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INTERACTION BETWEEN Ca^{2+} AND DIPALMITOYLPHOSPHATIDYLCHOLINE MEMBRANES

II. FLUORESCENCE ANISOTROPY STUDY

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The effect of Ca^{2+} on the molecular mobility in dipalmitoylphosphatidylcholine membranes was studied by steady-state and time-resolved measurements of fluorescence anisotropy. The fluorescence anisotropy decay of 1,6-diphenyl-1,3,5-hexatriene in the hydrocarbon region indicated that the free volume of molecular rotation became more restricted when the Ca^{2+} concentration was increased. The decrease of the molecular mobility was observed from 1 mM Ca^{2+}, at which the number of bound Ca^{2+} is much less than that of the total lipid molecules. A distinct difference between Ca^{2+} and Mg^{2+} effects suggested that the change in various membrane properties was induced by the binding of these ions. From these results we propose a long-range attractive interaction between bound Ca^{2+} and the polar head groups of distant phosphatidylcholine molecules.

1. Introduction

The interaction between divalent cations and phosphatidylcholine membranes has been studied from two aspects: intermembrane and intramembrane structures. The intermembrane spacing varies drastically according to the concentration of divalent cations [1]. Theoretical calculations of electrostatic interaction have revealed that the change in the intermembrane spacing is explained well by binding of ions with a binding constant of 21 M^{-1} for Ca^{2+} and 2.5 M^{-1} for Mg^{2+} [2,3]. The intramembrane structure has been studied by calorimetry and ultrasonic measurements, indicating that the gel-to-liquid crystal transition temperature increases monotonically [4,5], whereas the critical phenomenon has a maximum at about 10 mM Ca^{2+} [5]. However, little molecular information about the Ca^{2+}-phosphatidylcholine interaction is available because the intramembrane properties have been studied mainly by thermodynamic measurements. We consider that the following questions should be answered. How does the molecular motion of a polar head group as well as a hydrocarbon chain vary on addition of Ca^{2+}? Is the structural change really caused by the binding of Ca^{2+} and not by free Ca^{2+} in the solvent?

These questions may be answered by dynamic as well as steady-state fluorescence anisotropy measurements which are suitable for monitoring the molecular motions in membranes [6]. There are many fluorescent probes containing, e.g., hydrophobic probes and lipid-like probes with fluorophores in the polar head region. Therefore, an

appropriate combination of fluorescent probes should reveal the difference in dynamic properties between different parts of membranes. Furthermore, the fluorescence technique has the advantage that the restriction of molecular motion (the cone angle of barrier against wobbling motion) as well as the rate of rotation (wobbling diffusion constant) may be determined separately [6,7].

We have measured the steady-state and dynamic fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) and dansylphosphatidylethanolamine (DPE) in dipalmitoylphosphatidylcholine membranes. Ca^{2+} reduced the cone angle of DPH in the hydrocarbon region, monotonically increasing the anisotropy even at a concentration as low as 1 mM. On the other hand, the anisotropy of DPE had a shallow minimum. The comparison between Ca^{2+} and Mg^{2+} demonstrated clearly that the binding of the ions causes the intramembrane structural change.

2. Materials and methods

Synthetic L-α-dipalmitoylphosphatidylcholine (DPPC) was purchased from Sigma and used without further purification. DPH was the product of Molecular Probes and DPE was synthesized by Dr. Y. Kimura.

We prepared dilute suspensions of multilamellar liposomes as described previously [8,9]. Vigorous stirring was avoided to preserve large multilamellar liposomes. The dry weight concentration of lipid was 0.028 mg/ml (turbidity: 0.05 at 360 nm) for steady-state measurement and 0.055 mg/ml (turbidity 0.2–0.25) for time-resolved fluorescence anisotropy measurement. The molar ratio of fluorescent probe to DPPC was 1 : 1000 (steady-state measurement) and 1 : 500 (dynamic measurement).

The steady-state as well as time-resolved fluorescence anisotropy of DPH was measured by a computer-aided multipath nanosecond fluorometer at different Ca^{2+} and Mg^{2+} concentrations and temperatures. The concentration of Ca^{2+} and Mg^{2+} was changed by titrating with condensed stock solutions. The suspension was incubated at 42–44°C for 30 min after the titration in order to ensure that Ca^{2+} and Mg^{2+} were distributed uniformly in the multilamellar system. The incubation temperature was selected so that the permeability of the membrane became maximum [10,11], which was confirmed from the time course of the fluorescence anisotropy as well as the ultrasonic properties, so that equilibrium was reached within the incubation time. The rate of heating and cooling in the temperature-dependence measurement of steady-state anisotropy was 15 and 30°C/h, respectively.

The time-resolved anisotropy decay was analyzed by a wobbling-in-cone model which was developed by Kinosita et al. [6,7]. The anisotropy was separated into two terms: a relaxation term and an equilibrium term.

$$\frac{r(t)}{r_0} = \frac{r_\infty}{r_0} + \left(1 - \frac{r_\infty}{r_0}\right) \exp\left(-\frac{t}{\phi}\right), \tag{1}$$

$$\frac{r_\infty}{r_0} = S^2 = \left\{\tfrac{1}{2} \cos\theta_c (1 + \cos\theta_c)\right\}^2, \tag{2}$$

$$\phi = \frac{\langle\sigma\rangle}{D_w}, \tag{3}$$

in which r_0 and r_∞ are the anisotropy at the time of excitation and the stationary anisotropy, respectively, and ϕ denotes the wobbling relaxation time. θ_c is a cone angle in which probes may rotate and D_w represents a wobbling diffusion constant in the limited angel of θ_c. $\langle\sigma\rangle$ is a parameter depending on θ_c alone. The parameter S denotes the average orientational order parameter. The influence of Ca^{2+} on the cone angle and the wobbling diffusion constant was examined separately.

3. Results

We first measured the dynamic anisotropy decay in order to elucidate the details of the Ca^{2+} effects on the molecular mobility of phosphatidylcholine bilayers. Fig. 1 shows the time-resolved anisotropy decay of DPH in a DPPC membrane at 43.8°C in the absence and presence of Ca^{2+} at 300 mM. The anisotropy decay curves are characterized by an initial fast decay together with a constant anisotropy after a sufficiently long time,

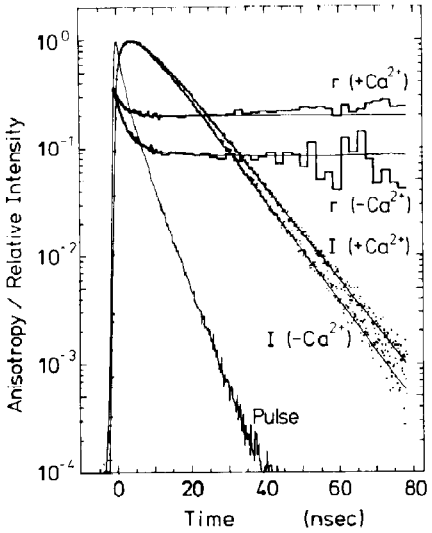

Fig. 1. Fluorescence anisotropy decay (r) of DPH in DPPC membranes in the presence ($+Ca^{2+}$) and absence ($-Ca^{2+}$) of 0.3 M $CaCl_2$. The anisotropy decay curves (r, zigzag lines) were analyzed by a wobbling-in-cone model, as indicated by smooth lines. Total fluorescence intensity (I, dots) and light pulse are also shown.

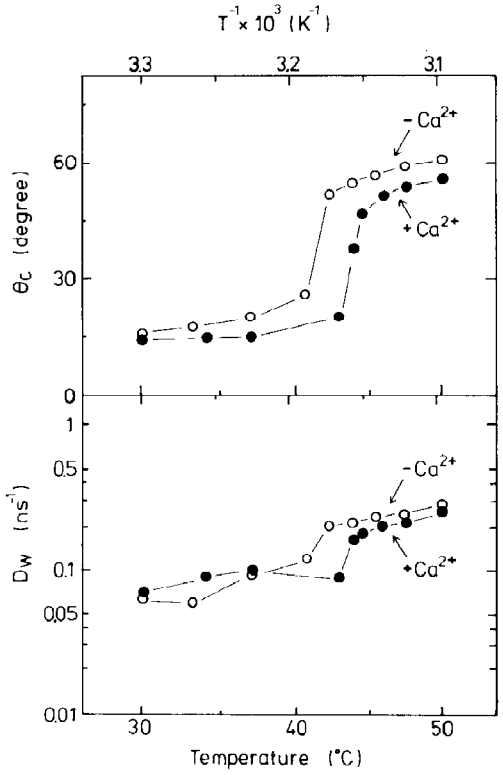

Fig. 2. Cone angle θ_c and wobbling diffusion constant D_w of DPH in DPPC membranes as a function of temperature. (●, ○) Suspension in 0.3 M $CaCl_2$ solution and in pure water, respectively.

as described by Kawato et al. [6]. Solid lines indicate the result of the analysis by eqs. 1–3 with a least-squares method, assuming the wobbling-in-cone model. The agreement between the analyzed curves with the experimental data is good enough demonstrating that DPH molecules rotate in a limited angle in the presence as well as absence of Ca^{2+}. The stationary value of the anisotropy apparently increased due to the addition of Ca^{2+}, suggesting that the rotational motion of DPH was more restricted.

The time-resolved anisotropy decay was measured with and without Ca^{2+} as a function of temperature in the range 30–50°C. The cone angle and the wobbling diffusion constant analyzed with the use of eqs. 1–3 are plotted as a function of temperature in fig. 2. The characteristic features of the temperature dependence are as follows: Firstly, the temperature at which θ_c and D_w changed sharply shifted by about 2.5°C to higher temperature in the presence of Ca^{2+}. This is in accordance with the increase in transition temperature as observed with calorimetry [4] and ultrasonic measurements [5]. Secondly, the cone angle was reduced by the addition of Ca^{2+}, particularly in the higher temperature region, whereas the change in wobbling diffusion constant was not significant in the temperature region far from the transition point. This feature of the Ca^{2+} effect on the mobility of DPH molecules indicates that the free volume for the molecular rotation is restricted by Ca^{2+} binding, while the rate of molecular motion is not changed significantly.

Since the wobbling diffusion constant remains constant with respect to Ca^{2+} concentration except in the transition region, it should be reasonable to study the interaction between divalent cations and DPPC bilayers in terms of steady-state anisotropy which reflects mainly r_∞. When the wobbling relaxation time ϕ is much smaller than the fluores-

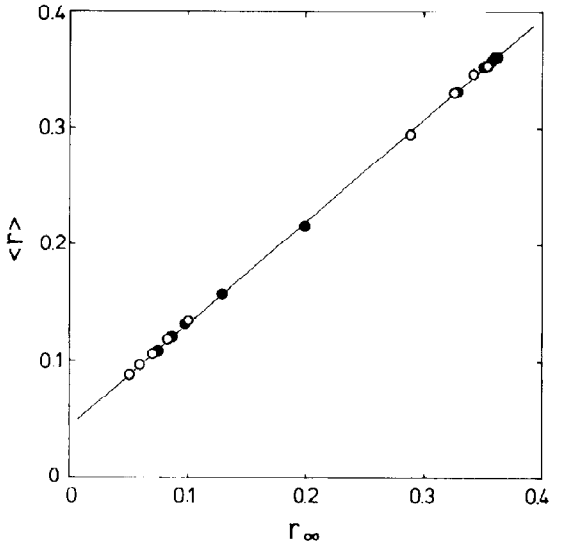

Fig. 3. Relationship between steady-state anisotropy, $\langle r \rangle$, and equilibrium anisotropy, r_∞, in the presence (●) and absence (○) of 0.3 M $CaCl_2$.

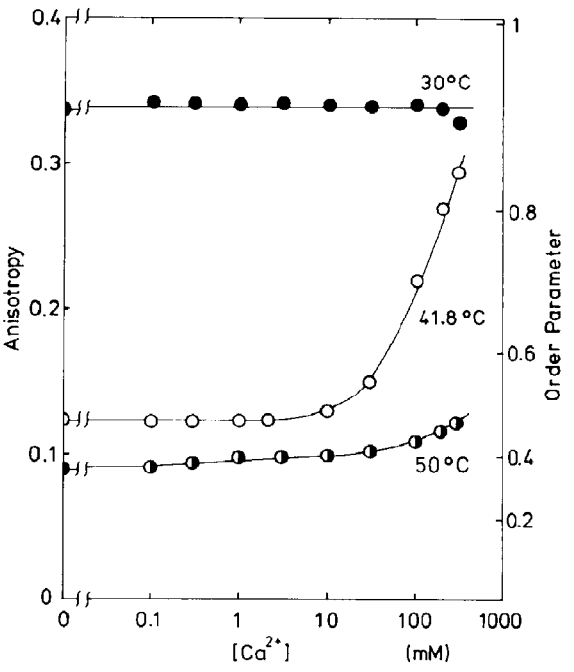

Fig. 4. Steady-state anisotropy (and order parameter) of DPH in DPPC membrane as a function of Ca^{2+} concentration at 30 (●), 41.8 (○) and 50°C (◑)

cence lifetime without quencher, τ_0, which is valid in DPPC membranes for DPH whose optical transition moments are parallel to the probe axis [6], the average anisotropy, i.e., the steady-state anisotropy, is approximately proportional to r_∞ [12]:

$$\langle r \rangle = \frac{\phi}{\tau_0 + \phi} r_0 + \frac{\tau_0}{\tau_0 + \phi} r_\infty, \qquad (4)$$

In fact, the linear relationship, $\langle r \rangle = 0.041 + 0.887 r_\infty$, was obtained from the measurement of the temperature dependence of the decay parameters irrespective of Ca^{2+} concentration as shown in fig. 3. Therefore, we carried out further studies of various effects of divalent cations in terms of the steady-state anisotropy.

Fig. 4 shows the Ca^{2+} concentration dependence of the steady-state anisotropy of DPH in DPPC membranes at 30, 41.8 and 50°C. The steady-state anisotropy, $\langle r \rangle$, represents the order of the hydrocarbon chains in lipids, because r_∞ is directly proportional to the order parameter of the lipid hydrocarbon chains (eq. 2) [12–15]. The values of the order parameter are also shown in figs. 4, 6 and 7. The lipid hydrocarbon chains were in the ordered state at 30°C irrespective of the Ca^{2+} concentration while they were in the disordered state at 50°C. Drastic change in the anisotropy was observed above 10 mM Ca^{2+} at the temperature (41.8°C) indicating the Ca^{2+}-induced phase transition in DPPC membranes, which was in accordance with the increase in transition temperature [4,5]. It should also be pointed out that the anisotropy in the liquid crystalline phase (50°C) exhibited a small but definite increase at a Ca^{2+} concentration as low as 1 mM. Because the Ca^{2+}-DPPC complex comprises only 0.4% of the total number of DPPC molecules at 1 mM Ca^{2+} as calculated in the preceding paper [5], this result indicates clearly that the order parameter of hydrocarbons is influenced by a long-range interaction between bound Ca^{2+} and DPPC molecules within the Debye screening length.

In order to compare the Ca^{2+} effect on the hydrocarbon region to that on the polar group, we carried out analogous measurements using DPE instead of DPH in DPPC membranes. A shown in

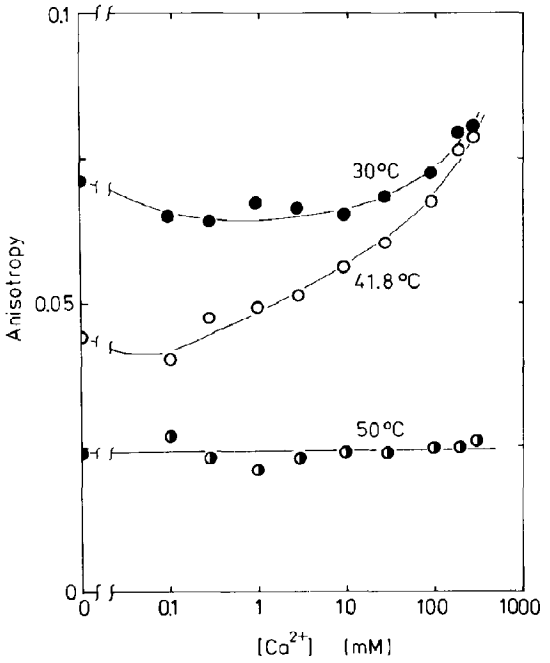

Fig. 5. Steady-state anisotropy of DPE in DPPC membrane as a function of Ca^{2+} concentration at 30 (●), 41.8 (○) and 50°C (◑).

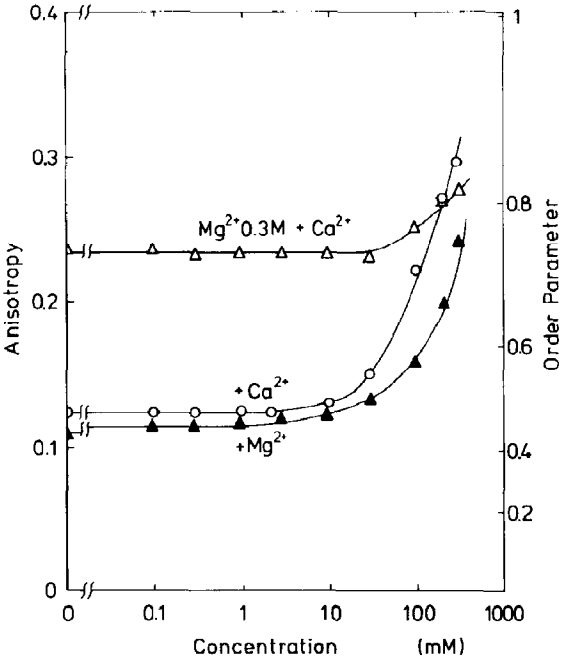

Fig. 7. Steady-state anisotropy (and order parameter) of DPH in DPPC membranes at 41.8°C as a function of the concentration of divalent cations; Ca^{2+} (○), Mg^{2+} (△) and Ca^{2+} in the presence of 0.3 M Mg^{2+} (▲).

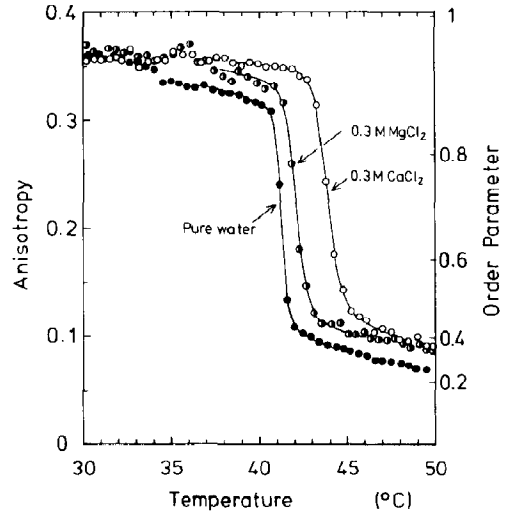

Fig. 6. Temperature dependence of steady-state anisotropy of DPH in DPPC membrane suspended in pure water (●), 0.3 M $CaCl_2$ (○) and 0.3 M $MgCl_2$ (◑).

fig. 5, the behavior of DPE is somewhat different from that of DPH. Firstly, the magnitude of the anisotropy of DPE is smaller than DPH, suggesting a larger free rotation of the dansyl group in the polar region. Secondly, the anisotropy of DPE appears to decrease in the Ca^{2+} concentration range lower than 0.1 mM at 30.0°C. This suggests the occurrence of long-range interaction between the bound Ca^{2+} and the distant polar head groups of lipids, because the bound Ca^{2+} amounts to less than 1% of the lipid molecules in this Ca^{2+} concentration range.

Fig. 6 shows the temperature dependence of the steady-state anisotropy of DPH as a function of Ca^{2+} and Mg^{2+} concentrations. The transition temperature increased in the presence of both Ca^{2+} and Mg^{2+}. However, the increase in the transition temperature due to Mg^{2+} was much smaller than that with Ca^{2+}. A small decrease in the anisotropy at the pretransition of DPPC of 34°C was still observable at higher temperature in the presence

of 0.3 M $MgCl_2$ while it disappeared at 0.3 M $CaCl_2$. Also, the increase in anisotropy in the liquid crystalline phase appears to be smaller with Mg^{2+} than with Ca^{2+}. The concentration dependence of the anisotropy was measured in detail at 41.8°C for both Ca^{2+} and Mg^{2+} (fig. 7). It is apparent from fig. 7 that the increase in the anisotropy is much larger for Ca^{2+}. Because the ionic concentration in the solvent is the same and there is little difference in the solution properties between these cations, the different effects must be due to the different binding constants: 21 M^{-1} for Ca^{2+} and 2.5 M^{-1} for Mg^{2+} [3]. When Ca^{2+} was added to the DPPC liposome suspension in the presence of 300 mM Mg^{2+}, the increase of anisotropy was smaller than that in the absence of Mg^{2+}. Since Ca^{2+} and Mg^{2+} show apparent competitive binding to DPPC membranes due to electrostatic interaction [3], the reduction of the Ca^{2+} effect due to Mg^{2+}, also proves that the Ca^{2+} effects are caused by binding of the ions.

4. Discussion

The present results can be summarized as follows: (1) the effects of Ca^{2+} binding on the molecular mobility in the lipid hydrocarbon region are characterized mainly by the reduction of the range of free rotation (the cone angle). (2) The Ca^{2+} effects, i.e., the increase of the anisotropy as well as the transition temperature, are observed at Ca^{2+} concentration even below 1 mM. (3) The effects of Ca^{2+} are significantly different from those of Mg^{2+}.

Kinosita et al. [7] first analyzed the rotational motion of fluorescent probes in terms of the wobbling-in-cone model in the absence of divalent cations. They have shown that the angle of the rotation is limited in a certain range (cone angle) which is affected by various factors [16]. The smaller cone angle indicates the closer packing of the hydrocarbon chains. Thus, the decrease in the cone angle due to Ca^{2+} suggests that the hydrocarbon chains are more ordered and packed in the presence of Ca^{2+}, forming a narrower potential well for the rotational motion of DPH.

There are, in principle, two possible mechanisms to explain the Ca^{2+} effects at very low ionic concentration: the interaction between Ca^{2+} in the solvent and the polar head groups of DPPC molecules and the long-range interaction between bound Ca^{2+} and DPPC molecules. However, the large difference between Ca^{2+} and Mg^{2+} excludes the possibility of the former, because the properties of these ions in solution are very similar. If only short-range interaction occurs between bound Ca^{2+} and DPPC molecules, a two-step decrease in the anisotropy should be observed in the temperature dependence: one from lipid molecules within the influence of bound Ca^{2+} and the other from free lipid molecules which are far from bound Ca^{2+}. The experimental results, however, indicated a sharp single-step change, leading to the conclusion that all lipid molecules are almost equivalent, even when the concentration of bound Ca^{2+} is less than 10 mM, where the bound Ca^{2+} amounts to about 2% of lipid molecules (data not shown). Therefore, the present results strongly suggest the presence of long-range interaction between bound Ca^{2+} and the surrounding DPPC molecules. It seems that the decrease in anisotropy of DPE at Ca^{2+} concentrations below 0.1 mM is also due to long-range interaction in the polar region.

Since direct interaction between DPH and Ca^{2+} or between hydrocarbon chains and Ca^{2+} is inconceivable, the interaction between the polar head group of the DPPC molecule and Ca^{2+}, which is electrostatic in nature, must be the main cause of the closer packing of hydrocarbon region. The fact that the wobbling diffusion constant was hardly affected by the addition of Ca^{2+} also appears to indicate the interaction mechanism through the polar head group: If there is direct interaction between the bound Ca^{2+} and hydrocarbons, the rotational diffusion constant of DPH should be influenced by the Ca^{2+}. Thus, the present results demonstrate the Ca^{2+}-phosphatidylcholine interaction in which the binding of Ca^{2+} to the polar head group induces the long-range interaction in the polar region and causes tightening of the hydrocarbon chain packing throughout the DPPC membrane.

In order to explain the long-range interaction in the polar head group region as well as the closer packing in the hydrocarbon region in the presence

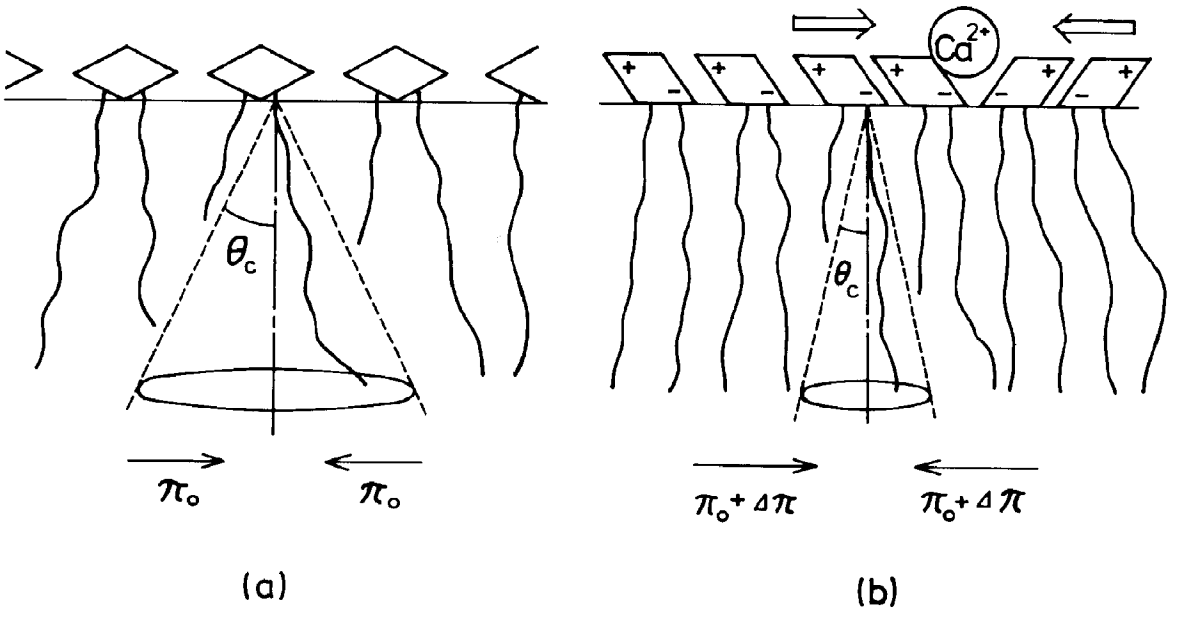

Fig. 8. Schematic diagram of the molecular model of the Ca^{2+}-phosphatidylcholine interaction. Ca^{2+} binds to a negatively charged group of the zwitterionic head group and attracts the polar head groups of surrounding phosphatidylcholine molecules. The pressure is changed from π_0 to $\pi_0 + \Delta\pi$ by the addition of $CaCl_2$.

of Ca^{2+}, we propose a molecular model of the Ca^{2+}-phosphatidylcholine interaction, as shown schematically in fig. 8 [5]. A Ca^{2+} binds to a negatively charged group of the zwitterionic head group of DPPC. Then, the positive net charge of the Ca^{2+}-DPPC complex produces an electric field not only in the direction perpendicular to the membrane but also in the plane of the membrane. Due to the electric field in the plane of the membrane, the zwitterions of the surrounding DPPC molecules are rearranged due to the electric field in the plane of the membrane so that the negatively charged groups are oriented to the bound Ca^{2+}. Consequently, bound Ca^{2+} and the surrounding DPPC molecules attract each other, and produce a larger lateral pressure, $\pi_0 + \Delta\pi$, than that in the absence of Ca^{2+}, π_0, and a closer packing of hydrocarbon chains. This explains the decrease of the cone angle, θ_c, in the presence of Ca^{2+}. The long-range nature of this kind of interaction was discussed in the preceding paper and it is quite plausible that the electric field of bound Ca^{2+} covers the whole membrane surface [5].

In the preceding paper, we measured the ultrasonic properties of DPPC membranes and found that the critical phenomenon became maximum at a Ca^{2+} concentration of about 10 mM, whereas the transition temperature increased monotonically. These types of transition behavior were discussed in detail and explained well by the same molecular model as shown in fig. 8. Therefore, the Ca^{2+} effects on the molecular motion as well as on the thermodynamic properties of DPPC membranes are consistently explained by the long-range attraction between bound Ca^{2+} and the polar head groups of DPPC molecules which increases the lateral pressure. We have discussed the Ca^{2+}-phosphatidylcholine interaction only qualitatively. The quantitative calculation will be reported elsewhere.

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