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Ca^{2+}-INDUCED LATERAL PHASE SEPARATIONS IN PHOSPHATIDIC ACID–PHOSPHATIDYLCHOLINE MEMBRANES

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SUMMARY

The effects of Ca^{2+} on phosphatidic acid-phosphatidylcholine membranes have been studied using phospholipid spin labels. ESR spectra of spin-labeled phosphatidic acid–phosphatidylcholine membranes and phosphatidic acid–spin-labeled phosphatidylcholine membranes are exchange-broadened immediately upon addition of $CaCl_2$. These changes directly and conclusively indicate Ca^{2+}-induced clustering of spin-labeled phosphatidylcholine and aggregation of spin-labeled phosphatidic acid bridged by Ca^{2+}-chelation in the binary phospholipid membranes. In the Ca^{2+}-chelated aggregates, the motions of the alkyl chains of phosphatidic acid are greatly reduced and the lipid molecules are more closely packed. The clusters and aggregates are formed in patches and the sizes are dependent on the fractions. Ba^{2+} and Sr^{2+} induce the lateral phase separations to the same extent as Ca^{2+}. Mg^{2+} is also effective but to a lesser extent. In acid solutions (pH 5.5), the Ca^{2+}-induced lateral phase separations are of slightly lesser extent than in alkaline solution (pH 7.9). These results are compared with those for phosphatidylserine–phosphatidylcholine membranes reported previously and necessary conditions for the lateral phase separations are discussed.

INTRODUCTION

Ca^{2+} plays an essential rôle in many cellular functions involving membrane systems, such as nerve excitation, cell adhesion, hormone release, etc. The effects have been extensively studied and analyzed mostly from phenomenological aspects [1–3]. Structural studies on the Ca^{2+} effects are relatively few, however, and the molecular mechanisms underlying the Ca^{2+}-requiring physiological phenomena are not yet clear. We have carried out structural studies using phospholipid spin-labels and found Ca^{2+}-induced lateral phase separations in some binary phospholipid membranes. In phosphatidylserine–phosphatidylcholine membranes, Ca^{2+} caused rapid and reversible aggregation of phosphatidylserine molecules by intermolecular chelation and concomitant clustering of phosphatidylcholine molecules [4, 5]. Ba^{2+} and Sr^{2+} were also able to induce the lateral phase separations but to lesser extents,

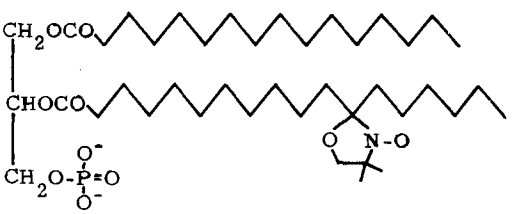
while Mg^{2+} was completely ineffective. In the present paper, we study effects of Ca^{2+} on phosphatidic acid–phosphatidylcholine membranes using spin-labeled phosphatidylcholine and phosphatidic acid. The lateral phase separations are clearly demonstrated by Ca^{2+}-induced exchange-broadening in ESR spectra of phosphatidic acid–spin-labeled phosphatidylcholine and spin-labeled phosphatidic acid–phosphatidylcholine membranes. The effects are quite similar to those on phosphatidylserine–phosphatidylcholine membranes except for one marked difference in the ionic selectivity. The factors leading to the lateral phase separations are discussed from these results.

Binding of Ca^{2+} to pure phospholipid membranes has been studied by many authors with monomolecular films and lipid vesicles [6]. Some of these results are discussed in relation to the present findings.

EXPERIMENTAL

Materials

Phosphatidylcholine was obtained from egg yolk by the method of Singleton et al. [7]. Phosphatidic acid was prepared by the action of phospholipase D on the phosphatidylcholine and purified according to the procedure by Kornberg and McConnell [8]. Phospholipase D was prepared from cabbage inner leaves and purified to purification step 3 (acetone precipitation) as described by Yang [9]. Spin-labeled phosphatidylcholine was synthesized by acylation of egg lysolecithin with anhydride of 4′,4′-dimethyloxazolidine-*N*-oxyl derivative of 12-keto stearic acid by the method of Hubbell and McConnell [10]. Spin-labeled phosphatidic acid (see



Spin-labeled phosphatidic acid

formula) was prepared as the Na^+ salt by hydrolysis of spin-labeled phosphatidylcholine with phospholipase D. A mixture of 20 mg of the labeled phosphatidylcholine, 20 mg of freshly prepared phospholipase D in 10 ml of 0.1 M acetate buffer (pH 5.5), 1 ml of 0.1 M $CaCl_2$ and 1.5 ml of diethyl ether was shaken for 3 h at 37 °C and added to 180 mg of citric acid monohydrate. The mixture was then added to 2.5 ml of chloroform, shaken and centrifuged. The chloroform phase and the precipitate were collected and the aqueous phase was further extracted twice with 2.5-ml portions of chloroform. The combined chloroform phase and the precipitate were washed with 2.5 ml of 0.1 M citrate and, after removal of precipitate, concentrated by evaporation under N_2 to 4 ml. The chloroform phase was then shaken with 1 ml of 0.1 M sodium citrate buffer (pH 5.2), washed with 1 ml of water, dried and evaporated. Silica thin-layer chromatograms were developed in chloroform–methanol–28% NH_4OH (70 : 26 : 4, by vol.). No spots corresponding to phosphatidylcholine

and lysophosphatidic acid were observed. Some traces of spot were discernible at the top. However, no further purification was performed.

Phospholipid membranes

Phospholipid membranes were prepared on Millipore filter SMWP 02500 with average pore diameter 5 μm as described in previous papers [4, 5]. A mixture of phospholipid and the label was dissolved in benzene, the filters cut in pieces of approx. 0.5 cm $\times$ 0.4 cm and dipped into the mixture. The average amount of impregnated lipids was approx. 0.6 mg per piece. The dried filters were then soaked in 100 mM KCl solution for at least 2 h for conditioning. The conditioned filters were transfered to 5 ml of the desired salt solutions and their ESR spectra measured at 25 °C. All the solutions contained 100 mM KCl in addition to the indicated salts. The pH of the salt solutions was adjusted with 50 mM Tris–HCl buffer in the slightly alkaline region and with 50 mM acetate buffer in the acidic region. It is suggested that the phospholipid membranes thus obtained have a bilayer structure, by analogy of their ESR spectra with those of sonicated phospholipid vesicles [5].

RESULTS

Ca^{2+}-induced lateral phase separations as observed with phosphatidic acid–spin-labeled phosphatidylcholine membranes

When the conditioned membranes were transfered to aqueous salt solutions containing 10 mM $CaCl_2$, their ESR spectra became immediately exchange-broadened. As an example, Fig. 1 shows the spectral change at pH 7.9 for phosphatidic acid–spin-labeled phosphatidylcholine (9 : 1) membrane. The change was rapidly reversed by addition of EDTA. The exchange-broadening was dependent on the contents of the labeled phosphatidylcholine in the membranes, those with higher contents giving larger broadening. All the characteristics of the Ca^{2+}-induced changes were quite similar to those of phosphatidylserine–spin-labeled phosphatidylcholine membranes. The same discussions [5] therefore hold for the phosphatidic acid–spin-labeled phosphatidylcholine membranes and lead to the conclusion that Ca^{2+} causes clustering of the labeled phosphatidylcholine molecules in patches in the bilayer membrane.

Ca^{2+}-induced lateral phase separations as observed with spin-labeled phosphatidic acid–phosphatidylcholine membranes

The Ca^{2+} effects on the binary phospholipid membranes were investigated on the part of the phospholipid molecules to be aggregated by Ca^{2+}. Figs 2 and 3 show the Ca^{2+}-induced spectral changes in spin-labeled phosphatidic acid–phosphatidylcholine (1 : 9 and 1 : 4) membranes, respectively. The Ca^{2+} spectra consist of two components, sharp and broad background. Decomposition into the two components was made by subtraction of the Ca^{2+} spectra by a model spectrum with no exchange-broadening (see Fig. 4). The results yielded Spectra a and b indicated in Fig. 4. The broad component comprised approx. 86% of the total intensity for the (1 : 9) membrane and approx. 95% for the (1 : 4) membrane. The broadening in the spectra is mainly due to the intermolecular spin–spin interactions, exchange and dipolar interactions. The latter contribution could be considerable in this case since the

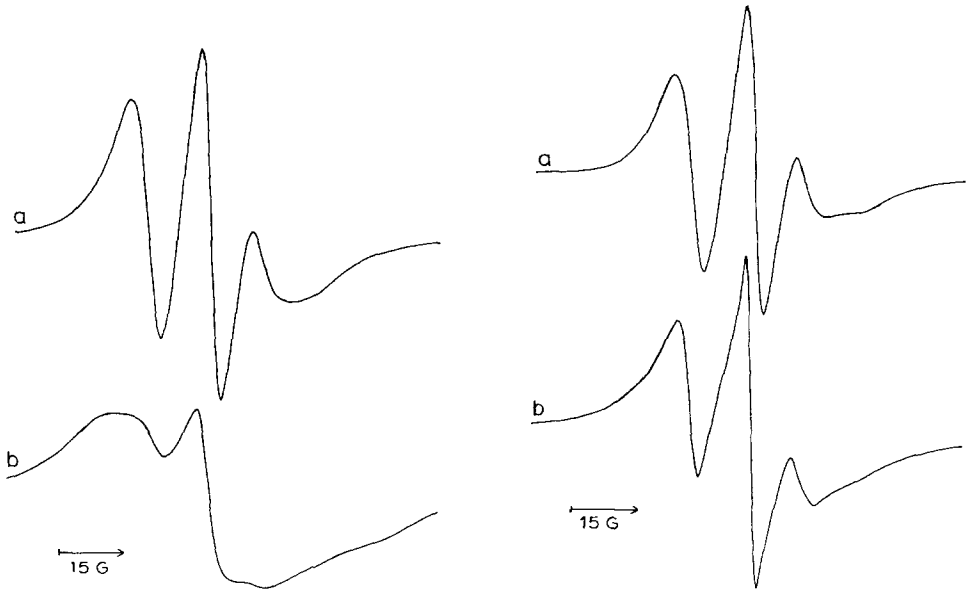


Fig. 1. ESR spectra of phosphatidic acid-spin-labeled phosphatidylcholine (9 : 1) membrane in (a) 10 mM EDTA and (b) 10 mM $CaCl_2$. The solutions contain additionally 100 mM KCl and 50 mM Tris–HCl buffer (pH 8.0).

Fig. 2. ESR spectra of spin-labeled phosphatidic acid–phosphatidylcholine (1 : 9) membrane in (a) 10 mM EDTA and (b) 10 mM $CaCl_2$. The solutions contain additionally 100 mM KCl and 50 mM Tris–HCl buffer (pH 8.0).

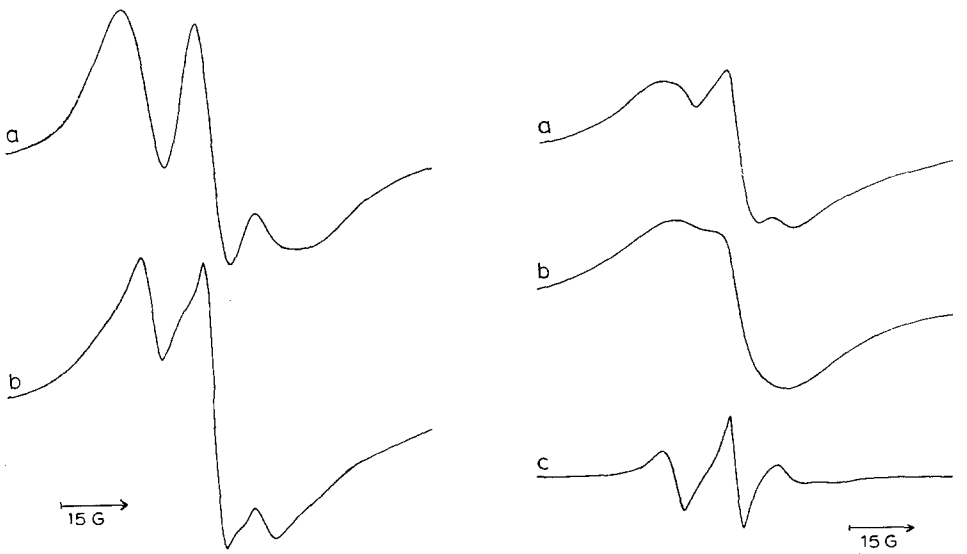


Fig. 3. ESR spectra of spin-labeled phosphatidic acid–phosphatidylcholine (1 : 4) membrane in (a) 10 mM EDTA and (b) 10 mM $CaCl_2$. The solutions contain additionally 100 mM KCl and 50 mM Tris–HCl buffer (pH 7.7).

Fig. 4. Subtraction of ESR spectra of spin-labeled phosphatidic acid–phosphatidylcholine membranes in the presence of Ca^{2+} with a Model Spectrum c. (a) Spectrum of the (1 : 9) membrane in 10 mM $CaCl_2$ (Fig. 2b) is subtracted by the Model Spectrum c. (b) Spectrum of the (1 : 4) membrane in 10 mM $CaCl_2$ (Fig. 3b) is subtracted by the Spectrum c. (c) Spectrum of the (1 : 50) membrane in the absence of Ca^{2+}.

motions of lipid alkyl chains are greatly reduced in the Ca^{2+}-chelated aggregates of phosphatidic acid as described later. The broadening was dependent on the contents of the labeled phosphatidic acid in the membranes, larger for higher contents (cf. Figs 4a with 4b). These results indicate that spin-labeled phosphatidic acid molecules are aggregated by Ca^{2+} in patches in the binary membranes and the average size of the patches is larger for the membranes with higher contents.

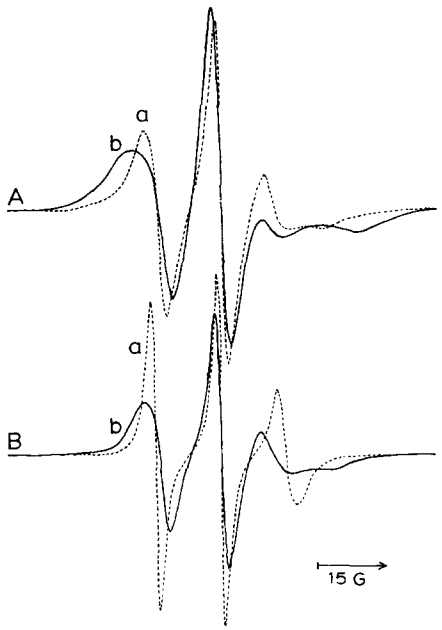
The sharp components in the Ca^{2+} spectra are ascribable to the labeled phosphatidic acid in the fluid phosphatidylcholine phase. These components amounted to only small percentages. In the examples shown in Figs 2 and 3, the spectra before addition of $CaCl_2$ were somewhat exchange-broadened owing to the high concentrations of the labeled phosphatidic acid. The initial exchange-broadenings were decreased to essentially zero by Ca^{2+} since most of the labeled phosphatidic acid molecules were wiped out to form the aggregates in the membranes. The results on the spin-labeled phosphatidic acid–phosphatidylcholine membranes therefore confirm and strengthen the conclusions on the Ca^{2+}-induced phase separations. In these membranes, the lateral phase separations are observed as a large increase in the exchange-broadening for the majority of the spin-labeled phosphatidic acid on one hand and elimination of the exchange-broadening for small fractions of the phospholipid molecules on the other.

The aggregate formation was observed for the membranes containing 10% of the labeled phosphatidic acid. However, when the membranes contained very small fractions of the anionic phospholipid to be aggregated, no exchange-broadenings were discernible. For example, the ESR spectrum of the membrane containing only 2% of spin-labeled phosphatidic acid was not affected by Ca^{2+} addition. Under such conditions, the volume fraction of the aggregate phase and therefore the patch size of the aggregates would be so small that no noticeable exchange-broadening was observed. Alternatively, no aggregate phase would even exist in the phase diagram.

Reduced mobility of lipid alkyl chains in Ca^{2+}-chelated aggregates

Effects of Ca^{2+} on phosphatidic acid membranes containing 2% of spin-labeled phosphatidic acid were investigated to obtain information on the Ca^{2+}-chelated aggregates. As shown in Fig. 5, Ca^{2+} largely broadened the ESR spectra of the membrane. The broadening observed here arises from intramolecular dipolar broadening due to reduced mobility of the lipid alkyl chains. The perpendicular principal value $2T_{\perp}$ was decreased at 20 °C to 17.9 G from 21.3 G in the absence of Ca^{2+}. The corresponding large increase in the parallel principal value $2T_{||}$ can be easily seen from the outward shift of the high-field bump. However, the splitting value was not able to be measured since the low-field peak was not resolved. The order parameter was estimated from the observed $T_{\perp}$ value and the calculated $T_{||}$ value using the relation $T_{||} = 3a - 2T_{\perp}$ where a, the isotropic hyperfine splitting, is taken to be 14.7 G. The parameter increased from 0.46 to 0.67 (42%) by Ca^{2+} chelation (see Fig. 6). Such reduced mobility of lipid alkyl chains generally occurred when the temperature was lowered. By comparison with low-temperature spectra of the membrane in the absence of Ca^{2+}, the Ca^{2+}-induced reduction in mobility is equivalent to more than 20 °C cooling. The Ca^{2+} spectrum at 60 °C was still broader than that in the absence of Ca^{2+} at 20 °C (Fig. 5B).

The reduction in the mobility and the concomitant increase in the order



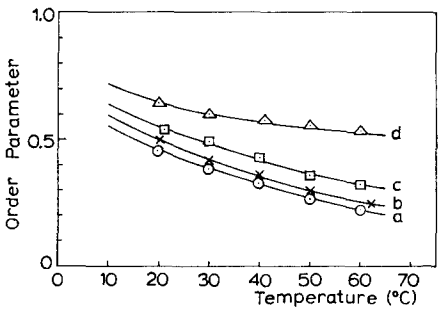


Fig. 5. ESR spectra of phosphatidic acid membrane containing 2 % of spin-labeled phosphatidic acid in (a) 10 mM EDTA (dotted line) and (b) 50 mM $CaCl_2$. Temperature: A) 22 °C; B) 60 °C. The solutions contain additionally 100 mM KCl and 50 mM Tris–HCl, pH 7.9.

Fig. 6. The order parameter as a function of temperature for phosphatidic acid membrane containing 2 % of spin-labeled phosphatidic acid in (a) 10 mM EDTA, pH 7.9 (○), (b) 10 mM EDTA, pH 5.6 (×), (c) 50 mM $MgCl_2$, pH 7.9 (□), and (d) 50 mM $CaCl_2$, pH 7.9 (△). The solutions contain additionally 100 mM KCl and 50 mM Tris–HCl buffer (pH 7.9) or KCl and 50 mM potassium acetate buffer (pH 5.6); K^+ concn = 100 mM.

parameter indicate closer packing of the anionic phospholipid molecules in the Ca^{2+}-chelated aggregates. This is consistent with the observation by Papahadjopoulos [11] that Ca^{2+} caused approx. 30% decrease in the surface pressure of monomolecular film of phosphatidic acid at pH 7.6.

Mg^{2+} also caused motional freezing of lipid alkyl chains in the phosphatidic acid membranes (see Fig. 6). However, the extent of reduction in the mobility was smaller. The increase in the order parameter was from 0.46 to 0.54 (17%) at 20 °C.

Effects of various divalent cations on phosphatidic acid–phosphatidylcholine membranes

Effects of Mg^{2+}, Ba^{2+}, and Sr^{2+} on phosphatidic acid–spin-labeled phosphatidylcholine membranes were investigated for comparison with the effect induced by Ca^{2+}. Fig. 7 compares ESR spectra of the membranes (7 : 1) in the presence of 50 mM of $MgCl_2$, $BaCl_2$, or $SrCl_2$. It is clearly demonstrated that Ba^{2+} and Sr^{2+} induce almost same strong exchange-broadening in the spectrum. The broadenings were also nearly same as that induced by Ca^{2+}. Mg^{2+} was also effective in causing exchange-broadening but its magnitude was smaller than those induced by the other divalent cations. The ionic selectivity for the phosphatidic acid membranes was thus remarkably different from that for phosphatidylserine–spin-labeled phosphatidyl-

choline membranes where Ca^{2+} was most prominent in inducing exchange-broadening, Ba^{2+} and Sr^{2+} were weaker, decreasing in that order, and Mg^{2+} was completely ineffective.

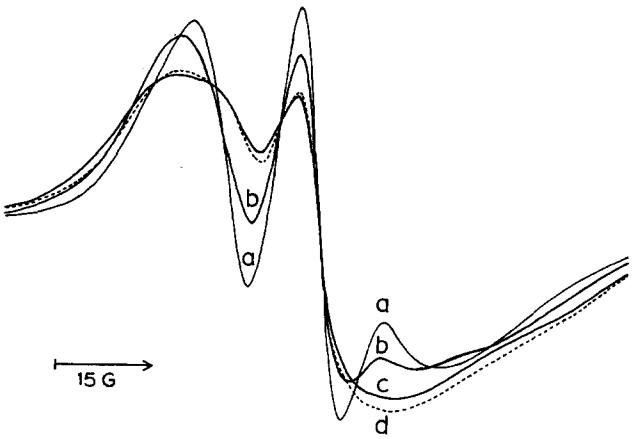


Fig. 7. ESR spectra of phosphatidic acid–spin-labeled phosphatidylcholine (7 : 1) membranes in (a) 10 mM EDTA, (b) 50 mM $MgCl_2$, (c) 50 mM $BaCl_2$ and (d) 50 mM $SrCl_2$ (dotted line). The solutions contain additionally 100 mM KCl and 50 mM Tris–HCl buffer (pH 7.9).

Ca^{2+}-induced lateral phase separations in acidic solution

All the above results were obtained in slightly alkaline solutions where the phosphate group in phosphatidic acid can be assumed to bear dinegative charges. When the bathing salt solutions were acidified, the perpendicular principal value of phosphatidic acid membrane containing 2% of spin-labeled phosphatidic acid was decreased slightly but definitely. $2T_{\perp}$ at pH 5.6 was 20.5 G while the value at pH 7.9 was 21.3 G (see Fig. 6). The slight reduction in the mobility in acidic solution reflects slightly closer packing of phosphatidic acid molecules in the membrane brought about by decreased negative charges on the phosphate groups.

Ca^{2+}-induced lateral phase separations were also observed in acidic solutions. ESR spectra of phosphatidic acid–spin-labeled phosphatidylcholine (18 : 1 and 7 : 1) membranes were rapidly exchange-broadened upon addition of 5–10 mM $CaCl_2$ at pH 4–5. However, the magnitude of the exchange-broadening was somewhat smaller than in alkaline solutions. This could be due to formation of smaller patches of the labeled phosphatidylcholine molecules in the membranes at lower pH.

In acid solutions, phosphatidic acid bears mononegative charge at the phosphate group according to the reported pK values, $pK_1 = 3.0$ and $pK_2 = 8.0$ in 100 mM KCl [12]. It is very likely that Ca^{2+} displaces the ionizations to lower pH. Abramson et al. [12] reported that Ca^{2+} released H^+ from phosphatidic acid by a nearly equivalent amount. Hauser and Dawson [13] observed that a monolayer of phosphatidic acid on distilled water (pH 5.5) adsorbed $^{45}Ca^{2+}$ as if it were completely dissociated.

DISCUSSION

The present results provide conclusive evidence for the Ca^{2+}-induced lateral phase separations in phosphatidic acid–phosphatidylcholine membranes. The phase separations are directly indicated by Ca^{2+}-induced aggregate formation of phosphatidic acid molecules on one hand and by Ca^{2+}-induced clustering of phosphatidylcholine molecules on the other. The same phase separations were observed for phosphatidylserine–phosphatidylcholine membranes [4, 5]. Ca^{2+} was not capable, however, of inducing the lateral phase separations in phosphatidylethanolamine–, phosphatidylinositol–, and cardiolipin–phosphatidylcholine membranes [5]. The capability of the other divalent cations of inducing the phase separations in the phosphatidic acid and the phosphatidylserine membranes was characteristically different.

The lateral phase separations occur when the enthalpy decrease due to formation of the M^{2+}-chelated aggregates surmounts the entropy decrease. The chelation energies and the Van der Waals energies among the lipid alkyl chains in the closely-packed aggregates can become sufficiently large. The chelations should be sufficiently strong and the ionic radius should be appropriate for bringing about the closely-packed structure. The M^{2+}-chelation energies are larger for more negative head groups since the interactions are mainly Coulombic. The Pauling–Corey–Koltun molecular models arranged in the bilayer structure based on X-ray analysis [14] suggest that M^{2+} can chelate to a number of oxygen atoms and connect two lateral lines of phospholipid molecules in the closely packed structure. The polar head groups could sterically affect the structure and give rise to the characteristic ionic selectivity of the individual lipids. For the case of phosphatidylserine, the carboxyls are also involved in the chelation, and the head groups bearing the carboxyls would be well located for the aggregate formation so as to induce the phase separations in spite of the net mononegative charge. The lateral phase separations were not observed in the cardiolipin–phosphatidylcholine membrane because the dinegative head group was attached to the four alkyl chains.

The biological significance of the Ca^{2+}-induced lateral phase separations was discussed in a previous paper [5]. It is very probable that such phase separations may affect the lateral and transverse motions of phospholipid molecules as well as proteins in biological membranes. This notion is supported and generalized by recent observation of lateral phase separations in binary phospholipid mixtures in the absence of Ca^{2+} [15, 16].

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