Binding of Local Anesthetic Tetracaine to Phospholipid Mixed Vesicles

Kohsuke FUKUSHIMA,* Masayuki SOMEYA, and Ryosuke SHIMOZAWA
Department of Chemistry, Faculty of Science, Fukuoka University, Nanakuma, Fukuoka 814-80
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We measured the binding isotherms of a local anesthetic, tetracaine, to dilauroylphosphatidic acid (DLPA) and dilauroylphosphatidylcholine (DLPC) mixed vesicles along with their pure vesicles. The binding isotherms were constructed by the equilibrium dialysis method at different pH (5.5, 7.0, 8.0) at 35 °C. The binding isotherms were analyzed based on the equations combined with Langmuir and Hill-type isotherms, and the parameters characterizing the binding isotherms were evaluated. The thus-obtained binding parameters for pure vesicles well reproduce the binding isotherms for the mixed vesicles by taking account of the mixing ratios. This indicates that DLPA and DLPC also provide independent binding sites for tetracaine, as for previously reported dibucaine. The estimated values of the binding parameters for these two anesthetics, i.e., tetracaine and dibucaine, were compared in terms of the strength of the anesthetic action as well as the molecular structure.

In the latter half of the 19th century, cocaine, which was extracted from the leaves of coca shipped from South America, had been found to have an action as local anesthesia. So far, many local anesthetics have been developed targeting strong anesthesia with less toxicity and addiction. These days, local anesthetics have been widely used from clinical spinal anesthesia to the relief of pain due to a scratch. Local anesthetics have a common structure involving a ternary amine, and change their charged states depending on the bulk pH holding a neurological activity, irrespective of the charged states. Tetracaine belongs to aminobenzoate-type local anesthetics, which were developed in the early 20th century as one of the procaine family, and has been widely used in the clinical stage. In spite of the wide use and successive effort to understand anesthesia from both neurological and physicochemical view points, the mechanism of anesthesia is still not clear.

Before discussing the neurological action of local anesthetics, we are aware that the binding or partitioning of the drug to cells is the first event to interact, in any case, regardless of being specific or non-specific. It is therefore desirable to evaluate the binding mode of the drugs to cells in order to understand the anesthetic potencies.

A cell is constructed with a phospholipid bilayer, where modified proteins and lipids are embedded on functioning cellular activities, such as metabolism and self-reproduction. Such phospholipid bilayers are the matrix of the cell structure through plants and animals. As the first step, we choose a phospholipid bilayer as a simple model of cells on focusing our attention exclusively on the binding of anesthetics to the bilayer. Phospholipid-local anesthetic interactions have been investigated regarding both static and dynamic aspects, such as binding or partitioning,[1—5] membrane fluidity,[6] depression of the phase-transition temperature of bilayers,[7,8] and fluorescence[9] and NMR[10—12] spectroscopic behaviors. We report here on the binding of tetracaine to phospholipid mixed vesicles having different compositions at three different bulk values of the pH.

In this experiment, two types of phospholipids (dilauroylphosphatidic acid (DLPA) and dilauroylphosphatidylcholine (DLPC)), which have common acyl chains and different head groups in the charged state and size, were used to prepare the vesicles. The binding isotherms of tetracaine to the vesicles were constructed by a dialysis method. In addition to pure vesicles, mixed vesicles having different compositions of the respective phospholipids were prepared; the binding isotherms were also constructed in order to compare the effect of phospholipid mixing on the binding. The obtained binding parameters were compared with those for previously reported dibucaine,[13] which is a different type of local anesthetic with a quinoline ring instead of an anilino moiety.

Experimental

DLPA sodium salt and DLPC, whose nominal purities are 98 and 99%, respectively, were purchased from Sigma, and used without further purification. Vesicles were prepared by an extrusion method so as to have homogeneous single-compartment vesicles, as before. The thus-prepared stock suspensions were used up within two days in order to prevent aggregation. Mixed vesicles with different compositions were prepared from a premixed chloroform solution by the same procedure as that used for single-component vesicles. The phospholipid concentrations of the stock solutions were determined by inorganic phosphate assays after hydrolysis.[14] The phospholipid concentration in the stock suspension was around 2×10^{-4} M (M=mol dm^{-3}). A comparison of the phospholipid concentrations before and after dialysis showed no difference within the experimental error, indicating a negligible binding of the phospholipids both to the dialysis cells and the semipermeable membrane.

Three buffer solutions with different pH were used: pH 5.5, 7.0, and 8.0 containing 100 mM NaCl, respectively. Tetracaine (Wako Pure Chemical Co.), which is shown in Fig. 1 together with dibucaine, was dissolved in buffer solutions of different pH, and the molar-extinction coefficients were determined to be 23000 M^{-1} cm^{-1} at 310 nm, substantially irrespective of the pH.

Binding isotherms were constructed by the dialysis

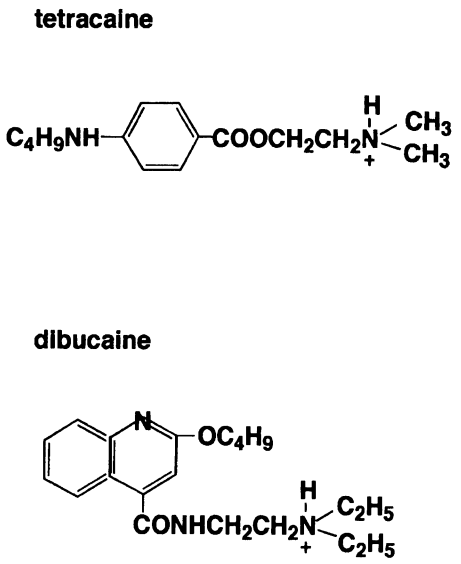

Fig. 1. The molecular structures (charged forms) of tetracaine and dibucaine.

method utilizing a conventional ten-unit cell with a 5 ml compartment each. A dialysis cell containing tetracaine and vesicle suspension separated by a semipermeable membrane was incubated in a shaking bath at 35 °C for over 12 h to ensure the complete equilibrium. After incubation, the tetracaine concentrations were determined spectrophotometrically; the calculated binding number of tetracaine to the vesicles is expressed as mol tetracaine/mol phopholipid after a proper correction for the inherent binding of tetracaine to the cell walls.

Results and Discussion

Binding to Pure DLPA Vesicles. Figure 2(a) shows the binding isotherm of tetracaine to pure DLPA vesicles at pH 5.5, where the binding number in mol/mol unit is plotted against the equilibrium tetracaine concentration. The binding starts from the origin, followed by a gradual increase up to a binding number of about 0.6, and then tends to level off at around 4—6×10^{-4} M. In contrast to dibucaine binding, tetracaine does not exhibit a cooperative initial binding, reflecting its smaller hydrophobic part compared with that of dibucaine. In addition to the less cooperative binding, the binding profile is characterized by a lower binding number compared with that of dibucaine, where the binding number almost attains unity at the corresponding equilibrium concentration. Because the prepared vesicles were of a single compartment, the ultimate binding number in the employed tetracaine concentration corresponds well to the molecular ratio of the outer phospholipids of a curved vesicle with a single bilayer.

The binding isotherm at pH 7.0 is shown in Fig. 2(b). Binding starts at zero concentration and attains a binding number of 0.8 after passing through a narrow shoulder. The binding profile of the initial stage resembles that at pH 5.5, suggesting a Langmuir-type binding during the initial stage. Two-step binding with the asymptotic region becomes pronounced; a further increase in the concentration leads to a higher binding number of 0.8 at 6×10^{-4} M than the corresponding value of pH 5.5.

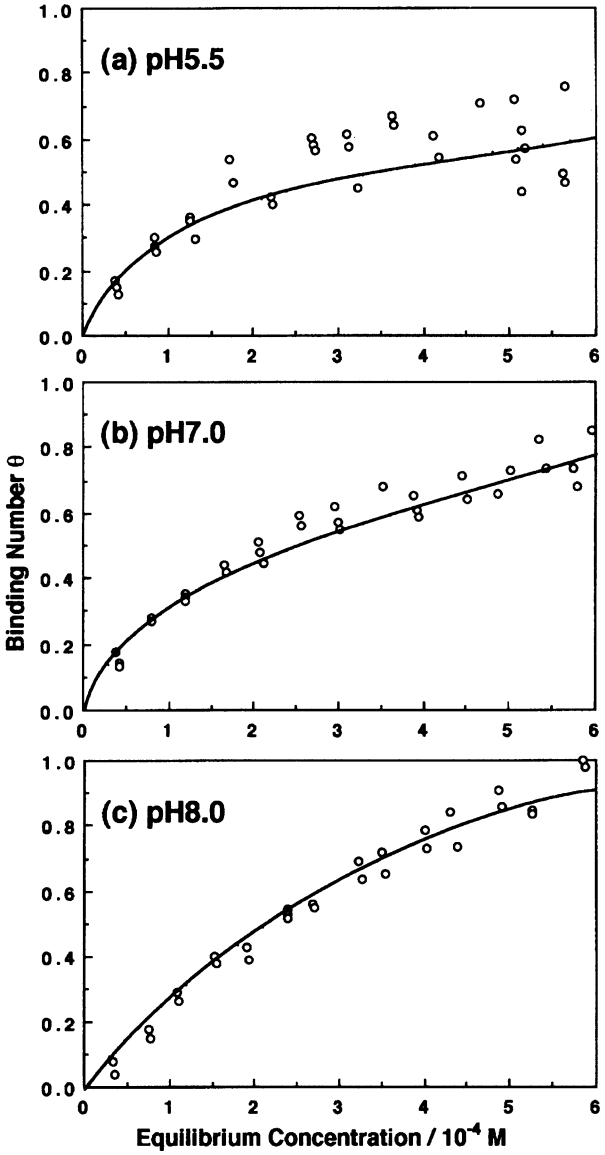

Fig. 2. Binding isotherms of tetracaine to pure DLPA vesicles at pH 5.5 (a), pH 7.0 (b), and pH 8.0(C) where θ is expressed as mol/mol unit. Solid curves denote the calculated values derived from the best-fit parameters.

Fig. 2(c) depicts the binding isotherm at pH 8.0. At a glance, a gentle increase in the initial binding can be seen, followed by a steep increase in the binding number holding the character of binding from zero concentration. For this reason, the shoulder is not clear. Ultimately, the binding number at 6×10^{-4} M reaches unity. From the pH variation, it is revealed that the pH change has different effects on either the initial or successive binding: a low pH facilitates the first-step

binding; on the contrary, a high pH favors the second-step binding.

The degradation of an amine-type local anesthetic in alkaline buffers has been pointed out.[7] To check the degradation during incubation, we measured the time profile of tetracaine absorbance at pH 8.0 for over 100 h, and found that the absorbance is retained at about 90% of a freshly prepared one even after 100 h, where no peak shift at maximum wavelength was observed.

The thus-obtained binding isotherms were analyzed in terms of a combination of Langmuir and Hill-type equations in order to reproduce the two-step binding starting from zero concentration. In the Hill-type equation, we assume multiple-site binding to the independent and equivalent sites at a time. In other words, the Langmuir equation is a special case of the Hill equation, where a single site binding proceeds at a time. The equation used for analysis is expressed in two terms having different n and K, as follows:

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

where n and [D] represent the binding number of the ligand at one-step and the equilibrium tetracaine 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. Subscripts 1 and 2 represent the first and second binding, respectively. The total number of primary sites was fixed at 0.6, as before, because the outer phospholipid molecules in the bilayer are looked upon as being primary sites. The introduction of the second term to the equation is based on the consideration that the second binding takes place after completion of the first binding, i.e., the first binding sites are phospholipid molecules themselves belonging to the outer surface of vesicles, and the second sites comprise tetracaine-bound phospholipids.

The binding parameters were estimated by an iteration method to reproduce the experimental values over the entire concentration range. Parameter extraction has been frequently performed by linearization of the equations using reciprocal plots. However, this procedure often introduces a serious error arising from a partial fit to a few data points. We therefore adopted an iteration method to evaluate the binding parameter characterizing the binding profiles as a whole. The thus-obtained calculated binding numbers are expressed as solid curves in the respective figures.

The estimated parameters (listed in Table 1) well reproduce the experimental binding isotherms over the entire concentration range. The value of K, whose significant figure is one digit, reflects the steepness and leveling-off concentration of the isotherm. On the other hand, the choice of n is sensitive to the initiation of binding, whether the binding starts from zero or a finite concentration.

The best-fit value for n_1 is unity for all of the pH examined, reflecting a linear increase in the binding number from zero concentration. The estimated K_1's for pH 5.5 and 7.0 are identical, and that for pH 8.0 is lower than the other two pH, as expected from gentle increase in the binding number during the initial binding. For the second sites, the n_2's at three pH are 2, as a result of the cooperative nature for the second binding. The K_2's increase at four-fold with increasing pH from 5.5 to 7.0 and from 7.0 to 8.0, respectively.

We now discuss the binding affinity of tetracaine to the DLPA vesicle in terms of n and K as a function of the pH. Because the n's are identical to the respective pH, the binding affinity is directly estimated from the value of K. Since we assume negatively charged phospholipid head groups as the first binding sites, K_1 is regarded as being the binding constant representing the electrostatic interaction between positively charged tetracaine and the negatively charged phospholipid head group.[13] Judging from the reported dissociation constant of tetracaine, pK_a 8.0—8.5,[15] it is fully protonated at pH 5.5; a further increase in the pH leads to a reduction in charged tetracaine, for example, one or five of the ten molecules to be deprotonated at pH 7.0 or 8.0, respectively. Having a charged state in mind, the binding parameter is well understood in that an identical value of K_1 for pH 5.5 and 7.0 reflects the almost same charged state of tetracaine, expecting an equal electrostatic interaction at both pH; the lower value for pH 8.0 is attributed to the reduced number of positively charged tetracaine, which may increase in a hydrophobic interaction rather than in an electrostatic one.

For the second binding site, n_2 exhibits a common value of 2 at the respective pH; the corresponding K_2 increases four-fold with increasing 1.5 or 1 pH unit. As discussed in the previous paper,[13] we take the second binding sites as being newly formed sites after completion of the first tetracaine binding. The new second binding sites are therefore tetracaine bound phospholipids, to which successive binding proceeds putting the tetracaine molecule side by side in between the head groups of the phospholipids, not stacking up the vesicle surfaces. Hence, the second binding takes place on the outer bilayer of a DLPA vesicle which has been fully or partly neutralized by the first binding. The driving force to the second binding is attributed to the hydrophobic interaction between tetracaine and the tetracaine-bound phospholipid. From the view point of hydrophobicity, the binding to such a neutral surface favors neutral tetracaine rather than a positively charged one. On the contrary, charged tetracaine results in an electrical repulsion to reduce the second binding. This picture may explain the regular increase in K_2 with increasing pH.

Binding to Pure DLPC Vesicles. As the next

Table 1. The Binging Parameters

Anesthetic	pH	$K_{1(DLPA)}$	n_1	$K_{2(DLPA)}$	n_2	$K_{3(DLPC)}$	n_3
Tetracaine	5.5	1×10^{4} M^{-1}	1	5×10^{5} M^{-2}	2	1×10^{9} M^{-3}	3
	7.0	1×10^{4} M^{-1}	1	2×10^{6} M^{-2}	2	3×10^{9} M^{-3}	3
	8.0	6×10^{3} M^{-1}	1	8×10^{6} M^{-2}	2	6×10^{9} M^{-3}	3
Dibucaine[a)]	5.5	4×10^{8} M^{-2}	2	3×10^{13} M^{-4}	4	5×10^{9} M^{-3}	3
	7.0	1×10^{4} M^{-1}	1	1.2×10^{14} M^{-4}	4	5×10^{9} M^{-3}	3

a) Ref. 13.

step, we measured the binding isotherms to zwitter ionic DLPC vesicles over the same concentration range as for DLPA. Figure 3 shows the binding isotherms of tetracaine to DLPC vesicles at the respective pH. The binding isotherms are characterized by the initiation of

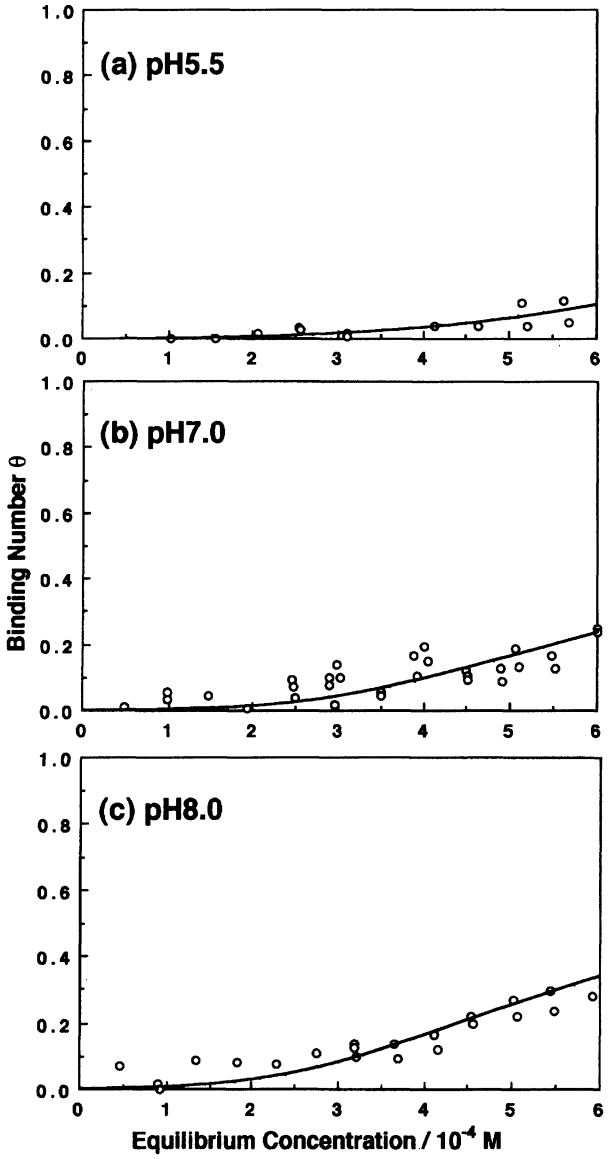

Fig. 3. Binding isotherms of tetracaine to pure DLPC vesicles at pH 5.5 (a),pH 7.0(b), and pH 8.0(c). Solid curves denote the calculated values derived from the best-fit parameters.

binding at a relatively high concentration, and a lower binding number at any concentration compared with those for DLPA. For example, at pH 7.0 the binding starts from 2×10^{-4} M, and the saturated binding number is only 0.2 even at 7×10^{-4} M. Here, we observe the pH dependence upon binding; the higher is the pH, the larger is the number of binding.

These isotherms were also reproduced using the Hill equation, in which a single term was employed to account for the simple isotherm for DLPC. The binding parameters designated by subscript 3 for DLPC binding show a rather large value of n_3 of 3 and a small binding constant, K_3, of 1×10^{9} M^{-3} at pH 5.5 as the result of a delayed onset and the low binding number. The observed binding isotherms were reproduced by a regular increase in K_3, holding n_3 constant as the pH increased. The solid curves in Fig. 3 represent the calculated binding number evaluated from the best-fit values. The regular increase in K_3 indicates a hydrophobic interaction of tetracaine with DLPC vesicles, i.e., deprotonated tetracaine favorably penetrates deeper into the bilayer than does the protonated one. It can be expected that such penetration of tetracaine takes place, taking into account the slender hydrophobic part of the molecule.

Binding to DLPA/DLPC Mixed Vesicles. The binding isotherms for DLPA/DLPC mixed vesicles with various compositions were measured at three corresponding pH. Figure 4 shows the binding isotherms of tetracaine to mixed vesicles with three different compositions: DLPA : DLPC=3 : 1, 1 : 1, and 1 : 3 at pH 5.5. For DLPA : DLPC=3 : 1, the binding isotherm exhibits a gradual increase from zero concentration, followed by a flat region at 2—3×10^{-4} M. The ultimate binding number reaches 0.45 at 6×10^{-4} M. The binding number over the entire concentration range is smaller than that for pure DLPA at the corresponding concentration as a result of DLPC mixing. The binding isotherms for the mixed vesicles with higher DLPC ratios show essentially the same features as DLPA : DLPC=3 : 1, except that the binding number becomes smaller along with an increase in the DLPC fraction in the vesicle.

Figure 5 shows the binding isotherms for the mixed vesicles at pH 7.0. These isotherms are characterized by a monotonous increase in the binding number during the initial stage, followed by successive binding with different slopes. The flat region is more obscure than

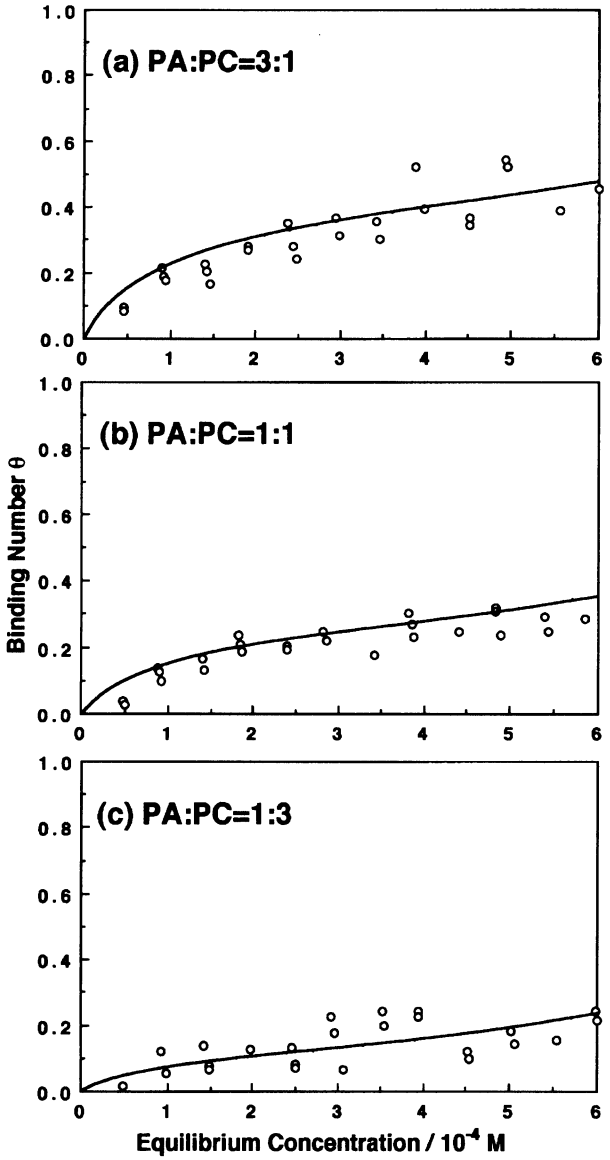

Fig. 4. Binding isotherms of tetracaine to DLPA/DLPC mixed vesicles at pH 5.5 with mixing ratios DLPA : DLPC = 3 : 1 (a), 1 : 1 (b), 1 : 3 (c). Solid curves are drawn based on the estimated parameters for pure DLPA and DLPC at pH 5.5.

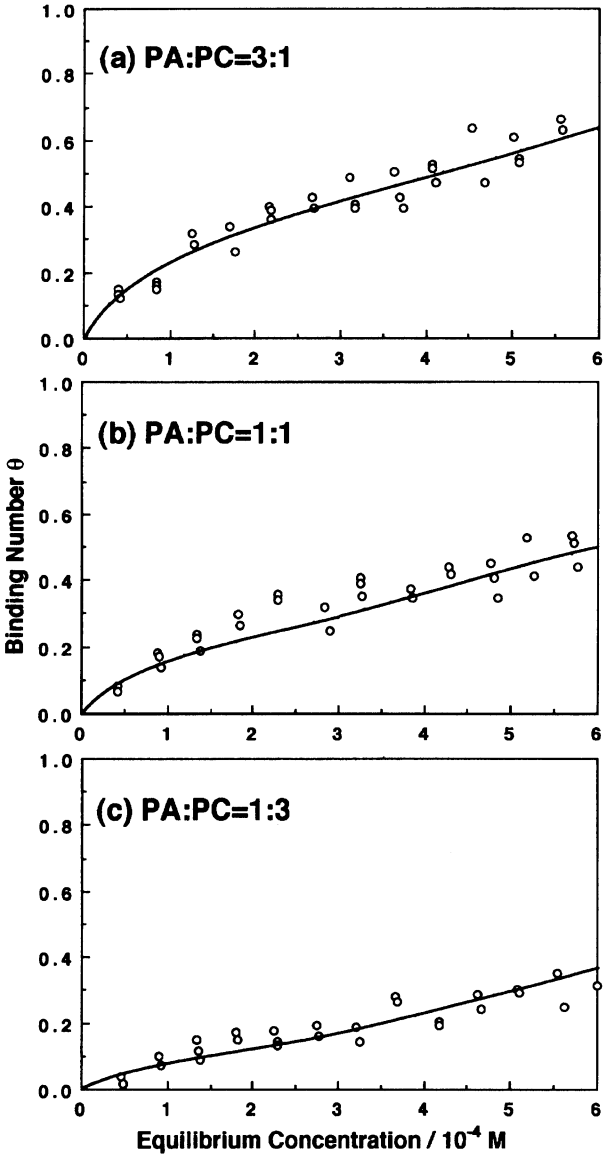

Fig. 5. Binding isotherms of tetracaine to DLPA/DLPC mixed vesicles at pH 7.0 with mixing ratios DLPA : DLPC = 3 : 1 (a), 1 : 1 (b), and 1 : 3 (c). Solid curves are drawn based on the estimated parameters for pure DLPA and DLPC at pH 7.0.

that of pH 5.5. The binding number over the concentration range becomes smaller compared to those of pure DLPA vesicles owing to DLPC mixing. The binding isotherms for mixed vesicles with a DLPA-rich composition resemble that for pure DLPA vesicles, and vice versa. The ultimate binding number is larger than that at pH 5.5, as expected from the larger binding number for the respective pure phospholipid vesicles at pH 7.0.

The binding isotherms for mixed vesicles at pH 8.0 are depicted in Fig. 6. The binding isotherms are characterized by an enhanced binding number at any mixing ratio compared with the previous two pH, reflecting the large binding affinity of tetracaine to the pure vesicles.

The binding isotherms for DLPA/DLPC mixed vesicles were reproduced by a linear combination of the equations with the same value of parameters adopted for the respective pure phospholipid vesicles. To reproduce the variation in the isotherms with the composition of the mixed vesicle, the respective terms originated from pure vesicles were multiplied by the mole fractions of each phospholipid in the mixed vesicles; i.e., we regard the DLPA and DLPC molecules in the mixed vesicles as being independent binding sites to tetracaine. The estimated binding numbers, assuming independent sites to DLPA and DLPC molecules in the mixed vesicle, are indicated in the respective figures by solid curves, and satisfactory trace the observed values for all cases.

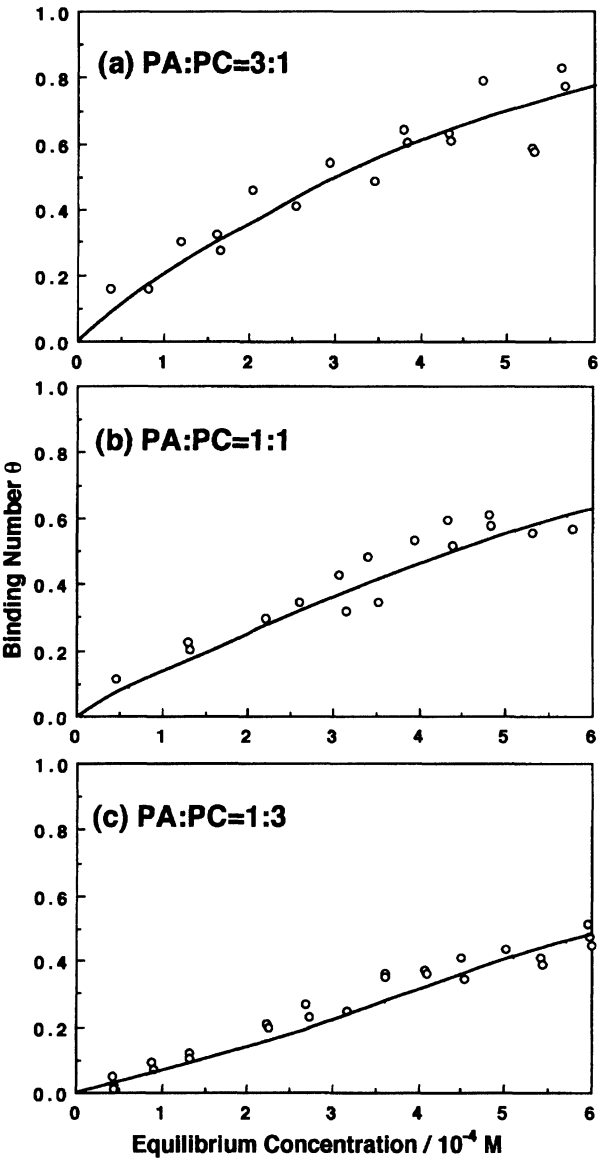

Fig. 6. Binding isotherms of tetracaine to DLPA/DLPC mixed vesicles at pH 8.0 with mixing ratios DLPA : DLPC=3 : 1 (a), 1 : 1 (b), and 1 : 3 (c). Solid curves are drawn based on the estimated parameters for pure DLPA and DLPC at pH 8.0.

The Binding Parameters. The binding parameters obtained for tetracaine are listed in Table 1 together with the previously reported values for dibucaine.[13] We first discuss the first binding to DLPA vesicles whose parameters are expressed by subscript 1. For tetracaine, the cooperative parameter (n_1) is unity at any pH, indicating the site binding of one molecule at a time. Such Langmuir-type binding is seen for dibucaine at pH 7.0. The binding constant (K_1) is equal at pH 5.5 and 7.0 and shows a lower value at pH 8.0. Because the driving force of the first binding results from an electrostatic interaction, as in the case for dibucaine, such a decrease in the binding constant with increasing pH can be understood in terms of losing the charge in alkaline pH. There is a difference in the binding mode between tetracaine and dibucaine at pH 5.5, where dibucaine exhibits cooperative binding with $n_2=2$, accompanied by a larger binding constant than that for tetracaine. On the contrary, at pH 7.0, the values of K_1 and n_1 are identical for both anesthetics. This indicates, in particular for dibucaine, a subtle balance between the electrostatic and hydrophobic interactions affected by a slight pH change around the physiological condition. Such a change in pH would lead to an apparent identical K_1 and n_1 for both anesthetics at pH 7.0, suggesting the existence of the corresponding pH to the binding number for different anesthetics.

For the second binding for DLPA vesicles, the K_2's increase about four times with each pH unit holding $n_2=2$. In view of the hydrophobic nature for the second binding, whose binding site should be tetracaine-bound phospholipid, the increase in K_2 is reasonably ascribed to the enhanced hydrophobicity due to a discharge in tetracaine with increasing pH. From n and the nth root of the binding constants for both anesthetics, we point out the larger cooperativity and corresponding larger binding constants of dibucaine, while the binding is less sensitive to a pH change than that for tetracaine. This tendency was also observed for the binding to zwitter ionic DLPC vesicles, where the binding force is attributed to a hydrophobic interaction because of the zero net charge of the DLPC molecules.

The difference in the binding behavior between tetracaine and dibucaine should be explained in terms of the molecular structure. These two local anesthetics have a common structure, possessing tertiary amines as polar part as well as a hydrophobic part constructed with an aromatic ring. When the local anesthetics interact with the hydrophobic part of the vesicle, the polar parts are forced to stay in the bulk phase. With this particular orientation in mind, we view the molecules through their molecular shapes and dimensions. Dibucaine, for example, has a widely spread molecular structure due to a bulky quinoline ring when putting the polar part upward. Tetracaine, on the other hand, has a rather longitudinal body, which is brought about by a benzoyl ring with a para substituted hydrocarbon. The pH dependence of tetracaine on binding to the hydrophobic region of the vesicle is interpreted as being a favorable insertion of tetracaine to the vesicle interior with increasing pH, because of its wedge-like structure. On the contrary, dibucaine may be prevented to enter interior deeply owing to its wide-spread body, which results in less sensitive binding constants to the hydrophobic part against the pH. In spite of the shallow insertion of dibucaine like a boat floating on water, the absolute values of K_2 and K_3 are larger than those for tetracaine, which may reflect larger contact area of dibucaine with the vesicle.

The binding constants obtained here are expected to be related to anesthesia (anesthetic potency) of local

anesthetics. The potency of anesthesia is evaluated based on the concentration of anesthetics to have the same depth of anesthesia in addition to the period of duration; high potency comes from a low concentration and long period of duration to keep the standard depth of anesthesia. Regarding these view points, dibucaine has been reported to display a potency that is as twice as that of tetracaine in clinical usage. The binding constants obtained in this experiment tell us of preferential binding of dibucaine compared with tetracaine at any stage of binding to the phospholipid vesicles.

In this experiment, we constructed binding isotherms of tetracaine to DLPA, DLPC, and their mixed vesicles, respectively. The binding isotherms were analyzed by assuming two-step binding to the DLPA vesicles and single-step binding to DLPC vesicles. The binding parameters obtained from the isotherms to the respective pure vesicles well produce the isotherms to their mixed vesicles, indicating that DLPA and DLPC afford independent sites for tetracaine in the pH range examined. A comparison with the binding parameters for tetracaine and dibucaine shows a close relation between the binding constant and the anesthesia as well as that the pH dependence on the binding constant can be interpreted in terms of the molecular structure of the local anesthetics.

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