J. Membrane Biol. 177, 117–128 (2000)
DOI: 10.1007/s002320001105

The Journal of
Membrane
Biology

Orientations and Locations of Local Anesthetics Benzocaine and Butamben in Phospholipid Membranes as Studied by ^{2}H NMR Spectroscopy

Y. Kuroda[1], H. Nasu[1], Y. Fujiwara[2], T. Nakagawa[1]
[1]Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, 606-8501, Japan
[2]Kyoto Pharmaceutical University, Kyoto, 607-8414, Japan

Received: 22 March 2000/Revised: 20 June 2000

Abstract. This paper presents experimental evidence that an aromatic compound that has a quadrupole moment locates in a polar headgroup region in the lipid membranes, but not in a membrane interior hydrophobic region, and discusses correlation to the site of action of benzocaine and butamben on sodium channels. The ^{2}H NMR spectra of benzocaine-d_4, benzocaine-d_5, butamben-d_4, and butamben-d_9 in the model membranes were observed. The ^{2}H NMR spectra of perdeuterated palmitic acid and selectively deuterated palmitic acids at C2, C3, C5, C6, C9, or C10, which were inserted into the lipid membranes, were also observed. The phosphatidylserine (PS), phosphatidylcholine (PC), and liquid mixtures composed of PS, PC, and phosphatidylethanolamine (PE), which contain or do not contain cholesterol, were employed. A moment analysis was applied to the ^{2}H NMR spectra of palmitic-d_{31} acid. An order parameter, S_{CD}, for each carbon segment was calculated from the observed quadruple splitting. We concluded that in the lipid mixture containing cholesterol, the aromatic rings of benzocaine and butamben locate around the glycerol moiety of the lipids and that when there exists no cholesterol, they locate a little more inside from the headgroup region, directing, in both cases, their amino groups upward (polar region) and the ethyl and butyl groups downward (hydrophobic region). These data cast a question on the site of action of the neutral local anesthetics in the sodium channels.

Key words: Sodium channels — Local anesthetics — Benzocaine — Butamben — Phospholipid membranes — ^{2}H NMR

Correspondence to: Y. Kuroda

Introduction

Local anesthetics are compounds that block the propagation of action potentials by acting on the sodium (Na) channels in excitable membranes (Ritchie & Greene, 1985; Strichartz & Ritchie, 1987: Courtney & Strichartz, 1987). Most of the clinically useful local anesthetics are composed of an aromatic ring, a tertiary amine nitrogen, and an amide or an ester linkage that joins the aromatic ring and the tertiary amine nitrogen moiety. These amine-type local anesthetics can more or less be ionized at physiological pH depending on their pKa and the environmental pH. Benzocaine and its butyl homologue, butamben, are clinically useful anesthetics. They exist as neutral molecules at physiological pH, since in addition to the aromatic ring, they possess a primary amine nitrogen (pKa = 2.5–2.6) instead of a tertiary amine nitrogen. The molecular mechanisms of the action of these local anesthetics on the Na channels remain unclear. The Hille's modulated receptor hypothesis states that: (i) all the local anesthetics including charged and uncharged amine-type anesthetics, and neutral-type anesthetics share a single binding site, (ii) lipid-soluble drugs come and go from the receptor via a hydrophobic region of the membrane, while charged drugs pass via a hydrophilic region, and (iii) the receptor changes its configuration and the resulting binding affinity against the drug (Hille, 1977). The local anesthetic binding site for at least charged amine-type anesthetics is thought to be on the cytoplasmic side of the Na channel protein α subunit (Narahashi, Frazier & Yamada, 1970). The Na channel protein α subunit is composed of four homologous domains, I–IV, and each domain consists of six transmembrane segments, S1–S6 (Numa & Noda, 1986; Catterall, 1995; Marban, Yamagishi & Tomaselli, 1998). By employing etidocaine as an example for the amine-type local anesthetics, Ragsdale et al. (1994) have shown

that amino acids, F1764 and Y1771, which locate near the center of the transmembrane helix in domain IV-segment 6 (IVS6) of the rat brain type IIA Na channel α subunit, are determinants of the local anesthetic binding site. The driving forces of binding seem to be cation (drug)-π orbital (F1764) (Dougherty, 1996) and π orbital (drug)-π orbital (Y1771) stacking (Hunter & Sanders, 1990) interactions. However, it is not yet settled whether uncharged drugs, especially the neutral-type anesthetics as benzocaine and butamben, are binding at the same receptor site as the charged amine-type anesthetics. Recently, a notable paper has been published by Wang, Quan & Wang (1998) that prompted us to study the location of benzocaine in lipid bilayers. They showed for rat skeletal muscle μ1 Na channels that both benzocaine and etidocaine bind at the same site formed by including F1579 in IVS6 as a key determinant of the receptor; the F1579 is equivalent to F1764 in the rat brain type IIA Na channel. Taken together, F1764 (F1579) in IVS6 seems to be included to form a single local anesthetic receptor for the different drug types, agreeing well with Hille's modulated receptor hypothesis. We noticed that F1764 can be considered to take a position in the center of the transmembrane helix which is immersed in the lipid bilayers. Moreover, since the aqueous solubility of benzocaine is very low, that is, *ca.* 6 mM (Lalor, Flynn & Weiner, 1995), benzocaine should inevitably reside within the lipids or inside the hydrophobic pocket formed by some hydrophobic amino acid residues which may include F1764 (F1579). This hydrophobic pocket may also be immersed in lipids. Thus, it seems to be important to investigate the location of benzocaine in the lipid bilayers; if the lipids surrounding the transmembrane segment IVS6 are organized as bilayers, benzocaine should locate in the center of the bilayer to interact, at least directly, with F1764 (F1579). Arias et al. (1994) have shown that merocyanine 540 and benzocaine compete in binding to the nicotinic acetylcholine receptor membranes. They deduced the benzocaine binding site to be the region where the hydrophobic portion of the acyl chains meet the interfacial polar headgroup of the phospholipids. By intuition, we imagine the location of benzocaine to be in the center of the bilayers because of its low solubility with water and small size of the molecule. This is especially so for butamben because of its increased hydrophobicity of the butyl group. On the other hand, it is also expected to locate at the polar headgroup region as suggested by Arias et al. (1994). A benzene ring of benzocaine has no permanent dipole, but it has a quadruple moment that may require it to locate within such a relatively polar media as in a lipid headgroup region. In the present investigation, we elucidated the location of benzocaine and butamben in various lipid bilayers by ^{2}H NMR spectroscopy and proved that they locate in the lipid headgroup region in the mixed lipids composed of PC, PS and PE and also in the pure PS lipids; in pure PC lipids, they locate in a lipid interior region.

Materials and Methods

Materials

Benzocaine-d_4 (4-aminobenzoic-d_4 acid ethyl ester) and benzocaine-d_5 (4-aminobenzoic acid ethyl-d_5 ester) were synthesized according to the schemes shown in Fig. 1*a* and *b*, respectively; we followed the method described by Le Count and Reid and to convert toluene-d_8 to p-nitrotoluene-d_7 (Le Count & Reid, 1968). Butamben-d_4 (4-aminobenzoic-d_4 acid butyl ester) and butamben-d_9 (4-aminobenzoic acid butyl-d_9 ester) were synthesized using butanol (butanol-d_9) instead of ethanol (ethanol-d_5) in the corresponding schemes shown in Fig. 1. The percentages of deuteration of these anesthetics were checked by ^{1}H NMR spectra and found to be greater than 95%. Palmitic-d_{31} acid was obtained from MSD Isotopes (Montreal, Quebec, Canada). Palmitic-2,2-d_2 acid, palmitic-3,3-d_2 acid, and palmitic-5,5,6,6-d_4 acid were obtained from C/D/N Isotope (Vaudreuil, Quebec, Canada). Palmitic-9,9-d_2 acid and palmitic-10,10-d_2 acid were synthesized starting from a relevant α-deuterated fatty acid and a dicarboxylic monomethyl ester by a 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%. Egg yolk L-α-phosphatidylcholine (PC), bovine brain L-α-phosphatidylserine (PS), egg yolk L-α-phosphatidylethanolamine (PE), and cholesterol were purchased from Sigma and used without further purification. Deuterium-depleted water ($<5 \times 10^{-5}$ atom %D) was obtained from ISOTEC (Miamisburg, OH).

Lipid Suspensions Containing Deuterated Benzocaine or Butamben

The thin films of PC (130 mM), PS (130 mM), or the mixtures of PC, PS, and PE (PC:PS:PE = 1:1:2.5 molar ratio, 130 mM) with or without cholesterol (0 mM ~55.7 mM) were prepared by concentrating their chloroform/methanol solution with a rotary evaporator and then by vacuum evaporation overnight. A weighed amount of deuterated benzocaine (20 mM) or butamben (20 mM) was added to the chloroform/methanol solution before preparing the thin films. Their multilamellar dispersions were prepared by vigorously vortexing the round-bottomed flask containing the thin films and an isotonic (310 mOsm, 150 mM, pH 7.0) phosphate buffer in deuterium-depleted water for 30 min. To equilibrate the state of anesthetics interacting with the lipids, the multilamellar dispersions were subjected to five freeze-thaw-vortex cycles (Westman et al., 1982).

Lipid Suspensions Containing Deuterated Palmitic Acid

The PC (60 mM), PS (60 mM), or the mixtures of PC, PS, and PE (PC:PS:PE = 1:1:2.5, 60 mM) in a chloroform/methanol solution were mixed with cholesterol (25.7 mM) and deuterated palmitic acid (20 mM). A weighed amount of benzocaine (20 mM) was added to the

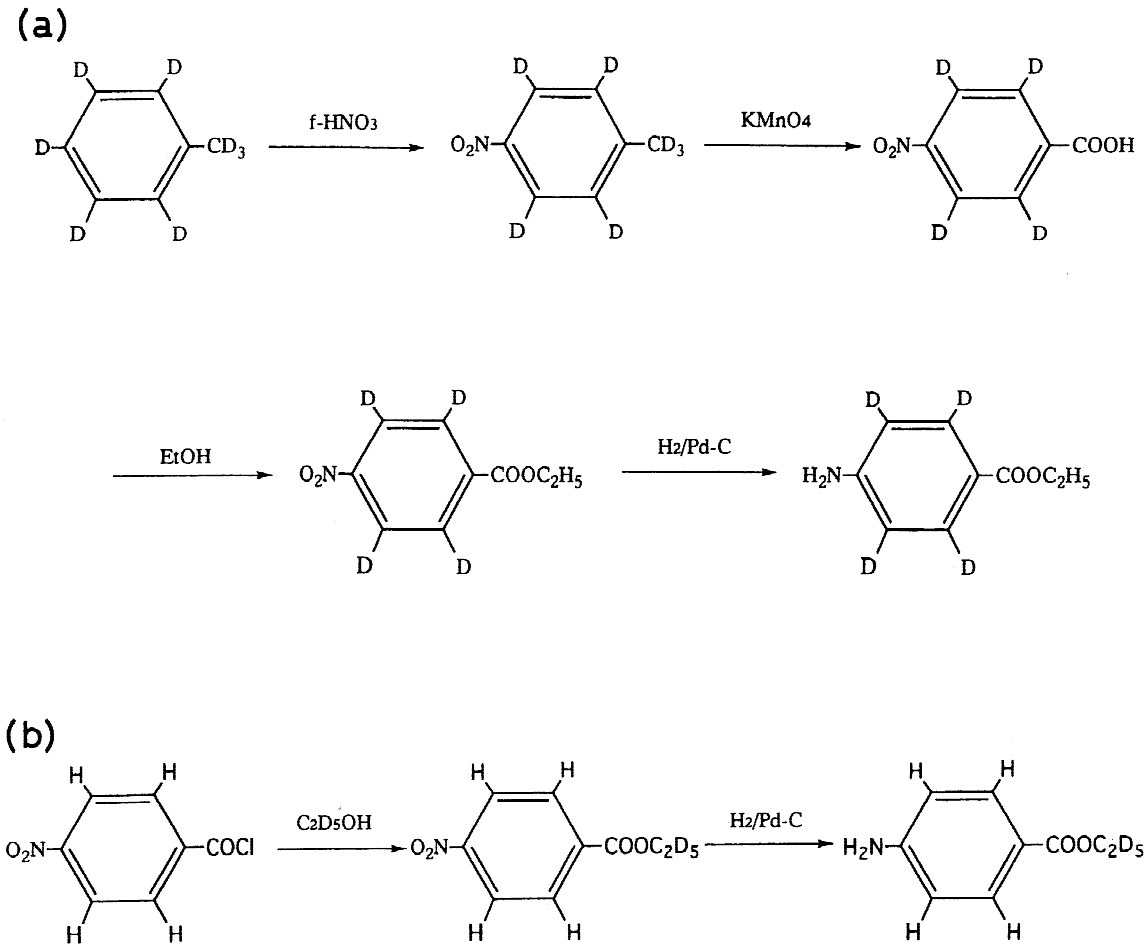

Fig. 1. Reaction scheme to synthesize (*a*) benzocaine-d_4 and (*b*) benzocaine-d_5. Butamben-d_4 and butamben-d_9 are synthesized similarly by using butanol and butanol-d_9 instead of ethanol and ethanol-d_5 in (*a*) and (*b*), respectively.

chloroform/methanol solution. The solvent was evaporated using a rotary evaporator to produce the thin films of the lipids. The thin films were dried in a vacuum overnight, dispersed into the deuterium-depleted water (310 mOsm, pH 7.0) by vortexing, and then subjected to five freeze-thaw-cycles.

Measurements

The ^{2}H NMR spectra of deuterated benzocaine and butamben were observed at 46.0 MHz on a Varian UNITYplus-300 spectrometer using a wideline nmr probe for a 5-mm sample tube. The quadrupole echo sequence was employed (Davis et al., 1976); $\pi/2 = 2.4$ μs and its pulse spacing was 50 μs. The observed spectral width was 100 kHz for 16K data points. The recycle time was 0.282 sec and the acquired number of transients was 130,000–180,000 (*ca.* 10–14 hr) for a sample solution containing 20 mM benzocaine or butamben. ^{2}H NMR spectra of deuterated palmitic acids were observed at 92.1 MHz on a Bruker AM-600 spectrometer using a broad band probe for a 5-mm sample tube. The quadrupole echo sequence was employed (Davis et al., 1976); $\pi/2 = 10.5$ μs and its pulse spacing was 50 μs. A typical observing spectral width was 100 kHz for 32K data points. The recycle time was 0.26 sec and the acquired number of transients was about 250,000 (*ca.* 20 hr) for a sample solution containing 20 mM deuterated palmitic acid. All the measurements were performed at 300 K. Moments of the ^{2}H NMR spectra (Bloom, Davis & Dahlquist, 1978; Davis et al., 1979; Nichol et al., 1980) for suspensions containing palmitic-d_{31} acid were calculated as described in our previous paper (Kuroda et al., 1996).

Results

^{2}H-NMR Spectra of Benzocaine-D_4

Figure 2*a* and *b* shows the ^{2}H NMR spectra of benzocaine-d_4-lipid mixture suspensions in the absence and presence of cholesterol, respectively. The composition of the lipid mixture (PC:PS:PE = 1:1:2.5 molar ratio) mimics that of the inside of erythrocyte membranes (Op den Kamp, 1979). The ^{2}H NMR spectra consist of two Pake patterns and one isotropic line. The isotropic line at around 0 kHz is due to the residual HO^{2}H resonance of the solvent and also due to small amounts of benzocaine-d_4 molecules tumbling isotropically in suspensions. In

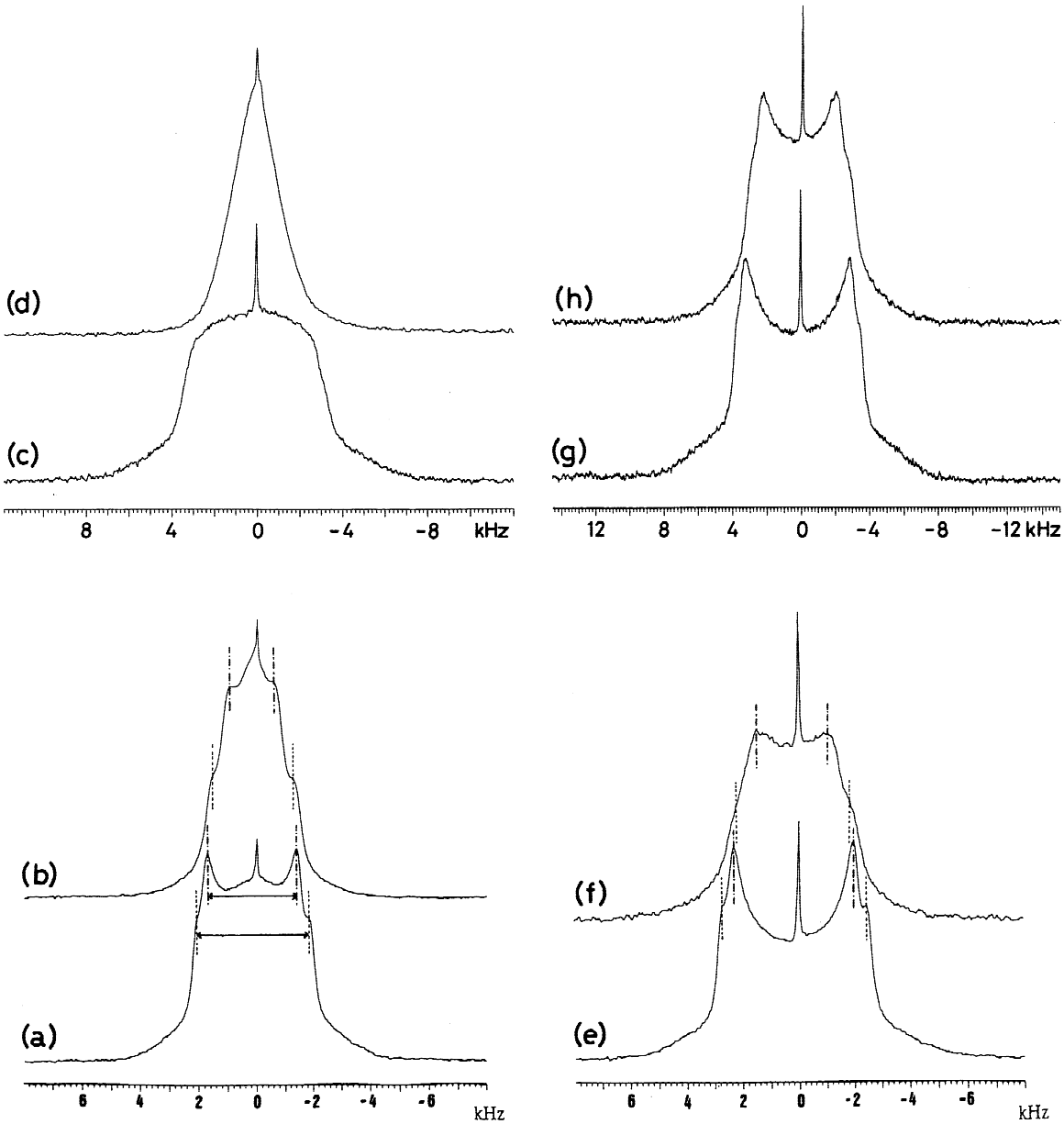

Fig. 2. ^{2}H-NMR spectra of benzocaine-d_4-lipid mixture suspensions (*a*) in the absence and (*b*) presence of cholesterol (55.7 mM); ^{2}H-NMR spectra of benzocaine-d_4-PS suspensions (*c*) in the absence and (*d*) presence of cholesterol (55.7 mM); ^{2}H-NMR spectra of butamben-d_4-lipid mixture suspensions (*e*) in the absence and (*f*) presence of cholesterol (55.7 mM); ^{2}H-NMR spectra of butamben-d_4-PS suspensions (*g*) in the absence and (*h*) presence of cholesterol (55.7 mM).

Fig. 2*a*, one Pake pattern has a quadrupolar splitting of 3.92 kHz and another one is 3.08 kHz. These quadrupolar splittings decreased to 2.80 and 1.60 kHz, respectively, when the lipid membrane contained cholesterol (Fig. 2*b*). The assignments of the two Pake patterns to the position of deuteration on the aromatic ring are at present unknown.

Figure 2*c* and *d* shows the ^{2}H NMR spectra of benzocaine-d_4-PS suspensions in the absence and presence of cholesterol, respectively. In contrast to the spectra in the lipid mixture, the ^{2}H NMR spectrum became broad and showed a humplike form (Fig. 2*c*) with an approximate half-height width of 6.7 kHz. This line width decreased (~2.5 kHz) when the membrane contained cholesterol (Fig. 2*d*) and is roughly comparable to that in the lipid mixture solution shown in Fig. 2b. It is suggested from the ^{2}H NMR data that the PS headgroup has a relatively rigid structure as compared with that of PC or

PE (Browning & Seelig, 1980). This rigid structure of the headgroup might have caused intermediate time scale motions of the anesthetic molecules and resulted in the humplike form in the spectral line shape. To mix PC and PE with PS is expected to soften the headgroup packing among PS molecules. Therefore, the change in the ^{2}H NMR spectral pattern from Fig. 2*a* to *c* suggests that the aromatic ring of benzocaine locates around the headgroups of the lipids. For multilamellar membranes formed by egg PC, X-ray diffraction (Franks, 1976), neutron diffraction (Worcester & Franks, 1976), and magic-angle-sample-spinning NMR (Villalain, 1996) data show that the hydroxyl group of cholesterol locates in close vicinity to the phospholipid ester carbonyl groups orienting its steroid ring parallel to the hydrocarbon chains of the membrane phospholipids. Because of this location, orientation and the polycyclic ring structure, cholesterol induces a high degree of order in the acyl chains of the liquid-crystalline phospholipid bilayers (Stockton & Smith, 1976; Sankaram & Thompson, 1990) and also the rapid axially symmetric reorientation (Vist & Davis, 1990; Weisz et al., 1992) of the acyl chains. The latter effect may be a result of the increased *trans* populations of the methylene chains and/or increased separations among the headgroups of lipids as a result of "a spacer effect" by the presence of cholesterol. The decreased quadrupole splittings or the decreased linewidths shown in Fig. 2*b* and *d* can thus be considered to be caused by this increased headgroup separation which may result in unordered binding and/or changes in the mean orientation of the aromatic rings of the benzocaine molecules that locate at the headgroup region.

The ^{2}H NMR spectra of benzocaine-d_4-PC suspensions were also obtained. The overall line width was narrower than those in the lipid mixture and PS. Observed quadrupole splittings were 2.11 and 1.14 kHz; these splittings were decreased by the presence of cholesterol to 1.49 and 0.75 kHz, respectively. All the observed quadrupole splittings of benzocaine-d_4 are summarized in Table 1.

Table 1. Observed quadrupole splittings ($\Delta\nu q$) of ^{2}H-NMR spectra of benzocaine-d_4 and butamben-d_4 in lipids suspensions

Lipid	Cholesterol (mM)	$\Delta\nu$q(kHz)	
Benzocaine-d_4			
Mixed lipids	0.0	3.08	3.92
	55.7	1.60	2.80
PS	0.0	6.70[a]	
	55.7	2.50[a]	
PC	0.0	1.14	2.11
	55.7	0.75	1.49
Butamben-d_4			
Mixed lipids	0.0	4.26	5.12
	55.7	2.50	3.91
PS	0.0	6.11	7.03
	55.7	4.20	5.58
PC	0.0	1.70	2.48
	55.7	1.23	2.34

[a] Half-height width.

^{2}H NMR Spectra of Butamben-D_4

Figure 2*e* and *f* shows the ^{2}H NMR spectra of the butamben-d_4-lipid mixture suspensions in the absence and presence of cholesterol, respectively. Two Pake patterns, as observed in benzocaine-d_4, were also observed in butamben-d_4, although their assignments to each deuteron in the aromatic ring are not clear. Their quadrupole splittings were 5.12 and 4.26 kHz in the absence of cholesterol (Fig. 2*e*) and 3.91 and 2.50 kHz in the presence of cholesterol (Fig. 2*f*). These quadrupole splittings were larger than the corresponding ones shown by benzocaine-d_4, suggesting a more ordered binding of the butamben molecule to the lipids. Evidently, this seems to be due to the increased hydrophobicity of the butyl group as compared with that of the ethyl group of benzocaine. The butyl group of butamben may be penetrating through the glycerol moiety of the lipids and locates along the acyl chains, thus firmly anchoring its aromatic ring. This interpretation was evidenced by the ^{2}H NMR spectra of butamben-d_9 presented below.

Figure 2*g* and *h* shows ^{2}H NMR spectra of butamben-d_4-PS suspensions in the absence and presence of cholesterol, respectively. In contrast to the ^{2}H NMR spectra of benzocaine, butamben gave clear axially symmetric powder patterns, indicating again the more ordered binding to the lipids than that of benzocaine, although two Pake patterns were not very clearly resolved. The Pake pattern with a wider quadrupole splitting (7.03 kHz) was barely detected as a shoulder of the more intense peak having a quadrupole splitting of 6.11 kHz. These quadrupole splittings were decreased by cholesterol to 5.58 and 4.20 kHz, respectively (Fig. 2*h*). Accordingly, the aromatic ring of butamben appears to also locate in the headgroup region of the lipids.

In PC membranes, butamben-d_4 showed the smallest quadrupole splittings of 2.48 and 1.70 kHz as compared with those shown in the lipid mixture and PS, as in the cases of benzocaine-d_4 (Table 1). Interestingly, these splittings were little affected by cholesterol; they decreased to 2.34 and 1.23 kHz, respectively. We can consider two possibilities for the location of benzocaine and butamben in PC. One possibility is that they stay in the headgroup region of the lipids, as in the cases of the lipid mixture and PS; we ascribe the small quadrupole splittings to the relatively unordered binding of these drugs to the headgroup moiety for some reason. Another possibility is that they penetrate more deeply through the glycerol moiety and reside in the acyl chains of the lipids; the small quadrupole splittings can easily be ascribed to their

relatively unconstrained motion within the acyl chain moiety. In conclusion, we wish to favor the latter location, at least for the PC without cholesterol, because benzocaine was found to increase the order of acyl chains of palmitic-d_{31} acid probes as will be described later.

All the observed quadrupole splittings of butamben-d_4 in the lipid mixture, PS, and PC suspensions are summarized in Table 1.

^{2}H-NMR Spectra of Benzocaine-D_5

Figure 3 shows the ^{2}H NMR spectra of benzocaine-d_5-lipid mixture suspensions with increasing amounts of cholesterol (*a–d*); *e, f, g,* and *h,* respectively, show only the ±3 kHz region and with reduced peak intensities as compared to those shown in *a, b, c,* and *d.* The ^{2}H NMR spectra consist of two Pake patterns and one isotropic peak. The Pake pattern with a larger quadrupole splitting (15.86 kHz, Fig. 3*a*) is evidently due to the CD_2 and that with a smaller one (1.08 kHz, Fig. 3*e*) to the CD_3. The observed quadrupole splittings are summarized in Table 2. Interestingly, the quadrupole splitting due to the CD_2 increased with increasing amounts of cholesterol, whereas that due to the CD_3 decreased. These results indicate that the mean orientation of the C(=CO)-O-CD_2-CD_3 chain in a lipid bilayer was changed by cholesterol. Benzocaine can thus be considered to be binding to lipids, directing its amino group upward (polar region) and ethyl group downward (hydrophobic region). This situation can be understood more clearly from the following results for butamben-d_9.

^{2}H NMR Spectra of Butamben-D_9

Figure 4 shows the ^{2}H NMR spectra of butamben-d_9-lipid mixture suspensions with increasing amounts of cholesterol (*a–d*). Evidently, butamben-d_9 showed four Pake patterns and one isotropic line, as expected. The observed quadrupole splittings are summarized in Table 3 together with those observed in PS and PC solutions. In contrast to the cases for benzocaine-d_5, the quadrupole splittings due to the CD_3 group increased with increasing amounts of cholesterol. This result clearly means that the terminal CD_3 in the butyl group of butamben is locating more deeply than that in the ethyl group of benzocaine, suffering from the ordering effect exerted from cholesterol. It is also noteworthy that the quadrupole splittings in the PC membranes were not very different from those in the lipid mixture or in PS, although the absolute magnitudes themselves were slightly smaller than those in the lipid mixture or in PS. This finding means that the dynamic environment within the acyl chains of lipids resembles one another regardless of the differences in the kinds of lipids.

^{2}H NMR Spectra of Palmitic Acid (PA) Probes Intercalated in Phospholipid Bilayers

To obtain information on the effects of benzocaine on the orientational order of the methylene groups of multilamellar dispersions of phospholipids, we observed the ^{2}H NMR spectra for deuterated palmitic acids (PA-d_{31}) incorporated into the lipid dispersions (Kuroda et al., 1996). The addition of benzocaine to the lipid mixture narrowed the spectrum. To quantitatively follow the changes in the spectral lineshape, we have calculated the moments for these spectra. The usefulness of the deuterated fatty acid probes for determining the order of the phospholipid membranes is well demonstrated (Stockton et al., 1974, 1976; Pauls, McKay & Bloom, 1983; Vogt, Killian & De Kruijff, 1994). A close resemblance between the ^{2}H NMR spectra of the perdeuterated dipalmitoylphosphatidylcholine (DPPC-d_{62}) bilayers, which include the free fatty acid, and the DPPC bilayers, which include perdeuterated free fatty acid (PA-d_{31}), suggests that the free acid is mixed with lipids in such a manner and orientation that reorients its acyl chain like those of the lipid acyl chains (Pauls et al., 1983).

In Table 4, we summarized the first (M_1) and the second (M_2) moments, mean order parameter (S_{CD}), and its dispersion (Δ_2) for the lipid mixture and also for the lipid mixture containing cholesterol, each with and without benzocaine. In addition to these lipid mixture suspensions, we observed the ^{2}H NMR spectra for the lipid membranes composed of PS and PC and calculated their moment parameters. These results are also summarized in Table 4. In all of the cases, except for the PC containing no cholesterol, M_1, M_2, and S_{CD} decreased and Δ_2 increased with benzocaine. The decreases in M_1 (S_{CD}) and M_2 mean increased molecular motion and the resulting decrease in the order of the probe caused by benzocaine, while the increase in Δ_2 means increased mean square deviation of the order parameter or the decreased degree of homogeneity of the acyl chain packing. The results for the lipid mixtures and PS evidently show that benzocaine fluidized the lipid membranes. This fluidizing effect of benzocaine originates from its location in the lipids, that is, the aromatic ring of benzocaine is located at the lipid headgroup region as in the amine-type local anesthetics (Kuroda et al., 1996). In the PC containing no cholesterol, however, the reverse trend was found in the moment parameters; M_1, M_2, and S_{CD} were increased and Δ_2 was decreased. In this case, the aromatic ring of benzocaine is considered to be surrounded by the acyl chains of the lipids and it hindered their dynamic motions. This binding situation of benzocaine was reflected in the quadrupole splittings due to the aro-

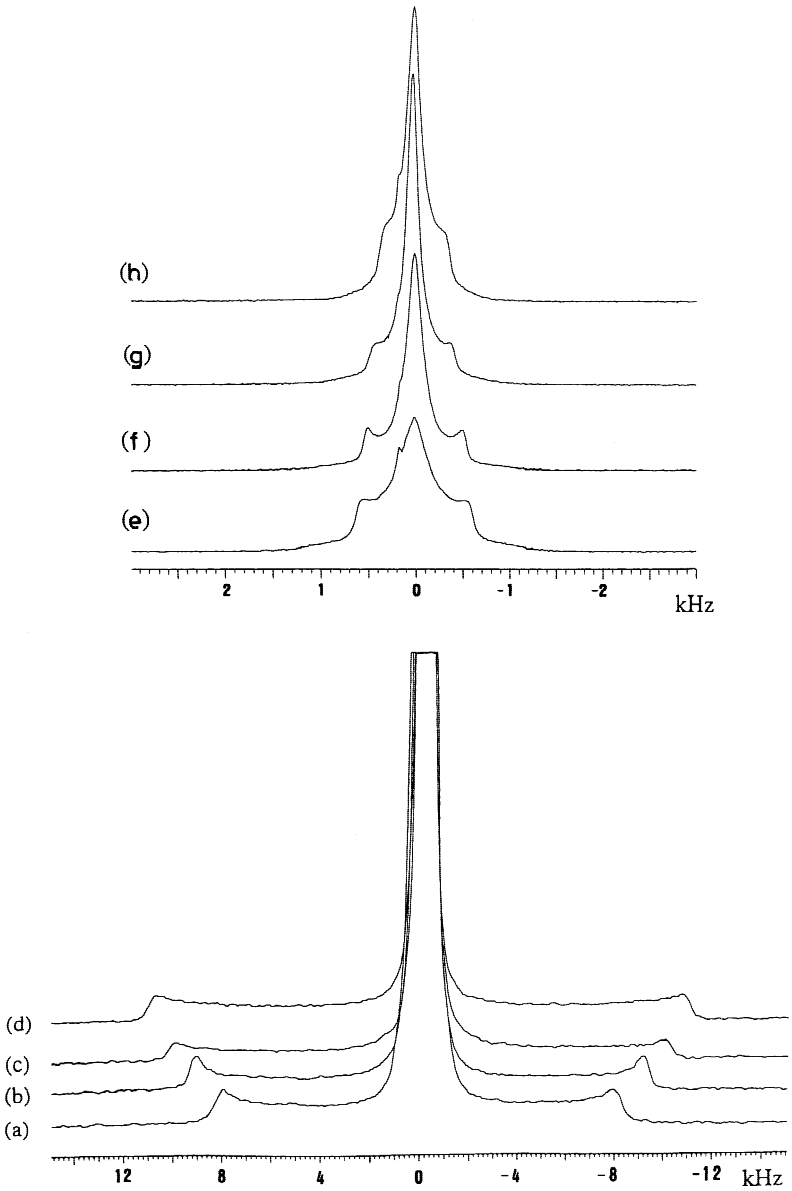

Fig. 3. (*a–d; bottom*) ^{2}H-NMR spectra of benzocaine-d_5-lipid mixture suspensions with increasing amounts of cholesterol: (*a*) 0 mM; (*b*) 18.6 mM; (*c*) 37.1 mM; (*d*) 55.7 mM. (*e–h; top*) as in (*a–d*), respectively, but show only ±3 kHz region with reduced peak intensities.

Table 2. Effect of addition of cholesterol on quadrupole splittings ($\Delta\nu q$) of ^{2}H NMR spectra of benzocaine-d_5 in mixed lipids suspension

Cholesterol (mM)		$\Delta\nu q$(kHz)
0.0	1.08	15.86
18.6	1.00	18.19
37.1	0.77	19.92
55.7	0.58	21.59

matic ring deuterons; the quadrupole splittings in PC were smaller than those in the lipid mixtures and PS membranes as described above (Table 1). In the PC containing cholesterol, benzocaine again decreased the order of acyl chains of the lipids and increased the Δ_2 value. This result can be interpreted as the benzocaine locating among the acyl chains of the lipids reduced the ordering effect of cholesterol on the lipid chains.

Order Parameter Profiles for Palmitic Acids (PA) Intercalated in Phospholipid Bilayers

To draw the acyl chain segmental order parameter profile of palmitic acids which were inserted into the lipid bilayers as a probe to inquire into the orientational order of the hydrocarbon chains of the phospholipids, we further observed the ^{2}H NMR spectra of selectively deuterated palmitic acids. The order parameter (S_{CD}) for each chain

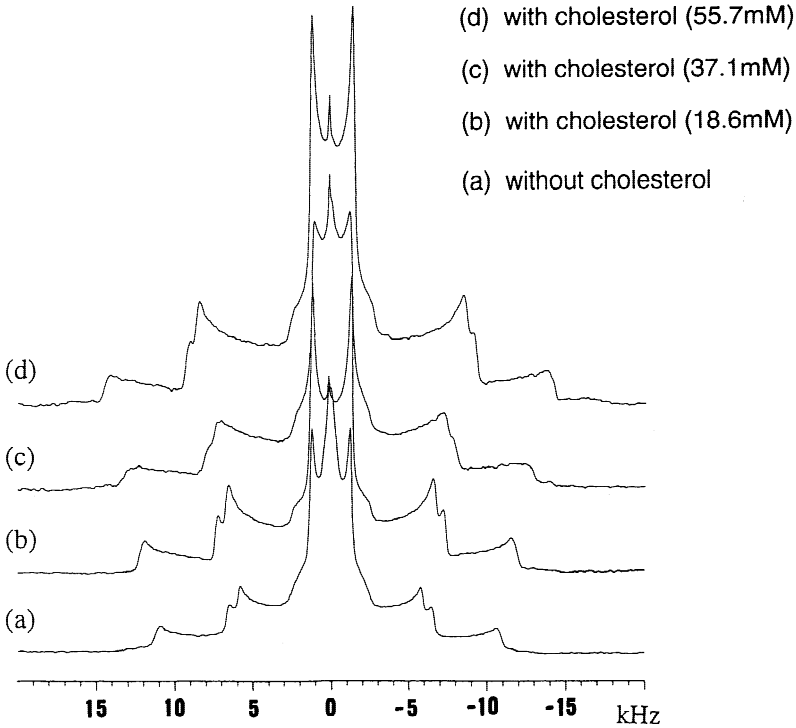

Fig. 4. ^{2}H-NMR spectra of butamben-d_9-lipid mixture suspensions with increasing amounts of cholesterol: (*a*) 0 mM; (*b*) 18.6 mM; (*c*) 37.1 mM; (*d*) 55.7 mM.

Table 3. Observed quadrupole splittings ($\Delta\nu q$) of ^{2}H NMR spectra of butamben-d_9 in lipids suspension

Lipid	Cholesterol (mM)	$\Delta\nu q$(kHz)			
Mixed lipids	0.0	2.43	11.53	12.91	21.47
	18.6	2.47	13.06	14.40	23.39
	37.1	2.49	14.25	15.66	24.57
	55.7	2.60	16.92	18.08	27.93
PS	0.0	2.47	11.27	12.87	21.55
	55.7	2.63	17.56	18.66	28.95
PC	0.0	2.15	10.18	11.27	19.46
	55.7	2.51	15.75	16.89	26.72

segment was calculated by correlating the observed $\Delta\nu\ q$ (in units of kilohertz) to S_{CD} according to the following equation:

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

where e^2qQ/h is the static quadrupole coupling constant and a value of 170 kHz was assumed (Stockton et al., 1976). We plotted the S_{CD} values of the PA in the lipid mixture and PS, respectively, in Figs. 5 and 6 against the chain segment number. The quadrupole splitting values at C16, C15, and C14 were observed with increasing magnitude of splitting, respectively, starting from the innermost doublet in the ^{2}H NMR spectrum of PA-d_{31}. The order parameter profile drawn for the lipid mixture and PS with no cholesterol significantly resembled that of PA in dimyristoylphoshatidylcholine (DMPC) bilayers reported by Vogt et al. (1994). Overall, the S_{CD} values ranged from about 0.25 (C2~C9) to 0.04 (C16), regardless of the differences in the kinds of lipids. However, we found some dissimilarities between our and the Vogt et al. (1994) order parameter profiles. Vogt et al. have shown almost the same S_{CD} value from C2 to C8 (a plateau region); this trend has been generally found so far for the deuterated fatty acid probes (Stockton et al., 1976; Stockton & Smith, 1976) and for the deuterium-labeled phospholipids (Seelig & Seelig, 1980; Sankaram & Thompson, 1990; de Planque et al., 1998). In our present data, the S_{CD} values at C3 in both the lipid mixture and PS dispersions slightly decreased compared with the S_{CD} value at C2 or C5. Moreover, the S_{CD} values at C5, C6, and C9 became slightly larger than the S_{CD} value at C2. Interestingly, this latter tendency was amplified when the lipid mixture contained cholesterol and the S_{CD} values monotonically increased from C2 to C9 (Fig. 5). The addition of benzocaine to the lipid dispersions differentially decreased the S_{CD} values among the positions of each chain segment (Figs. 5 and 6). We note that in the presence of cholesterol, the effect of benzocaine on the S_{CD} values emerged instantly at around the C2–C3 moiety of the acyl chain, whereas in the absence of cholesterol, there is a lag (C2–C6) in the appearance of the effect. This lag might have arisen owing to the mutual cancellation between the fluidizing and

Table 4. Effect of addition of benzocaine on the first (M_1) and second (M_2) moments, S_{CD}, and Δ_2 of ^{2}H NMR spectra of palmitic-d_{31} acid incorporated into the lipid bilayers

Lipid	Benzocaine (mM)	$M_1 \times 10^{-5}$ (Rad/sec)	$M_2 \times 10^{-10}$ (Rad^2/sec^2)	S_{CD}	Δ_2
Mixed lipids	0.0	0.686	0.664	0.226	0.0458
	20.0	0.678	0.660	0.224	0.0615
Mixed lipids + cholesterol	0.0	0.785	0.837	0.259	0.0052
	20.0	0.738	0.755	0.244	0.0282
PS	0.0	0.724	0.736	0.239	0.0420
	20.0	0.660	0.633	0.218	0.0744
PS + cholesterol	0.0	0.792	0.854	0.261	0.0085
	20.0	0.780	0.849	0.258	0.0328
PC	0.0	0.637	0.601	0.210	0.0962
	20.0	0.648	0.611	0.214	0.0774
PC + cholesterol	0.0	0.784	0.862	0.259	0.0394
	20.0	0.754	0.809	0.249	0.0535

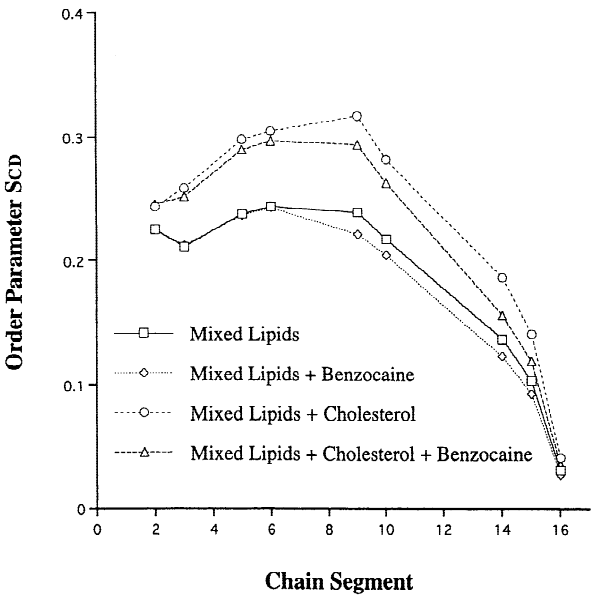

Fig. 5. Plot of S_{CD} against positions of deuteration for specifically deuterated palmitic acid probes intercalated in the lipid mixture with cholesterol and/or benzocaine.

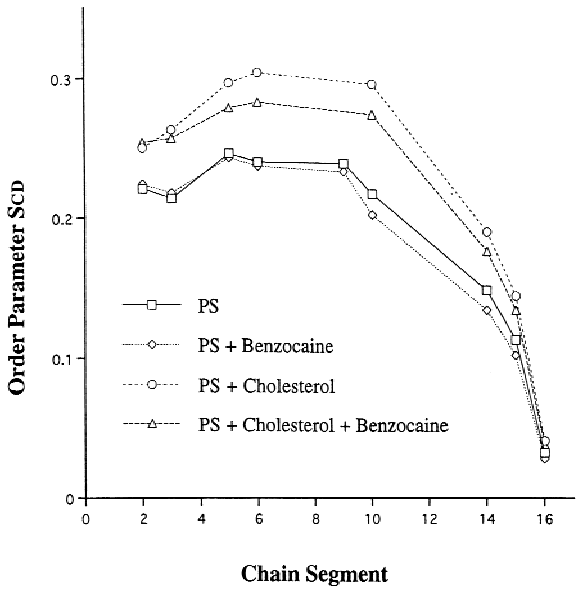

Fig. 6. Plot of S_{CD} against positions of deuteration for specifically deuterated palmitic acid probes intercalated in PS with cholesterol and/or benzocaine.

ordering effects of benzocaine at around the C2–C6 moiety. In the presence of cholesterol, we also note that the effect of benzocaine on the S_{CD} values is stronger in PS than in the lipid mixture. Thus we may be allowed to consider as follows. (i) In the absence of cholesterol, benzocaine resides by spreading its aromatic ring around the C2–C6 moiety; (ii) in the presence of cholesterol, the aromatic ring resides in a more polar region, that is, around the C1–C2 moiety; and (iii) in the case of (ii), benzocaine is situated in a more polar region in PS than in the lipid mixture.

In conclusion, in Fig. 7 we showed schematically the location and orientation of benzocaine in the lipid mixture and PS, (*a*) with and (*b*) without cholesterol. A plausible hydrogen bonding, which may stabilize the binding of benzocaine to the lipids, is indicated by the dotted lines. In PC membranes, the aromatic ring of benzocaine resides in a more hydrophobic lipid interior region.

Discussion

Nerve membranes are composed of lipids that include phospholipids, cholesterol, and free fatty acids; the phospholipids are composed of PC, PE, PS, sphingomyelin,

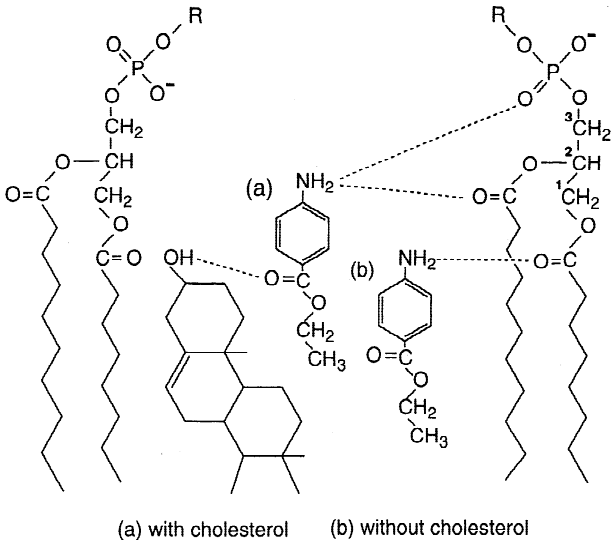

Fig. 7. Location and orientation of benzocaine in the lipid mixture and PS: (*a*) with and (*b*) without cholesterol.

Lyso-PE, and Lyso-PC (Zambrano, Cellino & Canessa-Fischer, 1971). Moreover, it is also suggested that the Na channel is surrounded by PS (Cook, Low & Ishijimi, 1972; Hille et al., 1975). Thus the presently employed mixed lipids and PS with and without cholesterol can be good model membranes for an actual nerve membrane that involves the Na channel protein. The present investigation indicated that in the presence of cholesterol, benzocaine and butamben reside in a lipid headgroup region and that in the absence of cholesterol, they reside in a slightly more hydrophobic region (Fig. 7). These results agree well with the location deduced for benzocaine from the binding competition experiments between benzocaine and merocyanine 540 with nicotinic acetylcholine receptor membranes (Arias et al., 1994). It was shown by Auger, Jarrell & Smith (1988) that the location of the uncharged form of tetracaine in the cholesterol-containing system is higher (polar) than that found in pure phosphatidylcholine bilayers. Thus cholesterol may have a general propensity to squeeze out anesthetics into the aqueous interface of the bilayer.

Wang et al. (1998) proposed a common local anesthetic receptor for benzocaine and etidocaine in voltage-gated rat skeletal muscle μ1 Na channels. They have shown that the receptor locates near the domain IV-segment 6 (IVS6) and it is constructed by including F1579 which is situated in the center of the transmembrane helix, S6. The IVS6 in rat brain type IIA Na channels has already been pointed out by Ragsdale et al. (1996) as a common receptor site among amine-type local anesthetics, antiarrhythmics, and anticonvulsants. They showed that F1764, which is equivalent to the F1579 in μ1 Na channels, is the most important determinant of the binding site. Since F1764 (F1579) is situated in a center part of S6, if the lipids surrounding S6 are organized as a bilayer, benzocaine (and butamben) should locate at the center of the bilayers to directly interact with the receptor consisting of F1764. This requirement, of course, assumes that the S6 takes a typical helical structure and is immersed in bilayers parallel to the bilayer normal. Evidently, the locations of benzocaine and butamben presently determined cannot reach F1764. This finding does not always mean that IVS6 is not the receptor site for benzocaine, because (i) the real structure of S6 in membranes is not known, (ii) a local anesthetic action on the Na channel can be allosterically exerted, for example, by way of the "receptor site" of IVS6 to enhance Na channel inactivation (Marban et al., 1998; Balser et al., 1996), and (iii) lipids surrounding the S6 can also be organized as a hexagonal phase (Matsuzaki et al., 1998; Dibble & Feigenson, 1994; Tournois et al., 1987). Matsuzaki et al. (1998) have shown that the lipids organized as bilayers change their organization into a hexagonal-like structure near the peptides forming a pore. In this case, benzocaine bound to the headgroup region of the lipid bilayers (and many amine-type local anesthetics which may also exist at the lipid headgroup region) can easily approach F1764 in IVS6 by getting into the flow of the lipids lateral diffusion, supporting views presented by Wang et al. (1998), Ragsdale et al. (1996), and Hille's modulated receptor hypothesis (Hille, 1977).

Quite recently, Okamura and Nakahara (1999) have reported the locations of n-propylbenzene (PrBe) and benzyl alcohol (BzOH) in small unilamellar egg-PC vesicles by ^{1}H and ^{13}C NMR. They found that PrBe is located at the hydrophobic chain region of the bilayer core, whereas BzOH is located at the level of the lipid headgroups. Evidently, further work will be necessary to clarify how the substituent group at an aromatic ring alters the location of the aromatic ring depending on the kinds of lipids.

References

Arias, H.R., Romanowski, S.A., Disalvo, E.A., Barrantes, F.J. 1994. Interaction of merocyanine 540 with nicotinic acetylcholine receptor membranes from *Discopyge tschudii* electric organ. *Biochim. Biophys. Acta* **1190:**393–401

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

Balser, J.R., Nuss, H.B., Orais, D.W., Johns, D.C., Marban, E., Tomaselli, G.F. 1996. Local anesthetics as effectors of allosteric gating. Lidocaine effects on inactivation-deficient rat skeletal muscle Na channels. *J. Clin. Invest.* **98:**2874–2886

Bloom, M., Davis, J.H., Dahlquist, F.W. 1978. Determination of orientational order in bilayer systems using moments of deuterium magnetic resonance spectra. *In:* Xxth Ampere Congress Proceed-

ings, Tallinn, Estonia. E. Kundla, E. Lippmann, and T. Saluvere, editors. pp. 551. Springer-Verlag, New York

Browning, J.L., Seelig, J. 1980. Bilayers of phosphatidylserine: a deuterium and phosphorus nuclear magnetic resonance study. *Biochem.* **19:**1262–1270

Catterall, W.A. 1995. Structure and function of voltage-gated ion channels. *Ann. Rev. Biochem.* **64:**493–531

Cook, A.M., Low, E., Ishijimi, M. 1972. Effect of phosphatidyl serine decarboxylase on neural excitation. *Nature New Biol.* **239:**150–151

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

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

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

de Planque, M.R.R., Greathouse, D.V., Koeppe II, R.E., Schafer, H., Marsh, D., Killian, J.A. 1998. Influence of lipid/peptide hydrophobic mismatch on the thickness of diacylphosphatidylcholine bilayers. A ^{2}H NMR and ESR study using designed transmembrane α-helical peptides and gramicidin A. *Biochem.* **37:**9333–9345

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

Dougherty, D.A. 1996. Cation-π interactions in chemistry and biology: A new view of benzene, Phe, Tyr, and Trp. *Science* **271:**163–168

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

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

Hille, B., Woodhull, A.M., Shapiro, B.I. 1975. Negative surface charge near sodium channels of nerve. Divalent ions, monovalent ions, and pH. *Phil. Trans. R. Soc. London Ser. B.* **270(908):**301–318

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

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

Kuroda, Y., Ogawa, M., Nasu, H., Terashima, M., Kasahara, M., Kiyama, Y., Wakita, M., Fujiwara, Y., Fujii, N., Nakagawa, T. 1996. Locations of local anesthetic dibucaine in model membranes and the interaction between dibucaine and a Na^+ channel inactivation gate peptide as studied by ^{2}H- and ^{1}H-NMR spectroscopies. *Biophys. J.* **71:**1191–1207

Lalor, C.B., Flynn, G.L., Weiner, N. 1995. Formulation factors affecting release of drug from topical vehicles. II. Effect of solubility on in vitro delivery of a series of n-alkyl p-aminobenzoates. *J. Pharm. Sci.* **84:**673–676

Le Count, D.J., Reid, J.A.W. 1968. Novel process cycle for para-substituted benzene derivatives. *J. Appl. Chem.* **18:**108–110

Marban, E., Yamagishi, T., Tomaselli, G.F. 1998. Structure and function of voltage-gated sodium channels. *J. Physiol.* **508:**647–657

Matsuzaki, K., Sugishita, K., Ishibe, N., Ueha, M., Nakata, S., Miyajima, K., Epand, R.M. 1998. Relationship of membrane curvature to the formation of pores by magainin 2. *Biochem.* **37:**11856–11863

Narahashi, T., Frazier, D., Yamada, M. 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., Davis, J.H., Weeks, G., Bloom, M. 1980. Quantitative study of the fluidity of *Escherichia coli* membranes using deuterium magnetic resonance. *Biochem.* **19:**451–457

Numa, S., Noda, M. 1986. Molecular structure of sodium channels. *Ann. N.Y. Acad. Sci.* **479:**338–355

Okamura, E., Nakahara, M. 1999. NMR study directly determining drug delivery sites in phospholipid bilayer membranes. *J. Phys. Chem. B.* **103:**3505–3509

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

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

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

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

Ragsdale, D.S., McPhee, J.C., Scheuer, T., Catterall, W.A. 1996. Common molecular determinants of local anesthetic, antiarrhythmic, and anticonvulsant block of voltage-gated Na^+ channels. *Proc. Natl. Acad. Sci. USA* **93:**9270–9275

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

Sankaram, M.B., Thompson, T.E. 1990. Modulation of phospholipid acyl chain order by cholesterol. A solid-state ^{2}H nuclear magnetic resonance study. *Biochem.* **29:**10676–10684

Seelig, J., Seelig, A. 1980. Lipid conformation in model membranes and biological membranes. *Q. Rev. Biophys.* **13:**19–61

Stockton, G.W., Polnaszek, C.F., Leitch, L.C., Tulloch, A.P., Smith, I.C.P. 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., Polnaszek, C.F., Tulloch, A.P., Hasan, F., Smith, I.C.P. 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. *Biochem.* **15:**954–966

Stockton, G.W., Smith, I.C.P. 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., Ritchie, J.M. 1987. The action of local anesthetics on ion channels of excitable tissues. *In:* Local Anesthetics. G.R. Strichartz, editor. pp. 21–52. Springer-Verlag, New York

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

Villalain, J. 1996. Location of cholesterol in model membranes by magic-angle-sample-spinning NMR. *Eur. J. Biochem.* **241:**586–593

Vist, M.R., Davis, J.H. 1990. Phase equilibria of cholesterol/dipalmitoylphosphatidylcholine mixtures: ^{2}H nuclear magnetic resonance and differential scanning calorimetry. *Biochem.* **29:**451–464

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

Wang, G.K., Quan, C., Wang, S-Y. 1998. A common local anesthetic receptor for benzocaine and etidocaine in voltage-gated μ1 Na^+ channels. *Pfluegers Arch.* **435:**293–302

Weisz, K., Grobner, G., Mayer, C., Stohrer, J., Kothe, G. 1992. Deuteron nuclear magnetic resonance study of the dynamic organization of phospholipid/cholesterol bilayer membranes: molecular properties and viscoelastic behavior. *Biochem.* **31:**1100–1112

Westman, J., Boulanger, Y., Ehrenberg, A., Smith, I.C.P. 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., Franks, N.P. 1976. Structural analysis of hydrated egg lecithin and cholesterol bilayers. II. Neutron diffraction. *J. Mol. Biol.* **100:**359–378

Zambrano, F., Cellino, M., Canessa-Fischer, M. 1971. The molecular organization of nerve membranes IV. The lipid composition of plasma membranes from squid retinal axons. *J. Membrane Biol.* **6:**289–303