Binding of Dibucaine to Phospholipid Mixed Vesicles

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(Received December 28, 1992)

Binding isotherms of a local anesthetic, dibucaine, to phospholipid vesicles were measured using a dialysis method. Two types of phospholipids, dilauroylphosphatidic acid (DLPA) and dilauroylphosphatidylcholine (DLPC), were used to prepare vesicles. The binding isotherms for pure DLPA and DLPC vesicles and their mixtures were analyzed with the Hill equation. The binding parameters obtained for pure phospholipid vesicles reproduce the binding isotherms for the mixed vesicles by taking account of the mixing ratio; this indicates the presence of independent binding sites, corresponding to the DLPA and DLPC molecule, in the mixed vesicle.

A biological cell is isolated from the outer environment by a phospholipid bilayer in which proteins and other lipids are embedded to make a functional membrane. In addition to the role of a wall, the membrane has a gate-like character through which substances and information are exchanged with the outer environment. Substances, in some cases, are transported through partition to the membrane caused by a concentration gradient rather than the active transport mediated by enzymes. As well as biological substances, it is expected that some drugs will also show pharmacological actions through partitioning or binding to membranes.[1)]

Local anesthetics, which are generally tertiary amines, change their forms between charged and uncharged states depending on the pH of the medium, and both forms are neurologically active. So far the interaction of local anesthetics with phospholipid membranes has been widely investigated in terms of binding or partitioning,[2—5)] membrane fluidity,[6)] surface pressure of monolayer,[7)] the phospholipid head group conformation,[8)] membrane effect on the apparent pK of the local anesthetic,[9)] and NMR behavior.[10)]

As mentioned above, biomembranes are mainly constructed with phospholipids and proteins. Thus it is desirable to discriminate the specific binding to a membrane protein and the nonspecific binding to a phosholipid bilayer. In order to study the two types of binding separately, binding measurements for phospholipid vesicles with and without a protein should be done. As a first step, we measured the binding of a local anesthetic, dibucaine, to phospholipid vesicles to investigate the nonspecific binding to a phospholipid bilayer.

In this experiment, we used two types of phospholipids, dilauroylphosphatidic acid (DLPA) and dilauroylphosphatidylcholine (DLPC). They have common acyl chains but head groups different in sizes and charged states. It is expected that the two types of phospholipids will give different binding sites for dibucaine because of different charged states of the phospholipids. On the other hand, a pH change results in altering the charged state of dibucaine, which allows us to estimate the electrostatic contribution to the dibucaine binding in addition to the hydrophobic contribution.

Binding isotherms were measured for pure DLPA and DLPC vesicles and their mixtures with different mixing ratios using a dialysis method. Two buffer solutions of different pH were employed. The binding isotherms were analyzed in terms of the Hill equation based on multiple binding at a time, and it is shown that the isotherms obtained for the mixed vesicles are well reproduced using the binding parameters estimated from pure phospholipid vesicles only changing the compositional factor of the mixed vesicles.

Experimental

DLPA sodium salt and DLPC were purchased from Sigma, and used without further purification. Vesicle suspensions were prepared as follows. The weighed phospholipid was once dissolved in chloroform, which was then evaporated in vacuo. After swelling in a buffer solution, the phospholipid was sonicated in a bath type sonicator under controlled temperature prior to extrusion. To obtain homogeneous single-compartment vesicles, the sonicated suspension was passed through porous membranes several times by applying pressurized gaseous N_2 (extrusion method). The stock suspensions were used up within two days after preparation to prevent aggregation. The mixed vesicles were prepared by premixing the phospholipids at prescribed ratios in chloroform followed by the same procedure as that for pure vesicles. The concentration of the phospholipid was determined by the inorganic phosphate assay after hydrolysis.[11)] The phospholipid concentration in the stock suspension thus prepared was about 2×10^{-4} M (M=mol dm^{-3}).

Dibucaine (Wako Pure Chemical Co.) was dissolved in a buffer solution and its concentration was calibrated spectrophotometrically by using preconstructed curves. Two types of buffer solutions were used; pH 5.5 (50 mM 2-Morpholinoethanesulfonic acid, MES) and pH 7.0 (50 mM 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid, TES) both containing 100 mM NaCl. The molar extinction coefficient of dibucaine at 325 nm was taken as 4180 M^{-1} cm^{-1} for both buffers.

Binding isotherms were constructed by the dialysis method. Dialysis cells with a 5 ml compartment each, separated by a semipermeable membrane, were filled with the vesicle suspension and dibucaine, respectively, and were incubated in a shaking bath at 35 °C. In order to accelerate stirring, a grass ball was put in each compartment. Dialysis took more than 6 h for complete equilibrium, as judged from the time profile of pre-examinations. Then the cells were shaken for 12 h prior to the dibucaine assay. To esti-

mate the dibucaine binding to the cell wall, dialysis without vesicles was carried out in advance to the binding measurements. The binding number of dibucaine to the vesicle is expressed as mol dibucaine/mol phospholipid after correction for the cell wall binding.

Results and Discussion

Binding to Pure DLPA Vesicles. Figure 1(a) shows the binding isotherm of dibucaine to pure DLPA vesicles at pH 5.5, where the binding number in mol/mol unit is plotted against the equilibrium dibucaine concentration. The binding profile is characterized by a cooperative initial binding followed by an asymptotic region at (2—3)$\times 10^{-4}$ M. Further increase in the dibucaine concentration leads to successive binding up to the binding number of about 1. The onset of binding happens at a certain dibucaine concentration, indicating a non-Langmuir type of binding. Because the vesicles prepared were single compartment ones, the plateau, i.e. the asymptotic region of the binding number about 0.6, well corresponds the molecular ratio of the outer phospholipid of a curved vesicle composed of the single wall. The successive binding at concentrations above 3×10^{-4} M gradually increases in the binding number, and reaches 1. This increase in the binding number in higher concentrations suggests the presence of a second site with different affinity from the initial binding site.

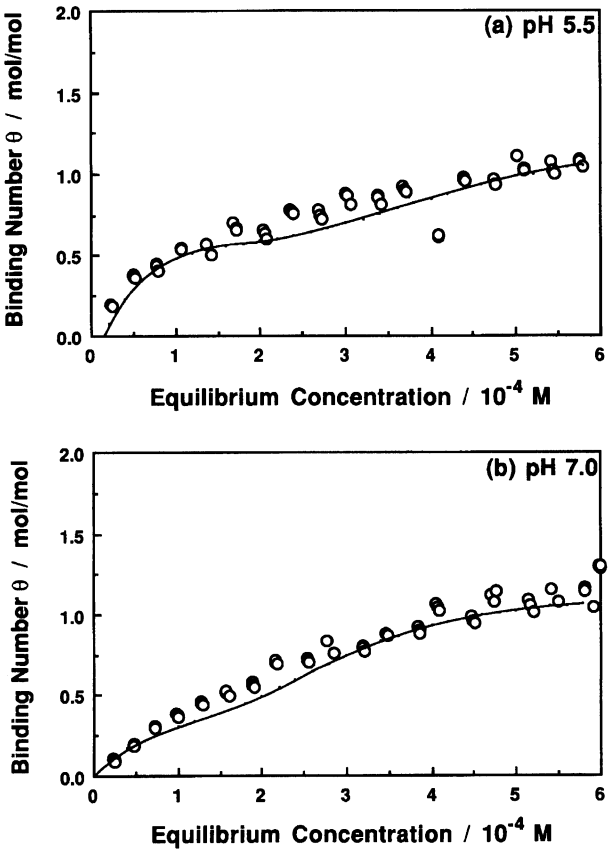

Fig. 1. Binding isotherms of dibucaine to pure DLPA vesicles at pH 5.5 (a) and pH 7.0 (b). Solid curves denote the calculated values derived from the Hill equation with the following parameters: $n_1=2$, $K_1=4\times 10^8$ M^{-2}, $n_2=4$, $K_2=3\times 10^{13}$ M^{-4}, for pH 5.5; $n_1=1$, $K_1=1\times 10^4$ M^{-1}, $n_2=4$, $K_2=1.2\times 10^{14}$ M^{-4} for pH 7.0.

The binding isotherm at pH 7.0 is shown in Fig. 1(b). Binding starts at zero concentration and attains the binding number of 1 after passing through a narrow shoulder. The binding profile is different from that at pH 5.5 regarding the onset of binding at zero concentration and the linear increase in the binding number at the initial stage. However, the two-step binding and the saturated binding number of 1 at 6×10^{-4} M are also observed even at pH 7.0. The linear increase in the binding number from zero concentration suggests, if anything, the occurrence of the Langmuir type binding in the initial stage.

The binding isotherms thus obtained were analyzed in terms of the Hill equation, in which we assume multiple-site binding rather than single ligand binding to the independent and equivalent sites at a time. Because the binding isotherms obtained for pH 5.5 and 7.0 are in line with two-step binding, we analyzed them using the Hill equations composed of two terms having different n and K as follows,

$$\Theta = 0.6\left(\frac{K_1[\mathrm{D}]^{n_1}}{1+K_1[\mathrm{D}]^{n_1}} + \frac{K_2[\mathrm{D}]^{n_2}}{1+K_2[\mathrm{D}]^{n_2}}\right),$$

where n and [D] represent the binding number of ligand at one-step and the equilibrium dibucaine concentration, respectively, and K is the binding constant for such n-site binding at a time. The value of n, therefore, shows the index of cooperativity of binding, and the specific case of $n=1$ corresponds to the Langmuir equation. The subscripts 1 and 2 represent the first and second binding sites, respectively. The total number of the primary sites was fixed at 0.6 taking account of the outer phospholipid molecules in the bilayer as primary sites. In fact, this value corresponds to the first saturated binding number observed as the plateau in Figs. 1(a) and (b). The binding parameters were estimated by iterating them to reproduce the experimental values over the whole range. We did not adopt linearization of the Hill equation by reciprocal plots because this procedure sometimes introduces a serious error. The binding numbers thus obtained are given in the respective figures as solid curves.

The estimated parameters well reproduce the experimental binding isotherms in the whole concentration range. At pH 5.5, the binding parameters characterizing the initial binding, i.e., n_1 and K_1, are 2 and 4×10^8 M^{-2}, respectively. At pH 7.0, the corresponding values are 1 and 1×10^4 M^{-1}. The error for K does not exceed the order of the significant figure. However, the choice of n is sensitive whether the binding starts from the origin or from the finite value. The shape of the

initial binding reflects the estimated n value whether the binding is cooperative (at pH 5.5) or linear (at pH 7.0). For the second sites, n_2's for both pH are equal to each other, indicating that the binding mode to second sites is common. Introduction of the second term to the equation is based on the consideration that the second binding takes place after saturation of the first binding, where the binding sites are phospholipid molecules themselves belonging to the outer surface of vesicles. The second binding, on the other hand, proceeds to the sites composed of dibucaine-bound phospholipid molecules. It should be noted that the introduction of the second terms does not imply the second binding taking place after completion of the first binding explicity. However, the second term substantially contributes to the binding number after completion of the first binding as a result of large n_2 and K_2. Hence it is reasonable that the estimated parameters for the second term are identical for each pH, because the charged state of the second binding sites is considered to be neutralized by the first dibucaine binding. Then the binding mode for the second sites seems to be less sensitive to the charged state of dibucaine.

To discuss the binding affinity of dibucaine to the DLPA vesicle, it is desirable to compare the binding constants. It is, however, not adequate to compare the respective K_1's directly between the two different pH, because n is not identical. If we extract the nth root of K_1 for respective pH instead of direct comparison, those values can represent a measure of the binding affinity. When we compared the value based on the nth root of K_1, it is revealed that the value at pH 5.5 is as twice as that at pH 7.0. For the second sites, we can compare K_2 directly because of the identical value of n. Here, on the contrary, the value at pH 5.5 is about fourfold smaller than that at pH 7.0.

The binding affinity of dibucaine in different buffer solutions will be explained in terms of the charged state of dibucaine. The dissociation constant of dibucaine has been reported as 8.0—8.5.[3,12] Thus dibucaine is regarded to be fully protonated at pH 5.5, and about 10% is neutral at pH 7.0. Because DLPA has a negative charge through pH 5.5—7.0, the preferential binding at pH 5.5 than at pH 7.0 is ascribed to an electrostatic interaction between positive dibucaine and negative DLPA in vesicles as a first approximation. However, the charged states of dibucaine at both pH's are not so critically different as to explain the difference in n and K in terms of the electrostatic interaction. A bulky charged molecule such as dibucaine must be sensitive to small change in pH because it dissolves in water under a subtle balance between the hydrophobicity and hydrophilicity. From pharmacological point of view, it is interesting to note that the binding mode of dibucaine is fairly sensitive to pH even below the dissociation pH.

For the second binding site, n_2 exhibits a common value at both pH's, and the corresponding K_2 at pH 5.5 was evaluated to be four times smaller than that at pH 7.0, in contrast to K_1 for the initial binding which is twice as large at pH 5.5 as that at pH 7.0. As described above, we take the second binding sites as newly formed sites after the completion of the first dibucaine binding. In other words, the new second binding sites are dibucaine bound phospholipids. Hence, the second binding takes place on DLPA at on outer vesicle surface which has been fully or partly neutralized by the first binding. We speculate that the second binding sites are located in between the phospholipid head groups already occupied by the first bound dibucaine. Hence, the second binding can be depicted as to proceed putting dibucaine molecule side by side in between the head group not stacking outward the vesicle surface. It is, therefore, expectable that the binding to such a neutral surface favors neutral dibucaine rather than positively charged one, because successive binding of positively charged dibucaine results in an electrical repulsion between them. This may explain the larger K_2 at pH 7.0 than that at pH 5.5.

Binding to Pure DLPC Vesicles. We then measured the binding isotherms to zwitter ionic DLPC vesicles over the same concentration range as for DLPA. Figure 2 shows the binding isotherms of dibucaine to DLPC vesicles at pH 5.5 and 7.0. In contrast to the binding isotherms of DLPA, the binding to DLPC gives essentially identical binding isotherms at both pH's. The binding isotherms are characterized by initiation of the binding at a relatively high concentration and the lower binding number at any concentrations compared with those for DLPA. The saturated binding number is only 0.4 even at 7×10^{-4} M. These isotherms were also reproduced with the Hill equation using the best fit parameters listed in the figure caption, in which a single term was employed to account for the simple isotherm for DLPC. The binding parameter n_3, 3, is rather large and K_3, 5×10^{9} M^{-3}, is small due to the delayed onset and the low binding number. It is acceptable that the binding of the dibucaine to neutral DLPC vesicles shows no pH dependence at low binding number, because the driving force to zwitter ionic phopholipid is considered to be equivalent irrespective of the charged state of dibucaine. Furthermore, with such a low binding number, the electrostatic interaction between neighboring dibucaine molecules in different charged states seems to be so small that no serious difference in the equivalent binding parameters appears as a function of pH.

Binding to DLPA/DLPC Mixed Vesicles. The binding isotherms for DLPA/DLPC mixed vesicles with different compositions were measured at pH 5.5 and 7.0. Figure 3 shows the binding isotherms of dibucaine to the mixed vesicles with three different compositions, DLPA : DLPC = 2 : 1, 1 : 1, and 1 : 3 at pH 5.5. For DLPA : DLPC = 2 : 1, the initiation of binding happens at non-zero concentration followed by a sharp increase

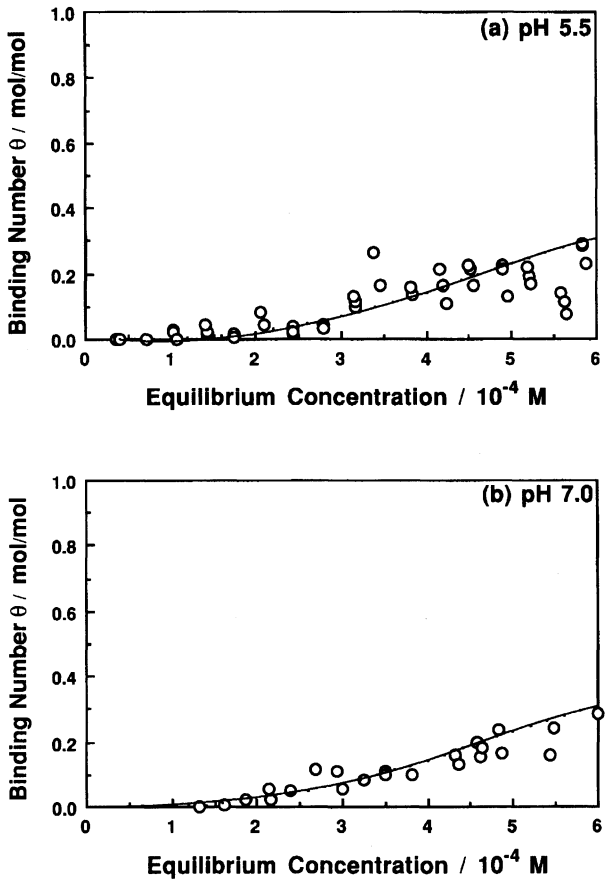

Fig. 2. Binding isotherms of dibucaine to pure DLPC vesicles at pH 5.5 (a) and pH 7.0 (b). Solid curves are drawn for both pH's based on parameters: $n_3=3$ and $K_3=5\times10^9$ M^{-3}.

in the binding number. After that, the binding number gradually increases to 0.5, exhibiting a leveling off region at about 2×10^{-4} M. Hence, the binding isotherm is characterized by two-step binding, which suggests independent binding to DLPA and DLPC. The total binding number is smaller than that for pure DLPA due to the mixing with DLPC. The binding isotherms for the mixed vesicles with higher DLPC ratios exhibit essentially the same features as DLPA : DLPC=2 : 1 although the binding number is getting smaller with an increase in the DLPC fraction in the vesicle. The variation of the vesicle compositions is, however, less effective on the binding number at higher equilibrium concentrations such as 4×10^{-4} M.

Figure 4 shows the binding isotherms for the mixed vesicles at pH 7.0. These isotherms are characterized by a linear increase in the binding number at the initial stage followed by successive binding with different slopes. Because of the DLPC mixing, the binding numbers at various concentrations become smaller compared to those of pure DLPA vesicles. At high DLPA composition such as the mixing ratio of 2 : 1, the binding isotherm hold the character similar to that for the pure DLPA vesicle. At a low DLPA composition of 1 : 3,

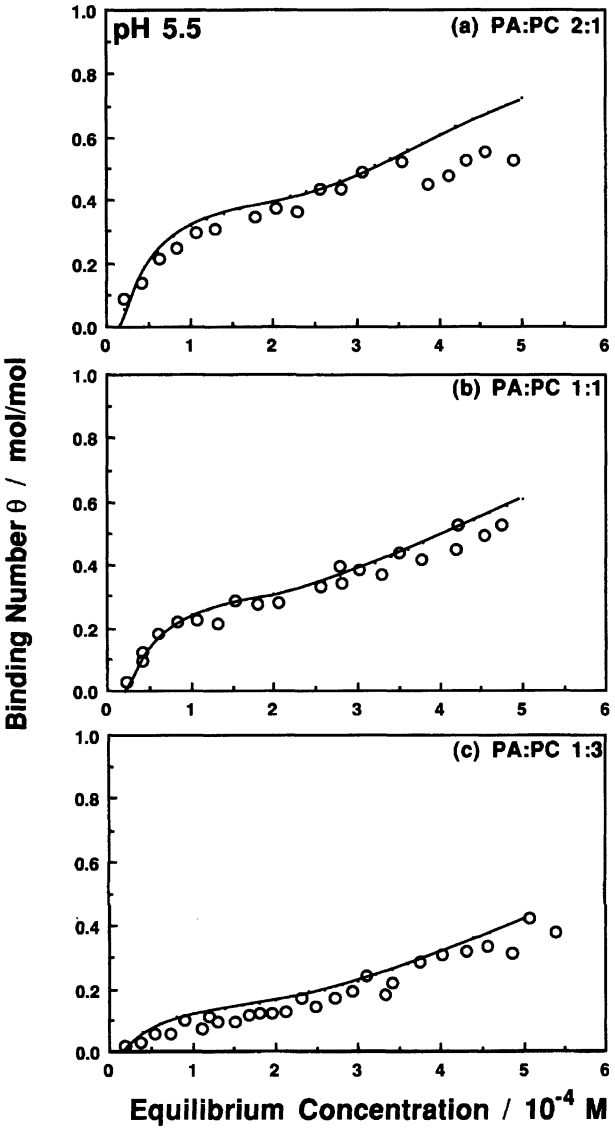

Fig. 3. Binding isotherms of dibucaine to DLPA/DLPC mixed vesicles at pH 5.5 with mixing ratios DLPA : DLPC=2 : 1 (a), 1 : 1 (b), 1 : 3 (c). Solid curves are drawn based on the estimated parameters for pure DLPA and DLPC at pH 5.5.

on the contrary, the isotherm has a character similar to the pure DLPC vesicles.

The binding isotherms obtained for DLPA/DLPC mixed vesicles were analyzed with the same equation having common parameters used for the respective pure phospholipid vesicles. In order to apply the equation to the mixed vesicles, the respective terms which represent the binding to pure DLPA and DLPC vesicles were mutiplied by the mole fractions of each phospholipid molecule in the mixed vesicles. In the case of DLPA : DLPC=2 : 1, for example, the equation was composed of the terms for pure DLPA and DLPC binding with weighted factor of 0.67 and 0.33, respectively. This means that we regard the DLPA and DLPC molecules in the mixed vesicles as independent binding

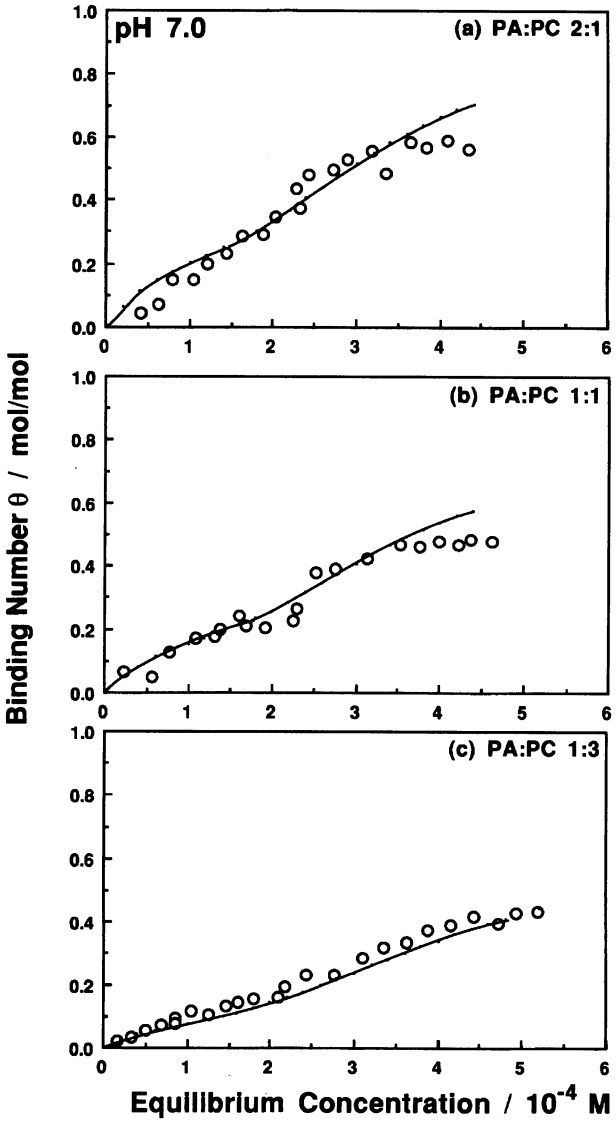

Fig. 4. Binding isotherms of dibucaine to DLPA/DLPC mixed vesicles at pH 7.0 with mixing ratios DLPA : DLPC 2 : 1 (a), 1 : 1 (b), and 1 : 3 (c). Solid curves are drawn based on the estimated parameters for pure DLPA and DLPC at pH 7.0.

sites to dibucaine. The estimated values are shown in the respective figures by solid curves.

The calculated binding numbers assessed by assuming independent sites for DLPA and DLPC molecules satisfactorily correspond to the observed values in each pH. In particular, the initial stage of binding and the following plateau observed at pH 5.5 are well reproduced by the calculated values. However, we note a slight positive deviation from the observed values at higher dibucaine concentrations in each pH. Such positive deviations are ascribed to overestimation of the second term contribution for pure DLPA vesicles. In order to clarify the contribution, we should examine the origin of the second term. This term comes from the idea that the successive binding takes place after completion of the binding to the primary sites composed of DLPA molecules on the outer vesicle surface. Hence the possible location of the second site is in between the dibucaine-bound DLPA molecule. In the case of the mixed vesicle, the neighboring DLPA molecules are separated by DLPC. Thus the number of neighboring DLPA is reduced by mixing with DLPC molecules. Such a reduction in the neighboring pair results in a smaller contribution of the second term to the total binding number than that estimated from pure DLPA visicles, which leads to a positive deviation from the observed value at higher phospholipid concentration.

From a viewpoint of pharmacology, it is desirable to separate drug partition to the cell into the contribution of respective constituent biological substances in it. For dibucaine, except for higher dibucaine concentration described above, the calculated isotherms reproduce the observed isotherms for the mixed vesicle with different compositions. This indicates that dibucaine binds to DLPA and DLPC independently with the same affinity as to pure phospholipids. This additivity facilitates the estimation of drug partition to biological substances in the cell.

This work was supported in part by the Central Research Institute of Fukuoka University.

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