Interactions between Local Anesthetic Dibucaine and Pig Erythrocyte Membranes as Studied by Proton and Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy

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The interactions between amine local anesthetic dibucaine and pig erythrocyte membranes have been studied by proton and phosphorus-31 nuclear magnetic resonance (^{1}H- and ^{31}P-NMR) spectroscopy. It was found that dibucaine, bound to the membranes, increases the mobility of the hydrophobic acyl chains of the phospholipids, but that it decreases the mobility and/or changes the structure of the polar headgroups. The interactions with peripheral membrane proteins, *i.e.*, spectrin and actin, were found to be weak. These observations indicate that the dibucaine locates across the polar and hydrophobic areas of the lipid phase of the membranes by both electrostatic and hydrophobic interactions. It is assumed that the changes in the mobility and/or the conformation of the phospholipids residing around the Na channel protein are essential in causing anesthesia.

Keywords local anesthetic; dibucaine; erythrocyte membrane; ^{1}H-NMR; ^{31}P-NMR

The basic questions underlying studies on the molecular mechanism of local anesthesia have been where local anesthetic molecules are bound and how the state of the binding site changes. From the viewpoint focussed on lipids, lots of attempts have been made to prove the hypothesis that the perturbation of the lipid bilayer structure by local anesthetics alters the structure of the Na channel protein and results in anesthesia. They have, however, failed to solve the problem as to whether or not major structural changes in the lipid bilayer can be induced at physiologically relevant concentrations of anesthetics.[1] Recent studies have shown that local anesthetics interact with some membrane proteins, $(Ca^{2+}-Mg^{2+})$-adenosine triphosphatase (ATPase),[2] (Na^+-K^+)-ATPase,[3] and calmodulin.[4] Moreover, Greenberg and Tsong have demonstrated that the membrane protein provides a local anesthetic receptor site in the axonal membranes.[5] However, the interaction with membrane lipids is still well worth investigating to understand the mechanism of anesthesia at a molecular level. This is because the local anesthetics are generally accepted to exert their effects when they are present at the cytoplasmic side of axonal membranes, requiring transbilayer movement of the drugs having polar groups.[6] In addition, axonal membranes are quite different from artificial lipid membranes in that they have heterogeneous properties. Various kinds of phospholipids and membrane proteins distribute asymmetrically between the outer and inner layers.[7] Also, it is generally known that in an intact membrane there are thermotropic lateral phase separations into crystalline and liquid-crystalline phases.[8] The intact membranes which have these heterogeneous properties are expected to provide a specific binding site for a local anesthetic molecule. From this point of view, we have investigated the interaction between local anesthetic dibucaine and pig erythrocyte membranes. The analogy between the membrane actions of anesthetics on excitable membranes and on the erythrocyte membranes is reported.[9] In addition, Roth *et al.* showed that the minimal blocking concentrations of local anesthetics acting on nerve fibers are virtually the same as those which cause anti-haemolysis of erythrocytes.[10] Recently, it is also reported that Na channels in the brain are linked to ankyrin and subsequently to spectrin.[11] It is well-known that in the erythrocyte membranes, anion channels known as the band 3 protein are linked to the ankyrin and spectrin.[12] Moreover, Christophersen *et al.* showed that there is a voltage-gated, non-selective cation channel in the human red cell membrane.[13] Accordingly, these structural similarities between the erythrocyte membranes which include the anion channel and the excitable membranes which include the Na channel are also fascinating for employing the erythrocyte membranes. These are the reasons why we have chosen pig erythrocyte membranes to study the interaction with local anesthetics. In order to obtain information on interactions at a molecular level, proton and phosphorus-31 nuclear magnetic resonance (^{1}H- and ^{31}P-NMR) spectroscopy have been employed.

Experimental

Materials Fresh pig blood was employed to prepare erythrocyte membranes which are usually called ghosts. Ethylenediaminetetraacetic acid (EDTA) was used as an anticoagulant. Dibucaine hydrochloride (Fig. 1) was purchased from Sigma and used without further purification. Acrylamide slab gel (8—16%) were purchased from TEFCO Corporation.

Preparation of Erythrocyte Ghosts Pig erythrocyte ghosts were prepared by a modified Dodge procedure.[14] Ghosts for ^{1}H-NMR measurements were treated with phosphate buffer (pH 7.4), and for ^{31}P-NMR measurements were treated with Tris–HCl buffer (pH 7.4). The following procedures were common to both preparations. Pig erythrocytes were isolated from the EDTA-treated blood by centrifugation for 10 min at $4000 \times g$. Cells were washed three times with isotonic 310 ideal milliosmolar (imOsm) buffer, and then were hemolysed with 20 volumes of 10 imOsm buffer. Ghosts were recovered from the hemolysate by centrifugation for 20 min at $20000 \times g$. The packed cells thus obtained were washed four to five times with 10 imOsm buffer until they became pearly white or pale pinkish in color. From 3 ml of the blood, we obtained about 1 ml packed cells; these cells were lysed in the 10 imOsm buffer. The 1 ml packed cells were found to contain about 2.3 mg proteins by the Lowry method.[15] If we assume the weight ratio between the proteins and lipids to be 1.1,[16] and also if we assume the percentage of the phospholipids in the total lipids to be 56%,[17] the 1 ml packed cells were found from calculation to contain 1.16 mg phospholipids; consequently, by assuming the average molecular weight of the phospholipids

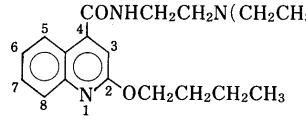

Fig. 1. Structure of Dibucaine Hydrochloride and Numbering Scheme in Its Aromatic Ring

to be 750, the concentration of the phospholipids was calculated to be 1.55 mM. All experiments were performed at 0 to 4°C.

Preparation of Samples for NMR Measurements Erythrocyte ghosts were isolated by centrifugation for 30 min at 20000 × *g*, and washed with 10 imOsm buffer in 99.75% D_2O. The samples for ^{1}H-NMR measurements were prepared from about 1 ml of the packed cells suspended in 2.5 ml of the buffer. The samples for ^{31}P-NMR measurements were prepared from ghost suspension which is twice to three times as concentrated as those employed for ^{1}H-NMR measurements, since the NMR sensitivity of the ^{31}P nucleus is lower than that of proton. Sonicated samples were prepared with an ultrasonic generator irradiating the ghost suspension in an ice/water bath for 30 s. The sonication was performed to such an extent that no original red blood cell ghosts were observed in a phase-contrast optical microscope at a 1500-fold magnification. The supernatant of the ghost suspension in phosphate buffer was prepared by collecting by centrifugation for 30 min at 20000 × *g*. Proteins dissolved in the supernatant were identified by dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) which was modified by Fairbanks *et al.* so as to be applicable for membrane proteins.[18] A weighed amount of a drug, dissolved or not dissolved in a buffer, was added to the solution of the ghost suspension.

NMR Measurements ^{1}H-NMR measurements were performed on a Bruker AM-600 spectrometer at a resonance frequency of 600 MHz. All spectra were measured with suppression of the water resonance by presaturation. A presaturation time was 1 s. The irradiation power was kept constant throughout the present work. The chemical shifts were referenced to the residual HDO proton (4.8 ppm). ^{31}P-NMR measurements were carried out on a Bruker AC-300 spectrometer at a resonance frequency of 121 MHz and with a broadband decoupling. The observing spectral width was 242 ppm and the acquisition time was 0.557 s. The chemical shifts were referenced to external 85% H_3PO_4. In all the ^{1}H- and ^{31}P-NMR measurements, 90° pulses were employed and the delay times for relaxation were longer than 2 s. All experiments were performed at 300 K. The recycling delay time of 2.557 s for the measurements of ^{31}P-NMR spectra would be reasonable by considering the spin-lattice (T_1) relaxation time of the ^{31}P nucleus at 300 K in 1,2-dioleoyl-*sn*-glycero-3-phosphocholine multilayered vesicles (T_1 = 1.1 s at 121.4 MHz resonance frequency).[19]

Results

^{1}H-NMR Spectrum of Erythrocyte Ghosts Figure 2a shows the ^{1}H-NMR spectrum of intact pig erythrocyte ghosts in 10 imOsm phosphate buffer. In the conditions of low ionic strength, spectrin and erythrocyte actin, which are membrane skeletal proteins at the cytoplasmic surface, are well-known to be easily released from erythrocyte membranes.[20] We confirmed by SDS-PAGE that the supernatant of the ghost suspension really contained the spectrin (bands 1 and 2) and erythrocyte actin (band 5). This result suggests that the signals in the spectrum of ghost suspension in Fig. 2a originate not only from the membranes but also from the proteins dissolved in the buffer. The ^{1}H-NMR spectrum of the supernatant is shown in Fig. 2b. Compared with Fig. 2a, two very broad envelopes of overlapping signals at 0.5 to 2.5 ppm and at 3.0 to 4.5 ppm were hardly observed in this Fig. 2b. This difference in the appearance of the two spectra suggests that the former very broad peak is due to the methylene protons of the acyl chains of lipids and that the latter very broad peak is due to the protons of the sugar residues of glycoproteins. These peaks also associate with the side chains and α-hydrogens of proteins remaining in or on the membranes. Figure 2c shows the spectrum obtained from the free induction decay (FID) by subtracting the FID of the supernatant from that of the ghost suspension. The difference in appearance is hardly discernible between Figs. 2a and 2c. Therefore, we concluded that the contribution from the dissolved proteins to the spectrum of the ghost suspension is small enough to be neglected.

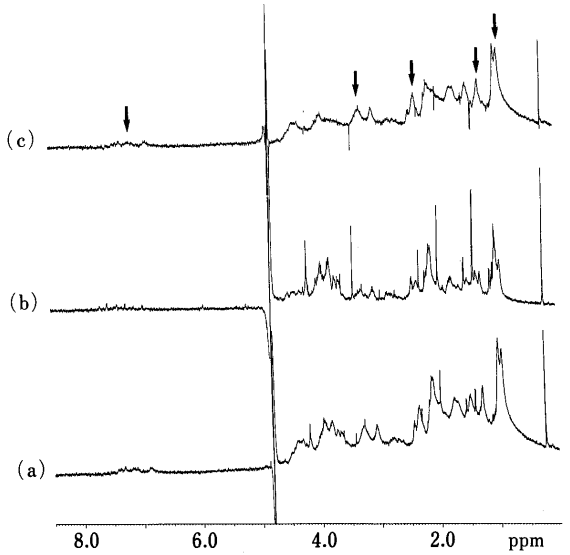

Fig. 2. (a) ^{1}H-NMR Spectrum (600 MHz) of Pig Erythrocyte Ghost Suspension
(b) ^{1}H-NMR Spectrum (600 MHz) of the Supernatant of the Ghost Suspension
(c) ^{1}H-NMR Spectrum (600 MHz) Obtained by Subtracting the Free Induction Decay (FID) of the Supernatant from the FID of the Ghost Suspension
The FIDs in (a) and (b) were obtained from the first increment of a two-dimensional NOESY experiment with presaturation during relaxation (1.0 s) and mixing (0.01 s) periods. The signals which are indicated by an arrow were assigned in the text. The sharp signals at 0.3, 1.4, 1.6, 2.0, 3.4 and 4.2 ppm are due to impurities in the phosphate buffer.

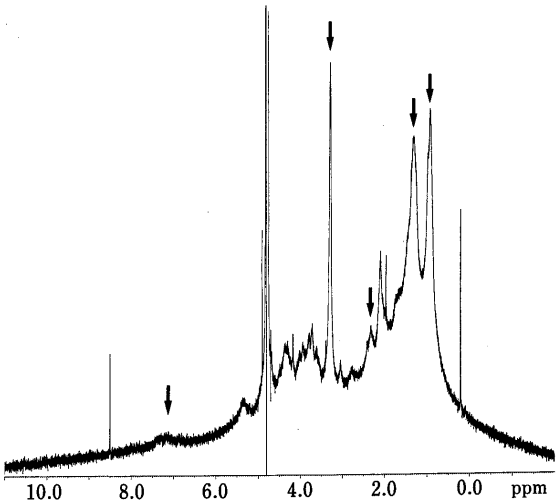

Fig. 3. ^{1}H-NMR Spectrum (600 MHz) of Sonicated Ghosts
The spectrum was obtained by using a simple presaturation method. The signals which are indicated by an arrow were assigned in the text. The sharp signals at 0.3, 2.0, 4.2 and 8.5 ppm are due to impurities in the phosphate buffer.

The ^{1}H-NMR spectrum of sonicated ghosts is shown in Fig. 3. Sonication destroyed the structure of the ghosts as can be seen from significant changes in spectral shape or reduction in turbidity of the suspension. However, fragmentation or rearrangement into small particles often provides useful information on the assignment of the signals. The signals at 0.9, 1.3, and 3.3 ppm became sharper. Moreover, a new signal appeared at 5.3 ppm which may be due to the unsaturated CH=CH groups in the acyl

chains of the lipids. Based on the previous reports,[21,22] we assigned the peaks which are indicated by an arrow in both Figs. 2c and 3 as follows:

(i) A signal at 0.9 ppm is due to the CH_3 protons of lipids, cholesterol, and proteins.

(ii) A signal at 1.3 ppm is associated with the $(CH_2)_n$ protons of acyl chains of lipids.

(iii) A signal at 2.2 ppm is due to the *N*-acetyl protons ($NCOCH_3$) of sugar residues and proteins.

(iv) A signal at 3.3 ppm, the intensity of which is very weak in Fig. 2b, whereas significantly sharp and strong in Fig. 3, is due to the protons of the choline groups ($N^+(CH_3)_3$) of the lipids.

(v) The broad and weak peaks observed at 6.8 to 8.4 ppm are associated with the ring hydrogens of aromatic amino acids in the membrane proteins.

Effects of Dibucaine on the ^{1}H-NMR Spectrum of the Ghost Suspension ^{1}H-NMR spectra of the ghost suspension with and without dibucaine are shown in Fig. 4. In order to assign the signals of dibucaine, we measured the ^{1}H-NMR spectrum of 3 mM dibucaine solution prepared using the same buffer as that of the ghost suspension. Five signals due to the ring hydrogens of dibucaine appeared in the region between 7 and 8 ppm (Fig. 4b), where ghosts show no clear signals (Fig. 4a). As dibucaine was added to the ghost suspension, the broad envelope which ranges from 0.5 to 2.5 ppm disappeared followed by increased peak height at 1.3 ppm; the peak at 1.3 ppm was assigned to $(CH_2)_n$ of lipids (Fig. 3). The signals due to the dibucaine, on the other hand, dramatically broadened, as can be seen clearly by comparing the spectra in the aromatic region (Figs. 4b and 4c). These observations indicate that the strong interaction of dibucaine with membranes decreased the mobility of dibucaine molecules; whereas, it increased the mobility of the acyl chains of lipids. The immobilization of dibucaine can be considered to mainly arise from the electrostatic interaction, namely, between cationic dibucaine and negatively charged membrane components, especially the interaction with acidic phospholipids.

Effects of Dibucaine on the ^{31}P-NMR Spectrum of the Ghost Suspension For the purpose of revealing the changes in the mobility and/or the structure of the polar head groups of lipids induced by dibucaine, ^{31}P-NMR spectra were observed. Figure 5 shows the spectra of the ghost suspension in the absence (Fig. 5a) and in the

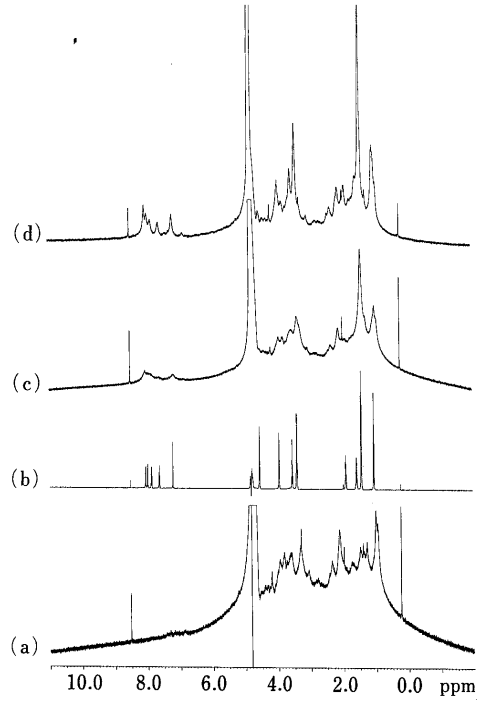

Fig. 4. (a) ^{1}H-NMR Spectrum (600 MHz) of Pig Erythrocyte Ghost Suspension
(b) ^{1}H-NMR Spectrum (600 MHz) of 3 mM Dibucaine Hydrochloride Solution Prepared Using the Same Buffer as That of Ghost Suspension
(c) ^{1}H-NMR Spectrum (600 MHz) of Pig Erythrocyte Ghost Suspension with 1 mM Dibucaine Hydrochloride
(d) ^{1}H-NMR Spectrum (600 MHz) of Pig Erythrocyte Ghost Suspension with 3 mM Dibucaine Hydrochloride

The spectra were obtained by using a simple presaturation method. The sharp signals observed at 0.3, 2.0, 4.2 and 8.5 ppm are due to impurities in the buffer and at 3.3 ppm is due to EDTA.

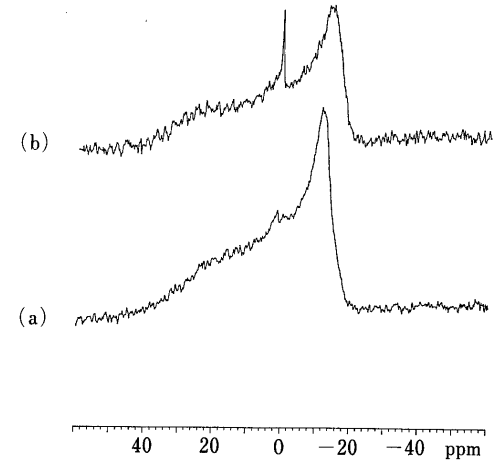

Fig. 5. ^{31}P-NMR Spectra (121 MHz) of the Pig Erythrocyte Ghost Suspension (a) in the Absence and (b) in the Presence of Dibucaine Hydrochloride (10 mM)

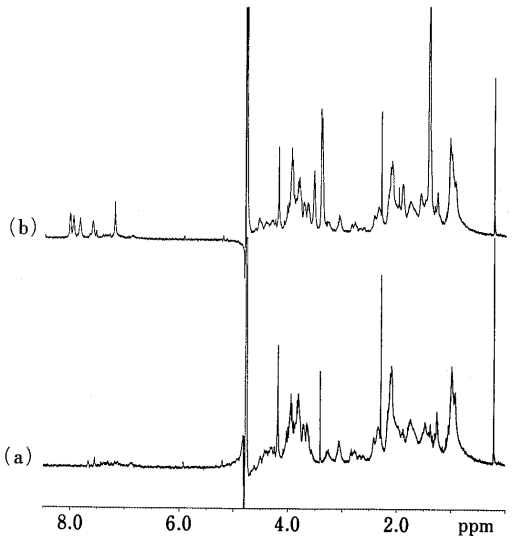

Fig. 6. ^{1}H-NMR Spectra (600 MHz) of the Supernatant (a) in the Absence and (b) in the Presence of Dibucaine Hydrochloride (0.5 mM)

The spectra were obtained by using the NOESY pulse sequence as in Fig. 2. The sharp signals at 0.3, 1.4, 2.0, 2.3, 3.4 and 4.2 ppm are due to impurities in the buffer.

presence (Fig. 5b) of dibucaine. The ^{31}P-NMR spectrum of the ghost showed a shoulder at the low-field and a strong peak at the high-field. Such a spectrum is characteristic for membranes in which phospholipids are arranged as a bilayer structure.[23] The chemical shift anisotropy, being related to the order in the polar head groups, was determined as the chemical shift difference between the low-field shoulder and the high-field peak. The value was 34 ppm in Fig. 5a and 45 ppm in Fig. 5b, indicating that the addition of dibucaine decreased the mobility and/or changed the structure of the polar head groups of the phospholipids. Interestingly, at 0 ppm, where the signal due to the isotropically rotating ^{31}P nucleus should appear, a sharp signal was observed (Fig. 5b). This peak may be due to the formation of mixed micelles with dibucaine.

Effects of Dibucaine on the ^{1}H-NMR Spectrum of the Supernatant In order to investigate whether dibucaine interacts directly with cytoskeletal membrane proteins, 0.5 mM dibucaine was added to the supernatant containing spectrin and actin. The results are shown in Fig. 6. Evidently, no appreciable effects were seen on the spectrum of these membrane proteins by the addition of the dibucaine. This finding indicates that the interaction between dibucaine and the cytoskeletal membrane proteins is weak, if any.

Discussion

The present experiments indicate that the local anesthetic dibucaine increased the mobility of the acyl chains of phospholipids. This result agrees with the previous observations in the study made by using liposomes as a model membrane.[24] In contrast, ^{31}P-NMR measurements suggest that dibucaine immobilized and/or changed the structure of the polar head groups of phospholipids. We interpret these changes in the membrane fluidity and also in the structure of the phospholipid headgroup as follows: Under the experimental condition of pH 7.4, dibucaine exists predominantly as a cationic form, since its pK_a value is 8.54.[25] On account of an electrostatic interaction, the positively charged diethylamino group of dibucaine preferentially binds to the negatively charged phosphate group of lipids; consequently, the polar surface of the membranes would be immobilized. This binding may also accompany the structural changes at the polar headgroup. Beyer attributed the enhanced absolute value of ^{31}P chemical shift anisotropy in egg phosphatidylcholine bilayers, which is caused by positively charged amphiphilic molecules, to the conformational changes in the phospholipid headgroup.[26] He interpreted the increased chemical shift anisotropy as being due to tilting the phospholipid dipole from the membrane parallel into a more upright orientation.[26] It should be stressed that the same trend was noted in the intact erythrocyte membranes. On the other hand, the hydrophobic part of dibucaine is expected to locate at the hydrophobic area of lipids. It is reasonable for such an amphiphilic molecule to lie across the polar and hydrophobic areas. A similar model for dibucaine binding with egg phosphatidylcholine bilayers is reported in our previous paper.[27] The hydrophobic part of the cationic dibucaine cannot penetrate deeply into the membrane on account of the constraint imposed by the molecular structure. Thus, this type of binding can be considered to produce a gap which allows the acyl chains of lipids to move more freely.

Recently we found that dibucaine molecules are present as a dimer in egg phosphatidylcholine bilayers.[28] It would be reasonable to imagine that in the presently employed erythrocyte membranes the dibucaine also exists as a dimer, producing a larger gap within the hydrophobic area of lipids than does a monomer molecule because of bulkiness of the dimerized structure.[28]

In the present NMR experiments, the interactions between the water-soluble peripheral membrane proteins, *i.e.*, spectrin and actin, and the cationic dibucaine were found to be weak. In addition, no direct information was obtained relating to the interaction with anion channels. In the erythrocyte membranes, it is known that phosphatidylserine (PS) solely resides inside the bilayer membrane.[29] Since the partition coefficient of dibucaine for the PS is at least 20-fold larger than that for neutral phospholipids,[24] it is conceivable that dibucaine mainly exists inside of the ghosts. It has been suggested that Na channels are surrounded by the PS.[30,31] Thus, if we dare to extend discussion to neuronal membranes, it seems that anesthetic molecules bind to the boundary acidic lipids and then change the mobility and conformation of the lipids; this perturbation for the lipids would anesthesia through the lipids (PS)–protein (Na channel) interactions.

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