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PHYSICAL PROPERTIES OF PHOSPHATIDYLCHOLINE-PHOSPHATIDYLINOSITOL LIPOSOMES IN RELATION TO A CALCIUM EFFECT

KAZUO OHKI, TAKASHI SEKIYA, TAEKO YAMAUCHI and YOSHINORI NOZAWA

Department of Biochemistry, Gifu University School of Medicine, Tsukasamachi 40, Gifu (Japan)

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Physical properties of binary mixtures of dipalmitoylphosphatidylcholine and yeast phosphatidylinositol were studied by ESR analysis using TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) and lipid spin probes, freeze-fracture electronmicroscopy and particle microelectrophoresis, and they were compared with those of phosphatidylcholine/bovine brain phosphatidylserine mixtures. The phase diagram of the binary mixtures of dipalmitoylphosphatidylcholine and phosphatidylinositol was obtained from the thermal features of TEMPO spectral parameter in the lipid mixtures. The phase diagram provided evidence that these two phospholipids in various combinations were miscible in the crystalline state. The addition of 10 mM Ca^{2+} slightly shifted the phase diagram upward. TEMPO titration of the binary mixture of dipalmitoylphosphatidylcholine and bovine brain phosphatidylserine revealed that 10 mM Ca^{2+} caused the complete phase separation of this lipid mixture. Studies of phase separations using phosphatidylcholine spin probe manifested that 10 mM Ca^{2+} induced almost complete phase separation in egg yolk phosphatidylcholine/bovine brain phosphatidylserine mixtures but only slight phase separation in egg yolk phosphatidylcholine/yeast phosphatidylinositol mixtures. However, some phase changes around the fluidus and the solidus curves were visualized by the freeze-fracture electronmicroscopy. The molecular motion of lipid spin probe was decreased by the addition of Ca^{2+} in the liposomes containing phosphatidylinositol. The temperature dependence of electrophoretic mobility was also examined in the absence and presence of 1 mM Ca^{2+}. Liposomes of dipalmitoylphosphatidylcholine-phosphatidylinositol (90 : 10, mol/mol) exhibited a clear transition in the thermal features of electrophoretic mobilities. Raising the phosphatidylinositol content up to 25 mol% rendered the transition broad and unclear. The addition of 1 mM Ca^{2+} decreased the electrophoretic mobility but did not change its general profile of the thermal dependence. These results suggest that the addition of calcium ions induced a small phase change in the binary mixture of phosphatidylcholine and phosphatidylinositol while Ca^{2+} causes a remarkable phase separation in phosphatidylcholine/phosphatidylserine mixture. The physical role of phosphatidylinositol is discussed related to the formation of diacylglycerol.

Introduction

It has been accepted that most of biological functions in cells are related to membranes. Interaction between proteins and lipids occupied a main part of the regulation mechanism of membrane functions, in fact, most of membrane proteins are modulated in their activities by lipids [1]. Some cells altered the lipid composition using their adaptation mechanism corresponding to the change in environmental conditions [2,3]. Hokin and Hokin [4] discovered that some cells enhanced the turnover of the phosphati-

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl.

dylinositol in response to a stimulus. This phenomenon has been observed in many types of cells, e.g., secretory cells, platelets, lymphocytes, fibloblasts, leucocytes and synaptosomes, in response to a variety of stimuli mediated by membranes. The basic mechanism of the enhanced turnover of phosphatidylinositol has been elucidated by biochemical approaches [5]. To our knowledge, however, there are few reports about the phsyical properties of phosphatidylinositol in spite of its important role in biological functions. However, phosphatidylserine, another acidic phospholipid, was extensively studied by various physical techniques, such as spin labeling [6–8], differential scanning calorimetry [9–11] and freeze-fracture electronmicroscopy [11]. These studies revealed the lateral phase separation of phosphatidylserine from phosphatidylcholine induced by calcium ions or basic proteins. Ca^{2+}-induced occurrence of lipidic particle was found in phosphatidylethanolamine-phosphatidylserine [12], phosphatidylcholine-cardiolipin liposomes [13] and other phospholipid compositions [14]. The biological significances of lipidic particles were proved for the membrane fusion [15] and the flip-flop movement of membrane lipids [16]. Recently, Kishimoto et al. [17] revealed that the activities of Ca^{2+}-activated protein kinases from various mammalian tissues were enhanced by diacylglycerol which was produced from phosphatidylinositol by phospholipase C. Therefore, for further knowledge regarding the functions of acidic phospholipids in membranes, the examination of the functions of phosphatidylinositol is required.

In this paper, the basic properties of phosphatidylinositol were studied by various physical techniques; ESR using TEMPO and lipid spin probes, a freeze-fracture electronmicroscopy and a particle microelectrophoresis. The properties of phosphatidylcholine/phosphatidylinoistol mixtures in relation to temperature and calcium ions were characterized by these techniques, and compared with those of phosphatidylcholine/phosphatidylserine mixtures.

Materials and Methods

Synthetic 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (DPPC) was purchased from Sigma Chemical Co. (St. Louis, MO). The purity of fatty acyl chains was determined to be over 98.0% by gas-liquid chromatography. Phosphatidylinositol (PI) was extracted from Baker's yeast (Oriental Yeast Co. Ltd., Tokyo) as described by Trevelyan [18], and stored as ammonium salt in benzene solution at −20°C. Phosphatidylserine (PS) was extracted from bovine brain as described by Sanders [19], and stored as benzene solution at −20°C. Phosphatidylcholine (PC) was extracted from egg yolk as described by Singleton et al. [20], and stored as benzene solution at −20°C. Each phospholipid gave a single spot on the silica gel H thin-layer plate developed by $CHCl_3$/CH_3OH/CH_3COOH/H_2O (50 : 30 : 8 : 4, v/v). 5-Nitroxide stearic acid spin probe (5SAL) was purchased from Syva Associates (Palo Alto, CA). 2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO) spin probe and phosphatidylcholine spin probe with a 12-nitroxide stearic acid at 2 position (PC*) were kindly provided by Dr. S. Ohnishi of Kyoto University.

Multilamellar liposomes were prepared as follows. Desired amount of phospholipid, determined by phosphorus assay according to Bartlett [21], was taken from the benzene solution and the solvent was evaporated first in a nitrogen stream and then by evacuation overnight. Buffer solution and several glass beads were added and vortexed for 3 min with a Vortex-Genie mixer (Scientific Industry, Bohemia, N.Y.) above the phase transition temperature (50°C). Buffer A (150 mM NaCl/50 mM Tris-HCl, pH 7.5) was used for spin label and freeze-fracture studies, and buffer B (25 mM NaCl/5 mM sodium phosphate, pH 7.5) for particle microelectrophoresis.

For studies of phase separation using phosphatidylcholine spin probe, phospholipid multilayered membranes were prepared in a Millipore filter with average pore diameter of 5 μm, SMWP01300 [22]. The filter was dipped in benzene solution of egg PC/PI/PC* (4 : 5 : 1) or egg PC/PS/PC* (4 : 5 : 1) of 40 mg/ml and then dried under vacuum for 3 h. The dried filter was soaked in 20 ml buffer A containing 10 mM $CaCl_2$ or 10 mM Na_2EDTA for 5 h.

The lipid dispersion was taken into glass capillary and the filter was set on a Teflon holder. The ESR spectra were measured at various temperatures using a commercial X-band spectrometer, JEOL FE-1X (JEOL, Tokyo) equipped with a variable temperature control. The rate of temperature decrease was no greater than 0.5°C per min. The TEMPO spectral parameter, f, and the high and low temperature

breaks in the slope of f vs. $1/T$ curves were obtained according to the procedure of Shimschick and McConnell [23]. The spectral parameter, α, of the exchange-broadened spectrum was calculated according to the definition by Devaux and McConnell [24]. The order parameter, S, was calculated as described previously [25].

For the preparation of freeze-fractured samples, a small volume of dispersions was pipetted into a specimen holder after incubation of the samples for 10 min at the desired temperature. The holder placed on an aluminium vessel was further incubated at the same temperature for 5 min. The sample was frozen immediately in liquid Freon 12 and transferred to liquid nitrogen and then fractured in a freeze-etching device, HFZ-1 (Hitachi Co., Hitachi) at −110°C. Replica, prepared by platinum-carbon followed by carbon shadowing, was floated onto hypochlorous acid (HClO) and cleaned by distilled water. The replica was then collected in 300 mesh grids and observed by an electronmicroscopy, JEM-U (JEOL, Tokyo) [26].

Electrophoretic mobilities were measured using a particle microelectrophoresis apparatus, Mark II (Rank Brothers, Cambridge, U.K.). A thin-walled cylindrical cell (inner diameter, 1850 μm) was supplied with four platinum electrodes. Two of them were used for supplying constant current (1 mA) and the other for measuring potential differences with a high input resistance voltmeter. The movement of liposomes was observed in a lower stationary level under the illumination of 3 mW He/Ne laser (Scientifica and Cook Electronics Ltd.). The temperature was controlled by the equipped temperature controller and the external coolnit Cl-19 (Taiyo Ltd., Tokyo). The rate of temperature change was not greater than 0.1°C/min. The electrophoretic mobility, u, was calculated according to the following equation from 40 measurements:

$$u = \frac{v}{E}$$

$$= \frac{1 \cdot 10^{-4}\ \text{cm}/t\ \text{s}}{\Delta V\ \text{volts}/l\ \text{cm}}$$

where v is the particle velocity obtained from the time t, to run across a distance of $1 \cdot 10^{-4}$ cm. E is the applied strength represented by the potential difference, ΔV, devided by the effective interprobe electrode distance, l. An l value of 9.94 cm was obtained for the cylindrical cell used in this experiment.

Results

In the present study, phosphatidylinositol from yeast, phosphatidylserine from bovine brain and phosphatidylcholine from egg yolk were used as the representatives of these phospholipid sepcies. The fatty acid compositions of these phospholipids were very different as shown in Table I. However, the contents of unsaturated fatty acids were rather similar; 61.7, 50.8 and 50.8% for phosphatidylinositol, phosphatidylserine and phosphatidylcholine, respectively.

In the ESR spectrum of the TEMPO spin probe, the high-magnetic-field peak is splitted into two peaks according to the location of the probe, in water or in

TABLE I

FATTY ACID COMPOSITIONS OF PURIFIED PHOSPHOLIPIDS

Phosphatidylinositol, phosphatidylserine and phosphatidylcholine were extracted from yeast, bovine brain and egg yolk by the method of Trevelyan [18], Sanders [19] and Singleton et al. [20], respectively. The methyl esters were prepared by the method of Morrison and Smith [27] and analyzed with a Model GC-6 gas chromatograph (Shimadzu, Kyoto). Peaks of individual fatty acids were quantified by comparison of retention time with those of authentic standards.

Fatty acid	Percent of total fatty acids in		
	Phosphatidyl-inositol	Phosphatidyl-serine	Phosphatidyl-choline
C16 : 0	21.3	1.3	33.5
C16 : 1	17.0	0.7	0.6
C17 : 0		0.9	
C18 : 0	15.1	40.4	14.0
C18 : 1	44.7	31.0	28.6
C18 : 2			17.9
C20 : 0	0.6		
C20 : 1		5.6	
C20 : 4		1.0	4.3
C22 : 1		1.0	
C20 : 5		6.1	
C24 : 0		3.5	
C24 : 1		3.0	
C22 : 6		2.3	

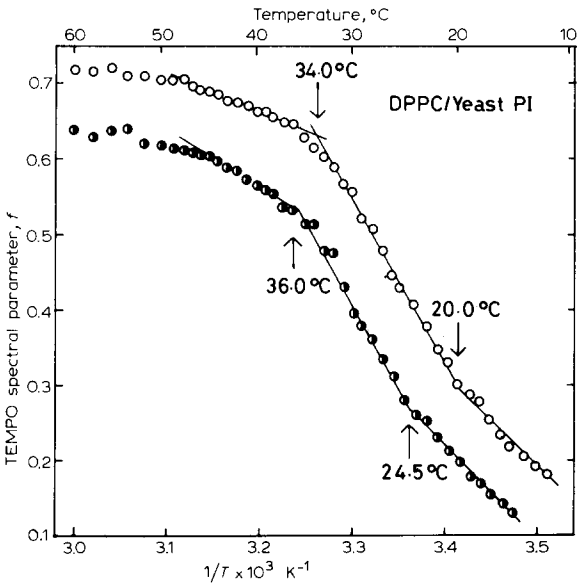

Fig. 1. TEMPO parameter, f, vs. $1/T$ for DPPC-PI (67 : 33) liposomes in the absence (control) (o———o) and presence (●———●) of 10 mM Ca^{2+}. The dispersion was prepared by vortexing with glass beads. After addition of 4 mM TEMPO (40 μl) and 1 M $CaCl_2$ in buffer A (1 μl) into the lipid dispersion (400 μl), it was concentrated by centrifugation (35 000 × g; 10 min) to approx. 200 mg/ml for ESR measurements.

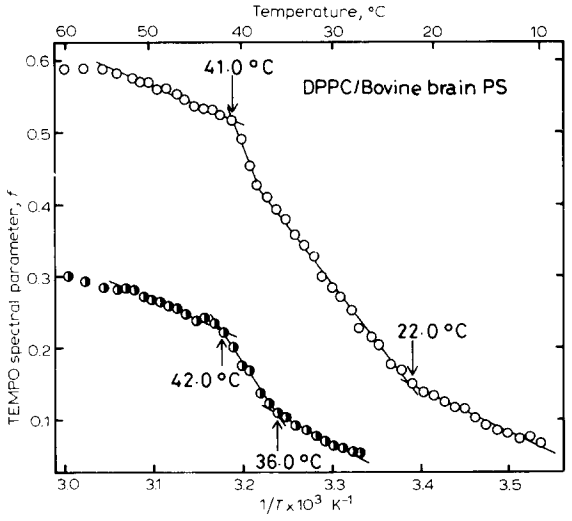

Fig. 2. TEMPO parameter, f, vs. $1/T$ for DPPC-PS (67 : 33) liposomes in the absence (control) (o———o) and presence (●———●) of 10 mM Ca^{2+}. The lipid dispersion was prepared by the same method as described in the legend of Fig. 1.

lipid bilayers, by environmental polarity. The TEMPO parameter, f, represents the partition of the probe between the water and the lipid phase, which reflects the physical state of liposomes, especially the phase transition and the phase separation [23]. As the DPPC liposomes changed from the liquid crystalline state to crystalline state, f decreased abruptly at the phase transition temperature of 41°C. The pretransition was observed as a small decrease of f near 30°C. The addition of 10 mM Ca^{2+} caused no change in the theremal feature of the TEMPO parameter in DPPC liposomes (data not shown). PI included in DPPC liposomes lowered the phase transition temperature and also broadened the temperature range of the transition (Fig. 1). In DPPC-PI (67 : 33, mol/mol) liposomes, the transition of liquid crystalline to crystalline state occurred over a wide range of temperature; the onset temperature of transition (T_h) was 34.0°C and the end-point temperature (T_l) was 20.0°C. Upon addition of 10 mM Ca^{2+}, the thermal characteristics of DPPC-PI liposomes were changed so that the effect of PI became less marked. The temperature range (T_h ~

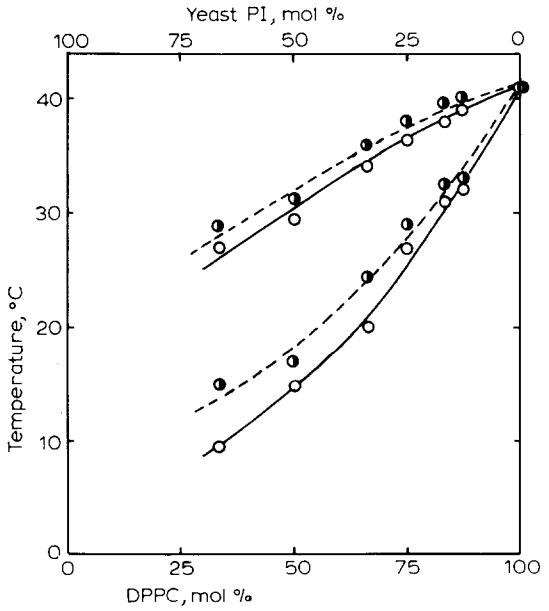

Fig. 3. The phase diagram of the binary mixture of DPPC and yeast PI. Liposomes were prepared in buffer A by vortexing with glass beads above the phase transition temperature. The fluidus and solidus points were obtained as shown in Fig. 1 in the absence (control) (o———o) and presence (●———●) of 10 mM Ca^{2+}.

T_l) of the phase transition was decreased to 11.5°C; T_h and T_l were raised to 36 and 24.5°C, respectively.

As shown in Fig. 2, PS also broadened the temperature range of DPPC phase transition by lowering the end-point temperature. In DPPC-PS (67 : 33) liposomes, T_h and T_l were 41.0 and 22.0°C, respectively. Addition of 10 mM Ca^{2+} raised the T_l up to 36.0°C while T_h was slightly shifted to 42.0°C. Stewart et al. [11] have reported the phase diagram of the binary mixture of DPPC and bovine brain PS in the absence of calcium ions using calorimetry and X-ray diffraction techniques. The T_h and T_l temperatures obtained in the present experiment using TEMPO spin probe were observed to coincide with their results. On the other hand, the T_h and T_l temperatures in the presence of 10 mM Ca^{2+} were assigned around those

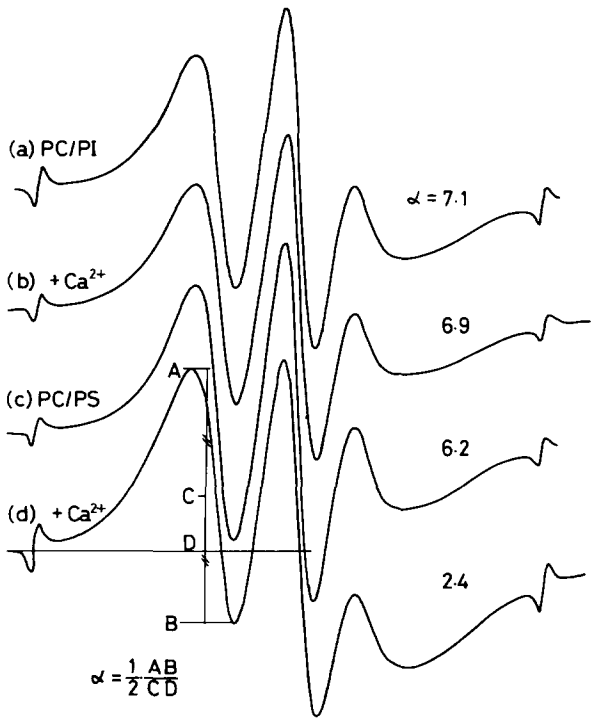

Fig. 4. ESR spectra of phosphatidylcholine spin probes (PC*) in phospholipid multilayered membranes prepared in a Millipore filter. The filter was set along the magnetic field by a Teflon holder and ESR measurements were carried out at 25°C. Spectral parameter, α, was calculated as shown in (d). C is the midpoint of AB. Egg PC/PI/PC* (4 : 5 : 1) mixture in the presence of (a) 10 mM EDTA and (b) 10 mM Ca^{2+} in buffer A. Egg PC/PS/PC* (4 : 5 : 1) mixture in the presence of (c) 10 mM EDTA and (d) 10 mM Ca^{2+} in buffer A.

of DPPC-PS (90 : 10) in the phase diagram. This strongly suggests that Ca^{2+} induces phase separation in DPPC-PS liposomes.

To examine in more detail the effect of calcium ions, the properties of phase transitions were observed in liposomes composed of various ratios of DPPC to PI in the presence and the absence of 10 mM Ca^{2+}. The phase transition temperatures obtained from the thermal feature of the TEMPO spectral parameter gave a phase diagram of a binary mixture of DPPC and PI (Fig. 3). The phase diagram was not completed because the phase transition became unclear as the content of PI increased. Upon the addition of 10 mM Ca^{2+}, the phase diagram was shifted to higher temperature while the transition temperature of DPPC liposome alone was unchanged. The Ca^{2+} effect on DPPC-PI liposomes was very small in comparison with its effect on DPPC-PS liposomes. For example, the T_h and T_l temperatures of DPPC-PI (67 : 33) liposomes were slightly shifted approximately to those of DPPC-PI (73 : 27) upon the addition of 10 mM Ca^{2+}.

Another technique which indicates phase separation more directly [6] was adopted to confirm whether the phase separation is induced by calcium ions in PC-PI liposomes. This technique employs the principle that the ESR spectrum is changed by spin-spin exchange interaction depending on the concentration of spin probes. The ESR spectra shown in Fig. 4 manifest typical examples of an exchange broadening. The decrease in the spectral parameter, α, denotes that spin probes are segregated by phase separation to give a higher concentration from the homogeneous distribution in the lipid mixture. In the mixture of egg yolk PC/PI/PC* (4 : 5 : 1), the α parameter was slightly reduced by 10 mM Ca^{2+} (from 7.1 to 6.9). On the other hand, in the mixture of egg yolk PC/PS/PC* (4 : 5 : 1), 10 mM Ca^{2+} remarkably decreased the α parameter from 6.2 to 2.4. These direct measurements also indicated that the degree of Ca^{2+}-induced phase separation in PC-PI liposomes was very small compared with that of PC-PS liposomes.

Also, the Ca^{2+} effect on molecular motions of liposomal lipids was studied using a stearic acid spin probe (5SAL). In DPPC liposomes, the abrupt change in order parameters was shown at the phase transition temperature (41°C), but the thermotropic feature was not affected by the addition of 10 mM Ca^{2+}

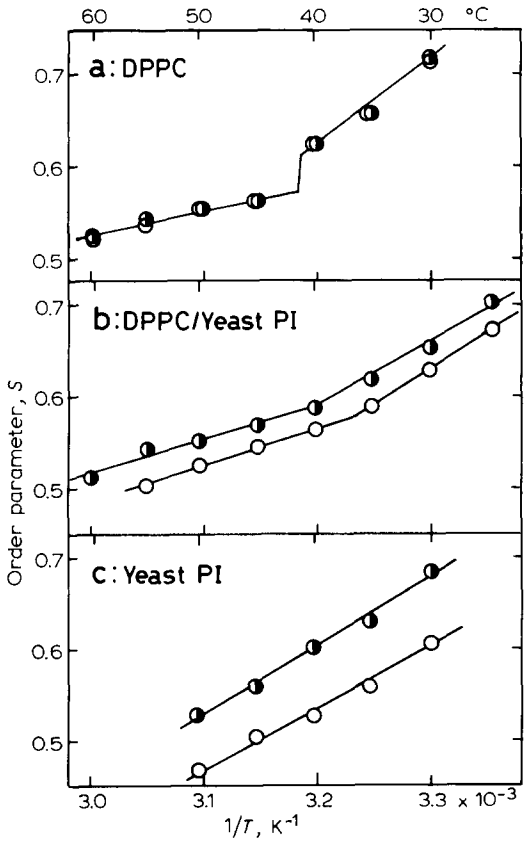

Fig. 5. Order parameters vs. $1/T$ for (a) DPPC, (b) DPPC-PI (75 : 25) and (c) yeast PI liposomes in the absence (control) (o———o) and presence (●———●) of 10 mM Ca^{2+}. The liposomes were prepared in buffer A by vortexing with glass beads. Stearic acid spin probe (5SAL) was included in the liposomes.

(Fig. 5a). However, the plots of order parameters of PI liposomes against temperatures (30–50°C) depicted no transition. Upon the addition of 10 mM Ca^{2+}, the order parameters were markedly increased in these temperatures (Fig. 5c). In DPPC-PI (75 : 25) liposomes, there was a break at approx. 40°C which is, however, less marked than the increase observed in PI liposomes (Fig. 5b). Similar results were obtained in these three types of liposomes when a phosphatidylcholine spin probe esterified with a 5-nitroxide stearic acid was used (data not shown).

Furthermore, the Ca^{2+} effect on the phase properties of DPPC-PI liposomes were examined by freeze-fracture electronmicroscopy. In the phase diagram (Fig. 3), upon addition of 10 mM Ca^{2+}, the physical state of the region between two fluidus curves (the upper solid and dotted curves) is changed from the liquid crystalline phase (L_α) to the coexisting phase of L_α and P_β'. P_β' is a phase of the crystalline state. Similarly, the change from this coexisting phase to the P_β' phase of crystalline state occurs in the area between two solidus curves (the lower solid and dotted curves) upon the addition of Ca^{2+}. If the temperature for fracturing was well controlled, these phase changes would be expected to be, for example, for DPPC-PI (75 : 25) liposomes at 38 and 28°C for the fluidus and the solidus curves, respectively. Since it was proved that our technique of freeze-fracture could detect at least structural alterations due to the difference of 2°C in the phase transition of DPPC liposomes (data not shown), freeze-fracture studies were carried out for DPPC-PI (75 : 25) liposomes at 38 and 28°C. Fig. 6a shows a freeze-fracture electronmicrograph of DPPC-PI (75 : 25) liposomes quenched from 38°C. A jumbled structure, which is characteristic of the liquid crystalline (L_α) phase [28], was observed in the fractured face. On the other hand, in the presence of 10 mM Ca^{2+}, both the banded structure and the jumbled structure, which is partially modified into a rivuletted pattern [29], were observed to coexist in the same fractured face (Fig. 6b). The banded structure was reported to represent the P_β' phase of the crystalline state [26,28]. Quenched from 28°C in the absence of calcium, a thin jumbled-like structure was seen in areas of the few banded structures (Fig. 6c). Upon addition of 10 mM Ca^{2+}, the jumbled-like pattern disappeared in the fractured face and instead a rivuletted structure appeared (Fig. 6d). Similar freeze-fracture electronmicrographs were observed in dimyristoylphosphatidylcholine (DMPC)-PI (67 : 33) liposomes quenched from 20 or 10°C in the absence or presence of 10 mM Ca^{2+} (data not shown). Since PI is known to be negatively charged at physiological pH, DPPC-PI liposomes were expected to produce a negative zeta potential and move to the anode electrophoretically. Fig. 7 gives the temperature dependence of the electrophoretic mobility of DPPC-PI liposomes, which was examined by microparticle electrophoresis. DPPC liposomes containing 10 mol% PI showed two breaks in the plots, one being sharp at 35°C and the other at around 39°C. These break points were identical, irrespective of the direction of temperature change; decreasing or

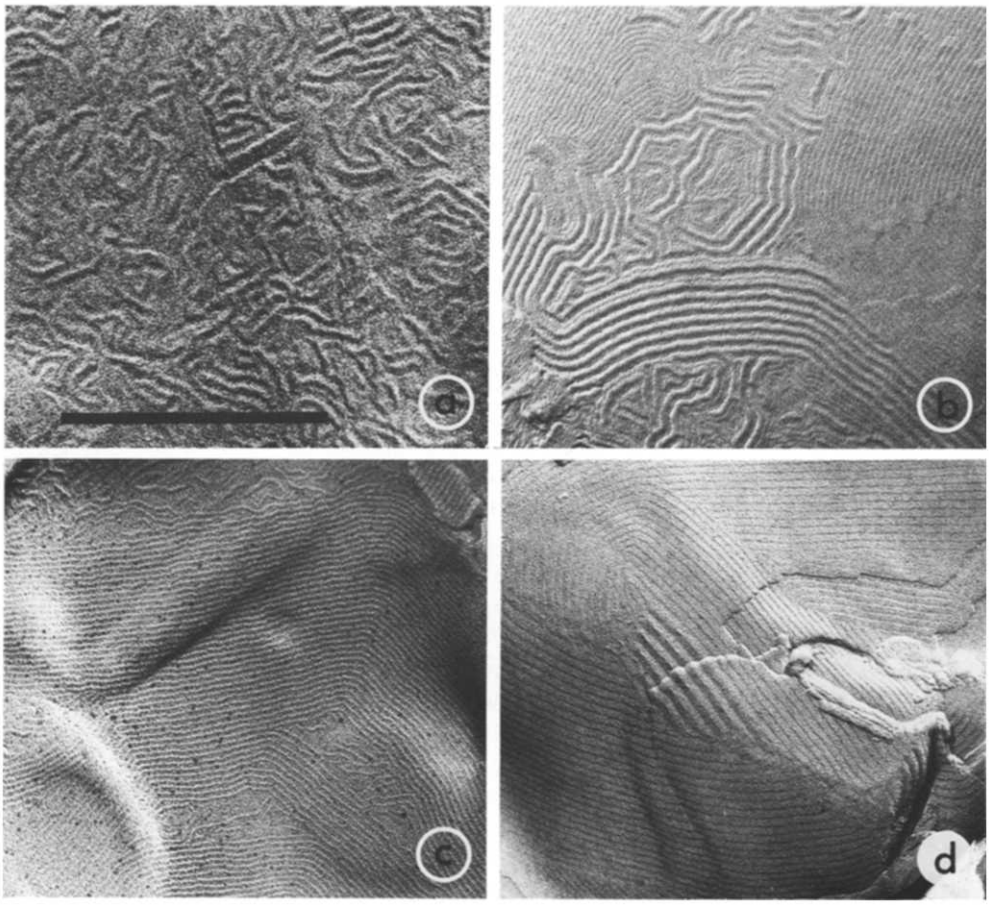

Fig. 6. The freeze-fracture electronmicrographs of DPPC-PI (75 : 25) liposomes. Liposomes were prepared in buffer A by vortexing with glass beads above 50°C. The dispersion (approx. 10 mg/ml) was quenched from 38°C (a) in the absence and (b) in the presence of 10 mM Ca^{2+}, or quenched from 28°C (c) in the absence and (d) in the presence of 10 mM Ca^{2+}. Magnifications are the same in all micrographs (a–d). Bar indicates 500 nm. The direction of shadowing is from the bottom to the top.

increasing. The phase change affected the zeta potential of liposomes including small amounts of PI to reduce the effective charge, probably resulting from segregating of PI from DPPC [30]. As shown in Fig. 3, the incorporation of PI into DPPC liposomes lowered and broadened the phase transition temperature. The sharp transition, which was observed in DPPC-PI (90 : 10) liposomes, disappeared in DPPC-PI (75 : 25) liposomes, and break points appeared at around 44 and 18°C. The higher electrophoretic mobility was assumed to reflect larger surface density of negative charge which was due to the greater content of PI. Again, in order to examine the effect of calcium ions on the liposome mobility, 1 mM Ca^{2+} was added into the DPPC-PI (75 : 25) liposomal system, resulting in decreased mobility in the temperatures tested. However, the general profile of thermal dependence of the mobility was not altered by the addition of calcium ions, which provides another evidence that calcium ions would not induce the remarkable phase separation in DPPC-PI lipsomes.

Discussions

Physiological roles of calcium have been studied in various cells: for example membrane excitability, calmodulin [31], release of catecholamines, and enzyme activities. Most of the biological functions

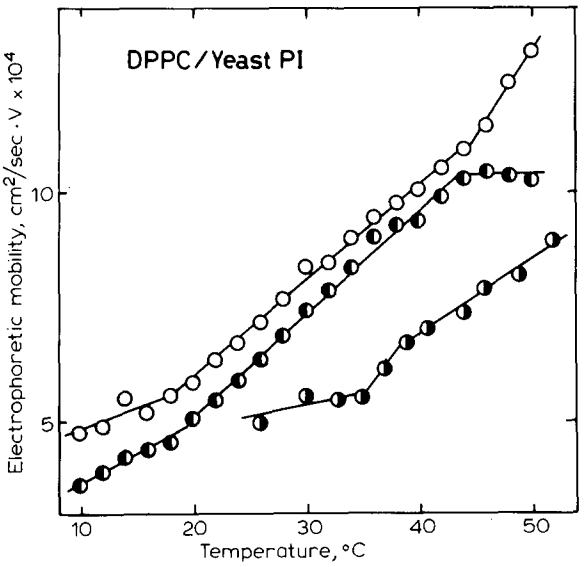

Fig. 7. Electrophoretic mobility vs. temperature for DPPC-PI liposomes. The liposomes were prepared by vortexing with glass beads above the phase transition temperature in buffer B (approx. 0.5 mg/ml). The mobilities were obtained from 40 alternative measurements in the opposite direction in three independent experiments. The averaged values are plotted in the figure. DPPC-PI (90 : 10) liposomes (○———○); DPPC-PI (75 : 25) liposomes in the absence (◑———◑) and presence (●———●) of 1 mM Ca^{2+}.

related to calcium are known to be associated with membranes. Main constituents of membranes are lipids and proteins, and some lipids are negatively charged; such as phosphatidylserine, phosphatidylinositol, phosphatidylglycerol and cardiolipin. Membrane lipids are composed of phospholipids which differ in polar head group and fatty acyl chains, together with sterols and glycolipids. Binary mixtures of lipids have principally been used for studies on interaction between different lipid species [23,32–35].

Phase separation, induced by calcium ions, seems to play an important role in membranes, including negatively charged lipids. Jacobson and Papahadjopoulos [9], demonstrated by differential scanning calorimetry that DPPC-bovine brain PS (1 : 2) exerted a broad transition and that the addition of 10 mM Ca^{2+} raised the endothermic peak and separated it into two peaks, one of which corresponded to the transition of pure DPPC. In the present experiment, DPPC-yeast PI (33 : 67) liposomes still showed a broad transition in the presence of 10 mM Ca^{2+}. And it was revealed that Ca^{2+}-induced phase separation is not pronounced in PC-PI liposomes. In Fig. 4, an attempt to estimate the PI amount segregated in 10 mM Ca^{2+} was made using standard exchange-broadened spectra [36], and it was assumed that only about 5% of total PI in the egg PC/PI/PC* (4 : 5 : 1) mixture was segregated in contrast to 85% PS in the egg PC/PS/PC* (4 : 5 : 1) mixture.

Most of the important porperties of the binary mixture are reflected in the phase diagram. The phase diagram can be made from various measurements, e.g., differential scanning calorimetry, X-ray diffraction and TEMPO spin probe. Each technique has an advantage and disadvantage. The phase diagram of DPPC-bovine brain PS was drawn from differential scanning calorimetry and X-ray diffraction including a pretransition in addition to a main transition [11]. Although the effect of calcium on the phase diagram was not examined, the shift of transition temperature induced by calcium ions was observed by differential scanning calorimetry in DPPC-bovine brain PS liposomes [9]. The shift in the phase diagram of DPPC-bovine brain PS liposomes was observed to occur upon the addition of lipophillin, a hydrophobic myelin protein which binds preferentially to PS [33]. The phase diagram of the binary mixtures of synthetic phosphatidylglycerol and PC was studied in the presence of calcium ions; dimyristoylphosphatidylglycerol (DMPG)-dilauroylphosphatidylcholine (DLPC) showed a phase separation, whereas dipalmitoylphosphatidylglycerol (DPPG)-dipalmitoylphosphatidylcholine (DPPC) and DMPG-DPPC liposomes gave a single narrow transition [34]. The phase diagram of binary mixture of DPPC-PI (Fig. 3) showed that DPPC and yeast PI are miscible in the crystalline phase [23], and that calcium ions (10 mM) slightly shifted the phase diagram upward. The transition temperatures (T_h and T_l) of DPPC-PI (67 : 33) liposomes in the presence of 10 mM Ca^{2+} was found to correspond to the T_h and T_l of the control liposomes (without Ca^{2+}) consisting of about 73 mol% DPPC and 27 mol% PI. Estimated from this shift of the transition temperature, it is presumed that about 8% of PI was segregated by 10 mM Ca^{2+} in DPPC-PI (67 : 33) liposomes. If the same estimation method was applied to the phase diagram of the liposomes of DPPC-bovine brain PS (Fig. 2 in Ref. 11), about 85% of PS is thought to be segregated by

Ca^{2+} (10 mM) in DPPC-PS (67 : 33) liposomes. These results are compatible with those obtained from the studies using PC spin probe in egg PC/PI and egg PC/PS mixtures.

Interaction between calcium ions and anionic phospholipids is suggested to include at least two types of effects. One enhances the intermolecular interactions by crosslinking the anionic phospholipids mediated by calcium ions. As shown in Fig. 5. the decrease of the fatty acyl chain motion may be due to this effect, which is specific for PI, and calcium ions never decreased the molecular motion of DPPC. The other effect is induction of lateral phase separation. Calcium ions gather the anionic phospholipids and result in the formation of the anionic phospholipid-poor regions in the lipid mixtures. This segregating effect of Ca^{2+} was weak for the PC/PI mixture. It was demonstrated by freeze-fracture electron-microscopic studies that the separated areas composed of only PI were not produced by the addition of 10 mM Ca^{2+} in DPPC-PI liposomes. Pure PI liposomes in the presence of 10 mM Ca^{2+} did not produce any banded structure but smooth surfaces even at 4°C. Smooth areas as seen in PI liposomes were not observed in the freeze-fracture face of DPPC-PI liposomes in the presence of 10 mM Ca^{2+} (Fig. 6).

Recently, certain mechanisms are proposed for the enhancement of phosphatidylinositol turnover during cell responses toward stimuli. PI is thought to be a donor of diacylglycerol which is produced by phospholipase C, and phosphatidylserine, diacylglycerol and calcium ions are required for the activation of a protein kinase [17]. As an initial step to approach the underlying molecular mechanism for PI response, we made some attempts to demonstrate the effect of calcium ions on the phase separation of egg PC/PS/diacylglycerol and egg PC/PS/PI using PC spin probes. Our preliminary results indicated that the phase separation was induced by a lower Ca^{2+} concentration in the egg PC/PS/diacylglycerol mixture than in the egg PC/PS/PI mixture. This finding would be interpreted by either the concept that PI suppresses the Ca^{2+}-induced phase separation or that diacylglycerol enhances the phase separation [37].

The results obtained in the present study demonstrated the basic properties of PC-PI liposomes, including the effect of calcium ions, and would provide some useful information for better understanding of the biological functions related to Ca^{2+}-PI interactions.

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References

1 Sandermann, H., Jr. (1978) Biochim. Biophys. Acta 515, 209–237
2 Nozawa, Y., Iida, H., Fukushima, H., Ohki, K. and Ohnishi, S. (1974) Biochim. Biophys. Acta 367, 134–147
3 Ohki, K., Kasai, R. and Nozawa, Y. (1979) Biochim. Biophys. Acta 558, 273–281
4 Hokin, M.R. and Hokin, L.E. (1953) J. Biol. Chem. 203, 967–977
5 Michell, R.H. (1975) Biochim. Biophys. Acta 415, 81–174
6 Ohnishi, S. and Ito, T. (1974) Biochemistry 13, 881–887
7 Tokutomi, S., Ohki, K. and Ohnishi, S. (1980) Biochim. Biophys. Acta 596, 192–200
8 Schnepel, G.H., Hegner, D. and Schummer, U. (1974) Biochim. Biophys. Acta 367, 67–74
9 Jacobson, K. and Papahadjopoulos, D. (1975) Biochemistry 14, 152–161
10 Boggs, J.M., Wood, D.D., Moscarello, M.A. and Papahadjopoulos, D. (1977) Biochemistry 16, 2325–2329
11 Stewart, T.P., Hui, S.W., Portis, A.R., Jr. and Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 556, 1–16
12 De Kruijff, B., Verkleij, A.J., van Echteld, D.J.A., Gerritsen, W.J., Mombers, C., Noordam, P.C. and de Gier, J. (1979) Biochim. Biophys. Acta 555, 200–209
13 Cullis, P.R. and Verkleij, A.J. (1979) Biochim. Biophys. Acta 552, 546–551
14 Cullis, P.R. and de Kruijff, B. (1979) Biochim. Biophys. Acta 559, 339–420
15 Verkleij, A.J., Mombers, C., Gerritsen, W.J., Leunissen-Bijvelt, L. and Cullis, P.R. (1979) Biochim. Biophys. Acta 555, 358–361
16 Gerritsen, W.J., de Kruijff, B., Verkleij, A.J., de Gier, J. and van Deenen, L.L.M. (1980) Biochim. Biophys. Acta 598, 554–560
17 Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y. (1980) J. Biol. Chem. 255, 2273–2276
18 Trevelyan, W.E. (1966) J. Lipids Res. 7, 445–447
19 Sanders, H. (1967) Biochim. Biophys. Acta 144, 485–487
20 Singleton, W.S., Gray, M.S., Brown, W.L. and White, J.L.

(1965) J. Am. Oil. Chem. Soc. 42, 53–56
21 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468
22 Tokutomi, S., Eguchi, G. and Ohnishi, S. (1979) Biochim. Biophys. Acta 552, 78–88
23 Shimschick, E. and McConnell, H.M. (1973) Biochemistry 12, 2351–2360
24 Devaux, P. and McConnell, H.M. (1972) J. Am. Chem. Soc. 94, 4475–4481
25 Ohki, K., Imai, A. and Nozawa, Y. (1980) Biochem. Biophys. Res. Commun. 94, 1249–1255
26 Inoko, Y., Mitsui, T., Ohki, K., Sekiya, T. and Nozawa, Y. (1980) Phys. Stat. Solid 61, 115–121
27 Morrison, W.R. and Smith, L.M. (1964) J. Lipid Res. 5, 600–608
28 Luna, E.J. nd McConnell, H.M. (1978) Biochim. Biophys. Acta 509, 462–473
29 Lentz, B.R., Barrow, D.A. and Hoechli, M. (1980) Biochemistry 19, 1943–1954
30 Hauser, H., Guyer, W. and Howell, K. (1979) Biochemistry 18, 3285–3291
31 Cheung, W.Y. (1980) Science 207, 19–27
32 Von Dreele, P.H. (1978) Biochemistry 17, 3939–3943
33 Van Dijck, P.W.M., Kaper, A.J., Oonk, H.A.J. and de Gier, J. (1977) Biochim. Biophys. Acta 470, 58–69
34 Findlay, E.J. and Barton, P.G. (1978) Biochemistry 17, 2400–2405
35 Boggs, J.M., Moscarello, M.A. and Papahadjopoulos, D. (1977) Biochemistry 16, 5420–5426
36 Ohnishi, S. and Tokutomi, S. (1980) Biol. Magn. Resonance 3, 121–153
37 Ohki, K., Yamauchi, Y., Banno, Y. and Nozawa, Y. (1981) Biochem. Biophys. Res. Commun., in the press