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Local anesthetics destabilize lipid membranes by breaking hydration shell: infrared and calorimetry studies

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Abstract

Differential scanning calorimetry (DSC) showed that local anesthetics decreased the pretransition ($L_{\beta'} \rightarrow P_{\beta'}$) temperature of dipalmitoylphosphatidylcholine (DPPC) vesicle membranes four- to five-fold more than the main transition ($P_{\beta'} \rightarrow L_{\alpha}$) temperature. Because pretransition is mainly a change in the hydrophilic head property (tilted-rippled), the stronger effect on the pretransition suggests that the primary action site of local anesthetics is the lipid-water interface. The interfacial effect was analyzed by Fourier-transform infrared spectroscopy (FTIR) in water-in-oil (CCl_4) reversed micelles. FTIR showed that the local anesthetics released hydrogen-bonded water molecules from the phosphate (P = O bands) and glycerol (*sn*-2 C = O) moieties. The N-H stretching band of the local anesthetics was deconvoluted into two bands: hydrogen bonded to the phosphate moiety of the lipid and free (unbound to lipid). The formation constants between lipid P = O and anesthetic N-H were estimated in CCl_4 from the spectral changes: 110 M^{-1} for lidocaine and 250 M^{-1} for dibucaine. This small difference in the formation constants cannot explain the ten-fold stronger effect on the phase-transition temperature of dibucaine over lidocaine. By comparing the local anesthetic adsorption to the air/water interface in the presence and absence of lipid monolayers, we have previously shown (Lin et al. (1980) Biochim. Biophys. Acta 598, 51–65) that lipid–anesthetics interaction involves three forces: lipophilic effect, hydrophobic effect, and anesthetic–anesthetic interaction. The anesthetic potency depends mainly on the hydrophobic effect (the difference in the standard molar free energies of local anesthetics in water and at the interface) and anesthetic–anesthetic interaction energy. The anesthetic–anesthetic interaction means cooperativity of local anesthetics for the interfacial density: local anesthetics condense at the membrane surface when there are enough anesthetic molecules present at the interface to attract more anesthetics. The present data suggest that anesthetic action is directed to the interface between water and macromolecule, whether it is lipid membranes or proteins.

Key words: Local anesthetic; Lipid bilayer; Hydrogen bond; Hydrophobic effect; FTIR; Calorimetry

1. Introduction

The binding of local anesthetics with lipid membranes has been demonstrated by a number of methods: ESR [1], NMR [2–7], X-ray diffraction [8,9], infrared [10,11], UV-spectroscopy [12–18], surface tension [19–21], etc. Local anesthetics expand [22] and fluidize [1] lipid membranes. Melting of lipid membranes from the solid-gel to liquid-crystalline states by local anesthetics is well documented [12–18]. Numerous reports described the anesthetic effects on membrane fluidity. However, it has been clearly demonstrated that the same magnitude of fluidity can be achieved by a temperature elevation less than one degree [23,24]. The recent trend is to discredit lipid membranes as a site of anesthetic action because elevation of the temperature of this magnitude does not induce anesthesia.

The membrane fluidizing theory of anesthesia is complicated with two problems: (i) the lack of rigorous definition of the *membrane fluidity* [25], and (ii) difficulty in comparing anesthetic effects with heat effects. The heat-induced fluidity (disorder) is not identical to the anesthetic-induced disorder. The heat-induced disorder is kinetic and is caused by the increase in the thermal motion of lipid molecules (thermal entropy), while the anesthetic-induced disorder (mixing or cratic

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entropy) is configurational and is caused by the spatial displacement of lipid molecules by the presence of anesthetic molecules. Heat increases enzyme reaction rates and metabolism, while anesthetics decrease the reaction rates and metabolism. Heat is a force; anesthetic is a matter. Although both anesthetics and heat affects lipid membranes similarly, they cannot be compared on the same standard.

In physics, fluidity is defined as the reciprocal of viscosity. Viscosity is flow resistance, expressed in appropriate dimensions. Membrane fluidity is a dimensionless property. *Microviscosity*, expressed by the ease of movements of probe molecules in membranes, is not exactly *viscosity* because no flow occurs in membrane molecules. Nevertheless, membrane fluidity conveys intuitively the state and property of membranes and is a useful term when applied with caution.

Because of the ambiguity, membrane fluidity is expressed by various terms, depending upon the method of estimation. It is usually related to the state of hydrocarbon tails. Nuclear magnetic resonance (NMR) studies [2–7] revealed that local anesthetics interact with the interfacial region and change the conformations of head-group [2–7] and glycerol skeleton [4], without significantly affecting the property of hydrocarbon tails.

In phosphatidylcholine membranes, the main transition of lipid membranes between L_α is a core property, whereas pre-transition between $P_{\beta'}$ and $L_{\beta'}$ is a surface property. General anesthetics [26,27] and alcohols [28] were shown to depress the pretransition temperature stronger than the main transition temperature of phosphatidylcholine membranes. The higher sensitivity of pretransition is attributed to the lower enthalpy value of pretransition than main transition [29]. This study probed anesthetic-lipid interactions with differential scanning calorimetry (DSC) on the pre- and main phase transition temperatures. Fourier-transform infrared (FTIR) spectroscopy analyzed the hydrogen bonds of lipid membranes.

Clinical local anesthetics exist as a mixture of charged and uncharged forms at physiological pH. It is often assumed that charged forms are the active species, but uncharged benzocaine is also a highly potent local anesthetic. Boulanger et al. [2] reported that both species interact with lipid membranes and uncharged local anesthetics penetrate deeper into lipid bilayers. This study analyzed interaction mode of uncharged local anesthetics with net uncharged phosphatidylcholine membranes.

2. Materials and methods

Synthetic dipalmitoylphosphatidylcholine (DPPC, 99% + pure), tetracaine, and dibucaine were obtained from Sigma. Lidocaine base was a gift from Astra (Södertälje, Sweden). Water was distilled and treated by a Sybron/Barnstead water purifier (Boston, MA) consisting of two mixed-bed ion-exchanger columns, an activated carbon column, and an ultrafilter.

The DPPC vesicle membranes for differential scanning calorimetry (DSC) were prepared by sonicating DPPC 3.0 mM in water at a temperature above the main transition in the cuphorn of a Branson Sonifier Model 185 (Danbury, CT). The unilamellar vesicle suspension was aged for a week at 4°C to obtain a relatively homogeneous size distribution [30]. Crystalline anesthetics were added to the liposome. The amount of the local anesthetics added to the system was measured by weighing with a Perkin-Elmer Ultramicrobalance AD-2D (Norwalk, CT). To estimate the interaction mode of uncharged form of local anesthetics, the DSC study was performed at alkaline conditions by adding small amount of 0.1 N NaOH to pH 9 or over. By nonlogarithmic linear titration method we reported that the pK_a values of lidocaine, tetracaine, and dibucaine are 7.91_9, 8.46_3, and 8.72_9, respectively at 25°C [31].

The thermotropic phase transition of DPPC vesicle membranes was monitored by a MicroCal MC-2 differential scanning calorimeter (Northampton, MA) interfaced with an IBM personal computer. A computer-controlled Haake F3-CH heating-cooling water bath (Berlin, Germany) was used to scan the temperature. To prevent bubble formation in the DSC cell, the sample was degassed under vacuum with stirring for 5 min before transfer into the scanning cell. The DSC cell was pressurized by nitrogen gas at 15 psi. The heating and cooling scanning rates were 30°C per h. The DSC thermogram was analyzed by a MicroCal Origin software package.

The FTIR study probed the DPPC-water interface in a DPPC-H_2O-CCl_4 reversed micellar system to avoid large signal from water. The advantage of using reversed micelles for the study of interfacial bound water has been well documented [32–34]. The reversed micellar system was prepared by dispersing 6 mM DPPC in H_2O-CCl_4 mixture which contained 36 mM H_2O (15 wt%, water : lipid) in the cuphorn of the Branson sonicator. At this water-to-lipid ratio, all water molecules are bound to the DPPC headgroup.

A Perkin-Elmer Model 1750 FTIR spectrophotometer interfaced with a Model 7300 computer was used for analysis. The cell window was a fixed thickness zinc-selenide crystal with 1.25 mm pathlength (Spectra-Tech, Stamford, CT). A triglycine sulfate detector was used. The temperature was maintained at 22°C by circulating water from a constant temperature water bath, and was monitored by a United System (Dayton, OH) Digitec Model 5810 thermistor thermometer with 0.01°C resolution. Each sample was

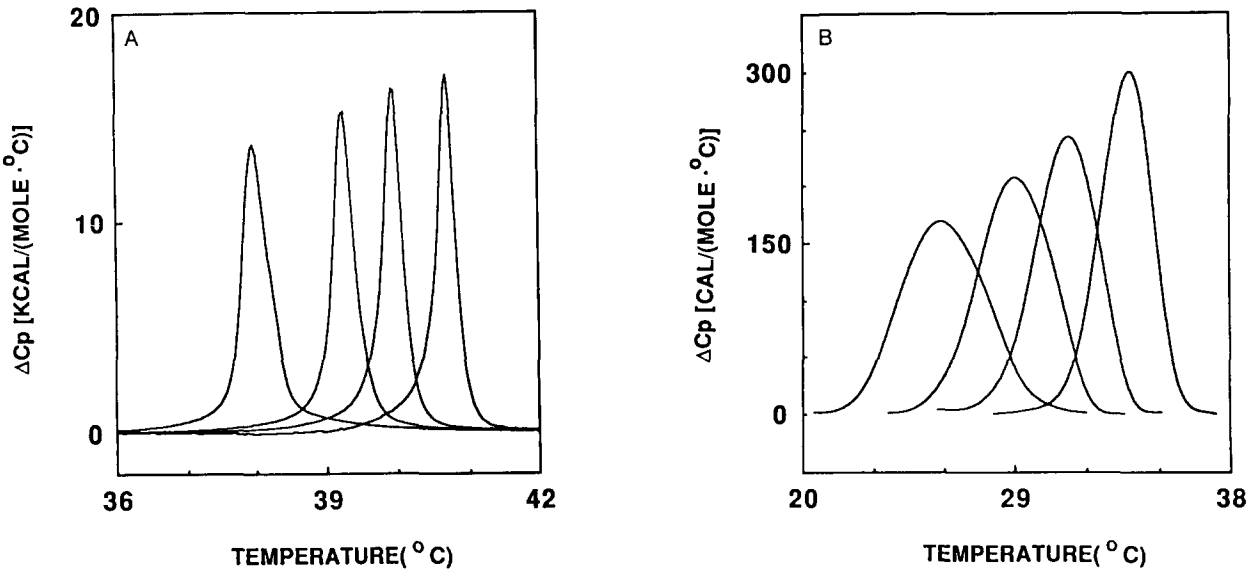

Fig. 1. (A) DSC thermogram of the lidocaine effects on the main transition of DPPC. The lidocaine concentrations are from the right; zero control, 1, 2, and 5 mM. (B) DSC thermogram of the lidocaine effects on the pretransition of DPPC. The lidocaine concentrations are from the right; zero control, 1, 2, and 5 mM.

scanned over the frequency range of 4000–450 cm^{-1}, and the spectra were obtained by averaging 50 scans.

Subtraction of spectra, estimation of the peak position, deconvolution, and estimation of the peak area were performed by a computer software, Perkin-Elmer IR Data Manager. The accuracy of the peak position was ± 0.1 cm^{-1}. The deconvoluted spectra of the C = O stretching band were obtained with a half-width smoothing function of 18 cm^{-1} and a narrowing factor of 2.25.

3. Results

Phase transition

Fig. 1 shows the effect of lidocaine 1.0–5.0 mM on the DSC thermogram for the pre- and main transitions of DPPC. Fig. 1A is the main transition, and Fig. 1B is the pretransition. Lidocaine decreased the transition temperature dose-dependently. The peak height decreased according to the increase in the anesthetic concentration, but the excess heat flow stayed at a constant value of 8.2 kcal/mol for the main transition and 815 cal/mol for the pretransition. Fig. 2 shows the dose-dependent decrease in the transition temperature by lidocaine. The anesthetic concentrations that decreased the transition temperature 1.0°C were lidocaine 0.36 mM and dibucaine 0.028 mM for the pretransition, and lidocaine 1.37 mM and dibucaine 0.148 mM for the main transition. The anesthetic effect was stronger on the pretransition temperature.

Hydrogen bonding

Fig. 3 is the difference spectra of the DPPC in H_2O-in-CCl_4 reversed micellar systems after subtracting the CCl_4 absorption bands. Fig. 3a is with 10 mM lidocaine and Fig. 3b is the control without lidocaine. Assignments of the major peaks are shown in the figure legend.

Fig. 4 shows the effect of lidocaine on DPPC between 3800 cm^{-1} and 3000 cm^{-1}, where the control reversed micelle (DPPC-H_2O-CCl_4) spectrum and the

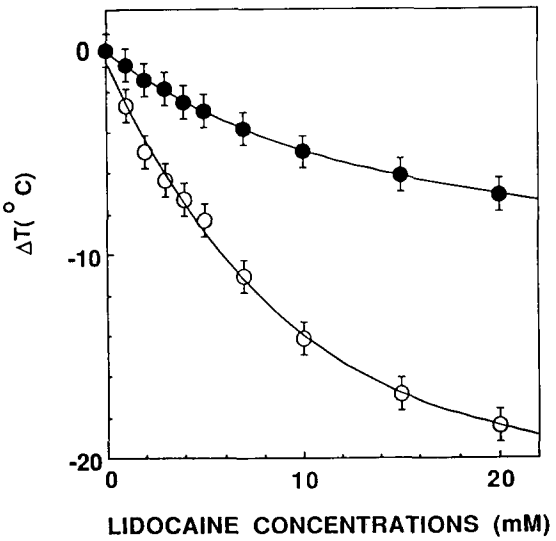

Fig. 2. Decrement of the transition temperature by lidocaine. Open circles are for the pretransition and closed circles are for the main transition.

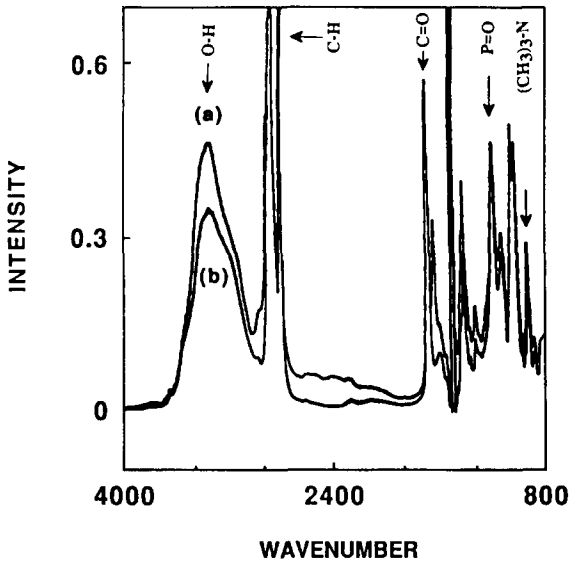

Fig. 3. Difference IR spectra of the DPPC-H_2O-CCl_4 reversed micelles. The CCl_4 absorption bands are subtracted. The system contained DPPC 6 mM and H_2O 36 mM in CCl_4. Tracing (a) is with 10 mM lidocaine and tracing (b) is control without lidocaine. The assignments of major peaks are: $(CH_3)_3$-N^+ stretching of the choline head at 970 cm^{-1}, P = O stretching at 1238 cm^{-1}, C = O stretching of the ester group at 1734 cm^{-1}, N-H stretching of the bound lidocaine at 3384 cm^{-1}, N-H stretching of the trans and cis forms of lidocaine at 3170 cm^{-1} and 3130 cm^{-1}, respectively, and O-H stretchings of the bound H_2O at 3400 cm^{-1} and unbound H_2O at 3480 cm^{-1}.

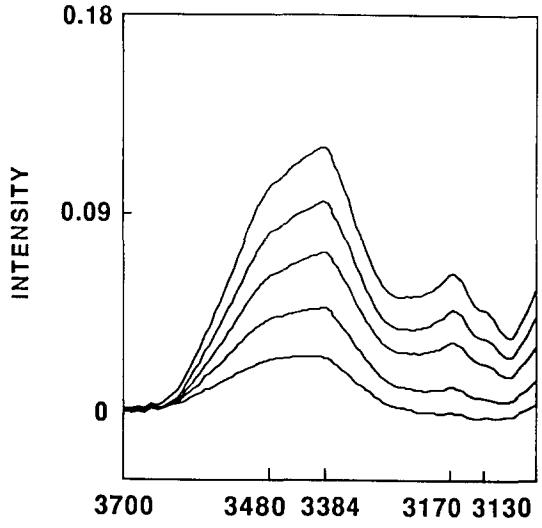

Fig. 4. Lidocaine interaction with DPPC. Truncated difference spectra of the lidocaine effects on the reversed micelles between 3800 cm^{-1} and 3000 cm^{-1}. The lidocaine-CCl_4 and DPPC-H_2O-CCl_4 spectra are subtracted. The lidocaine concentrations are from the top: 10, 7, 5, 3, and 1 mM. Four bands appeared at 3480 cm^{-1} (shoulder), 3384 cm^{-1}, 3170 cm^{-1} and 3130 cm^{-1} (shoulder). The assignments of peaks are (see text): N-H stretching of the cis and trans forms of lidocaine at 3130 cm^{-1} and 3170 cm^{-1}, respectively, N-H stretching of the bound lidocaine at 3384 cm^{-1}, and O-H stretchings of the bound H_2O at 3400 cm^{-1} and unbound H_2O at 3480 cm^{-1}.

lidocaine-DPPC-H_2O-CCl_4 mixture spectrum were subtracted. The difference spectra show four bands: 3480 cm^{-1} (shoulder), 3384 cm^{-1}, 3170 cm^{-1}, and 3130 cm^{-1} (shoulder). Similar results were obtained with dibucaine: N-H stretching at 3384 cm^{-1} for the bound and 3190 cm^{-1} for the unbound (spectra not shown).

To identify the above peaks, the anesthetic effects on DPPC in CCl_4 in the absence of H_2O were studied (Fig. 5). These are the difference IR spectra after subtracting the spectra of lidocaine-CCl_4 and DPPC-CCl_4 from the lidocaine-DPPC-CCl_4 spectrum at various lidocaine concentrations. In the absence of water, the 3480 cm^{-1} shoulder in Fig. 4 disappeared and only three bands were observed at 3384 cm^{-1}, 3170 cm^{-1}, and 3130 cm^{-1} (shoulder). This identifies that the 3480 cm^{-1} band is the O-H stretching of water, bound to lidocaine. The peak at 3384 cm^{-1} is the N-H stretching of lidocaine, bound to DPPC. The figure for dibucaine is not shown because the effects were similar to lidocaine.

The bound N-H stretching peak at 3384 cm^{-1} in Fig. 4 is deconvoluted in Fig. 6 as a Gaussian peak with 164 cm^{-1} half-width (Fig. 6b). Using this parameter, the unbound water peak at 3480 cm^{-1} (shoulder) is deconvoluted with 132 cm^{-1} half-width (Fig. 6c).

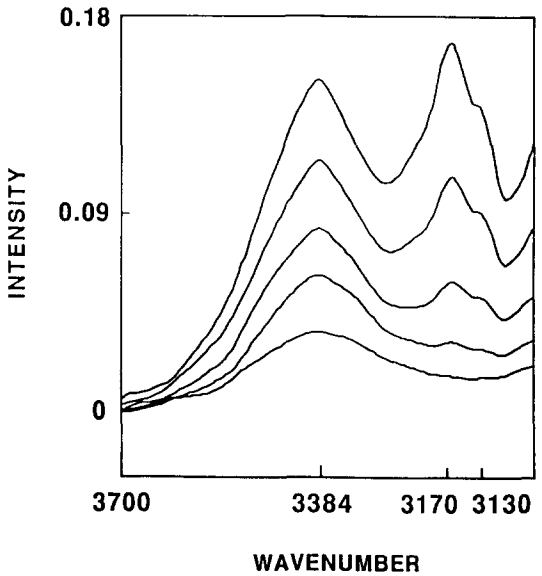

Fig. 5. Difference IR spectra of the lidocaine-DPPC-CCl_4 solution without water. The DPPC-CCl_4 and lidocaine-CCl_4 spectra are subtracted. The lidocaine concentrations are from the top; 10, 7, 5, 3, and 1 mM. Three bands appeared at 3384 cm^{-1}, 3170 cm^{-1} and 3130 cm^{-1}. The 3480 cm^{-1} peak disappeared, which is the free (unbound) water.

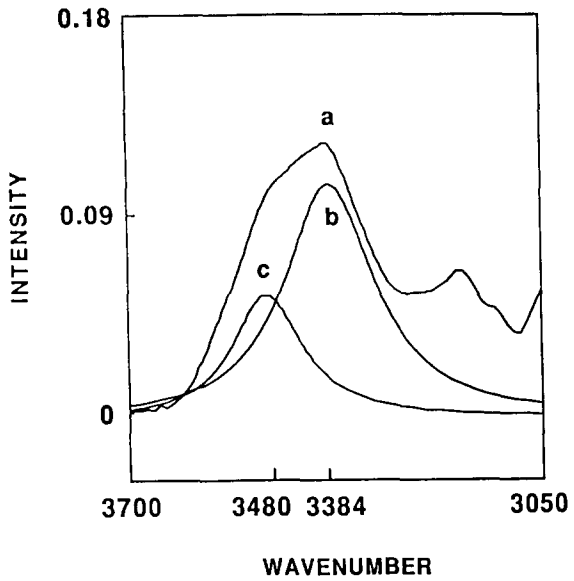

Fig. 6. Deconvoluted spectra of the lidocaine-DPPC-H_2O-CCl_4 reversed micelles. The lidocaine-CCl_4 and DPPC-H_2O-CCl_4 spectra are subtracted. Curve a, Original IR spectrum; curve b, bound N-H stretching of local anesthetic-lipid deconvoluted band (3384 cm^{-1}), and curve c, free O-H stretching band of the water-anesthetic deconvoluted band (3480 cm^{-1}).

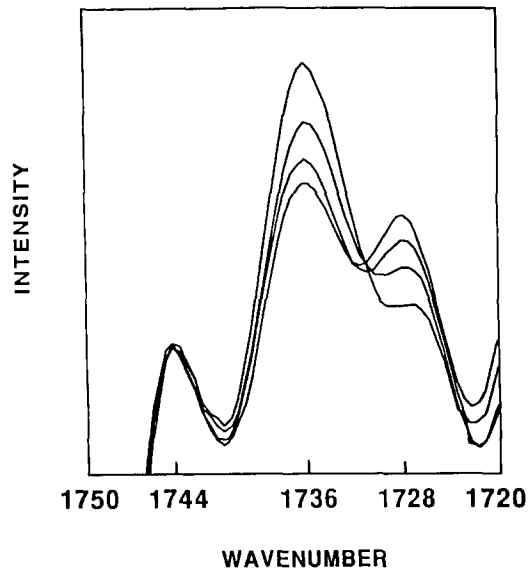

Fig. 7. Lidocaine effects on the deconvoluted C = O stretching band. The lidocaine concentrations are from the bottom at 1736 cm^{-1}: zero control, 3, 5, and 10 mM. The 1736 cm^{-1} band is the free *sn*-2, the 1728 cm^{-1} band is the hydrogen-bonded *sn*-2, and the 1744 cm^{-1} band is the free *sn*-1.

C = O stretching. There are two C = O groups in DPPC: *sn*-1 is the C = O group closer to the hydrophobic core, and *sn*-2 is closer to the polar head. These two C = O stretching bands overlap in the IR spectrum and appear as a single peak at 1734 cm^{-1} (not shown). Lidocaine shifted the C = O stretching band to 1740 cm^{-1}. The blue-shift indicates dehydration of this region [35–37].

Wong and Mantsch [35] deconvoluted the C = O stretching band of the partially hydrated DPPC into three components: the unhydrated free *sn*-1 band, the unhydrated free *sn*-2 band, and the hydrogen-bonded *sn*-2 band. In a fully hydrated system, only the free *sn*-1

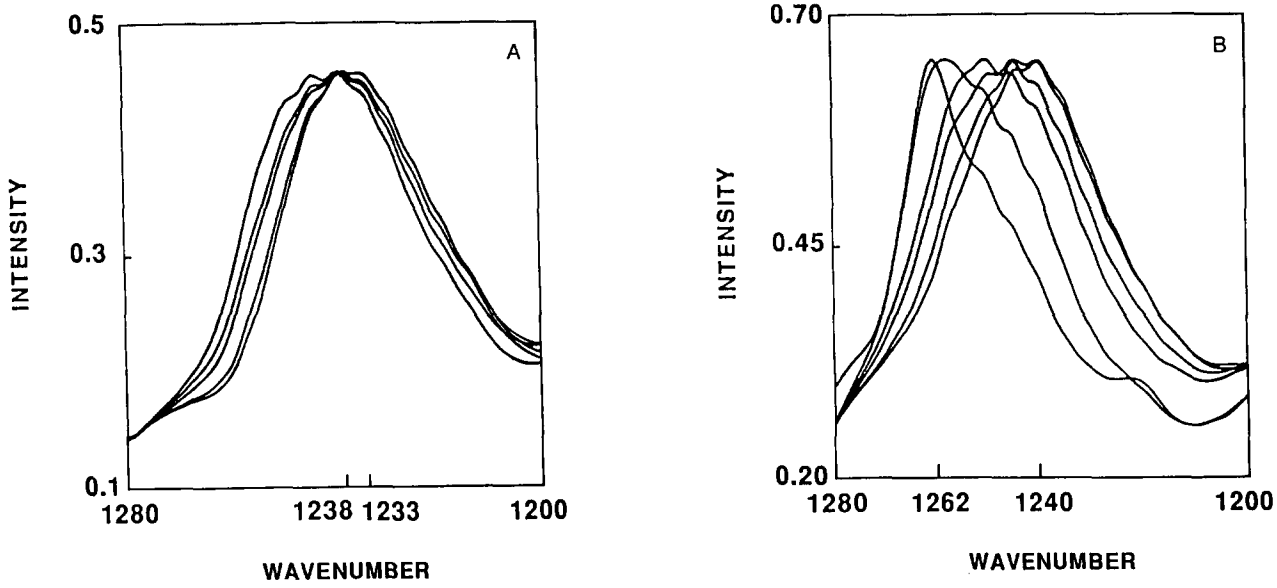

Fig. 8. (A) Lidocaine effects on the P = O stretching band in the reversed micelles. The lidocaine-CCl_4 and DPPC-H_2O-CCl_4 spectra are subtracted. The lidocaine concentrations are from the left: zero control at 1238 cm^{-1} 3, 5, 7, and 10 mM at 1233 cm^{-1}. (B) Lidocaine effects on the P = O stretching band in the DPPC-CCl_4 mixture without water. The DPPC-CCl_4 and lidocaine-CCl_4 spectra are subtracted. The lidocaine concentrations are from the left; zero control at 1262 cm^{-1}, 1, 3, 5, 7, and 10 mM at 1240 cm^{-1}.

band and the hydrogen-bonded *sn*-2 band were observed [35].

Fig. 7 is obtained by deconvoluting C = O bands. The band positions of free *sn*-1 (1744 cm^{-1}), free *sn*-2 (1736 cm^{-1}), and hydrogen-bonded *sn*-2 (1728 cm^{-1}) agree with the report by Wong and Mantsch [35]. When lidocaine concentration was increased, the intensity of the free *sn*-2 band (1736 cm^{-1}) increased while that of the bound *sn*-2 band (1728 cm^{-1}) decreased. The free *sn*-1 band (1744 cm^{-1}) did not change.

P = O stretching. The P = O band shifts to lower frequency when the P = O group forms hydrogen bonds with molecules containing OH or NH groups [37]. The wide P = O band with moderate intensity splits into several components [35–38]. Chiou et al. [37] discussed the possible causes of this splitting, but the exact mechanism remains to be elucidated.

Fig. 8A and 8B show lidocaine effects on the P = O stretching of DPPC in the presence (A) and absence (B) of water. The red shift of the P = O band indicates that lidocaine molecules bind to the phosphate moiety. The change in the presence of water is smaller than the change in the absence of water, because in the presence of water, phosphates were already bound to water molecules and local anesthetic molecules displaced the water molecules. The spectra in CCl_4 were used to estimate the binding constant between uncharged local anesthetic molecules and P = O moiety of DPPC (see below).

$(CH_3)_3$-N^+ stretching. In agreement with our studies on volatile anesthetics [39,40] and alcohols [37,38], the local anesthetics showed no effects on the $(CH_3)_3$-N^+ stretching band (data not shown).

Formation constants between phosphate group of lipid and local anesthetics

From the change in the spectral intensity of P = O stretching, the formation constant between the local anesthetics and DPPC is estimated in CCl_4 without water. Let L be the uncharged local anesthetic and M be DPPC. At low concentrations of anesthetics,

$$[L] + [M] \rightleftharpoons [LM]$$

and

$$K = \frac{C_{LM}}{C_L C_M}$$

where K is the formation constant of the anesthetic-lipid complex, and C_{LM}, C_L, and C_M are the concentrations of the complex, local anesthetics, and lipid, respectively. The observed absorbance, A, per unit cell length for this system is expressed,

$$A = \epsilon_L C_L + \epsilon_M C_M + \epsilon_{LM} C_{LM}$$

where ϵ_{LM}, ϵ_L and ϵ_M are the respective molar absorptivity of the complex, anesthetic, and lipid.

By designating ΔA as the change in the absorbance of the association band relative to pure L and M, the above equation is written as

$$\begin{aligned}\Delta A &= A - \epsilon_L C_L - \epsilon_M C_M \\ &= C_{LM}(\epsilon_{LM} - \epsilon_L - \epsilon_M) \\ &= \Delta\epsilon_{LM} C_{LM}\end{aligned}$$

When $C_L < C_M$, we have, approximately,

$$\Delta A = \frac{\Delta\epsilon_{LM} K C_L C_M}{1 + K C_M}$$

where $\Delta\epsilon_{LM}$ is the change in the absorptivity of the anesthetic-DPPC complex. The association band arises from N-H $\cdots$ P = O hydrogen bonding.

K is obtained by changing the concentration of local anesthetics at two DPPC concentrations. The ratio between the slopes plotted between ΔA and C_L at two DPPC concentrations is

$$\begin{aligned}\frac{\text{Slope 1}}{\text{Slope 2}} &= \frac{1 + K C_{M_2}}{1 + K C_{M_1}} \cdot \frac{\Delta\epsilon_{LM_1} K C_{M_1}}{\Delta\epsilon_{LM_2} K C_{M_2}} \\ &= \frac{(1 + K C_{M_2}) C_{M_1}}{(1 + K C_{M_1}) C_{M_2}}\end{aligned}$$

where subscripts 1 and 2 signify the two concentrations of DPPC. Because these two DPPC values are known, K is obtained.

The DPPC-lidocaine formation constant was 110 M^{-1} and the DPPC-dibucaine formation constant was 250 M^{-1}. To verify that these values represent the binding between the P = O moiety of the phospholipid and the N-H moiety of local anesthetics, the change in the intensity of the bound N-H stretching spectra of local anesthetics was analyzed by this equation. The values obtained from the N-H stretching data agreed with the above values.

4. Discussion

The present study revealed that the response of pretransition temperature to local anesthetics was much stronger than the main transition temperature in agreement with the reports on general anesthetics [26,27] and ethanol [28]. Pretransition is related to the reorientation of the DPPC head group and water, while the main transition to the orientational disorder of the hydrocarbon chains [41]. The interface-targeted action of local anesthetics is predictable because local anesthetics are amphiphilic molecules.

The main hydrogen bonding site of DPPC was the phosphate moiety. In the absence of water, the P = O stretching band appears at 1262 cm^{-1}. The peak shifts

to 1238 cm^{-1} by adding water, and further to 1233 cm^{-1} by adding lidocaine. The choline head cannot form hydrogen bonds because it lacks protons to donate, and the positive charge prevents accepting protons. The hydration of the choline head is weak because the charge is covered by the three hydrophobic CH_3 groups. Our FTIR studies [37–40] also failed to show any change in the choline head signal by volatile anesthetics and alcohols.

We [25,37,43] proposed that anesthetics destabilize membranes and proteins by releasing surface-bound water molecules. The hydrogen bond breaking activity of anesthetics was proposed by Eyring et al. [44] based on the large entropy increase when anesthetics bound to firefly luciferase [45]. Sandorfy and coworkers [46–49] found that partially fluorinated carbons broke hydrogen bonds. They [48,49] further demonstrated that anesthetics in general, with or without fluorine atoms, break macromolecule-water hydrogen bonds ($X\text{-}H_2O$) by forming competitive X-anesthetic hydrogen bonds and release water molecules from the binding site.

The present FTIR study demonstrates that local anesthetics release water molecules from the phosphate and C = O moieties of DPPC molecules. The new band at 3384 cm^{-1} is attributable to the N-H stretching of local anesthetics that bound to the phosphate group of DPPC.

The 'free' water peak (3480 cm^{-1}, unbound to DPPC) in Fig. 4 is deconvoluted in Fig. 6. There is no general agreement about the real state of unbound water because of the strong hydrogen bonding capability of water molecules. The peak probably represents water-anesthetic complex and is not free rotating water monomer [50–52]. Nevertheless, this peak demonstrates that local anesthetics released bound water molecules from the DPPC hydrophilic surface.

The present result demonstrates that the site of local anesthetics on lipid membranes is primarily the water-membrane interface. The NMR studies [2–6] unanimously reported that local anesthetics affect ^{31}P-NMR signal. By nuclear Overhauser effect ^{1}H-NMR, Kuroda and Fujiwara [5] reported a similar conclusion that local anesthetic potency is not related to membrane fluidity, but to the ability of binding with lipids at the polar head group of the bilayer.

The hydrophobic effect is often confused with lipophilic effect. This is especially true for anesthetics because the potency follows the Meyer-Overton rule, where the potency is correlated to the olive oil/water partition coefficients. The 'oil' phase is often misinterpreted to mean the hydrocarbon core of lipid membranes, but olive oil is not like apolar hydrocarbon solvents. The dielectric constant of olive oil is close to 1-octanol, which is 10. Because the dielectric constant of the lipid core is about 1.9, the binding site is the interfacial region of lipid membranes, or it can be hydrophobic surface on proteins. The concept of binding of amphiphilic molecules at the surface of lipid membranes without losing contact with water gained general acceptance in theory and experimental results (see, for instance [53]).

The mode of interaction of local anesthetics with lipid membranes remains controversial with negative enthalpy of binding reported by Seelig and Ganz [54]. They used titration calorimetry to estimate the binding of dibucaine to 1-palmitoyl-2-oleoyl-3-phosphocholine (POPC) vesicle membranes, and reported that the binding enthalpy was negative -1.5 kcal/mol, which is against the concept of classical hydrophobic effect [54]. However, our study with ^{14}C-labeled local anesthetics [55] showed that their binding to the membrane fraction of bovine brain homogenate carried positive ΔH of + 7.0 kcal/mol for procaine and + 3.4 kcal/mol for lidocaine at pH 7.4, respectively, indicating an entropy-driven interactions [54]. The cause of this discrepancy is unknown. Wimley and White [53] contested the 'nonclassical hydrophobic effect' proposed by Seelig and Ganz [54].

By comparing adsorption onto clear water surface and the surface covered with a DPPC monolayer measured by surface tension, we [21] separated the anesthetic binding into three forces: (i) lipid–anesthetic interaction (lipophilic effect), (ii) the tendency to escape from water (hydrophobic effect), and (iii) anesthetic–anesthetic interaction. The lipid–anesthetic interaction energy (lipophilic effect) was estimated from the increment of anesthetic adsorption at the air/water interface by the presence of lipid monolayer [21]. The values were $-2.95\ kT$ for procaine and $-2.99\ kT$ for tetracaine, where k is the Boltzmann constant and $T = 298$ K. The nerve blocking potency of tetracaine is about ten-fold stronger than procaine [56]. The differences in the lipid–anesthetic interaction energy was too small to account for the stronger nerve blocking potency of tetracaine over lidocaine. In contrast, the interaction energies among anesthetic molecules were $-0.056\ kT$ for procaine and $-0.397\ kT$ for tetracaine [21]. The adsorption of local anesthetics to the air/water interface was cooperative. The anesthetic–anesthetic interaction at the interface determines the cooperativity. The cooperativity means that local anesthetics do not condense at the interface until there are enough molecules at the interface close enough together to attract more anesthetics from the bulk. The difference in anesthetic binding is caused mainly by the anesthetic–anesthetic interaction energy and the tendency to escape from the water phase (the difference in the standard molar free energies of anesthetics at the interface and in water). The tendency to be excluded from the water determines the anesthetic concentration where the interfacial anesthetic molecules start to condense. The principal mechanism to deter-

mine anesthetic potency is the interfacial condensation of local anesthetic molecules, rather than the membrane–anesthetic attraction. This does not mean that lipophilic effect is negligible. Incorporation of cholesterol into phospholipid bilayers changes the affinity to local anesthetics [7] by the lipophilic interaction.

In the present study, the formation constant of dibucaine (250 M^{-1}) between the N-H group of the local anesthetics and P = O group of the DPPC head group differed little from that of lidocaine (110 M^{-1}). In contrast, there is a ten-fold difference in the effect on the phase transition temperatures. The formation constants were measured in an aprotic solvent, CCl_4, where hydrophobic effect is absent. The phase transition temperature was measured in water. Hydrophobic effect and anesthetic–anesthetic interaction, which also requires water, contributes a great deal for the anesthetic potency. If the principal mechanisms of local anesthetic binding is the hydrophobic effect and anesthetic–anesthetic interaction, the mechanism of anesthetic interaction with proteins may not be too different from that with lipid membranes.

5. Acknowledgments

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6. References

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