Clustering of Lecithin Molecules in Phosphatidylserine Membranes Induced by Calcium Ion Binding to Phosphatidylserine

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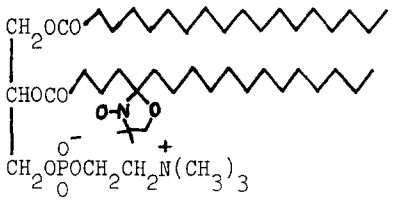
SUMMARY: The effects of calcium ion on phosphatidyl L-serine (PS) have been studied with PS membranes containing a lecithin spin label (L*). The calcium ion makes the ESR spectra of the L* in PS membranes broadened owing to the intermolecular spin-spin exchange interactions. The results indicate that the calcium ion binds to PS molecules to form rapidly rigid calcium ion-bound PS aggregates, the lecithin molecules being thereby separated from the host PS bilayers to form clusters. The magnesium ion is ineffective for the aggregation and exerts a quite different effect only at much higher concentrations.

INTRODUCTION: Calcium ion is known to show profound physiological effects on many biological membranes and some efforts have been made to elucidate their molecular mechanism from structural aspects.[1,2] However, the molecular basis for the effects is not yet clear. In the present communication, we briefly present results of our study on the effect of calcium ion on phosphatidylserine (PS) membranes containing a lecithin spin label I. The lecithin label (L*) was chosen because it is a neutral lipid and calcium ion has no effects on the lecithin membranes. The ESR spectra of the L* in PS membranes become, on addition of calcium ion, broadened owing to the intermolecular spin-spin exchange interactions and the results strongly suggest the phase separation of lecithin-PS membranes, induced by calcium ion, into the lecithin clusters and the PS aggregates bound by calcium ions. We employed Millipore filters to support lipid membranes. The lipid-Millipore filter has been used in the studies of electrical properties of lipid membranes[3] and will be particularly useful for comparative studies. Various spin labels give identical ESR spectra whether they are in aqueous dispersions or in the filters in the presence of aqueous solutions. Moreover, some lipid-filter systems such as L* in lecithin show anisotropic ESR spectra.

(I) 

EXPERIMENTAL: PS was extracted from beef brain white matter and purified according to the procedure described by Sanders.[4] Lecithin (L) was obtained from egg yolk.[5] The lecithin label was synthesized from the lysolecithin and 5-nitroxide stearic acid by the method of Hubbell and McConnell.[6] The Millipore filters with average pore diameter 5μ (SMWP 02500, Millipore Filter Corp.) were dipped in benzene solutions of PS, L, and L* at various molar ratios and then dried. The lipid concentrations used were 6 to 25mg/0.2ml of benzene and the amount of the impregnated lipids in the filters ranged from 0.3 to 0.6 mg/0.15cm^2. The dried filters were soaked overnight in 100mM KCl solution and then transferred to various salt solutions (pH 5~6). ESR spectra of the filters were measured with a tissue cell in the aqueous solutions. The spectrometer was a commercial one (JES-ME-2X) with variable temperature accessories.

RESULTS AND DISCUSSION: Fig. 1a shows an example of the ESR spectra of the L* in PS membranes in 100mM KCl solution (PS:L* = 200:1). The spectra can be well approximated by the axially symmetric spin Hamiltonian.[6] The principal values are dependent on temperature; the parallel value $2T'_{//}$ (the outermost splitting) decreases and the $2T'_{\perp}$ value increases with temperature.[7] This spectrum does not exhibit anisotropy, while the spectra of L* in L membranes do show the anisotropy indicating preferred orientation of the alkyl chains perpendicular to the filter plane. The difference implies some difference in the mode of packing between PS and L membranes.

On addition of calcium ion, the ESR spectra of L* in PS membranes rapidly change into the broadened ones such as shown in Fig. 1b (PS:L* = 200:1), the outermost splitting remaining almost unchanged (~57G). This change is completely reversible when the filter was resoaked in a EGTA (ethylene glycol bis

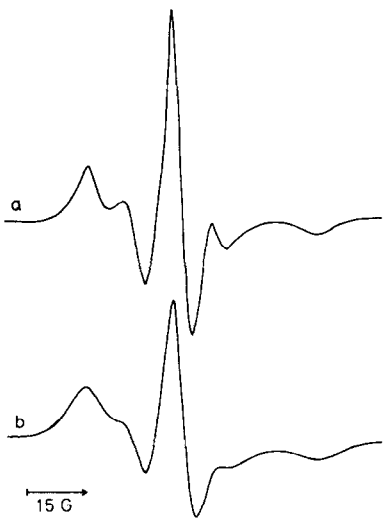


Fig. 1.

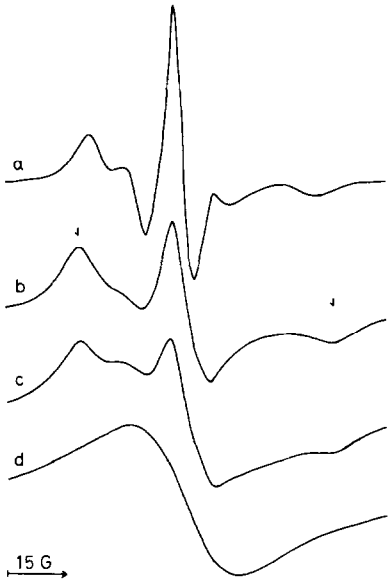


Fig. 2.

Fig. 1: ESR spectra of the lecithin label in PS membrane a) in 100mM KCl and b) in 10mM $CaCl_2$ and 100mM KCl solutions (22°C). PS:L* = 200:1.

Fig. 2: ESR spectra of the lecithin label in PS membranes in 10mM $CaCl_2$ and 90mM KCl solution with various PS:L:L* ratios (22°C); a) 180:19:1, b) 180:10:10, and c) 180:0:20. The spectrum d) is for the pure lecithin label membrane in 100mM KCl solution.

(β-aminoethylether)-N,N,N',N'-tetraacetic acid) solution. The change was rapid in either direction; the spectra measured after 10 minutes were found to have changed already, though no attempt was made to determine its rate in the present study. The reverse change was too slow, however, in KCl solutions even at saturated concentration. The broadening depends on the calcium ion concentration as well as on the KCl concentration. In 100mM KCl, the broadening almost saturates at 1mM Ca^{2+} ion, while, in a more dilute KCl solution (2mM), the effect already saturates at 0.5mM.

The magnesium ion at a concentration of 1mM in 100mM KCl gives no effects on the L* in PS membranes. At higher concentrations, the spectra become affected, but in a quite different way. The outermost splitting of the spectrum in 50mM Mg^{2+} in 100mM KCl solution increased to 60G and the spectrum appeared

similar to a spectrum of the membrane taken at a low temperature (14°C) in the absence of Mg^{2+} ion. Another change noted is that the spectrum comes to show anisotropy similar to that of L* in L membrane, indicating an increased order in the membrane structure. The magnesium ion, however, does not induce the broadening as observed in the case of calcium ion. The spectra of L* in L membranes were not affected by calcium ion at all.

The calcium effect on the L* in PS membranes was examined keeping the PS to L+L* ratio constant and changing the L* to L ratio. The broadening progressively increases with the L* fraction. As an example, the spectra obtained with a high L+L* content are reproduced in Fig. 2a, b, c(PS:L:L* =180:20-x:x). The spectra of the membranes with high L* contents show quite pronounced broadening and the outermost splitting also increases noticeably (63G, see arrows in Fig. 2b and c).

The broadening cannot be ascribed to the motional effects of the lecithin labels in PS membranes since the spectra of dilute L* in PS measured at lower temperatures show different features. Instead, the broadening can be reasonably accounted for by the intermolecular spin-spin exchange effects. In fact, the experimental spectra measured with membranes containing high L* contents in L well simulate the main features of the broadened spectra. Fig. 2d shows the extreme of the exchange-broadened spectrum obtained for pure L* membrane. Exchange broadening of the spectra has been studied with androstan spin label at high contents in dipalmitoyl phosphatidylcholine.[8] The spectra of L* in PS in the absence of calcium ion, on the other hand, show essentially no evidence of exchange broadening, indicating uniform distributions of L* in PS. Of course, the membranes with higher L* contents (for example, PS:L* = 180:20) exhibit some exchange broadening. However, uniform distribution of L* in PS are also retained for these membranes since the spectrum is almost the same as that of the L:L* = 180:20 membrane. The calcium ion-induced broadening indicates, therefore, that the lecithin molecules, which have been uniformly distributed in PS membrane, separate from the host PS bilayers to form lecithin clusters when the calcium ion binds to PS.

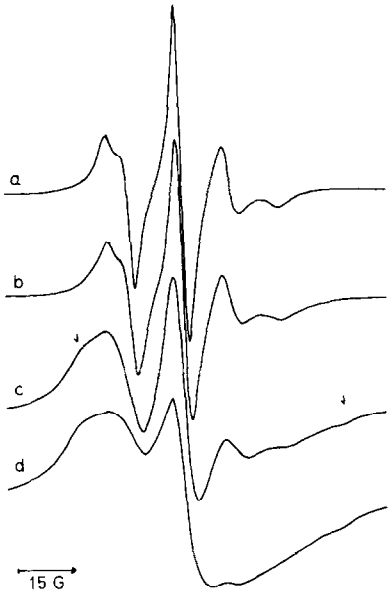


Fig. 3: ESR spectra at 70°C of the lecithin label in PS membranes with various PS:L:L* ratios. a) 180:0:20 in 100mM KCl solution. b) 200:0:1, c) 180:10:10, and d) 180:0:20 in 10mM $CaCl_2$ and 90mM KCl solution.

The broadening induced by calcium ion depends on the L* contents in PS. The membranes containing higher L* contents exhibit larger exchange broadening. This can be seen if we compare, for example, Fig. 2c for PS:L* = 180:20 with Fig. 1b for 200:1. The exchange interaction in the highly broadened spectrum (Fig. 2c) is still smaller than that of pure L* membrane (Fig. 2d). These results suggest that the lecithin clusters do not solely consist of lecithins but are contaminated by PS molecules. The sizes of clusters are considered to be smaller in the PS membranes with lower contents of lecithin and, therefore, the dilution effect by PS molecules becomes relatively larger. If we make a simple estimate, the L* clusters in the PS:L* = 200:1 membrane contain approximately 9 times as many PS molecules since the broadening is similar to that of the L:L* = 9:1 or PS:L* = 9:1 membranes in the absence of calcium ion.

The spectra at higher temperatures are also consistent with the interpretation. These spectra give decreased $2T'_{//}$ values and are exchange-broadened (Fig. 3). The membrane PS:L* = 200:1 in the presence of calcium ion shows

almost the same spectrum as that of the membrane PS:L* = 180:20 in the absence of calcium ion. Strong exchange broadening induced by calcium ion can again be seen by comparison of Fig. 3d with 3a. In the spectra of the membranes with high L* contents (PS:L:L* = 180:10:10 and 180:0:20), we observe quite broadened peaks at the decreased $2T'_{//}$ positions and, in addition, the low field shoulders or peaks and the corresponding high field peaks (see arrows in Fig. 3c and d) at almost the same positions as observed in the room temperature spectra (Fig. 2b and c). Since the exchange-broadened spectra of homogeneous system such as L* in L or L* in PS without calcium do not show such shoulders or peaks, the heterogeneous spectra suggest that the lecithin molecules can exist, besides in the form of the L* clusters, in some regions where the motional freedom is hindered and shows very little temperature dependence. A possible model for such regions is the lecithin molecules entrapped in the PS aggregates bound by calcium ions. The probability for such entrapping would be higher for the membranes with higher contents of L or L* in PS. The membranes with low contents of L or L* in PS, therefore, show exchange-broadened spectra with no appreciable shoulders or peaks.

CONCLUSIONS: The conclusions and suggestions drawn from the present study can be summarized as follows. Calcium ions, when added to PS membranes containing lecithin molecules, bind to PS molecules to form the PS aggregates, the lecithin molecules being thereby separated from the host PS bilayers to form the clusters. This phase separation occurs rapidly and this is reasonable in view of the rapid lateral diffusion of lipid molecules in the bilayer as discovered by McConnell et al.[9] The PS aggregates bound by calcium ions are rigid. The motional freedom of the alkyl chains is frozen and the lateral diffusion within the aggregates is probably slow. The magnesium ion is completely ineffective for the aggregation and exerts quite different effects only at much higher concentrations. The effect is such that the motions of the alkyl chains are somewhat frozen and the ordering of the membranes increases. We think that these

findings would be significant in some physiological events such as permeability change, excitation, etc. More detailed analysis and further studies with anionic phospholipid spin labels are being undertaken on model membranes as well as biological membranes.

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