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Determination of binding constants of Ca^{2+}, Na^{+}, and Cl^{-} ions to liposomal membranes of dipalmitoylphosphatidylcholine at gel phase by particle electrophoresis

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Abstract

The zeta potentials of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) liposomes were measured at a gel phase as a function of $CaCl_2$ concentration (0–200 mM) in a solution containing different NaCl concentrations (0–200 mM). The data obtained were analyzed with the diffuse double layer theory including the Graham theory. The intrinsic binding constants of ions to DPPC membranes and the distance of the shear plane have been determined independent of both the concentration of $CaCl_2$ and that of NaCl. The values of the constants were 37 M^{-1} for Ca^{2+}, 0.28 M^{-1} for Cl^{-}, and 0.25 M^{-1} for Na^{+}; the distance was 0.24 nm under the assumption of binding stoichiometry of Ca^{2+}/DPPC = 1:1.

Keywords: Dipalmitoylphosphatidylcholine; Calcium ion; Binding constant; Electrophoresis; Liposome; Shear plane

1. Introduction

Calcium ions have an especially important role in many cellular processes [1]. The ions bind naturally to negatively charged phospholipids as phosphatidylserine [2–4] and phosphatidylglycerol [5,6] but rather weakly to zwitterionic lipids as phosphatidylcholine (PC) and phosphatidylethanolamine. In order to study the binding mechanism of Ca^{2+} to PC, several techniques have been employed: X-ray diffraction [7–11], calorimetry [7], NMR [12–14], myelin form formation [15,16], force measuring method [17], ultrasonic technique [18–20], fluorescence anisotropy [21], and particle electrophoresis [22–25]. Though some studies have determined the intrinsic binding constants of Ca^{2+} to PC, the reported values have been scattered in the order of 1–100 M^{-1} [14].

Among those techniques the particle electrophoresis gives us electrical information about the intact surface of phospholipid liposomes. Recently developed equipments of the particle electrophoresis made it possible for us to measure the zeta potentials (the potentials at the shear plane) of many liposomes in a short period of time. But in order to know the binding constants of ions to lipid membranes, we must determine the surface potentials of the liposomes. The potentials are determined from the zeta potential if the distance of the shear plane is determined.

This paper is the first trial to determine both the binding constants of ions for DPPC membranes and the distance of the shear plane only by the use of the particle electrophoresis technique. The zeta potentials of DPPC liposomes were measured at a gel phase as a function of $CaCl_2$ concentration in buffered solutions containing different NaCl concentration. Data were analyzed on the basis of the double layer theory including the Graham theory to determine the binding constants of Ca^{2+}, Na^{+}, and Cl^{-} to DPPC membranes and the distance of the shear plane.

2. Materials and methods

2.1. Sample preparation

Synthetic DPPC (99%, by TLC) was purchased from Sigma. The electrolytes of $CaCl_2$ (anhydrous, Kanto), NaCl (Wako Pure Chem), Tris-(hydroxymethyl)amino-

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methane (Nakarai), and HCl (Kanto) were of reagent grade. These chemicals were used without further purification. The $CaCl_2$ was baked at 160°C for 6.5 h to remove any organic contaminants. Distilled, deionized water was freshly distilled on use in all experiments. Aqueous solution was buffered to pH 7.2 with 5 mM Tris (4.5 mM HCl). The solution was filtered through millipore filter of 0.22 μm to remove dust.

The powder of DPPC was dispersed in the solution to form liposomes at 60°C which is above the main phase transition temperature of DPPC in excess water (T_c = 41°C) [26]. Then the dispersion of liposomes was incubated at that temperature for over 10 min, and shaken by hand. Furthermore, two kinds of liposomes were prepared as follows:

Sonicated liposomes. The incubated liposomes were softly sonicated at the incubated temperature with ultrasonic vibrator (Handy Sonic UR-20P, Tomy Seiko, Tokyo). Sonication was done at the power of 22 W for 3 min at 55–60°C. The concentration of DPPC was 2.7 mM for solution in the absence of NaCl; 2 mM for 25 mM NaCl, and 0.68 mM for both 100 mM and 200 mM NaCl.

Extruded liposomes. The incubated liposomes were extruded through polycarbonate membrane filters by a Extruder (Lipex Biomembranes) at 55°C. In the case of extrusion through 0.4 μm filters the concentration of DPPC was 1.36 mM and the extrusion was done four times at the pressure of 0.3 kgf/cm^2. In the case of 0.1 μm filters the concentration was 5.44 mM and the extrusions were done ten times at 18 kgf/cm^2. And in the case of 0.2 μm filters the concentration was 2.72 mM and the extrusion was done one to three times at 6 kgf/cm^2.

Both liposomes were considered to be multilayer. The extruder was used in order to disperse DPPC powder when the sonication at 22 W was not strong enough to disperse the powder.

The dispersions of the liposomes were left in air at room temperature till measurements which were done 3–30 h after the preparations. Calcium ions never induced fusion but serve to prevent aggregation by adding positive charges on the surface of the liposomes. On the measurements of zeta potentials the sonicated dispersions were diluted to the ratio of 1/3–1/30, and the extruded dispersions to 1–1/3, depending on salt concentration.

2.2. Measurements of zeta potentials

The zeta potentials of liposomes were measured with a particle electrophoresis apparatus of Penkem SYSTEM 3000. All measurements were done at 25°C which was controlled with the precision of 0.1°C. The liposomes were moved under the electric field of below 52 V/cm for 16 returns. The measurements were repeated usually three times and the averaged values were used.

Zeta potentials ζ is calculated with the following Helmholtz-Smolukowski equation (expressed in SI units) [27] from statistically averaged mobility μ:

$$\zeta = \mu\eta/\varepsilon = (v/E)\eta/\varepsilon \quad (1)$$

where η and ε, are the viscosity and the dielectric constant of solution respectively, v, the average velocity of liposomes, and E, the applied electric field. We used the values of $\eta = 8.950 \cdot 10^{-4}$ N · s/m^2 (= 0.8950 cP), and $\varepsilon = 7.854 \cdot 10^{-10}$ F/m (= 78.54 CGSesu) at 25°C, which had been set in computer software of System 3000.

This Eq. (1) was deduced under some assumptions [28]. Among them two are important for our experiments.

One assumption is that moved particles are rigid bodies and electrically insulators. It is reasonable to consider that the liposomes used satisfy this assumption. No deformation of liposomes would occur while being moved by an electric field since the applied field was low and the liposomes composed of multilayer of bilayer membranes. The membranes would be regarded as insulator in bulk electrolyte solution. According to Sakurai et al. [29] the apparent specific conductivity along the interface between a DPPC monolayer and a surface of pure water has been measured to be order of 1 S/m at 25°C. Though this value is several hundred times as large as that of 10 mM KCl solution [30], the thickness of bilayer membrane is so small at the order of 10 nm [31] enough for the membrane to be regarded as insulator in bulk electrolyte solution.

The other assumption is that the diameter of particles are sufficiently larger than the Debye length λ_D and the surface of the particles is considered as a flat plane. In order to satisfy this assumption we selected the dispersions of liposomes that the ratio of the average radius to λ_D was over 27. By this selection the error that comes from this assumption is reduced to be less than 8% according to the calculation by O'Brien and White [32]. An actual error would be smaller since the contribution of larger liposomes to average mobility would be larger than that of smaller ones in laser doppler system of PEN KEM SYSTEM 3000c.

The size distributions of liposomes were measured with Malvern System 4700c sub-micron particle analyzer in which dynamic light scattering was in use. Since the liposomes prepared were distributed in size, we used 'z average mean size' which is a representative value of radius for many liposomes in solution. The average values of the mean size were used as the average radius of liposomes.

The dispersions used contained various size of liposomes and the size distribution of the dispersions depended on the concentration of $CaCl_2$. However the zeta potentials obtained had a single sharp peak. And both kinds of liposomes, which naturally had different size distributions, showed almost the same zeta potential as will be shown later in Fig. 4. Those facts mean that the size distribution did not seriously affect on the mobility of the liposomes in our condition of measurements. This will be understood

since the mobility is proportional to the surface charge density never on the size in Debye–Hückel approximation.

2.3. Basic theory for data analysis

We consider PC liposomes in unbuffered aqueous solution that contains Ca^{2+}, Na^+, and Cl^-. Buffer effects will be taken into consideration in the next section. In addition to the assumptions introduced in deducing Eq. (1) we assume as follows:

1. The ions of Ca^{2+} and Cl^- bind to PC independently.
2. The cations of Ca^{2+} and Na^+ bind to PC competitively.
3. The surface potentials of one bilayer was not affected from other bilayers, and one side of the bilayer from the other side.
4. The bilayer membranes of PC are charged only by ion binding.
5. The depletion of ions in bulk solution by ion binding is neglected.
6. The binding stoichiometry of Ca^{2+}/PC is $1{:}n$, that of Cl^-/PC, 1:1, and that of Na^+/PC, 1:1. We will assume $n = 1$ in our analysis in Section 3.

The 1st and 2nd assumptions are thought to be reasonable. In fact those have been used in other theoretical treatments [6,33–35]. The importance of the binding of Cl^- and Na^+ will be shown in Section 3.1. The 3rd have been used also in other theoretical works [8,10,11]. The 4th will be satisfied if attentions are paid for DPPC not to be degraded to produce fatty acids which has negative charge. The 5th is satisfied in our experiment as will be shown in Section 3.2. In the 6th assumption the value of n will be discussed in Section 4.1.

Though X-ray diffraction showed the dependence of the repeated distance between lipid bilayers on the concentration of Ca^{2+} [8,10], there is no need to think about the effects of Ca^{2+} on the structure of liposomes in our analysis since we have concern with the surface of the bilayers.

Let N_{Ca}, N_{Cl}, and N_{Na} denote the number densities of lipids bound with Ca^{2+}, Cl^-, and Na^+ in the membrane (in m^{-2}), respectively. Then the surface charge density σ (C/m^2) is expressed as

$$\sigma = e\left((2/n)N_{Ca} - N_{Cl} + N_{Na}\right) \tag{2}$$

where e is the elementary electric charge. The number densities are related to the intrinsic binding constants of Ca^{2+}, Cl^-, and Na^+ to C, K_{ca}, K_{Na}, and K_{Cl} as follows;

$$N_{Ca} = K_{Ca}(N - nN_{Ca} - N_{Na})C^{o}_{Ca} \tag{3}$$

$$N_{Na} = K_{Na}(N - N_{Na} - nN_{Ca})C^{o}_{Na} \tag{4}$$

$$N_{Cl} = K_{Cl}(N - N_{Cl})C^{o}_{Cl} \tag{5}$$

where C^{o}_{i} is the concentrations of ions of type i at the surface, and N, the number density of PC molecules in the bilayer membranes (in m^{-2}). From those equations the number densities are obtained as follows:

$$N_{Ca} = \frac{NK_{Ca}C^{o}_{Ca}}{1 + nK_{Ca}C^{o}_{Ca} + K_{Na}C^{o}_{Na}} \tag{6}$$

$$N_{Na} = \frac{NK_{Na}C^{o}_{Na}}{1 + nK_{Ca}C^{o}_{Ca} + K_{Na}C^{o}_{Na}} \tag{7}$$

$$N_{Cl} = \frac{NK_{Cl}C^{o}_{Cl}}{1 + K_{Cl}C^{o}_{Cl}} \tag{8}$$

The surface charge density σ is also expressed by the Graham equation as follows [27]:

$$\sigma = \text{sign}\,\sigma\left(2000\varepsilon N_A kT\sum_i C_i(\exp(-z_i e\phi_o/kT) - 1)\right)^{1/2} \tag{9}$$

$$= \text{sign}\,\sigma\left(2000\varepsilon N_A kT(C_{Ca}L1_o + C_{Na}L2_o)\right)^{1/2} \tag{10}$$

where sign σ is the sign of σ, ε, the dielectric constant of solution, N_A, Avogadro number, k, Boltzmann constant, T, the temperature, C_i (in M), the concentration of ions of type i at bulk solution, z_i, the valence of ions of type i. and ϕ_o, the surface electric potential of the liposomes. The effects of ions on ε [36] are neglected. In Eq. (10) we rewrote as follows:

$$L1_o = Y_o^2 + 2Y_o^{-1} - 3 \tag{11}$$

$$L2_o = Y_o + Y_o^{-1} - 2 \tag{12}$$

with

$$Y_o = \exp(-e\phi_o/kT) \tag{13}$$

The combination of Eqs. (2) and (10) is the basic equation for our analysis. We do not use the Debye–Hückel approximation where the second and the higher order of $(e\phi_o/kT)$ are neglected.

Since K_{Ca} is fairly larger than K_{Cl} and K_{Na} as we will see in Section 3.1, we set the values of K_{Cl} and K_{Na} to be 0 as the first approximation. Then the basic equation is written as:

$$n^2J^2X^2 + 2J(n - 2PJ/n^2)X + 1 = 0 \tag{14}$$

where

$$X = C_{Ca},\ J = K_{Ca}Y_o^2,\ P = P1/(L1_o + QL2_o) \tag{15}$$

with

$$P1 = (eN)^2/(2000\varepsilon N_A kT),\ Q = C_{Na}/X \tag{16}$$

J is the so-called apparent binding constant of Ca^{2+}.

Next we neglect the binding of Na^+ but take that of Ca^{2+} and Cl^- into consideration as the second approximation. In this approximation we set $K_{Na} = 0$ in the basic

equation and obtained the binding constant of Cl^- as follows:

$$K_{Cl} = \left\{\left(1 + K_{Ca}Y_o^2\left(nX - \mathrm{sign}\sigma(2/n)(PX)^{1/2}\right)\right)/(2+Q)\right\}/\left\{\mathrm{sign}\sigma(PX)^{1/2}\left((2/n-1)K_{Ca}X/Y_o - 1/Y_o\right) - \left(nK_{Ca}XY_o + 1/Y_o\right)X\right\} \quad (17)$$

For solution without Ca^{2+} we got the following formula:

$$K_{Cl} = \frac{(L2_o)^{1/2}Y_o}{(P1C_{Cl})^{1/2} - (L2_o)^{1/2}C_{Cl}} \quad (18)$$

Naturally, there is no approximation in the analysis with Eqs. (17) and (18) for solution without Na^+.

It is difficult to take the binding of Cl^- and Na^+ into consideration accurately in the analysis for solution that contains Ca^{2+}, Na^+ and Cl^-. Fortunately, the data show that the value of K_{Na} is considered to be near to that of K_{Cl} as wii be seen in Section 3.1. Hence, we approximate to be $K_{Na} = K_{Cl}$ to analyze this case. Then K_{Cl} is obtained as a solution of the following quadratic equation if the concentration of Ca^{2+}, X, is not 0.

$$aK_{Cl}^2 + bK_{Cl} + c = 0 \quad (19)$$

where

$$a = Q(2+Q)(X^3/P)^{1/2}$$

$$b = (QY_o + A(2+Q)/Y_o)(X/P)^{1/2} - QY_o - (2/n)K_{Ca}X(2+Q)Y_o + (2+Q)A/Y_o$$

$$c = A/(PX)^{1/2} - (2/n)K_{Ca}Y_o^2$$

with

$$A = 1 + nK_{Ca}XY_o^2 \quad (20)$$

For aqueous solution that do not contain Ca^{2+} ($X = 0$) but Na^+ and Cl^-, the equation for K_{Na} will be shown in the next section. Naturally Eq. (19) is reduced to Eq. (17) when K_{Na} is set to be 0, and Eq. (17) to Eq. (14) when K_{Cl} to be 0.

2.4. Buffer effects

We consider the buffer effects in all calculations. Since the aqueous solution was buffered to pH 7.2 with 5 mM Tris-4.5 mM HCl, the solution contain 0.5 mM Tris, 4.5 mM Tris cation ($HTris^+$), 4.5 mM Cl^- and negligible amount of H^+ ($10^{-4.2}$ mM) and OH^- ($10^{-3.8}$ mM) [37]. There is a possibility for Tris cations to bind to lipid phosphate groups. However, since Tris cations are larger than Na^+ ions, they are considered to be more difficult to penetrate into a binding site of the phosphate groups than Na^+ do. Hence, we assumed that Tris cations do not bind to PC but only serve to increase the ionic strength of the solutions.

Under this assumption, for the solution of $CaCl_2$ without Na^+, we treat the effects of Tris buffer to zeta potentials accurately by setting the value of C_{Na} to be the concentration of Tris cation in Eqs. (17) and (18). For the solution of NaCl without Ca^{2+}, we treat the buffer effects in the same manner. In this case the binding constant of Na^+ is obtained from the basic equation as

$$K_{Na} = \frac{(1-D)K_{Cl}C_{Cl}/Y_o - D}{K_{Cl}C_{Na}Y_o(1+D) + DK_{Cl}^2C_{Na}C_{Cl}} \quad (21)$$

where

$$D = (C_{Cl}L2_o/P1)^{1/2} \quad (22)$$

In calculations for aqueous solution with Ca^{2+}, Na^+, and Cl^-, we set C_{Na} as the sum of the concentration of Na^+ and Tris cation (4.5 mM) in Eq. (19) in order to simplify the calculation. In this treatment Tris cation is not distinguished from Na^+. Therefore, in this case the results of calculations with Eq. (19) is approximate. But significant error will occur only in the low concentration of $CaCl_2$ (< 0.05 mM) as we will mention in Section 3.1.

2.5. Numerical calculations

In the analysis in the next section we will assume $n = 1$ and set the surface area occupied by one molecule, B ($= 1/N$), to be $4.37 \cdot 10^{-19}$ m^2, which has been obtained by Furuya et al. [38] using X-ray analysis at 20°C. Calculations were done at the $CaCl_2$ concentrations where the zeta potentials were measured, and the lines in graphs were drawn through the calculated points with a spline program.

In calculating electric potential ϕ as a function of the space coordinate perpendicular to the surface x, we used the following Poisson-Boltzmann equation for a flat plane [27].

$$\left(\frac{d\phi}{dx}\right)_x = -\mathrm{sign}\sigma\left(\frac{2000N_A kT}{\varepsilon}\sum_i C_i \times\left(\exp(-z_i e\phi/kT) - 1\right)\right)^{1/2} \quad (23)$$

We used a numerical resolution technique, which is the same manner of Amory and Dufey [35]. We used a distance increment of 0.01 nm for the calculation of zeta potentials from surface potentials and that of 0.001 nm for surface potentials from zeta potentials.

3. Results

3.1. Determination of binding constants

The marks in Fig. 1 show the data of zeta potentials of DPPC liposomes as a function of $CaCl_2$ concentration with the use of the sonicated liposomes. The average

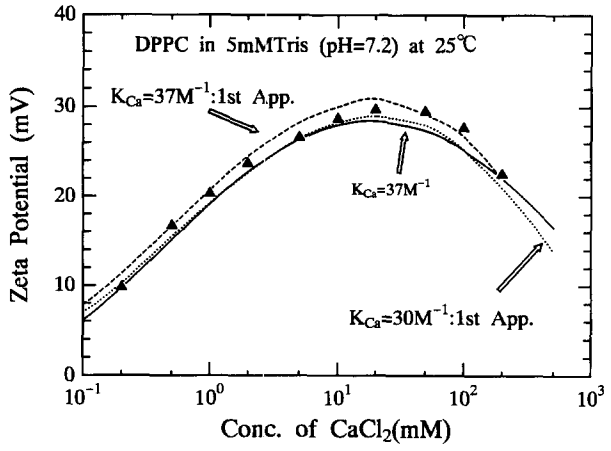

Fig. 1. The zeta potentials of DPPC liposomes as a function of $CaCl_2$ concentration at 25°C. The marks (▲) are the data points obtained with the sonicated liposomes. The dashed and the broken lines were the results of calculations in the first approximation in which the binding of Cl^- and Na^+ was neglected. The dashed line is the results of the calculation with $K_{Ca} = 30\ M^{-1}$ and $\Delta = 0.35$ nm, and the dotted line with $K_{Ca} = 30\ M^{-1}$ and $\Delta = 0.35$ nm. As a refererice the results of calculation by Eqs. (17) and (23) with the obtained parameters; $K_{Ca} = 37\ M^{-1}$, $K_{Cl} = 0.28\ M^{-1}$ and $\Delta = 0.24$ nm in Section 3.1.

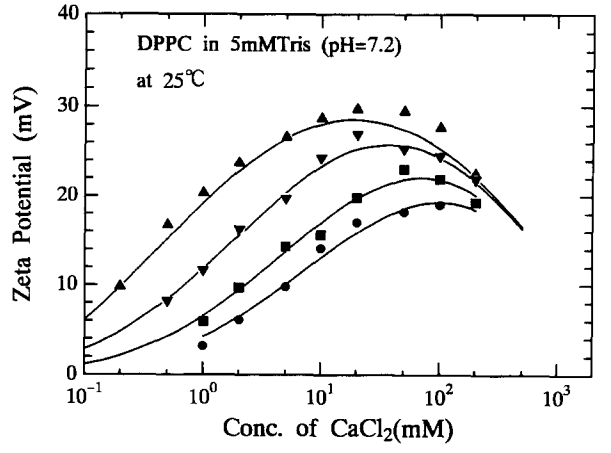

Fig. 2. The effects of NaCl on the zeta potentials of DPPC liposomes in solution of $CaCl_2$ at 25°C. The data points of (▲) were obtained for solution in the absence of NaCl, (▼) (■) and (●) in the presence of 25 mM, 100 mM and 200 mM NaCl respectively. The sonicated liposomes were used. The unbroken lines were the results of calculations. The line for solution without NaCl is the same one in Fig. 1. Another three lines show the results by Eqs. (19) and (23) with the values of $K_{Ca} = 37\ M^{-1}$, $K_{Cl} = K_{Na} = 0.28\ M^{-1}$ and $\Delta = 0.24$ nm.

radius of liposomes was over 150 nm. The dashed and dotted lines show the results of calculations in the first approximation with Eqs. (14) and (23). The values of K_{Ca} and the distance of the shear plane, Δ, were used as parameters in order to fit calculated values to the data. The values of the fitting parameters were $K_{Ca} = 37$–$30\ M^{-1}$ and $\Delta = 0.3$–0.4 nm. This approximation helps us to fix our aim in estimating their values for the best fit.

In the first approximation the binding of Cl^- ions is neglected. Actually the binding of Cl^- drops the surface potential. Hence, the value of K_{Ca} is naturally estimated lower. It will be shown later that the higher value of 37 M^{-1} gave better fitting than the lower value of 30 M^{-1} did in a wide $CaCl_2$ concentration range when we took the binding of Ca^{2+}, Cl^- and Na^+ into consideration. If we deal only with liposomes in solution shown in Fig. 1 we cannot notice the importance of the binding of Cl^- and Na^+.

Fig. 2 shows the effects of NaCl on the zeta potentials of DPPC liposomes in the solutions of $CaCl_2$. For solutions with 100 mM NaCl the average radius of the liposomes was 145–1000 nm depending on $CaCl_2$ concentration. We could fit the calculated values to the data points in the range of 0.5–200 mM $CaCl_2$ even in the first approximation. However, the value of fitting parameter K_{Ca} decreased with NaCl concentration as shown in Table 1 (Method A). On the other hand, if we assume the value of K_{Ca} to be constant, the value of Δ obtained will increase with NaCl concentration. Such unreasonable results come mainly from the neglect of Cl^- binding.

Next we analyzed the data in Fig. 2 by Eq. (17) of the second approximation where the binding of Ca^{2+} and Cl^- were taken into consideration. The value of $\Delta = 0.28$ nm gave the constant value of $K_{Cl} = 0.15\ M^{-1}$ independent of

Table 1
Fitting methods and values obtained

Method	n	K_{Na}(/M)	K_{Cl}(/M)	Δ (nm)	K_{Ca}(/M)			
					Sol. 1	Sol. 2	Sol. 3	Sol. 4
A	1 *	0 *	0 *	0.35	37	30	25	23.5
B	1 *	0 *	0.15	0.28	37 *	37 *	37 *	37 *
C	1 *	0.25	0.28	0.24	37 *	37 *	37 *	37 *
D	2 *	0 *	0.17	0.20	74	–	–	–
E	23	0 *	0 *	0 *	750	–	320	320

The values in the table were obtained by a best fit analysis in fitting the calculated zeta potentials to the measured potentials for dipalmitoylphosphatidylcholine (DPPC) liposomes. The measurements were done for the solution without NaCl (Sol. 1) and the solutions containing 25 mM NaCl (Sol. 2.), 100 mM (Sol. 3), and 200 mM (Sol. 4). In the table n represents the binding ratio, i.e., Ca^{2+}/DPPC = 1:n, and Δ, the distance of the shear plane. The symbols of K_{Na}, K_{Cl}, and K_{Ca} are the intrinsic binding constant of Na^+, Cl^-, and Ca^{2+} ions to DPPC membranes, respectively. The figures with the '*' marks show the postulated values. The '–' marks show that calculation was not done. This table shows that Method C gives us reasonable results.

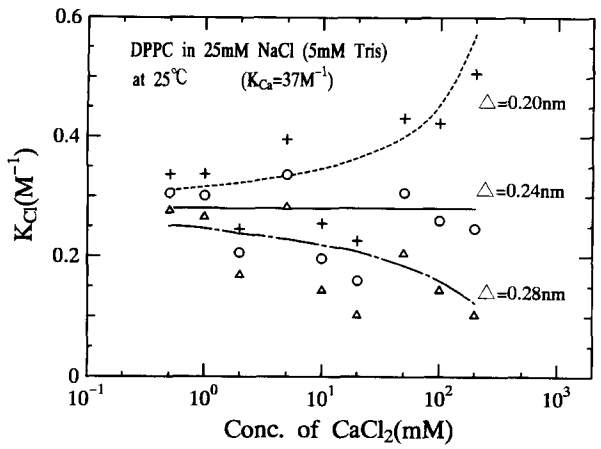

Fig. 3. The determination of the distance of the shear plane, Δ, for liposomes in a $CaCl_2$ solution with 25 mM NaCl at 25°C. The marks were obtained by calculations with the measured values of zeta potentials by the use of Eq. (19). The values of K_{Cl} were constant for $\Delta = 0.24$ nm (○), but decreased for $\Delta = 0.28$ nm (Δ) and increased for $\Delta = 0.20$ nm (+). The lines were obtained by calculations by the use of the calculated values of zeta potentials with the values of $K_{Ca} = 37\ M^{-1}$, $K_{Cl} = K_{Na} = 0.28\ M^{-1}$, and $\Delta = 0.24$ nm; the unbroken line for $\Delta = 0.24$ nm, the dotted chain for $\Delta = 0.28$ nm, and the broken line for $\Delta = 0.20$ nm.

concentrations of both $CaCl_2$ and NaCl (Method B in Table 1). And the fitting to the data was satisfactory in the range above 1 mM $CaCl_2$. However, in this case, the calculated values at the very low concentration of $CaCl_2$ below 0.01 mM were conflicting with the data. The analysis with Eq. (18) for 0 mM $CaCl_2$ showed that the zeta potential were negative and decreased with NaCl concentration as follows: −2.4 mV at 0 mM, −3.06 mV at 25 mM and −4.5 mV at 100 mM. On the contrary, the measured zeta potentials at 0 mM $CaCl_2$ were negative and showed a tendency to increase with NaCl concentration: −2.57 mV at 0 mM, −1.0 mV at 25 mM and −0.83 mV at 100 mM. These results show that the binding of Na^+ should not be neglected and that the binding constant is a little bit smaller than that of Cl^-.

Next we analyzed the data in Fig. 2 with Eq. (19) where $K_{Cl} = K_{Na}$ was assumed. We succeeded to obtain the value of Δ that gave the constant value of K_{Cl} independent of the concentration of both $CaCl_2$ and NaCl. For $K_{Ca} = 37\ M^{-1}$ we obtained the value as $\Delta = 0.24 \pm 0.04$ nm as shown in Fig. 3. The figure shows the results of calculations for 25 mM NaCl. The marks show the results with the measured values of zeta potentials. The average value of K_{Cl} was 0.26 M^{-1} for 25 mM NaCl, 0.30 M^{-1} for 100 mM NaCl, and 0.27 M^{-1} for 200 mM NaCl. We obtained the averaged value of $K_{Cl} = K_{Na} = 0.28 \pm 0.02\ M^{-1}$. The lines in Fig. 3 show the results of calculations by the use of calculated zeta potentials with the values of parameters obtained, $K_{Ca} = 37\ M^{-1}$, $K_{Cl} = K_{Na} = 0.28$, and $\Delta = 0.24$ nm. These lines explain the tendency of the marks though the marks scattered a little. In Fig. 2 the unbroken lines show the results of calculations with those values. The fitting to the data is fairly good. For $K_{Ca} = 30\ M^{-1}$ we obtained $\Delta = 0.28$ nm and $K_{Cl} = K_{Na} = 0.087\ M^{-1}$ by the same procedure.

Fig. 4(a) shows the difference between the fittings with $K_{Ca} = 37\ M^{-1}$ and with $K_{Ca} = 30\ M^{-1}$ to the data for solution without NaCl. The unbroken line shows the results of calculations with $K_{Ca} = 37\ M^{-1}$, and the dotted

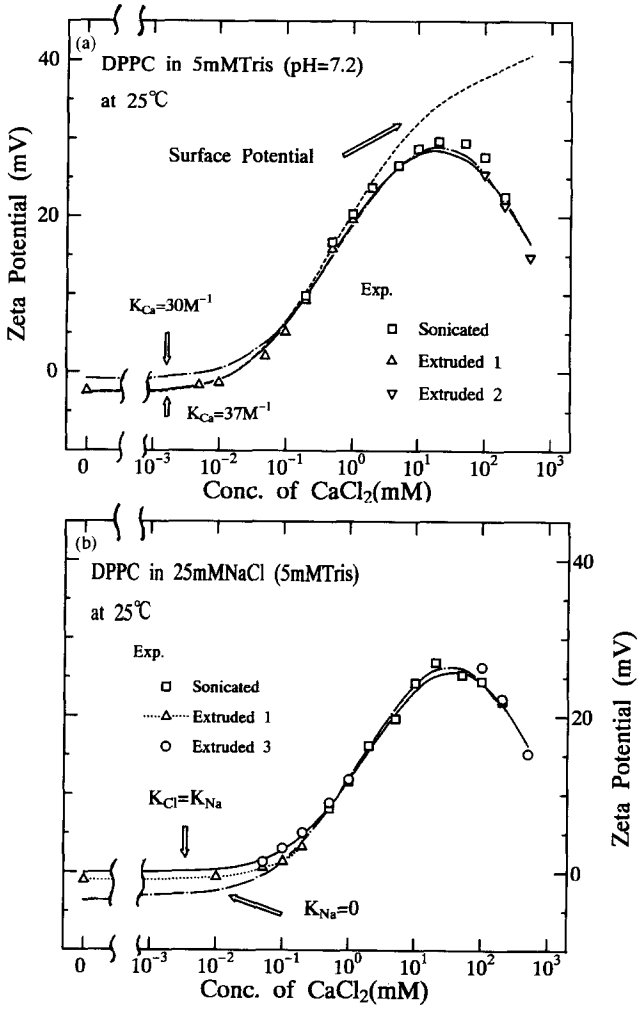

Fig. 4. The zeta potentials of DPPC liposomes as a function of $CaCl_2$ concentration at 25°C. (a) In the absence of NaCl: (□) sonicated liposomes (those data are the same as shown in Fig. 1), (Δ) extruded liposomes through 0.4 μm filters and (▽) extruded liposomes through 0.1 μm filters were used. The unbroken and the dotted chain lines shows the results of calculations with the values of $K_{Ca} = 37\ M^{-1}$, $K_{Cl} = 0.28\ M^{-1}$ and $\Delta = 0.24$ nm, and with $K_{Ca} = 30\ M^{-1}$, $K_{Cl} = 0.087\ M^{-1}$ and $\Delta = 0.28$ nm, respectively. The broken line shows surface potential for the former set of the values. The calculations were done with Eqs. (17), (18) and (23). (b) In the presence of 25 mM NaCl: (□) sonicated liposomes (those data are the same as shown in Fig. 2). (Δ) Extruded liposomes through 0.4 μm filters and (▽) extruded liposomes through 0.2 μm filters were used. The dotted line was drawn through data points below 0.2 mM for comparison with calculated lines. The unbroken line was the results of calculations by Eqs. (19) and (23) with the values of $K_{Ca} = 37\ M^{-1}$, $K_{Cl} = K_{Na} = 0.28\ M^{-1}$ and $\Delta = 0.24$ nm. The dotted chain line was the results with the same values but by the use of Eqs. (17) and (18) in which the binding of Na^+ was neglected.

chain line with $K_{Ca} = 30\ M^{-1}$. Both lines were similar over 0.1 mM $CaCl_2$ but they separate below 0.1 mM. The data below 0.005 mM $CaCl_2$ were near to the solid line. It shows that $K_{Ca} = 37\ M^{-1}$ would be the best selection.

Fig. 4(b) shows the importance of taking Na^+ binding into consideration for 25 mM NaCl solution. The unbroken line is the results of calculations with $K_{Ca} = 37\ M^{-1}$ by the use of Eqs. (19) and (23). Fitting the calculated values to the data is fairly well at the concentration of over 1 mM. However, below 0.5 mM the measured zeta potentials were slightly (0.5 mV) smaller than the calculated ones. This would come mainly from the assumption of $K_{Na} = K_{Cl}$ and partly from regarding Tris cation as Na^+ as explained in Section 2.4. The discrepancy between the measured data and the calculated line below 0.5 mM suggests that the value of K_{Na} is slightly smaller than that of K_{Cl}. In this figure, the dotted chain line shows the calculated potentials by use of Eqs. (17) and (18) where Na^+ binding is ignored. Those three lines in this figure show the importance of the binding of Na^+.

In order to obtain the value of K_{Na} by Eq. (21), the zeta potentials at 0 mM $CaCl_2$ were measured. The results were −1.0 mV for 25 mM NaCl and −0.83 mV for 100 mM NaCl. From those potentials we obtained $K_{Na} = 0.25\ M^{-1}$ with $K_{Ca} = 37\ M^{-1}$ (Method C in Table 1).

Since the calculations with $K_{Ca} = 30\ M^{-1}$ gave almost the same line as the line in Fig. 4(b), the determination of the value of K_{Ca} should be done from the analysis for solutions without NaCl.

3.2. State of liposomal surface

In Fig. 4(a) the surface potentials were shown for the liposomes in solution of $CaCl_2$ without NaCl as a function of $CaCl_2$ concentration. Though the surface potential continued to increase with the concentration, the value of the zeta potentials had maximum at about 20 mM $CaCl_2$. The depression of zeta potentials over 20 mM came mainly from the screening effect of ions. The dependence of potentials on the distance from the surface or the ion binding plane is easily calculated with Eq. (23) and explain the depression.

With the value of the surface potential, we calculated surface charge density and the ratio of ion bound PC molecules to whole PC in the membrane of liposomes. The calculations were done with Eqs. (6)–(8). Fig. 5(a) shows the number ratios of Ca^{2+} bound PC, N_{Ca}/N, and that of Cl^- bound PC, N_{Cl}/M as a function of $CaCl_2$ concentration for solution without NaCl. In the low concentration below 0.02 mM the value of N_{Cl}/N is almost constant of 0.11% and the value of N_{Cl} is larger than that of N_{Ca} below 0.05 mM. This explains that, in Fig. 4(a), the values of surface and zeta potentials were minus and almost constant in this concentration range. At this range Cl^- ions bound to PC come mainly from HCl of the buffer. It is very interesting that Cl^- ions bind to PC almost at the

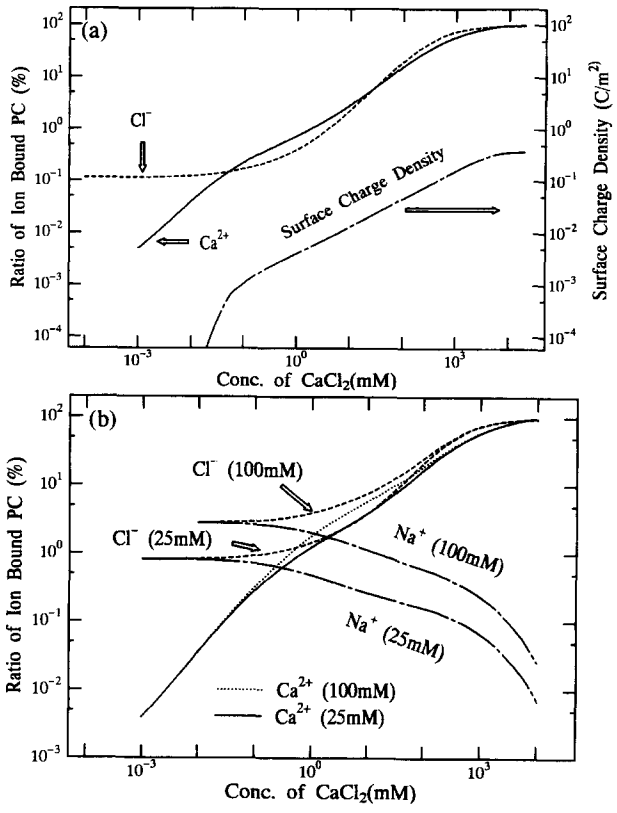

Fig. 5. The number ratios of ion bound phosphatidylcholine (PC) molecules to the whole PC in liposomal membranes as a function of $CaCl_2$ concentration. The calculations were done by Eqs. (6)–(8) with the values of $K_{Ca} = 37\ M^{-1}$, and $K_{Cl} = K_{Na} = 0.28\ M^{-1}$. (a) In the absence of NaCl. The solid line is for Ca^{2+}, and the dashed line for Cl^-. The surface charge density of the membranes is shown as a reference. (b) In the presence of 25 mM NaCl and 100 mM. The solid line and the dotted one are for Ca^{2+}, the former is in solution with 25 mM NaCl, and the later in solution with 100 mM NaCl. The broken lines are for Cl^- and the dashed chain lines are for Na^+.

same rate as Ca^{2+} ions do above 0.1 mM in spite of the low value of K_{Cl}. This will be explained as follows: N_{Cl} is proportional to C^o_{Cl} in Eq. (5) and C^o_{Cl} increases by Ca^{2+} ion binding. In Fig. 5(a) the surface charge density is also shown above 0.02 mM $CaCl_2$ where the density was positive.

Fig. 5(a) gives us the validity of the 5th assumption introduced in Section 2.3. When we take an example of 10 mM $CaCl_2$ solution, the figure shows that Ca^{2+} bound lipids is 3–6%. Since the lipids concentration was about 1 mM, the depletion of Ca^{2+} in the solution is 0.03 mM, which is 0.3% of 10 mM. This meets the assumption that the depletion of ions in solutions by ion binding is neglected. In much the same way as this example the validity of the assumption for any other $CaCl_2$ concentrations is shown.

Fig. 5(b) shows the dependence of N_{Ca}/N, N_{Cl}/N, and N_{Na}/N on the concentration of $CaCl_2$ for the liposomes in solution with NaCl. As this figure shows N_{Na} decreased

with $CaCl_2$ concentration while N_{Cl} increased as N_{Ca} did. This is because Cl^- was considered to bind independently of Ca^{2+} despite Na^+ competitively with Ca^{2+}. This figure also explains that the binding of Na^+ is effective only in the low concentration below 0.5 mM $CaCl_2$.

4. Discussion

4.1. Methods of fitting

As shown in Section 3.1 and Table 1 there are several ways of fitting the calculated values to the data.

In the analysis we assumed a stoichiometry of $Ca^{2+}/PC = 1:1$, i.e., $n = 1$, according to the following authors: Grasdalen et al. [13], and McLaughlin et al. [2], who worked on EggPC; Ohshima et al. [8], Lis et al. [10], and Marra and Israelachvili [17] who worked on DPPC. However, Altenbach and Seelig [14], who worked on 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), concluded $Ca^{2+}/PC = 1:2$, i.e., $n = 2$. Under the assumption of $n = 2$, we tried to fit calculated values to the data of Fig. 4(a) in the range of 0.5 mM to 100 mM by using Eq. (17). The best fit was done with the values of $K_{Ca} = 74\ M^{-1}$, $K_{Cl} = 0.17\ M^{-1}$, and $\Delta = 0.20$ nm (Method D in Table 1). The value of K_{Ca} was approximately doubled, which is anticipated since the binding cite density was reduced to half. Accordingly it was difficult to determine the stoichiometry only by our method.

The value of n can be used as a fitting parameter if we add the assumption of $\Delta = 0$ to the first approximation. This kind of fitting have been done by Tatulian [25], who obtained the value of $K_{Ca} = 441\ M^{-1}$, and $n \times B = 7.2$ nm^2 for DPPC at the temperature between the pretransition temperature ($T_P = 35°C$) and the main transition one ($T_C = 41.4°C$) [39]. With our data the obtained values of fitting parameters were $K_{Ca} = 750\ M^{-1}$ and $n = 23$ ($n \times B = 10$ nm^2) at 25°C. In fact the fitting to the data was not substantially worse. Under this assumption the drop of zeta potentials at the concentration over 20 mM $CaCl_2$ is explained mainly by the saturation of the binding cites and partly by screening effects. However, the values of n and K_{Ca} are about 20-times those shown in the previous section (or Method C in Table 1). Moreover, since we assumed $K_{Cl} = 0$ in this case, the value of K_{Ca} decreased with the concentration of NaCl (Method E). This is analogous to the case of Method A. These unreasonable results came from the assumption of $\Delta = 0$. Therefore, the assumption is unreasonable.

As shown in Table 1 and above when we neglected the binding of Cl^- we obtained the unreasonable results. But there may be a question. Is it possible to explain the results of Fig. 2 by using the proper charge density σ_0 instead of K_{Cl} as a parameter? There is a possibility for DPPC to degrade to produce fatty acids which would bring the negative surface charge density at the low concentration of $CaCl_2$. But the data in the figure show that the $CaCl_2$ concentration of zeta potential peak moves from low to high as the concentration of NaCl concentration increases. This behavior will not be explained only by the existence of such negatively charged components in the surface. This behavior will be explained only by taking the binding of Cl^- into consideration as described in this paper.

4.2. Binding constants

There has been a very wide spread in the reported values of the intrinsic binding constant of Ca^{2+} to PC, K_{Ca}, as pointed out in Section 1. Lis et al. [10] tried to determine the value of K_{Ca} by the osmotic stress method with X-ray analysis. But they obtained the values of 10 to several hundred M^{-1} depending upon the distance between bilayers and the ionic strength of solution. They suggested the change of the polar group orientation relative to the plane of the bilayer to explain their results [11]. Marra and Israelachvili [17] measured the force between the bilayer-coated surfaces and obtained $K_{Ca} = 120\ M^{-1}$. This higher value may come from the difference in the conditions of lipids and in the experimental method. Ohshima et al. [8] analyzed the effects of $CaCl_2$ on the repeat distance of lamellar and obtained the value of $K_{Ca} = 21 \pm 9\ M^{-1}$ at 5°C though they ignored the binding of Cl^-. Their value is very near to our result of $K_{Ca} = 37\ M^{-1}$.

When we consider the binding constant of ions to PC membranes we must pay attention to the fact that the ions interact with the lipids in membranes not with naked lipids. Since the ions interact with the head group of lipids, the configuration of the head group would affect the binding constants of ions. And the configuration should depend naturally on the state of assembly of the molecules. Hence, the binding constant for a lipid in bilayer membranes is naturally different from that in monolayer one. And even in bilayer membranes, the binding constants of ions to pure phospholipids, e.g., PC, may differ from those to PC in the mixtures of PC and another kind of phospholipids, e.g., phosphatidylglycerol (PG) [6]. In particular chemically synthesized phospholipids like DPPC show distinct changes in the state of assembly at their phase transition temperatures [39]. Recently Dufourc et al. [40] have reported that the tilt angle of DPPC molecules were 30° at the gel phase and 0° at the fluid phase while the geometrical parameters of the head group remained constant throughout the different phases. This means that the angle of head group of DPPC to the membrane surface at a liquid crystalline phase is larger than that at a gel phase. This would result in the lower binding constant of cations to DPPC at a liquid crystalline phase. In fact we obtained the values of K_{Ca} for DPPC at a liquid crystalline phase, $10\ M^{-1}$ at 44°C (Satoh, K. and Mishima, K., unpublished data), which were about one forth of those at a gel phase,

and the value of K_{Ca} for EggPC, which is at fluid phase at room temperature, about 5 M^{-1} at 25°C (Satoh, K. and Mishima, K., unpublished data). Several reports have been published to show the dependence of zeta potentials of PC liposomes in ionic solutions on the phases [9,22,24,41]. We are preparing a paper about the effects of the phase difference of PC membranes on the binding constants of ions.

To our knowledge, there have been no report about the values of K_{Na} and K_{Cl} for DPPC at gel phase. As for K_{Na} for PC, Seelig group [6,33,34] reported the value of K_{Na} = 0.15–0.85 M^{-1} for POPC, which was in a fluid state at 25°C. They obtained the value from the computer simulation of Ca^{2+} binding isotherms, which was obtained by ^{2}H-NMR by taking into account the competitive binding of Na^{+} but neglecting Cl^{-} binding. As for K_{Cl} for EggPC, Grasdalen et al. [13] reported the value of K_{Cl} = 0.065 M^{-1}, Westman and Eriksson [42], $K_{Cl} = 0.9$ M^{-1}, both at 30°C by using ^{31}P-NMR. Afterwards Eriksson and Westman [43] reported the value of $K_{Cl} = 0.15$ M^{-1} at 25°C by using EPR. Hahn et al. [44] reported the influence of Cl^{-} on the binding of divalent cations to PC by the osmotic pressure technique but they did not obtain the value of K_{Cl}. Those reported values of K_{Na} and K_{Cl} are the same order as our results.

4.3. Distance of shear plane

There have been no report about the location of hydrodynamic plane of shear for phospholipid liposomes at gel phase. However, as for the liposomes at fluid phase McLaughlin group [45] showed the evidence that the shear plane exists at 0.2 nm outer from ion binding plane. They used negatively charged phospholipid of bovine brain phosphatidylserine (PS) and EggPG in 0.1 M NaCl at 25°C. And the same group reported later [2] that the distance of the shear plane, Δ, decreased with the concentration of NaCl by use of the liposomes formed from 5:1 mixtures of EggPC and bovine brain PS, but they could not show the reason. This unreasonable results may come from their ignoring the binding of Cl^{-}. There is a possibility that the value of Δ depend on the surface condition of liposomes and viscosity of the solution. Since the influence of ions on those will be small, it is reasonable that we got the results of $\Delta = 0.24$ nm independence of ionic concentration.

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