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The Effect of Tetracaine·HCl on Rotational Mobility of *n*-(9-Anthroyloxy) Stearic Acid in Outer Monolayers of Neuronal and Model Membranes

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To provide a basis for studying the pharmacological actions of tetracaine•HCl, we analyzed the membrane activities of this local anesthetic. The n-(9-anthroyloxy) stearic and palmitic acid (n-AS) probes (n = 2, 6, 9, 12 and 16) have been used previously to examine fluorescence polarization gradients. These probes can report the environment at a graded series of depths from the surface to the center of the membrane bilayer structure. In a dose-dependent manner, tetracaine•HCl decreased the anisotropies of 6-AS, 9-AS, 12-AS and 16-AP in the hydrocarbon interior of synaptosomal plasma membrane vesicles isolated from bovine cerebral cortex (SPMV), and liposomes derived from total lipids (SPMVTL) and phospholipids (SPMVPL) extracted from the SPMV. However, this compound increased the anisotropy of 2-AS at the membrane interface. The magnitude of the membrane rotational mobility reflects the carbon atom numbers of the phospholipids comprising SPMV, SPMVTL and SPMVPL and was in the order of the 16, 12, 9, 6, and 2 positions of the aliphatic chains. The sensitivity of the effects of tetracaine•HCl on the rotational mobility of the hydrocarbon interior or surface region was dependent on the carbon atom numbers in the descending order 16-AP, 12-AS, 9-AS, 6-AS and 2-AS and on whether neuronal or model membranes were involved in the descending order SPMV, SPMVPL and SPMVTL.

Key words : neuronal and model membranes, tetracaine •HCl, fluorescence probe technique, hydrocarbon interior, membrane interface, rotational mobility

Introduction

There are two proposals for the mechanism of pharmacological action of local anesthetics. One proposal is specific receptor theory. For a protein to be a feasible site for anesthetic binding one would anticipate that a hydrophobic pocket is required (Strichartz, 1973; Strichartz and Richie, 1987; Butterworth and Strichartz, 1990; Cutterall, 2000). The second proposal is membrane expansion theory (Seeman, 1972; Lee, 1976; Singer, 1977; Yun *et al.*, 1987, 2002; Smith *et al.*, 1991; Shibata *et al.*, 1995; Welin-Berger *et al.*, 2002; Mizogami *et al.*, 2002; Tsuchia *et al.*, 2005; Lee *et al.*, 2007). A number of theories propose various perturbations of bulk physical properties of the lipids of cell membranes as the primary event leading to inebriation or local anesthesia. One of the major lipid bilayer properties that have been proposed to be relevant to local anesthesia is membrane fluidity, which describes the motional and structural properties of the phospholipid acyl chains. Thus the activities of a number of membrane enzymes have been correlated with spectroscopic parameters that are modified by local anesthetics, observations that suggest a route by which these compounds could modulate membrane protein function (Lee, 1976; Yun *et al.*, 2002; Lee *et al.*, 2007). Membrane proteins are also sensitive to changes

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in membrane dimensions, such as the thickness of the lipid bilayer, which is also influenced by local anesthetics (Yun *et al.*, 1987). In the case of membrane fluidity, it is worth nothing that the effects varied considerably according to the lipid composition of the membrane. However, in spite of intense efforts its mechanism of pharmacological action at the molecular level has remained elusive.

The physical state of membrane lipids has been shown to influence such membrane enzymes as Na, K-ATPase (Chong *et al.*, 1985), hormone-responsive adenylate cyclase (Dipple and Houslay, 1978), and membrane transport processes such as glucose and amino acid uptake (Balcar *et al.*, 1980; Carrière and Le Grimellec, 1986). Membrane lipids also play an important role in membrane permeability to sodium, calcium, and potassium (Green *et al.*, 1980).

Effects of local anesthetics on motion, order and phase transitions of bulk bilayer systems of native or model membranes have received considerable attention in past decades. This is due to the interest in biological membranes as well as the unique information on intermolecular interactions that can be derived from the investigation of volume changes. It is known that the potency of an anesthetic increases roughly in proportion with its lipid/water partition coefficient, strongly suggesting an amphiphilic site for anesthetic molecules (Yun *et al.*, 1987; Miller *et al.*, 1986; Yun *et al.*, 1994; Kang *et al.*, 1996). Yun *et al.* (1987) reported that local anesthetics decreased microviscosity of synaptosomal plasma membrane vesicles isolated from the bovine cerebral cortex (SPMV). In addition, differential scanning thermograms of dimyristoylphosphatidylcholine multilamellar liposomes showed that local anesthetics significantly lowered the phase transition temperature, broadened the thermogram peaks, and reduced the size of the cooperative unit. Sweet *et al.* (1987) reported that prilocaine·HCl preferentially reduced the limiting anisotropy of 1,6-diphenyl-1,3,5-hexatriene in the inner monolayer of LM fibroblast plasma membrane. However, it was also true that local anesthetics had a fluidizing effect on the outer monolayer of membrane, although the effect was smaller than that of the inner monolayer. Recent fluorescence measurements showed that the highest affinity site for dibucaine in sarcoplasmic reticulum vesicles (SRV) is a lipid site near the membrane surface (Louro *et al.*, 1994). Furthermore, dibucaine·HCl has significant disordering effects on hydrocarbon interior of neuronal and model membranes, while it has significant ordering effects on the membrane interface of the membranes (Lee *et al.*, 2007). Most of the accumulated results for the analysis of local anesthetics' effect on the cell membrane fluidity used a single molecular probe for estimations of bulk membrane fluidity, and thus obtained information about one region (or average). However, the membrane fluidity may vary at different positions.

Previous studies have shown that the fluorophores of anthroyloxy derivatives locate at a graded series of levels from the surface to the center of the lipid bilayer structure (or a series of anthroyloxy fatty acids indicates that the depth of the group is almost linearly related to the number of carbon atoms between it and carboxyl group) (Thulborn and Sawyer, 1978; Tilley *et al.*, 1979; Villalaín and Prieto, 1991; Abrams and London, 1993; Mason, 1994; Tricerri *et al.*, 1994). The fluorophores of anthroyloxy derivatives can also be used to differentiate whether the bilayer has a fluidity gradient across it, as the anthroyloxy group can be positioned at different positions of the stearic acid moiety (Thulborn and Sawyer, 1978; Tilley *et al.*, 1979; Tricerri *et al.*, 1994; Schacter *et al.*, 1982; Collins *et al.*, 1990; Yun and Kang, 1990). These probes have been suggested to measure primarily the dynamic component of membrane fluidity (Schacter *et al.*, 1982; Vincent *et al.*, 1982; Schachter, 1984; Brasitus and Dudeja, 1985; Brasitus *et al.*, 1986).

The aim of this research is to provide a basis for studying the molecular mechanism of pharmacological action of tetracaine·HCl. This study was done through investigation of the effect of tetracaine·HCl on rotational mobility of the hydrocarbon interior and polar region (membranes interface, surface region) in the native and model membranes which differ in fluidity, and was done through investigation of magnitude of differential sensitivity between native and model membranes to the fluidizing or ordering effect of tetracaine·HCl. The study was carried out using 16-(9-anthroyloxy)palmitic acid (16-AP), 12-(9-anthroyloxy)stearic acid (12-AS), 9-(9-anthroyloxy)stearic acid (9-AS), 6-(9-anthroyloxy)stearic acid (6-AS) and 2-(9-anthroyloxy)stearic acid (2-AS) those reflecting rotational mobility at the 16, 12, 9, 6 and 2 position of aliphatic chains present in phospholipids of neuronal and model membranes.

Materials and Methods

Chemicals

The fluorescent anthroyloxy palmitate or stearate probes, 16-AP, 12-AS, 9-AS, 6-AS and 2-AS were purchased from Molecular Probes, Inc. (Junction City, OR, USA). Tetracaine·HCl, *N*-2-hydroxyethyl-piperazine-*N*'-2-ethanesulfonic acid (Hepes) and bovine serum albumin (BSA) were purchased from Sigma Chemical (St. Louis, MO, USA). All other reagents were purchased commercially and were of the highest quality available. Water was deionized.

SPMV preparation

The SPMV were prepared according to the procedure reported from earlier studies (Yun and Kang, 1990; Yun *et al.*, 1990). The specific activities of Na, K-ATPase, acetylcholinesterase and 5'-nucleotidase in the plasma membrane fraction were approximately 4-, 2.5- and 3-times higher than those in crude homogenates. The electron microscopic examination of the prepared SPMV showed very high purity. The vesicles, which were separated according to size, demonstrated homogeneous distribution and no longer showed the presence of intracellular organelles or leakage. The protein concentra-

tion was determined by the method of Lowry *et al.* (1951) using BSA as a standard.

Liposome preparation

Total lipids were extracted from the SPMV as previously described (Yun and Kang, 1990). Cholesterol content of the extracted total lipids was determined according to the Liebermann-Buchard reaction (Huang *et al.*, 1961). Phospholipids were quantitated by measuring the amounts of inorganic phosphate (Bartlett, 1959) after hydrolysis of the phospholipids at 180°C in 70% $HClO_4$ (Madeira and Antunes-Madeira, 1976). The SPMV had a high lipid to protein ratio (0.942 mg total lipids/1 mg protein) and a low cholesterol to phospholipid molar ratio. The value was 0.593 ± 0.011 (cholesterol 0.208 ± 0.010, phospholipids 0.702 ± 0.025). An average molecular weight of 775 for phospholipids is assumed and the molecular weight of cholesterol is 387 for the calculation. Phospholipids were composed (mol%) of phosphatidylcholine (41.55 ± 0.91), phosphatidylethanolamine (36.83 ± 0.48), phosphatidylserine (13.60 ± 0.26), sphingomyelin (4.15 ± 0.16), phosphatidylinositol (2.90 ± 0.09) and lysophosphatidylcholine (0.97 ± 0.03).

The characteristics of the lipid samples, such as size, lamellarity, radius of curvature, and shape, are strongly dependent on the method used to form the vesicles (Lee *et al.*, 2007; Lasic, 1988; Bagatolli and Gratton, 2000). As a consequence of the preparation method, the parameters that characterize the lipid phase equilibrium in lipid mixtures are affected by the lipid sample characteristics. Because the size of GUVs is on the same order as the size of cells, GUVs are becoming objects of intense scrutiny in diverse areas that focus on membrane behavior (Lee *et al.*, 2007; Bagatolli and Gratton, 2000; Menger and Keiper, 1998). Stock solutions of total lipids or phospholipids were made in chloroform. The concentration of the lipid stock solutions was 0.2 mg/ml. GUVs (SPMVTL or SPMVPL) with a mean diameter of 45 μm were prepared by the method developed by Angelova and Dimitrov (Angelova and Dimitrov, 1986; Dimitrov and Angelova, 1987; Angelova *et al.*, 1992). To grow the GUVs, a special temperature-controlled chamber, which was previously described (Bagatolli and Gratton, 1999; 2000), was used. The experiments were carried out in the same chamber after the vesicle formation, using an inverted microscope (Axiovert35: Zeiss, Thornwood, NY). The following steps were used to prepare the GUVs: 1) ~3 μl of the lipid stock solution was spread on each Pt wire under a stream on N_2. To remove the residues of organic solvent we put the chamber in a liophilizer for ~2 h. 2) To add the aqueous solvent inside the chamber (Millipore water 17.5 MΩ/cm), the bottom part of the chamber was sealed with a coverslip. The Millipore water was previously heated to the desired temperature (80°C for SPMVTL, 60°C for SPMVPL), and then sufficient water was added to cover the Pt wires. Just after this step, the Pt wires were connected to a function generator (Hewlett-Packard, Santa Clara, CA), and a low-frequency AC field (sinusoidal wave function with a frequency of 10 Hz and an amplitude of 3 V) was applied for 90 min. After the vesicle formation, the AC field was turned off.

Fluorescence measurements

The fluorescence measurements were taken using a modified method of earlier studies (Yun *et al.*, 2002; Lee *et al.*, 2007; Molitoris and Hoilien, 1987). The SPMV were suspended in phosphate-buffered saline (PBS) to concentration 50 μg of protein/ml. The liposomes (SPMVTL and SPMVPL) were suspended in PBS to give a concentration 0.2 mg of total lipids or total phospholipids/ml. Stock solutions of the 16-AP, 12-AS, 9-AS, 6-AS and 2-AS in methanol (2×10^{-5} M) were prepared and kept in a cold and dark place. Aliquots were added to the solutions of the native and model membranes so that the final concentrations of the 16-AP, 12-AS, 9-AS, 6-AS and 2-AS became 4×10^{-8} M (in the case of SPMV) or 2×10^{-8} M (in the cases of SPMVTL and SPMVPL) incorporated the probes. The mixture was stirred for 20 min at room temperature in order to reduce the concentration of methanol that might alter the rotational mobility of the SPMV, SPMVTL and SPMVPL. Also, the mixture was bubbled by dry nitrogen for 1 min with 20 min intervals in order to eliminate oxygen that might act as a quencher. To ensure complete removal of methanol residue in the mixture, the prepared mixtures were subjected to exhausted stirring for more than 2 hr which have shown the same results as the mixtures stirred for 20 min. Concentrated solution of tetracaine·HCl was prepared in PBS and added to the labeled membrane suspension to give the desired concentration of anesthetic. The pH of the buffered sample was not changed significantly by addition of tetracaine·HCl.

Fluorescence measurements were carried out with a Multi Frequency Cross-Correlation Phase and Modulation Fluorometer (ISS K2-003), equipped with a thermostated cell holder and performed at pH 7.4 (37 ± 0.1°C). The fluorescent probes, 16-AP, 12-AS, 9-AS, 6-AS and 2-AS, were excited at 360 nm (4 nm slit width) and those emissions recorded at 445 nm (8 nm slit width) through a sharp cut-off filter (Schott KV418). Corrections for light scattering (membrane suspensions without fluorescent probes) and for fluorescence in the ambient medium (quantified by pelleting the membranes after each estimation) were made routinely, and the combined corrections were less than 9% of the total fluorescence intensity observed for anthroyloxy palmitate or stearate-loaded suspensions. The intensity of the components of the fluorescence which were parallel ($I_{//}$) and perpendicular ($I_{\perp}$) to the direction of the vertically polarized excitation light, was determined by measuring the emitted light through polarizers oriented vertically and horizontally. Polarization (P) was obtained from intensity measurements using $P = (I_{//} - GI_{\perp})/(I_{//} + GI_{\perp})$, where G is a grating correction factor for the monochromator's transmission efficiency for vertically and horizontally polarized light. This value is given by the ratio of the fluorescence intensities of the vertical to horizontal

components when the exciting light is polarized in the horizontal direction. The polarization was expressed as anisotropy [$r = 2P/(3 - P)$] of 16-AP, 12-AS, 9-AS, 6-AS and 2-AS.

Results

In the present study, using fluorescence probe technique, we examined the amphiphilic effects of cation tetracaine·HCl on the differential rotational mobility between interface and hydrocarbon interior of SPMV, SPMVTL and SPMVPL. And we examined the differential effect of tetracaine·HCl on the different rotational mobility at the 16, 12, 9, 6 and 2 position of aliphatic chain present in phospholipids of neuronal and model membranes. Furthermore, we examined the different sensitivity to the increasing or decreasing effect of the rotational mobility of hydrocarbon interior or membrane interface by the tetracaine·HCl on the native and model membranes. In order to determine effects of the local anesthetic on the aforementioned rotational mobility, it was first necessary to demonstrate that the drug did not interact directly with fluorescent probes and thereby quench its fluorescence. Quenching of absorbance-corrected fluorescence intensity by the local anesthetic is not observed at all of the concentration levels where tetracaine·HCl was tested. Furthermore, if direct quenching of fluorescence of 16-AP, 12-AS, 9-AS, 6-AS and 2-AS by the local anesthetic occurred, fluorescence lifetime would decrease. However, the fluorescence lifetime of 16-AP was not changed by the local anesthetic in the SPMV. For example, the lifetime of 16-AP in the SPMV was 11.4 ± 0.2 (n = 5), 11.3 ± 0.1 (n = 5), 11.5 ± 0.3 (n = 5), 11.6 ± 0.1 (n = 5) and 11.2 ± 0.2 (n = 5) ns at 0.01, 0.1, 0.2, 1 and 2 mM tetracaine·HCl, respectively. Similar results were with 12-AS, 9-AS, 6-AS and 2-AS. Direct quenching of probe fluorescence by the local anesthetic used in the present experiments was ruled out.

The anisotropy (*r*) values of 16-AP for hydrocarbon interior of intact SPMV, SPMVTL and SPMVPL were 0.092 ± 0.002 (n = 5), 0.070 ± 0.002 (n = 5) and 0.047 ± 0.001 (n = 5) at 37°C (pH 7.4) respectively (Table 1). In contrast, the values of 2-AS for interface of intact SPMV, SPMVTL and SPMVPL were 0.126 ± 0.002 (n = 5), 0.114 ± 0.001 (n = 5) and 0.096 ± 0.001 (n = 5) at 37°C (pH 7.4) respectively (Table 1). This means that rotational mobility of hydrocarbon interior is faster than that of membrane interface. The rotational mobility's degrees of SPMV, SPMVTL and SPMVPL differed depending on the phospholipids, cholesterol and phospholipids are co-present and the proteins, cholesterol and phospholipids are co-present in the descending order of the SPMVPL, SPMVTL and SPMV.

The local anesthetic may induce disordering or ordering in their host lipids. Ordering takes place in membrane interface, whereas disordering occurs deep within the acyl chains. Although the local anesthetic has strong disordering effects on hydrocarbon interior of native and model membranes, ordering effects of local anesthetic on membrane interface are minor (pH 7.4, 37°C). This is consistent with the findings of Smith *et al.* (1991) and Lee *et al.* (2007). This has been interpreted in terms of different locations within the bilayer for the charged and neutral forms of the anesthetic; the neutral form is located deeper within the acyl chains and consequently the local anesthetic has a lesser effect on the head group (Smith *et al.*, 1991;Lee *et al.*, 2007).

Disordering effects of tetracaine·HCl on the rotational mobility of the hydrocarbon interior

The effect of increasing concentrations of the tetracaine·HCl on the anisotropy (*r*) of the 16-AP in the hydrocarbon interior of SPMV, SPMVTL and SPMVPL is shown in Figs. 1-3. The tetracaine·HCl decreased the anisotropy (*r*) of the 16-AP (increased rotational mobility) in a concentration-dependent manner. The significant decreases in the anisotropy (*r*) values by the tetracaine·HCl in the SPMV, SPMVTL and SPMVPL were observed even at such low concentrations as 0.02, 0.05 and 0.02 mM, respectively (Figs. 1-3). The effect of increasing concentrations of the local anesthetic on the anisotropy (*r*) of the 12-AS in the hydrocarbon interior of SPMV, SPMVTL and SPMVPL are shown in Figs. 1-3. The tetracaine·HCl decreased the anisotropy (*r*) of the 12-AS (increased rotational mobility) in a concentration-dependent manner. The significant decreases in the anisotropy (*r*) values by the tetracaine·HCl in the SPMV, SPMVTL and SPMVPL were observed even at such low concentrations as 0.02, 0.2 and 0.02 mM, respectively (Figs. 1-3). The effect of increasing concentrations of the local anesthetic on the anisotropy (*r*) of the 9-AS in the hydrocarbon interior of SPMV, SPMVTL and SPMVPL is shown in Figs. 1-3. The tetracaine·HCl decreased the anisotropy (*r*) of the 9-AS (increased rotational mobility) in a concentration-dependent manner. The significant decreases in the anisotropy (*r*) values by the tetracaine·HCl in the SPMV, SPMVTL and SPMVPL were observed at concentrations as 0.2, 0.5 and

Table 1. Fluorescence parameters of 16-AP, 12-AS, 9-AS, 6-AS and 2-AS in SPMV and SPMVTL and SPMVPL

Membranes	Parameter	16-AP	12-AS	9-AS
SPMV	Anisotropy	0.092 ± 0.002	0.099 ± 0.002	0.108 ± 0.001
SPMVTL	Anisotropy	0.070 ± 0.002	0.078 ± 0.001	0.098 ± 0.001
SPMVPL	Anisotropy	0.047 ± 0.001	0.051 ± 0.001	0.082 ± 0.001

Fluorescence measurements were performed at 37°C (pH 7.4). Values represent the mean ± SEM of 5 sample determinations.

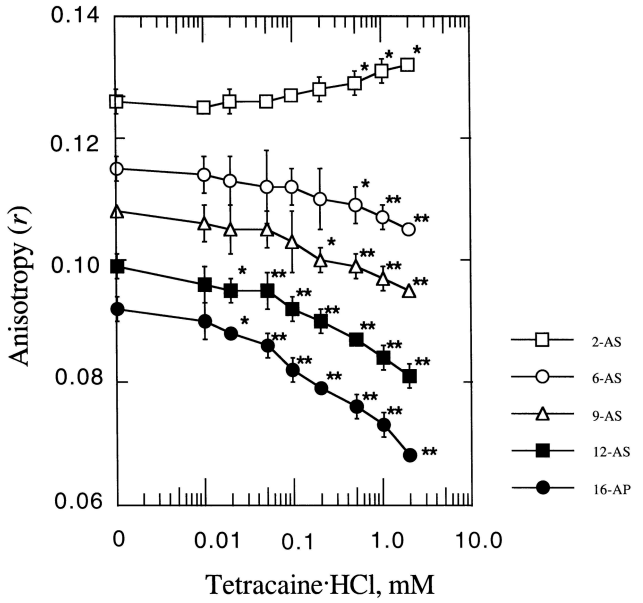

Fig. 1. Effects of tetracaine·HCl on the anisotropy (r) of 16-AP, 12-AS, 9-AS, 6-AS and 2-AS in SPMV. Fluorescence measurements were performed at 37°C (pH 7.4). Each point represents the mean ± SEM of 5 sample determinations. An asterisk and double asterisk signify $P < 0.05$ and $P < 0.01$, respectively, compared to control according to Student's t-test.

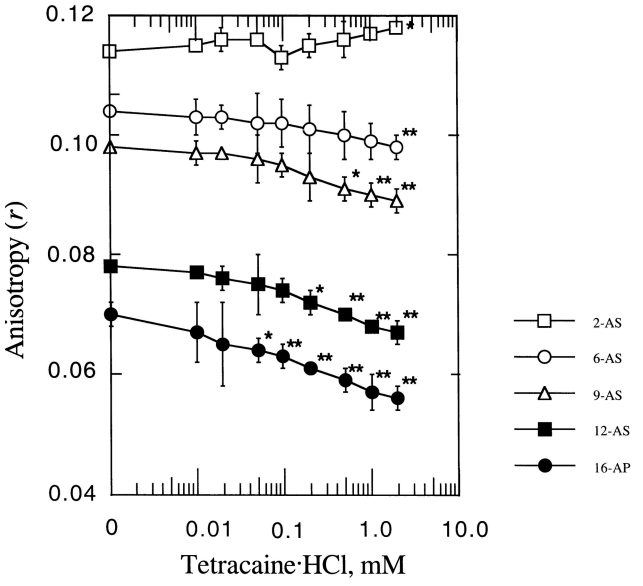

Fig. 2. Effects of tetracaine·HCl on the anisotropy (r) of 16-AP, 12-AS, 9-AS, 6-AS and 2-AS in SPMVTL. Fluorescence measurements were performed at 37°C (pH 7.4). Each point represents the mean ± SEM of 5 sample determinations. An asterisk and double asterisk signify $P < 0.05$ and $P < 0.01$, respectively, compared to control according to Student's t-test.

0.2 mM, respectively (Figs. 1-3). The effect of increasing concentrations of the local anesthetic on the anisotropy (r) of the 6-AS in the hydrocarbon interior of SPMV, SPMVTL and SPMVPL is shown in Figs. 1-3. The local anesthetic decreased the anisotropy (r) of the 6-AS in a dose-dependent

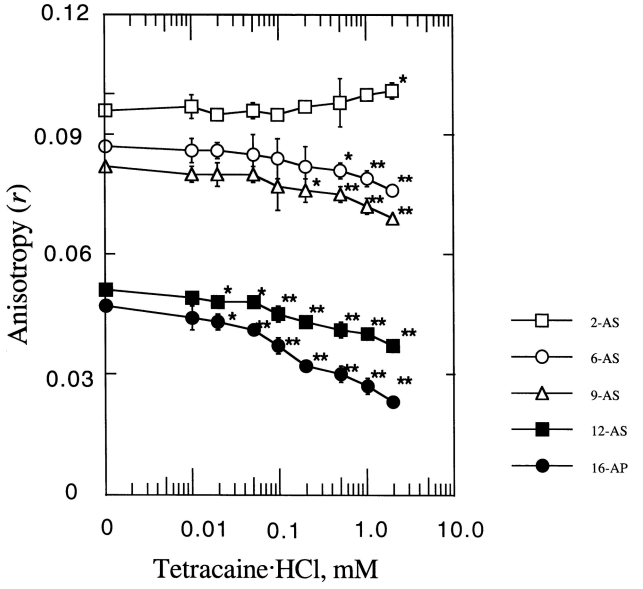

Fig. 3. Effects of tetracaine·HCl on the anisotropy (r) of 16-AP, 12-AS, 9-AS, 6-AS and 2-AS in SPMVPL. Fluorescence measurements were performed at 37°C (pH 7.4). Each point represents the mean ± SEM of 5 sample determinations. An asterisk and double asterisk signify $P < 0.05$ and $P < 0.01$, respectively, compared to control according to Student's t-test.

manner and the decrease in anisotropy (r) values in the SPMV, SPMVTL and SPMVPL by the tetracaine·HCl was at the concentration of 0.5, 2.0 and 0.5 mM, respectively (Figs. 1-3). The magnitude of the increased rotational mobility by the tetracaine·HCl was in the order at the position of 16, 12, 9 and 6 of aliphatic chains in phospholipids of neuronal and model membranes.

The differences in the anisotropy (r) values of the 16-AP found in hydrocarbon interior of SPMV, SPMVTL and SPMVPL before and after adding 0.1 mM tetracaine·HCl were 0.010, 0.007 and 0.010, respectively. These can be illustrated by comparing effects of temperature on this parameter. The anisotropy (r) of the 16-AP in hydrocarbon interior of SPMV, SPMVTL and SPMVPL are 0.092 ± 0.002 (n = 5), 0.070 ± 0.002 (n = 5) and 0.047 ± 0.001 (n = 5) at 37°C (pH 7.4), respectively. The anisotropy (r) values of the 16-AP in hydrocarbon interior of SPMV, SPMVTL and SPMVPL are 0.122 ± 0.003 (n = 5), 0.106 ± 0.001 (n = 5) and 0.086 ± 0.001 (n = 5) at 25°C (pH 7.4), respectively. Thus, the differences in the anisotropy (r) values at the position of 16 of carbon atom number in hydrocarbon interior of SPMV, SPMVTL and SPMVPL before and after adding 0.1 mM tetracaine·HCl were 0.010, 0.007 and 0.010 respectively, which were as large as those produced by the temperature raises of approximate 4.0, 2.3 and 3.1°C respectively.

Ordering effects of tetracaine·HCl on the rotational mobility of the membrane interface

The effect of the local anesthetic on the anisotropy (r) values of the 2-AS in the interface of SPMV, SPMVTL and

SPMVPL are shown in Figs. 1-3. The tetracaine·HCl increased the anisotropy (*r*) values of the 2-AS (decreased rotational mobility) in interface of SPMV, SPMVTL and SPMVPL in a concentration-dependent manner. The significant increase in the anisotropy (*r*) value in interface SPMV by tetracaine·HCl was observed even at 0.5 mM (Fig. 1). The significant increase of the anisotropy (r) value in interface of SPMVTL and SPMVPL by tetracaine·HCl was observed at 2.0 and 1.0 mM, respectively (Figs. 2-3). The anisotropy (*r*) values of the 2-AS in interface of SPMV, SPMVTL and SPMVPL raised by 0.006, 0.004 and 0.005 respectively, which values are larger than 2.0 mM tetracaine·HCl was added. Variations in the anisotropy (*r*) values were also noticed by the change in temperature as mentioned earlier. At 37°C (pH 7.4), the anisotropy (*r*) values of the 2-AS in interface of SPMV, SPMVTL and SPMVPL are 0.126 ± 0.002 (n = 5), 0.114 ± 0.001 (n = 5) and 0.096 ± 0.001 (n = 5), respectively. On the other hand, at 25°C (pH 7.4), the anisotropy (*r*) values of the 2-AS in interface of SPMV, SPMVTL and SPMVPL are 0.165 ± 0.003 (n = 5), 0.157 ± 0.002 (n = 5) and 0.142 ± 0.001 (n = 5), respectively. Based on the results obtained at the two different temperatures, the observed effects by the addition of 2.0 mM tetracaine·HCl, different values 0.006, 0.004 and 0.005 were comparable to the effect of the temperature changes by approximately 1.8, 1.1 and 1.3°C.

Discussion

We paid a special attention to the study by Villalaín and Prieto (1991) among several studies (Thulborn and Sawyer, 1978; Tilley *et al.*, 1979; Villalaín and Prieto, 1991; Abrams and London, 1993; Mason, 1994; Tricerri *et al.*, 1994) in regard to 2-AS distribution in membranes. The study (Villalaín and Prieto, 1991) clearly indicated the distribution region. It was reported that the 2-AS is a peculiar member of the family of probes, as the chromophore is adsorbed on the membrane interface (Villalaín and Prieto, 1991). Membrane interface is difficult to define precisely and it certainly includes the polar region near the phospholipid headgroups, or even the carbonyl groups, which are largely involved in hydrogen bonds. The degree of rotational mobility in according with the carbon numbers of phospholipids comprising of neuronal and model membranes was in the order at the position of 16, 12, 9, 6, and 2 of aliphatic chains in phospholipids, respectively. Using the membrane interface probe, 2-AS, we found that tetracaine·HCl decreased the rotational mobility of lipids in the interface of neuronal and model membranes. On the contrary, using the hydrophobic interior probes, 16-AP, 12-AS, 9-AS and 6-AS, we found that tetracaine·HCl increased rotational mobility of lipids in hydrophobic region of neuronal and model membranes. Furthermore, we found that in terms of increase or decrease of mobility of the neuronal and model membrane lipid bilayers by tetracaine·HCl, the magnitude of effects of tetracaine·HCl was greater in neuronal membranes than model membranes.

The potency of local anesthetics is generally defined as the dose necessary to achieve a specified end point. Clinically, the potency is usually the total mass (or moles) of drug required to relieve or prevent pain, produce tactile numbness, or effect inhibition of sympathetic or motor activity. By comparison, dibucaine·HCl is the most potent and one of the most toxic of the long-acting local anesthetics, and dibucaine·HCl is 15 to 20 times more potent and 15 times more toxic than procaine·HCl (Swinyard, 1985), tetracaine is approximately 10 times more potent as procaine (Yagiela, 1986; Mitchell, 1982). The physicochemical behavior of local anesthetics is a consistent and somewhat predictable function of their structure (Kim *et al*., 2007). Local anesthetic molecules with larger alkyl groups on both the tertiary amine nitrogen and the aromatic moiety show greater hydrophobicity.

In a dose-dependent manner, the tetracaine·HCl used in the present study lowered the anisotropy (*r*) values of 16-AP, 12-AS, 9-AS and 6-AS in hydrocarbon interior of SPMV, SPMVTL and SPMVPL but increased anisotropies of 2-AS in interface of the native and model membranes. When we take the results of this study and our previous study (Lee *et al.*, 2007), the disordering or ordering effects of the dibucaine·HCl on the lipid bilayer occurred in the order of dibucaine (ester type) > tetracaine (ester type), which is in accordance with the clinical potency of the local anesthetics.

Our data on tetracaine·HCl suggest that the observed anisotropy (*r*) values reflect differences in disordering or ordering constraints in hydrocarbon interior and interface of SPMV, SPMVTL and SPMVPL. This is due to differences in the intrinsic component and/or the structure in interface and hydrocarbon interior of the native and model membranes. The mechanism of the action(s) of tetracaine·HCl on disordering and ordering effects on the neuronal and model membranes is not well-understood. Nuclear magnetic resonance (NMR) studies have demonstrated that local anesthetic dibucaine (Louro *et al.*, 1994) and tetracaine is located closer to the head groups than to the center of hydrocarbon region and that the charged form of tetracaine is effective in changing the head group conformation (Boulanger *et al.*, 1981; Kelusky *et al.*, 1986; Seeling *et al.*, 1988; Hong *et al*., 2010). The magnitude of these effects depends on the nature and phase behavior of the phospholipid and on whether the anesthetic is charged (Smith *et al.*, 1991; Auger *et al.*, 1988). Water associates with the head group region of phospholipids via hydrogen bonding (Boggs, 1987). Local anesthetics bind (the competitive binding of the local anesthetics and water) strongly to the phosphate moiety of the phospholipids in membrane inter- face and weakly to the carbonyl group in competition with water, and effectively establish formation of hydrogen bonds with the carbonyl moiety, which is associated with a significant change in hydration of the local anesthetic molecules themselves (Shibata *et al.*, 1995). Incorporation of local anesthetics into the native and model

membranes cause alterations of the interface's charge density of the membrane, and a conformational change in phospholipid head groups (Shibata *et al.*, 1995). At the same time, local anesthetics may exert a significant influence on hydration of the lipid bilayer. As a result, such competitive binding decreases rotational mobility and increases hydrophobicity (Shibata *et al.*, 1995). The interaction of the local anesthetics with the hydrocarbon interior will generate rearrangements of the intermolecular hydrogen-bonded network among phospholipid molecules and/or protein molecules that are associated with the liberation of hydrated water molecules on the native and model membranes (Shibata *et al.*, 1995). The interaction will also change the orientation of the P-N dipole of phospholipid molecules (Scherer and Seeling, 1989). These changes should cause disordering of the hydrocarbon interior, and thus they could affect the transport of Na^+ and K^+ in nerve membranes, leading to anesthetic action.

The sensitivities to the increasing effect of the rotational mobility of the hydrocarbon interior by the tetracaine·HCl differed depending on the native and model membranes in the descending order of the SPMV, SPMVPL and SPMVTL. When we take the results of this study and our previous study (Lee *et al.*, 2007), it is without a doubt that the local anesthetic agent increases the rotational mobility of the hydrocarbon interior of the membrane. What could be the reason that the effects on neuronal membranes where phospholipids, cholesterol and proteins are co-present are greater compared to the effect on model membranes where the protein is not co-present? It is presumed because proteins magnify the effect of tetracaine·HCl on lipids through protein-lipid interaction. These effects are not solely due to the influence of the local anesthetic on lipids, but they are magnified by the interaction among lipids, proteins and water. Water plays a fundamental role in cell membrane structure in that it drives the formation of the lipid bilayer, with a polar surface facing the aqueous environment and a hydrophobic interior containing the fatty acyl chains and transmembrane proteins. In general, the structure and dynamics of proteins are also to a large extent governed by interactions with water (Teeter, 1991). Water penetrates into lipid bilayers at least as far as the glycerol backbone and also deeper between fatty acyl chain packing defects. Water at the protein-lipid interface is an additional factor involved in influencing the lipid bilayer structure. The introduction of small peptides, consisting of three amino acids, can cause a shift of water deeper into the bilayer, indicating increased hydration (Jacobs and White, 1989). Altered hydration may have marked effects on membrane protein/lipid functioning, possibly due to the formation of hydrogen bonds between the interchain water and protein amino acid side chains facing/lipid acyl chains facing into the hydrophobic interior of the membrane. It is possible that the proteins organize the lipid in a way that makes them more susceptible to the anesthetic.

Opinions have been divided as to whether local anesthetic interfered with membrane protein function by directly binding to the proteins, or whether the main modes of action occurred indirectly through a change in the physicochemical properties of the lipid membranes into which the local anesthetic readily diffused. Because biological membranes are of highly complex composition, it has not been feasible to monitor changes in the local lipid environment and to determine its effect on the membrane protein function at the same time. It is possible to explain the multiplication effects citing the increased mobility of protein triggered by lipids, but the reverse case of protein triggering change in lipids is more likely. It is certain that tetracaine·HCl increases the mobility of the neuronal lipid bilayer but the direct effects of tetracaine·HCl on protein appear to have magnified such effects on the lipid. In conclusion, the present data suggest that tetracaine·HCl, in addition to its direct interaction with sodium channels, concurrently interact with membrane lipids, affecting fluidity of the neuronal membranes.

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