLPE or DMPC, the threshold concentrations of Na^+ for vesicle aggregation were higher. The threshold concentration for aggregation of DMPC-DPPA vesicles was greater than that for aggregations of DPPE-, DMPE- or DLPE-DPPA vesicles. Moreover, the threshold concentrations for vesicles composed of DPPA alone and its mixture with DPPE were changed by varying either the pH or temperature, while that for mixed DMPC-DPPA vesicles was essentially independent of the pH and temperature. Although phosphatidylethanolamine and phosphatidylcholine retain similar charges at neutral pH, they differ in their polar head-group structures and extents of hydration: phosphatidylcholine produces a rapid uptake of water [35] and has a higher affinity for water [36], and phospholipids having bulk hydrated groups would interfere with the close approach of the membranes [37]. Therefore, the extent of hydration could be an important factor in aggregation of mixed vesicles induced by monovalent cations. This idea is also consistent with the fact that the effects of the head groups of phosphatidylethanolamine and phosphatidylcholine on aggregation of phosphatidic acid vesicles are quite similar to those on fusion of phosphatidic acid and phosphatidylserine vesicles [25–27], since the role of neutral phospholipids in vesicle fusion is related to their effects on the formation of dehydrated intermembrane complexes with divalent cations [25,26,38]. However, even taking the hydration effect into account, the reason for the difference in the pH and temperature dependences of aggregation of phosphatidic acid vesicles containing phosphatidylethanolamine and containing phosphatidylcholine is unclear, implying that the two vesicles aggregate by somewhat different mechanisms. More systematic studies are necessary to elucidate the effect of the phospholipid head group on monovalent cation-induced aggregation of phosphatidic acid vesicles.

Our results show that monovalent cations induce aggregation of anionic vesicles. Theoretical and experimental studies on vesicle aggregation have been reported [7,39,40], and the present system should be useful for further studies on this subject.

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