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Ca^{2+}-INDUCED PHASE SEPARATION IN PHOSPHATIDYLSERINE, PHOSPHATIDYLETHANOLAMINE AND PHOSPHATIDYLCHOLINE MIXED MEMBRANES

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Summary

Ca^{2+}-induced phase separation in phosphatidylserine/phosphatidylethanolamine and phosphatidylserine/phosphatidylethanolamine/phosphatidylcholine model membranes was studied using spin-labeled phosphatidylethanolamine and phosphatidylcholine and compared with that in phosphatidylserine/phosphatidylcholine model membranes studied previously. The phosphatidylethanolamine-containing membranes behaved in qualitatively the same way as did phosphatidylserine/phosphatidylcholine model membranes. There were some quantitative differences between them. The degree of phase separation was higher in the phosphatidylethanolamine-containing membranes. For example, the degree of phase separation in phosphatidylserine/phosphatidylethanolamine membranes containing various mole fractions of phosphatidylserine was 94—100% at 23°C and 84—88% at 40°C, while the corresponding value for phosphatidylserine/phosphatidylcholine membranes was 74—85% at 23°C and 61—79% at 40°C. Ca^{2+} concentration required for the phase separation was lower for phosphatidylserine/phosphatidylethanolamine than that for phosphatidylserine/phosphatidylcholine membranes; concentration to cause a half-maximal phase separation was $1.4 \cdot 10^{-7}$ M for phosphatidylserine-phosphatidylethanolamine and $1.2 \cdot 10^{-6}$ M for phosphatidylserine/phosphatidylcholine membranes. The phase diagram of phosphatidylserine/phosphatidylethanolamine membranes in the presence of Ca^{2+} was also qualitatively the same as that of phosphatidylserine/phosphatidylcholine except for the dif-

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Abbreviations: PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PE* and PC*, spin-labeled PE and PC, respectively; GEDTA, glycoletherdiaminetetraacetic acid.

ferent phase transition temperatures of phosphatidylethanolamine (17°C) and phosphatidylcholine (—15°C). These differences were explained in terms of a greater tendency for phosphatidylethanolamine, compared to phosphatidylcholine, to form its own fluid phase separated from the Ca^{2+}-chelated solid-phase phosphatidylserine domain.

Introduction

There has been increasing interest in phase transitions and separations in bilayer membranes in recent years [1,2]. Ionotropic phase separations in isothermal conditions may have biological significance. Using spin-labeled phosphatidylcholine (PC*) in PS/PC model membranes, Ohnishi and Ito [3,4] found that Ca^{2+} causes PS to form a separate domain within the membrane. Jacobson and Papahadjopoulos [5] and van Dijk et al. [6] also showed Ca^{2+}-induced phase separation by scanning calorimetry and electron microscopy. Recently, it has been shown that acidic pH as well as low ionic strength cause a similar type of phase separation in PS/PC model membranes [7]. Ca^{2+}- and H^+-induced phase separations have recently been reviewed and their possible biological significance discussed [8].

In the present study, we used spin-labeled phosphatidylethanolamine (PE*) to study Ca^{2+}-induced phase separation in two (PS/PE) and three (PS/PE/PC) component model membrane systems and the results were compared with those for PS/PC membranes [8]. The PE-containing membranes were studied because in certain biological membranes Ca^{2+}-induced phase separation may cause the transition of PE to an inverted micelle form, perturbing membrane structure and thus causing fusion [9,10].

Materials and Methods

PC was obtained from egg yolk and PS from bovine brain. PE was synthesized from egg yolk PC using phospholipase D [11]. PC* and PE* were labeled at the 2-position with 12-doxyl stearate [12].

Millipore filters with average pore diameter of 5 μm were impregnated with phospholipid mixture in benzene, dried in vacuum, then hydrated in 50 mM Tris-HCl/100 mM KCl buffer at pH 7.1 with or without 10 mM $CaCl_2$ [13]. 10 mol% of phospholipid spin label was included in the phospholipid mixture for ESR spectroscopy. In three component model membranes, PC* and PE* were both used in separate experiments. Ca^{2+} concentration-dependence of the phase separation was studied in a Ca^{2+} buffer using GEDTA to adjust an initial Ca^{2+} concentration of 1 mM in 20 mM Tris-HCl/100 mM KCl buffer at pH 7.2. Ca^{2+} concentration was calculated using the apparent binding constant of GEDTA with Ca^{2+} reported by Ogawa [14]. The value is $3.1 \cdot 10^6\ M^{-1}$ at pH 7.2. The range in the experimental values causes a variation in the calculated concentration of about 30%.

ESR spectra were measured with the filter paper in a flat quartz cell using an X-band spectrometer (JEOLCO Model ME-2X). Spectra were measured at room temperature and at 40°C. The degree of phase separation was estimated from

ESR spectral broadening as described elsewhere [8]. The method utilizes spectral broadening with an increase of spin label concentration in membranes. A spectral parameter defined as 1/2 (*AB/CD*) (see inset in Fig. 1) was used to quantify change in the spectral broadening caused by Ca^{2+}.

The solid-to-fluid phase-transition temperature of PE was determined using differential scanning calorimetry (SEIKO Model SSC/560) by courtesy of Dr. M. Kito, Institute for Food Science, Kyoto University. 45 mM PE in 20 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) and 100 mM NaCl, adjusted to pH 7.0, was vortex-mixed exhaustively. A heating rate of 1 K/min was used. Cooling scans were not done.

Results

A graph of the spectral parameter α over PE* concentration in PE/PE* model membranes is shown in Fig. 1. This parameter describes the degree of spectral broadening caused by spin-spin interactions. It is a function of label concentration as well as temperature-related motion of phospholipids in the membranes. The relationship between α parameter and spin label concentration is also shown for PC/PC* model membranes [8]. In both PE/PE* and PC/PC* mem-

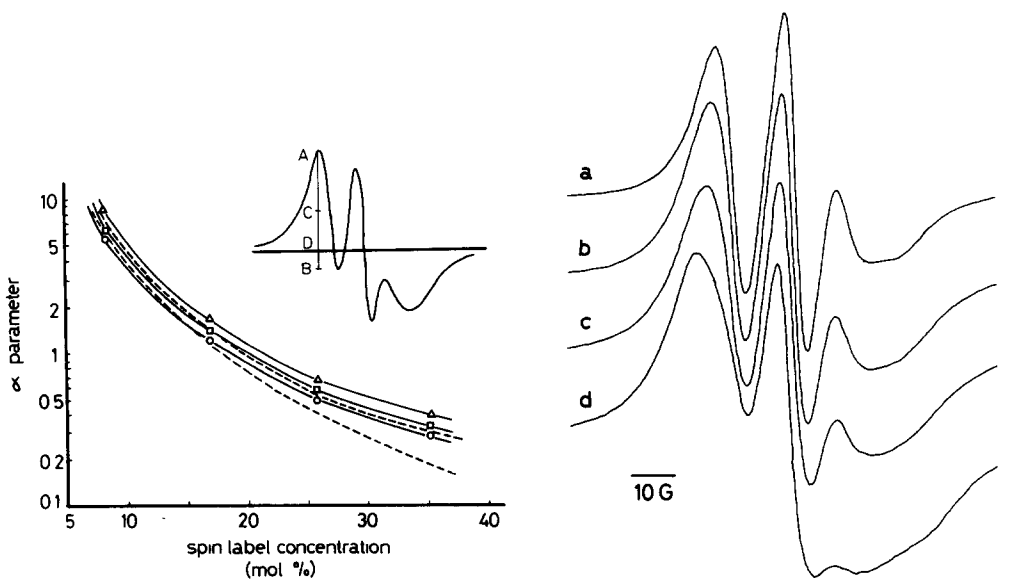

Fig. 1. The spectral parameter α as a function of spin label concentration in PE/PE* (solid lines) and PC/PC* (dashed lines) model membranes. The parameter is defined as 1/2 (*AB/CD*) (see inset) where *C* is the midpoint of *AB*; smaller values represent more broadening. □, at 23°C; ○, at 40°C; and △, in the presence of $CaCl_2$ at 23°C. The upper dotted line is PC/PC* membranes at 23°C and the lower dotted line at 40°C. The PC/PC* data are taken from Ref. 8.

Fig. 2. Effect of Ca^{2+} on ESR spectrum of PS/PE model membranes containing 10 mol% PE*. Spectrum a is a PS/PE (33 : 67) membrane in the absence of Ca^{2+} and spectrum b is in the presence of Ca^{2+} (10 mM). Spectrum c and d are, respectively, PS/PE (50 : 50) and (67 : 33) membranes, both in the presence of Ca^{2+}. Spectra in the absence of Ca^{2+} for these membranes are quite similar to spectrum a. Temperature was 23°C.

branes, higher temperature causes an increase in spectral broadening. Only in the case of PE/PE* model membranes does Ca^{2+} cause a slight decrease in spectral broadening.

The presence of Ca^{2+} causes a marked increase in spectral broadening in a two component system of PS and PE. The degree of broadening increases with increasing PS mole fractions (Fig. 2). This Ca^{2+}-induced phase separation can be quantified using the relationship

$$1 - ([PE^*]/[PE^*]_{Ca^{2+}}) = 1 - x_{fl} = x_s$$

where x_{fl} and x_s are the mole fractions of fluid and solid phospholipids, respectively. $[PE^*]$ and $[PE^*]_{Ca^{2+}}$ are the apparent spin label concentrations in the absence and presence of Ca^{2+}, respectively, and can be extrapolated from the relationship between α parameter and spin label concentration in Fig. 1 [8]. Calculations of x_{fl} using this method are shown in Table I for different PS mole fractions at room temperature and at 40° C. Assuming that only PS is chelated by Ca^{2+} to form separate solid-phase domain, the degree of phase separation can be calculated by: degree of phase separation = $(1 - x_{fl})/x_{PS}$. The calculations are summarized in Table II, together with those obtained previously for PS-PC membranes [8].

The same type of experiments and analysis were made with the three component model membranes using PC* and PE*. The x_{fl} value and degree of phase separation are given in Table II. Data obtained with PC* and PE* agreed rather well. In membranes containing smaller PS fractions, the calculation gave a degree of phase separation higher than 100%. The reason for these higher values is not clear at present.

All the data for the two and three component model membranes indicate that the degree of phase separation in the PE-containing membranes is always higher than that for the corresponding PS/PC model membranes. The degree of phase separation becomes lower at higher temperature.

A PS/PE/PC (28 : 47 : 25) model membrane, with a composition similar to the inner layer of human erythrocyte membrane, but lacking sphingomyelin, was also studied for the phase separation. The degree of phase separation was

TABLE I

MOLE FRACTION OF FLUID PHOSPHOLIPID, x_{fl}, IN PS/PE MODEL MEMBRANES IN THE PRESENCE OF Ca^{2+}

$[PE^*]$ and $[PE^*]_{Ca^{2+}}$ are the apparent PE spin label concentrations before and after addition of Ca^{2+} (10 mM), respectively. x_{fl} is given by $[PE^*]/[PE^*]_{Ca^{2+}}$.

PS : PE	T (°C)	α	$[PE^*]$ (%)	$\alpha_{Ca^{2+}}$	$[PE^*]_{Ca^{2+}}$ (%)	x_{fl}
33 : 67	23	5.4	8.9	2.3	13.1	0.68
	40	5.0	9.0	2.3	12.6	0.71
50 : 50	23	6.2	8.4	1.3	17.6	0.48
	40	4.3	9.6	1.4	16.4	0.59
67 . 33	23	6.2	8.4	0.77	22.6	0.37
	40	4.9	9.2	0.81	21.0	0.44

TABLE II

DEGREE OF Ca^{2+}-INDUCED PHASE SEPARATION, d, IN PS/PC, PS/PE AND PS/PE/PC MODEL MEMBRANES

Assuming Ca^{2+} complexes only with PS, the degree of phase separation is equal to $(1 - x_{fl})/x_{PS}$, where x_{fl} and x_{PS} are the fluid and PS mole fractions, respectively. In the case of PS/PE/PC, the mole fractions of PC and PE were equal.

x_{PS}	T (°C)	PS/PC		PS/PE		PS/PE/PC	
		x_{fl}	d (%)	x_{fl}	d (%)	x_{fl}	d (%)
0.33	23	0.75	76	0.68	96	0.51 *	140
						0.56 **	
	40	0.80	61	0.71	88	0.61	118
						0.61	
0.50	23	0.63	74	0.50	100	0.48	101
						0.51	
	40	0.66	68	0.56	88	0.61	80
						0.59	
0.68	23	0.43	85	0.37	94	0.35	81
						0.45	
	40	0.47	79	0.44	84	0.40	81
						0.50	

* Spin-labeled PC used.
** Spin-labeled PE used.

found to be 84% (PC*) and 76% (PE*) at 23°C and 83% (PC*) and 77% (PE*) at 40°C.

Ca^{2+} concentration dependence of phase separation was determined for PS/PE model membranes. The result is shown in Fig. 3, compared to that for PS/PC membrane [8]. Ca^{2+} of the order of 10^{-7} M is enough to cause the phase separation in PS/PE membrane. A higher concentration of Ca^{2+} is required for

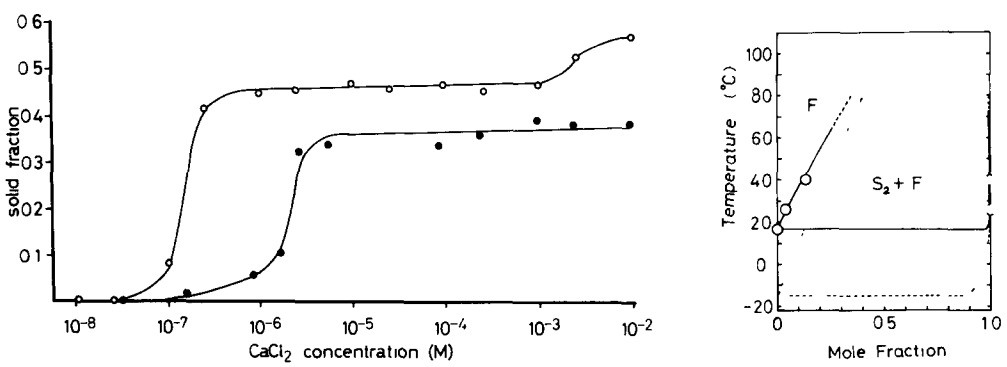

Fig. 3. Ca^{2+} concentration dependence of phase separation in PS/PE (50 : 50) and PS/PC (50 : 50) model membranes (23°C). Open circles show PS/PE membranes, and closed circles PS/PC membranes. Calculation of Ca^{2+} concentration in bathing medium is explained in the text. The data for PS/PC membranes are taken from Ref. 8.

Fig. 4. Phase diagram for PS/PE model membranes in the presence of Ca^{2+}. F, fluid phase; S_2, solid phase (Ca^{2+}-chelated PS). At temperatures lower than 17°C, two solid phases S_1 (PE) and S_2 co-exist. The dotted lines reproduce phase diagram for PS/PC membranes in the presence of Ca^{2+} [8].

the phase separation in PS/PC. Ca^{2+} concentration to cause a half-maximal phase separation is an order of magnitude different: $1.4 \cdot 10^{-7}$ M for PS/PE and $1.2 \cdot 10^{-6}$ M for PS/PC. The higher degree of phase separation in PS/PE than in PS/PC membranes is again demonstrated in Fig. 3 (see also Table II).

The phase diagram for PS/PE model membranes in the presence of Ca^{2+} is drawn based on the data in Table II (see Fig. 4). It is assumed that only PS forms a solid phase chelated by Ca^{2+}. The fluid phase is, therefore, composed of PE at a mole fraction of x_{PE}/x_{fl} and PS at $1 - (x_{PE}/x_{fl})$ (see Ref. 8 for a more detailed discussion). The data point at $x_{PS} = 0$ is based on the calorimetric determination of phase transition temperature of PE (17°C). The phase diagram for PS/PC membranes is also reproduced in Fig. 4. It is seen that both membrane systems behave in essentially the same way in the presence of Ca^{2+}. The only difference arises from difference in the phase-transition temperature: −15°C for PC [15] and 17°C for PE.

Discussion

PS/PE model membranes respond to Ca^{2+} in basically the same way as do PS/PC membranes. This is to be expected since both PE and PC are neutral at the pH used in this study.

There are some differences between the two systems, however; the higher degree of phase separation and the lower Ca^{2+} concentration required for phase separation in PS/PE compared to PS/PC membranes. These differences indicate that PE has more tendency to form its own fluid phase separated from the Ca^{2+}-chelated PS domain. This may be related to the higher phase transition temperature of PE compared to that of PC. A simple explanation for the higher degree of phase separation can be made with reference to the phase diagram (Fig. 4). The fluidus-solidus curve has a different starting point on the left ordinate due to the different transition temperatures, and must go to the same ending point on the right ordinate which is the phase-transition temperature of Ca^{2+}-chelated PS aggregates (over 100°C). Therefore, the fluidus-solidus curve for PS/PE must run on the left side of that for PS/PC, if the curves do not cross. This results in higher degree of phase separation for PS/PE membranes at all temperatures.

Phase separation in PS-PE model membranes occurs at a Ca^{2+} concentration an order of magnitude lower than that in PS/PC membranes. This apparent larger binding constant of Ca^{2+} to PS/PE membranes can be explained if we assume the Gibbs free energy change ΔG accompanied by the phase separation is larger (more negative) for PS/PE than for PS/PC membranes, since the binding constant is proportional to $\exp(-\Delta G/RT)$. A more negative ΔG value is possible since PE has more tendency to form a fluid phase separated from the PS domain. Among various enthalpic and entropic factors contributing to the smaller free energy, we can point out a larger interaction energy between the ethanolamine head groups, including hydrogen bonding.

In the scanning thermogram for PE, we noted a very broad transition around 30°C, in addition to the pronounced exothermic peak at 17°C due to the solid to fluid bilayer phase transition. This thermogram for PE derived from egg yolk PC is quite similar to that reported for egg yolk PE [9]. The authors assigned

the small exothermic peak around 30°C to the transition from bilayer to hexagonal H_{II} phase. It is interesting to ask whether the hexagonal phase formation assists the Ca^{2+}-induced phase separation in PS/PE membranes. Our data (Table II) show no marked difference in the degree of phase separation at 23 and 40°C, i.e. below and above the transition temperature. This may be due to very little difference between the effect of the bilayer and hexagonal structures on the phase separation or, since our sample is multilayered membranes supported on a Millipore filter pore surface [13], the bilayer structure may have been stabilized even at 40°C. This problem is to be studied by ^{31}P-NMR. Cullis and Verkleij [16] found by ^{31}P-NMR that Ca^{2+} triggered transition of PS/PE membrane from bilayer to hexagonal H_{II} phase at 37°C. Hope and Cullis [17] observed a similar transformation in PS/PE/PC/sphingomyelin liposome membrane.

Ca^{2+}-induced phase separation occurs at Ca^{2+} concentrations as low as 10^{-7}—10^{-6} M, which are close to cytoplasmic Ca^{2+} level. There is a strong possibility, therefore, that this ionotropic phase separation is involved in Ca^{2+}-dependent cellular control mechanisms either by itself or in conjunction with proteins. The present study does show that, when the inner layer of human erythrocyte membrane is exposed to Ca^{2+}, the phospholipids can be separated into Ca^{2+}-chelated PS domains and fluid bilayer phase. In actual erythrocyte membranes, there are integral proteins, as well as cytoskeletal spectrin, actin and the anchoring ankyrin, which may modify the phase behavior of phospholipids. Intracellular Ca^{2+} crucially affects cellular activities. For human erythrocytes, it drastically changes cell shape, decreases cell deformability, and increases membrane fluidity. We would strongly suggest that PS, among others, is a probable receiving site for intracellularly driven Ca^{2+}.

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