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Differential Effects of Local Anesthetics on Rate of Rotational Mobility between Hydrocarbon Interior and Surface Region of Model Membrane Outer Monolayer

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Using fluorescence polarization of 12-(9-anthroyloxy)stearic acid (12-AS) and 2-(9-anthroyloxy)stearic acid (2-AS), we evaluated the differential effects of local anesthetics on differential rotational rate between the surface (in carbon number 2 and its surroundings including the head group) and the hydrocarbon interior (in carbon number 12 and its surroundings) of the outer monolayer of the total lipid fraction liposome extracted from synaptosomal plasma membrane vesicles. The anisotropy (*r*) values for the hydrocarbon interior and the surface region of the liposome outer monolayer were 0.078 ± 0.001 and 0.114 ± 0.001, respectively. This means that the rate of rotational mobility in the hydrocarbon interior is faster than that of the surface region. In a dose-dependent manner, the local anesthetics decreased the anisotropy of 12-AS in the hydrocarbon interior of the liposome outer monolayer but increased the anisotropy of 2-AS in the surface region of the monolayer. These results indicate that local anesthetics have significant disordering effects on the hydrocarbon interior but have significant ordering effects on the surface region of the liposome outer monolayer.

Key Words: Fluorescence probe technique, Rate of rotational mobility, Local anesthetics, Model membranes

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INTRODUCTION

Several theories have been suggested to explain the molecular mechanism of pharmacological action of local anesthetics but so far none of them has been fully accepted. However, a number of research groups reported that local anesthetics increased rotational motion of bulk bilayer system of native and model membranes. The effects of local anesthetics on motions, order and phase transitions of bulk bilayer systems of native or model membranes have received considerable attention in the past two decades because of the interest in biological membranes and the unique information on intermolecular interactions that can be derived from the investigation of volume changes. The most compelling evidence was the excellent correlation between the lipid solubility and anesthetic potency (Yun et al, 1987, 1993, 1994; Kang et al, 1996).

Sweet et al (1987) reported that prilocaine preferentially reduced the limiting anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) in the inner monolayer

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of LM fibroblast plasma membranes. In our laboratory, Park (1996) reported that tetracaineHCl, bupivacaineHCl, lidocaineHCl and prilocaineHCl preferentially increased the rotational and lateral diffusion of inner monolayer of synaptosomal plasma membrane vesicles (SPMV) and increased annular lipid fluidity of SPMV. It was reported in that study that the local anesthetics caused protein clustering in SPMV and induced interdigitation of the lipid bilayers. But it was also true that local anesthetics had a fluidizing effect on the membrane outer monolayer, although the effect was smaller than that on the inner monolayer.

We carried out this study to evaluate the role of lipids, especially cholesterol, in the fluidizing effects of local anesthetics. The aim of this research is two-fold: (i) to provide a basis for studying the molecular mechanism of action of local anesthetics through the investigation of differential effects of local anesthetics on the rotational rate of hydrocarbon interior and surface region of outer monolayer of liposome of total lipid fraction (SPMVTL) extracted from SPMV which differ in fluidity; and (ii) to develop a fluorescence spectroscopic method which can detect the differences in fluidity between hydrocarbon interior and surface region of biological outer monolayer.

METHODS

Chemicals

The fluorescent probes, 12-(9-anthroyl)stearic acid (12-AS) and 2-(9-anthroyl)stearic acid (2-AS) were purchased from Molecular Probes, Inc. (Junction City, OR., USA). Local anesthetics and N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (Hepes) were purchased from Sigma Chemical Co. (St. Louis, MO., USA). All other reagents were purchased commercially and were of the highest quality available. Water was deionized.

Preparation of the neuronal plasma membrane

SPMV was prepared according to the procedure of Yun's group (Yun & Kang, 1990; Yun et al, 1990). The specific activities of Na,K-ATPase, acetylcholinesterase and 5'-nucleotidase were approximately 4-, 2.5- and 3-times, respectively, higher than those in the plasma membrane fraction with respect to crude homogenates. The electron microscopic examination of the prepared SPMV showed very high purity. The same sizes which were separated vesicles showed a homogeneous distribution and no longer showed the presence of intracellular organelles or leakage.

Preparation of liposome

Total lipids were extracted from the SPMV as previously described (Yun & Kang, 1990). The cholesterol 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 & Antunes-Madeira, 1976). The SPMV had a high lipid to protein ratio (0.942 mg/total lipids/mg protein) and a low cholesterol to phospholipid ratio (0.60 ± 0.01). Large unilamellar liposomes (SPMVTL; 0.7 mg of total lipids/ml, pH 7.4) were prepared by the method described previously (Yun & Kang, 1992). The extracted total lipids in chloroform solution were deposited on the sides of a round-bottom flask by removing the organic solvent through rotary evaporation. The lipids were then redissolved in diethyl ether which had been redistilled in the presence of $NaHSO_3$ immediately prior to use. Phosphate-buffered saline (PBS: 8 g/l NaCl, 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 1.15 g/l $Na_2HPO_4 \cdot 7H_2O$, 0.48 g/l Hepes, pH 7.4) was added to the solution of lipids and the organic/aqueous mixture was placed in an ultrasonic processor (Sonics & Materials, Inc., Danbury, CT, USA) under N_2 at 30°C. It was sonicated for 5 min to form a milky white, homogeneous emulsion. The emulsion was then transferred to a rotary evaporator and the organic solvent was removed under reduced pressure. During evaporation of the solvent, the system foamed. As the process continued, progressively higher vacuum was needed to maintain foaming. As the majority of the solvent was removed, the material first formed a viscous gel and subsequently (within $5 \sim 10$ min) it became an aqueous suspension. At this time, additional PBS was added, the preparation foamed and was vented again several times until the foaming ceased. The procedure was finished when no foaming occurred. The preparation was then dialyzed and passed through a Sepharose 4B column.

Fluorescence measurements

The fluorescence measurements were carried out using a modified method of earlier studies (Molitoris et al, 1985; Molitoris & Hoilien, 1987). The SPMVTL were suspended in PBS to give concentration 0.7 mg of total lipids/ml. Stock solutions of 12-AS and 2-AS in ethanol (1 mM) were prepared and kept in a cold dark place. The probes were incorporated by adding aliquots of the solutions to the liposomes so that the final concentrations of 12-AS and 2-AS were 10 μM. The mixture was stirred for 20 min at room temperature in order to reduce the concentration of ethanol that might alter the rotational rate of the SPMVTL lipid bilayer. Also, the mixture was bubbled by dry nitrogen for 1 min with 20 min intervals in order to eliminate oxygen that might be a quencher and denaturalize SPMVTL. Concentrated solutions of the local anesthetics were prepared in PBS and added to the labelled membrane suspension to give the desired concentration of the anesthetics. The pH of the buffered sample was not changed significantly by addition of local anesthetics.

Fluorescence measurements were carried out with Multi Frequency Cross-Correlation Phase and Modulation Fluorometer (ISS K2-003), equipped with a thermostated cell holder and performed at pH 7.4 (37 $\pm$0.1°C). The fluorescent probes, 12-AS and 2-AS, were excited at 386 nm and those emission were recorded at 440 nm. Blanks (SPMVTL suspensions without fluorescent probes), prepared under identical conditions, served as controls for the fluorometric measurements. The intensity, of the components of the fluorescence that were parallel ($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. The 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 the anisotropy [$r = 2P/(3-P)$].

RESULTS

Using fluorescence technique, we determined the differential effects of cations of tetracaine · HCl, bupivacaine · HCl, lidocaine · HCl, prilocaine · HCl and procaine · HCl on the differential rate of rotational mobility between the surface (in carbon number 2 and its surroundings including the head group) and the hydrocarbon interior (in carbon number 12 and its surroundings) of the outer monolayer of SPMVTL extracted from the SPMV. Significant changes in fluorescence intensities of the probes in the SPMVTL by the drugs were not detected over the entire concentration range used tetracaine · HCl, bupivacaine · HCl, lidocaine · HCl, prilocaine · HCl and procaine · HCl. Hence, the possibility of direct quenching of fluorescence of the probes by local anesthetics is ruled out.

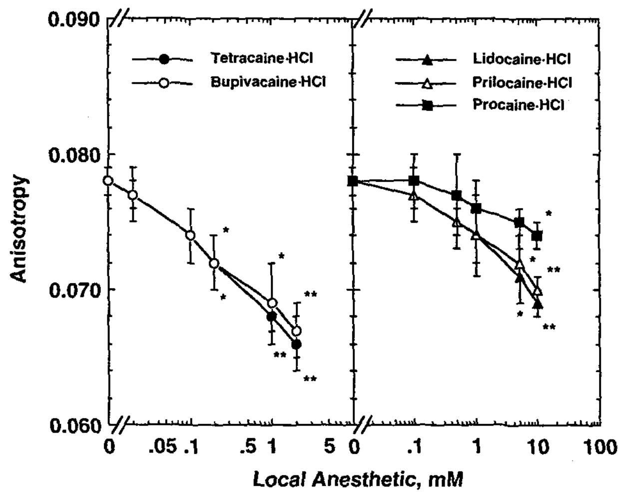
The polarization and anisotropy values of 12-AS for the hydrocarbon interior of the intact SPMVTL outer monolayer were 0.113$\pm$0.002 (n=5) and 0.078 $\pm$0.001 (n=5), respectively, and those values of 2-AS for the surface region of the monolayer were 0.162$\pm$ 0.002 (n=5) and 0.114$\pm$0.001 (n=5), respectively (Table 1). This means that the rate of rotational mobility of the hydrocarbon interior is faster than that of the surface region.

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 (Boulanger et al, 1981; Kelusky et al, 1986; Seeling et al, 1988). The magnitudes of these effects depend on the nature and phase behavior of the phospholipid and on whether the anesthetic is

Table 1. Fluorescence parameters of 12-(9-anthroyloxy) stearic acid (12-AS) and 2-(9-anthroyloxy)stearic acid (2-AS) in model membranes of total lipids extracted from synaptosomal plasma membrane vesicles

Parameter	12-AS	2-AS
Polarization	0.113$\pm$0.002	0.162$\pm$0.002
Anisotropy	0.078$\pm$0.001	0.114$\pm$0.001

Fluorescence measurements were performed at 37°C (pH 7.4). Values represents the mean$\pm$SEM of 5 determinations.



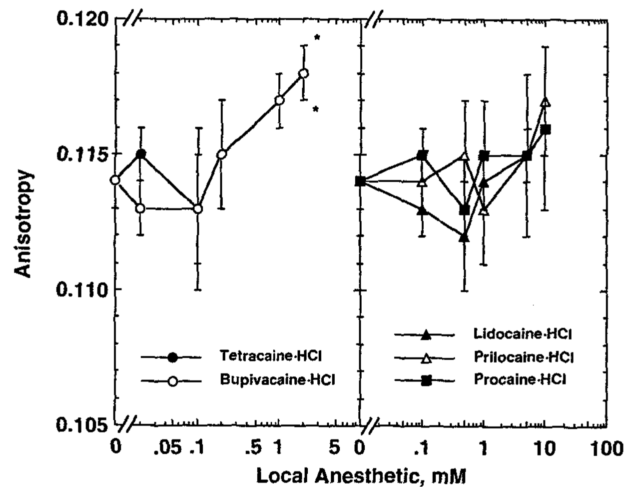


Fig. 1. Effects of local anesthetics on the anisotropy (*r*) of 12-(9-anthroyloxy)stearic acid (12-AS) in model membranes of total lipids extracted from synaptosomal plasma membrane vesicles. Flurorescence measurements were performed at 37°C (pH 7.4). Each point represents the mean ± SEM of 5 determinations. 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 2-(9-anthroyloxy)stearic acid (2-AS) in model membranes of total lipids extracted from synaptosomal plasma membrane vesicles. Flurorescence measurements were performed at 37°C (pH 7.4). Each point represents the mean ± SEM of 5 determinations. An asterisk signifies $P<0.05$ compared to control according to Student's *t*-test.

charged (Auger et al, 1988; Smith et al, 1991). The effects of increasing concentrations of local anesthetics on the anisotropy of 12-AS and 2-AS are shown in Fig. 1-2. Local anesthetics decreased the anisotropy of 12-AS (increased rate of rotational mobility) in a concentration dependent manner. The significant decreases in anisotropy values by tetracaine · HCl, bupivacaine · HCl, lidocaine · HCl, prilocaine · HCl and procaine · HCl were observed even at 0.2, 0.2, 5, 5 and 10 mM, respectively (Fig. 1). However, the important point is the different potencies among local anesthetics, in terms of minimal drug concentrations for the significant decreases in the anisotropy values. In contrast, both tetracaine · HCl and bupivacaine · HCl increased the anisotropy of 2-AS (decreased rate of rotational mobility). The significant increases in anisotropy values by tetracaine · HCl and bupivacaine · HCl were observed at 2.0 mM and 2.0 mM, respectively. But the anisotropy of 2-AS was not changed significantly by over the entire concentration range (0.1 ~ 10 mM) used lidocaine · HCl, prilocaine · HCl and procaine · HCl (Fig. 2). The important point is the different potencies among local anesthetics, in terms of minimal drug concentrations for the significant increases in the values.

Using the EPR technique, de Paula and Schreier (1995) reported that the ester type anesthetics were seen to be more active (membrane fluidizing effects) than the amides. In a dose-dependent manner, the local anesthetics decreased the anisotropy values of 12-AS in the hydrocarbon interior of SPMVTL outer monolayer but increased the values of 2-AS in the surface region of the monolayer. The disordering or ordering potencies of local anesthetics on the monolayer occurred in the following order: tetracaine · HCl (ester type) ≥ bupivacaine · HCl (amide type) > lidocaine · HCl (amide type) ≥ prilocaine · HCl (amide type) > procaine · HCl (ester type) and the same order was observed when these local anesthetics were clinically used. The order of membrane disordering potencies of local anesthetics was not consistent. The reasons for these differences cannot be fully clarified but they can be explained in part. This discrepancy is due to differences in the type of probe molecules used in the EPR and fluorescence study. In addition, due to the lower sensitivity of EPR, relatively high probe concentrations are required and the probe may itself perturb the membrane and thus alter the drug effects.

DISCUSSION

The difference between the values of the anisotropy (r) of 12-AS found in the outer monolayer hydrocarbon interior of SPMVTL with and without the addition of 2 mM tetracaineHCl is 0.012, illustrated by comparing the effects of temperature on this parameter. The anisotropy (r) of 12-AS in the hydrocarbon interior is 0.105 ± 0.002 (n=5) and 0.078 ± 0.002 (n=5) at 25 and 37°C (pH 7.4), respectively. Thus, the difference in the anisotropy (r) value of the 12-AS in the outer monolayer hydrocarbon interior of the SPMVTL before and after adding 2 mM tetracaine · HCl on the same region, namely, 0.012 was as large as that produced by approximately a 5.2°C change in temperature. The anisotropy (r) value of the 2-AS in the intact surface region is 0.004 lower than that in the same region when 2 mM tetracaine · HCl is added. The significance of such difference in the anisotropy (r) value 0.004, can also be interpreted in terms of change in temperature. The anisotropy (r) of 2-AS in the surface region is 0.148 ± 0.002 (n=5) and 0.114 ± 0.001 (n=5) at 25 and 37°C (pH 7.4), respectively. When converted to temperature this value (0.004) is approximately 1.4°C.

We confirmed that local anesthetics tetracaine · HCl, bupivacaine · HCl, lidocaine · HCl, prilocaine · HCl and procaine · HCl increased the rotational rate of the hydrocarbon interior of the SPMVTL outer monolayer to a great degree that the tetracaine · HCl and bupivacaine · HCl decreased the rotational rate of the surface region of the monolayer. Here, we present data for local anesthetics suggesting that the observed anisotropy (r) values reflect the differences in ordering or fluidizing constraints in the two regions. This is due to differences in the intrinsic component and/or the structure in the surface and the hydrocarbon region of the SPMVTL outer monolayer and which, in turn, results in different electrostatic interactions and structured water effects on the surface and hydrocarbon (acyl chain) interior of the SPMVTL outer monolayer (Table 1, Fig. 1-2). However the clear mechanism of action of local anesthetics in the ordering (surface of SPMVTL outer monolayer) and fluidizing (acyl chain SPMVTL outer monolayer) effects on the SPMVTL outer monolayer is unknown.

The ordering effects of local anesthetics on the surface region of the SPMVTL outer monolayer were probably due to the competitive binding of local anesthetics and water to phosphate moiety of phospholipids in the SPMVTL outer monolayer. The incorporation of the local anesthetics into SPMVTL follows that alterations of the membrane outer monolayer's surface charge density by charged local anesthetics cause a conformational change in phospholipid head groups (ordering effects of local anesthetics) and, at the same time, may exert a significant influence on hydration of the lipid bilayer (dehydration effects of local anesthetics). The overall effects of the local anesthetic cations can be explained by the following reactions: for the phospholipid molecules in the SPMVTL outer monolayer, local anesthetic cations bind weakly to the phosphate moiety and effectively establish formation of hydrogen bonds with the carbonyl moiety. The present results show that the cationic form of the local anesthetics may interact with the phosphate moieties of negatively charged phospholipids and the ester carbonyl moieties of neutral phospholipids in the monolayer surface. The interaction of the local anesthetic cations with the outer monolayer's hydrocarbon region will generate rearrangements of the intermolecular hydrogen-bonded network among phospholipid molecules and/or hydrated water molecules on the surface of the membranes, and change the orientation of the P-N dipole of phospholipid molecules. These changes should cause disordering of the monolayer hydrocarbon region, and thus they could affect the transport of Na^+ and K^+ in nerve membranes, leading to the anesthetic action.

Using fluorescence quenching techniques, Park (1996) evaluated effects of local anesthetics on the annular lipid fluidity of SPMV. The fact that the degree of fluidity induced by all of the local anesthetics tetracaine · HCl, bupivacaine · HCl, lidocaine · HCl, prilocaine · HCl and procaine · HCl was far greater on annular lipid fluidity than SPMV bulk fluidity, deserves special attention. When we consider the results of our study, it is without a doubt that local anesthetic agents increase the rotational rate of the membrane monolayer hydrocarbon interior. However, the most significant finding is that the increasing effects on the annular lipid fluidity were far greater in SPMV than the increasing effects on the rotational rate of the hydrocarbon interior in SPMV (unpublished data) and SPMVTL (in this study). These effects are not solely due to the influence of local anesthetics on lipids, but they are magnified by the interaction between lipids and sodium channel protein.

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 outer monolayer but the direct effects of local anesthetics on protein appear to have magnified such effects on the lipid. This conclusion can be drawn because we confirmed that the increase on the rotational rate of the SPMV outer monolayer induced by local anesthetics was significantly less than the increase on the annular lipid fluidity.

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