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DISAPPEARANCE OF CALCIUM-INDUCED PHASE SEPARATION IN PHOSPHATIDYLSERINE-PHOSPHATIDYLCHOLINE MEMBRANES CAUSED BY PROTONATION AND BY ELECTRIC CURRENT

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Summary

Disappearance of Ca^{2+}-induced phase separation in phosphatidylserine-phosphatidylcholine membranes has been studied under several conditions by monitoring electron spin resonance spectrum of spin-labeled phosphatidylcholine. The membranes were prepared in Millipore filters. Electron micrographs of the preparations showed formation of multilayered structures lined on the pore surface. The phase separation was disappeared when the membrane was soaked in non-buffered salt solution (100 ml KCl, pH 5.5). It was markedly contrasting that when the bathing salt solution was buffered no disappearance was observed. Disappearance of the phase separation was also observed when the Ca^{2+}-treated membrane was transferred to acidic salt solutions ($\leqslant$pH 2.5) or to low ionic strength media ($\leqslant$10 mM) buffered at pH 5.5, and then to the buffered salt solution (100 mM KCl, pH 5.5). These are due to replacement of Ca^{2+} by proton, proton-induced separation, followed by disappearance of the phase separation in the buffered salt solution. Biological significance of the competition between Ca^{2+} and proton for the phase separation or domain formation in the membranes was emphasized.

Introduction

Ca^{2+}-induced lateral phase separations are an isothermal ionotropic process that may be involved in Ca^{2+}-triggered physiological membrane phenomena. The phase separation was first observed in phosphatidylserine-phosphatidylcholine membranes using spin-labeled phospholipid [1], and then extensively studied by spin label technique [2—5] and by differential scanning calorimetry [6]. Similar ionotropic phase separations were also induced by basic proteins [7,8]. Regulation of the thermal phase transition and phase separation by uni-

and divalent cations has also been demonstrated [9—11]. The ionotropic regulation of charged membranes is thus receiving more attention with regard to physiological significance.

In the present study, we have attempted to find factors regulating the Ca^{2+}-induced phase separation and found involvement of H^+ in replacing Ca^{2+}. We used phospholipid membranes prepared in Millipore filter paper that were first introduced by Tobias et al. [12,13]. We have also studied structural characteristics of the membrane preparation by electron microscopy, electric resistance measurement, and spin label technique.

Materials and Methods

Phosphatidylserine from bovine brain white matter [14], phosphatidylcholine from egg yolk [15], spin-labeled phosphatidylcholine with 12-nitroxide stearic acid at 2-position [16], and 12-nitroxide stearic acid [4] were prepared as described in the literature.

As a backbone for phospholipid membranes, we used Millipore filter (Millipore Filter Corp.) with three different pore sizes; SMWP 02500 (average pore diameter $5 \pm 1.2\ \mu m$), HAWP 02500 ($0.45 \pm 0.02\ \mu m$), and VSWP 02500 ($0.025 \pm 0.002\ \mu m$). The filter papers were washed with benzene to remove detergent. Phospholipid-absorbed Millipore filters were prepared as described previously [1—5]. Typically, the filter was dipped in benzene solution of phosphatidylserine-spin-labeled phosphatidylcholine (9 : 1) at 40 mg/ml for 10 min and then dried. The amount of adsorbed lipids ranged from 1.6 to 2.1 mg/cm^2. The dried filter was soaked in 50 ml 100 mM KCl and stored at 5°C overnight. The bathing salt solution was not buffered usually and the pH ranged from 5.4 to 5.8. When necessary, the solution was buffered with 50 mM each of Tris-HCl (pH 7.1), potassium acetate/acetate (pH 3.5—5.8), or potassium acetate/HCl (pH 1.8—2.5).

For ESR measurements, a small piece of the phospholipid-adsorbed filter was put into a quartz flat cell with remaining mother liquor. The spectrum was measured at room temperature (22°C) with an X-band spectrometer (JEOL Model ME-2X). Spectrum computor (JEC-6 and EC-100) and automatic chart reader (JEC CR-114) were used for subtraction and superposition of spectra.

For electron microscopy, the phospholipid-adsorbed filter was fixed in 1% O_sO_4 and dried by evacuation. Dehydration in alcohol was avoided since it dissolved the filter paper. The fixed specimen was embedded in Epon 812 and gold to silver thin sections were cut transversely to the filter plane with OmU_2 ultramicrotome (C. Reichert Optische Werke). The sections were viewed under a JEM-7A electron microscope at 80 kV. Staining with uranyl acetate and lead citrate was omitted because the treatment did not affect the electron micrographs.

For the electric resistance measurements, the phospholipid-adsorbed filter was set in a glass cell and small electric current (20, 40, 60 μA) was supplied through a pair of Ag/AgCl electrodes connected to the cell with salt bridges. Voltage drop across the filter was read with a microvoltmeter (Ohkura Electric Co, Model AM 1002) using a pair of calomel electrodes. The voltage drop and applied current generally followed Ohm's law.

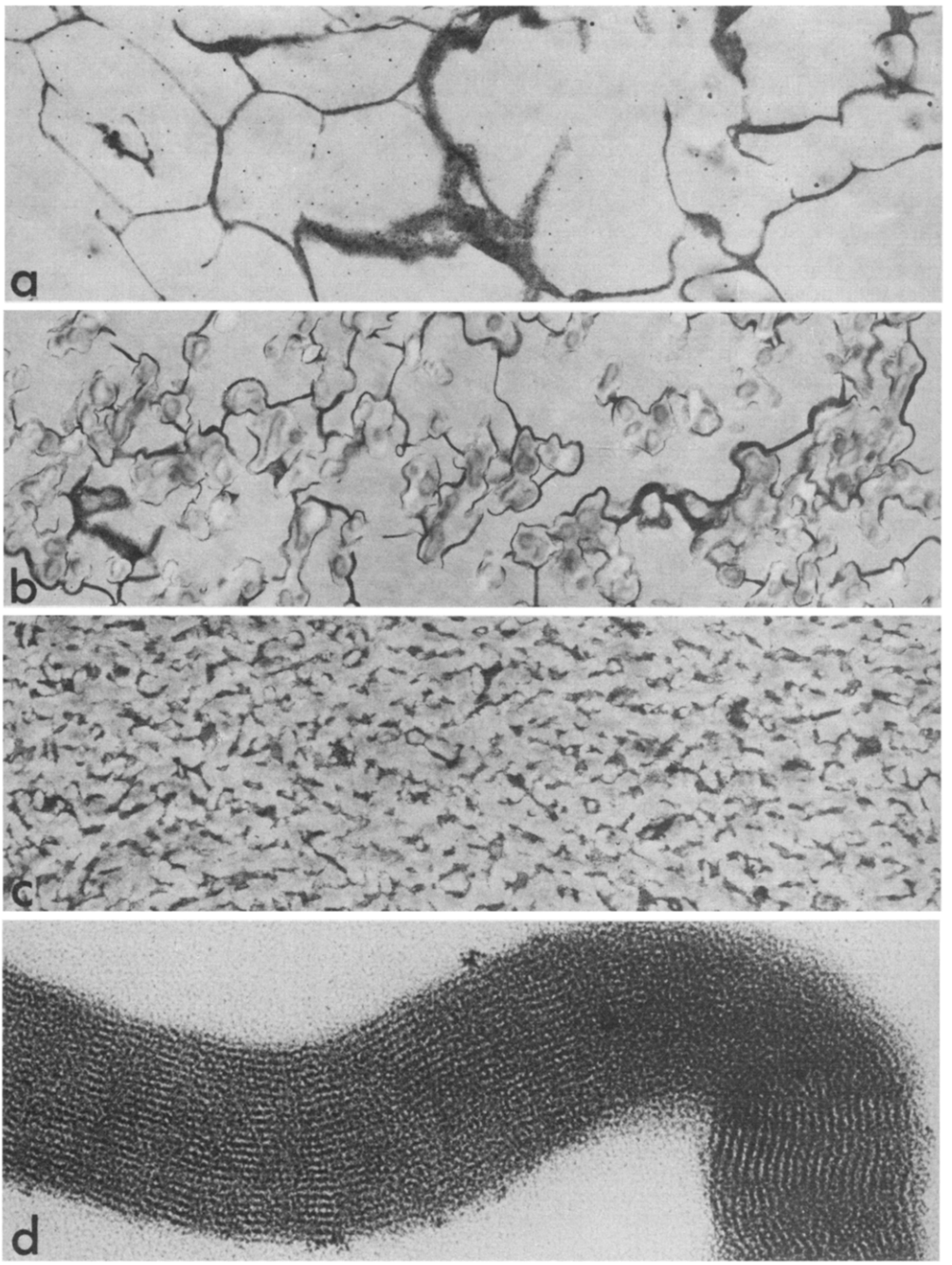

Fig. 1. Electron micrographs of thin sections of phospholipid-adsorbed Millipore filters with average pore diameter of 5 μm (a), 0.45 μm (b), and 0.025 μm (c) at a magnification of 10 000 X. (d) Phospholipid-adsorbed Millipore filter (5 μM) at a higher magnification (X250 000). Phosphatidylserine-spin-labeled phosphatidylcholine mixtures (3 : 2 for a—c and 9 : 1 for d) were deposited in the Millipore filters, conditioned in 100 mM KCl for a—c, and then treated with 10 mM $CaCl_2$/100 mM KCl for d. The samples were fixed and thin-sectioned as described in Materials and Methods.

Results

Electron micrograph of phospholipid membranes prepared in Millipore filter

Fig. 1 shows electron micrographs of phosphatidylserine-phosphatidylcholine membranes prepared in Millipore filters with three different poor sizes. At lower magnification, network figures were observed (Figs. 1a—1c). The average network dimension was roughly proportional to the pore size. At higher magnification (Fig. 1d), the network lines showed myeline figures with a repeating period of 2.9 nm. Since the average thickness of the multilamellar structures was 0.01—0.1 μm, there was still large lipid-free space in the 5- and 0.45-μm pores, whereas only limited space in the 0.025 μm pore of the filters.

Similar electron micrographs were observed for the single component membranes as well as their mixtures with various ratios. High contents of phosphatidylserine and pretreatment with Ca^{2+} favored for maintaining ordered multilamellar structure. The filter paper itself was not stained and did not give the network structure. The myelinic figures were similar to that of phospholipid extract from brain tissue [17], although the repeating period obtained in that study (4 nm) was somewhat larger than the present value.

Electric resistance of phospholipid-adsorbed Millipore filter

Electric resistance of the filters was measured with or without adsorbed phospholipid and in the presence or absence of Ca^{2+}. Only the smallest pore (0.025 μm) filter with phospholipids showed significantly higher resistance than that of bathing salt solution (100 mM KCl). This is consistent with the presence of lipid-free channel in the larger pores. The resistance of the filter deposited with phosphatidylserine-spin-labeled phosphatidylcholine (9 : 1) at 2 mg/cm^2 was 100—300 Ω/cm^2. The resistance was increased to 3000—5000 Ω/cm^2 when the membrane was treated with 10 mM $CaCl_2$. This change was reversed by EDTA. The electric resistance of phosphatidylserine-phosphatidylcholine membranes in the absence of Ca^{2+} was dependent on the molar ratio and became smaller with the increase of the anionic lipid content. Pure phosphatidylserine membrane had a resistance of 50—100 Ω/cm^2, about one sixth of pure phosphatidylcholine membrane.

ESR spectra of phosphatidylserine-spin-labeled phosphatidylcholine membrane in Millipore filters

ESR spectra of spin-labeled phospholipids in membranes prepared in Millipore filter were superposable to those of liposomes. However, in the spectra of smaller pore filters, shoulders were observed at high- and low-field positions (see arrows in Fig. 2). The relative intensity was larger for the filters with smaller pores, and for the same pore size filter, larger for smaller amounts of the deposited lipid. The shoulder component was not reduced by ascorbate. Therefore, when the phospholipid-deposited filter was soaked in ascorbate solution overnight, only the shoulder component remained (Fig. 2d). The overall splitting 67 G indicates strong immobilization of the phospholipid alkyl chains and suggests the signal to be due to phospholipid spin labels strongly bound to the filter material.

Osnium treatment caused an increase in the overall splitting of the spectrum

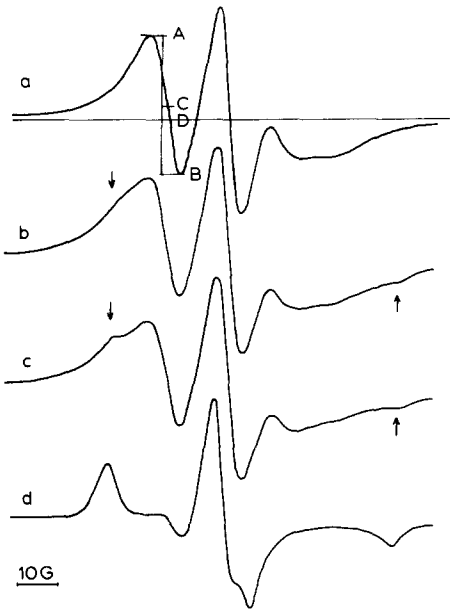

Fig. 2. ESR spectra of phosphatidylserine-spin-labeled phosphatidylcholine (9 : 1) membrane prepared in Millipore filters with pore diameter of 5 μm (a), 0.45 μm (b), and 0.025 μm (c) bathed in 100 mM KCl, pH 5.5. (d) The spectrum remained after reduction of the membrane preparation (0.025 μm) with ascorbate (200 mM, pH 7.1) overnight. In a, the peak heights for definition of α are shown.

for phosphatidylserine-spin-labeled phosphatidylcholine (9 : 1) membrane. The splitting value approx. 60 G indicated moderate immobilization of the alkyl chains. Addition of Ca^{2+} did not cause any further change in ESR spectrum. The osmium treatment thus fixed the membrane to prevent Ca^{2+}-induced phase separation. When the membrane was first treated with Ca^{2+} and then with osmium, the exchange broadening due to the phase separation remained. There was some increase in the overall splitting. The moderate immobilization by osmium appears to be consistent with its fixation mechanism through crosslinking of unsaturation bonds. Since most of the spin labels lack the unsaturation, osmium would mainly crosslink phosphatidylserine acyl chains leaving spin labels and their clusters surrounded by the crosslinked networks. This result is consistent with that of Jost and Griffith [18].

Ca^{2+}-induced phase separation and its disappearance in non-buffered salt solution

Ca^{2+} caused exchange broadening in the ESR spectrum of phosphatidylserine-spin-labeled phosphatidylcholine owing to clustering of the spin labels in the membranes [1—3]. A spectral parameter α defined as (1/2)(AB/CD) (see Fig. 2a) was used to represent the broadening [19]. This parameter is convenient to use since it is dependent only on the spectral shape, sensitive to the broadening, and relatively free from the immobilized component. Correction for the immobilized component was only necessary for the membranes prepared in the 0.025 μm pore filter. For homogeneous phosphatidylserine-spin-labeled phosphatidylcholine mixtures, logarithm of α was found nearly propor-

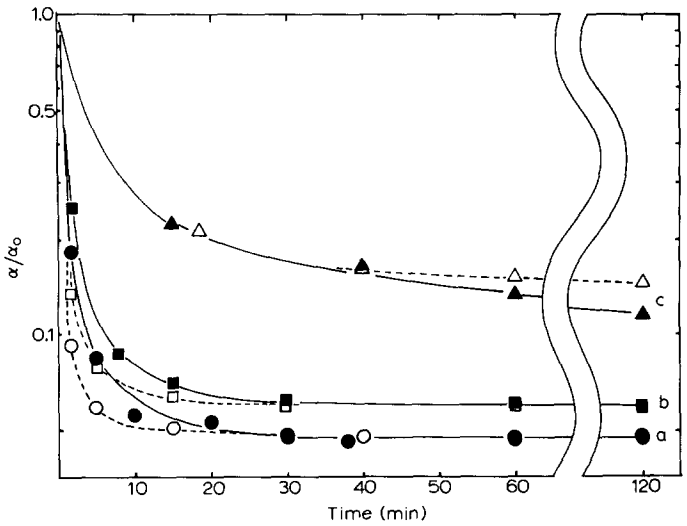

Fig. 3. Time course of Ca^{2+}-induced phase separation in phosphatidylserine-spin-labeled phosphatidylcholine (9 : 1) membrane prepared in Millipore filters with 5-μm (a), 0.45-μm (b), and 0.025-μm (c) pores. Log α/α_0 was plotted against soaking time in non-buffered (———) or buffered (- - - - -) 10 mM $CaCl_2$/100 mM KCl, pH 5.5. α_0 is the α value in the absence of Ca^{2+}.

tional to the spin label mol fraction [phosphatidylcholine *] in a limited range of [phosphatidylcholine *] from 0.1 to 0.3; log $\alpha = -8.2$ [phosphatidylcholine *] + 1.54. However, when Ca^{2+} was added to the membranes, the ESR spectrum became sum of variously broadened spectra [1,3] and the parameter would only approximate the value for the average size cluster.

Fig. 3 shows time course of the phase separation in phosphatidylserine-spin-labeled phosphatidylcholine (9 : 1) membrane by plotting log α/α_0 against time in 10 mM $CaCl_2$/100 mM KCl. It is seen that the rate of phase separation was dependent on the pore size of the supporting filter, faster for the larger pore filters. The phase separation in the buffered solution was slightly faster than that in non-buffered one.

The phase separation was disappeared when the Ca^{2+}-treated membrane was soaked in non-buffered salt solution (100 mM KCl, pH 5.4—5.8). The parameter α/α_0 increased with soaking time owing to gradual declustering and randomization of spin-labeled phosphatidylcholine in the membrane (see Fig. 4). The disappearance was the fastest in the 5 μm pore filter, moderately fast in the 0.45 μm pore filter, but very slow in the 0.025 μm pore filter. The disappearance rate was also dependent on the time of previous Ca^{2+} treatment (Fig. 4B). The disappearance in the 5-min treated membrane was faster than that in the 10-min treated one. It was too slow to be detected in the 5-h treated membrane.

The phase separation in the 0.025 μm pore filter was very slowly affected in the non-buffered 100 mM KCl solution. However, it was rapidly disappeared when electric current was supplied through the filter in the salt solution. Fig. 5 shows the current-induced declustering of spin-labeled phosphatidylcholine in the binary membrane. The disappearance was faster on application of larger current; almost complete disappearance in 10 min at 15 mA. The electric resis-

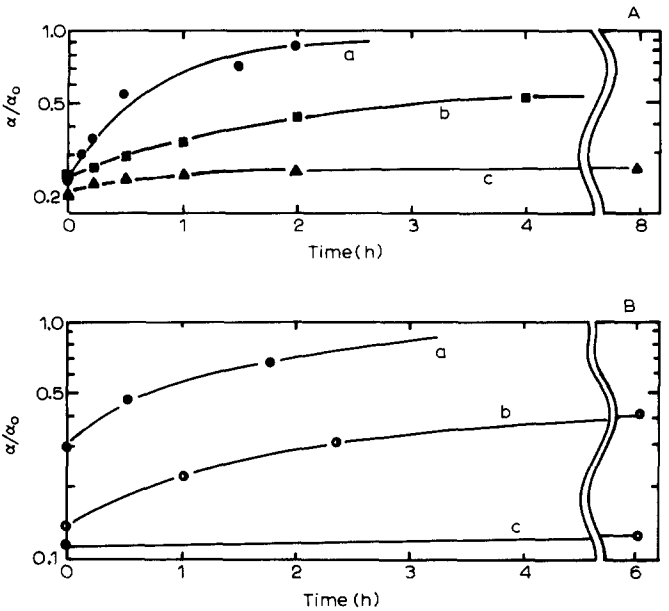

Fig. 4. Disappearance of Ca^{2+}-induced phase separation in phosphatidylserine-spin-labeled phosphatidylcholine (9 : 1) membrane on soaking in non-buffered salt solution (100 mM KCl, pH 5.5). Log α/α_0 was plotted against soaking time. (A) Dependence on the pore size of the backbone Millipore filter: a, 5 μm; b, 0.45 μm; and c, 0.025 μm. The membranes were pretreated with 10 mM $CaCl_2$/100 mM KCl for 10 min (a and b) and 1 h (c). (B) Dependence on the pretreatment time of the membranes with Ca^{2+}: a, 5 min; b, 10 min, and c, 5 h. The membranes were prepared in 5 μm pore filter.

tance of the phospholipid-deposited filter was also decreased on application of the current (Fig. 5). The decrease in the resistance was more rapid than the decrease in the broadening parameter with respect to the time. These changes were reversed by retreatment with Ca^{2+}.

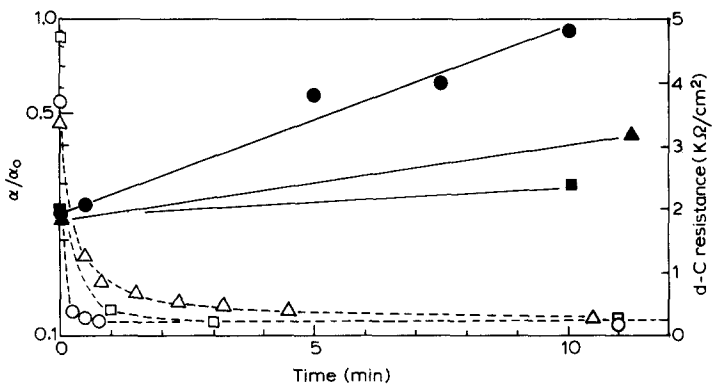

Fig. 5. Disappearance of Ca^{2+}-induced phase separation (———) and decrease in electric resistance (- - - - -) on application of electric current through Millipore filter (0.025 μm) deposited with phosphatidylserine-spin-labeled phosphatidylcholine (9 : 1) membrane. The membrane preparation was pretreated with Ca^{2+} and electric current was supplied in 100 mM KCl for varying time at 1.5 mA (□), 7.5 mA (△), and 15 mA (○). The filled symbols are for the broadening parameter α/α_0 and the open symbols for the resistance.

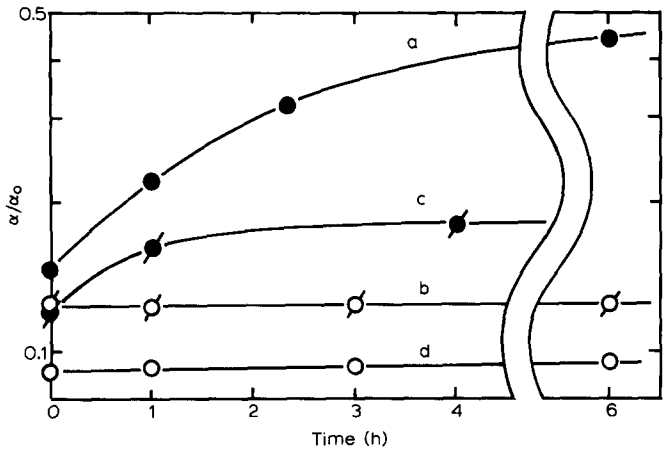

Fig. 6. No disappearance of Ca^{2+}-induced phase separation in buffered salt solution (100 mM KCl, 50 mM acetate buffer (pH 5.8) or 50 mM Tris-HCl (pH 7.1)). Phosphatidylserine-spin-labeled phosphatidylcholine (9 : 1) membrane in the 5 μm pore filter was pretreated with Ca^{2+} for 5 min and soaked in non-buffered pH 5.8 (a), buffered pH 5.8 (b), non-buffered pH 7.1 (c), and buffered pH 7.1 (d).

Disappearance of Ca^{2+}-induced phase separation triggered by protonation

The Ca^{2+}-induced phase separation was disappeared when the membrane was soaked in non-buffered salt solution as described in the previous section. However, it is remarkably contrasting that the phase separation was not disappeared

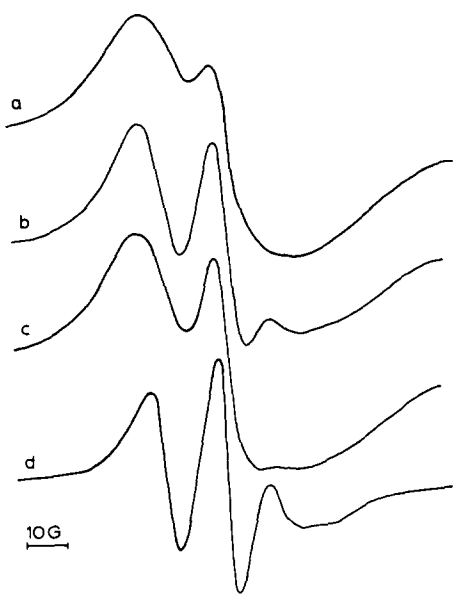

Fig. 7. Change in ESR spectrum of Ca^{2+}-treated phosphatidylserine-spin-labeled phosphatidylcholine (9 : 1) membrane on soaking in acidic salt solution (100 mM KCl, 50 mM potassium acetate/HCl pH 1.8). (a) Control in 100 mM KCl, 10 mM $CaCl_2$, 50 mM acetate buffer, pH 5.8. (b) 50 min and (c) 6 h after soaking in the acid solution. (d) The membrane after 6 h was taken out, washed with and soaked in the buffered salt solution (pH 5.8) for 30 min. The broadening parameter α was 0.35, 1.11, 0.53, and 7.83 for a, b, c, and d, respectively. During soaking in pH 1.8 solution, a small fraction of spin labels was reduced but the reduction did not appreciably affect the major features.

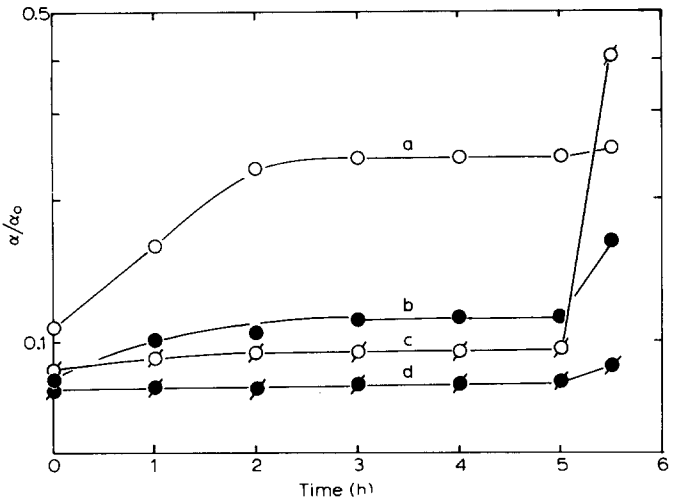

Fig. 8. Disappearance of Ca^{2+}-induced phase separation in low ionic strength media at pH 5.5—6.0: a, 100 mM KCl; b, 10 mM KCl; c, water, and d, 3 M KCl. The broadening parameter was plotted against soaking time. After 5 h soaking the membranes were transferred to 100 mM KCl, 50 mM acetate buffer, pH 5.5, for 30 min and the parameter was measured. Phosphatidylserine-spin-labeled phosphatidylcholine (9 : 1) membrane in the 5 μm filter was pretreated with Ca^{2+}.

on soaking in buffered salt solution (100 mM KCl, 50 mM acetate buffer, pH 5.8). Fig. 6 shows the dramatic difference. The broadening parameter remained unchanged when the Ca^{2+}-treated membranes were soaked in the buffered salt solution, in contrast to the increase in the non-buffered salt solution. No disappearance was observed also when both Ca^{2+} treatment and subsequent soaking were done in the buffered solution, and also when Ca^{2+} treatment in buffer and soaking in non-buffer.

When the Ca^{2+}-treated membranes were first transferred to acidic salt solutions (pH 1.8—2.5) and then to the pH 5.8 buffered salt solution, the phase separation disappeared rapidly. If we follow the spectral change at the acid pH, the parameter α was increased at first and then decreased (see Fig. 7). The change can be attributed to replacement of Ca^{2+} with H^+. The first increase in α is assigned to detachment of Ca^{2+} causing declustering and the following decrease to the phase separation induced by protonation at the serine head group. When the membranes were brought back to pH 5.8, the proton-induced phase separation was disappeared (Fig. 7d). The proton-induced phase separation in acidic salt solution and its disappearance in pH 5.8 salt solution have been demonstrated by the spin label technique (manuscript in preparation).

Effect of lowering the ionic strength of the bathing salt solution was also striking (see Fig. 8). The parameter α did not apparently change when the Ca^{2+}-treated membrane was soaked in water or low ionic strength solution (e.g. 10 mM KCl) at pH 5.5. However, the parameter increased rapidly when the membrane was transferred to 100 mM KCl solution (pH 5.5). Therefore, the unchanged broadening parameter does not mean persistence of the Ca^{2+}-induced phase separation in the membrane. It is very likely that, during soaking in the low ionic strength media, Ca^{2+} had been replaced by H^+ and the proton-induced phase separation had occurred. The phase separation in phosphatidylserine-

spin-labeled phosphatidylcholine membranes in low ionic strength media has been demonstrated by the spin label technique (manuscript in preparation). Finally, raising the ionic strength of the bathing solution to 3 M KCl was not so effective for disappearance of the Ca^{2+}-induced phase separation (Fig. 8d).

Discussion

It is shown that Millipore filter pores provide a stable support for phospholipid multilayered membranes although some limited fractions of phospholipid bind strongly to the filter material. The membrane preparation in the larger pore filters (e.g. 5 μm) is useful for studying effect of changing aqueous environments. The preparation in the smaller pore filters (e.g. 0.025 μm), however, had only small aqueous channels and therefore the membrane surface properties would become important. For example, the decrease in the electric resistance with the increase of phosphatidylserine content in phosphatidylserine-phosphatidylcholine membranes may be ascribed to concentration of K^+ due to the increase in the surface potential. The large increase in the electric resistance by treatment with Ca^{2+} may be due to the increase in the surface hydrophobicity [5,12]. Entrance of the ionic species to the small hydrophobic pores would be hampered. If we compare the rate of Ca^{2+}-induced phase separation with that of disappearance in the 0.025 μm pore filter, we notice much slower disappearance (Figs. 3 and 4). The phase separation reached a half of the final level in 5 min, while only very small disappearance occurred even after 8 h. The marked difference may also be explained for the most part by the increased hydrophobicity. The rapid disappearance of the phase separation by electric current can be attributed to electrophoretic propelling of K^+ into the small hydrophobic pores and carrying away of Ca^{2+} out of the pores. As one more related phenomenon, we have observed reduction of the head group spin-labeled phosphatidylcholine by Fe^{3+} and cysteine in phosphatidylserine membranes prepared in the 0.025 μm pore filter. The rate of reduction became much slower when the membranes were treated with Ca^{2+}.

The Ca^{2+}-induced phase separation was disappeared under several conditions. The trigger for the disappearance is attributed to replacement of Ca^{2+} with H^+. Thus the phase separation was disappeared when the Ca^{2+}-treated membranes were soaked in acidic salt solution with pH lower than pK of the serine head group in the membrane (4.0) or in low ionic strength media at pH 5.5. In the latter the pK of the head group will increase and can be as high as 6 in 1 mM KCl [10]. The disappearance in the non-buffered salt solution (pH 5.5) can also be attributed to replacement of Ca^{2+} with H^+. In the buffered salt solution, however, the phase separation was not disappeared. This marked difference indicates that the proton concentration or effective pK on the membrane surface in the non-buffered media can be different from the bulk value. The present study therefore strongly suggests that phase separation or domain formation in biological membranes containing phosphatidylserine or phosphatidic acid can be regulated by a competition between Ca^{2+} and H^+. On the membrane surface, various physical parameters such as charge density, ionic strength, proton concentration can be locally different.

Dependence of the disappearance rate on the time of Ca^{2+} pretreatment (Fig. 4b) can be explained based on completeness of the two-dimensional crystallization of phosphatidylserine in the mixed membranes.

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