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EFFECT OF PHOSPHATIDYLINOSITOL REPLACEMENT BY DIACYLGLYCEROL ON VARIOUS PHYSICAL PROPERTIES OF ARTIFICIAL MEMBRANES WITH RESPECT TO THE ROLE OF PHOSPHATIDYLINOSITOL RESPONSE

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In an attempt to gain insight into the physiological role of phosphatidylinositol turnover enhanced by extracellular stimuli, the physical properties of artificial membranes (egg yolk phosphatidylcholine/bovine brain phosphatidylserine) containing phosphatidylinositol or diacylglycerol were studied by ESR using spin probes and freeze-fracture electron microscopy. Diacylglycerol lost both the ability to form lipid bilayer structures and its susceptibility to calcium ions. Yeast phosphatidylinositol included in dipalmitoylphosphatidylcholine liposomes lowered the phase transition temperature of dipalmitoylphosphatidylcholine and expanded the temperature range of phase transition. However, diacylglycerol at the same concentration did not undergo the effects caused by phosphatidylinositol but the phase transition temperature was slightly raised. Phase separation of phosphatidylserine induced by calcium ions was enhanced when the phosphatidylinositol was replaced by diacylglycerol in phosphatidylcholine/phosphatidylserine/phosphatidylinositol (3:5:2, by molar ratio) mixtures. The mobility of phosphatidylcholine spin probe was decreased in phosphatidylcholine/phosphatidylserine/diacylglycerol (3:5:2, by molar ratio) liposomes compared with phosphatidylcholine/phosphatidylserine/phosphatidylinositol (3:5:2, by molar ratio) liposomes. An additional component from protonated stearic acid spin probes was observed in phosphatidylcholine/phosphatidylinositol (8:2, by molar ratio) liposomes at 40°C, whereas the component was not seen in phosphatidylcholine/diacylglycerol (8:2, by molar ratio) liposomes. This may indicate the alteration of surface charge induced by the replacement of phosphatidylinositol by diacylglycerol. Indeed, in the presence of 1 mM Ca^{2+}, the additional component was removed by an electrostatic interaction between Ca^{2+} and phosphatidylinositol molecules in phosphatidylcholine/phosphatidylinositol liposomes at 40°C. These results support the hypothesis that the enhanced turnover of phosphatidylinositol may play a triggering role for various cellular responses to exogenous stimuli by altering membrane physical states.

Introduction

It is widely accepted that membrane lipids play an important role in the various functions of biomembranes [1–3]. Since physical properties of membranes are related to the constituents of membranes, alteration of lipid composition is considered to affect membrane functions. A unicellular eukaryote, *Tetrahymena*, modulates the lipid composition of its membranes to survive in a new environment, e.g., in low temperature [4]. This is a slow process of membrane alteration. On the other hand, enhanced turnover is observed as a fast process in many types of animal cells in response

Abbreviations: TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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to various stimuli and is followed by a wide variety of cellular functions [5]. For example, enhanced turnover of phosphatidylinositol is induced in platelets activated by thrombin [6], adipose tissue stimulated by insulin [7], cerebral cortex excited by dopamine [8], submaxillary gland pulsed by electric current [9] and other tissues influenced by various stimuli. The initial step of phosphatidylinositol turnover is hydrolysis of phosphatidylinositol to diacylglycerol catalyzed by phosphatidylinositol-specific phospholipase C. These changes in membranes may trigger the following responses. Indeed, it was shown that diacylglycerol activates Ca^{2+}-dependent protein kinase in the presence of phosphatidylserine and Ca^{2+} [10,11]. Since protein kinases, in general, are implicated in phosphorylation of various proteins, activation of Ca^{2+}-protein kinase is a useful system for studying the coupling of phosphatidylinositol effects with cell functions. However, the physiological role of the enhanced turnover of phosphatidylinositol is not as yet elucidated in spite of numerous studies on the metabolism of phosphatidylinositol during stimulation. The effects of phosphatidylinositol turnover on the physical properties of membranes are also unclear since it is difficult to detect rapid and local changes of membranes. To overcome this difficulty, we have adopted artificial membrane systems in previous reports: physical properties of phosphatidylinositol in membranes were investigated in the presence of Ca^{2+} [12], and the effects of diacylglycerol on Ca^{2+}-induced phase separation of phosphatidylserine were also examined in phosphatidylcholine/phosphatidylserine mixture [13].

The intention of the present work was to extend these previous studies and to investigate the alterations in membrane physical states caused by the replacement of phosphatidylinositol by diacylglycerol. The results obtained here demonstrated that membrane stability, phase separation, phase transition, fluidity and membrane potential were affected by this lipid replacement.

Materials and Methods

Phosphatidylinositol was purified from baker's yeast (Oriental Yeast Co. Ltd., Tokyo) as described by Trevelyan [14], and stored as an ammonium salt in benzene solution at $-20°C$. In order to prepare diacylglcyerol with the same fatty acyl composition, yeast phosphatidylinositol was hydrolyzed by phosphatidylinositol-specific phospholipase C which was isolated from *Bacillus cereus* by the method of Ikezawa et al. [15]. 1,2-Diacylglycerol was separated on Silica gel H thin-layer plates developed by petroleum ether/diethyl ether/acetic acid (70 : 30 : 1, by vol). Egg yolk phosphatidylcholine and bovine brain phosphatidylserine were purified as described by Singleton et al. [16] and Sanders [17], respectively, 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, by vol). Phosphatidylcholine spin probe with a 12-nitroxide stearic acid at the 2 position of glycerol was kindly provided by Dr. Ohnishi of Kyoto University. 5-Nitroxide stearic acid spin probe and 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) were purchased from Syva Assoicates (Palo Alto, CA).

The phospholipid content was determined by phosphorus assay according to Bartlett [18]. The content of diacylglycerol was determined from the quantitative analysis of fatty acyl chains by gas-liquid chromatography using erucic acid as a standard.

For the preparation of multilamellar liposomes with the desired lipid composition, each lipid was taken from its benzene solution into a small test tube. The solvent was evaporated first under a nitrogen stream and then under reduced pressure overnight. Tris-buffered saline (150 mM NaCl/50 mM Tris-HCl, pH 7.5) containing EDTA or $CaCl_2$ was added with several glass beads, and vortexed for 3 min above 50°C. Lipid spin probe (phosphatidylcholine or stearic acid) was added to the lipid mixture before the evaporation of solvent, and TEMPO spin probe was added to the lipid dispersion after the vortexing. For the preparation of sonicated liposomes, the lipids in buffer were sonicated for 30 s with the microtip of a Branson sonifier (B-12) set at level 3.

The studies of Ca^{2+}-induced phase separation of phosphatidylserine were carried out as described in a previous paper [13]. Multilayered membranes were prepared in a pore of a Millipore filter, SWMPO 1300 (average pore diamter 5 μm).

The filter was dipped in the benzene solution of a lipid mixture of about 40 mg/ml and dried by evacuation for 3 h. The dried filter was soaked in 25 mM Tris-HCl buffer (pH 6.8) containing $CaCl_2$ and EGTA (ethyleneglycol bis (β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid) overnight at 25°C. Estimation of the concentration of free Ca^{2+} was based on an apparent binding constant of Ca^{2+}-EGTA of $8 \cdot 10^5$ M^{-1} [19]. Ca^{2+}-induced phase separation of phosphatidylserine was also studied in a system of sonicated liposomes prepared as described above.

For the ESR measurement, the lipid dispersion was taken into a glass capillary and the filter was set on a Teflon holder. The ESR spectra were measured using a commercial X-band spectrometer (JEOL FE-1X) equipped with a variable temperature control (JEOL, Tokyo). The rate of temperature decrease was no greater than 0.5 K/min.

The TEMPO spectral parameter calculated from high-magnetic field peaks of ESR spectrum according to the procedure of Shimshick and McConnell [20]. Then, in order to remove the effect of lipid concentration, TEMPO spectral parameter was transferred to fluid phase parameter using the following equation.

$$\text{Fluid phase parameter} = f_t / f_{60}$$

where f_t and f_{60} are TEMPO spectral parameters at t°C and 60°C, respectively. It is assumed that lipid dispersions are in a complete fluid phase at 60°C. Alpha spectral parameter, α, was calculated according to the definition by Devaux and McConnell [21], and the degree of phase separation was estimated by the method reported in a previous paper [13]. The order parameter, *S*, was calculated as described previously [22].

Freeze-fracture samples were prepared according to the procedure described in a previous paper [23]. A small volume of dispersions pipetted into a specimen holder placed on an aluminum vessel was further incubated in a water bath at the same temperature for 5 min. The sample was frozen immediately with liquid Freon 12 and transferred to liquid nitrogen and then fractured in a freeze-etching device, HZ-1 (Hitachi Co., Hitachi) at −110°C. Replica, prepared by platinum-carbon followed by carbon shadowing, was floated on to hypochlorous acid (HClO) and cleaned with distilled water. The replica was then collected on 300-mesh grids and observed by an electron microscope, JEM-U (JEOL, Tokyo).

Results

Artificial membrane systems used in the present experiment were composed of phospholipids from various sources. Phosphatidylinositol was purified from baker's yeast, and diacylglycerol was prepared by the hydrolysis of phosphatidylinositol by bacterial phospahtidylinositol-specific phospholipase C. As shown in Table I, the fatty acid compositions of phosphatidylinositol and diacylglycerol derived from phosphatidylinositol were practically the same. Therefore, it was possible to compare the effects of polar head groups, ignoring the effects of fatty acyl chains. Phosphatidylcholine and phosphatidylserine were purified from egg yolk of chicken and bovine brain, respectively.

The ability to form bilayer structure was compared between phosphatidylinositol and diacylglycerol. Phosphatidylinositol was easily dispersed in water by vortexing with glass beads, and stearate spin probe in the dispersion gave an ESR

TABLE I

FATTY ACID COMPOSITION OF YEAST PHOSPHATIDYLINOSITOL AND ITS DERIVED DIACYLGLYCEROL

Phosphatidylinositol was extracted from yeast by the method of Trevelyan [14]. Diacylglcyerol was prepared from phosphatidylinositol by hydrolysis using bacterial phosphatidylinositol-specific phospholipase C. The methyl esters were prepared in methanol solution of boron trifluoride at 100°C for 10 min and analyzed with a Model GC-6 gas-liquid chromatograph (Shimadzu, Kyoto). Peaks of individual fatty acids were quantified by comparison of retention time with those of authentic standards. Averaged values of three independent analyses are listed in the table.

Fatty acid	% Content of fatty acid	
	Phosphatidyl-inositol	Diacylglycerol
C16:0	17.2	19.3
C16:1	12.4	14.1
C18:0	18.5	17.1
C18:1	50.1	48.0

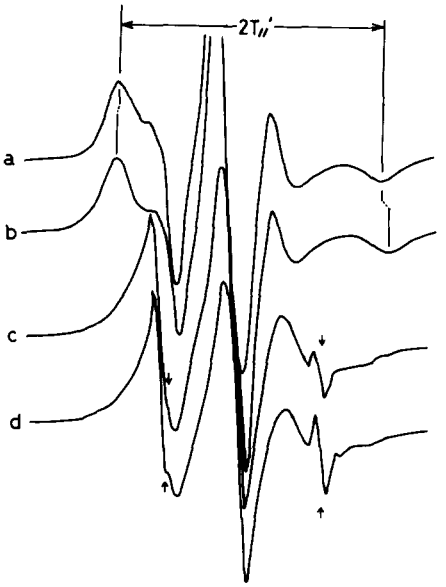

Fig. 1. ESR spectra of stearic acid spin probes incorporated into phosphatidylinositol dispersions in the absence (a) and in the presence (b) of 10 mM $CaCl_2$, and into diacylglycerol dispersions in the absence (c) and in the presence (d) of 10 mM $CaCl_2$. Lipid dispersions were prepared in Tris-buffered saline (150 mM NaCl/50 mM Tris-HCl, pH 7.5) by vortexing with glass beads. The parallel principal values of the hyperfine tensor of an axially symmetrical spin Hamiltonian ($T'_{//}$) and ESR signals from stearate spin probes partitioned in water (↑,↓) were indicated in figures.

spectrum derived from axial symmetric rotation in bilayer structure of lipids (Fig. 1a and 1b). On the other hand, diacylglycerol was hardly dispersed in water even by prolonged sonication and formed oil droplets immediately after cessation of sonication. As shown in Fig. 1c and 1d, ESR spectra of stearate spin probe in diacylglycerol showed the patterns characteristic of an isotropic motion. There was also a minor component (indicated by the arrows in the figures) due to stearate spin probe partitioned in water. The structure formed by diacylglcyerol in water was further examined by freeze-fracture electron microscopy. The fractured face of the sonicated dispersion of diacylglcyerol was rather flat, and the cumulated layers characteristic of multilammellae were not observed (Fig. 2a). In contrast, the convex and concave fractured faces showing multilayers were manifested in egg phosphatidylcholine dispersion containing 33 mol% of diacylglcyerol (Fig. 2b).

As shown in Figs. 1 and 2, unlike phosphatidylinositol, diacylglycerol which was derived from this phospholipid was no longer able to produce a bilayer structure. We have then examined whether diacylglycerol was actually incorporated into the bilayer structure of phospholipids. The sonicated

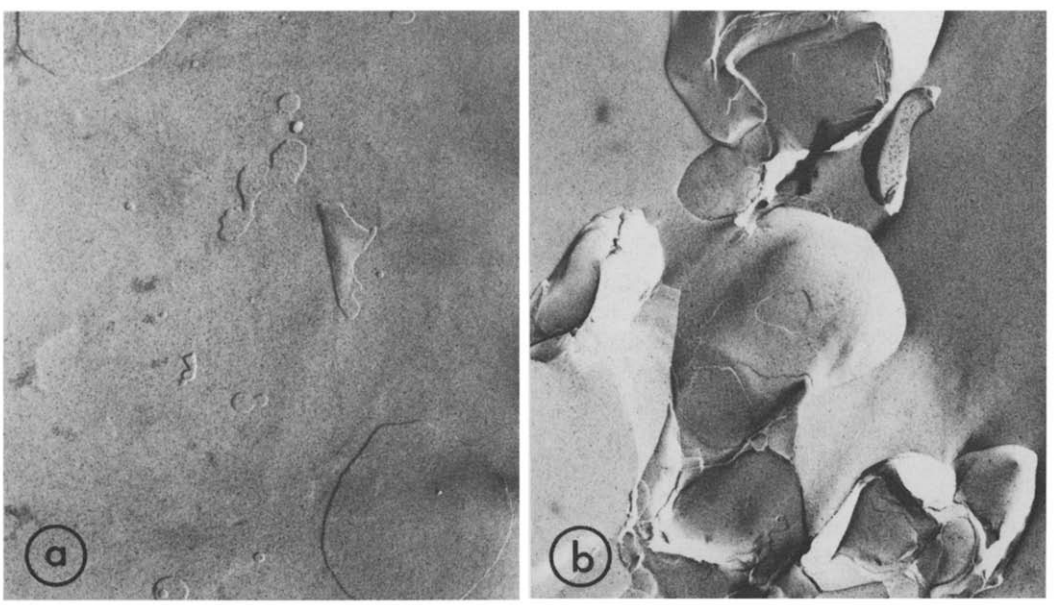

Fig. 2. Freeze-fracture electron micrographs of diacylglycerol (a) and egg yolk phosphatidylcholine/diacylglycerol (2 : 1, by molar ratio) (b) dispersions. Lipid dispersions were prepared in Tris-buffered saline (150 mM NaCl/50 mM Tris-HCl, pH 7.5) by sonication. The dispersions were quenched from 25°C. The magnification is 27000×.

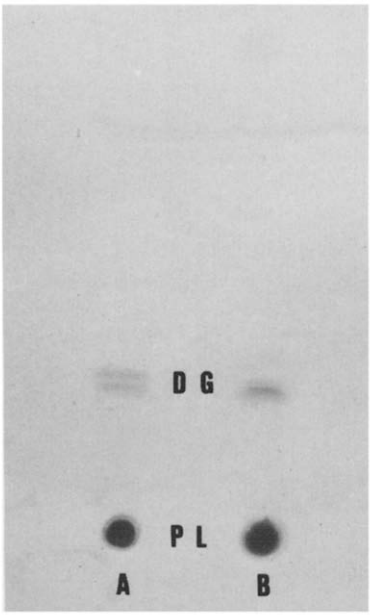

Fig. 3. Diacylglcyerol (DG) separated on thin-layer plate. (A) Benzene solution of phosphatidylcholine/phosphatidylserine/diacylglycerol (3:5:1, by molar ratio) (B). Extracted lipids of liposomes passed through Sepharose 6B column (1×20 cm). The liposomes were prepared from the benzene solution of phosphatidylcholine/phosphatidylserine/diacylglycerol (3:5:1, by molar ratio) as described in Materials and Methods. The lipids were extracted from liposomal fraction by the method of Bligh and Dyer [24]. Lipids containing 60 nmol phospholipid phosphorus (PL) were spotted on a silica gel plate then developed by petroleum ether/diethyl ether/acetic acid (80:30:1, by vol).

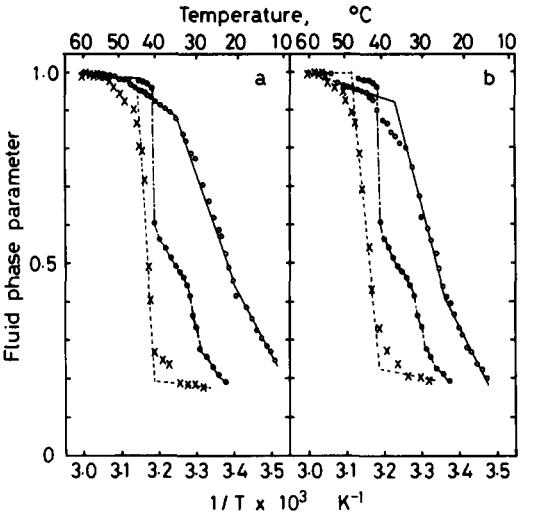

Fig. 4. Fluid phase parameter vs. $1/T$ for dipalmitoylphosphatidylcholine/phosphatidylinositol (2:1, by molar ratio) (○——○), dipalmitoylphosphatidylcholine/diacylglycerol (2:1, by molar ratio) (×------×) and dipalmitoylphosphatidylcholine (●- — - -●) liposomes in the absence (a) and in the presence (b) of 10 mM $CaCl_2$. The liposomes were prepared by sonication. 4 mM TEMPO (40 μl) was added to the dispersion (400 μl) (4 μl of 1 M $CaCl_2$ was added in the case of (b)), and concentrated by centrifugation (35000×g; 10 min) to approx. 200 mg/ml for ESR measurements. Fluid phase parameters were calculated from TEMPO spectral parameters as described in Materials and Methods.

liposomes (phosphatidylcholine/phosphatidylserine/diacylglcyerol (3:5:2, by molar ratio)) were passed through a Sepharose 6B column to remove unincorporated lipids. The existence of diacylglycerol in sonicated liposomes was confirmed by thin-layer chromatography (Fig. 3). The liposomes passed through the Sepharose 6B column were found to contain almost the same content of diacylglycerol as the original lipid benzene solution.

Phase transition of dipalmitoylphosphatidylcholine occurs sharply at 41°C, which divides the physical state of dipalmitoylphosphatidylcholine into liquid crystalline and crystalline phases. In addition to the main transition at 41°C, a pretransition is observed at 35°C [25]. The phase transition is well monitored by TEMPO spin probes which are partitioned between a lipid phase and an aqueous phase, and these probes give signals at different positions of the magnetic field according to the solvent polarity [20]. In the present experiment, a fluid phase parameter was introduced as a ratio of TEMPO spectral parameter at each temperature to that at 60°C. Addition of phosphatidylinositol (33 mol%) to dipalmitoylphosphatidylcholine lowered the transition temperature and expanded the temperature range of transition (Fig. 4). As shown in Fig. 4a, such effects were not distinct in the presence of 10 mM Ca^{2+} since some phosphatidylinositol molecules were segregated by Ca^{2+} [12]. On the other hand, the same content of diacylglcyerol recovered the sharp transition at a temperature higher than that of pure diplamitoylphosphatidylcholine by 3 K, but the pretransition was not observed despite the clear main transition. The addition of 10 mM Ca^{2+} did not influence the effect of diacylglycerol.

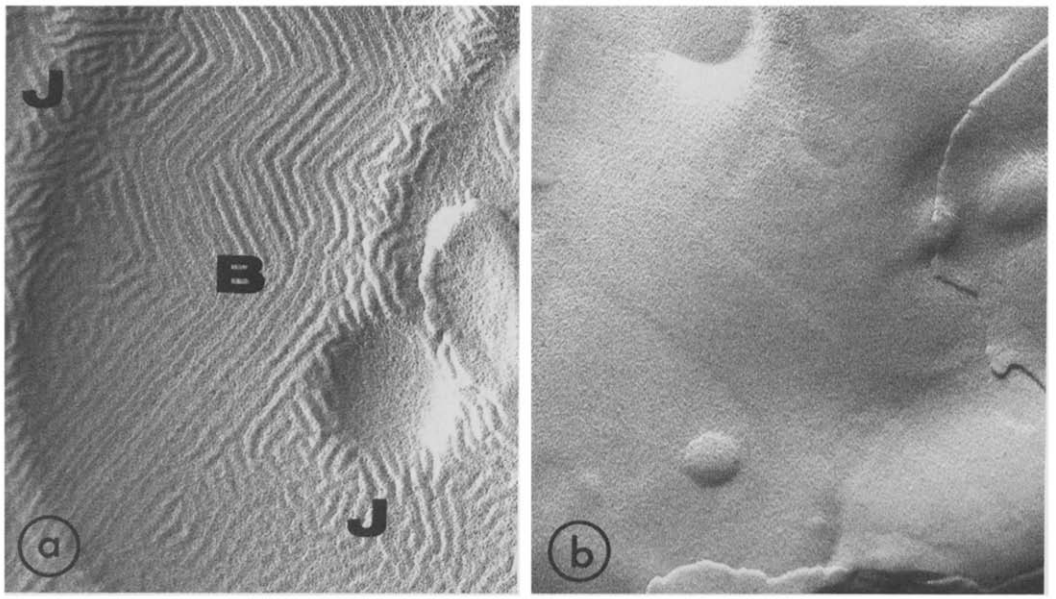

Fig. 5. Freeze-fracture electron micrographs of dipalmitoylphosphatidylcholine/phosphatidylserine/phosphatidylinositol (3 : 1 : 1, by molar ratio) (a) and dipalmitoylphosphatidylcholine/phosphatidylserine/diacylglycerol (3 : 1 : 1, by molar ratio) (b) liposomes prepared in 25 mM Tris-HCl buffer (pH 6.8) containing 1 μM free Ca^{2+} by vortexing with glass beads. The liposomes were quenched from 25°C. Jumbled (J) and banded (B) patterns are indicated in the figure. Magnification is 60000×.

The loss of susceptibility of diacylglcyerol to Ca^{2+} was considered to result from cleavage of phosphoinositol. As shown in Fig. 1, the added Ca^{2+} increased the hyperfine splitting, $2T_{II}'$, of phosphatidylinositol liposomes, but did not affect the ESR spectrum of diacylglycerol dispersions.

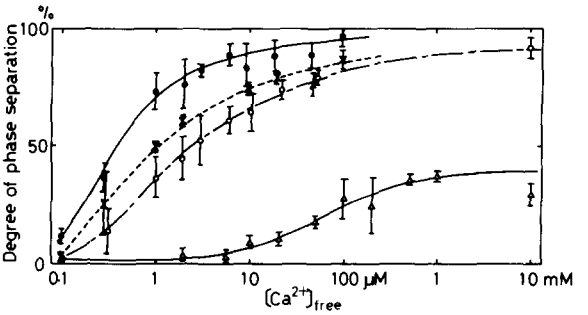

Fig. 6. Degree of phase separation vs. concentration of free calcium ions for phosphatidylcholine/phosphatidylinositol (1 : 1) (△———△), phosphatidylcholine/phosphatidylserine (1 : 1) (○- - - -○), phosphatidylcholine/phosphatidylserine/phosphatidylinositol (3 : 5 : 2) (×- - - - -×) and phosphatidylcholine/phosphatidylserine/diacylglycerol (3 : 5 : 2) (●———●) liposomes. Liposomes include 10 mol% of phosphatidylcholine spin probe. Degree of phase separation was obtained from the change in spin-spin exchange broadening of ESR spectrum according to the method reported previously [13]. Plots are expressed as mean ±S.D. obtained from 3–6 independent experiments.

The phase transition of phospholipids was characterized by the freeze-fracture faces [26]. Jumbled, banded and terrace patterns observed by freeze-fracture electron microscopy were identified to be L_α, $P_{\beta'}$ and $L_{\beta'}$ phases obtained by X-ray diffraction, respectively [27]. The main transition from the liquid crystalline to the crystalline phase was depicted as a transformation from the jumbled to the banded pattern, and the pretransition as a transformation from the banded to the terrace pattern. As shown in Fig. 5a, both jumbled and banded patterns were observed on the fractured face of dipalmitoylphosphatidylcholine/bovine brain phosphatidylserine/yeast phosphatidylinositol (3 : 1 : 1, by molar ratio) at 25°C in the presence of 1 μM Ca^{2+}. Although liquid crystalline and crystalline phases coexist under these conditions, such patterns were found to disappear by the replacement of phosphatidylinositol by diacylglcyerol (Fig. 5b). This indicates the shift from the coexisting state of L_α and $P_{\beta'}$ to the crystalline state of $L_{\beta'}$.

It is well established that membrane lipids are

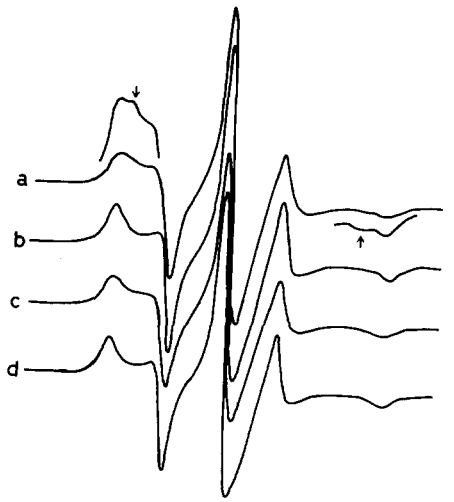

Fig. 7. ESR spectra of stearic acid spin probes incorporated into phosphatidylcholine/phosphatidylinositol (4:1, by molar ratio) in the absence (a) and in the presence (b) of 1 mM $CaCl_2$ and into phosphatidylcholine/diacylglycerol (4:1, by molar ratio) in the absence (c) and in the presence (d) of 1 mM $CaCl_2$. Liposomes were prepared in Tris-buffered saline (150 mM NaCl/50 mM Tris-HCl, pH 7.5) by vortexing with glass beads. ESR signals due to higher molecular motion are indicated by arrows.

distributed heterogeneously in biological membranes [28]. As possible mechanisms by which the heterogeneity is maintained, lipid-protein interaction, turnover of lipids and biogenesis of membranes are considered. Phase separation, which is induced by Ca^{2+} [29], proteins [30], pH [31] etc., may be one of the mechanisms for heterogeneous lipid distribution. The effect of replacement of phosphatidylinositol by diacylglycerol was extensively examined in the phosphatidylcholine/phosphatidylserine system in Ca^{2+}-EGTA buffer. In Fig. 6, the degree of phase separation in four different lipid systems was plotted at various concentrations of calcium ions. In a phosphatidylcholine/phospahtidylinositol (1:1, by molar ratio) system, phase separation was not observed below 10 μM Ca^{2+}, and even at 10 mM Ca^{2+} approx. 30% phosphatidylinositol of total was segregated. Phase separation of phosphatidylinositol did not appear at a physiological concentration of Ca^{2+}. In a phosphatidylcholine/phosphatidylserine (1:1, by molar ratio) system, Ca^{2+}-induced phase separation of phosphatidylserine was distinct below 1 μM Ca^{2+} and was much pronounced (80%) at 100 μM Ca^{2+}. When 40% of phosphatidylcholine was replaced by phosphatidylinositol; phosphatidylcholine/phosphatidylserine/phosphatidylinositol (3:5:2, by molar ratio), the degree of Ca^{2+}-induced phase separation was increased by only several percent. However, when phosphatidylinositol was replaced by diacylglycerol derived from phosphatidylinositol, the phase separation was augmented to a greater extent. For example, at 1 μM Ca^{2+}, the replacement of phosphatidylinositol by diacylglycerol increased the degree of phase separation from 46.5% to 72.5%. Such enhancement of phase separation was observed not only for lipid bilayers formed in a Millipore filter but also for sonicated dispersions with the same lipid composition. At 1 μM Ca^{2+}, the degree of phase separation was increased from 42% to 67% by the replacement of phosphatidylinositol by diacylglycerol. These findings provide suggestive evidence that at physiological concentrations of Ca^{2+}, phase separation can be induced by conversion from phosphatidylinositol to diacylglycerol in biological membranes.

In order to examine other physical properties, stearic acid spin probe which has a nitroxide radical at C-5 position was introduced into phosphatidylcholine/phosphatidylinositol and phosphatidylcholine/diacylglycerol liposomes. Fig. 7 shows the ESR spectra in the presence and absence of 1 mM Ca^{2+} at 40°C. In the absence of free Ca^{2+} (in buffer containing 1 mM EDTA), the ESR spectrum of phosphatidylcholine/phosphatidylinositol (4:1, by molar ratio) liposomes was composed of two components (Fig. 7a). The component due to higher molecular motion (indicated by arrows in Fig. 7a) was not observed in other samples (Fig. 7b, c and d), which suggests that the surface charge produced by phosphatidylinositol was important for the appearance of the component. The component has been attributed to the ESR signal from the stearic acid spin probe whose carboxyl group is protonated [32,33]. The protonation of the carboxyl group depends on the local concentration of H^+ or pH. The local pH was lowered by phosphatidylinositol but increased by diacylglycerol. In the presence of 1 mM Ca^{2+}, the component of protonated stearate spin probe was not observed in the liposomes including phosphatidylinositol. Also Ca^{2+} diminished the pH-

TABLE II

ORDER PARAMETER OF PHOSPHATIDYLCHOLINE SPIN PROBE IN LIPOSOMES CONTAINING PHOSPHATIDYLINOSITOL AND DIACYLGLYCEROL

Liposomes were prepared in Tris-buffered saline (150 mM NaCl/50 mM Tris-HCl, pH 7.5) containing 1 mM EDTA or 1 mM $CaCl_2$ by vortexing with glass beads. Order parameters were obtained from ESR spectra of phosphatidylcholine spin probes incorporated into liposomes. Values are expressed as mean ± S.D. from three experiments. PC, phosphatidylcholine, PS, phosphatidylserine; PI, phosphatidylinositol; DG, diacylglycerol.

	Order parameter			
	at 20°C		at 40°C	
	1 mM EDTA	1 mM $CaCl_2$	1 mM EDTA	1 mM $CaCl_2$
PC/PS/PI (3:5:2)	0.373 ± 0.008	0.384 ± 0.005	0.210 ± 0.008	0.218 ± 0.007
PC/PS/DG (3:5:2)	0.402 ± 0.003	0.406 ± 0.004	0.238 ± 0.002	0.241 ± 0.002

lowering effect of phosphatidylinositol (Fig. 7b).

By the replacement of phosphatidylinositol by diacylglycerol, the molecular motion of lipid spin probe in liposomes was reduced regardless of the presence or absence of Ca^{2+}. Table I summarizes the effects of the lipid replacement on the order parameter of phosphatidylcholine spin probe in phosphatidylcholine/phosphatidylserine liposomes. In both the homogeneously distributed state (1 mM EDTA) and the phase-separated state (1 mM $CaCl_2$), the order parameters of liposomes containing diacylglycerol were greater than those of liposomes containing phosphatidylinositol. While in the presence of 1 mM EDTA, phosphatidylcholine, phosphatidylserine and phosphatidylinositol (or diacylglcyerol) were distributed homogeneously in membranes, 1 mM Ca^{2+} caused separation of phosphatidylserine from either phosphatidylcholine/phosphatidylinositol or phosphatidylcholine/diacylglcyerol. Phosphatidylcholine spin probe gave information mainly about the latter area rather than the phosphatidylserine-rich area.

Discussions

Hydrolysis of phosphatidylinositol to form diacylglcyerol is thought to be the initial step of phosphatidylinositol turnover. Enhanced turnover of phosphatidylinositol has been observed in diverse cell types exerting the rapid responses to stimuli. Actually diacylglycerol is produced within several seconds after human platelets are exposed to thrombin [6]. Such rapid lipid alteration in membranes seems to play an important role for cellular functions. In the model membrane systems adopted for the present study, most of the physical properties of membranes, such as phase separation, phase transition, surface charge and membrane fluidity, were to various extents affected by the transient lipid modification.

Phosphatidylinositol could easily form a lipid bilayer structure, and the ability to form the bilayer structure was lost during its conversion to diacylglycerol by a phospholipase C (Fig. 1). Diacylglycerol was readily incorporated into the bilayer structure of liposomes (Fig. 3), and egg yolk phosphatidylcholine containing 33% of diacylglycerol formed a bilayer structure (Fig. 2). These results suggest that the bilayer structure of phospholipids was maintained when diacylglycerol molecules were distributed homogeneously among phospholipids. Recently, much attention has been paid to the formation of non-bilayer structures in membranes since the discovery of inverted micelle and hexagonal II phase of membranes [34]. The non-bilayer structures were suggested to be closely related to such membrane functions as ion transport [35], membrane fusion [36] and flip-flop of membrane lipids [37]. Although any direct evidence has not yet been obtained that diacylglycerol participates in formation of inverted micelle and hexagonal II phase in membranes, it can be expected that this neutral lipid may form

these specific membrane structures, considering the inverted cone structure [38].

Liposomes of a single phospholipid component undergo a thermal phase transition in a narrow temperature range [39]. Addition of other lipids influences the phase transition in various ways. Cholesterol expands the temperature range of phase transition and finally diminishes the transition [40,41]. In a binary mixture of synthetic phospholipids, in the miscible combination only a single transition appears between the transition temperatures of each phospholipid, whereas in an immiscible system transitions emerge around the transition temperatures of two phospholipids [42]. Natural phospholipids, such as egg yolk phosphatidylcholine and yeast phosphatidylinositol employed in the present experiments, are heterogeneous molecules, because of their different fatty acyl chain composition, demonstrating phase transition temperatures below 0°C for the high level of unsaturated fatty acids. Addition of yeast phosphatidylinositol to dipalmitoylphosphatidylcholine decreased and expanded the phase transition temperature markedly, and the effect was reduced in the presence of calcium ions (Fig. 4). In this context, phosphatidylinositol has a miscible property for the phase transition of dipalmitoylphosphatidylcholine. On the other hand, diacylglcyerol shows rather an immiscible property. Therefore, conversion from phosphatidylinositol to diacylglycerol is supposed to induce a phase change in membranes.

Lateral diffusion of membrane components is required for some functions of biological membranes, e.g. activation of adenylate cyclase by the β-adrenergic receptor [43]. Membrane proteins are surrounded by a special layer of phospholipids or boundary (annular) lipids, whose molecular motion is more restricted than bulk lipids [44]. Specific interaction of lipids and proteins are important for the functions of membrane proteins [1]. Membrane functions seem to be influenced by the change in the lateral distribution of the membrane component, i.e. lateral phase separation. In the present experiment, evidence was offered which demonstrates that conversion from phosphatidylinositol to diacylglycerol enhances the Ca^{2+}-induced phase separation of phosphatidylserine. Many types of cells are known to accelerate the phosphatidylinositol turnover in response to various stimuli, and phosphatidylinositol is at first converted to diacylglycerol by a phospholipase C. Ca^{2+}-dependent protein kinase requires phosphatidylserine, diacylglycerol and Ca^{2+} for its full activation [10]. The protein kinase is a cytosol enzyme which posseses a hydrophobic part. Complexes of calcium ion and phosphatidylserine in the phase separated region becomes hydrophobic since bound water is released by calcium ions [45]. Therefore, the hydrophobic interaction between the enzyme and the specified membrane site may contribute to its activation.

The electrical property was drastically changed by the replacement because phosphatidylinositol lost its charged moiety to become a neutral molecule, diacylglycerol. The change in surface potential was detected by fatty acid spin probe, using the protonation of the carboxyl group. The surface potential and the H^+ concentration of membrane surface may affect the various functions of membranes. Indeed, the apparent K_m of D-β-hydroxybutyrate dehydrogenase was enhanced by the increase of electrostatic potential caused by treatment with phospholipase D [46].

The phosphatidylinositol-diacylglycerol replacement decreased the membrane fluidity (Table II). Diacylglcyerol has a small polar head, which seems to allow the lipid molecules closely to contact each other.

In cell membranes, phosphatidylinositol content is rather small; at most about 5% of total phospholipids. The transformation of phosphatidylinositol to diacylglcyerol would not affect the physical properties of membranes as a whole. However, the hetergeneous distribution of membrane lipids is widely accepted; some phospholipids are specifically located around specific membrane proteins as boundary lipids [44,47], and asymmetric distribution of phospholipids is reported in biological membranes [28,48]. It is tempting to speculate that such transient lipid conversion might regulate membrane functions taking place in the local domains where phosphatidylinositol molecules are present.

In addition to phosphatidylinositol turnover, methylation of phosphatidylethanolamine to phosphatidylcholine was also observed to occur in a variety of cells when stimulated by various stimuli

[49]. In the presence of phosphatidylethanolamine, Ca^{2+}-induced phase separation of phosphatidylserine was enhanced in a model membrane system [50]. Increase of membrane fluidity coupled with the methylation of phosphatidylethanolamine was observed in red blood cells [51]. These findings, toghether with the conversion from phosphatidylinositol to diacylglycerol, suggest that the rapid alteration in membrane lipids triggered by stimulation would be an initial and pivotal event for further cellular processes.

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