Binding of Local Anesthetic Tetracaine to Phosphatidylinositol/Dipalmitoylphosphatidylcholine Mixed Vesicles

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We measured the binding of local anesthetic tetracaine to phosphatidylinositol (PI), dipalmitoylphoshpatidylcholine (DPPC) and PI/DPPC mixed vesicles by an equilibrium dialysis method at different pH (5.5, 7.0, 8.0) at 35 °C. The results obtained for the pure phospholipid vesicles show that tetracaine binds to PI with the polyhydroxy headgroup, rather than to twitter ionic DPPC. For mixed vesicles, the higher is the ratio of the PI in mixed vesicles, the higher is the binding number. The change in pH slightly influences the binding to PI, while it strongly influences the binding to DPPC. We calculated the binding parameters which characterize the binding isotherms based on Langmuir-type and Hill equations. The binding parameters for pure PI and DPPC vesicles well reproduce the binding isotherms for PI/DPPC mixed vesicles with different mixing ratios. The results for pure PI were compared with those previously obtained for dilauroylphosphatidic acid (DLPA) in order to examine the influence of inositol in the head group of PI on the binding of tetracaine to phospholipid vesicles.

Local anesthetics have been widely used in medicine as a spinal anesthesia, surface anesthesia, conduction anesthesia and so on. The tetracaine used in this experiments is mainly used as a surface and conduction anesthesia, and belongs to the procaine family, which was developed as alternatives of cocaine that was used as the first anesthetic. The anesthesia quality and toxicity of local anesthetics depend on their structures. Therefore, intensive efforts have been made to develop new anesthetics with high anesthesia potency and low toxicity.

Although the mechanism of a local anesthetic has been intensively investigated,[1—4] it is far from being unified. With the action mechanism though to be multistep, we look upon the binding of anesthetics to cells as the first step of anesthesia.[5,6] Because the major components of the cell membranes are lipids and proteins, like phospholipids, cholesterol, glycolipids, lipoproteins, glycoproteins, etc., lipids and/or proteins should be candidates for the binding sites. If we focus our attention to the phospholipids, it is important to evaluate the contribution of the phospholipid head group to the binding of anesthetics. In this experiment, we chose two phospholipids with different head groups, phosphatidylinositol (PI) with a polyhydroxy head group (which has been investigated in terms of its fusion action of microsomes[7] and its interaction with biofilms,[8]) and twitter ionic dipalmitoylphoshpatidylcholine (DPPC), as the components of mixed phospholipid vesicles.

Previously, we had examined the binding of tetracaine to the vesicles comprising neutral dilauroylphosphatidylcholine (DLPC) having a bulky choline group and acidic dilauroylphosphatidic acid (DLPA) with the same saturated acyl chain length.[6] The results obtained for different pH showed that the initial binding of tetracaine is driven by an electrostatic force, and the following second binding is driven by a hydrophobic force.

In this study we measured the binding of tetracaine to the vesicles of PI with a polyhydroxy head group (Fig. 1), while focusing attention on the effect of inositol as well as the negative charge, and by comparing the results with those for negatively charged DLPA, we expect to clarify the influence of inositol to tetracaine binding.

Since pK_a of tetracaines have been reported as 8.38±0.03

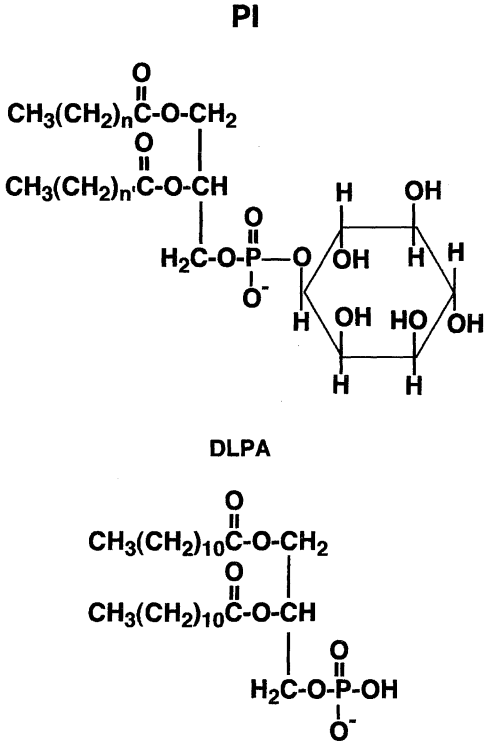

Fig. 1. The molecular structures (charged form) of PI and DLPA.

at 36 °C,[9] tetracaine is positively charged under physiological conditions. A further increase in the pH leads to an increase in the fraction of neutral tetracaine; at pH 8.38, for example, the ratio of positive : neutral becomes 1 : 1. Therefore, the pH change allows one to estimate the contribution of the electrostatic and hydrophobic binding to the total binding number.

Experimental

PI sodium salt and DPPC were purchased from Sigma and Nippon Oil and Fats Co., respectively, and were used without further purification. Single-compartment vesicles were prepared by sonication and followed by an extrusion method. Mixed vesicles with different mixing ratios were prepared by the same method as pure vesicles after premixing in chloroform. The prepared vesicles stocks for binding experiments were used up in two days. To calculate the binding number per phospholipid, the phospholipid concentrations for vesicle solutions were determined by an inorganic phosphate assay after hydrolysis.[10] The phospholipid concentration was kept at about 2.0×10^{-4} M (M=mol dm^{-3}) throughout the experiment.

The local anesthetic tetracaine was purchased from Wako Pure Chemical Co., and was used without further purification. The concentrations of tetracaine were calibrated spectrophotometrically using preconstructed lines. In this experiment the molar extinction coefficient of tetracaine at 310 nm was taken to be 23000 M^{-1} cm^{-1}.

The binding isotherms were constructed by an equilibrium dialysis method at 35 °C. The binding numbers per phospholipid were calculated from the concentration difference before and after the dialysis for 20 h, based on the phospholipid concentration. In order to calculate the true binding number, the bindings of the tetracaine to dialysis cells and a permeable membrane were subtracted from the apparent binding number.

In all of the experiments three buffers were used: 50 mM (2-morpholinoethanesulfonic acid MES, pH 5.5) and 50 mM (2-{[tris-(hydroxymethyl) methyl] amino} ethanesulfonic acid, TES, pH 7.0 and 8.0), each including 100 mM NaCl.

Results and Discussion

Binding to Pure PI Vesicles. In advance to PI/DPPC mixed vesicles we measured the binding of tetracaine to pure PI vesicles. Figure 2(a) shows the binding isotherm of tetracaine for pure PI vesicles at pH 5.5, where the binding number in mol/mol unit is plotted against the tetracaine equilibrium concentration. The binding isotherm starts from 0 M equilibrium concentration, followed by a gradual increase in the binding number up to 0.55 at 4.5×10^{-4} M. This type of isotherm is well reproduced with the Langmuir-type binding isotherm, because the binding isotherm starts from the origin, and has a single saturated value.

The binding isotherm at pH 7.0 is shown in Fig. 2(b). The binding isotherm rises up from 0 M like that for pH 5.5, and shows an almost identical slope to that for pH 5.5. However, saturation takes place at around 3.5×10^{-4} M, while maintaining a binding number of about 0.55.

The result for pH 8.0 is shown in Fig. 2(c). This binding isotherm, also having a character of an onset from 0 M, has a less-steep slope compared to those at lower pH, and reaches to as much as 0.6 at 6.0×10^{-4} M.

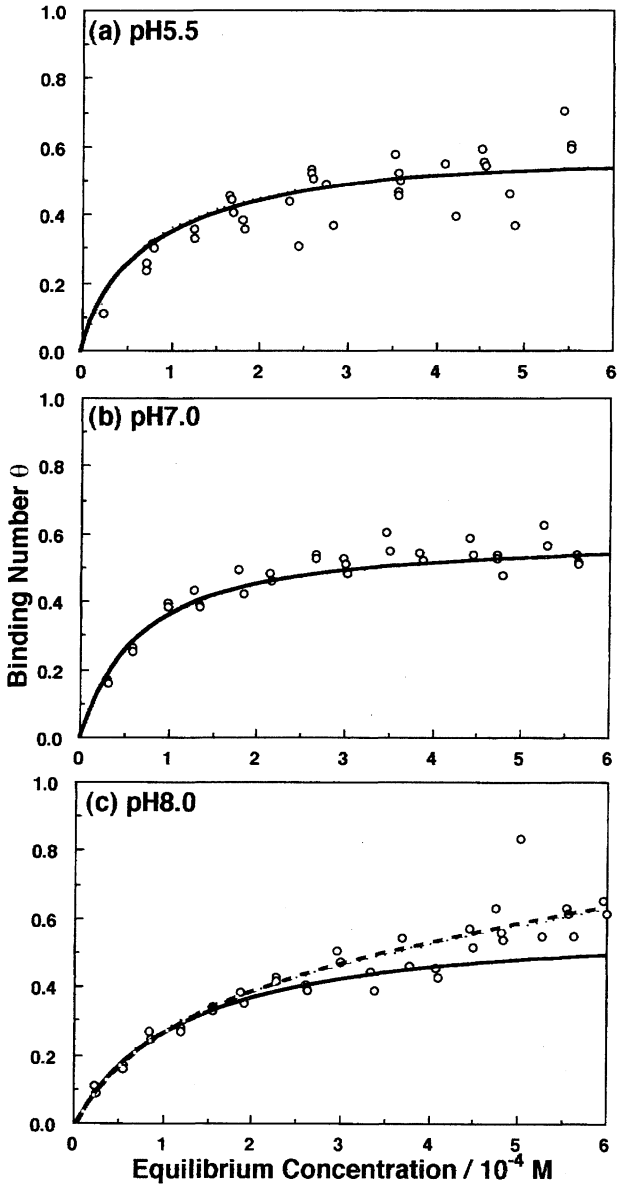

Fig. 2. Binding isotherms of tetracaine to pure PI vesicles at pH 5.5 (a), pH 7.0 (b), and pH 8.0 (c) where θ is expressed as mol/mol unit. Solid and dashed curves denote the calculated values derived from the best-fit parameters based on Eq. 1 and Eq. 2, respectively.

The binding isotherms for pH 5.5 and 7.0, which exhibit a steeper slope than that for pH 8.0, have similar binding profiles of the saturated binding number, being about 0.55 at around 4.0×10^{-4} M. On the other hand, the binding isotherm for pH 8.0 continues to increase the binding number until the highest concentration examined, and attains to a binding number of up to 0.6.

We analyzed the binding isotherms using an equation based on the Langmuir-type equation, judging from the obtained binding profiles. Further, we also set the saturated binding number to be 0.6, as before,[5] taking into account the outer phospholipid population in the bilayer. Thus, the binding number expressed in mol/mol unit is

$$\theta = 0.6\frac{K_1[T]^{n_1}}{1+K_1[T]^{n_1}}, \quad (1)$$

where [T] represents the tetracaine equilibrium concentration, and K_1 and n_1 are the binding constant and the number of tetracaine bound at a time, respectively. When n is equal to unity, the binding isotherm is reduced to the Langmuir isotherm. Here, since K_1 and n_1 reflect the strength of the binding and cooperativity, respectively, they are estimated by an iteration method so as to reproduce the experimental values over the entire concentration range examined. Parameter extraction has been performed by linearization of the equations using reciprocal plots. However, this procedure frequently introduces a serious error arising from a partial fit to a few data points. We therefore adopted an iteration method in order to evaluate the binding parameters so as to characterize the binding profiles as a whole. Thus, the obtained binding isotherms are expressed as solid curves in the respective figures.

The binding isotherms for pH 5.5 and 7.0 are well reproduced by the estimated parameters listed in Table 1 over the entire concentration range. On the other hand, that for pH 8.0 shows a positive deviation from the reproduced binding isotherm in a higher concentration range. Because the solid curve is produced by using the Langmuir equation, while exclusively focusing the attention on reproducing the initial binding, in the case of successive additive binding after tentative saturation, such as the binding at pH 8.0, the binding isotherm deviates positively. This successive binding suggests the second binding site where tetracaine has already bound in a low concentration range.

In order to evaluate the positive deviation of the binding isotherm at pH 8.0 we introduce the following additive term into Eq. 1:

$$\theta = 0.6\left(\frac{K_1[T]^{n_1}}{1+K_1[T]^{n_1}} + \frac{K_2[T]^{n_2}}{1+K_2[T]^{n_2}}\right), \quad (2)$$

where K_2 and n_2 are the binding constant and cooperative parameter for the second binding, respectively. The reproduced curve based on Eq. 2 is depicted with a broken line in Fig. 2(c), and the evaluated parameters are listed in Table 1.

Binding to DPPC Vesicles. As the second step, we measured the binding of tetracaine to zwitter ionic DPPC vesicles. Figure 3 shows the binding isotherms for three different pH. The binding isotherms are characterized by a very low binding number, and a delay of the onset of the binding, in contrast with those for PI where the bindings start from the origin. For example, the binding isotherm to DPPC at pH 5.5 gradually takes off at around 2.5×10^{-4} M; the binding number reaches 0.05 at most at the highest concentration examined. The increase in the pH of the buffer solutions causes an increase in the binding number over the concentration range.

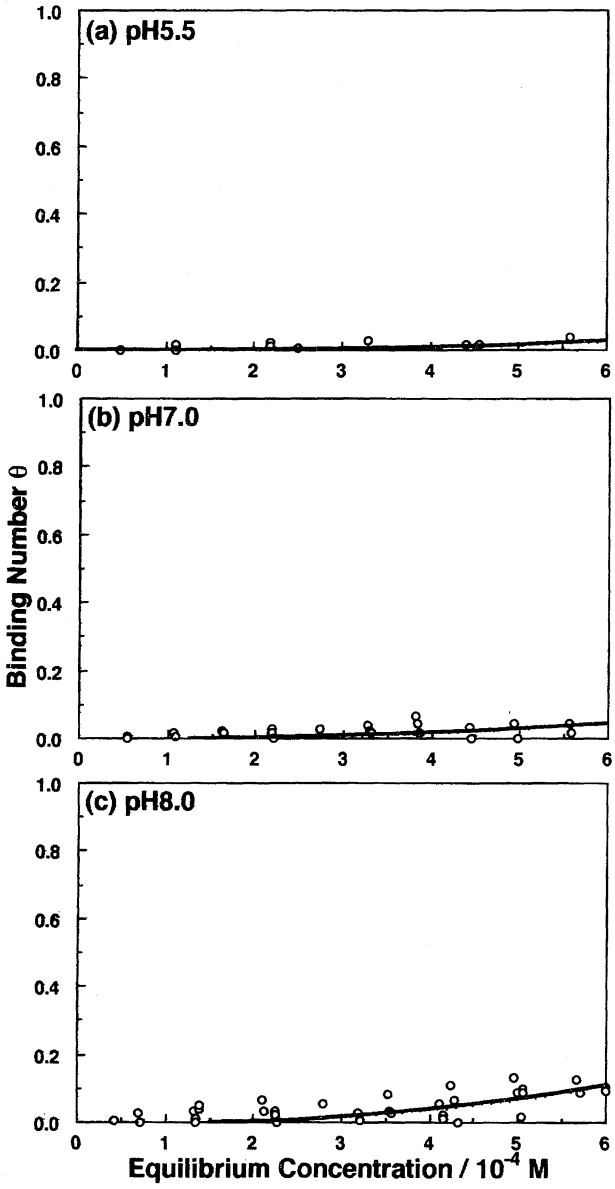

Fig. 3. Binding isotherms of tetracaine to pure DPPC vesicles at pH 5.5 (a), pH 7.0 (b), and pH 8.0 (c). Solid curves denote the calculated values derived from the best-fit parameters.

To analyze the binding isotherms with such a cooperative nature, we use the Hill equation, which has a parameter of cooperativity. Setting the saturated binding number to be

Table 1. The Binding Parameters

	$K_{1(PI)}/M^{-1}$	n_1	$K_{2(PI)}/M^{-2}$	n_2	$K_{(DPPC)}/M^{-3}$	n	$K_{1(DLPA)}{}^{a)}/M^{-1}$	$n_1{}^{a)}$	$K_{2(DLPA)}{}^{a)}/M^{-2}$	$n_2{}^{a)}$
pH 5.5	1.5×10^4	1	—	—	2×10^8	3	1×10^4	1	5×10^5	2
7.0	1.5×10^4	1	—	—	4×10^8	3	1×10^4	1	2×10^6	2
8.0	8×10^3	1	8×10^5	2	1×10^9	3	6×10^3	1	8×10^6	2

a) Ref. 6

0.6, the binding number of θ is expressed as

$$\theta = 0.6\frac{K[\mathrm{T}]^n}{1+K[\mathrm{T}]^n}, \quad (3)$$

where n is the index of cooperativity. The binding parameters are estimated by iteration method, as described above. The estimated K and n for respective pH (Table 1) well reproduce the binding isotherms for DPPC vesicles.

We can directly compare the K_{DPPC} for different three pH, because each binding isotherm has an identical n value, i.e., n=3. The K becomes larger as the pH increases in parallel with an increase in the deprotonated form of tetracaine, suggesting a hydrophobic interaction between tetracaine and neutral DPPC.

Because K in the Hill equation is estimated for multisite binding at a time, K's differing in n can not be compared directly. In order to compare the K's evaluated for different n, we must take the nth root of K. For instance, the nth root of the$K$'s for PI and DPPC at pH 8.0 are equal to 8×10^3 M^{-1} and 1×10^3 M^{-1}, respectively. The difference reflects the stronger binding affinity of tetracaine to PI than to DPPC. In other words, the binding affinity is extracted from the onset concentration and steepness of the slope of an isotherm, rather than cooperativity by itself.

In a previous paper we reported on the binding parameters of tetracaine to DLPC vesicles.[6] Because n for DLPC is identical with that for DPPC, the K values can be compared directly. A comparison reveals that $K_{\mathrm{DLPC}} > K_{\mathrm{DPPC}}$ at the respective pH. These relations can be explained in terms of the state of the phospholipid bilayers. DLPC has a shorter length of the acyl chains by two C_2H_4 units than does DPPC. Under the experimental conditions employed in these experiments, this difference of the acyl chain length leads to different states of the bilayers, such as in liquid-crystalline and gel states for DLPC (the phase transition temperature, T_m=−4.3°C) and DPPC (T_m=41.3°C), respectively. In general, the binding (partition) of local anesthetics to phospholipid bilayers is favorable to the liquid-crystalline state than the gel state.[11,12] Concerning tetracaine binding to PC with different acyl chain lengths, a parallel relation is also observed.

Binding to PI/DPPC Mixed Vesicles. Finally, we measured the binding of tetracaine to PI/DPPC mixed vesicles with different compositions. Figure 4 shows the binding isotherms of tetracaine to mixed vesicles with different compositions: PI : DPPC=3 : 1, 1 : 1, 1 : 3 at pH 5.5. The saturation tendency can be seen for all of the compositions examined, just as that for pure PI, except for the lower binding numbers compared to that for pure PI throughout the concentration range. The binding numbers of three binding isotherms increase in the order PI : DPPC=1 : 3, 1 : 1, and 3 : 1 at any equilibrium concentration, which corresponds to the order of increase in PI in mixed vesicles.

The binding isotherms for mixed vesicles at pH 7.0 are depicted in Fig. 5. The binding isotherms for the respective compositions have a saturation tendency similar to that for pure PI in a concentration range of around 3.0×10^{-4} M. However, the binding numbers are reduced due to DPPC

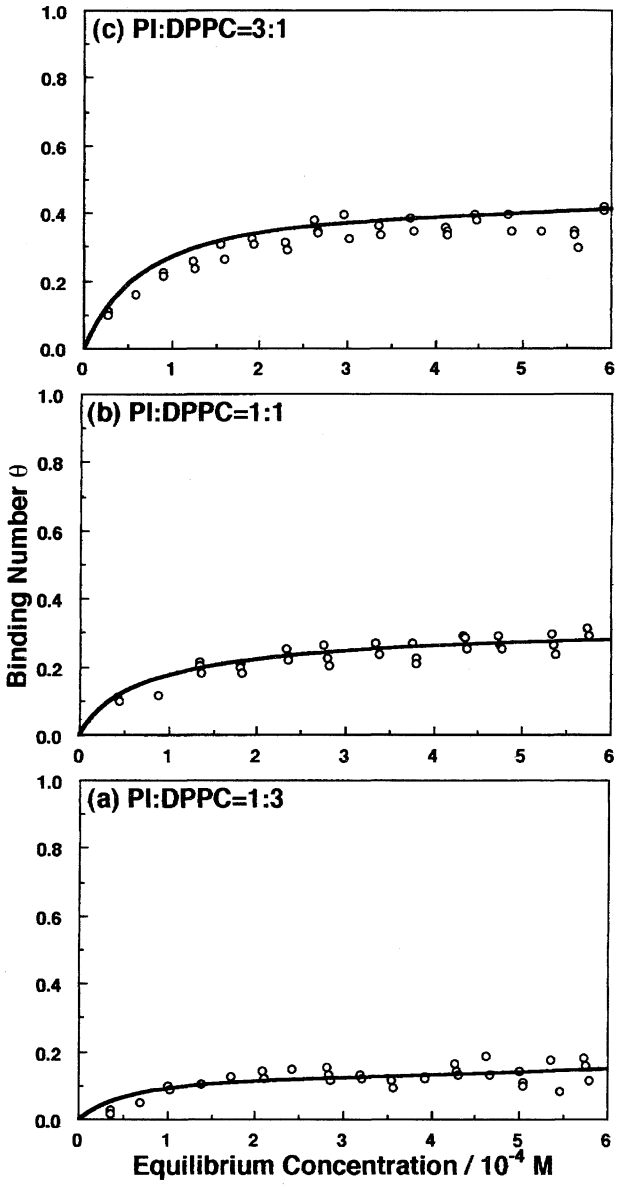

Fig. 4. Binding isotherms of tetracaine to PI/DPPC mixed vesicles with mixing ratios PI/DPPC=3 : 1 (a), 1 : 1 (b), and 1 : 3 (c). Solid curves are drawn based on the estimated parameters for pure PI and DPPC at pH 5.5.

mixing. Figure 6 shows the binding isotherms for mixed vesicles at pH 8.0, where, as in the case of a lower pH, the binding isotherm is lowered as the result of DPPC mixing, maintaining the character of the original binding isotherm for pure PI vesicles.

The binding isotherms for PI/DPPC mixed vesicles are reproduced by a linear combination of the equations with the same value of parameters adopted for the respective constituent phospholipid vesicles. To reproduce the variation in the isotherms with the composition of the mixed vesicle, the respective terms originating from pure vesicles were multiplied by the mole fractions of each phospholipid in the vesicles; i.e., we regard the PI and DPPC molecules in the mixed vesicles as being independent binding sites to tetracaine. The

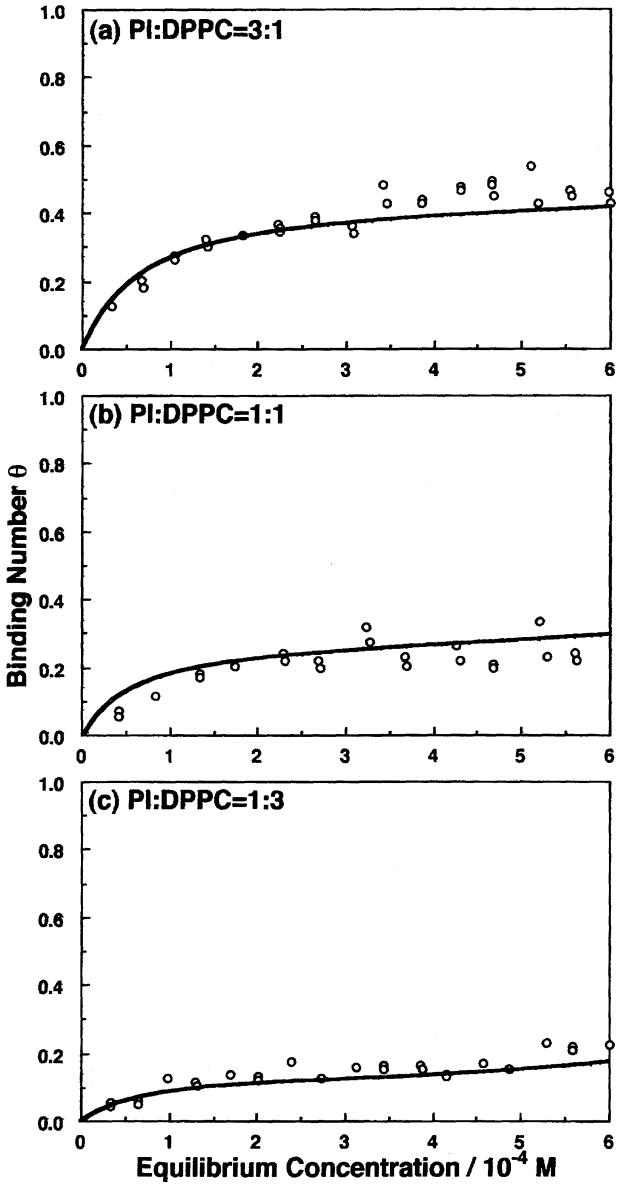

Fig. 5. Binding isotherms of tetracaine to PI/DPPC mixed vesicles with mixing ratios PI/DPPC=3:1 (a), 1:1 (b), and 1:3 (c). Solid curves are drawn based on the estimated parameters for pure PI and DPPC at pH 7.0.

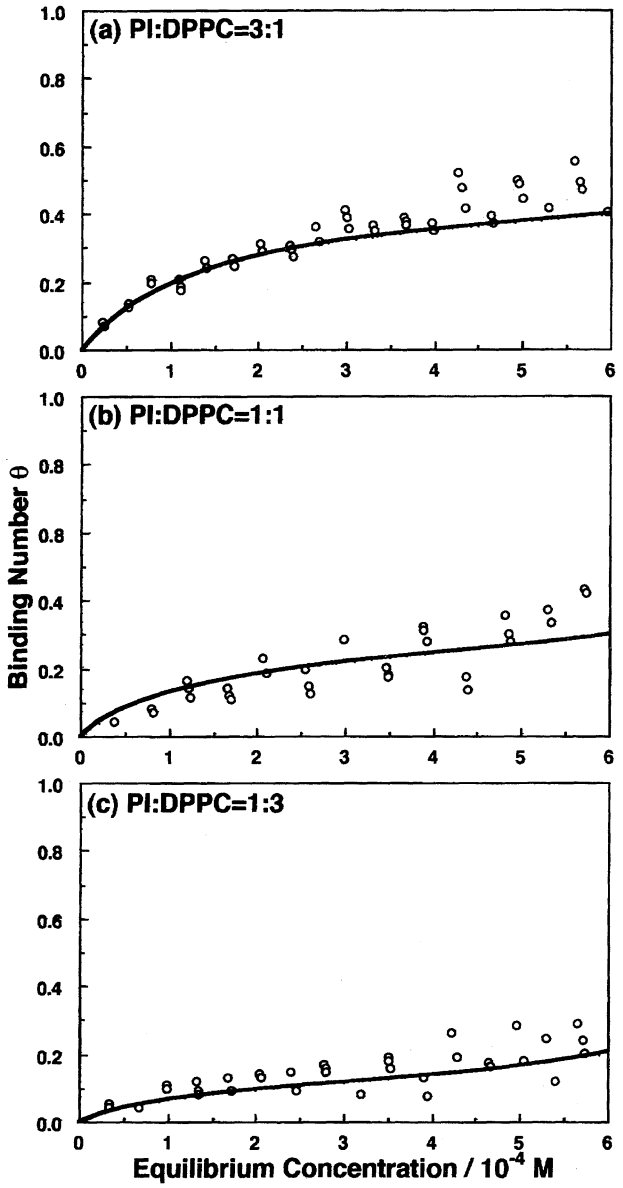

Fig. 6. Binding isotherms of tetracaine to PI/DPPC mixed vesicles with mixing ratios PI/DPPC=3:1 (a), 1:1 (b), and 1:3 (c). Solid curves are drawn based on the estimated parameters for pure PI and DPPC at pH 8.0.

calculated binding numbers based on mole-fraction basis in mixed vesicles are indicated in the respective figures by the solid curves.

Comparison between the Bindings to PI and DLPA. We previously examined the binding of tetracaine to DLPA, having the simplest head group, i.e., phosphate. In this experiment the binding of tetracaine to PI, which has inositol in addition to phosphate in the head group, was examined. Based on the results for the binding to DLPA, an electrostatic interaction between the head groups of phospholipids and tetracaine has been suggested.[6] It is therefore interesting to compare the effect of the structure of the head group on the binding.

We reported that the binding to DLPA shows two-step binding,[6] in contrast to the binding to PI, which is a single step, except for that at pH 8.0. We thus reproduced each binding isotherm for DLPA using K_1, n_1 and K_2, n_2, as shown in Eq. 2, where subscript 1 and 2 were referred to the initial and second binding. The binding parameters for PI and DLPA are listed in Table 1.

The initial binding constants of tetracaine for PI are larger than those for DLPA at corresponding pH. First of all, we shall consider the packing of PI and DLPA in vesicles in order to explain the above results. In both cases the separation between neighboring phospholipid molecules in the vesicles are mainly controlled by the repulsive force between negatively charged head groups, and with the cross-sectional area of the acyl chains, rather than the acyl chain length.

Because PI has a biological origin, its acyl chain length and the degree of saturation are unknown. However, no phase transition from liquid-crystalline to the gel phase above 0 °C has been observed on differential scanning calorimetry. Therefore, we may look up PI and DLPA (T_m=32°C[13]) in the liquid-crystalline state under the experimental conditions. With DLPA and PI being a liquid-crystalline state at 35 °C in mind, provided that the cross sectional areas of the acyl chains in these two phospholipid are equivalent, the repulsive force exclusively controls the separation. How should we consider the effect of the inositol residue on the separation between the PI molecule in the vesicles? Although the interaction between the inositol residues is attractive in themselves, originating from the ability to form a hydrogen bond, their effect on shortening the separation would be negligible compared to the electrostatic repulsion between the negatively charged phosphates in DLPA and PI. Thus, the difference in the separations between neighboring phospholipids in the DLPA and PI vesicles may not be very serious.

We next consider the difference between the packing states of the DLPA and PI vesicles after tetracaine binding. The binding of positively charged tetracaine reduces the repulsive force between negatively charged phosphates as a result of charge neutralization. This makes the neighboring phospholipids closer. This situation is favorable to PI molecules, because the attractive force between the inositol residues is a function of distance; the shorter, the stronger. In such a way, the favorable initial binding to PI vesicles may be explained in terms of the more energetically stable of PI–tetracaine complex than that of the DLPA–tetracaine complex.

The second binding for DLPA after the plateau comes out in any pH examined, where K for the second binding becomes larger with pH, in other words, with an increase in the deprotonated form of tetracaine. On the other hand, the second binding for PI is seen only at pH 8.0, and the binding affinity is one order weaker than that for DLPA, compared at the same pH. After accomplishing the initial binding, the second binding is subjected to a counter effect that lowers the binding affinity to a PI compared to that for DLPA. This behavior should be explained in line with the attractive nature of the inositol residues, as explained for the initial binding. The second binding introduces hydrophobic tetracaine into the PI vesicles, which results in an extension of the separation between the neighboring PI. Such an additional binding to the neutralized vesicle surface is favorable for uncharged tetracaine, such as at pH 8.0. An extension of the separation between the neighboring PI makes the inositol residue apart. This work must be done against the attractive force between the inositol residues, leading to a lower binding affinity to PI than that for DLPA.

In these experiments, the bindings of tetracaine to PI, DPPC, and PI/DPPC mixed vesicles were examined. PI was employed in order to examine the influence of inositol in the head group on the binding of tetracaine. The binding isotherms were analyzed by the Langmuir-type equation or the Hill equation. The binding parameters obtained from the isotherms of the respective pure vesicles well produce the isotherms of their mixed vesicles, indicating that PI and DPPC afford independent sites for tetracaine. A comparison with the binding parameter of tetracaine for PI and DLPA shows that the inositol residue enhances the initial binding of tetracaine to the PI vesicles and reduces the second binding to PI vesicles. The influence of the inositol residue is explained in terms of the attractive nature between the OH groups in the inositol residue.

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