Locations of Local Anesthetic Dibucaine in Model Membranes and the Interaction between Dibucaine and a Na^+ Channel Inactivation Gate Peptide as Studied by ^2H- and ^1H-NMR Spectroscopies

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ABSTRACT To study the molecular mechanisms of local anesthesia, locations of local anesthetic dibucaine in model membranes and the interactions of dibucaine with a Na^+ channel inactivation gate peptide have been studied by ^2H- and ^1H-NMR spectroscopies. The ^2H-NMR spectra of dibucaine-d_9 and dibucaine-d_1, which are deuterated at the butoxy group and at the 3 position in its quinoline ring, respectively, have been observed in multilamellar dispersions of the lipid mixture composed of phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine. ^2H-NMR spectra of deuterated palmitic acids incorporated, as a probe, into the lipid mixture containing cholesterol have also been observed. An order parameter, S_{CD}, for each carbon segment was calculated from the observed quadrupole splittings. Combining these results, we concluded that first, the butoxy group of dibucaine is penetrating between the acyl chains of lipids in the model membranes, and second, the quinoline ring of dibucaine is located at the polar region of lipids but not at the hydrophobic acyl chain moiety. These results mean that dibucaine is situated in a favorable position that permits it to interact with a cluster of hydrophobic amino acids (Ile-Phe-Met) within the intracellular linker between domains III and IV of Na^+ channel protein, which functions as an inactivation gate. To confirm whether the dibucaine molecule at the surface region of lipids can really interact with the hydrophobic amino acids, we synthesized a model peptide that includes the hydrophobic amino acids (Ac-GGQDIFMTEEQK-OH, MP-1), the amino acid sequence of which corresponds to the linker part of rat brain type IIA Na^+ channel, and the one in which Phe has been substituted by Gln (MP-2), and measured ^1H-NMR spectra in both phosphate buffer and phosphatidylserine liposomes. It was found that the quinoline ring of dibucaine can interact with the aromatic ring of Phe by stacking of the rings; moreover, the interaction can be reinforced by the presence of lipids. In conclusion, we wish to propose that local anesthesia originates from the π-stacking interaction between aromatic rings of an anesthetic molecule located at the polar headgroup region of the so-called boundary lipids and of the Phe in the intracellular linker between domains III and IV of the Na^+ channel protein, prolonging the inactivated state and consequently making it impossible to proceed to the resting state.

INTRODUCTION

Since the discovery by Sigmund Freud in Heidelberg of local anesthesia by cocaine, many chemicals have been proposed as local anesthetics (Ritchie and Greene, 1985; Covino, 1987). Their molecular structures have a similarity in that they have the following chemical arrangement: an aromatic ring, an intermediate chain including an amide or an ester linkage, and a tertiary amine nitrogen. Thus these chemicals are amphiphilic in nature, and owing to the presence of the tertiary amine nitrogen, they can exist as both cationic and uncharged molecular forms, depending on their pK_a and the pH of the fluid surrounding nerve membranes. The mechanism of action of the local anesthetics upon excitable membranes, which appears to be well established so far, is 1) diffusion of the uncharged form of the local anesthetic across the nerve sheath and nerve membrane; 2) reequilibration between the uncharged and cationic forms on the axoplasmic surface of the nerve membrane; and 3) penetration into and attachment to a receptor at a site within the sodium channel (Covino, 1987; Strichartz and Ritchie, 1987). However, the molecular nature of the local anesthetic site(s) of action remains unclear. Debate has focused on whether such sites are purely lipid in nature or whether protein targets may be involved. A good correlation between the potency of local anesthetics and their ability to penetrate artificial lipid membranes gives support for the former view (Trudell, 1977, 1980), whereas the use-dependent anesthetic block, the stereospecific effects of local anesthetics, and the higher affinity binding of anesthetic for the inactivated channels than for the activated ones support the latter (Strichartz and Ritchie, 1987; Courtney and Strichartz, 1987).

Recently two notable papers that urge us to study the molecular mechanisms of local anesthesia have been published. One is related to the Na^+ channel inactivation gate in rat brain type IIA Na^+ channel by Catterall et al. (Patton et al., 1992; West et al., 1992), and the other is related to the amine blockers of the cytoplasmic mouth of Na^+ channels by Zamponi and French (1994). Catterall et al. have reported that they successfully specified the amino acid residues required for fast Na^+ channel inactivation and have proposed a "hinged-lid" model of the Na^+ channel inacti-

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vation gate (Patton et al., 1992; West et al., 1992). In this model, a cluster of hydrophobic amino acids, Ile-1488, Phe-1489, and Met-1490 within the intracellular linker between domains III and IV of the channel protein (Fig. 1), occludes the intracelluar mouth of the activated Na^+ channel (Fig. 2) and stabilizes the inactivated state, making use of Gly-1484 (or Gly-1485, or both) and Pro-1509 residues on either side of the IFM domain as hinge points. On the other hand, Zamponi and French have reported that for a drug to function as an inactivation enhancer, it is necessary that the aromatic ring of the drug bind to a hydrophobic domain (Fig. 2) within the internal mouth of the channel (Zamponi and French, 1994).

By taking these two reports into consideration and by noting that there are negatively charged amino acids only on both sides of the IFM amino acid residues (Asp-1487, Glu-1492, and Glu-1493; see Figs. 1 and 2) in the linker, we hypothesized as follows: 1) local anesthetics locate within lipid membranes surrounding domains III and IV, allowing their hydrophobic moieties to interact simultaneously with both lipids and one or two of the three hydrophobic amino acids of Ile-1488, Phe-1489, or Met-1490; 2) the tertiary amine nitrogen, especially in its protonated quaternary ammonium form, interacts electrostatically with one of the three negatively charged amino acids that occur on both sides of the three hydrophobic amino acids. These assumptions can explain all of the reasoning involved in the two conflicting models of molecular mechanisms of local anesthesia. However, for these assumptions to be proved correct, as a first step we should confirm whether all local anesthetic molecules reside at the polar headgroup region of the membranes, rather than at the hydrophobic acyl chain region. This is because the "hinged lid" occupies a position within the aqueous channel pore (Fig. 2), and thus local anesthetics should also locate at a site within the hydrophilic regions of the channel. We assumed that such an interfacial site between the hydrophobic site suggested by Zamponi and

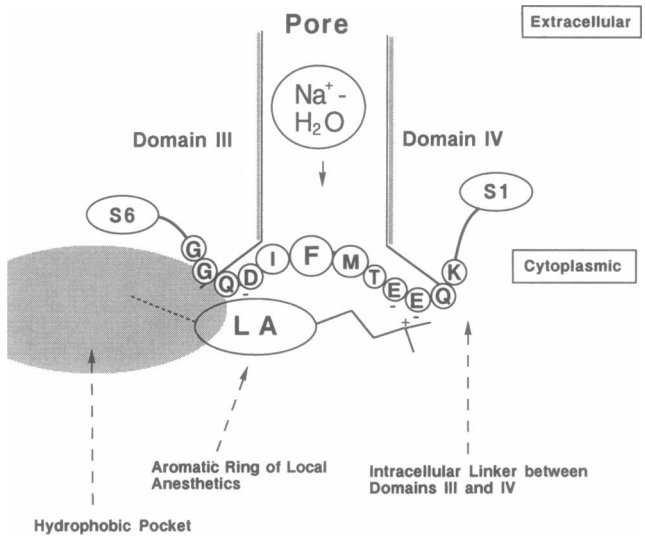

FIGURE 2 A hinged-lid model for Na^+ channel inactivation (Patton et al., 1992; West et al., 1992) and schematic representation of the interaction between a local anesthetic drug and the amino acid residues in the inactivation gate peptide.

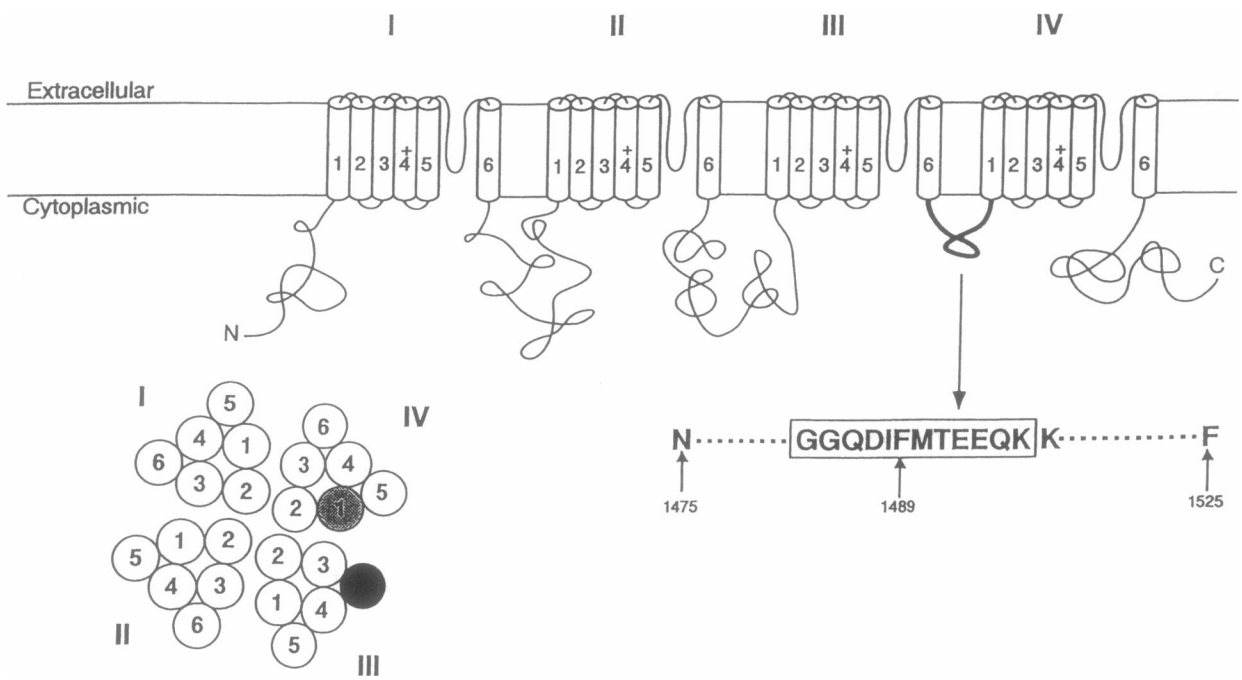

FIGURE 1 Schematic representation for the Na^+-channel α subunit. It is considered to be composed of four homologous domains (I–IV). Each domain has six α-helices (1–6). A space that is surrounded by the 24 α-helices is thought to be a pore through which the Na^+ ion passes (Noda et al., 1986). The numbering and the amino acid sequence at the intracelluar linker between domains III and IV is in the rat brain IIA Na^+ channel (Noda et al., 1986).

French and the hydrophilic region in which the hinged-lid exists is provided by the polar headgroup region of the so-called boundary lipids, and not by the channel protein.

The local anesthetic dibucaine (Fig. 3) was selected for use in the present experiments. The molecule consists of a big quinoline ring, which seems to be the most hydrophobic aromatic ring among the clinically used local anesthetics. Therefore, elucidating its location in lipid membranes can be a promising means of testing the above assumptions. ^{2}H-NMR spectroscopy has been employed to investigate the location of the dibucaine molecule (Seelig, 1977). The ^{2}H-NMR spectra of the deuterated dibucaine at the 3 position in its quinoline ring and at the butoxy group in multilamellar dispersions of phospholipids, composed of phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylethanolamine (PE, PC:PS:PE = 1:1:2.5 molar ratio), which contain or do not contain cholesterol (phospholipids:cholesterol = 7:3 molar ratio), have been observed. The ratio of the amount of each lipid (PC:PS:PE = 1:1:2.5 molar ratio) is that reported for the phospholipids at the inside of human erythrocyte ghost (Op den Kamp, 1979), whereas the ratio of the amount of the total phospholipids to cholesterol (7:3 molar ratio) is approximately one-half that reported for the total phospholipids and cholesterol of the whole human ghost (Maraviglia et al., 1982), but approximately equal to that reported for the excitable membranes of the garfish olfactory nerve (Chacko et al., 1976). Moment analyses (Bloom et al., 1978; Davis et al., 1979; Nichol et al., 1980) have been applied to the observed ^{2}H-NMR spectra, because the observed spectra are made up by the superposition of spectra due to variously oriented dibucaine molecules.

Effects of dibucaine on the orientational order of methylene groups of the multilamellar dispersions of phospholipids have been studied by ^{2}H-NMR spectroscopy. Deuterated palmitic acid was incorporated into the lipid mixture as a probe of the lipid structure. An order parameter S_{CD} for each carbon segment was calculated from the quadrupole splitting in the ^{2}H-NMR spectra. By taking all of these ^{2}H-NMR spectroscopic data into account, we deduced the location of dibucaine in membranes and concluded that the quinoline ring of dibucaine is located at the polar headgroup region of lipids.

To ascertain whether the dibucaine molecule located at the polar headgroup region of lipids can actually interact with the hydrophobic amino acids, especially with Phe, we synthesized a model peptide that includes the IFM domain in the linker (Ac-GGQDIFMTEEQK-OH) named MP-1 and another in which Phe has been substituted by Gln (MP-2). ^{1}H-NMR spectra of dibucaine in the presence of MP-1 or MP-2 in both phosphate buffer and phosphatidylserine liposomes have been observed to obtain information on the interactions. Finally, we discuss all of the results in relation to the interaction between local anesthetics and the Na^+ channel inactivation gate to elucidate the molecular mechanisms of local anesthesia.

MATERIALS AND METHODS

Materials

Dibucaine hydrochloride deuterated at the 3 position in the quinoline ring (dibucaine-d_1) was synthesized from isatin according to the scheme shown in Fig. 4 (Miescher, 1932) (m.p. 91–96°C). The percentage of deuteration was checked by ^{1}H-NMR spectroscopy and was found to be 83%; this rather low percentage of deuteration was later found to be due to the use of C_4H_9OH instead of C_4H_9OD at the final stage of the reaction. The dibucaine hydrochloride deuterated at the butoxy group was synthesized by using C_4D_9OH instead of C_4H_9OH (Fig. 4). The percentage deuteration was greater than 99% (m.p. 91–96°C). Palmitic-d_2 acids deuterated at various positions (C4, C6, C9, or C10) were synthesized starting from relevant α-deuterated fatty acids and dicarboxylic acid monomethyl esters by the method of Kolbe electrolysis (Greaves et al., 1950); we followed the method described by Oldfield et al. (1978). The percentages of deuteration were checked by ^{1}H-NMR spectra and found to be greater than 85% for each palmitic-d_2 acid. Palmitic-d_{31} acid was obtained from MSD Isotopes (Montreal, Quebec, Canada). Palmitic-2,2-d_2 acid and palmitic-3,3-d_2 acid were obtained from C/D/N Isotope Inc. (Vaudreuil, Quebec, Canada). Peptides MP-1 (Ac-GGQDIFMTEEQK-OH) and MP-2 (Ac-GGQDIQMTEEQK-OH) were synthesized by the solid phase method using Fmoc chemistry; their N-termini were acetylated (denoted by Ac-), and their C-termini were free carboxylic acids (denoted by -OH). They were purified on a reverse-phase C_{18} high-performance liquid chromatography column using a gradient of 85% A, 15% B to 60% A, 40% B, where A is 0.1% trifluoroacetic acid (TFA) in water and B is 0.1% TFA in acetonitrile; the rate of decrease in A was 25%/50 min. They were characterized by determination of amino acid composition by ion spray mass spectrometry.

Egg yolk L-α-phosphatidylcholine (egg PC), bovine brain L-α-phosphatidylserine (PS), egg yolk L-α-phosphatidylethanolamine (PE), and cholesterol were obtained from Sigma and used without further purification.

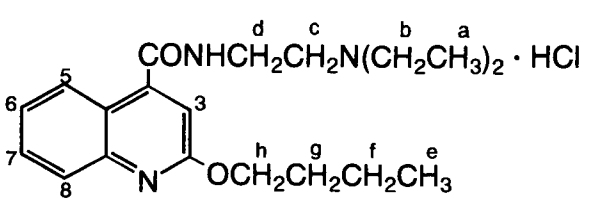

FIGURE 3 Chemical structure of dibucaine hydrochloride and the numbering scheme.

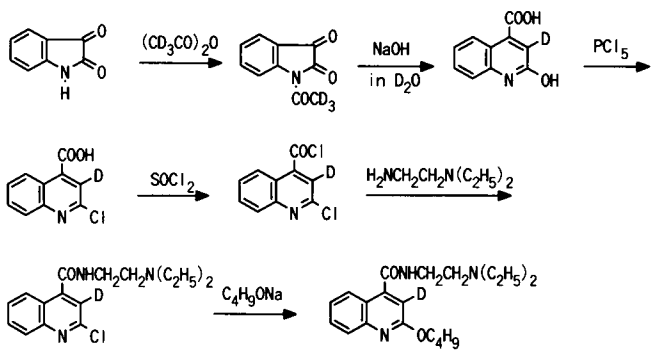

FIGURE 4 Reaction scheme to synthesize the dibucaine deuterated at the 3 position in the quinoline ring.

Deuterium-depleted water ($<5 \times 10^{-5}$ atom %D) was obtained from ISOTEC (Miamisburg, OH).

Determination of the gel-to-liquid crystalline phase transition temperature of multilamellar dispersions of the lipid mixture composed of PC, PS, and PE (PC:PS:PE = 1:1:2.5 molar ratio) was made by monitoring the lock signal provided from an isotonic (310 mOsm, 150 mM) phosphate buffer in ^2H_2O and was found to be 274 K.

The partition coefficients of dibucaine for the lipid mixture that contains cholesterol and palmitic acid and dispersed in the phosphate buffer (pH 7.2 and pH 5.2) were obtained by fluorometrically determining the concentrations of dibucaine not binding with the membranes. The measured solutions were obtained by ultrafiltration of the lipid mixture suspended in the phosphate buffer.

Preparation of sample solutions for ^{2}H-NMR measurements

Lipid mixture solutions

The thin films of the mixtures of PC, PS, and PE (PC:PS:PE = 1:1:2.5 molar ratio, 60–70 mM) that contain or do not contain cholesterol (phospholipids:cholesterol = 7:3 molar ratio) were prepared by concentrating their chloroform/methanol solution with a rotary evaporator and then pumping on a vacuum line overnight. Their multilamellar dispersions were prepared by vortexing vigorously the round-bottomed flask containing the thin films and an isotonic (310 mOsm, 150 mM, pH 7.4 or 5.6) phosphate buffer in deuterium-depleted water for 5 min. A weighed amount of dibucaine-d_1 (12 mM) or dibucaine-d_9 (3 mM) dissolved in the phosphate buffer was added before the vortexing. To equilibrate the state of anesthetics interacting with lipids completely, the multilamellar dispersions of model membranes were subjected to five freeze-thaw-vortex cycles (Westman et al., 1982). Finally, the pH of the sample solution was checked and adjusted to a desired value by adding a trace amount of Na_2HPO_4 or NaH_2PO_4.

Lipid mixture solutions with cholesterol and deuterated palmitic acid

The PC, PS, and PE in a chloroform/methanol solution (60 mM) were mixed with cholesterol (25 mM) and deuterated palmitic acid (20 mM), and then the solvent was evaporated by pumping with a vacuum line overnight. Thus obtained thin films were dispersed into the deuterium-depleted water (310 mOsm, pH 7.4 or 5.8) by vortexing. A weighed amount of dibucaine (3 mM) was dissolved in the suspension and then subjected to five freeze-thaw-vortex cycles.

Preparation of sample solutions for ^{1}H-NMR measurements

A weighed amount of dibucaine hydrochloride (3 mM) with or without peptide (3 mM) was dissolved in the phosphate buffer (10% ^2H_2O-90% H_2O, 310 mOsm, 150 mM), and the pH of the sample solution was adjusted to 7.4 by adding a trace amount of Na_2HPO_4 or NaH_2PO_4. Single bilayer vesicles (liposomes) of PS were prepared by ultrasonic irradiation of the phosphate buffer suspension (in ^2H_2O) of dried PS (15 mM) for 20 min, cooling in an ice/water bath, and bubbling with nitrogen gas. A weighed amount of dibucaine (3 mM) with or without peptide (3 mM) was added to the solution of preformed vesicles; the pH was adjusted to 7.4 by adding a trace amount of Na_2HPO_4 or NaH_2PO_4.

Measurements

^{2}H-NMR spectra were observed at 92 MHz on a Bruker AM-600 spectrometer equipped with a usual broadband probe for high-resolution NMR spectroscopy. The quadrupolar echo sequence was employed (Davis et al., 1976); $\pi/2 = 10$ μs, and its pulse spacing was 50 μs. A typical observing spectral width was 50,000 Hz for 16 K data points, and the recycle time was 0.26 s. The acquired number of transients was typically 300,000 (~22 h) for a sample solution containing dibucaine-d_1, 200,000 (~15 h) for a sample solution containing dibucaine-d_9, and 250,000 (~20 h) for a sample solution containing 20 mM deuterated palmitic acid. No symmetrization procedure was performed. An exponential weighting function with a line broadening factor of 50–100 Hz was applied to the free induction decay acquired with 16 K data points and zero-filled to 64 K. The quadrupole splittings reported in the following were read directly from the CRT display of an AM-600 spectrometer. ^{1}H-NMR spectra were observed on a Bruker AM-600 (600 MHz) spectrometer with a digital resolution of 0.18 Hz/point; the ambient probe temperature was 27°C. The chemical shifts were referenced to TSP (3-trimethylsilyl-propionic acid-d_4 sodium salt).

Moment analyses for ^{2}H-NMR spectra and calculations of order parameters, S_{CD}, for the segments of a flexible hydrocarbon chain

Moments of the ^{2}H-NMR spectra (Bloom et al., 1978; Davis et al., 1979; Nichol et al., 1980) were calculated according to

$$M_n = \int_0^\infty \omega^n f(\omega)\mathrm{d}\omega \Big/ \int_0^\infty f(\omega)\mathrm{d}\omega \qquad (1)$$

and by using IRIS Indigo for half the Fourier-transformed spectral data points (~16,000) written in the ASCII format according to the JCAMP-DX norm (McDonald and Wilks, 1988) in a Bruker X-32 computer. The half-spectra selected for calculations were from either the high- or the low-frequency sides, which do not include the resonance due to the solvent. Estimated errors in calculating the first (M_1) and the second (M_2) moments and the resulting mean order parameter (S_{CD}), which is defined by

$$S_{CD} = 2\sqrt{3}M_1/(e^2qQ/\hbar) \qquad (2)$$

and its dispersion (Δ_2), defined by

$$\Delta_2 = M_2/1.35M_1^2 - 1, \qquad (3)$$

where e^2qQ/h is the static quadrupole coupling constant, were within a few percent. The order parameter S_{CD} for each acyl chain carbon position (Stockton et al., 1976) was calculated by correlating the corresponding observed quadrupole splitting, $\Delta\nu q$ (in units of kilohertz), of specifically deuterated palmitic acids to the order parameter in the following way:

$$\Delta\nu q = 3/4(e^2qQ/h)S_{CD}. \qquad (4)$$

RESULTS

^{2}H-NMR spectra of dibucaine-d_1 in lipid mixtures

Fig. 5, *a* and *b*, shows ^{2}H-NMR spectra of dibucaine-d_1 (12 mM)-lipid mixture (70 mM) solutions at pH 7.4 and at 300 K in the absence and in the presence of cholesterol (phospholipids:cholesterol = 7:3 molar ratio), respectively. The dibucaine in the lipid mixture without cholesterol gave a trapezoid lineshape with an approximate maxium quadrupole splitting of 6.7 kHz. In contrast, this spectral feature drastically changed when the lipid mixture included cholesterol (spectrum b) and resulted in a narrow resonance with a half-height width of 0.6 kHz. The differences in these spectral features essentially disappeared, however, when the temperature was lowered to 279 K and resulted in closely similar spectra (spectra c and d); the

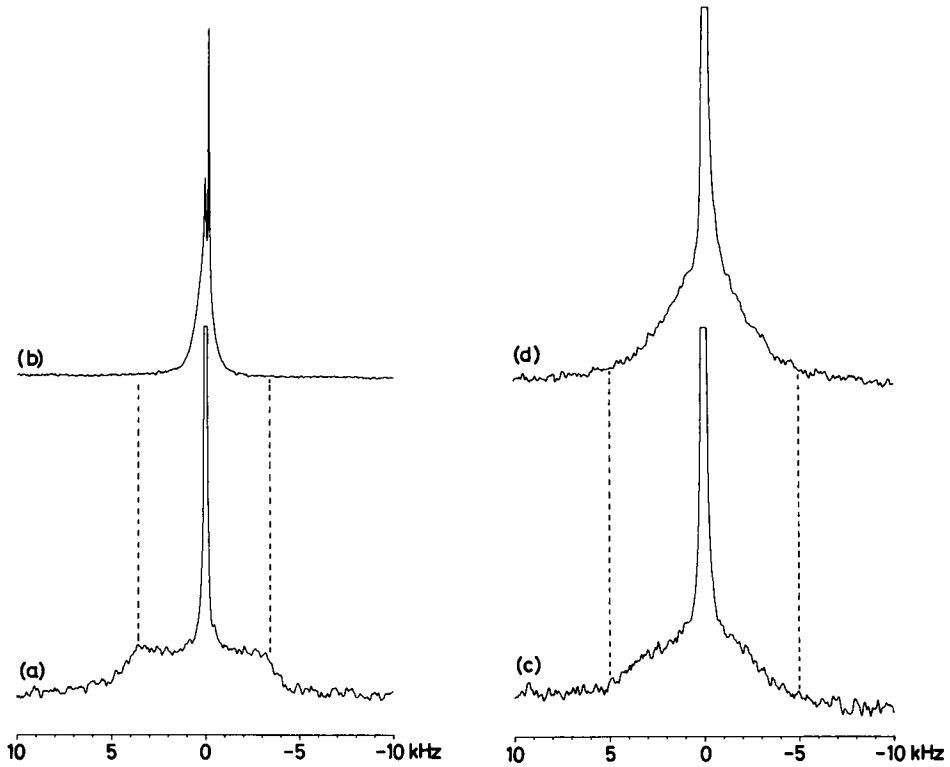

FIGURE 5 (*a*) ^{2}H-NMR spectrum of dibucaine-d_1 (12 mM) mixed lipid (PC:PS:PE = 1:1:2.5 molar ratio, 70 mM) solution at pH 7.4 and at 300 K. (*b*) As in *a*, but contains cholesterol (phospholipids:cholesterol = 7:3 molar ratio). (*c*) As in *a*, but at 279 K. (*d*) As in *b*, but at 279 K.

quadrupole splitting decreased in spectrum c, and the linewidth at half-height increased in spectrum d. Because the gel-to-liquid crystalline phase transition temperature of the lipid mixture with no cholesterol was 274 K, there is no doubt that the lipid mixture is in a liquid crystalline state. It is known that cholesterol increases the order of the acyl chains of lipid bilayers at temperatures above that of the gel-to-liquid crystalline phase transition (Stockton and Smith, 1976; Bloom and Mouritsen, 1988). Thus the spectral change from spectrum a to spectrum b can be considered to be due to changes in the location of dibucaine toward the polar region, which may result in decreased order of the quinoline rings of dibucaine molecules. On the other hand, the close resemblance between spectra c and d suggests that at a low temperature dibucaine molecules locate at a similar environment with a similar manner, irrespective of the presence or absence of cholesterol. The change in the lineshape from spectrum a to spectrum c implies that the quinoline ring of dibucaine was squeezed out of the original binding site to the more unordered binding site as a consequence of increased degree of order of the lipids owing to decreased temperature.

A similar observation was also noted at pH 5.8. The observed maximum quadrupole splitting was 4.6 kHz; this value was somewhat smaller than that of the neutral solution (6.7 kHz).

^{2}H-NMR spectra of dibucaine-d_9 in lipid mixtures

Fig. 6 shows ^{2}H-NMR spectra of dibucaine-d_9 (3 mM) in multilamellar dispersions of the lipid mixture in the absence (Fig. 6, *a* and *c*) and presence (Fig. 6, *b* and *d*) of cholesterol at pH 7.4; spectra a and b were observed at 300 K and spectra c and d at 279 K. As shown in spectrum a, each methylene group of dibucaine-d_9 showed clearly resolved quadrupolar splittings ranging from 12 to 4.5 kHz; however, the methyl group showed no resolved splitting. In contrast to the spectra for dibucaine-d_1, all of these splittings increased when the membrane contained cholesterol, and even the terminal methyl group showed a clear quadrupolar splitting of 1.8 kHz (spectrum b). When the temperature was lowered to 279 K, the quadrupolar splittings due to the methylene groups increased, ranging from 14.5 to 6.2 kHz (spectrum c); however, those lineshapes became rather featureless in appearance. This tendency became more prominent when the lipid mixture contained cholesterol (spectrum d). The quadrupolar splittings due to the three methylene groups coalesced to give a broad trapezoid lineshape with a width of about 14 kHz, and that due to the methyl group increased (3.0 kHz). These observations indicate that dibucaine molecules are binding with the lipid membrane in such a manner that its butoxy group is between the acyl chains of lipids.

Fig. 7 shows ^{2}H-NMR spectra observed at acidic conditions. The quadrupole splittings in spectrum a decreased by about 0.4 kHz in each methylene group as compared to the corresponding one in the neutral pH solution (Fig. 6 *a*). However, no appreciable difference was noted when the lipid mixture contained cholesterol (compare spectrum b with Fig. 6 *b*). The tendencies for the spectral changes with added cholesterol or a temperature lowered to 279 K were

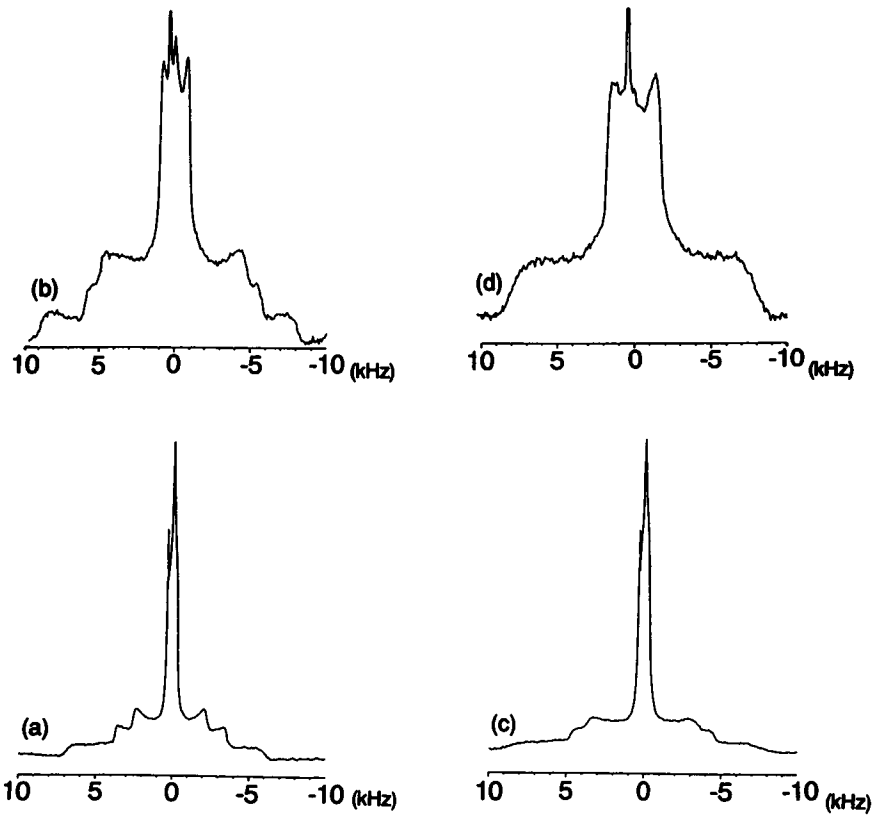

FIGURE 6 (*a*) ^{2}H-NMR spectrum of dibucaine-d_9 (3 mM) mixed lipid (PC:PS:PE = 1:1:2.5 molar ratio, 70 mM) solution at pH 7.4 and at 300 K. (*b*) As in *a*, but contains cholesterol (total lipids:cholesterol = 7:3 molar ratio). (*c*) As in *a*, but at 279 K. (*d*) As in *b*, but at 279 K.

the same as those noted for neutral pH solutions, except that spectrum c showed a more clear-cut quadrupole splitting for each methylene group; in fact, even the methyl group showd a small well-resolved quadrupole splitting (spectrum c).

Moment analyses for ^{2}H-NMR spectra of dibucaine-d_1 and dibucaine-d_9

Moment analyses (Bloom et al., 1978) have been applied to these ^{2}H-NMR spectra because the observed spectra are made up of a superposition of the spectra arising from variously oriented dibucaine molecules. Tables 1 and 2 show the first (M_1) and the second (M_2) moments, mean order parameter derived from the first moment (S_{CD}), and the mean square deviation of the order parameter (Δ_2) of dibucaine-d_1 and dibucaine-d_9, respectively. The static e^2qQ/h values assumed were 180 kHz (Burnett and Muller, 1971) in Table 1 and 167 kHz (Davis and Jeffrey, 1977) in Table 2; the former is a typical value for the C-^{2}H bond in an aromatic ring, and the latter is for the C-^{2}H bond in an aliphatic chain. All of the changes in spectral features described above were clearly reflected in these M_1, M_2, and S_{CD} values. Furthermore, we noticed that in dibucaine-d_1 the distribution of order parameters that is represented by the Δ_2 value increased when the lipid mixture contained cholesterol (from 0.379 to 0.887) or when the temperature was lowered (from 0.379 to 1.02); under acidic conditions the entirely same trend was noted. These findings mean that the order of dibucaine binding with lipid membranes was lowered by cholesterol and by lowering the temperature. On the other hand, in dibucaine-d_9, under both neutral and acidic conditions, the Δ_2 value decreased when the lipid mixture contained cholesterol, but again increased when the temperature was lowered. This former result means that in contrast to the case in dibucaine-d_1, the cholesterol increases the order of the butoxy group of dibucaine in the lipid bilayer. Because the butoxy group is attached to the carbon adjacent to the 3 position in the quinoline ring, this finding can be a good clue to the location of dibucaine.

^{2}H-NMR spectra of palmitic acid probes in the lipid mixture with cholesterol

To investigate the effects of dibucaine on the orientational order of methylene groups of multilamellar dispersions of phospholipids, we have observed ^{2}H-NMR spectra for deuterated palmitic acids incorporated into the lipid mixture with cholesterol. The usefulness of deuterated fatty acid probes for determining the order of phospholipid membranes is well demonstrated (Stockton et al., 1974, 1976; Pauls et al., 1983; Vogt et al., 1994). Fig. 8 shows ^{2}H-NMR spectra observed for palmitic-d_{31} acid (25 mol%), which is intercalated into multilamellar dispersions of the lipid mixture with cholesterol at pH 7.2; spectrum a was obtained in the presence of no dibucaine, whereas spectra b and c were obtained in the presence of 1 mM and 3 mM dibucaine, respectively. Each of these spectra had only six peaks, because the ^{2}H-NMR spectrum of palmitic-d_{31} acid is com-

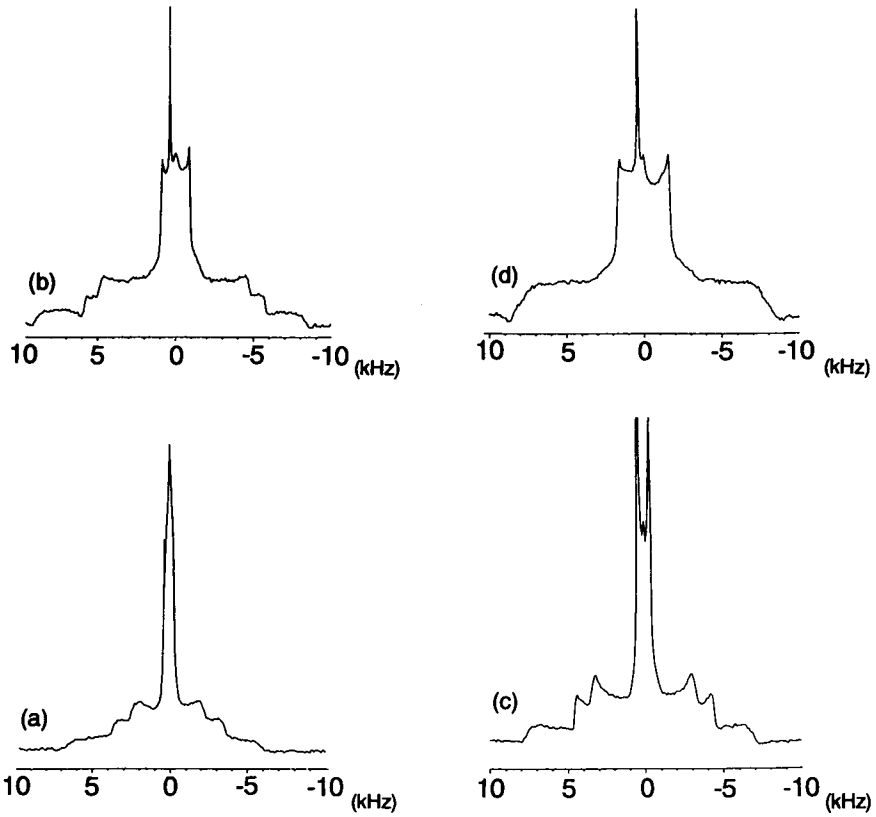

FIGURE 7 (*a*) ^{2}H-NMR spectrum of dibucaine-d_9 (3 mM) mixed lipid (PC:PS:PE = 1:1:2.5 molar ratio, 70 mM) solution at pH 4.8 and at 300 K. (*b*) As in *a*, but contains cholesterol at pH 4.6. (*c*) As in *a*, but at 279 K. (*d*) As in *b*, but at 279 K.

posed of many overlapping resonances; in an acidic condition (pH 5.2) the situation was the same as in the neutral condition. The sharp peak at around 0 kHz should be attributed to the residual HO^2H resonance of the solvent. The quadrupole splittings ($\Delta\nu q$) in spectrum a were decreased by adding dibucaine to the multilamellar dispersions. However, we found that the peaks showing the quadrupole splitting of 32.1 kHz (labeled by an asterisk in spectrum c) did not change on the addition of dibucaine; the peaks were later found to be assignable to the C2 methylene deuterons by comparison with the ^{2}H-NMR spectrum for palmitic 2,2-d_2 acid. For all methylene and methyl group deuterons, the spectrum observed at pH 7.2 was broader than that at pH 5.2. Thus it is conceivable that the order of the acyl chains of lipids under the acidic condition is lower than that under the neutral condition. The quadrupole splittings of 5.91, 20.5, and 26.6 kHz can be assigned to the terminal methyl (C16), methylene groups at 15- (C15), and 14- (C14) positions, respectively; the corresponding splittings under the acidic condition were 4.95, 17.9, and 23.8 kHz, respectively.

To obtain an order parameter for a specific carbon segment in the acyl chain, some palmitic acids that were deuterated at the specific methylene groups were incorporated into the lipid mixture. Fig. 9, *a* and *b*, shows ^{2}H-NMR spectra of palmitic-2,2-d_2 acid in the lipid mixture with and without dibucaine, respectively. A quadrupole splitting of 32.1 kHz was observed when the lipid mixture contained no dibucaine. However, notably, this quadrupole splitting scarcely changed, even when the lipid mixture contained dibucaine. A similar observation was noted for the probe under acidic conditions; a quadrupole splitting of 31.0 kHz was decreased very slightly (30.6 kHz) by the addition of dibucaine. In contrast, for example, as shown in Fig. 10, *a*

TABLE 1 Moment data for the dibucaine-d_1-mixed lipids (PC:PS:PE = 1:1:2.5 molar ratio) and dibucaine-d_1-mixed lipids-cholesterol (total lipids:cholesterol = 7:3 molar ratio) systems

Membrane	pH	Temperature	$M_1 \times 10^{-4}$(rad/s)	$M_2 \times 10^{-8}$(rad^2/s^2)	$S_{CD} \times 10^2$	Δ_2
Mixed lipids	7.4	300	1.16	2.51	3.56	0.379
		279	0.715	1.40	2.19	1.02
	5.8	300	1.03	1.64	3.16	0.141
		279	0.904	1.51	2.77	0.364
Mixed lipids + cholesterol	7.4	300	0.333	0.282	1.02	0.887
		279	0.707	1.14	2.17	0.689
	5.8	300	0.366	0.542	1.12	2.00
		279	0.955	1.69	2.93	0.375

TABLE 2 Moment data for the dibucaine-d_9-mixed lipids (PC:PS:PE = 1:1:2.5 molar ratio) and dibucaine-d_9-mixed lipids-cholesterol (total lipids:cholesterol = 7:3 molar ratio) systems

Membrane	pH	Temperature	$M_1 \times 10^{-4}$(rad/s)	$M_2 \times 10^{-8}$(rad^2/s^2)	$S_{CD} \times 10^2$	Δ_2
Mixed lipids	7.4	300	1.02	2.08	3.37	0.477
		279	1.20	3.02	3.96	0.558
	4.8	300	1.13	2.73	3.74	0.579
		279	1.22	2.71	4.02	0.360
Mixed lipids + cholesterol	7.4	300	1.55	4.18	5.11	0.293
		279	1.98	6.57	6.53	0.242
	4.6	300	1.44	3.70	4.76	0.322
		279	1.96	6.55	6.47	0.264

and *b*, dibucaine decreased the quadrupole splitting (39.2 kHz) in the palmitic-9,9-d_2 acid probe appreciably (37.1 kHz). Thus it seems that under both neutral and acidic conditions, dibucaine is situated in a position that does not so much affect the order parameter of the methylene groups near the polar region, but affects appreciably that of the methylene groups at an intermediate region of the acyl chain. Interestingly, Boulanger et al. (1981) have reported the same sort of finding in a tetracaine-deuterated PC dispersions system; they found that tetracaine affects only slightly the quarupole splittings of the deuterons at position 2 in the deuterated PC dispersions. They ascribed this finding to the location of the benzenoid group around the acyl chain position 2. To further understand the effect of dibucaine on the quadrupole splittings throughout the acyl chain methylene groups, we have observed ^{2}H-NMR spectra for the probes deuterated at C3, C4, C6, and C10. All of the observed quadrupole splittings at pH 7.2 and pH 5.2, with and without dibucaine, are summarized in Table 3.

Order parameter profiles for palmitic acids intercalated into the lipid mixture containing cholesterol

To discuss differences in the order among chain segments, we have calculated an order parameter (S_{CD}) for each carbon number of the palmitic acid probe, according to Eq. 4 (Stockton et al., 1976). The S_{CD} values for the neutral and acidic solutions, calculated by assuming the static e^2qQ/h value of 170 kHz (Stockton et al., 1976), are summarized in Table 3. To focus our attention on the changes in S_{CD} caused by 3 mM dibucaine (ΔS_{CD}) (Fig. 11), we plotted the ratios $\Delta S_{CD}/S_{CD}$ against carbon numbers. ΔS_{CD} represents the amount of decrease in the S_{CD} value caused by dibucaine. It appears that under both neutral and acidic condi-

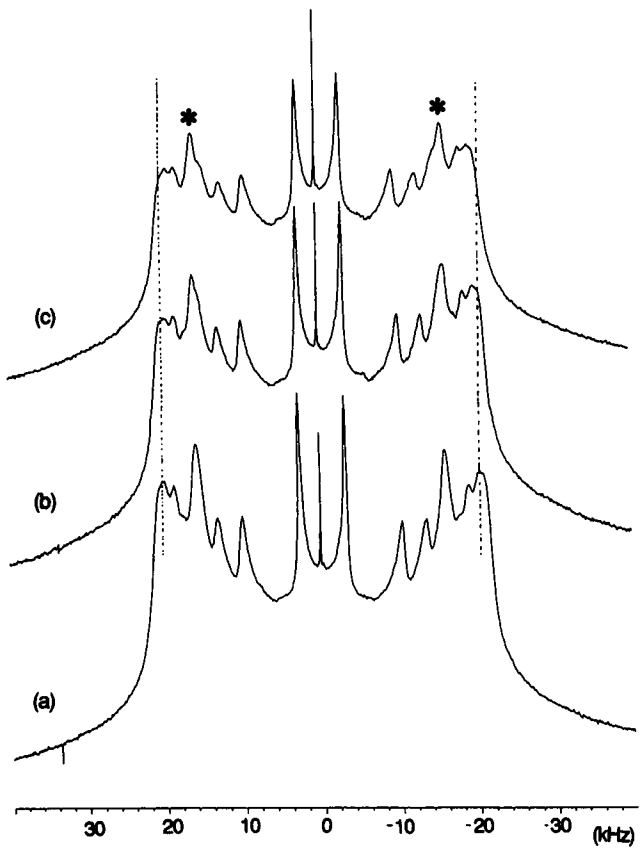

FIGURE 8 ^{2}H-NMR spectra of palmitic-d_{31} acid (20 mM) in mixed lipid (PC:PS:PE = 1:1:2.5 molar ratio, 60 mM) solution with cholesterol (total lipids:cholesterol = 7:3 molar ratio) at pH 7.2 and at 300 K. (*a*) Without dibucaine. (*b*) With dibucaine (1 mM). (*c*) With dibucaine (3 mM).

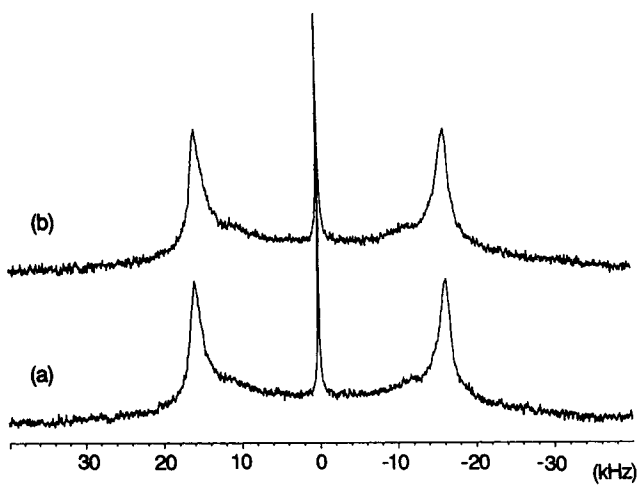

FIGURE 9 ^{2}H-NMR spectra of palmitic-2,2-d_2 acid (20 mM) in mixed lipid (PC:PS:PE = 1:1:2.5 molar ratio, 60 mM) solution with cholesterol (total lipids:cholesterol = 7:3 molar ratio) at pH 7.2 and at 300 K. (*a*) Without dibucaine. (*b*) With dibucaine (3 mM).

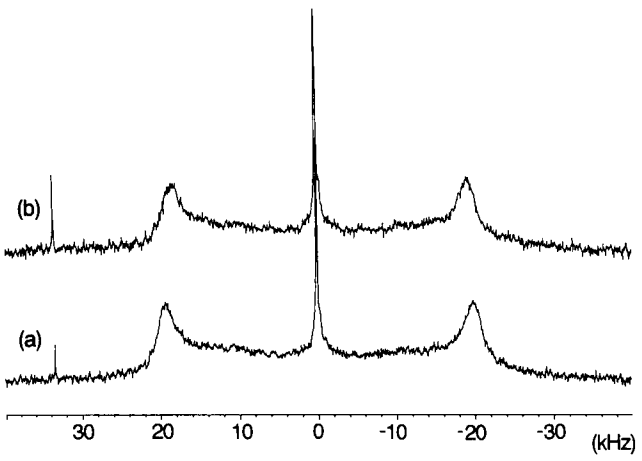

FIGURE 10 ^{2}H-NMR spectra of palmitic-9,9-d_2 acid (20 mM) in mixed lipids (PC:PS:PE = 1:1:2.5 molar ratio, 60 mM) with cholesterol (total lipids:cholesterol = 7:3 molar ratio) at pH 7.2 and at 300 K. (*a*) Without dibucaine. (*b*) With dibucaine (3 mM).

tions, the ratios monotonously increase from C2 to C10, and then gradually increase or remain approximately constant from C10 to the terminal methyl group, C16. Moreover, we notice that the ratio for the acidic solution is larger than that at the corresponding carbon number for the neutral solution. To confirm whether this finding is due to a larger amount of dibucaine partitioning into the lipid mixture under acidic than under neutral conditions, we have measured the partition coefficient K_p of dibucaine for the lipid mixture, which involves cholesterol and palmitic acid. The results are summarized in Table 4; the K_p value of 1108 at pH 7.2 compares favorably with that reported by Papahadjopoulos et al. (1975) for the lipid composition of PS/cholesterol (K_p = 1150). Evidently, the partition coefficient at pH 7.2 was greater than that at pH 5.2. Thus we should invoke another reason for the differences in the ratio. Interestingly, Boulanger et al. (1981) have reported a similar finding for tetracaine interacting with multilamellar dispersions of specifically deuterated phosphatidylcholine, namely that the charged tetracaine has a greater disordering effect on the phospholipid chain region and perturbs to a greater extent the dimensions of this region than does the uncharged one. The pK_a value of dibucaine reported is 8.0 (Kuroda et al., 1994) or 8.5 (Ritchie and Greengard, 1961). Furthermore, it is considered that the pK_a value of the tertiary amino group of the membrane bound drug would decrease by approximately 1 pK_a unit from the corresponding value in the aqueous phase (Lee, 1978). Accordingly, at pH 7.2, about half of the dibucaine molecules can be considered to be in an uncharged molecular form, whereas at pH 5.2 all of the dibucaine molecules are expected to exist as a cationic form. Consequently, the differences in the ratio $\Delta S_{CD}/S_{CD}$ between pH 7.2 and pH 5.2 should also be ascribed to the changes in the relative amount of uncharged versus charged species. Changes in the location due to protonation and/or electrostatic interactions between cationic drugs and lipid headgroups may be reasons for the finding in Fig. 11; in the Discussion, however, we favor only the latter view.

^{1}H-NMR spectra of dibucaine and Na^+ channel inactivation gate peptide (MP-1) in a phosphate buffer

To investigate a plausible interaction between dibucaine and MP-1, especially the expected π-stacking interaction (Hunter, 1993; Hunter and Sanders, 1990; Jorgensen and Severance, 1990) between the quinoline ring of dibucaine and the phenyl group of Phe, we have measured ^{1}H-NMR spectra of dibucaine in the absence and in the presence of MP-1. As a control experiment, we also measured ^{1}H-NMR spectra of dibucaine in the presence of MP-2, in which the Phe has been substituted by Gln. Fig. 12, *a*, *b*, and *c*, shows, respectively, ^{1}H-NMR spectra of MP-1 (3 mM), dibucaine (3 mM), and dibucaine (3 mM)-MP-1 (3 mM) solutions in a phosphate buffer at pH 7.4. In spectrum a the peaks in a range of 7.2–7.4 ppm are due to aromatic ring protons of Phe, and the other peaks are due to amide protons of the peptide. In spectrum b the quinoline ring proton resonances are assignable from high to low frequency, respectively, to the 5, 8, 7, 6, and 3 positions. As can be seen in spectrum c, it is evident that the quinoline ring protons at the 3 and 8 positions shifted appreciably to a lower frequency. Fig. 13, *a* and *b*, shows ^{1}H-NMR spectra of dibucaine (3 mM) and dibucaine (3 mM)-MP-2 (3 mM) solutions in a phosphate buffer at pH 7.4. Interestingly, in contrast to the observations in Fig. 12, no appreciable chemical shift changes were noted for the proton resonances at the 3 and 8 positions. In Fig. 14, we have schematically summarized observed changes in chemical shifts (in units of hertz at 600 MHz) of dibucaine protons as a result of interaction with MP-1 and MP-2. As can be seen in Fig. 14, both MP-1 and MP-2 shifted the resonances due to polar side-chain moiety (protons a–d) of dibucaine to a higher frequency. These high-frequency shifts can be ascribed to the electrostatic interaction between the positively charged quaternary ammonium group of dibucaine and the negatively charged carboxyl group of Asp, Glu, or the C-terminus. On the other hand, MP-1 shifted the resonances due to the butoxy group (protons f, g, and h) and all of the quinoline ring protons (protons 3, 5, 6, 7, and 8) to a lower frequency. These low-frequency shifts can be considered to be due to the ring-current effect (Pople et al., 1959) originating from the π-stacking interactions between the quinoline ring of dibucaine and the phenyl group of Phe in MP-1, because no such chemical shift changes were caused by the presence of MP-2 in the solution.

^{1}H-NMR spectra of dibucaine and a Na^+ channel inactivation gate peptide in phosphatidylserine liposomes

To determine whether the dibucaine molecules that are binding with lipids can interact with the Na^+ channel inac-

TABLE 3 Observed quadrupole splittings ($\Delta\nu_q$) and order parameters (S_{CD})* for deuterated palmitic acid probes intercalated in mixed lipids with cholesterol at pH 7.2 and 5.2

	pH 7.2				pH 5.2			
	Dibucaine 0 mM		Dibucaine 3 mM		Dibucaine 0 mM		Dibucaine 3 mM	
Chain segment	$\Delta\nu_q$(kHz)	S_{CD}	$\Delta\nu_q$(kHz)	S_{CD}	$\Delta\nu_q$(kHz)	S_{CD}	$\Delta\nu_q$(kHz)	S_{CD}
2	32.1	0.252	32.0	0.251	31.0	0.243	30.6	0.240
3	32.5	0.255	31.9	0.250	33.6	0.264	33.3	0.261
4	36.3	0.285	35.5	0.278	36.3	0.285	35.3	0.277
6	39.2	0.307	37.5	0.294	39.4	0.309	37.0	0.290
9	39.2	0.307	37.1	0.291	38.6	0.303	35.8	0.281
10	37.6	0.295	35.4	0.278	36.1	0.283	31.8	0.249
14#	26.6	0.209	24.9	0.195	23.8	0.187	20.9	0.164
15#	20.5	0.161	18.9	0.148	17.9	0.140	15.8	0.124
16#	5.91	0.046	5.49	0.043	4.95	0.039	4.38	0.034

*Calculated from the observed quadrupole splittings by using Eq. 4.
#Taken from the palmitic-d_{31} acid probe.

tivation gate peptide, we have observed ^{1}H-NMR spectra of dibucaine and the peptide in sonicated PS liposomes prepared in deuterated phosphate buffer at pH 7.4. Fig. 15, *a*, *b*, and *c*, shows, respectively, ^{1}H-NMR spectra of dibucaine (3 mM), dibucaine (3 mM)-MP-2 (3 mM), and dibucaine (3 mM)-MP-1 (3 mM) in the PS liposomes. As expected, the quinoline ring proton resonances of dibucaine appeared as broad resonances, reflecting interactions with the PS liposomes; the resonance at the 5 position shifted to a higher frequency, whereas all of the remaining resonances shifted to a lower frequency as compared to the corresponding chemical shift positions in the phosphate buffer solution. It is worth noting that all of the quinoline proton resonances shifted to a lower frequency, even on the addition of MP-2 (spectrum b). However, more remarkable is the fact that MP-1 caused a larger low-frequency shift to those resonances than did MP-2 (spectrum c). In Fig. 16 we show the changes in chemical shifts of quinoline ring proton resonances schematically. The reason for the changes in chemical shift on the addition of MP-2 is not clear at present; this should be explained together with the changes in chemical shift of those resonances as a result of interaction with PS liposomes. However, undoubtedly the larger low-frequency shift caused by MP-1 compared to that caused by MP-2 should be ascribed to the π-stacking interaction with the phenyl group of Phe. Moreover, we notice that the chemical shift differences between resonances in spectrum c and spectrum b are two to six times larger than observed changes in chemical shifts on the addition of MP-1 in the phosphate buffer (Fig. 14). This finding indicates that π-stacking interaction is enhanced when dibucaine molecules simultaneously interact with both lipids and MP-1.

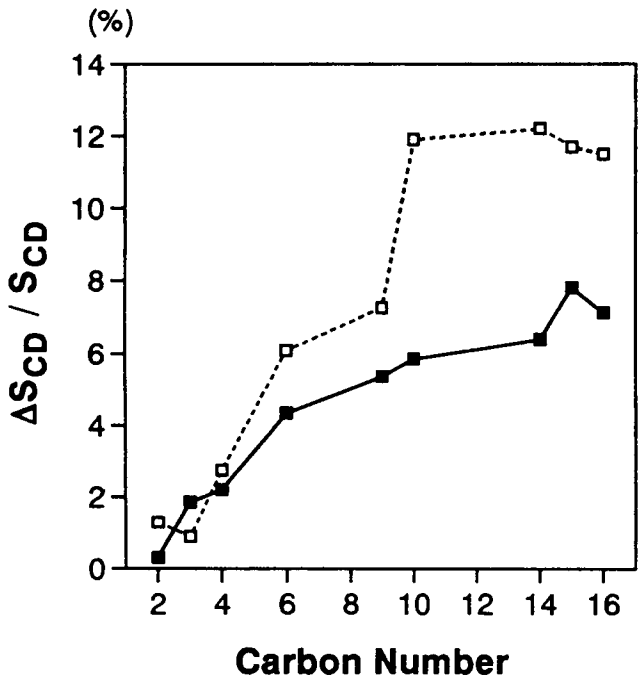

FIGURE 11 Plot of $\Delta S_{CD}/S_{CD}$ against positions of deuteration for specifically deuterated palmitic acid probes intercalated in mixed lipids with cholesterol at pH 7.2 (■) and pH 5.2 (□). ΔS_{CD} represents the amount of decrease in the S_{CD} value caused by 3 mM dibucaine.

^{1}H-NMR spectra of the phenyl group protons of phenylalanine in MP-1

Fig. 17, *a* and *b*, shows ^{1}H-NMR spectra of the phenyl group protons of Phe, in the absence and in the presence of dibucaine in the phosphate buffer, respectively. The spin-coupled peaks at around 7.36, 7.32, and 7.27 ppm are due to

TABLE 4 Partition coefficients of dibucaine in the bilayer of lipid mixture* with cholesterol# and palmitic acid§ at pH 7.2 and 5.2

	K_p	
Lipid	pH 7.2	pH 5.2
PC:PS:PE	1108	557.1

*PC:PS:PE = 1:1:2.5 molar ratio.
#Total lipids:cholesterol = 7:3 molar ratio.
§Total lipids:palmitic acid = 3:1 molar ratio.

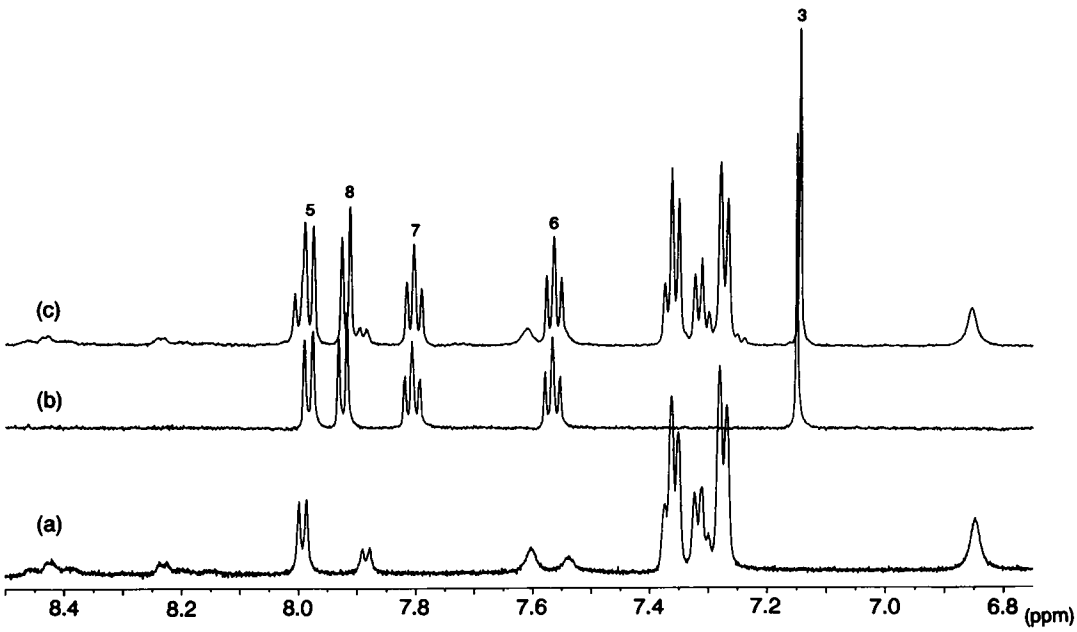

FIGURE 12 (*a*) ^{1}H-NMR spectrum of MP-1 (3 mM) in a phosphate buffer at pH 7.4. (*b*) ^{1}H-NMR spectrum of dibucaine (3 m) in a phosphate buffer at pH 7.4. (*c*) ^{1}H-NMR spectrum of dibucaine (3 mM)-MP-1 (3 mM) solution in a phosphate bufffer at pH 7.4. Assignments for the quinoline ring proton resonances are given on top of the spectrum (*c*).

meta, *para*, and *ortho* protons, respectively. It was found that dibucane shifted the phenyl group resonances very slightly to a lower frequency (0.4 Hz in *meta*, 0.0 Hz in *para*, and 1.3 Hz in *ortho* protons). Fig. 17, *c* and *d*, shows ^{1}H-NMR spectra of the phenyl group protons of Phe of MP-1 in PS liposomes. In Fig. 17 *c*, the chemical shifts of the phenyl group protons were nearly identical to those of the corresponding resonances in the phosphate buffer (Fig. 17 *a*), suggesting that the phenyl group of Phe locates at a region that does not receive any shielding or deshielding effect from the PS liposomes. However, in the presence of dibucaine, we noticed that in contrast to the large low-frequency shift of the quinoline ring proton resonances of dibucaine, the phenyl group resonances of MP-1 shifted slightly to a higher frequency (4.6 Hz in *meta* and *para* protons, and 5.0 Hz in *ortho* protons). This result indicates that the phenyl group protons are situated in positions with respect to the quinoline ring that receive deshielding field due to the ring current effect from the quinoline ring. According to the theory for the aromatic ring-current effect (Pople, 1959), this relative arrangement of the two aromatic rings can be considered to be such that the ring protons of Phe locate at the side of the quinoline ring of dibucaine and that the quinoline ring protons locate on the plane of the phenyl group of Phe. Although we cannot imagine the interaction model that satisfies these situations for all of the

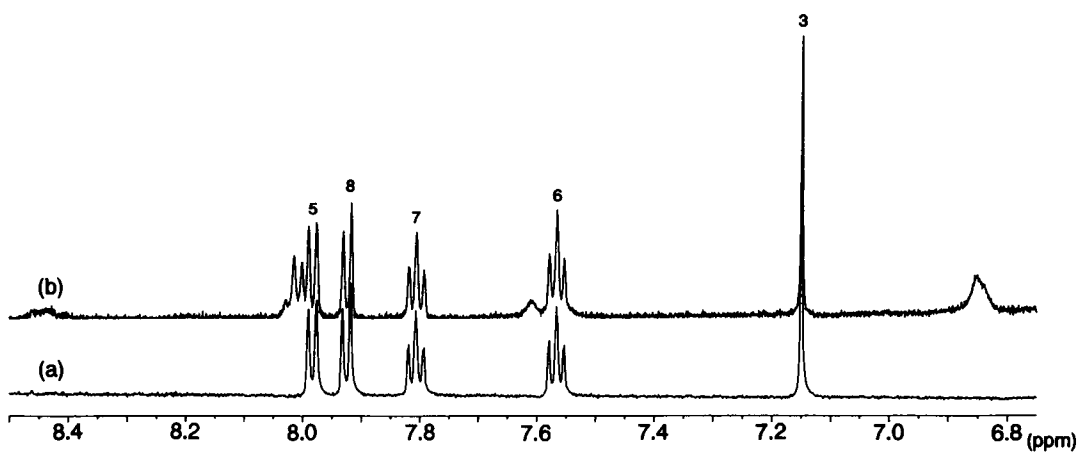

FIGURE 13 (*a*) ^{1}H-NMR spectrum of dibucaine (3 mM) in a phosphate buffer at pH 7.4. (*b*) ^{1}H-NMR spectrum of dibucaine (3 mM)-MP-2 (3 mM) solution in a phosphate buffer at pH 7.4. Assignments for the quinoline ring proton resonances are given on top of the spectrum (*b*).

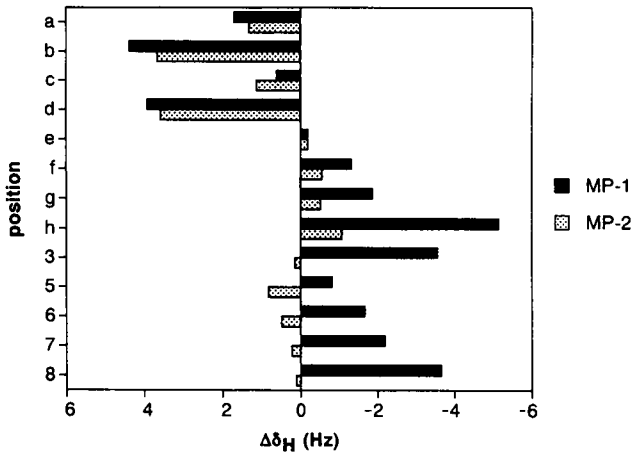

FIGURE 14 Changes in chemical shifts (in units of hertz at 600 MHz) of dibucaine as a result of interaction with MP-1 and MP-2 in a phosphate buffer at pH 7.4.

ring protons within one relative arrangement of the two aromatic rings, an edge-on or T-shaped geometry for the two π-stacking aromatic rings seems to be a reasonable one (Hunter and Sanders, 1990; Jorgensen and Severance, 1990). Taking rule 6 of Hunter et al. into account (Hunter and Sanders, 1990), the quinoline ring can be considered to be a "π-deficient atom" and the phenyl group to be a "π-rich atom"; this finding agrees well with the a priori expected result when we consider the differences in the substituents on the aromatic rings.

DISCUSSION

Locations of dibucaine in model membranes

In the present study the location of dibucaine in model membranes can be deduced by noting the following points: 1) changes in the quadrupole splittings of dibucaine-d_1 and dibucaine-d_9 by cholesterol, by temperature, and by pH; 2) changes in the quadrupole splittings of deuterated palmitic acids by dibucaine. In conclusion, the quinoline ring of dibucaine locates at a polar headgroup region, penetrating the butoxy group between the acyl chains of the lipids. In Fig. 18 we show this situation with computer graphics. The lipid demonstrated here is dipalmitoylphosphatidylserine, and its molecular structure is drawn tentatively, fully extending the acyl chains. The structure of cholesterol in egg phosphatidylcholine bilayers is well investigated by x-ray (Franks, 1976) and neutron diffraction (Worcester and Franks, 1976) studies. Thus, in Fig. 18, the cholesterol was so positioned as to locate its 3β-hydroxyl group in the vicinity of the acyl ester linkage or glycerol backbone, spanning the polycyclic ring up to the first 8 to 10 methylene segments of the acyl chains, and extending the side chain up to about the same depth in the bilayer (Stockton and Smith, 1976). The dibucaine is presented as taking a face-to-face π-stacked dimer, because it has been shown that dibucaine can exist as an aggregate in the lipid bilayer (Wakita et al., 1992; Kuroda et al., 1994). One of the most important results for deducing the location of dibucaine is that the quadrupole splitting of dibucaine-d_1 dramatically decreased when the multilamellar dispersions included cholesterol, whereas those of dibucaine-d_9 increased by including cholesterol. At first sight, the former observation appears to be explicable by changes in the membrane-aqueous partitioning of the anesthetic by cholesterol. In fact, it is reported that the partition coefficient of tetracaine between dimyristoylphosphatidylcholine and a buffer at pH 9.5 decreases from 200 to 110 by including a 7:3 molar ratio of cholesterol; at pH 5.5 it decreases from 21 to 8 (Auger et al., 1988). However, the same reasoning cannot be applied to the finding for the latter dibucaine-d_9 data, because the quadrupole splittings of methylene and methyl groups were increased by cholesterol. Because it is known that cholesterol induces a high degree of order for the acyl chains of phospholipids, it might be expected that if both the deuteron at the 3 position of the quinoline ring and the deuterons of the butoxy group that is attached to the 2 position locate inside the glycerol backbone, the magnitude of their quadrupole splittings in both dibucaine-d_1 and dibucaine-d_9 spectra would increase, reflecting the increased degree of order caused by cholesterol molecules. However, this was not the case; only the butoxy group deuterons showed increased quadrupole splittings by cholesterol. To settle this problem, it is inevitable that the carbon atom of the 3 position of the quinoline ring will be located near the acyl ester linkage or glycerol backbone, causing the butoxy chain to be approximately parallel to the acyl chains, as shown in Fig. 18. For this binding situation, we can ascribe the observed dramatic reduction in the quadrupole splitting of dibucaine-d_1 caused by cholesterol to the decreased order of the C-D vector at the 3 position; in fact, this was clearly reflected in the increased Δ_2 value shown in Table 1. The decrease in order of the C-D vector at the 3 position seems to arise from variations in the orientation of the quinoline ring plane relative to the bilayer normal. Order parameter profiles for palmitic acid probes shown in Fig. 11 give support to the deduced location of dibucaine. We might expect that if such a bulky group as a quinoline ring were located in the middle of the acyl chain, the relevant part would become rigid and result in an increased degree of order. However, this was not the case, because the S_{CD} values of all of the monitored chain segments were decreased by the addition of dibucaine. This implies that the bulky quinoline ring is located at the polar headgroup region and not between the hydrophobic acyl chains. This applies to both neutral and acidic conditions employed in this study. Under neutral conditions, about half of the dibucaine molecules can be considered to exist in an uncharged molecular form, whereas in the acidic condition, all of the dibucaine molecules exist in a cationic form. Boulanger et al. (1981) have shown that the positively charged tetracaine at low pH mostly locates at the PC headgroup level, whereas the uncharged form at high pH is intercalated partly in the

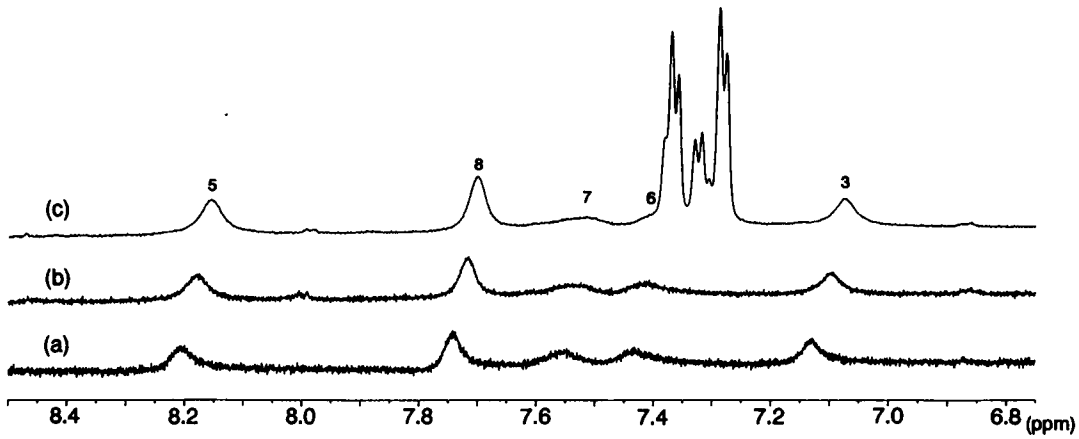

FIGURE 15 (*a*) ^{1}H-NMR spectrum of dibucaine (3 mM) in sonicated PS liposomes prepared in deuterated phosphate buffer at pH 7.4. (*b*) ^{1}H-NMR spectrum of dibucaine (3 mM)-MP-2 (3 mM) in the sonicated PS liposomes. (*c*) ^{1}H-NMR spectrum of dibucaine (3 mM)-MP-1 (3 mM) in the PS liposomes. Assignments for the quinoline proton resonances are given on top of the spectrum (*c*).

headgroup region and partly in the fatty acyl chains of the PC bilayers. The somewhat deeper binding of the neutral tetracaine is also pointed out by the fluorescence quenching experiment (Sikaris and Sawyer, 1982); the drug is oriented in the bilayer so that its aromatic amine is near carbon 9 of the fatty acyl chain of PC bilayers. For the PS bilayers, it is also concluded that the charged and uncharged tetracaine occupy different sites in the PS bilayer (Kelusky et al., 1986). In the present data, however, we find no definite reason to assume that the molecular form of dibucaine and its protonated form locate at different depths in the bilayer, at least to the precision of setting the carbon atom at the 3 position, based on the reasoning above. The differences in the ratio $\Delta S_{CD}/S_{CD}$ noted between pH 7.2 and pH 5.2 can be explained by the electrostatic interaction between the charged dibucaine and the polar headgroup; hence we need not invoke the changes in location of dibucaine upon protonation. The quadrupole splittings of both dibucaine-d_1 and dibucaine-d_9 are always smaller at the acidic condition than at the neutral condition. This can be explained by the increased freedom of movement of dibucaine at the polar headgroup region caused by the electrostatic interaction, which separates lipid molecules from each other and results in loose packing.

From the conclusions drawn about the location of dibucaine in model membranes, we may be allowed to conclude that all local anesthetic molecules reside at the polar headgroup region of the membranes, which is capable of interacting simultaneously with the hydrophobic amino acid

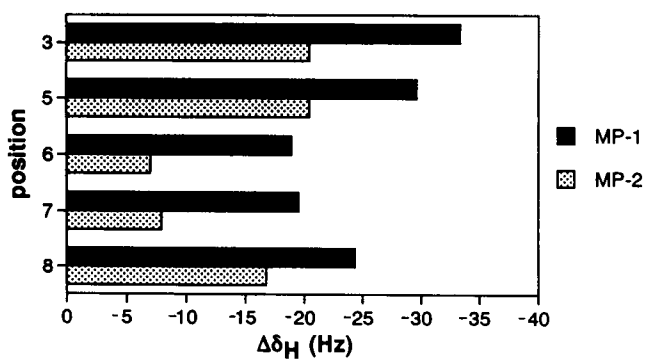

FIGURE 16 Changes in chemical shifts (in units of Hz at 600 MHz) of the quinoline proton resonances of dibucaine as a result of the interaction with MP-1 and MP-2 in the PS liposomes.

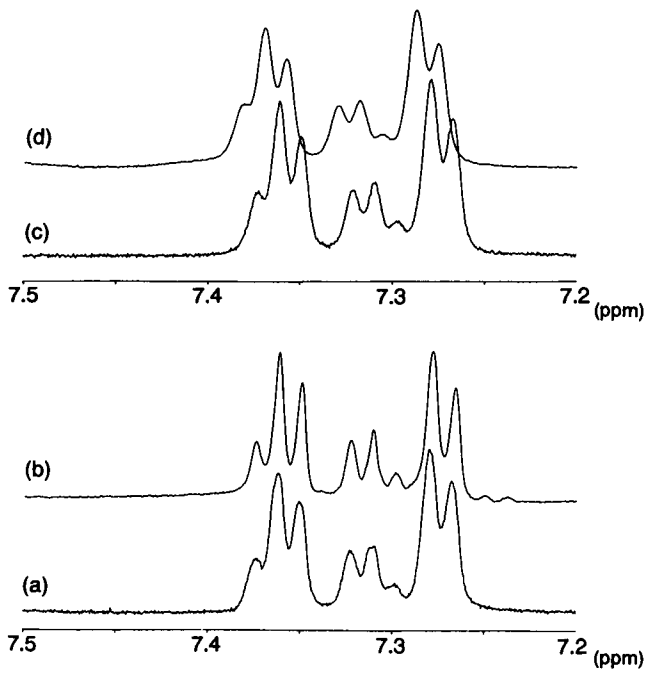

FIGURE 17 (*a*) ^{1}H-NMR spectrum of the phenyl group protons of MP-1 (3 mM) in a phosphate buffer at pH 7.4. (*b*) ^{1}H-NMR spectrum of the phenyl group protons of dibucaine (3 mM)-MP-1 (3 mM) solution in a phosphate buffer at pH 7.4. (*c*) ^{1}H-NMR spectrum of the phenyl group protons of MP-1 in the PS liposomes at pH 7.4. (*d*) ^{1}H-NMR spectrum of the phenyl group protons of dibucaine (3 mM)-MP-1 (3 mM) solution in the PS liposomes at pH 7.4.

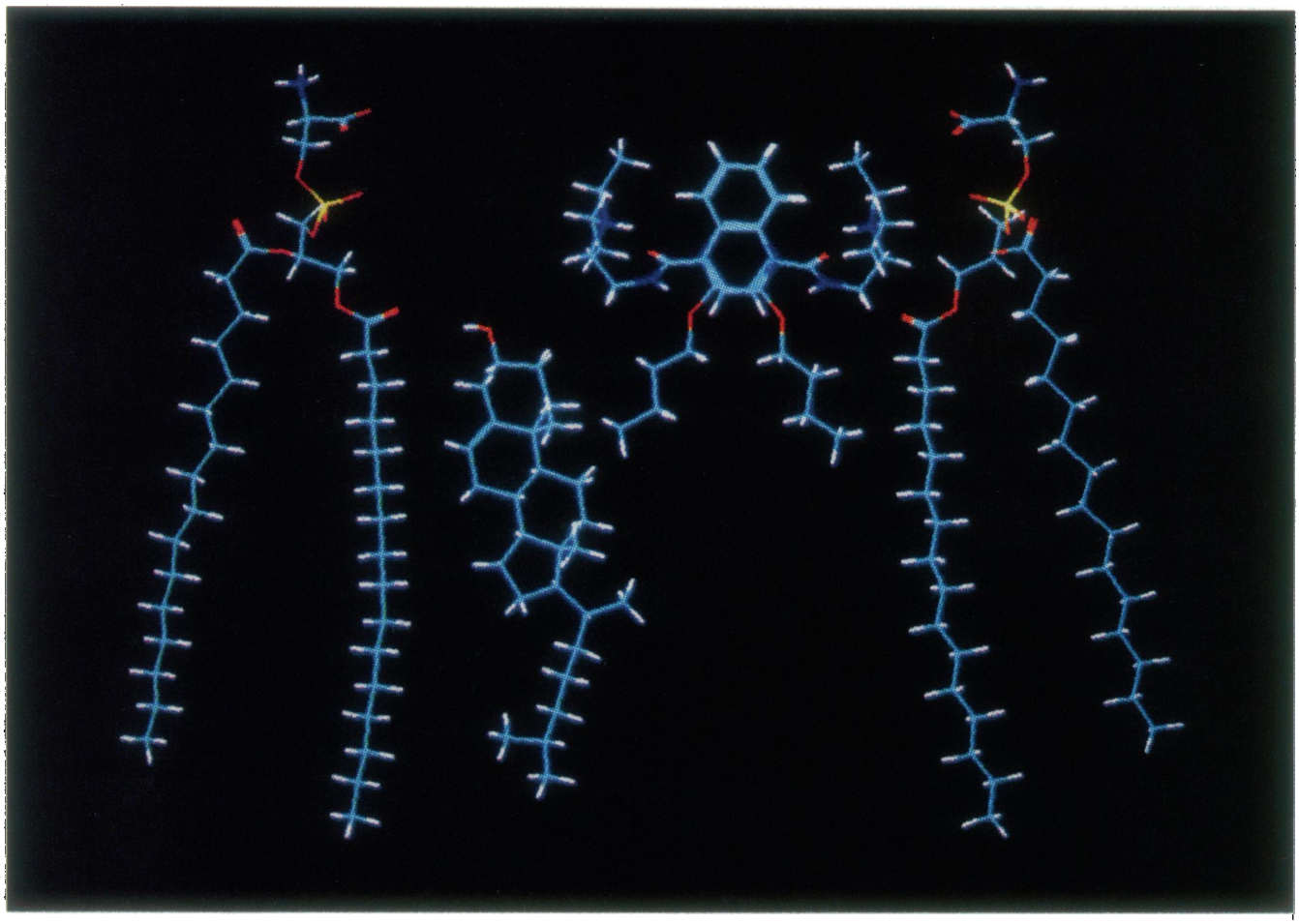

FIGURE 18 Computer graphics view of relative arrangements among local anesthetic dibucaine, cholesterol, and dipalmitoylphosphatidylserine. The dibucaine is presented as a face-to-face π-stacked dimer.

residues at the intracellular linker part (Fig. 2). Interestingly, Wang et al. have shown that there is a large hydrophobic region within the Na^+ channel pore in its cytoplasmic side, which accepts up to 18 methylene groups of the quaternary ammonium compounds; the hydrophobic domain also interacts with local anesthetics such as cocaine and mepivacaine (Wang et al., 1991). It seems very reasonable to assume that the hydrophobic domain is formed by the lipids surrounding the channel protein. Moreover, the lipids are not always required to be organized as a bilayer, but they can exist as a hexagonal phase (Dibble and Feigenson, 1994; Tournois et al., 1987), implying that the drug, especially its aromatic ring, can assume any convenient orientation to accommodate the three hydrophobic amino acids of the intracellular linker.

Molecular mechanisms of local anesthesia

Local anesthetics are chemicals that reversibly block action potentials in excitable membranes (Strichartz and Ritchie, 1987). They are usually composed of an aromatic ring, an intermediate chain including an amide or an ester linkage, and a tertiary amine nitrogen. They are amphiphilic in nature and can exist as either cationic or uncharged molecular forms, depending on the pK_a of the drugs and pH of the medium. As for the molecular mechanisms of anesthesia, it is generally established that tertiary amine-type local anesthetics penetrate into the nerve sheath and nerve membrane with their uncharged forms and bind to the Na^+ channel at the axoplasmic side of nerve membranes with their cationic forms (Narahashi et al., 1970). However, benzocaine, which lacks the ionizable tertiary amine at physiological pH, is also known to be a local anesthetic drug. Thus there still remain many controversial questions about the molecuar mechanisms of local anesthesia: What is the site of anesthetic action? Is the site within lipids or within the Na^+ channel protein? How many sites are there? Do both cationic and molecular forms of the drug bind at the same site? etc. In attempting to solve these problems, we noticed the publications on Na^+ channel inactivation gate (Patton et al., 1992; West et al., 1992), because it has already been shown that local anesthetics promote the channel to close its inactivation gate, stabilizing the inactivated state (Hille, 1977; Postma and Catterall, 1984). Accordingly, we supposed that

local anesthetics directly interact with the inactivation gate, especially with its phenylalanine residue in the three clustered hydrophobic amino acids of Ile-Phe-Met (Fig. 2), because the phenylalanine residue is playing an essential role in Na^+ channel inactivation (West et al., 1992). This assumption was also suggested to us by the fact that all local anesthetics essentially bear an aromatic ring in their structures. We believe that the aromatic ring might be a prerequisite group, not only for conferring lipophilicity on a drug that allows it to pass through membranes, but also for π-stacked binding with the phenyl group of the phenylalanine residue. Thus we studied the interaction of local anesthetic dibucaine and the Na^+ channel inactivation gate peptides MP-1 and MP-2. One of the most serious concerns, however, was whether dibucaine in a solution and in a lipid bilayer membrane really interacts with the peptide at its hydrophobic moieties. The present data shown in Figs. 14 and 16 clearly removed this concern. In addition, it was found that the presence of lipids enhances the interaction. This finding suggests firm evidence regarding the molecular mechanisms of local anesthesia that the drug residing at the polar headgroup region of the so-called boundary lipids in the vicinity of the Na^+ channel pore binds with the clustered hydrophobic amino acids, especially with the phenylalanine residue and results in stabilization of the inactivated state. The drug binding with the phenylalanine residue may also be facilitated by electrostatic interactions of its protonated nitrogen with the negatively charged amino acids on both sides of the IFM domain. Interestingly, Sheldon et al. have shown in their structure-activity relationship study on the lidocaine derivatives that there is an optimal distance between the aromatic ring and the tertiary amine nitrogen for binding with the Na^+ channel (Sheldon et al., 1991). Now we can assign the optimal length to a range of distances between the phenyl group of the phenylalanine residue and the negative charge on the carboxyl group of the aspartic acid, or between two glutamic acid residues that are situated in positions before and after the IFM domain, respectively (Fig. 2). Postma and Catterall (1984) and Wang et al. (1994) have shown from their Hill coefficient data that one local anesthetic drug binds with one inactivated channel. This finding lends further support to our view that a local anesthetic drug binds to the IFM domain in the manner described above. In Fig. 19 we show schematically the inter-

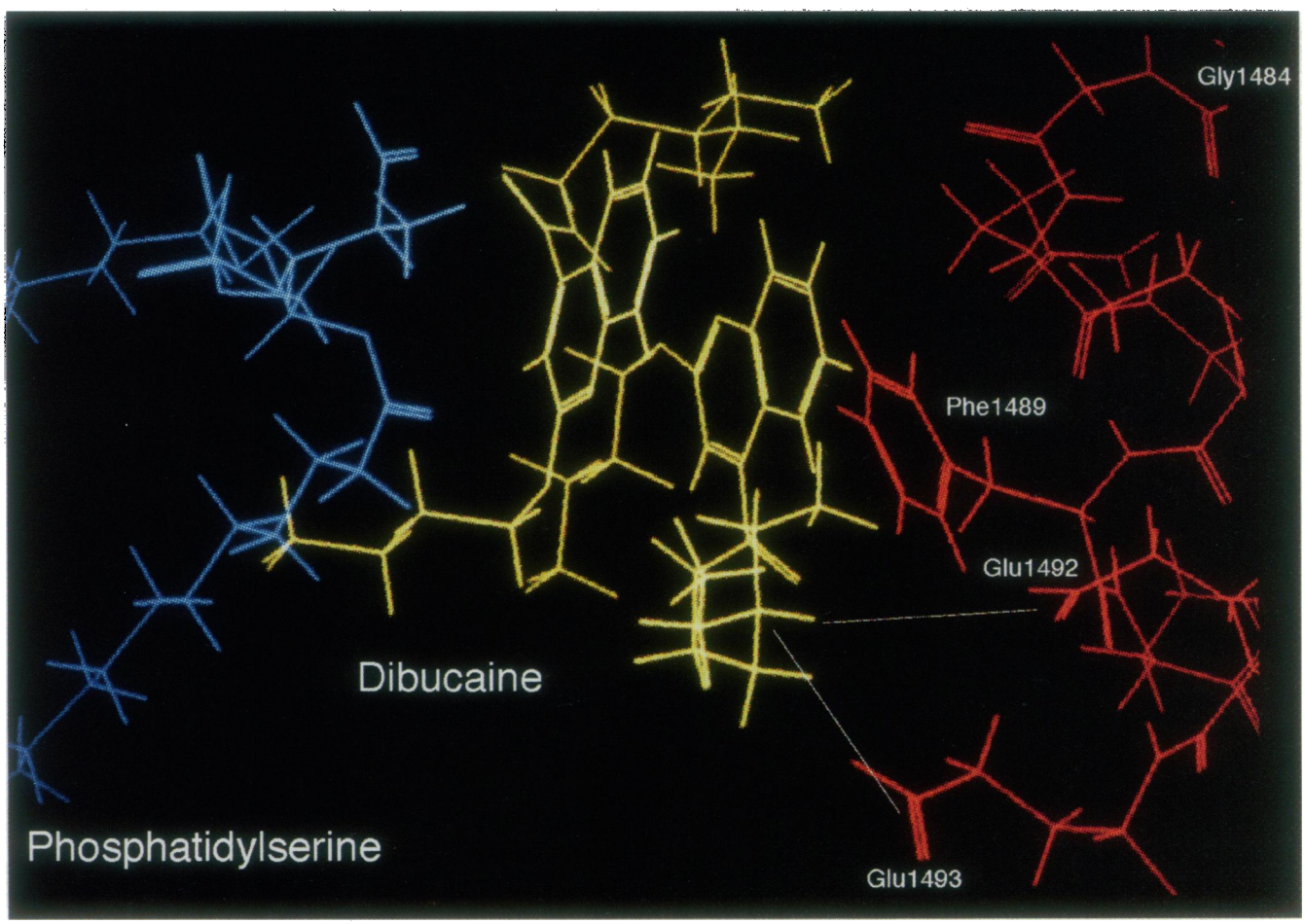

FIGURE 19 Computer graphics view for showing schematically the interaction between dibucaine, which resides at the polar headgroup region of phosphatidylserine, and the inactivation gate peptide. The dibucaine is shown as a face-to-face π-stacked dimer.

action between dibucaine and the inactivation gate peptide; one dibucaine dimer located at the polar headgroup region of phosphatidylserine, π-stacking face-to-face with the other, also interacts with the phenyl group of Phe-1489 by π-stacking interaction with a T-shaped geometry, allowing its protonated quaternary nitrogen to interact electrostatically with the negatively charged carboxyl groups of two adjacent glutamic acid residues of Glu-1492 and Glu-1493.

Finally, it should be added that Ragsdale et al. (1994) have recently assigned the Phe-1764 residue in the sixth segment of domain IV (Fig. 1) of rat brain type IIA Na^+ channel to a plausible site of action of the local anesthetic etidocaine. The Phe-1764 locates in the intermediate region of the transmembrane helix and plays an important role in fast inactivation of the Na^+ channel (McPhee et al., 1995). Interestingly, such a binding situation is reminiscent of the binding of local anesthetic agents acting as noncompetitive channel blockers for the nicotinic acetylcholine receptor (Giraudat et al., 1987; Charnet et al., 1990), in the sense that local anesthetic molecules can interact directly with transmembrane helices that form the lumen of the channel pore. More recently, on the other hand, Bennett et al. (1995) have indicated the importance of the IFM domain in the III-IV linker for the lidocaine block of the human cardiac Na^+ channel. This result strongly supports our conclusion. Evidently more work is required to enlighten us about the molecular mechanisms of local anesthesia.

REFERENCES

Auger, M., H. C. Jarrell, and I. C. P. Smith. 1988. Interactions of the local anesthetic tetracaine with membranes containing phosphatidylcholine and cholesterol: a ^{2}H-NMR study. *Biochemistry.* 27:4660–4667.

Bennett, P. B., C. Valenzuela, L.-Q. Chen, and R. G. Kallen. 1995. On the molecular nature of the lidocaine receptor of cardiac Na^+ channels. Modification of block by alterations in the α-subunit III-IV interdomain. *Circ. Res.* 77:584–592.

Bloom, M., J. H. Davis, and F. W. Dahlquist. 1978. Determination of orientational order in bilayer systems using moments of deuterium magnetic resonance spectra. *In* XXth Ampere Congress Proceedings, Tallinn, Estonia. E. Kundla, E. Lippmann, and T. Saluvere, editors. Springer-Verlag, New York. 551.

Bloom, M., and O. G. Mouritsen. 1988. The evolution of membranes. *Can. J. Chem.* 66:706–712.

Boulanger, Y., S. Schreier, and I. C. P. Smith. 1981. Molecular details of anesthetic-lipid interaction as seen by deuterium and phosphorus-31 nuclear magnetic resonance. *Biochemistry.* 20:6824–6830.

Burnett, L. J., and B. H. Muller. 1971. Deuteron quadrupole coupling constants in three solid deuterated paraffin hydrocarbons: C_2D_6, C_4D_{10}, C_6D_{14}. *J. Chem. Phys.* 55:5829–5831.

Chacko, G. K., G. M. Villegas, F. V. Barnola, R. Villegas, and D. E. Goldman. 1976. The polypeptide and the phospholipid components of axon plasma membranes. *Biochim. Biophys. Acta.* 443:19–32.

Charnet, P., C. Labarca, R. J. Leonard, N. J. Vogelaar, L. Czyzyk, A. Gouin, N. Davidson, and H. A. Lester. 1990. An open-channel blocker interacts with adjacent turns of α-helices in the nicotinic acetylcholine receptor. *Neuron.* 2:87–95.

Courtney, K. R., and G. R. Strichartz. 1987. Structural elements which determine local anesthetic activity. *In* Local Anesthetics. G. R. Strichartz, editor. Springer-Verlag, New York. 53–94.

Covino, B. G. 1987. Local anesthetic agents. *In* Practical Anesthetic Pharmacology. R. R. Attia, A. W. Grogono, and F. R. Domer, editors. Appleton-Century-Crofts, East Norwalk, CT. 55–77.

Davis, J. H., and K. R. Jeffrey. 1977. The temperature dependence of chain disorder in potassium palmitate-water. A deuterium NMR study. *Chem. Phys. Lipids.* 20:87–104.

Davis, J. H., K. R. Jeffrey, M. Bloom, M. I. Valic, and T. P. Higgs. 1976. Quadrupolar echo deuteron magnetic resonance spectroscopy in ordered hydrocarbon chains. *Chem. Phys. Lett.* 42:390–394.

Davis, J. H., B. Maraviglia, G. Weeks, and D. V. Godin. 1979. Bilayer rigidity of the erythrocyte membrane. ^{2}H-NMR of a perdeuterated palmitic acid probe. *Biochim. Biophys. Acta.* 550:362–366.

Dibble, A. R. G., and G. W. Feigenson. 1994. Detection of coexisting fluid phospholipid phases by equilibrium Ca^{2+} binding: peptide-poor L_α and peptide-rich H_{II} phase coexistence in gramicidin A′/phospholipid dispersions. *Biochemistry.* 33:12945–12953.

Franks, N. P. 1976. Structural analysis of hydrated egg lecithin and cholesterol bilayers. I. X-ray diffraction. *J. Mol. Biol.* 100:345–358.

Giraudat, J., M. Dennis, T. Heidmann, P.-Y. Haumont, F. Lederer, and J.-P. Changeux. 1987. Structure of the high-affinity binding site for noncompetitive blockers of the acetylcholine receptor: [^{3}H]chlorpromazine labels homologous residues in the β and δ chains. *Biochemistry.* 26:2410–2418.

Greaves, W. S., R. P. Linstead, B. R. Shephard, S. L. S. Thomas, and B. C. L. Weedon. 1950. Anodic syntheses. Part I. New syntheses of stearic, myristic, and other acids. *J. Chem. Soc.* 3326–3330.

Hille, B. 1977. Local anesthetics: hydrophilic and hydrophobic pathways for the drug-receptor reaction. *J. Gen. Physiol.* 69:497–515.

Hunter, C. A. 1993. Arene-arene interactions: electrostatic or charge transfer? *Angew. Chem. Int. Ed. Engl.* 32:1584–1586.

Hunter, C. A., and J. K. M. Sanders. 1990. The nature of π-π interactions. *J. Am. Chem. Soc.* 112:5525–5534.

Jorgensen, W. L., and D. L. Severance. 1990. Aromatic-aromatic interactions: free energy profiles for the benzene dimer in water, chloroform, and liquid benzene. *J. Am. Chem. Soc.* 112:4768–4774.

Kelusky, E. C., Y. Boulanger, S. Schreier, and I. C. P. Smith. 1986. A ^{2}H-NMR study on the interactions of the local anesthetic tetracaine with membranes containing phosphatidylserine. *Biochim. Biophys. Acta.* 856: 85–90.

Kuroda, Y., M. Wakita, and T. Nakagawa. 1994. Interaction between dibucaine and pig erythrocyte membranes as studied by NOESY experiments in ^{1}H-NMR spectroscopy. Which form of dibucaine interacts more strongly, cationic or uncharged? *Chem. Pharm. Bull.* 42: 2418–2425.

Lee, A. G. 1978. Effects of charged drugs on the phase transition temperatures of phospholipid bilayers. *Biochim. Biophys. Acta.* 514:95–104.

Maraviglia, B., J. H. Davis, M. Bloom, J. Westerman, and K. W. A. Wirtz. 1982. Human erythrocyte membranes are fluid down to −5°C. *Biochim. Biophys. Acta.* 686:137–140.

McDonald, R. S., and P. A. Wilks, Jr. 1988. JCAMP-DX: a standard form for exchange of infrared spectra in computer readable form. *Appl. Spectrosc.* 42:151–162.

McPhee, J. C., D. S. Ragsdale, T. Scheuer, and W. A. Catterall. 1995. A critical role for transmembrane segment IVS6 of the sodium channel α subunit in fast inactivation. *J. Biol. Chem.* 270:12025–12034.

Miescher, K. 1932. Studien über Lokalanasthetica. *Helv. Chim. Acta.* 15:163–190.

Narahashi, T., D. Frazier, and M. Yamada. 1970. The site of action and active form of local anesthetics. I. Theory and pH experiments with tertiary compounds. *J. Pharmacol. Exp. Ther.* 171:32–51.

Nichol, C. P., J. H. Davis, G. Weeks, and M. Bloom. 1980. Quantitative study of the fluidity of *Escherichia coli* membranes using deuterium magnetic resonance. *Biochemistry.* 19:451–457.

Noda, M., T. Ikeda, T. Kayano, H. Suzuki, H. Takeshima, M. Kurasaki, H. Takahashi, and S. Numa. 1986. Existence of distinct sodium channel messenger RNAs in rat brain. *Nature.* 320:188–192.

Oldfield, E., M. Meadows, D. Rice, and R. Jacobs. 1978. Spectroscopic studies of specifically deuterium labeled membrane systems. Nuclear magnetic resonance investigation of the effects of cholesterol in model systems. *Biochemistry.* 14:2727–2740.

Op den Kamp, J. A. F. 1979. Lipid asymmetry in membranes. *Annu. Rev. Biochem.* 48:47–71.

Papahadjopoulos, D., K. Jacobson, G. Poste, and G. Shepherd. 1975. Effects of local anesthetics on membrane properties. I. Changes in the fluidity of phospholipid bilayers. *Biochim. Biophys. Acta.* 394:504–519.

Patton, D. E., J. W. West, W. A. Catterall, and A. L. Goldin. 1992. Amino acid residues required for fast Na^+-channel inactivation: charge neutralizations and deletions in the III-IV linker. *Proc. Natl. Acad. Sci. USA.* 89:10905–10909.

Pauls, K. P., A. L. MacKay, and M. Bloom. 1983. Deuterium nuclear magnetic resonance study of the effects of palmitic acid on dipalmitoylphosphatidylcholine bilayers. *Biochemistry.* 22:6101–6109.

Pople, J. A., W. G. Schneider, and H. J. Bernstein. 1959. High-Resolution Nuclear Magnetic Resonance. McGraw-Hill, New York. 180–183.

Postma, S. W., and W. A. Catterall. 1984. Inhibition of binding of [^{3}H]batrachotoxinin A 20-α-benzoate to sodium channels by local anesthetics. *Mol. Pharmacol.* 25:219–227.

Ragsdale, D. S., J. C. McPhee, T. Scheuer, and W. A. Catterall. 1994. Molecular determinants of state-dependent block of Na^+ channels by local anesthetics. *Science.* 265:1724–1728.

Ritchie, J. M., and N. M. Greene. 1985. Local anesthetics. *In* The Pharmacological Basis of Therapeutics. A. G. Gilman, L. S. Goodman, T. W. Rall, and F. Murad, editors. Macmillan, New York. 302–321.

Ritchie, J. M., and P. Greengard. 1961. On the active structure of local anesthetics. *J. Pharmacol. Exp. Ther.* 133:241–245.

Seelig, J. 1977. Deuterium magnetic resonance: theory and application to lipid membranes. *Q. Rev. Biophys.* 10:353–418.

Sheldon, R. S., R. J. Hill, M. Taouis, and L. M. Wilson. 1991. Aminoalkyl structural requirements for interaction of lidocaine with the class I antiarrhythmic drug receptor on rat cardiac myocytes. *Mol. Pharmacol.* 39:609–614.

Sikaris, K. A., and W. H. Sawyer. 1982. The interaction of local anaesthetics with synthetic phospholipid bilayers. *Biochem. Pharmacol.* 16: 2625–2631.

Stockton, G. W., C. F. Polnaszek, L. C. Leitch, A. P. Tulloch, and I. C. P. Smith. 1974. A study of mobility and order in model membranes using ^{2}H-NMR relaxation rates and quadrupole splittings of specifically deuterated lipids. *Biochem. Biophys. Res. Commun.* 60:844–850.

Stockton, G. W., C. F. Polnaszek, A. P. Tulloch, F. Hasan, and I. C. P. Smith. 1976. Molecular motion and order in single-bilayer vesicles and multilamellar dispersions of egg lecithin and lecithin-cholesterol mixtures. A deuterium nuclear magnetic resonance study of specifically labeled lipids. *Biochemistry.* 15:954–966.

Stockton, G. W., and I. C. P. Smith. 1976. A deuterium nuclear magnetic resonance study of the condensing effect of cholesterol on egg phosphatidylcholine bilayer membranes. I. Perdeuterated fatty acid probes. *Chem. Phys. Lipids.* 17:251–263.

Strichartz, G. R., and J. M. Ritchie. 1987. The action of local anesthetics on ion channels of excitable tissues. *In* Local Anesthetics. G. R. Strichartz, editor. Springer-Verlag, New York. 21–52.

Tournois, H., J. Leunissen-Bijvelt, C. W. M. Haest, J. de Gier, and B. de Kruijff. 1987. Gramicidin-induced hexagonal H_{II} phase formation in erythrocyte membranes. *Biochemistry.* 26:6613–6621.

Trudell, J. R. 1977. A unitary theory of anesthesia based on lateral phase separations in nerve membranes. *Anesthesiology.* 46:5–10.

Trudell, J. R. 1980. Biophysical concepts in molecular mechanisms of anesthesia. *Prog. Anesthesiol.* 2:261–270.

Vogt, T. C. B., J. A. Killian, and B. de Kruijff. 1994. Structure and dynamics of the acyl chain of a transmembrane polypeptide. *Biochemistry.* 33:2063–2070.

Wakita, M., Y. Kuroda, Y. Fujiwara, and T. Nakagawa. 1992. Conformations of dibucaine and tetracaine in small unilamellar phosphatidylcholine vesicles as studied by nuclear Overhauser effects in ^{1}H nuclear magnetic resonance spectroscopy. *Chem. Phys. Lipids.* 62:45–54.

Wang, G. K., W. M. Mok, and S.-Y. Wang. 1994. Charged tetracaine as an inactivation enhancer in batrachotoxin-modified Na^+ channels. *Biophys. J.* 67:1851–1860.

Wang, G. K., R. Simon, and S.-Y. Wang. 1991. Quaternary ammonium compounds as structural probes of single batrachotoxin-activated Na^+ channels. *J. Gen. Physiol.* 98:1005–1024.

West, J. W., D. E. Patton, T. Scheuer, Y. Wang, A. L. Goldin, and W. A. Catterall. 1992. A cluster of hydrophobic amino acid residues required for fast Na^+-channel inactivation. *Proc. Natl. Acad. Sci. USA.* 89: 10910–10914.

Westman, J., Y. Boulanger, A. Ehrenberg, and I. C. P. Smith. 1982. Charge and pH dependent drug binding to model membranes. A. ^{2}H-NMR and light absorption study. *Biochim. Biophys. Acta.* 685:315–328.

Worcester, D. L., and N. P. Franks. 1976. Structural analysis of hydrated egg lecithin and cholesterol bilayers. II. Neutron diffraction. *J. Mol. Biol.* 100:359–378.

Zamponi, G. W., and R. J. French. 1994. Amine blockers of the cytoplasmic mouth of sodium channels: a small structural change can abolish voltage dependence. *Biophys. J.* 67:1015–1027.