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FUSION OF PHOSPHATIDYLSERINE AND MIXED PHOSPHATIDYLSERINE-PHOSPHATIDYLCHOLINE VESICLES

DEPENDENCE ON CALCIUM CONCENTRATION AND TEMPERATURE

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Summary

Dynamic light scattering has been used to study the temperature dependence of Ca^{2+}-induced fusion of phosphatidylserine vesicles and mixed vesicles containing phosphatidylserine and different phosphatidylcholines. The final vesicle size after Ca^{2+} and EDTA incubation serves as a measure of the extent of fusion. With phosphatidylserine vesicles, the extent of fusion shows a sharp maximum at an incubation temperature which depends on the Ca^{2+} concentration between 0.8 and 2 mM. The shift in the fusion peak temperature with Ca^{2+} concentration is similar to the typical shift in the phase transition temperature with divalent cation concentration in acidic phospholipids. The results suggest a direct correlation between the fusion peak temperature and the phase transition temperature in the presence of Ca^{2+} prior to fusion. With mixed vesicles containing up to 33% of a phosphatidylcholine in at least 2 mM Ca^{2+}, the extent of fusion as a function of incubation temperature also shows a maximum. The fusion peak temperature is essentially independent of the quantity and type of phosphatidylcholine and the Ca^{2+} concentration, and identical to that with pure phosphatidylserine in excess Ca^{2+}. The results imply that Ca^{2+}-induced molecular segregation occurs first, and fusion subsequently takes place between pure phosphatidylserine domains.

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Introduction

The study of the effect of divalent cations on acidic model phospholipid vesicles is of great current interest because of its relevance to biological membrane fusion induced by Ca^{2+} [1]. Irreversible morphological changes indicative of fusion have been observed in freeze-fracture electron microscopic studies in vesicles of phosphatidylserine [2] and dilauroyl phosphatidylglycerol [3] in the presence of Ca^{2+} above a threshold concentration. In the case of phosphatidylserine, large scroll-like cylinders, named cochleates, precipitated from a solution of sonicated vesicles after incubation in Ca^{2+}. Subsequent addition of the chelating agent EDTA resulted in the formation of large unilamellar vesicles 10—30 times larger than the original sonicated vesicles.

The fusion event in phosphatidylserine has been studied by using dynamic light scattering to measure vesicle size. We have studied the kinetics of fusion by following the time dependence of the average vesicle size after the addition of Ca^{2+} at a concentration slightly below threshold [4]. Furthermore, we have used the final vesicle size after subsequent EDTA addition as a measure of the extent of fusion. At a nominal Ca^{2+} concentration of 0.9 mM, this extent of fusion as a function of incubation temperature was found to show a dramatic maximum near 11°C [5]. It was suggested that the peak temperature of 11°C may be related to the phase transition temperature of the sonicated phosphatidylserine vesicles in the presence of Ca^{2+} prior to the fusion process. However, this phase transition temperature cannot be measured directly because of the rapid formation of cochleate cylinders. The cochleate structure is an extremely anhydrous association of phospholipid with Ca^{2+} [6,7] and therefore has a very high phase transition temperature [7].

In this paper we report the results of a systematic study of the temperature dependence of the extent of Ca^{2+}-induced fusion of phosphatidylserine and mixed phosphatidylserine-phosphatidylcholine vesicles. In pure phosphatidylserine vesicles, the effect of different Ca^{2+} concentrations on the temperature of maximum fusion supports the correlation between the fusion peak and the phase transition. Our results on mixed vesicles provide evidence for Ca^{2+}-induced molecular segregation prior to fusion.

Methods

Highly purified bovine brain phosphatidylserine, egg phosphatidylcholine, synthetic dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine were supplied to us by D. Papahadjopoulos (Department of Experimental Pathology, Roswell Park Memorial Institute, Buffalo, NY). The buffer used in all experiments contained 0.1 M NaCl, 2 mM L-histidine, 2 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, and 0.1 mM EDTA, adjusted to pH 7.4. In a typical experiment, 20 μmol of phospholipid in chloroform was evaporated to dryness, 2 ml of buffer was added and the dispersion was shaken mechanically on a vortex mixer for 10 min. The multilamellar vesicles were then sonicated by a tip sonifier for 30 min or in a bath-type sonifier for 1 h at a temperature a few degrees higher than the phase transition temperature of the lipid or mixture. The sample was kept under nitrogen at all times. Finally the

sample was centrifuged at 45 000 × *g* for 15 min to eliminate any large vesicles or titanium debris. The supernatant was then diluted ten-fold with buffer. In the studies with mixtures, the lipid species were first mixed thoroughly in chloroform before evaporation.

Incubation of vesicles in Ca^{2+} and subsequently in EDTA was done at temperatures controlled to ±0.1°C as previously described [5]. Typically, 0.5 ml of the vesicles in buffer was mixed with 0.5 ml of buffer with a chosen Ca^{2+} concentration. The nominal Ca^{2+} concentration referred to in the results is half the concentration prior to mixing. At the completion of Ca^{2+} incubation, excess EDTA in buffer was added, together with an appropriate amount of NaOH to maintain proper pH. The typical Ca^{2+} and EDTA incubation times were 3 h and 0.5 h, respectively. These were chosen to ensure the completion of the fusion and chelation processes, as evidenced by separate turbidity kinetics measurements.

The final vesicle size after Ca^{2+} and EDTA incubation at a given temperature was measured by using dynamic light scattering. The apparatus and experimental procedure have been described previously [4]. The Brownian motion of the vesicles caused temporal fluctuations in the scattered intensity. The average diffusion coefficient of the vesicles was obtained from the first-degree coefficient of a second-degree fit to the measured time-correlation function of the scattered intensity [5]. To obtain the average hydrodynamic diameter of the vesicles from the diffusion coefficient, we calibrated the instrument over the size range of interest using monodisperse polystyrene spheres of known diameters obtained from Dow Diagnostics.

Results and Discussion

Effect of Ca^{2+} concentration on fusion of phosphatidylserine vesicles

We have studied the temperature dependence of the extent of fusion of sonicated phosphatidylserine vesicles in Ca^{2+} concentrations ranging from 0.8 to 2 mM. A peak in the final vesicle size after Ca^{2+} and EDTA incubations as a function of the incubation temperature was found at all the Ca^{2+} concentrations studied. At 1.0 mM Ca^{2+} the maximum extent of fusion occurs around 14°C. At 1.6 mM Ca^{2+} the peak is around 21°C. Fig. 1 summarizes the peak temperature as a function of Ca^{2+} concentration. It can be seen that the peak temperature rises steadily from 8°C to 20°C as the Ca^{2+} concentration is increased from 0.8 mM to 1.4 mM. For Ca^{2+} concentrations above 1.4 mM, however, the peak temperature remains essentially constant at around 20°C.

The effect of divalent cations on the phase transition of acidic phospholipids has been the subject of several experimental [3,7—13] and theoretical [14,15] studies. In cases where there is no substantial fusion, the transition temperature first increases steadily with increasing cation concentration, and then remains constant after there is sufficient divalent cations to reach a 1 : 2 molar association of cations to phospholipids. The maximum upward shift in transition temperature is typically 6—20°C. If the fusion peak temperature in phosphatidylserine is related to the transition temperature in the presence of Ca^{2+} prior to fusion, it should change with Ca^{2+} concentration in a similar manner. This is qualitatively what is shown in Fig. 1. Moreover, the fusion peak

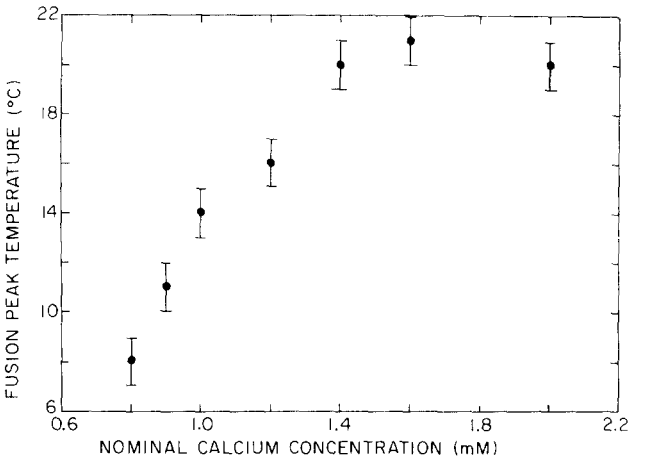

Fig. 1. Fusion peak temperature for sonicated phosphatidylserine vesicles as a function of nominal Ca^{2+} concentration. Sonicated vesicles were incubated for 3 h in Ca^{2+} and then in excess EDTA. The final vesicle size was measured by dynamic light scattering. The fusion peak temperature is the incubation temperature at which the final size shows a maximum.

tempeature stops increasing at a nominal Ca^{2+} concentration of 1.4 mM. This nominal Ca^{2+} concentration is calculated from the total amount of Ca^{2+} present in the sample and the phosphatidylserine concentration is estimated to be slightly below 0.5 mM. This implies a saturating 'free' Ca^{2+} concentration of about 1.2 mM to achieve 1 : 2 molar binding ratio of Ca^{2+} to phosphatidylserine, in reasonable agreement with binding data [10,16]. Thus the fusion peak temperature behaves in different Ca^{2+} concentrations in the same way the phase transition temperature would be expected to behave. This provides strong supporting evidence for the interpretation that an enhanced extent of fusion occurs near a temperature which is related to the phase transition temperature of phosphatidylserine vesicles in the presence of Ca^{2+} prior to fusion.

Temperature dependence of fusion of mixed vesicles

We have studied the temperature dependence of the extent of Ca^{2+}-induced fusion of vesicles made up of mixtures of phosphatidylserine and various phosphatidylcholines. Even at the lowest (16%) molar concentration of phosphatidylcholine we have examined, it was found that higher Ca^{2+} concentrations (typically at least 2 mM) were needed to induce significant fusion in the mixed vesicles than in pure phosphatidylserine vesicles. Fig. 2 shows the final vesicle size as a function of Ca^{2+} and EDTA incubation temperature for vesicles containing 33% distearoyl phosphatidylcholine and 67% phosphatidylserine in 5 mM Ca^{2+}. Two features of the results are noteworthy. There is a sharp peak around 20°C, and the maximum average final size is approximately half that obtained in pure phosphatidylserine at much lower Ca^{2+} concentrations.

Multilamellar vesicles of phosphatidylserine show a phase transition at 8°C [10], while those of distearoyl phosphatidylcholine show a phase transition at 58°C [17]. Our results on pure phosphatidylserine suggest a correlation

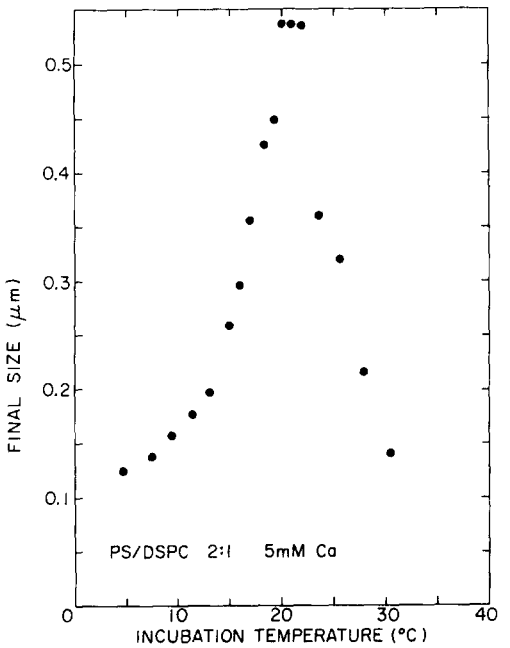

Fig. 2. Final average vesicle size as a function of Ca^{2+} and EDTA incubation temperature for mixed phosphatidylserine-phosphatidylcholine vesicles containing 33% distearoyl phosphatidylcholine in 5 mM Ca^{2+}.

between the temperature of maximum fusion and the phase transition temperature in the presence of Ca^{2+} prior to fusion. If the mixed vesicles had behaved as a miscible system in the presence of Ca^{2+} before fusion took place, one might expect a fusion peak temperature considerably higher than that obtained with pure phosphatidylserine vesicles. Instead, our results on the mixed vesicles show a fusion peak temperature around 20°C, which is essentially identical to that of pure phosphatidylserine in excess Ca^{2+}. This suggests that fusion took

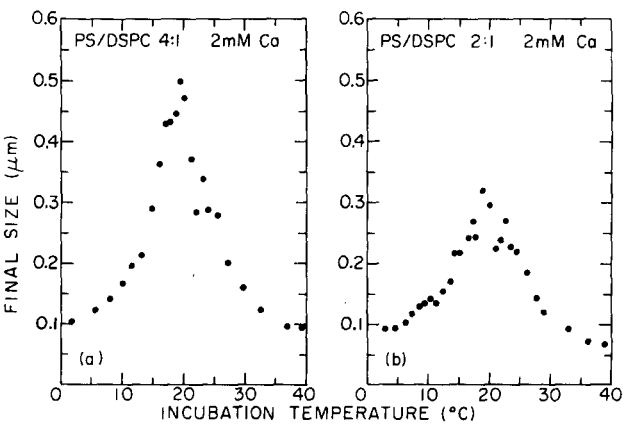

Fig. 3. Final average vesicle size as a function of Ca^{2+} and EDTA incubation temperature for mixed phosphatidylserine-phosphatidylcholine vesicles containing (a) 20% and (b) 33% distearoyl phosphatidylcholine in 2 mM Ca^{2+}.

TABLE I

PEAK TEMPERATURE AND MAXIMUM FINAL SIZE IN MIXED VESICLES

Sonicated vesicles containing phosphatidylserine (PS) and distearoyl phosphatidylcholine (DSPC) or dipalmitoyl phosphatidylcholine (DPPC) or egg phosphatidylcholine (egg PC) in the indicated molar ratio are incubated in Ca^{2+} and subsequently in excess EDTA. The peak temperature is the incubation temperature at which the average final vesicle size, as measured by dynamic light scattering, is maximum.

Lipids	Molar ratio	$\lvert Ca^{2+} \rvert$ (mM)	Peak temperature (°C)	Maximum average final size (nm)
PS/DSPC	4 : 1	2	20 ± 1	500
PS/DSPC	2 : 1	2	19 ± 1	320
PS/DSPC	2 : 1	5	20 ± 1	540
PS/DPPC	5 : 1	2	18 ± 2	530
PS/DPPC	2 : 1	5	16 ± 3	170
PS/egg PC	4 : 1	5	18 ± 3	630
PS/egg PC	2 : 1	25	19 ± 2	170

place between pure phosphatidylserine bilayers, and that Ca^{2+} induced molecular segregation into phosphatidylserine-rich and phosphatidylcholine-rich domains prior to fusion. The phenomenon of Ca^{2+}-induced molecular segregation in mixed vesicles has been examined using spin labelling [18,19], differential scanning calorimetry [10,20,21] and freeze-fracture electron microscopy [21,22]. There has also been speculation concerning the relationship between Ca^{2+}-induced molecular segregation and fusion [10,20,21,23]. Our results provide new evidence that molecular segregation occurs first, and then fusion between relatively pure phosphatidylserine domains follow. There is evidence that a certain degree of immiscibility occurs in vesicles wich charged and neutral phospholipids even in the absence of Ca^{2+} (Refs. 19 and 24; and Stewart, T., Hui, S.W., Portis, A.R., Jr. and Papahadjopoulos, D., personal communication). In referring to Ca^{2+}-induced molecular segregation, we include the case in which a binary system is not completely miscible initially and the addition of Ca^{2+} greatly enhances the extent of molecular segregation of the components.

We have made a systematic study of the temperature dependence of Ca^{2+}-induced fusion of mixed vesicles containing phosphatidylserine and varying quantities and types of phosphatidylcholines in different Ca^{2+} concentrations. Fig. 3 contains the results on vesicles containing (a) 20% and (b) 33% distearoyl phosphatidylcholine in the presence of 2 mM Ca^{2+}. Fig. 2 and Fig. 3b show that the fusion peak temperature is independent of Ca^{2+} concentration, while Fig. 3a and b imply that it is independent of the amount of distearoyl phosphatidylcholine. The maximum final size, however, decreases with increasing ratio of phosphatidylcholine to phosphatidylserine. Table I summarizes these results, as well as the results obtained with mixed vesicles containing dipalmitoyl phosphatidylcholine and egg phosphatidylcholine. For vesicles containing 33% dipalmitoyl phosphatidylcholine in 5 mM Ca^{2+}, or 33% egg phosphatidylcholine in 25 mM Ca^{2+}, the largest average final vesicle size is only 12% of that obtained with pure phosphatidylserine and only about three times the size of the original sonicated vesicles, but a weak maximum is still discern-

able. The relative insensitivity of the peak temperature to the amount and type of phosphatidylcholine and to the Ca^{2+} concentration supports our earlier conclusion about the occurrence of molecular segregation prior to fusion.

Our results also show an apparent pattern that the maximum final size under similar conditions is highest for mixed vesicles with distearoyl phosphatidylcholine, less for those with dipalmitoyl phosphatidylcholine and least for those with egg phosphatidylcholine. It has been found that a certain degree of molecular segregation occurs in mixed vesicles in the absence of Ca^{2+} [19,24]. The difference in the apparent extent of Ca^{2+}-induced fusion among the various mixed vesicles may be related to the difference in initial miscibility in the absence of Ca^{2+}. Under these assumption, our results would imply that distearoyl phosphatidylcholine is the least miscible with phosphatidylserine among the phosphatidylcholines studied, and therefore is most susceptible to Ca^{2+}-induced molecular segregation. We should mention, however, two possible complications that might affect any comparison of the behavior of various mixed vesicles. The first is the occurrence of any transbilayer asymmetry in the distribution of the charged and neutral phospholipids which may depend on the nature and concentration of the components [25]. The second is the possibility that some of the mixed vesicles do not fuse into cochleate structures. The final vesicles after EDTA incubation may then be multilamellar. This does not affect our conclusion about the temperature dependence of fusion of a particular type of mixed vesicles, but will complicate the relationship between the various maximum final sizes and the difference in the extent of fusion among mixed vesicles of different compositions.

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