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Phase Separation in Phosphatidylcholine/Anionic Phospholipid Membranes in the Liquid-Crystalline State Revealed with Fluorescent Probes[1]

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The mixing properties of anionic phospholipids such as phosphatidic acid (PA), phosphatidylserine (PS), and phosphatidylglycerol (PG) with phosphatidylcholine (PC) were examined in the liquid-crystalline state of membranes using extrinsic fluorescent probes incorporated into lipid bilayers. The excimer to monomer (E/M) fluorescence ratio of 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (PPC) was higher for the PA and PS matrices as compared to that for the PC matrix. When PC was replaced with PA or PS, the E/M ratio of PPC also increased in a concentration-dependent manner. When the concentration of PA or PS was increased in the PC membrane, the fluorescence of 1-palmitoyl-2-[12-[7-nitro-2,1,3-benzodiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine decreased, indicating the occurrence of lipid clustering. Direct evidence for the PA or PS-induced phase separation in the PC/PA or PC/PS system was provided by the resonance energy transfer between 2-(4,4-difluoro-5-methyl-4-boro-3*a*,4*a*-diaza-*s*-indacene-3-dodecanoly)-1-hexadecanoly-*sn*-glycero-3-phosphocholine and PPC. The fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene further supported the lateral organization of membranes by PA and PS. PA and PS also reduced the polarity of lipid bilayers, as measured by the emission fluorescence of 6-lauroyl-2-dimethylaminonaphthalene. On the other hand, PG had very little effect on the PC matrix, suggesting the ideal miscibility with PC molecules. The results suggest that the mixing properties of PA and PS in the PC matrix are not random but that phase separation occurs in the liquid-crystalline phase of membranes.

Key words: anionic phospholipids, liquid-crystalline phase, phase separation, phosphatidylcholin.

The current view of biological membranes emphasizes the dynamic coupling between the organization and function of membranes (*1, 2*). Several lines of evidence indicate that considerable heterogeneity in the lateral organization and domain formation of lipids and proteins may exist in biological membranes due to lipid-lipid, lipid-protein, and protein-protein interactions (*3*). The formation of these microdomains is believed to be relevant to the function of biological membranes, and to be linked to important cellular phenomena such as signal transduction, cholesterol efflux, and intracellular lipid and protein sorting (*4, 5*). However, the molecular interactions responsible for the formation of segregated lipid domains in natural or model lipid membranes are not fully understood.

Many non-ideal mixing properties of lipid molecules have been suggested in membranes containing artificial lipids. In general, a heterogeneous distribution of lipids can be defined as lipid-domains, lipid-clustering, phase separation, and so on. In particular, binary mixtures containing anionic lipids have been the subject of extensive study on lipid dynamics due to their important physiological roles in biological membranes. It has been shown that many peripheral and integral membrane proteins specifically recognize anionic lipids such as phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylglycerol (PG), and phosphatidylinositol (PI), and that their enzymatic activities and membrane topologies are regulated by these lipids (*6-8*). Several lines of evidence have been found for the lateral organization of anionic phospholipids in lipid bilayers. Ca^{2+} can induce the phase separation of anionic phospholipids through charge neutralization (*9, 10*). Polycations (*11*) and basic proteins, such as cytochrome *c* (*12*), annexin IV (*13*), and protein kinase C (*14*), have also been shown to cause

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Abbreviations: PC, phosphatidylcholine; PA, phosphatidic acid; PS, phosphatidylserine; PG, phosphatidylglycerol; LUVs, large unilamellar vesicles; PPC, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine; NBD-PC, 1-palmitoyl-2-[12-[7-nitro-2,1,3-benzodiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; BODIPY-PC, 2-(4,4-difluoro-5-methyl-4-boro-3*a*,4*a*-diaza-*s*-indacene-3-dodecanoly)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; Laurdan, 6-lauroyl-2-dimethylaminonaphthalene.

the clustering of anionic phospholipids. However, the self-ordering processes of these anionic phospholipids are not fully understood. It seems worthwhile, therefore, to study the mixing properties of anionic phospholipids in the liquid-crystalline phase of membranes. These properties may be important for understanding membrane functions.

In this study, we examined the mixing properties of anionic phospholipids, PA, PS, and PG, with phosphatidylcholine (PC) as binary mixtures using 1-palmitoyl-2-oleoyl phosphoglycerides, which are closer to the structures found in mammalian membranes. We found here that PA and PS exhibited non-random miscibility with PC in the liquid-crystalline phase of membranes but that PG did not show this property.

EXPERIMENTAL PROCEDURES

Materials—Most of the fluorescent probes [1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (PPC), 1,6-diphenyl-1,3,5-hexatriene (DPH), 2-(4,4-difluoro-5-methyl-4-boro-3*a*,4*a*-diaza-*s*-indacene-3-dodecanoly)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (BODIPY-PC), and 6-lauroyl-2-dimethylaminonaphthalene (Laurdan)], were from Molecular Probes (Eugene, OR). All of the unlabeled phospholipids and 1-palmitoyl-2-[12-[7-nitro-2,1,3-benzodiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) were obtained from Avanti Polar Lipids (Alabaster, AL), and used without further purification. The lipids were stored as chloroform solutions under argon at −20°C. Other chemicals were of the highest grade commercially available.

Liposome Preparation—To prepare vesicles containing pyrene-labeled lipid, 0.5-10 mol% of PPC was incorporated into liposomes. To determine the colocalization of probes, 1 mol% PPC and 1 mol% BODIPY-PC were incorporated into liposomes. To investigate fluorescence polarization (*P*), 0.5 mol% DPH was included in liposomes. Laurdan excitation and emission spectra were obtained using 0.2 mol% of the probe in large unilamellar vesicles (LUVs). After mixing of appropriate amounts of lipids in chloroform the solvent was evaporated under a stream of argon gas. The dry lipids were hydrated in a buffer solution (25 mM Tris-HCl, pH 7.4, containing 100 mM NaCl and 1 mM EDTA) by vortexing and subsequent sonication in a bath sonicator for 30 s. The dispersions were frozen and thawed five times, and then passed 25 times through two polycarbonate membranes (100 nm pore size) in an Extruder LiposoFast (Avestin, Ottawa, ON) as described (*15*). All LUVs used for this work were stable for at least 3 days. The change in the light scattering measured with a spectrofluorometer at 450 nm was less than 10% deviation during this time. The concentrations of nonfluorescent phospholipids were determined by means of a phosphorus assay (*16*). The concentrations of fluorescent probes were determined spectrophotometrically at 342 nm using 42,000 cm^{-1} for PPC, at 350 nm using 88,000 cm^{-1} for DPH, at 465 nm using 22,000 cm^{-1} for NBD-PC, and at 500 nm using 80,000 cm^{-1} for BODIPY-PC, and that of Laurdan at 364 nm using 20,000 cm^{-1} as the molar extinction coefficient.

Fluorescence Measurements—All experiments were performed at 35°C. Fluorescence spectra were recorded with a Shimadzu RF-5301 PC spectrofluorometer equipped with a thermostatted cuvette compartment. When the excimer to monomer (E/M) fluorescence ratio of pyrene-labeled phospholipids was determined, the excitation wavelength was 342 nm and the emission wavelength was in the range of 360-500 nm. The fluorescence intensities at 375 nm (for monomer) and 480 nm (for excimer) were selected to calculate the E/M ratio. The emission fluorescence of NBD-PC was measured at 534 nm an excitation wavelength of 465 nm. To determine the colocalization of fluorescent probes, the excimer fluorescence intensities of PPC were measured in the presence and absence of BODIPY-PC. To measure steady-state fluorescence polarization (*P*), the excitation wavelength of 350 nm and the emission wavelength of 452 nm were selected with monochromators, and *P* values were calculated with the following equation (*17*).

$$P=(I_{\parallel}-I_{\perp})/(I_{\parallel}+I_{\perp})$$

$I_{\parallel}$ and $I_{\perp}$ are the intensities measured with polarizers parallel and perpendicular to the vertically polarized exiting beam, respectively. For the Laurdan experiment, 364 and 480 nm were selected for the excitation and emission spectra, respectively. To prevent the excimer fluorescence quenching effect of oxygen (*18*), the buffer solution was saturated with argon gas for more than 2 h before use.

RESULTS

PPC Distribution in Anionic Phospholipids or the PC Matrix—Excited-state pyrene molecules give two characteristic emission fluorescence spectra for the monomer and excimer, respectively. The E/M ratio is strictly dependent on the rate of collision of the pyrene molecules. Consequently, in lipid bilayers, pyrene-labeled lipids reflect the lateral diffusion rate of the probes or their local concentration, or both. In order to determine the effects of anionic phospholipids on the lipid dynamics of PPC, the E/M ratio of PPC in anionic phospholipids or the PC matrix was determined as a function of the PPC concentration in the liquid-crystalline state of membranes. Figure 1 shows that the E/M ratio increased with increasing PPC concentration in all phospholipid matrices but the slope of the plot varied

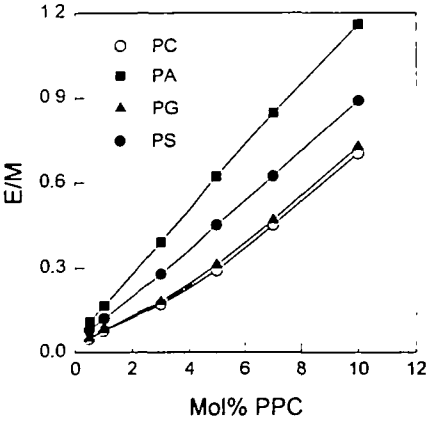

Fig. 1. **E/M ratio change in PC and anionic phospholipid matrices as a function of the PPC concentration.** Liposomes containing the indicated amounts of PPC in each phospholipid matrix were prepared, and then the E/M ratio was determined as described under "EXPERIMENTAL PROCEDURES."

with the type of phospholipid. All the anionic phospholipids examined here induced an increase in the slope, in the order of PA>PS>PG, compared to that for PC. This indicates that the mode of PPC distribution in phospholipid matrices is not random but largely dependent on the phospholipid head group species. This also suggests that the PPC segregation and/or the lateral diffusion rate of the probe are higher for anionic phospholipid matrices as compared to the PC matrix.

Effects of Anionic Phospholipids on the E/M Ratio of PPC—To determine the effect of the anionic phospholipid concentration on the excimer fluorescence of PPC, the E/M ratio was examined by replacing PC with PA, PS, or PG in liposomes that initially contained 99 mol% PC and 1 mol% PPC. When the concentration of anionic phospholipids was increased to 50 mol%, PA and PS increased the E/M ratio of PPC by 47 and 20%, respectively (Fig. 2). It was also found that the plot was progressive for the PC/PA system but linear for the PC/PS system. It seems that a certain critical concentration of PA is needed to increase the excimer fluorescence of PPC. On the other hand, PG had no effect on the E/M ratio of PPC. This finding also suggests that PA and PS molecules induce the local enrichment of PPC into domains and/or an increase in the mobility of the probe.

Effects of Anionic Phospholipids on the NBD-PC Fluorescence—In order to support that anionic phospholipids cause the lateral segregation of PPC, we utilized the self-quenching of the fluorescence of an NBD-labeled phospholipid (*19*) providing the information on lipid clustering in membranes. When PC was replaced with the anionic phospholipids, the fluorescence of NBD-PC decreased by about 18 and 10% with 50 mol% of PA and PS, respectively (Fig. 3). In contrast, PG had almost no effect on the quenching of NBD fluorescence. This indicates that distinct PC-rich and concomitant PA-rich (or PS-rich) phases appear in the PC/PA (or in the PC/PS) system, and supports that PA or PS induced-lateral separation of PPC into enriched domains contributed to the increase in the E/M ratio.

DPH Polarization—To determine whether or not anionic phospholipids induce an increase in the mobility of PPC in lipid bilayers, we measured the fluorescence polarization of DPH incorporated into vesicles by replacing PC with the anionic phospholipids. Figure 4 shows that all the anionic phospholipids examined here had, if any, little effect on the polarization. This indicates that the microviscosity and/or acyl chain order of lipids did not change by the presence of anionic phospholipids. Accordingly, this suggests that the PA or PS-induced segregation of PPC into enriched domains is a major reason for the increase in the E/M ratio. But we can not exclude the possibility that DPH only reflects the local properties of its microenvironment rather than those of macroscopic flow as suggested previously (*20*).

Colocalization of PPC and BODIPY-PC—To ascertain that phase separation of phospholipids in PC/PA and PC/PS binary mixtures is responsible for the increase in the E/M ratio of PPC and the fluorescence quenching of NBD-PC, we utilized resonance energy transfer between PPC and BODIPY-PC. As the dipyrromethene boron difluoride group exhibits a maximum absorption spectrum at around 500 nm (*21*), it was possible to make use of the energy transfer

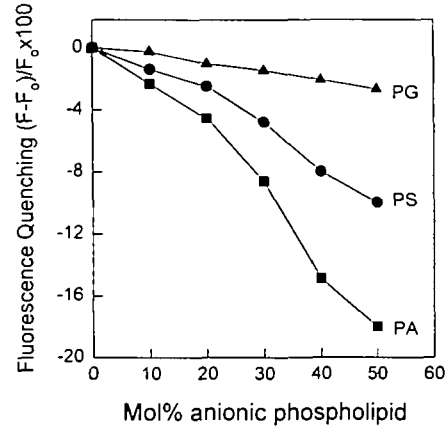

Fig. 3. **Quenching of NBD-PC fluorescence induced by anionic phospholipids.** 2 mol% of NBD-PC was included in all samples. F and F_o are the fluorescence intensities at 534 nm in the absence (F_o) or presence (F) of anionic phospholipids.

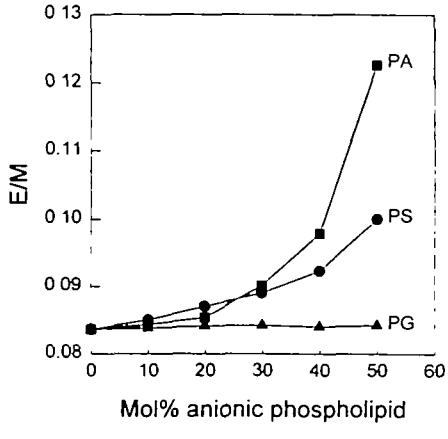

Fig. 2. **Effects of anionic phospholipid concentrations on the E/M ratio of PPC.** The fluorescence of PPC (1.5 mol%) incorporated into membranes was examined by replacing POPC with the anionic phospholipids, PA, PS, and PG. Data were obtained for two independent experiments.

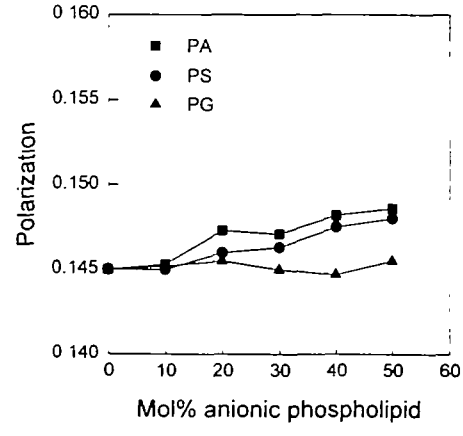

Fig. 4. **Dependence of the fluorescence polarization of DPH on the concentrations of anionic phospholipids.** 0.5 mol% DPH was incorporated into membrane vesicles and then fluorescence intensity was measured at 452 nm (excitation: 350 nm) with monochromators.

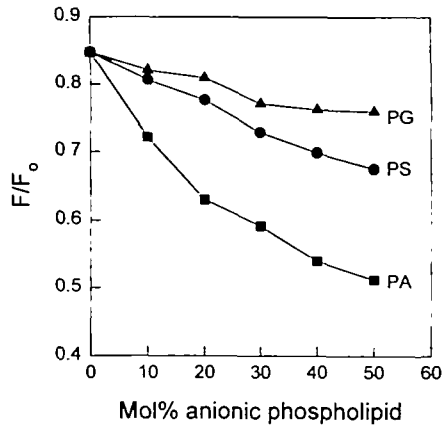

Fig. 5. **Efficiency of quenching of the excimer fluorescence of PPC by BODIPY-PC with increasing concentrations of anionic phospholipids.** F/F_o is the ratio of the fluorescence intensities at 480 nm for LUVs containing only 1 mol% PPC (F_o) or 1 mol% PPC and 1 mol% BODIPY-PC (F).

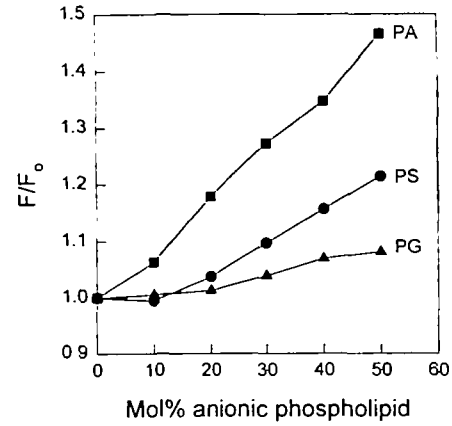

Fig. 7. **Dependence of the emission fluorescence of Laurdan at 420 nm on the concentrations of anionic phospholipids.** F/F_o represents the fluorescence intensity ratio in the presence (F) and absence (F_o) of anionic phospholipids. Other experimental conditions are the same as in Fig. 6.

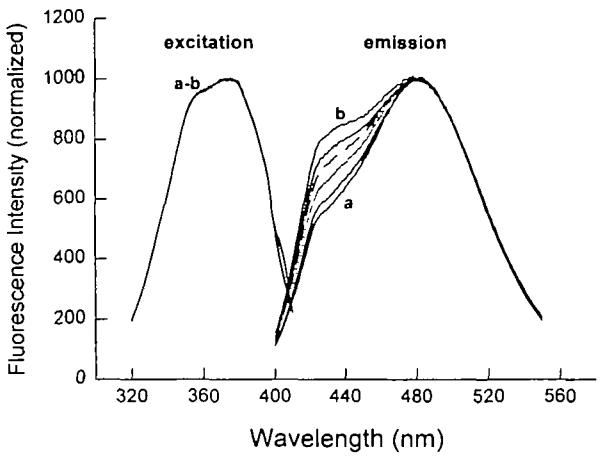

Fig. 6. **Change in the membrane polarity with PA concentration.** Laurdan excitation and emission spectra were normalized. PA mol% with respect to PC, from a to b: 0, 10, 20, 30, 40, and 50.

between these two fluorophores to estimate their colocalization. We determined the quenching efficiency (F/F_o) as a function of each anionic phospholipid concentration, where F and F_o are the intensities of excimer emission of PPC measured in the presence (F) and absence (F_o) of the quencher, BODIPY-PC. F/F_o ratios of 0 and 1 correspond to complete quenching of excimer fluorescence and no quenching, respectively.

When the concentration of PA, PS, or PG within the PC matrix was increased, the quenching was gradually enhanced but with different efficiencies (Fig. 5). 50 mol% of PA and PS decreased the F/F_o value by about 40 and 18%, respectively. However, only slight quenching of PPC fluorescence was seen when PG was used instead of PA or PS. This indicates that PPC and BODIPY-PC become enriched into domains or the distance between the two probes gets less with increasing PA or PS concentration. The colocalization also provides direct evidence that phase separation in PC/PA and PC/PS binary mixtures occurs in an anionic phospholipid concentration-dependent manner in the liquid crystalline state of membranes.

Measurement of Membrane Polarity with a Laurdan Probe—To obtain further insight into the molecular behavior of anionic phospholipids in the PC matrix, the excitation and emission fluorescence of Laurdan were utilized. It has been shown that Laurdan fluorescence is extremely sensitive to the polarity of the environment of its fluorescent moiety (*22*). Therefore, we could examine the effects of anionic phospholipids on the polarity of fluid membranes by using the fluorescent properties of the probe.

Figure 6 shows that the replacement of PC with PA resulted in an increase in the emission spectra of Laurdan. All the excitation and emission spectra of Laurdan display the typical features of the liquid-crystalline phase (*23*). An increase only in the blue part of the emission spectra (400-450 nm) with increasing PA concentration was shown but the excitation spectra did not change. PS and PG also induced a change in the emission fluorescence of Laurdan but not in its excitation fluorescence. Figure 7 shows the dependence of Laurdan emission fluorescence at 420 nm on the concentrations of anionic phospholipids in the PC matrix. PA had the most profound effect on the increase in the fluorescence, followed by PS. However, PG showed little effect. An increase in the intensity of the blue part of the emission spectra was observed in the case of decreased polarity (*22*). These results, therefore, indicate that PA and PS molecules within the PC matrix reduce the polarity of phospholipid bilayers and decrease the dipolar relaxation. This also means that the molecular motion of phospholipids in lipid bilayers decreases with increasing amounts of PA and PS, and this may be due to the increased packing order between PA or PS molecules.

DISCUSSION

In this study, we found that the mixing properties of PA and PS with PC in the liquid-crystalline phase of membranes are not random but that phase separation of phospholipids occurs with increasing concentrations of these anionic phospholipids in lipid bilayers. The PA or PS-induced colocalization of PPC and BODIPY-PC may be strong

evidence for the lateral separation of phospholipids in the PC/PA and PC/PS binary systems. The result of polarization by DPH further supports that phase separation of lipids caused by PA or PS is responsible for the increase in the E/M ratio of PPC, the decrease in NBD-PC fluorescence, and the colocalization of PPC with BODIPY-PC. However, PG had very little effect in these experiments, suggestive of almost ideal miscibility with PC molecules.

At present, we do not have a satisfactory explanation for the phase separation of PC/anionic phospholipids in the liquid-crystalline state of membranes. The formation of intermolecular hydrogen bonding between PA or PS molecules in spite of their negative charges, as suggested previously (for a review, see Ref. *24*), may be responsible for the non-random mixing properties of these anionic phospholipids with PC. This hypothesis is strongly supported by the fact that PA and PS exhibit higher gel to liquid-crystalline phase-transition temperatures at neutral pH as compared to that for PC, which was shown by means of a calorimetric study (*24*). In contrast, it was suggested that PG does not interact intermolecularly even though it has hydrogen bond-donating and -accepting groups. However, these investigations were focused on the packing and the interaction of phospholipids in the gel phase of membranes. Therefore, it may be important to understand the molecular behavior of phospholipids in the liquid-crystalline phase, in which most biological membranes are in a functionally active and physiological state.

The phase separation or lateral organization of lipids may be important in providing particular environments for reactions on or in the membrane. In particular, the phase separation could affect or control the protein functions in biological membranes. In relation to lipid domains enriched with anionic phospholipids, it was suggested that the formation of membrane domains containing PS is important for the activation of protein kinase C (*25*). Phospholipase A_2 from *Naja naja* was found to bind to membrane domains containing negatively-charged lipids (*26*). Closely relevant to biological significance of our finding, it was shown that PA and PS enhanced the activity of Ca^{2+}-independent protein kinase from human platelets in the order of PA > PS, but that PG was ineffective (*27*). PA also reduced the PS requirement and cooperativity in the activation of protein kinase C, but PG was less effective that PA (*28*). Therefore, our finding that phase separation occurred in PC/anionic phospholipid mixtures might be important for determining the biological functions of membranes *in vivo*. But at present, our results do not provide information on the phase behavior when additional lipid components such as PE or sterol, which are constituents of the biological cell membrane, are present in lipid bilayers. Also, the phase separation in liposomes composed of PC and anionic phospholipids seems to occur "partially," as judged from the result that the PA and PS-induced increases in the E/M ratio of PPC are about 47 and 20%, respectively. The self-quenching of NBD-PC fluorescence (about 18% by PA and 10% by PS), and the colocalization of PPC and BODIPY-PC (F/F_o about 0.5 at 50 mol% PA and 0.7 at 50 mol% PS) also suggest that "partial phase separation" occurs in PC/PA and PC/PS binary mixtures.

In conclusion, our results provide evidence concerning the lateral phase separation of anionic phospholipids in membranes. However, it should be further determined whether or not the use of fluorescent probes can reveal the real phase behavior of phospholipids in lipid bilayers because the probes carry bulky fluorescent moieties as indicators of possible segregation processes.

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