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ADSORPTION OF LOCAL ANESTHETICS ON PHOSPHOLIPID MEMBRANES

SHINPEI OHKI

Department of Biophysical Sciences, School of Medicine, State University of New York at Buffalo, Buffalo, NY 14214 (U.S.A.)

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In order to elucidate various types of adsorption modes of local anesthetics in membranes, a study of local anesthetic adsorption on lipid membranes was made by measuring electrophoretic mobility of phospholipid vesicles in the presence of local anesthetics of various concentrations in the vesicle suspension solution. The amounts of local anesthetics to be adsorbed on the membrane surface were deduced from the electrophoretic mobility of a phosphatidylcholine vesicle at various concentrations of the cationic form of local anesthetics. The order of surface adsorption of local anesthetic was dibucaine > tetracaine > procaine. A surface partition coefficient, $K^s = 1/AC^s$, was introduced, where A is the membrane surface area per local anesthetic molecule adsorbed and C^s the surface concentration of local anesthetics. The amounts of local anesthetic adsorbed on phosphatidylserine membrane were much greater than that of the phosphatidylcholine membrane. It was deduced that the major factor for this large adsorption was due to the enhancement of cationic forms of local anesthetic concentrations at the charged membrane surface. Divalent cations inhibited such surface adsorption of local anesthetics by reducing surface concentrations of local anesthetics where the surface potential of the negatively charged membrane surface was influenced by the presence of divalent cations in the solution as well as by the reduction of fixed surface charges due to divalent cation binding. Some association modes of local anesthetics on nerve membranes are discussed with the results obtained in the above adsorption study.

Introduction

A number of studies have been attempted to elucidate the influence of local anesthetics on membrane excitability. There are currently three general hypotheses explaining the mechanism of local anesthetic action on membrane excitability. One of these is based on the lipoid theory originally proposed by Overton in 1896 [1] and others [2–7], and it emphasizes the predominant role of the uncharged form of anesthetics. The anesthetics adsorb to hydrophobic phases of excitable membranes, expanding some critical regions in the membrane and thus preventing ionic permeability, thereby interfering with the action current. Another theory is concerned with the interaction of the cationic form of local anesthetics with certain charged sites (polar groups) of lipids or proteins in or on the membrane [6–16]. It has been reported that there is competitive interaction between local anesthetics and calcium ions which affects nerve cell excitability [11,12]. In connection with this theory involving the charge interaction mode, some have suggested that the local anesthetic may bind with negatively charged phospholipid polar group sites on the membrane surfaces [17–20], which may also interact competitively with divalent cat-

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ions. It is known that divalent cations are thought to have important roles as stabilizers as well as triggering factors for membrane excitation. Another hypothesis is based on the binding of local anesthetics with specific receptors such as ionic channels [21–23]. In this case, the action of the drug is direct and not mediated by some change in general membrane properties.

At this moment, all of these theories seem to have their own premises. However, the definite questions as to which of the forms (neutral or charged) of local anesthetic is neuroactive, where the action sites of these two forms are located, and whether the action sites for the two forms are the same or not, are not well answered as yet. The reason for this difficulty is probably due to the complexity of the action mode of local anesthetics, and three factors seem to be responsible for this. First, many local anesthetics are basically tertiary amine compounds and they can exist in two forms in the aqueous solution (cationic and neutral), and the distribution of these two forms in the solution depends on the pK_a of the anesthetic as well as the pH of the bathing solution. Second, since the neutral form is readily permeable through the membrane, the mode of interaction is a dynamic one containing diffusion processes in at least three phases (the outer solution, membrane and inner solution phases) [13–15]. Thirdly, since local anesthetic molecules are amphipathic, they tend to be adsorbed at the water/membrane interface in a manner different from bulk adsorption.

As for the third factor, there are a number of studies concerning adsorption and binding of various drugs and organic compounds on lipid membranes using electrophoresis [24], fluorescence [25–29] techniques for lipid vesicles and surface studies for lipid monolayers [6,8,30–32]. Studies of binding of local anesthetics of the charged form and uncharged form to lipid membranes have been done by analysing the shift of membrane phase transition by a fluorecent method [32] and also the surface potential of monolayers [31]. However, little work concerning the effect of adsorption of local anesthetic at the membrane surface on the nerve excitability has been discussed. Ueda et al. [33] have suggested that the interfacial water structure and its change by anesthetics may be major determinants of the state of the membrane activity. The adsorption of local anesthetics on the lipid bilayer was studied by measuring membrane conductance and it has been implicated for suppression of membrane excitability in terms of surface potential reduction due to the adsorption of local anesthetics on the membrane surface [34].

In order to give further insight into the mode of adsorption of local anesthetics onto the membranes, a study was made on the adsorption of the charged form of local anesthetics on the lipid membrane with the use of electrophoresis measurements of lipid vesicles and the ion-binding theory. From this adsorption study on model membranes, we have discussed various modes of adsorption of local anesthetic molecules onto lipid membrane and some discussion in relation to narcotic aciton on nerve membranes has been made.

Materials and Methods

Phosphatidylcholine (egg) and phosphatidylserine (bovine brain) were obtained from Avanti Biochemicals (Birmingham, AL). The phosphatidylcholine purchased was purified further by silicic acid chromatography in our laboratory, and showed a single spot on the silica-gel thin-layer chromatographic plate. The salts used ($CaCl_2$ and NaCl) were all of reagent grade from Fisher Chemical Co., and Hepes (Calbiochem, grade 1) was used to buffer the solutions. All local anesthetics used were of USP grade. Procaine-HCl and tetracaine-HCl were both obtained from Mann Research Lab., and dibucaine-HCl was purchased from K&K Labs. The experimental solutions consited of 0.1 M NaCl/10 mM Hepes and contained various amounts of local anesthetics. The pH of the experimental solutions were adjusted to 6.0 with NaOH or HCl.

Multilamellar lipid vesicles were prepared as follows: phospholipids were suspended at a concentration of about 10 μmol/ml in the NaCl buffer solution containing no local anesthetics and then vortexed for 10 min. The vortexed lipid suspension was used as a stock vesicle solution, and 20 μl of this stock vesicle solution was suspended in 3 ml of experimental solution.

Electrophoretic mobility of the lipid vesicles was measured with a microelectrophoresis apparatus (Mark II, Rank Bros., Bottisham, Cam-

bridge). Great care was taken to focus on the stationary layer and all experiments were done at room temperature (23 ± 1°C).

Experimental results

In the first series of experiments, we measured the electrophoretic mobility of multilamellar phosphatidylcholine vesicles suspended in 0.1 M NaCl as a function of various local anesthetic concentrations (Fig. 1). The electrophoretic mobility of phosphatidylcholine vesicles in 0.1 M NaCl buffer solution (pH 6.) was approx. zero. As the local anesthetic concentration was increased, the mobility of the lipid vesicle moving toward the cathodal side of the applied electric field increased, indicating an increase in the positive surface charge density of the vesicle membrane surface.

The degrees of increase in electrophoretic mobilities of the lipid vesicles at any given concentration for different local anesthetics were in the order of dibucaine > tetracaine > procaine. This was the same order as that of the narcotic potencies of these anesthetics on nerve membrane excitability [35], as well as that of the partition coefficients of the neutral forms of these local anesthetics partitioning into oil/water phases [5,36,37]. The increase in electrophoretic mobility of the lipid vesicle at 1 mM procaine in 0.1 M NaCl was 0.05 $\mu m \cdot cm \cdot V^{-1} \cdot s^{-1}$. The concentrations of other local anesthetics used to produce the same change in electrophoretic mobility as in the case of 1 mM procaine were 0.13 mM for tetracaine and 0.05 mM for dibucaine.

The second series of experiments was similar to the first series, except for the use of phosphatidylserine instead of phosphatidylcholine (Fig. 2). The elcetrophoretic mobility of a phosphatidylserine vesicle was negative in sign and its magnitude was approx. -4.2 $\mu m \cdot cm \cdot V^{-1} \cdot s^{-1}$ in 0.1 M NaCl suspension solution. As the concentration of anesthetics was increased, the mobility of the vesicles decreased in magnitude. For the cases of tetracaine and dibucaine, at a certain concentration of local anesthetic, the mobility became zero and a further increase in anesthetic concentration even altered the sign of the direction of movement of the vesicle in the electric field, so that the vesicle appeared to possess net positive surface charges. The change in mobility of the vesicles was monotonic with respect to the concentration of local anesthetics. The extent of reduction in electrophoretic mobility of the vesicles due to the other local anesthetics at any given concentration of local anesthetics was of the same order as that (Fig. 1) in the case of the phosphatidylcholine vesicle. Electrophoretic mobility of the phosphatidylserine vesicle in the presence of 1 mM procaine in 0.1 M NaCl at pH 6.0 was 3.9 $\mu m \cdot cm \cdot V^{-1} \cdot s^{-1}$, which resulted in the reduction of 0.35 $\mu m \cdot cm \cdot V^{-1} \cdot s^{-1}$ from that of

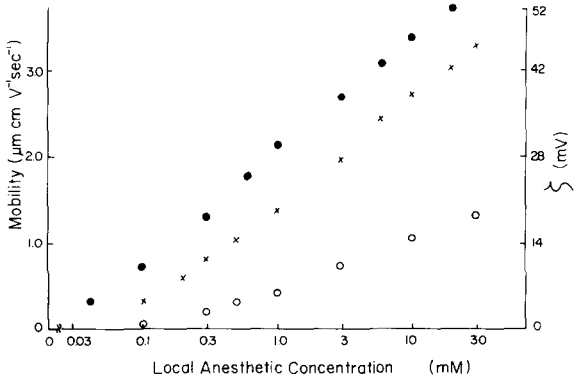

Fig. 1. Electrophoretic mobilites and ζ-potential of phosphatidylcholine multilamellar vesicles in 0.1 M NaCl suspenison solution (pH 6.0) as a function of local anesthetic concentrations. ○, Procaine; ×, tetracaine; ●, dibucaine.

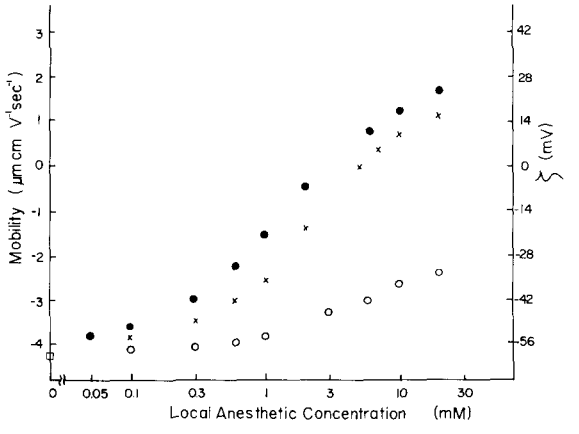

Fig. 2. Electrophoretic mobilites and ζ-potentials of phosphatidylserine multilamellar vesicles with variation of local anesthetic concentrations in 0.1 M NaCl suspension solution (pH 6.0). ○, Procaine; ×, tetracaine; ●, dibucaine.

no local anesthetic in the solution. The concentrations of the other local anesthetics needed to cause the same reduction in the mobility were 0.12 mM tetracaine and 0.04 mM dibucaine, which are the same magnitudes as those for the phosphatidylcholine case and which also correspond to the concentrations of those anesthetics needed to exert the same degree of narcotic acition on nerve axons.

In the third series of experiments, we examined the effect of Ca^{2+} on the electrophoretic mobilities of phosphatidylserine vesicles as a function of local anesthetic (tetracaine) concentration in 0.1 M NaCl (Fig. 3). The mobility of the vesicles was greatly affected by a small amount of Ca^{2+} present in the suspension solution. As Ca^{2+} concentration was increased, the mobility of a phosphatidylserine vesicle was further decreased (in magnitude). The change in mobility of the vesicle due to local anesthetics was also affected by the presence of Ca^{2+}. At a given Ca^{2+} concentration in the suspension media, the degree of the reduction in mobility of phosphatidylserine vesicles due to the change in local anesthetic concentration was smaller than that observed in the absence of Ca^{2+} in the solution, and this tendency was enhanced as the concentration of Ca^{2+} was increased (Fig. 3). Similar observations were also made for the other local anesthetics used, but the data are not shown.

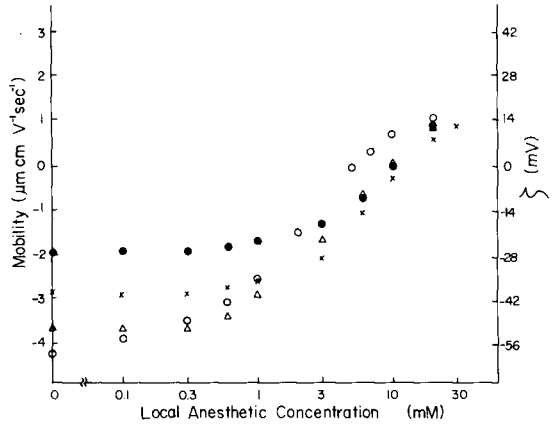

Fig. 3. Electrophoretic mobilities and ζ-potentials of phosphatidylserine multilamellar vesicles with variation of tetracaine concentration in 0.1 M NaCl suspension solution containing various $CaCl_2$ concentrations. □, No local anesthetic, no $CaCl_2$; ○, tetracaine, no $CaCl_2$; △, tetracaine, 0.1 mM $CaCl_2$; ×, tetracaine, 0.8 mM $CaCl_2$; ●, tetracaine, 3 mM $CaCl_2$.

Analysis of experimental results

Since the pK_a of all local anesthetic molecules used (procaine = 8.9, tetracaine = 8.4, dibucaine = 8.0) are much greater than 6.0, almost all the anesthetic molecules in our experimental solutions (pH = 6.0) are considered to have one positive charge per molecule, except for the anesthetic molecules near the lipid membranes which possess a relatively large net positive charge density (e.g., surface potential is greater than +25 mV or positive zeta potential is more than +20 mV). However, we will not consider the adsorption of local anesthetics onto the membrane in this region in our discussion hereafter. Therefore, the observed change in electrophoretic mobility of lipid vesicles which increases the surface charge density to a more positive quantity in the presence of local anesthetic, indicates that the absorption of a certain amount of such positively charged local anesthetic molecules onto the lipid membrane surface has taken place. The extent of alteration in the elctrophoretic mobility would therefore correspond to the amount of local anesthetic molecules adsorbed onto the lipid membrane surface.

Here, we assume that the cationic form of the local anesthetic will be adsorbed on the surface of the membrane so that the positively charged molecular group faces the polar-aqueous surface and the hydrophobic portion of the molecule is embedded in the membrane hydrocarbon phase [38], and also that the insertion of small amounts (compared with membrane lipid molecules) of such anesthetics would not alter the area per lipid molecule of the membrane. Subsequently, the amounts of adsorbed local anesthetics on the lipid membrane surface can be calculated with use of the theoretical relationships among ζ-potential, surface potential and the surface charge density given in the Appendix,

In the case of the phosphatidylcholine vesicle, the increase in ζ-potential with 1 mM procaine in the 0.1 M NaCl suspension solution at pH 6.0 was about +5 mV. It is calculated that this increase corresponds to the adsorption of one positively charged local anesthetic molecule per 3000 $Å^2$ of the membrane surface. As mentioned before, the concentration of the other local anesthetics used to produce the same change in ζ-potential (+5 mV)

was 0.13 mM for tetracaine and 0.05 mM for dibucaine (Fig. 1). These concentrations correspond somewhat to those of the same anesthetics required to block the nerve membrane excitability at the normal physiological environment [5,15,35,39]. If we assume that a membrane consists of a neutrally charged (e.g., phosphatidylcholine membrane) lipid bilayer and that local anesthetics of 1 mM exist only in the charged form in the solution, then the amount of the charged form of local anesthetics adsorbed onto the membrane at a physiological bulk solution which exerts the same narcotic action, is quite small, as mentioned above (one procaine molecule per 3000 $Å^2$ of the membrane surface).

Now let us estimate the bulk concentration of the uncharged form of local anesthetic in the hydrocarbon phase of the same (neutrally charged) membrane in the case where only the uncharged form of local anesthetic at the same physiological concentration is present in the solution, e.g., 1 mM procaine or 1 mM benzocaine. In the procaine case, the environmental pH should be higher than 10.0 so that all of the anesthetic molecules are in the neutral form. The partition coefficients of these molecules are about 40–50 at the oil/water phases [36,40]. In our calculation, we assume that the uncharged form of local anesthetics will be partitioned between the hydrocarbon phase of the lipid bilayer (50 Å in thickness) and the surrounding aqueous phases at the same ratio measured for the other oil/water phases [5,36,37]. Therefore, it is estimated that the neutral form of local anesthetics will be absorbed at a ratio of one local anesthetic molecule (uncharged) in the monolayer (half of a lipid bilayer) per about 1000 $Å^2$ membrane surface area. In addition, a certain amount of the neutral form local anesthetics will be adsorbed at a membrane/solution interface.

The above calculation shows that, in the case of neutrally charged lipid membrane, at 1 mM procaine of either all neutral forms or all charged forms, the neutral form will be adsorbed in the lipid membrane more than 3-times the charged form adsorbed. This difference may be due to the different distributions between the neutral and the charged forms of local anesthetics in the membrane (the neutral form can be adsorbed not only at the membrane/solution interface but absorbed also in the lipid bulk phase, whereas the charged form is adsorbed only at the membrane/aqueous interface).

In the case of the phosphatidylserine membrane (a negatively charged membrane), it was also observed that the presence of anesthetic molecules in the same vesicle suspension solution resulted in reduction of electrophoretic mobility in a manner of adding more positive charges onto the membrane surface (Fig. 2). The presence of 1 mM procaine in 0.1 M NaCl suspension solution altered the magnitude of electrophoretic mobility for the phosphatidylserine vesicle from 4.2 to 3.9 $\mu m \cdot cm \cdot V^{-1} \cdot s^{-1}$. It is calculated that this change in mobility corresponds to the adsorption of one positively charged local anesthetic per surface area of 274 $Å^2$ of a lipid monolayer which is half of a lipid bilayer, assuming that the negative surface charge density of the phosphatidylserine membrane was $-e/67$ $Å^2$, and the membrane did not expand inspite of adsorption of local anesthetic molecules onto the membrane surface. The binding constant of Na^+ to the phosphatidylserine membrane was calculated to be 0.7 M^{-1} from Eqns. 1–4 in the Appendix. The value of this binding constant is comparable with those (0.6–0.8 M^{-1}) obtained by others [41–43]. This large adsorption of the charged form of local anesthetics seems to be mostly due to the enhancement of the concentration of the charged form of local anesthetics in the solution near the membrane surface, which has a large negative surface potential. This will be analyzed later in this section.

In contrast to the previous case of the phosphatidylcholine membrane, the charged form of local anesthetics is more adsorbed (about 3-times more) onto the phosphatidylserine membrane surface than the neutral form, at the same bulk concentrations of local anesthetics in either form (charged or uncharged). In this case also, the concentrations of the other local anesthetics required to produce the same reduction in electrophoretic mobility of the phosphatidylserine membrane vesicle in 0.1 M NaCl at pH 6.0 equal to that obtained by 1 mM procaine, were 0.1 mM for tetracaine and 0.04 mM for dibucaine, which approximately corresponded to the order and magnitude of concentrations of these local anesthetics needed to induce the same narcotic action on

nerve membranes [5,15,39]. The same degree of reduction in the electrophoretic mobility would correspond to the same degree of adsorption of the charged form of local anesthetics on the membrane surfaces, since all anesthetic molecules are considered to be in the charged form at pH 6.0.

In order to ascertain the point mentioned above concerning the large adsorption of positively charged local anesthetic molecules on the phosphatidylserine membrane, we have performed the following analysis. First, we will consider the partition of the charged form of local anesthetics between the surface of a neutrally charged lipid (phosphatidylcholine) membrane and its aqueous phase near the membrane surface. Calculated values are shown in Table I. As seen, the partition (i.e., $K^s \equiv |\sigma/e|/C^s = 1/C^s A$, where C^s is the anesthetic concentration at the membrane surface and A is the area of the monolayer per anesthetic molecule adsorbed) of the charged form of local anesthetics between the phosphatidylcholine membrane surface and the aqueous phase near the membrane surface was approximatly a constant within a low concentration range (10^{-4}–10^{-3} M) of local anesthetics. Each anesthetic has its own surface partition coefficient K^s_{pc} for the phosphatidylcholine membrane ($1/(2\ \mathrm{M}\cdot\ \mathrm{Å}^2)$ for procaine, $1/(0.3\ \mathrm{M}\cdot\ \mathrm{Å}^2)$ for tetracaine and $1/(0.1\ \mathrm{M}\cdot\ \mathrm{Å}^2)$ for dibucaine). The greater the value of the surface partition coefficient, the greater the adsorption of molecules. Subsequently, using the surface partition coefficients obtained for each local anesthetic for the phosphatidylcholine (neutrally charged) membrane and the surface concentration, C^s, of the charged form of local anesthetic for the phosphatidylserine membrane, $C^s = C \exp(-e\psi(0)/kT)$, where C is the bulk concentration of the charged form of local anesthetic and $\psi(0)$ the surface potential (see Table II), we can calculate the amount of local anesthetics to be adsorbed onto the phosphatidylserine membrane surface ($K^s_{pc} \cdot C^s$). Calculated

TABLE I

The relations among bulk concentrations of local anesthetics, C^b, ζ-potential, surface potential, $\psi(0)$, area per unit electronic charge, A, surface concentrations of local anesthetics, C^s, and A_{cal}, the area per adsorbed local anesthetic molecule calculated by use of C^s and surface partition constant, $K^s \equiv 1/A \cdot C^s$.

Procaine				
C^b (M)	10^{-4}	$3\cdot10^{-4}$	10^{-3}	$3\cdot10^{-3}$
ζ (mV)	+0.86	+2.6	+6.0	+10.4
ψ_0 (mV)	+1.0	+3.2	+7.3	+12.7
A (Å^2)	21518	6831	2926	1689
C^s (M)	$0.96\cdot10^{-4}$	$2.9\cdot10^{-4}$	$7.5\cdot10^{-4}$	$16.6\cdot10^{-4}$
AC^s (MÅ^2)	2.1	2.0	2.2	2.8
$K^s_{pc} \equiv 1/AC^s$ (MÅ^2)$^{-1}$	0.48	0.50	0 45	0.36
Tetracaine				
C^b (M)	10^{-4}	$3\cdot10^{-4}$	10^{-3}	$2\cdot10^{-3}$
ζ (mV)	+5.2	+11.2	+19.8	+28.0
ψ_0 (mV)	+6.4	+13.7	+24	+36.0
A (Å^2)	3384	1570	861	543
C^s (M)	$0.78\cdot10^{-4}$	$1.92\cdot10^{-4}$	$3.78\cdot10^{-4}$	$7.1\cdot10^{-4}$
AC^s (MÅ^2)	0.31	0.30	0.32	0.38
$K^s_{pc} \equiv 1/AC^s$ (MÅ^2)$^{-1}$	3.0	3.3	3.1	2.6
Dibucaine				
C^b (M)	10^{-4}	$3\cdot10^{-4}$	10^{-3}	$2\cdot10^{-3}$
ζ (mV)	+10.3	+18.1	+29.2	+37.8
ψ_0 (mV)	+12.7	+22.2	+36.2	+47.4
A (Å^2)	1689	945	543	390
C^s (M)	$0.6\cdot10^{-4}$	$1.24\cdot10^{-4}$	$2.3\cdot10^{-4}$	$3\cdot10^{-4}$
AC_s (MÅ^2)	0.10	0.11	0.12	0.12
$K^s_{pc} \equiv 1/AC^s$ (MÅ^2)$^{-1}$	10	9.1	9.1	9.1

TABLE II

The values in parentheses are the areas per charged-form local anesthetic molecule adsorbed on the membrane surface. The calculation was made using area per lipid molecule (67 Å^2) for the lipid membrane. For further explanations, see Table I.

Procaine				
C^b (M)	10^{-4}	$3\cdot10^{-4}$	10^{-4}	$3\cdot10^{-3}$
ζ (mV)	−59.3	−58.5	−55.9	−48.1
ψ_0 (mV)	−77.6	−76.4	−72.5	−61.4
A (Å^2)	74.1 (700)	78.5 (451)	93.7 (235)	151.9 (119)
C^s (M)	$2.2\cdot10^{-3}$	$6\cdot10^{-3}$	$14.9\cdot10^{-3}$	$34.9\cdot10^{-3}$
AC^s (MÅ^2)	2.9	4.1	4.1	4.5
$A_{cal}=1/K^s_{pc}C^s$	(943)	(333)	(149)	(125)
Tetracaine				
C^b (M)	10^{-4}	$3\cdot10^{-4}$	10^{-3}	$3\cdot10^{-3}$
ζ (mV)	−55.9	−49.9	−36.1	−18.92
ψ_0 (mV)	−72.5	−63.8	−45.1	−23.2
A (Å^2)	93,7 (235)	137 (131)	299 (86.3)	499 (77.4)
C^s (M)	$1.82\cdot10^{-3}$	$3.8\cdot10^{-3}$	$6.07\cdot10^{-3}$	$7.59\cdot10^{-3}$
AC^s (Å^{2}M)	0.498	0.52	0.55	0.62
A_{cal} (Å^2)	(183)	(79,7)	(53)	(50)
Dibucaine				
C^b (M)	10^{-4}	$3\cdot10^{-4}$	10^{-3}	$3\cdot10^{-3}$
ζ (mV)	−51.6	−43	−22.4	−6.02
ψ_0 (mV)	−66.3	−54.3	−26.4	−7.35
A (Å^2)	123 (147)	202 (100)	648 (74.7)	2680 (67.9)
C^s (M)	$1.42\cdot10^{-3}$	$2.63\cdot10^{-3}$	$2.87\cdot10^{-3}$	$2.6\cdot10^{-3}$
AC^s (MÅ^2)	0.175	0.281	0.23	0.187
A_{cal} (Å^2)	(70.4)	(41.8)	(38)	(42)

values of the adsorbed local anesthetic by this method were approximately the same as those obtained from the surface charge density calculated using Eqns. 1–3 in the Appendix, for both procaine and tetracaine cases. When the amounts of adsorbed local anesthetics are too large to affect the molecular packing of membrane molecules greatly, these comparisons may not work well, as seen in the case of dibucaine or tetracaine at higher concentrations. However, within a small amount of adsorptions of anesthetic molecules, our analysis indicates that the surface partition constant for the charged form of a local anesthetic is approximately the same for both phosphatidylcholine and phosphatidylserine membranes, and that the large amounts of adsorbed local anesthetics on the phosphatidylserine membrane is due mainly to the enhanced surface concentrations of local anesthetics near the membrane surface, which is due to a large negative surface potential of the phosphatidylserine membrane.

Discussion

From the above study, we have obtained some important information concerning the different types of adsorptions for both charged and neutral forms of local anesthetics onto membrane: the charged form would be adsorbed at the membrane/water interface only and its adsorbed amount depends on the surface potential of the membrane and the surface partition coefficient, K^s, while the neutral form would be adsorbed not only at the membrane/water interface but also in the bulk membrane phase. Its adsorbed amount does not depend on the surface charge or surface potential of the membrane but does depend on the surface partition coefficient (K^s) and the bulk partition coefficient (K) between the water and membrane interior phases.

As seen in Analysis of experimental results, 1 mM benzocaine will be adsorbed in a lipid membrane at a ratio of one local anesthetic molecule

per about 1000 Å^2 membrane surface area in the monolayer and about one anesthetic molecule per 3000 Å^2 membrane surface area at the membrane/solution interface, which is deduced from the adsorbed amount of 1 mM procaine onto the phosphatidylcholine (electrically neutral) membrane surface. There may be some differences in adsorption between the charged and neutral forms [33]. As a net, the total amount of benzocaine molecules adsorbed would be at the ratio of one local anesthetic per 750 Å^2 of the membrane surface (4 benzocaine molecules per 3000 Å^2 membrane surface area) when the concentration of benzocaine is 1 mM in the bulk solution, while the charged form of procaine would be adsorbed at a ratio of one molecule per 3000 Å^2 of the membrane surface when the concentration of procaine in the bulk solution is 1 mM and all of the procaine molecules are in the charged form. However, when the procaine molecules of 1 mM are all in neutral form, the adsorbed amount of procaine molecules would be the same as that of benzocaine, since the bulk partition coefficients (K) for both procaine and benzocaine are approximately the same [36,40]. This situation may correspond to those observed for the narcotic action of benzocaine and procaine on nerve membranes.

It was found that the degree of narcotic action of 1 mM benzocaine for the nerve membrane excitability was independent of the external pH and was about equal to the corresponding values for procaine at pH 9.0 [44,45], where most procaine molecules are in the neutral form. Since both benzocaine and procaine have similar partition coefficients for the oil/water system [36,40], the same amount of the neutral form may be adsorbed into the membranes. On the other hand, when the pH of the extracellular solution is lowered below its pK_a values, the potency of procaine is reduced in proportion to $10^{pK_a - pH}$ where pK_a and pH are the negative logarithms of the dissociation constant of the local anesthetic and of the H^+ concentration in the bulk solution, respectively [3,15].

In the presence of 1 mM procaine in the extracellular solution at pH below its pK_a value, the concentration of the neutral form of procaine would not be sufficient to have enough adsorption into the membrane matrix, and the adsorbed amount of the charged form onto the membrane surface would not be enough in order to exert a narcotic action on the axon membrane, as was discussed in the preceeding paragraph.

Similar arguments can be made for tetracaine, dibucaine, etc., with respect to benzocaine, although it is expected that the bulk concentrations of tetracaine and dibucaine would be much smaller than that of procaine, because the partition coefficients of the basic forms of both anesthetics are much greater (273 for tetracaine, 500 * for dibucaine [5]) than that of procaine or benzocaine, 40.

Another aspect to be discussed is the effect of Ca^{2+} on adsorption of the charged form of local anesthetics to the membrane. The present experimental results as well as those of others [18–20] indicate that the presence of Ca^{2+} in the environmental solution of the negatively charged membrane inhibits the interaction of anesthetic molecules with the membrane. Fig. 3 shows that the absorption of anesthetic molecules onto the membrane surface is suppressed in the presence of Ca^{2+} in the solution in the region of low concentration of local anesthetics. However, at high enough concentrations, the adsorption of local anesthetics progressively increases, showing a sigmoid curve relationship between the adsorbed amounts and bulk concentration of local anesthetics. There may be at least two factors responsible for this inhibition: (1) the presence of even 1 mM Ca^{2+} in 0.1 M NaCl would substantially increase (decrease in magnitude) the ζ-potential for a phosphatidylserine membrane (from -60 mV for no $CaCl_2$ to -39 mV for 0.8 mM $CaCl_2$), which in turn would reduce the effective concentration of positively charged local anesthetic near the membrane surface; and (2) in many membrane systems, divalent cations, especially Ca^{2+}, have a strong binding affinity with negatively charged groups of membrane molecules in a manner analogous to chelation binding. Since such chelation binding is considerably stronger and makes the membrane rigid, the interaction of local anesthetic molecules with the membrane would be inhibited with respect to both binding to charged sites as

* The value was calculated from the experimental data [5] done at pH 7.0.

TABLE III

BINDING CONSTANTS K_{HA} OF THE CHARGED FORM OF LOCAL ANESTHETICS

	Procaine	Tetracaine	Dibucaine	Ref.
PC (egg)	26 (M^{-1})	256 (M^{-1})	720 (M^{-1})	present study [a]
DPPC	0	330		31
	14.3 ($\Delta pK = 1$)	1000 ($\Delta pK = 0$)		32
			555 ($\Delta pK = -1.5$)	24
PS	41	302	641	present study [b]
	10.8	650		31

[a] Calculation was done for the data at $\zeta \cong +10$ mV.

[b] Calculation was done for the data at $\zeta \cong -50$ mV. These calculations were done according to Rooney and Lee [24] assuming no adsorption of the uncharged form of local anesthetic on membranes and $\sigma_{max} = 1/70$ Å^2.

well as its physical penetration into the membrane phase.

The possible inhibition of nerve activity by local anesthetics has been suggested that the local anesthetics adsorbed on the membrane surface reduce the surface potential and, in turn, such a reduction in surface potential shifts the apparent transmembrane potential more to the hyperpolarization direction [34].

Although there may be factors other than the ones mentioned above concerning the effect of Ca^{2+}, the two factors mentioned would cause the inhibition of adsorption of the charged form of local anesthetics onto the negatively charged membrane surfaces (see Fig. 3). These could also be the reasons why the charged form of tertiary amine anesthetics at pH below its pK_a value does not affect anesthesia effectively from the extracellular phase of the axon membranes. Two factors are evident: (1) although the outer surface (possibly the surface of ionic channels) seems to have a considerably high charge density of negative sign [46,47,54], the high concentration of Ca^{2+} and other divalent cations near such a membrane surface [43] and their binding to the negatively charged sites reduces its surface potential substantially; and (2) the strong binding of Ca^{2+} to these negatively charged sites on the surface inhibits the adsorption of the monovalent cation form of local anesthetics on the outer surface (possibly the surface of ionic channels on the outer side of axon membranes). It should be noted, however, that several experimental results on axon membrane excitability suggest that the interaction of local anesthetics with the membrane is not competitive with extracellular Ca^{2+} [48–52]. Therefore, together with the argument mentioned above, the effect of Ca^{2+} on local anesthetic action of the nerve membrane seems to be due mainly to the first factor (alteration of surface concentration of the local anesthetics) rather than the second one (competitive binding of anesthetic molecules with the Ca^{2+}-binding sites at the membrane).

The main objective of this paper is not to determine the binding constants, per se, of local anesthetics to membranes, but rather to discuss various types of adsorptions (surface vs. bulk) of local anesthetics onto the membranes and compare the relative magnitudes of such adsorptions in relation to nerve membrane experiments. However, the calculated values of binding constants for the charged form of local anesthetics are listed in Table III in comparison with those obtained by others. The calculations done for the experimental data are within the validity of application of the adsorption formula given by Rooney and Lee [24] assuming no adsorption of the neutral form on membrane surfaces. Although the values of binding constants do not accord well for each anesthetic, the order of magnitude can be compared relatively well for the results obtained by three different experimental methods.

Thus, we have shown how local anesthetics are adsorbed on the membrane (surface as well as membrane bulk phase), and its adsorption is affected by the surface charge or surface potential of the membrane and also by the presence of monovalent and divalent cations. This type of analysis

may provide useful information as to which form of local anesthetic is adsorbed onto which part of the membrane and thus, for the elucidation of molecular mechanisms of narcotic action on nerve membrane excitability.

Appendix

The surface potential (ψ_o) of a charged membrane is described in the following equation [43]:

$$\sigma = \frac{1}{273}\left\{\sum_i C_i(\infty)\left(\exp\left(\frac{-ez_i\psi_0}{kT}\right)-1\right)\right\}^{1/2} \quad (1)$$

where σ is the surface charge density (charge per Å^2); $C_i(\infty)$ the molar concentration of the ith ionic species in the bulk phase; z_i the valency of the ith ion; ψ_0 the surface potential at the membrane interface, k the Boltzmann constant, and T the temperature, 23°C.

In the case of phosphatidylcholine membrane, we assumed that the degree of metal ions bound to the charged groups is small and can be neglected in 0.1 M NaCl at pH 6.0. On the other hand, in the case of phosphatidylserine, a monovalent cation may bind with one phospholipid polar group with a certain binding constant K. The binding constant K is expressed by

$$K = \frac{(C^+A)}{(C^+)(A^-)} \quad (2)$$

where (C^+) is the molar concentration of a positive ion in the bulk phase and (A^-) is the surface concentration of the lipid. Consequently, the surface charge density σ is expressed by

$$\sigma = \frac{\sigma^{int}}{1+K(C^+)^s} \quad (3)$$

where σ^{int} is the charge density of the membrane in the case of no ion binding, and $(C^+)^s = (C^+)\cdot \exp[-ez_i\psi_0/kT]$.

The ζ-potential is calculated from the measured value of the electrophoretic mobility, u, by the Helmholtz-Smoluchowski equation:

$$u = \zeta\epsilon_r\epsilon_0/\eta$$

where η is the viscosity of the aqueous phase, and ϵ_r, ϵ_0 are the permitivities of the aqueous medium and free space (vacuum), respectively.

The surface potential of the Gouy-Chapman diffused layer is expressed as [53]:

$$\psi(x) = \frac{2kT}{e}\ln\frac{1+\alpha\exp(-\kappa x)}{1-\alpha\exp(-\kappa x)} \quad (4)$$

where

$$\alpha = \frac{\exp(e\psi_0/2kT)-1}{\exp(e\psi_0/2kT)+1}$$

and

$$\kappa = \left(\frac{2e^2CNz^2}{\epsilon_r\epsilon_0 kT}\right)^{1/2}$$

With Eqns. 1, 4 and the ζ-potential obtained from the measured electrophoretic mobility, the surface potential ψ_0 can be calculated, where the plane of shear related to the ζ-potential was chosen to be 2.0 Å from the membrane charged surface. Also, the binding constant of Na^+ to phosphatidylserine membrane can be calculated from the obtained surface potential.

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