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# Amphiphilic effects of local anesthetics on rotational mobility in neuronal and model membranes

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**Abstract**

To provide a basis for studying the molecular mechanism of pharmacological action of local anesthetics, we carried out a study of the membrane actions of tetracaine, bupivacaine, lidocaine, prilocaine and procaine. Fluorescence polarization of 12-(9-anthroyloxy)stearic acid (12-AS) and 2-(9-anthroyloxy)stearic acid (2-AS) were used to examine the effects of local anesthetics on differential rotational mobility between polar region and hydrocarbon interior of synaptosomal plasma membrane vesicles (SPMV) isolated from bovine cerebral cortex, and liposomes of total lipids (SPMVTL) and phospholipids (SPMVPL) extracted from the SPMV. The two membrane components differed with respect to 2 and 12 anthroyloxy stearate (2-AS, 12-AS) probes, indicating that a difference in the membrane fluidity may be present. In a dose-dependent manner, tetracaine, bupivacaine, lidocaine, prilocaine and procaine decreased anisotropy of 12-AS in the hydrocarbon interior of the SPMV, SPMVTL and SPMVPL, but tetracaine, bupivacaine, lidocaine and prilocaine increased anisotropy of 2-AS in the membrane interface. These results indicate that local anesthetics have significant disordering effects on hydrocarbon interior of the SPMV, SPMVTL and SPMVPL, but have significant ordering effects on the membrane interface, and thus they could affect the transport of $Na^+$ and $K^+$ in nerve membranes, leading to anesthetic action. 

*Keywords:* Fluorescent probe technique; Rotational mobility; Hydrocarbon interior; Membrane interface; Local anesthetic; Biomembranes

## 1. Introduction

The molecular mechanism of pharmacological action of local anesthetics has long been a subject of great interest. Two general theories have been proposed to explain the action of local anesthetics on sodium channel: one considers a direct binding of local anesthetic molecules to specific receptors on sodium channels [1,2] and the other proposes the general perturbation of the bulk membrane structure by anesthetics and its consequences on channel function [3–6]. There is a large amount of evidence in support of the specific receptor hypothesis [7]. General membrane perturbation may also contribute to an explanation of anesthetic actions [7].

The physical state of membrane lipids has been shown to influence such membrane enzymes as NaK-ATPase [8], hormone-responsive adenylate cyclase [9], and membrane transport processes such as glucose and amino acid uptake [10,11]. Membrane lipids also play an important role in membrane permeability to sodium, calcium, and potassium [12].

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 inter-

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actions 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 [13–16]. Yun et al. [14] 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. [17] 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. Furthermore, recent fluorescence measurements showed that the highest affinity site for dibucaine in sarcoplasmic reticulum vesicles (SRV) is a lipid site near the membrane surface [18]. 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 the carboxyl group) [19–27]. 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 [25–44]. These probes have been suggested to measure primarily the dynamic component of membrane fluidity [39–44].

The aim of this research is to provide a basis for studying the molecular mechanism of pharmacological action of local anesthetics. This study was done through investigation of the effects of local anesthetics on rotational mobility of the hydrocarbon interior and polar 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 local anesthetics. In the present study, employing fluorescence polarization of 12-(9-anthroyloxy)stearic acid (12-AS) and 2-(9-anthroyloxy)stearic acid (2-AS), we investigated the amphiphilic effects of tetracaine, bupivacaine, lidocaine, prilocaine and procaine on hydrocarbon interior and polar region of SPMV isolated from bovine cerebral cortex and on liposomes of total lipids (SPMVTL) and phospholipids (SPMVPL) extracted from the SPMV.

## 2. Materials and methods

### 2.1. Materials

The fluorescent anthroyloxy stearate probes, 12-AS and 2-AS were purchased from Molecular Probes, Inc. (Junction City, OR, USA). Tetracaine·HCl, bupivacaine·HCl, lidocaine·HCl, prilocaine·HCl, procaine·HCl, *N*-2-Hydroxyethylpiperazine-*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.

### 2.2. SPMV preparation

The SPMV were prepared according to the procedure reported from earlier studies [45,46]. 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 concentration was determined by the method of Lowry et al. [47] using BSA as a standard.

### 2.3. Liposome preparation

Total lipids were extracted from the SPMV as previously described [45]. Cholesterol content of the extracted total lipids was determined according to the Liebermann–Burchard reaction [48]. Phospholipids were quantitated by measuring the amounts of inorganic phosphate [49] after hydrolysis of the phospholipids at 180°C in 70% $HClO_4$ [50]. 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 ($0.593 \pm 0.011$: cholesterol $0.208 \pm 0.010$, phospholipids $0.702 \pm 0.025$). 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 ($41.55 \pm 0.91$), phosphatidylethanolamine ($36.83 \pm 0.48$), phosphatidylserine ($13.60 \pm 0.26$), sphingomyelin ($4.15 \pm 0.16$), phosphatidylinositol ($2.90 \pm 0.09$) and lysophosphatidylcholine ($0.97 \pm 0.03$).

Stock solutions of total lipids or phospholipids were made in chloroform. The concentration of the lipid stock solutions was 0.2 mg/ml. Giant unilamellar vesicles (GUVs: SPMVTL or SPMVPL) with a mean diameter of 45 μm were prepared by the method developed by Angelova and Dimitrov [51–53]. To grow the GUVs, a special temperature-controlled chamber, which was previously described [54,55], 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.

### 2.4. Fluorescence measurements

The fluorescence measurements were taken using a modified method of earlier study [44]. The SPMV were suspended in 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 12-AS and 2-AS in methanol ($2\times10^{-5}$ 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 12-AS and 2-AS became $4\times10^{-8}$ M (in the case of SPMV) or $2\times10^{-8}$ 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, which 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. Concentrated solutions of local anesthetics were prepared in PBS and added to the labeled membrane suspension to give the desired concentration of anesthetics. The pH of the buffered sample was not changed significantly by addition of local anesthetics.

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, 12-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 anthroyloxystearate-loaded suspensions. The intensity of the components of the fluorescence which were parallel ($I_{\parallel}$) 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 $P=(I_{\parallel}-GI_{\perp})/(I_{\parallel}+GI_{\perp})$, 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 either 12-AS or 2-AS.

## 3. Results

In the present study, using the fluorescence probe technique, we examined the amphiphilic effects of cations of tetracaine, bupivacaine, lidocaine, prilocaine and procaine on the differential rotational mobility between the interface and hydrocarbon interior of SPMV, SPMVTL and SPMVPL. In order to determine effects of local anesthetics on the aforementioned rotational mobility, it was first necessary to demonstrate that the drugs did not interact directly with fluorescent probes and thereby quench its fluorescence. Quenching of absorbance-corrected fluorescence intensity by the local anesthetics is not observed at all of the concentration levels where tetracaine, bupivacaine, lidocaine, prilocaine and procaine were tested. Furthermore, if direct quenching of 12-AS and 2-AS by the local anesthetics occurred, fluorescence lifetime would decrease. However, the fluorescence lifetime of 12-AS was not changed by the local anesthetics in the SPMV. For example, the lifetime of 12-AS in the SPMV was 11.1±0.2, 11.5±0.1, 11.7±0.3, 11.3±0.1 and 11.4±0.2 ns at 0.01, 0.1, 0.2, 1 and 2 mM tetracaine, respectively. Similar results were with 2-AS. Direct quenching of probe fluorescence by the local anesthetics used in the present experiments was ruled out.

The anisotropy ($r$) values of 12-AS for hydrocarbon interior of intact SPMV, SPMVTL and SPMVPL were 0.099±0.002 ($n$=5), 0.078±0.001 ($n$=5) and 0.051±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

Table 1
Fluorescence parameters of 12-AS and 2-AS in SPMV and SPMVTL and SPMVPL extracted from SPMV

| Membranes | Parameter | 12-AS | 2-AS |
|---|---|---|---|
| SPMV | anisotropy | 0.099±0.002 | 0.126±0.002 |
| SPMVTL | anisotropy | 0.078±0.001 | 0.114±0.001 |
| SPMVPL | anisotropy | 0.051±0.001 | 0.096±0.001 |

Fluorescence measurements were performed at 37°C (pH 7.4). Values represents the mean±SE of five determinations.

(pH 7.4), respectively (Table 1). This means that the rotational mobility of the hydrocarbon interior is faster than that of the membrane interface. The rotational mobility's degrees of SPMV, SPMVTL and SPMVPL differed depending on the proteins and cholesterol in the descending order of the SPMVPL, SPMVTL and SPMV.

The local anesthetics may induce disordering or ordering in their host lipids. Ordering takes place in the membrane interface, whereas disordering occurs deep within the acyl chains. Although the local anesthetics have strong disordering effects on hydrocarbon interior of native and model membranes, ordering effects of local anesthetics on membrane interface are minor (pH 7.4, 37°C). This is consistent with the findings of Smith et al. [6]. This has been interpreted in terms of different locations within the bilayer for the charged and neutral forms of the anesthetics; the neutral form is located deeper within the acyl chains and consequently the local anesthetics have a lesser effect on the head group [6].

### *3.1. Disordering effects of local anesthetics on the rotational mobility of the hydrocarbon interior*

The effects of increasing concentrations of the local anesthetics on the anisotropy ($r$) of the 12-AS in the hydrocarbon interior of SPMV are shown in Fig. 1. The local anesthetics 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, bupivacaine, lidocaine, prilocaine and procaine were observed even at such low concentrations as 0.1, 0.1, 0.5, 0.5 and 1 mM, respectively (Fig. 1). The effects of increasing concentrations of the local anesthetics on the anisotropy ($r$) of the 12-AS in hydrocarbon interior of SPMVTL are shown in Fig. 2. The local anesthetics decreased the anisotropy ($r$) of the 12-AS in a concentration-dependent manner. The significant decreases in the anisotropy ($r$) values by the tetracaine, bupivacaine, lidocaine, prilocaine and procaine were observed to be significant at 0.2, 0.2, 5, 5 and 10 mM, respectively (Fig. 2). The effects of the local anesthetics on the anisotropy ($r$) of the 12-AS in hydrocarbon interior of SPMVPL are shown in Fig. 3. The local anesthetics decreased the anisotropy ($r$) of the 12-AS in a dose-dependent manner and the decreases in anisotropy ($r$) values by the tetracaine, bupivacaine, lidocaine, prilocaine and procaine were considerable at lower concentrations of 0.1, 0.1, 1, 1 and 5 mM, respectively (Fig. 3).

The differences in the anisotropy ($r$) values of the 12-AS found in the hydrocarbon interior of SPMV, SPMVTL and SPMVPL before and after adding 2 mM tetracaine·HCl were 0.018, 0.011 and 0.014, respectively. These can be illustrated by comparing effects of temperature on this parameter. The anisotropy ($r$) of the 12-AS in hydrocarbon interior of SPMV, SPMVTL and SPMVPL are $0.099\pm0.002$ ($n=5$), $0.078\pm0.001$ ($n=5$) and $0.051\pm0.001$ ($n=5$) at 37°C (pH 7.4), respectively. The anisotropy ($r$) of the 12-AS in hydrocarbon interior of SPMV, SPMVTL and SPMVPL are $0.128\pm0.003$ ($n=5$), $0.113\pm0.002$ ($n=5$) and $0.089\pm0.001$ ($n=5$) at 25°C (pH 7.4), respectively. Thus, the differences in the anisotropy ($r$) values of 12-AS

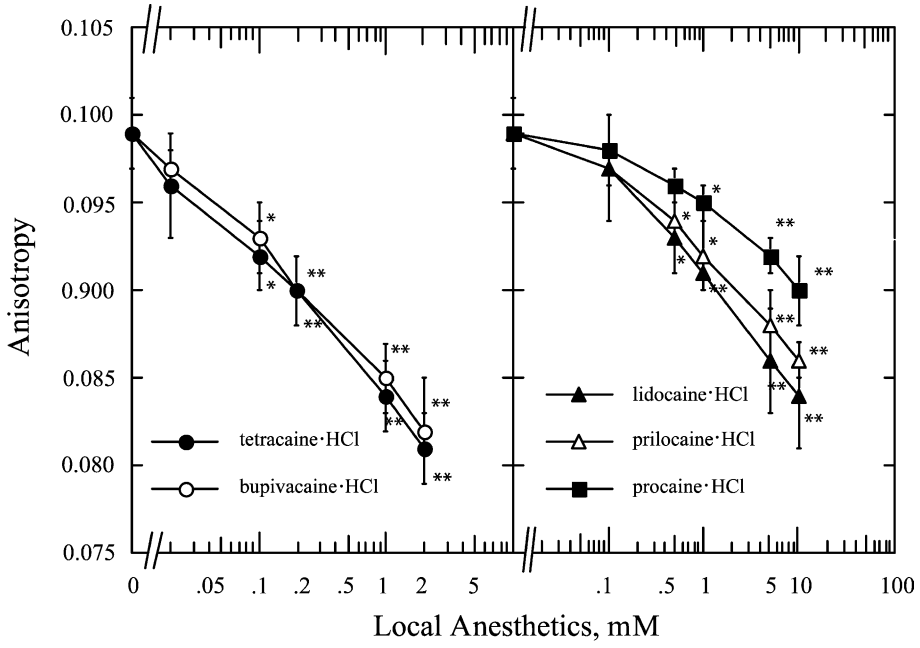


Fig. 1. Effects of local anesthetics on the anisotropy ($r$) of 12-AS in synaptosomal plasma membrane vesicles isolated from bovine cerebral cortex. Fluorescence measurements were performed at 37°C (pH 7.4). Each point represents the mean±SE of five determinations. An asterisk and double asterisk signify $P<0.05$ and $P<0.01$, respectively, compared to control according to Student's $t$-test.

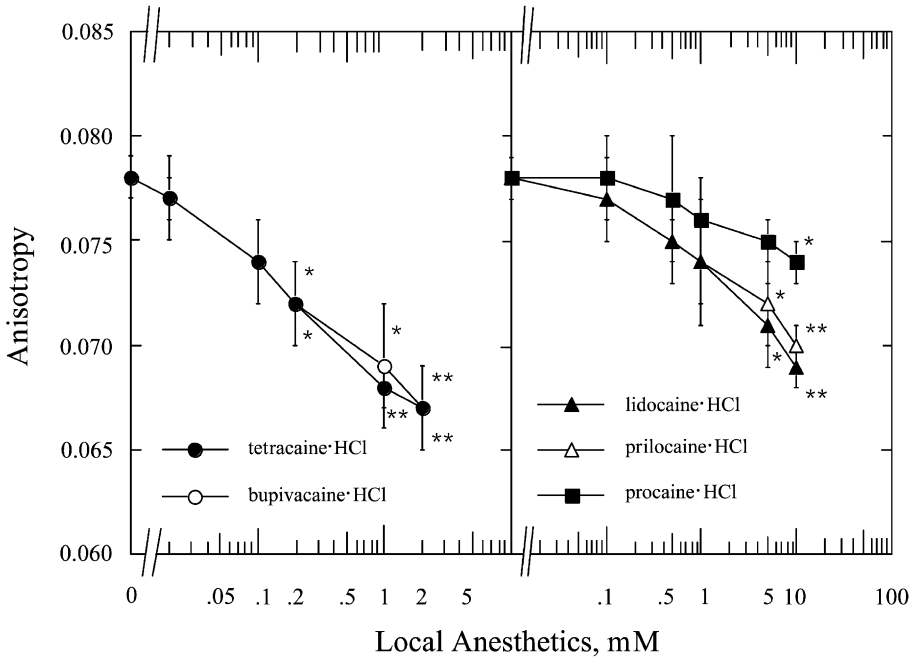


Fig. 2. Effects of local anesthetics on the anisotropy ($r$) of 12-AS in model membranes of total lipids extracted from synaptosomal plasma membrane vesicles. Fluorescence measurements were performed at 37°C (pH 7.4). Each point represents the mean±SE of five determinations. An asterisk and double asterisk signify $P<0.05$ and $P<0.01$, respectively, compared to control according to Student's $t$-test.

in hydrocarbon interior of SPMV, SPMVTL and SPMVPL before and after adding 2 mM tetracaine were 0.018, 0.011 and 0.014, respectively, which were as large as those produced by the temperature rises of approximately 7.4, 3.8 and 4.4°C, respectively.

### 3.2. Ordering effects of local anesthetics on the rotational mobility of the membrane interface

The effects of the local anesthetics on the anisotropy ($r$) of the 2-AS in the interface of SPMV are shown in Fig. 4. The

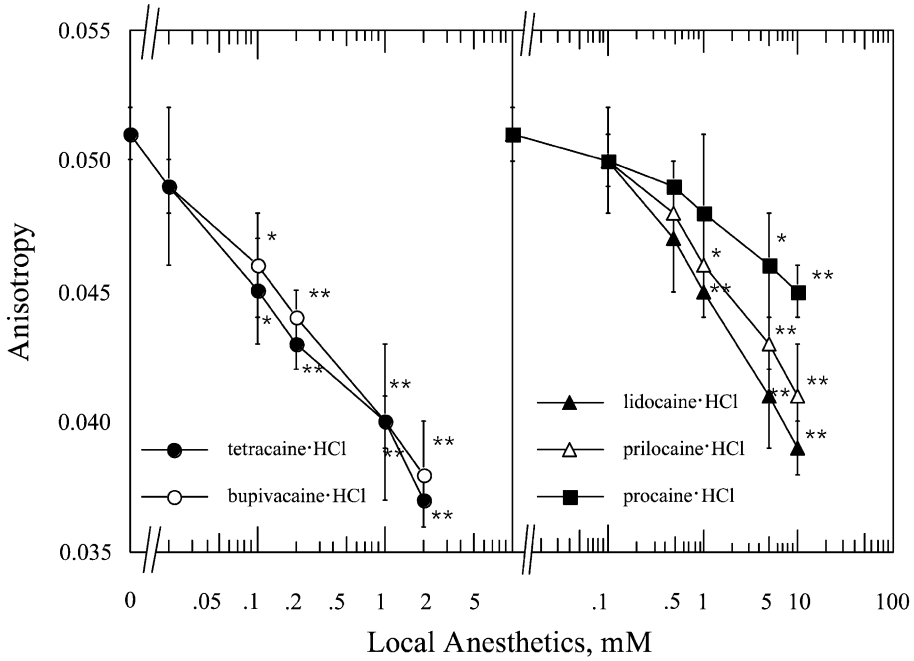


Fig. 3. Effects of local anesthetics on the anisotropy ($r$) of 12-AS in model membranes of total phospholipids extracted from synaptosomal plasma membrane vesicles. Fluorescence measurements were performed at 37°C (pH 7.4). Each point represents the mean±SE of five determinations. An asterisk and double asterisk signify $P<0.05$ and $P<0.01$, respectively, compared to control according to Student's $t$-test.

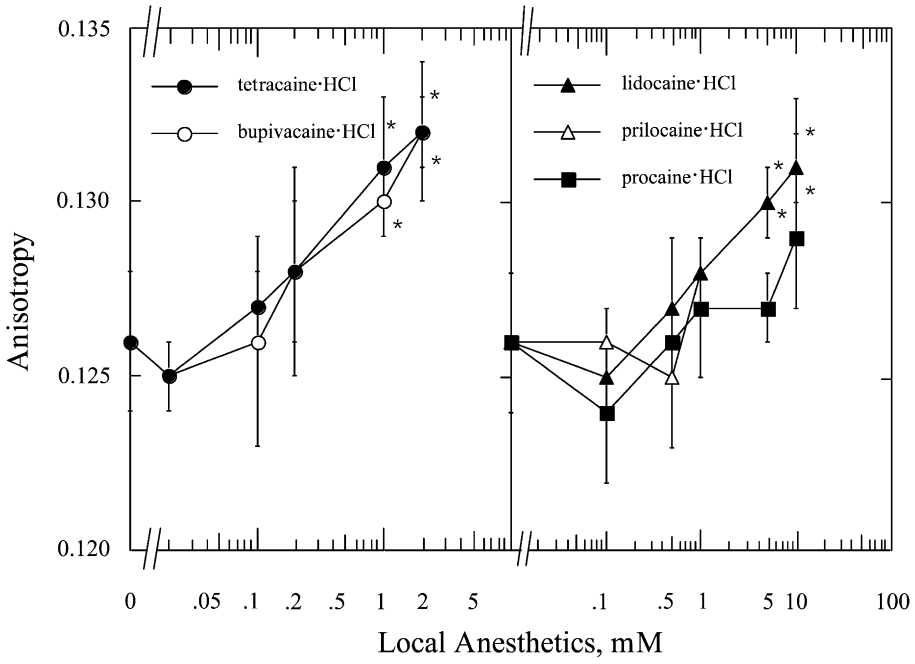


Fig. 4. Effects of local anesthetics on the anisotropy ($r$) of 2-AS in synaptosomal plasma membrane vesicles isolated from bovine cerebral cortex. Fluorescence measurements were performed at 37 °C (pH 7.4). Each point represents the mean±SE of five determinations. An asterisk signifies $P<0.05$ compared to control according to Student's $t$-test.

local anesthetics increased the anisotropy ($r$) of the 2-AS (decreased rotational mobility) in interface of SPMV in a concentration-dependent manner. The significant increases in the anisotropy ($r$) values by tetracaine, bupivacaine, lidocaine and prilocaine were observed even at 1, 1, 5 and 5 mM, respectively (Fig. 4). However, the anisotropy ($r$) of the 2-AS in membrane interface was not changed significantly in the entire concentration range (0.1–10 mM) when procaine was used, as seen in Fig. 4. The significant increases of the anisotropy ($r$) values in interface of

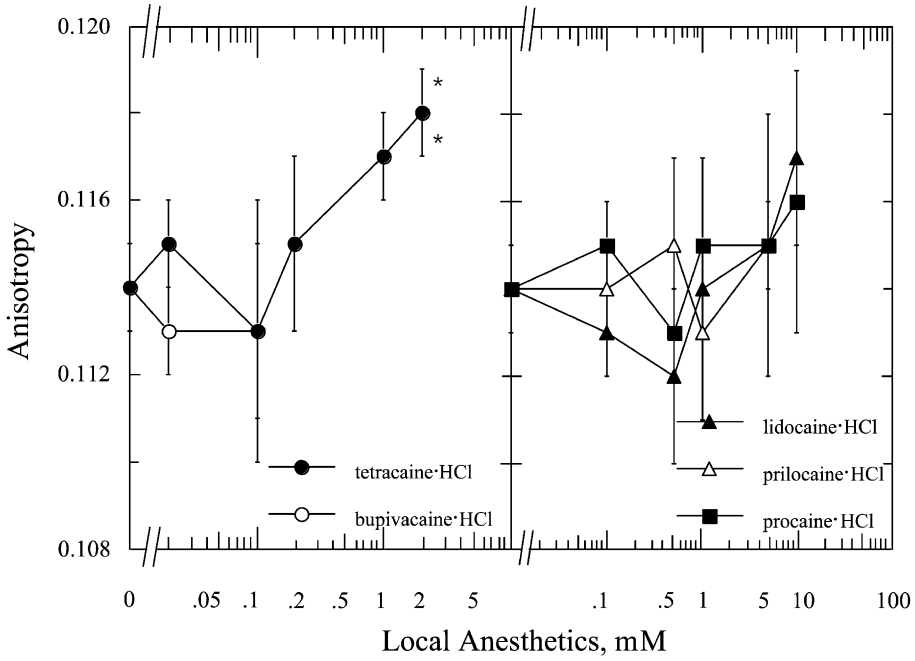


Fig. 5. Effects of local anesthetics on the anisotropy ($r$) of 2-AS in model membranes of total lipids extracted from synaptosomal plasma membrane vesicles. Fluorescence measurements were performed at 37°C (pH 7.4). Each point represents the mean±SE of five determinations. An asterisk signifies $P<0.05$ compared to control according to Student's $t$-test.

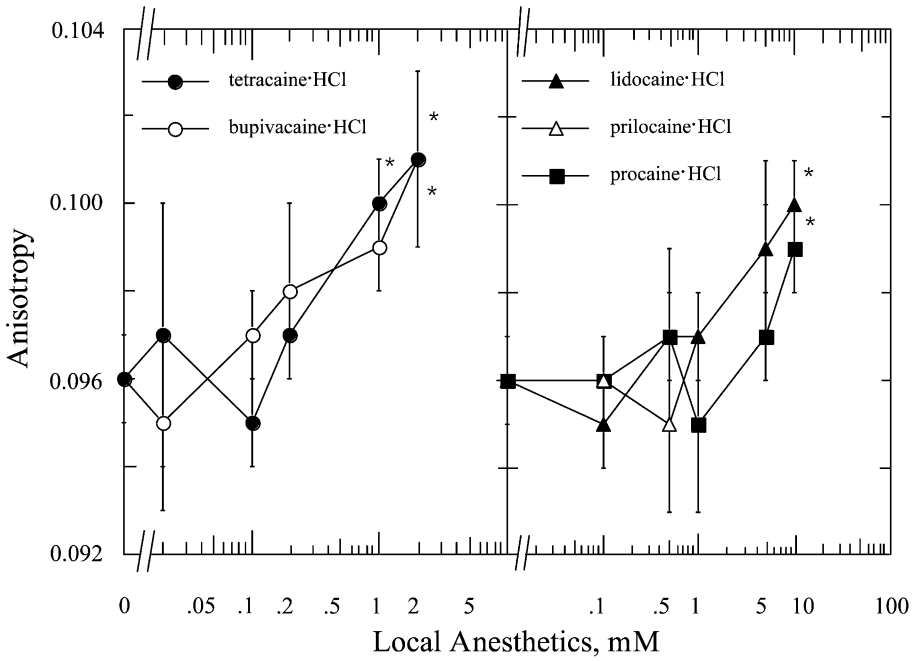


Fig. 6. Effects of local anesthetics on the anisotropy ($r$) of 2-AS in model membranes of total phospholipids extracted from synaptosomal plasma membrane vesicles. Fluorescence measurements were performed at 37°C (pH 7.4). Each point represents the mean±SE of five determinations. An asterisk signifies $P<0.05$ compared to control according to Student's $t$-test.

SPMVTL by tetracaine and bupivacaine were observed at 2 and 2 mM, respectively (Fig. 5). However, the lidocaine, prilocaine and procaine produced no noticeable effects, as seen in Fig. 5. The significant increases in the anisotropy ($r$) values in interface of SPMVPL by the tetracaine, bupivacaine, lidocaine and prilocaine were observed at 1, 2, 10 and 10 mM, respectively (Fig. 6). On the other hand, procaine did not make a significant effect, as seen Fig. 6.

The anisotropy ($r$) values of the 2-AS in interface of SPMV, SPMVTL and SPMVPL were lower by 0.006, 0.004 and 0.005, respectively, than those in the same regions when 2 mM tetracaine 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 ($r$) of the 2-AS in interface of SPMV, SPMVTL and SPMVPL are $0.126\pm0.002$ ($n=5$), $0.114\pm0.001$ ($n=5$) and $0.096\pm0.001$ ($n=5$), respectively. On the other hand, at 25°C (pH 7.4), the anisotropy ($r$) of the 2-AS in interface of SPMV, SPMVTL and SPMVPL are $0.165\pm0.003$ ($n=5$), $0.157\pm0.002$ ($n=5$) and $0.142\pm0.001$ ($n=5$), respectively. Based on the results obtained at the two different temperatures, the observed effects by the addition of 2 mM tetracaine, different values 0.006, 0.004 and 0.005 were comparable to the effects of the temperature changes by approximately 1.8, 1.1 and 1.3°C.

## 4. Discussion

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 [56]. As a consequence of the preparation method, the parameters that characterize the lipid phase equilibrium in lipid mixtures are affected by lipid sample characteristics. Because the size of the GUVs is on the same order as the size of cells, the vesicles are becoming objects of intense scrutiny in diverse areas that focus on membrane behavior [57].

We paid special attention to the study by Villalaín and Prieto [19] among several studies [19–27] in regard to 2-AS distribution in membranes. The study [19] clearly indicated the distribution region. It was reported that 2-AS is a peculiar member of the family of probes, as the chromophore is adsorbed on the membrane interface [19]. Membrane interface is difficult to define precisely and it certainly includes the polar region near the phospholipid head groups, or even the carbonyl groups, which are largely involved in hydrogen bonds. Using the membrane interface probe 2-AS, we found that local anesthetics decreased the rotational mobility of lipids in the interface of neuronal and model membranes. On the contrary, using the hydrophobic interior probe 12-AS, we found that local anesthetics increased rotational mobility of lipids in the 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 local anesthetics, the magnitude of effects of local anesthetics 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, tetracaine is approximately 10 times more potent as procaine, lidocaine has approximately 2 times the potency of procaine, prilocaine is somewhat less potent than lidocaine and bupivacaine is approximate 4 times more potent as lidocaine [58,59]. Tetracaine is an ester derivative of *p*-aminobenzoic acid in which a butyl chain replaces one of the hydrogens on the *p*-amino group. Bupivacaine is a homologue of mepivacaine rendered highly lipid soluble by replacement of the *N*-methyl group with a butyl chain. Lidocaine is an aminoethylamide derivative of xylidine. Prilocaine is a secondary amino derivative of toluidine. Procaine is the ester derivative of *p*-aminobenzoic acid and diethylaminoethanol. The physicochemical behavior of local anesthetics is a consistent and somewhat predictable function of their structure. 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 local anesthetics used in the present study lowered the anisotropy (*r*) values of 12-AS in hydrocarbon interior of SPMV, SPMVTL and SPMVPL but increased the anisotropies of 2-AS in interface of the native and model membranes. The disordering or ordering effects of the local anesthetics on the lipid bilayer occurred in the order of tetracaine (ester type)≥bupivacaine (amide type) > lidocaine (amide type)≥prilocaine (amide type)> procaine (ester type), which is in accordance with the clinical potency of the local anesthetics. The local anesthetics, tetracaine and bupivacaine showed the highest potency, while lidocaine, prilocaine and procaine showed lower potencies. Using EPR, de Paula and Schreier [60] reported that the ester-type anesthetics were seen to have greater active fluidizing effects than the amide-type ones. Our data are not in agreement with those findings. Differences between the two studies may be due in part to differences in the type of probe molecules used and to the differences between multilamellar liposomes and giant unilamellar liposomes in the EPR and fluorescence study. In addition, due to the lower sensitivity of EPR, relatively high probe concentrations (2% of total lipid, in moles) are required that could perturb the membrane and thus alter drug effects. The concentration of 5-doxylstearic acid and methyl ester of 5-doxylstearic acid, the probes used in the measurement through EPR, was 80 times higher than that of 12-AS and 2-AS, the fluorescence probes used in this study.

Our data on local anesthetics 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 local anesthetics 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 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 [61–63]. The magnitude of these effects depends on the nature and phase behavior of the phospholipid and on whether the anesthetic is charged [6,64]. Water associates with the head group region of phospholipids via hydrogen bonding [65]. Local anesthetics bind (the competitive binding of the local anesthetics and water) strongly to the phosphate moiety of the phospholipids in membrane interface 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 [66]. 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 [66]. 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 [66]. 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 [66]. The interaction will also change the orientation of the P–N dipole of phospholipid molecules [67]. 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 local anesthetics 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, it is without a doubt that the local anesthetic agents increase the rotational mobility of the hydrocarbon interior of the membrane. These effects are not solely due to the influence of the local anesthetics on lipids, but they are magnified by the interaction between 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 [68]. 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 [69]. 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 anesthetics.

Opinions have been divided as to whether local anesthetics 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 anesthetics 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 local anesthetics increase the mobility of the neuronal lipid bilayer but the direct effects of local anesthetics on protein appear to have magnified such effects on the lipid. That is to say, before or during or even after the interaction of the local anesthetics with sodium channels, the fluidization of membrane lipids may provide an ideal microenvironment for optimum local anesthetic effects. In conclusion, the present data suggests that local anesthetics, in addition to its direct interaction with sodium channels, concurrently interact with membrane lipids, affecting the fluidity of the neuronal membrane.

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