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Short communication

Effects of Local Anesthetics on the Rate of Rotational Mobility of Phospholipid Liposomes

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Using fluorescence probes, 2-(9-anthroyloxy) stearic acid (2-AS) and 12-(9-anthroyloxy) stearic acid (12-AS), we determined the differential effects of local anesthetics (tetracaine·HCl, bupivacaine·HCl, lidocaine·HCl, prilocaine ·HCl and procaine·HCl) on the 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 phospholipid fraction liposome that is extracted from synaptosomal plasma membrane vesicles. The anisotropy (r) values for the hydrocarbon interior and the surface region of the liposome outer monolayer were 0.051 ± 0.001 and 0.096 ± 0.001, respectively. This means that the rate of rotational mobility in the hydrocarbon interior is faster than that of the surface region. Local anesthetics in a dose-dependent manner 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.

Keywords: Fluorescent probe technique, Local anesthetics, Phospholipid liposomes, Rotational rate of outer monolayer.

Introduction

The action of local anesthetics are thought to be primarily due to their fluidizing effects on bulk membrane phospholipids, although their interactions with some receptor proteins are proposed to be decisive. 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 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 (Yun *et al.*, 1987, 1993, 1994; Kang *et al.*, 1996). It is known that the potency of anesthetic increases roughly in proportion with its lipid/water partition coefficient. This strongly suggests an amphiphilic site for anesthetic molecules (Miller *et al.*, 1986).

Yun *et al.* (1987) reported that local anesthetics decreased the microviscosity of synaptosomal plasma membrane vesicles that are isolated from the bovine cerebral cortex (SPMV). In addition, the 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 preferentially reduced the limiting anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) in the inner monolayer of LM fibroblast plasma membranes. In our laboratory, Park (1996) reported that tetracaine·HCl, bupivacaine·HCl, lidocaine·HCl and prilocaine·HCl preferentially increased the rotational and lateral diffusion of the inner monolayer of synaptosomal plasma membrane vesicles (SPMV) and increased annular lipid fluidity of SPMV. Park (1996) also reported that local anesthetics caused protein clustering in SPMV and induced interdigitation of the lipid bilayers. However, 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

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monolayer. Furthermore, recent fluorescence measurements (Louro *et al.*, 1994) showed that the highest affinity site for dibucaine in sarcoplasmic reticulum vesicles (SRV) is a lipid site near the membrane surface. The accumulated results for the analysis of a local anesthetics effect on the cell membrane fluidity have been focused on the bulk bilayer, or transbilayer domains, not on the hydrocarbon interior and surface region of the outer monolayer of the native and model membranes.

This study evaluated the role of phospholipids in the fluidizing effects of local anesthetics. The research aim is two-fold: (i) To provide a basis for studying the molecular mechanism of pharmacological action of the local anesthetics. This was done through the investigation of the differential effects of local anesthetics on the rotational rate of the hydrocarbon interior and the surface region of the liposome outer monolayer of a total phospholipid fraction (SPMVPL) that was extracted from SPMV which differs in fluidity. (ii) To develop a fluorescence spectroscopic method which can detect the differences in the rotational rate between the hydrocarbon interior and the surface region of biological outer monolayer.

Materials and 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 SPMV Preparation of SPMV was performed according to the procedure from earlier studies (Yun and 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, enriched in the plasma membrane fraction with respect to crude homogenates. The electron microscopic examination of the prepared SPMV showed very high purity. The vesicles, which were separated according to size, demonstrated a homogeneous distribution and no longer showed the presence of intracellular organelles or leakage.

Preparation of total phospholipid liposome Total lipids were extracted from the SPMV as previously described (Yun and 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 and 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). Phospholipids were composed of phosphatidylcholine (PC, 43%), phosphatidylethanolamine (PE, 36%), sphingomyelin (SP, 4%), phosphatidylinositol (PI, 3%) and lysophosphatidylcholine (LPC, 1%).

Large unilamellar liposomes (SPMVPL; 0.7 mg of total phospholipids/ml, pH 7.4) were prepared by the method previously described (Yun and Kang, 1992). The total extracted lipids in the 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 that 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, a 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 5-10 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 taken using a modified method of earlier studies (Molitoris and Hoilien, 1987; Molitoris *et al.*, 1985). The SPMVPL were suspended in PBS to give a concentration of 0.7 mg of total phospholipids/ml. Stock solutions of 12-AS and 2-AS in ethanol (1 mM) were prepared and kept in a cold dark place. Aliquots were added to the solutions of the liposomes so that the final concentrations of 12-AS and 2-AS were 10 μM incorporated into the probes. 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 SPMVPL 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 SPMVPL. 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 the 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 386 nm and those emissions were recorded at 440 nm. Blanks (SPMVPL suspensions without fluorescent probes), prepared under identical conditions, served as controls for the fluorometric measurements. 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. The polarization (P) was obtained from intensity measurements using $P = (I_{//} - GI_{\perp})/(I_{//} + GI_{\perp})$, where G is a grating correction factor for

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

Parameter	12-AS	2-AS
Polarization	0.075 ± 0.001	0.137 ± 0.002
Anisotropy	0.051 ± 0.001	0.096 ± 0.001

Fluorescent probes, both the 12-AS (10 μM) and 2-AS (10 μM), were incorporated into SPMVPL (phospholipids 0.7 mg/ml). Fluorescence measurements were performed at 37°C (pH 7.4). Values represents the mean ± SEM of 5 determinations.

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 and Discussion

Using anisotropy (r) of fluorescent probes in the liposome outer monolayer, we determined the differential effects 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 SPMVPL that was extracted from the SPMV. In order to determine the effects of local anesthetics on the aforementioned rate of rotational mobility, it was first necessary to demonstrate that the drugs do not interact directly with fluorescent probes and thereby quench its fluorescence. Significant changes in the fluorescence intensities of the probes in the SPMVPL by the drugs were not detected over the entire concentration range used for tetracaine·HCl, bupivacaine·HCl, lidocaine·HCl, prilocaine·HCl and procaine·HCl. Hence, we ruled out the possibility of direct quenching of fluorescence of the probes by local anesthetics. The polarization and anisotropy values of 12-AS for the hydrocarbon interior of the intact SPMVPL outer monolayer were 0.075 ± 0.001 (n = 5) and 0.051 ± 0.001 (n = 5), respectively. The values of 2-AS for the surface region of the monolayer were 0.137 ± 0.002 (n = 5) and 0.096 ± 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.

Disordering or ordering effects of the local anesthetics on the rotational rate of the hydrocarbon interior and surface of SPMVPL outer monolayer We confirmed that the local anesthetics tetracaine·HCl, bupivacaine·HCl, lidocaine·HCl, prilocaine·HCl and procaine·HCl increased the rotational rate of the hydrocarbon interior of the SPMVPL outer monolayer

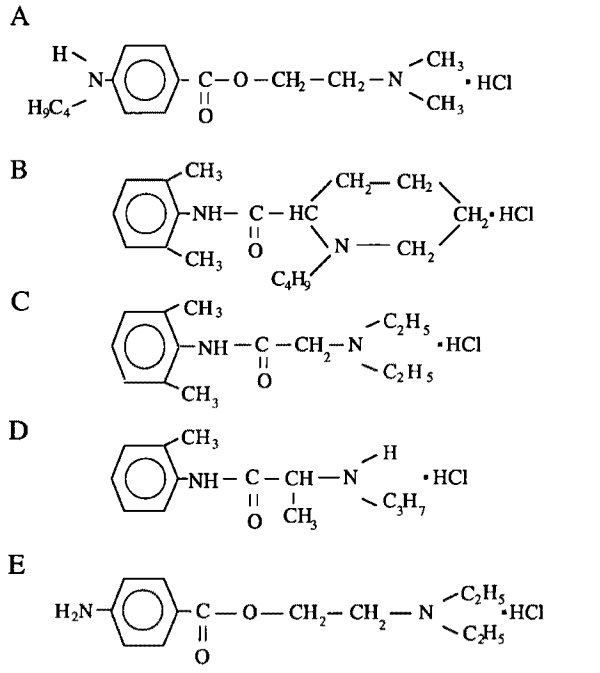
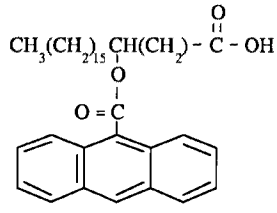
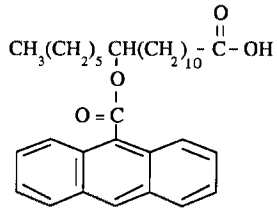


Fig. 1. The chemical structures of the local anesthetics, A; tetracaine·HCl, B; bupivacaine·HCl, C; lidocaine·HCl, D; prilocaine·HCl and E; procaine·HCl.



2-AS



12-AS

Fig. 2. The chemical structures of the fluorescent probes, 12-(9-anthroyloxy)stearic acid (12-AS) and 2-(9-anthroyloxy)stearic acid (2-AS).

to a great degree (Fig. 3). The tetracaine·HCl and bupivacaine·HCl decreased the rotational rate of the surface region of the monolayer (Fig. 4). The effects of increasing concentrations of the local anesthetics on the anisotropy (r) of 12-AS and 2-AS are shown in Figures 3 and 4. The local anesthetics decreased the anisotropy (r) of 12-AS (increased rate of rotational mobility) in a concentration dependent manner. The significant decreases in the anisotropy (r) values

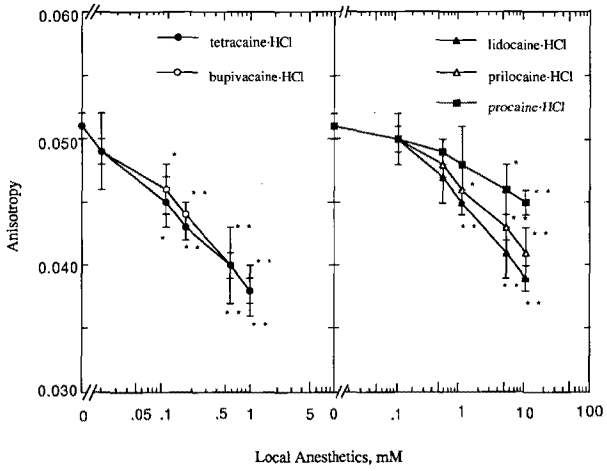


Fig. 3. Effects of local anesthetics on the anisotropy (r) of 12-(9-anthroyloxy)stearic acid (12-AS) in model membranes of phospholipids (SPMVPL) extracted from synaptosomal plasma membrane vesicles. Fluorescence measurements were performed at 37°C (pH 7.4). Each point represents the mean ± SEM of 5 determinations. An asterisk signifies $P<0.05$ compared to control according to Student's t-test.

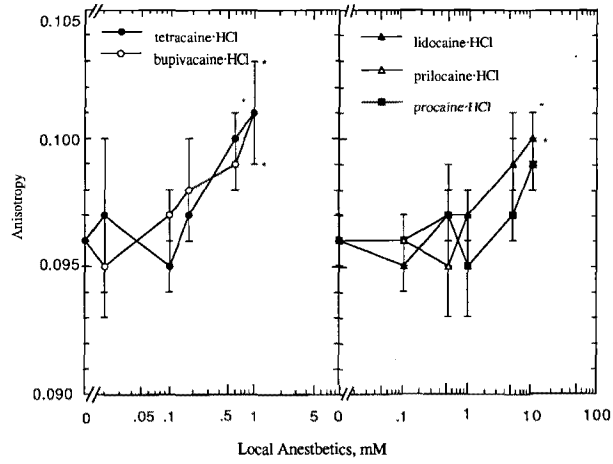


Fig. 4. Effects of local anesthetics on the anisotropy (r) of 2-(9-anthroyloxy)stearic acid (2-AS) in model membranes of phospholipids (SPMVPL) extracted from synaptosomal plasma membrane vesicles. Fluorescence measurements were performed at 37°C (pH 7.4). Each point represents the mean ± SEM of 5 determinations. An asterisk signifies $P<0.05$ compared to control according to Student's t-test.

by tetracaine·HCl, bupivacaine·HCl, lidocaine·HCl, prilocaine ·HCl and procaine·HCl were observed even at 0.1, 0.1, 1.0, 1.0 and 5.0 mM, respectively (Fig. 3). The difference in the anisotropy (r) values of the 12-AS, found in the hydrocarbon interior of the SPMVPL outer monolayer before and after adding 2 mM tetracaineHCl, was 0.013. This can be illustrated by comparing the effects of temperature on the parameter. The anisotropy (r) of 12-AS in the hydrocarbon interior was 0.079±0.001 (n = 5) and 0.051 ± 0.001 (n = 5) at 25 and 37°C (pH 7.4), respectively. Thus, the difference in the anisotropy (r) values of 12-AS in the hydrocarbon interior of the SPMVPL outer monolayer before and after adding 2 mM tetracaine·HCl, namely, 0.013, was as large as that produced by approximately a 5.6°C change in temperature.

In contrast, tetracaine·HCl, bupivacaine·HCl, lidocaine·HCl and prilocaine·HCl increased the anisotropy (r) of the 2-AS (decreased rate of rotational mobility). The significant increases in the anisotropy (r) values by tetracaine·HCl, bupivacaine·HCl, lidocaine·HCl and prilocaine·HCl were observed at 1.0, 2.0, 10.0 and 10.0 mM, respectively. But the anisotropy (r) of 2-AS in the surface region was not changed significantly over the entire concentration range (0.1-10 mM) when procaine·HCl (Fig. 4) was used.

The anisotropy (r) value of the 2-AS in the surface region of the SPMVPL outer monolayer was 0.005 lower than that in the same region when 2 mM tetracaine·HCl was added. The significance of such a difference in the anisotropy (r) value, 0.005, can also be interpreted in terms of a change in temperature. The anisotropy (r) of 2-AS in the surface region was 0.131 ± 0.001 (n = 5) and 0.096 ± 0.001 (n = 5) at 25 and 37°C (pH 7.4), respectively. This value, when converted to a change in temperature, was approximately 1.7°C.

The potency of the local anesthetics Figures 3 and 4 show that the important point is the different potencies among the local anesthetics in terms of minimal drug concentrations for the significant decreases or increases in the anisotropy (r) values. The disordering, or ordering potencies of the 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). The same order was observed when these local anesthetics were clinically used. More specifically, in terms of the magnitude of the increase or decrease on the outer monolayer rotational rate of SPMVPL by the local anesthetics, tetracaine·HCl showed the strongest effect followed by bupivacaine·HCl, lidocaine·HCl, prilocaine·HCl and procaine·HCl in descending order. Using the EPR technique, de Paula and Schreier (1995) reported that the ester type anesthetics appeared to be more active (membrane fluidizing effects) than the amides. The order of membrane disordering potencies of the local anesthetics was inconsistent. These differences cannot be fully clarified, but they can be depicted in part. The discrepancy is caused by 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. Also the probe may perturb the membrane and alter the drug effects.

Possible mechanisms of pharmacological actions of the local anesthetics Our data for local anesthetics suggests that observed anisotropy (r) values reflect the differences in ordering or disordering constraints in the hydrocarbon interior and surface region of SPMVPL. This is due to the differences in the intrinsic component and/or the structure in the surface

and the hydrocarbon region of the outer monolayer of the model membranes. These differences result in various electrostatic interactions and structured water effects on the surface and the hydrocarbon (acyl chain) interior of the monolayer of the model membranes. However, the clear mechanism of action of the local anesthetics in the ordering and disordering effects on the monolayer of the model membranes is unknown.

The ordering effects of the local anesthetics on the surface region of the outer monolayer of the model membranes were probably due to the competitive binding of the local anesthetics and water to the phosphate moiety of phospholipids in the monolayer of the model membranes. The incorporation of the local anesthetics into the model membranes then alter the membrane's outer monolayer surface charge density by charged local anesthetics that cause a conformational change in the phospholipid head groups (ordering effects of local anesthetics). At the same time, they may exert a significant influence on the hydration of the lipid bilayer (dehydration effects of local anesthetics). The overall effect of the local anesthetic cations can be explained by the following reactions: The phospholipid molecules in the monolayer of the model membranes, the local anesthetic cations, bind weakly to the phosphate moiety and effectively establish formation of hydrogen bonds with the carbonyl moiety. These results show that the cationic form of the local anesthetics may interact with the phosphate moieties of negatively charged phospholipids as well as 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 that are associated with the liberation of hydrated water molecules on the surface of the membranes. The interaction will also change the orientation of the P-N dipole of phospholipid molecules. These changes should cause disordering of the hydrocarbon interior of the monolayer.

Using fluorescence quenching techniques, Park (1996) evaluated the effects of the 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 the annular lipid fluidity than SPMV bulk fluidity deserves special attention. When we consider the results of this study along with Park's study (1996), there is no doubt that local anesthetic agents increase the rotational rate of the membrane outer 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 SPMVPL. These effects are not solely due to the influence of the local anesthetics on lipids; however, they are magnified by the interaction between lipids and the 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 a protein triggering change in lipids is more likely. It is certain that the local anesthetics increase the mobility of the neuronal outer monolayer. However, the direct effect of the local anesthetics on protein appears to have a magnified effect on the lipid. This conclusion was confirmed by our observation that the increase in the rotational rate of the SPMVPL outer monolayer (in this study) induced by the local anesthetics was significantly less than that of the annular lipid fluidity (Park, 1996).

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