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A POSSIBLE ROLE OF STIMULUS-ENHANCED PHOSPHATIDYLINOSITOL TURNOVER: CALCIUM-SPARING EFFECT OF DIACYLGLYCEROL IN INDUCING PHASE SEPARATION OF PHOSPHATIDYL-CHOLINE/PHOSPHATIDYLSERINE MIXTURES

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SUMMARY: A physiological role of phosphatidylinositol turnover accelerated by a stimulus was studied using a model system composed of egg yolk phosphatidylcholine (PC), bovine brain phosphatidylserine (PS), yeast phosphatidylinositol (PI) and diacylglycerol (DG) prepared from yeast PI by PI-specific phospholipase C of *Bacillus cereus*. The effects of PI and DG on Ca^{2+}-induced phase separation of PS were examined using the spin-spin exchange interaction of PC spin probes. DG enhanced Ca^{2+}-induced phase separation in PC/PS mixtures while PI had almost no effect on the phase separation. The results suggest a calcium-sparing effect of DG in inducing phase separation in the PS-containing membranes.

INTRODUCTION: Since the first discovery of the enhanced turnover of PI in response to a stimulus [1], this phenomenon has been observed in many types of cells and the basic mechanism(s) has been elucidated by many laboratories using biochemical approaches [2]. Several groups reported the hydrolysis of PI by phospholipase C and the consequent production of DG in activated platelets [3-5]. And the fluidity of platelet membranes increased remarkably in the activation [6]. However, the physiological role of the enhanced PI turnover was unknown in spite of numerous studies on the metabolism of PI. Recently, Kishimoto *et al.* found a Ca^{2+}-dependent protein kinase (protein kinase C) which requires PS, DG and calcium for its full activation [7]. They have shown that in human platelets the production of DG by exogenous phospholipase C is correlated with the activation of protein kinase C, and proposed a pos-

Abbreviations:

PC; phosphatidylcholine, PS; phosphatidylserine, PI; phosphatidylinositol DG; diacylglycerol.

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sible role of phospholipid turnover as a transmembrane signal [8]. But its mechanism remains unknown. To our knowledge, only scarce information is available about the physical properties of PI and DG despite their important roles in biological functions. In this communication, the effects of DG and PI on the Ca^{2+}-induced phase separation of PC/PS mixtures were studied using spin-spin exchange interactions of PC spin probes.

MATERIALS AND METHODS: PC was extracted from egg yolk according to the method of Singleton *et al.* [9]. PS was extracted from bovine brain as described by Sanders [10] and PI was extracted from baker's yeast as described by Trevelyan [11]. Each phospholipid gave a single spot on the silica gel H thin layer plate developed by $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (60:30:8:4, by vol). DG was prepared from PI by PI-specific phospholipase C which was purified from *Bacillus cereus* by the method of Ikezawa *et al.* [12]. 1,2-Diacylglycerol was separated on silica gel H thin layer plate developed by petroleum ether/diethylether/acetic acid (70:30:1, by vol). PC spin probe with a 12-nitroxide stearic acid at 2 position was kindly provided by Dr. S. Ohnishi of Kyoto University. The phospholipid content was determined by phosphorus assay according to Bartlett [13]. The content of DG was estimated from the quantitative analysis of fatty acyl chains by a gas-liquid chromatography using erucic acid as a standard.

For the studies of phase separation using a PC spin probe, multilayered lipid membranes were prepared in a Millipore filter with average pore diameter of 5 μm, SWP0130 [14]. The filter was dipped in benzene solution of a lipid mixture of about 40 mg/ml and then dried by vacuum for 3 hr. The dried filter was soaked into 25 mM Tris-HCl buffer (pH 6.8) containing Ca^{2+} and EGTA (Ethylene glycol bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid) overnight. Estimation of the concentration of free Ca^{2+} was based on an apparent binding constant of Ca^{2+}-EGTA of 8×10^5 M^{-1} [15].

The filter was set on a Teflon holder and the ESR spectrum was recorded at 23 °C by a commercial X-band spectrometer (JEOL FE-1X, JEOL, Tokyo) equipped with a variable temperature control. The spectral parameter, α, of exchange-broadened spectrum was calculated according to the definition by Devaux and McConnell [16].

RESULTS AND DISCUSSION: Mixtures of egg PC and bovine brain PS were adopted as model membranes, since Ca^{2+}-induced phase separation of PS was extensively studied in this system using a spin label technique [17-19] and differential scanning calorimetry [20].

Remarkable differences were observed in physical properties between PI and its derived DG. The PI and DG dispersions were examined by ESR using a stearic acid spin probe, and it was demonstrated that DG alone could not form bilayer structures, which was also confirmed by freeze-fracture electron micro-

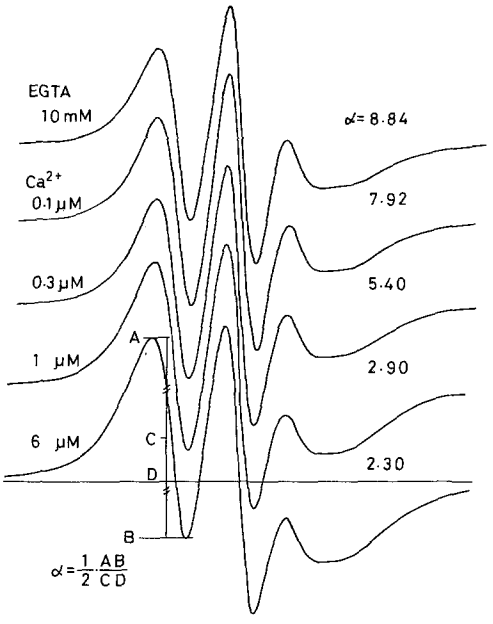

Fig. 1 ESR spectra of PC spin probe in PC/PS/DG (3:5:2, by molar ratio) at various concentrations of Ca^{2+}. The lipid mixture includes 7.8 mol % of PC spin probe. Multilayered membranes were prepared in pores of Millipore filter. The degree of spin-spin exchange broadening is estimated by α-parameter as shown in the figure.

scopy, and that DG no longer has susceptibility to calcium ions which PI possesses (unpublished data).

The use of spin-spin exchange broadening of ESR spectra is a suitable technique to detect the phase separation of phospholipids [17-19, 21]. In the present paper, the degree of phase separation was quantified by the method of Ohnishi and Tokutomi [22, 23], and was defined as the ratio of Ca^{2+}-PS complexes (solid phase) to total PS. Fig. 1 shows the ESR spectra of PC/PS/DG (3:5:2, by molar ratio) including 7.8 mol % of PC spin probe at various concentrations of calcium ions. The ESR spectra show increases in the degree of exchange-broadening in a Ca^{2+} concentration-dependent fashion which is characterized by α-parameter. Smaller value of α indicates a stronger spin-spin exchange interaction, *i.e.* the apparent concentration of PC spin probe is increased by phase separation. The addition of calcium ions into PC/PS mixtures caused the Ca^{2+}-induced phase separation. Ca^{2+} ions gather PS molecules

and consequently exclude PC and spin-labeled PC, which results in the higher concentration of PC spin probe in the segregated area. In order to obtain its apparent concentration, the α-parameters were measured for known composition of PC/PC spin probe mixtures (5 - 40 mol % of PC spin probe). The apparent concentration of PC spin probe was estimated from the standard curve of α *vs.* [PC spin probe] mol %. Then the degree of phase separation was quantified from the change in the apparent concentration of PC spin probe, assuming that PS molecules form solid regions by interaction with calcium ions and that the regions do not include any PC and PC spin probes. For example, when the concentration of PC spin probe obtained from α-parameter is 1.5 fold of the control value in the presence of 10 mM EGTA, the fluid area should be two third of the control, *i.e.* the apparent probe concentration and the size of fluid area are in inverse proportion because PC spin probes are excluded into the fluid area. Provided that the control mixture is completely fluid and homogeneous without Ca^{2+}, by the addition of Ca^{2+} one third (33 %) of the lipid mixture, *i.e.* the rest of fluid area, is expected to form solid domains composed of Ca^{2+}-PS complexes. As the mixture contains 50 mol % of PS, the degree of phase separation is calculated as $\frac{33\ \%}{50\ \%} \times 100\ \% = 66\ \%$ [23]. This method was applied for studying the Ca^{2+}-induced phase separation of PC/PS/PI and PC/PS/DG mixtures. Fig. 2 shows the degree of phase separations induced by Ca^{2+} in PC/PS mixtures containing PI or DG. The two curves are obviously different. The phase separation was occurred at lower Ca^{2+} concentrations in DG-containing lipid mixtures than PI-containing ones with no DG. Such Ca^{2+}-sparing effect of DG was further examined at various contents of DG in PC/PS mixtures (Fig. 3). By increasing the DG content, the curve in general shifted upward, especially at lower concentrations (0.3 ∿ 10 μM) of calcium ions. On the other hand, the curve of PC/PS mixtures containing PI in Fig. 2 almost fits the PC/PS mixtures in Fig. 3. This suggests that PI has little or no effect on the Ca^{2+}-induced phase separation. PI, as well as PS, is a negatively charged phospholipid. However, the behaviours of two phospholipids in

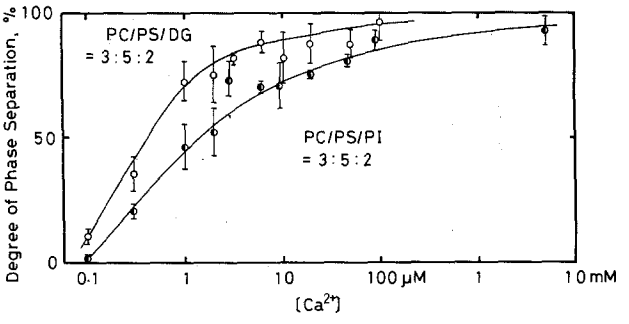

Fig. 2 Degree of phase separation of PS *vs.* Ca^{2+} concentration in PC/PS/DG (3:5:2) and PC/PS/PI (3:5:2) mixtures. The degree of phase separation was obtained as described in the text. Each plot shows the average and the standard deviation of three independent experiments.

inducing phase separation were very different [24]. At a concentration of 10 μM Ca^{2+}, only 7 % of total PI is phase-separated while phase-separated PS is much larger (75 %). Even at 10 mM Ca^{2+}, the degree of PI-phase separation is 35 % of total PI (data not shown). Therfore, the Ca^{2+}-induced phase separation of PI does not seem to affect practically the degree of phase separation of PS.

Ca^{2+}-induced phase separation would somehow alter the properties of membrane surface. The hydrophobic surface was produced on the Ca^{2+}-PS areas in the lipid bilayer, since bound water is replaced by calcium ions [25]. Such reorganization of membrane lipids may explain some underlying mechanism(s) of PI turnover-related functions in biological membranes. A typical example is the activation of calcium-dependent protein kinase (protein kinase C). This enzyme is water soluble and activated irreversibly by Ca^{2+}-dependent neutral protease [26-28]. Also reversible activation can be caused by PS, DG and calcium ions [7]. Now we would like to propose a possible mechanism for the reversible activation of the kinase. When cells are exposed to exogenous stimuli, PI-specific phospholipase C is activated leading to formation of DG. Then increased DG content in membranes triggers the Ca^{2+}-induced phase separation of PS by which hydrophobic domains are consequently formed. The newly produced hydrophobic regions may interact with hydrophobic part(s)

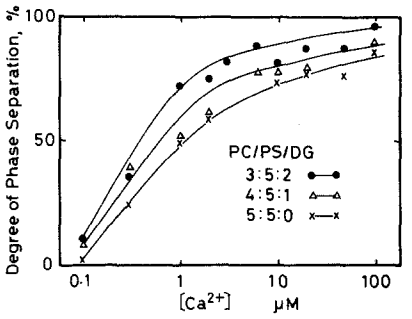

Fig. 3 Degree of phase separation of PS *vs.* Ca^{2+} concentration in PC/PS mixtures containing various amounts of DG. Each point shows the average of three independent experiments.

in protein kinase C molecule. The decrease in DG content through PI cycle (diacylglycerol → phosphatidic acid → phosphatidylinositol) would render the kinase inactivated. The proposed concept obtained with model membranes that DG may facilitate Ca^{2+}-induced phase separation of PS cannot be applied directly to biological membranes because the content of DG is much smaller in cell membranes. However, it is not unreasonable to think that small localized domains rich in DG might be produced by PI hydrolysis.

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