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Research Article

The Effect of Mepivacaine·HCl on the Physical Properties of Neuronal Membranes

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Abstract

Fluorescent probe techniques were used to evaluate the effect of mepivacaine·HCl on the physical properties (transbilayer asymmetric lateral and rotational nobilities, annular lipid fluidity and protein distribution) of Synaptosomal Plasma Membrane Vesicles (SPMVs) isolated from bovine cerebral cortex. An experimental procedure was used based on selective quenching of both 1,3-di(1-pyrenyl) propane (Py-3-Py) and 1,6-Diphenyl-1,3,5-Hexatriene (DPH) by trinitrophenyl groups, and radiation less energy transfer from the tryptophan of membrane proteins to Py-3-Py. Mepivacaine·HCl increased the bulk lateral and rotational mobilities, and annular lipid fluidity in SPMVs lipid bilayers, and had a greater fluidizing effect on the inner monolayer than the outer monolayer. The magnitude of increasing effect on annular lipid fluidity in SPMVs lipid bilayer induced by mepivacaine·HCl was significantly far greater than magnitude of increasing effect of the drug on the lateral and rotational mobilities of bulk SPMVS lipid bilayer. It also caused membrane proteins to cluster. These effects of mepivacaine·HCl on neuronal membranes may be responsible for some, though not all, of the local anesthetic actions of mepivacaine·HCl.

Keywords: Annular Lipid Fluidity; MepiVacaine.Hcl; Membrane Protein Clustering; Neuronal Membranes; Transbilayer Lateral; Rotational Motilities

Abbreviations:

BSA	:	Bovine Serum Albumin
DPH	:	1,6-Diphenyl-1,3,5-Hexatriene
Hepes	:	N-2- Hydroxyethyl-Piperazine-N'-2-Ethanesulfonic Acid
PBS	:	Phosphate Buffered Saline; Py-3-Py, 1,3-Di(1-Pyrenyl) Propane
SPMVs	:	Synaptosomal Plasma Membrane Vesicles Isolated from Bovine Cerebral Cortex
TNBS	:	2,4,6-Trinitrophenylbenzenesulfonic Acid

Introduction

The molecular mechanism of pharmacological action of local anesthetics has long been a subject of great interest. There are two general theories for the molecular mechanism of pharmacological action of local anesthetics. The specific receptor theory considers a direct binding of local anesthetic molecules to specific receptors on sodium channels [1-4]. There is a large amount of evidence in support of the specific receptor theory [5]. The other theory proposes the general perturbations of bulk physical properties of the lipids of cell membranes by local anesthetics and its consequences on channel function [6-12]. General membrane perturbation may also contribute to an explanation of anesthetic actions [5]. However, the precise location of molecular action has continued to be a subject of controversy to the present day.

The physical state of membrane lipids has been shown to influence such membrane enzymes as NaK-ATPase [13], hormone-responsive adenylate cyclase [14], and membrane transport processes such as glucose and amino acid uptake [15,16]. Membrane lipids also play an important role in membrane permeability to sodium, calcium, and potassium [17].

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 [9,18-20]. Yun et al. [9] reported that local anesthetics decreased micro viscosity of Synaptosomal Plasma Membrane Vesicles isolated from the bovine cerebral cortex (SPMVs). In addition, differential scanning thermograms of dimyristoylphosphatidylcholine multilamellar liposomes showed that local anesthetics significantly lowered the phase transition temperature, broadened the thermograms peaks, and reduced the size of the cooperative unit. Sweet, et al. [21] reported that prilocaine·HCl preferentially reduced the limiting anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) 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. Yun et al. [11] reported that local anesthetics increased the rotational mobility of SPMVS hydrocarbon interior, but the anesthetics decreased the mobility of SPMVs surface region (membrane interface). As mentioned above, there have been some sporadic studies which back up, although not fully satisfactorily, the membrane expansion theory. The membrane expansion theory is one of the more important theories regarding the mechanism of pharmacological action of local anesthetics.

If local anesthetics cause expansion of neuronal membranes, this expansion is probably due to the increased fluidity in neuronal membrane lipid bilayer induced by local anesthetics. Our questions were what the role of local anesthetics (which is believed to have more interaction with protein than other lipids) was and to what degree the neuronal membrane lipid bilayer was expanded by local anesthetics.

More specifically, our questions were; first, how much of an increase do local anesthetics bring to rotational and lateral diffusions of the neuronal membrane lipid bilayer; second, whether such increasing effects were shown evenly on both lipid bilayers or differently between inner and outer monolayers; third, it the degree of increase is different between the inner and outer monolayers, then which monolayer has been mostly affected; fourth, whether annular lipid fluidity of the neuronal membrane lipid bilayer is increased or decreased by local anesthetics, and whether the degree of such increase or decrease is approximately the same as or much greater than the degree of changes in rotational and lateral motilities. We are here to present the results of our study on how we solved the aforementioned questions by employing fluorescence technique, including the fluorescence quenching techniques.

Materials and Methods

The fluorescent probes, 1,3-di(1-pyrenyl) propane (Py-3-Py) and DPH were purchased from Molecular Probes, Inc. (Junction City, OR, USA). Mepivacaine·HCl, N-2-hydroxyethyl- piperazine-N'-2-ethanesulfonic acid (Hepes) and bovine serum albumin (BSA) were purchased from Sigma Chemical (St. Louis, MO, USA). 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was obtained from Fluka (Switzerland). All other reagents were purchased commercially and were of the highest quality available. Water was deionized.

Preparation of Synaptosomes and TNBS Labelling

Synaptosomes were prepared as described previously [19,22]. To determine the fluorescence parameters of probe molecules in each of the membrane monolayers, TNBS labeling reactions were performed as described [19,20,23-27] with a few modifications. The synaptosomal pellet was gently suspended in 50 ml of 4 mM TNBS in buffer A for 80 min (in the case of asymmetric lateral mobility) or 50 ml of 2 mM TNBS in buffer A for 40 min (in the case of asymmetric rotational mobility) or in buffer A alone. Buffer A contains of 30 mM NaCl, 120 mM $NaHCO_3$, 11 mM glucose and 1% Bovine Serum Albumin (BSA), and its pH was adjusted to 8.5 with NaOH. To assure complete exposure of all synaptosomal outer monolayers to TNBS, the pellet was passed slowly through an Eberbach tissue grinder (3 up and down strokes). Unless otherwise specified, treatment was carried out at 4°C. The TNBS labeling reaction was terminated by adding an equal volume of 1% BSA in 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).

Membrane Isolation

SPMVs were isolated according to the procedure reported from earlier studies [19,22,25-27]. Their protein concentration was measured following the method of Lowry, et al. [28] using BSA as a standard.

Fluorescence Measurements

The fluorescence measurements were taken using a modified method of earlier studies [20,24-27]. All fluorescence measurements were obtained with a Multi Frequency Cross-Correlation Phase and Modulation Fluorimeter (Model; ISS K2-003). Cuvette temperature was maintained at 37.0± 0.1°C in a circulating water bath (pH 7.4). Band pass slits were 10 nm on excitation and 5 nm on emission. Blanks, prepared under identical conditions without fluorescent probes, served as controls. Py-3-Py was incorporated by adding aliquots of a 5×10^{-3} M stock solution in absolute ethanol to the SPMVs, such that the final probe concentration was less than 5×10^{-7} M [20,24-27].

Mixtures were initially vigorously vortexed for 10 s at room temperature and then incubated at 4°C for 18 h with gentle stirring [20,24-27]. DPH was dissolved in tetrahydrofuran, and 0.5 µl tetrahydrofuran per ml of PBS was added directly to the membrane suspension to a concentration of 0.01 µg/50 µg membrane protein (fluorescent probe DPH 2: membrane protein 10,000) as described previously [20,23-27]. After probe incorporation the membrane suspension was placed in cuvettes, and control fluorescence was determined. Concentrated solutions of mepivacaine·HCl were prepared in 10 mM Tris-HCl (pH 7.4) and added to the labeled membrane suspension (or untreated SPMVs suspension) to give the desired concentration of mepivacaine·HCl (in this case, for 30 min incubation).

Excitation wavelengths were 286 nm for tryptophan and 330 nm for Py-3-Py. Emission wavelengths were 335 nm for tryptophan, 379 nm for Py-3-Py monomer and 480 nm for Py-3- Py excimer. For Py-3-Py excimer emission, a GG-455 cut-off filter was used. The excimer to monomer fluorescence intensity ratio, *I'/I*, was calculated from the 480 nm to 379 nm signal ratio. The excitation wavelength for DPH was 362 nm and emission wavelength was 424 nm.

Effect of Mepivacaine·HCl on the Structure of the Individual Monolayers of SPMVs: Selective Quenching of Py-3-Py

The experimental measurement of the structure of the individual monolayer of SPMVs is based on the method previously established for the SPMVs [25-27]. The method used is based on the assumption that the system is composed of fluorescing compartments that are differentially accessed by TNBS. The excimer to monomer fluorescence intensity ratios, *I'/I*, of Py-3-Py in bulk (inner plus outer), and in the inner and outer monolayers were calculated from the following equations:

$$(I'/I)_t = I'_t / I_t \quad \text{.....equation 1}$$

$$(I'/I)_i = I'_i / I_i \quad \text{.....equation 2}$$

$$(I'/I)_o = (I'_t - I_i) / (I_t - I_i) \quad \text{.....equation 3}$$

where $(I'/I)_t$, $(I'/I)_i$ and $(I'/I)_o$ are the excimer to monomer fluorescence intensity ratios of Py-3- Py (I'/I) in bulk, and in the inner and outer monolayers, respectively. The values of I'_t (excimer fluorescence intensity for inner plus outer monolayers) and I'_i (excimer fluorescence intensity for the inner monolayer) were determined for Py-3-Py from SPMVs incubated with buffer A and buffer A plus TNBS, respectively, at 4°C (pH 8.5) (non-penetrating conditions).

Determination of Annular Lipid Fluidity in SPMVs

The experimental determination of the annular lipid fluidity in SPMVs is based on a method previously established for synaptic plasma membrane [29,30] and SPMVs [25-27]. Incorporated Py-3-Py in the SPMVs was excited by radiation less energy transfer (RET) from tryptophan (excitation at 286 nm) and the excimer to monomer fluorescence intensity ratio (*I'/I*) of Py-3-Py was calculated from the ratio 480 nm to 379 nm signal. Taking into account that the Förster radius (the RET-limiting distance) for the tryptophan-Py-3-Py donor-acceptor pair is 3 nm [25-27,31], only Py-3-Py located in annular lipids (close to proteins) was excited, and the fluidity of annular lipids was considered proportional to *I'/I* [20,23-27].

Determination of Protein Distribution in the SPMVs Lipid Bilayer

This was based on a method previously established for membranes [25-27,29,30]. The fluorescence intensity of endogenous tryptophan in SPMVs was determined. Thereafter the Py-3-Py probe was incorporated at a concentration of 5×10^{-7} M (in absolute ethanol), and after 10 min, tryptophan emission fluorescence intensity was measured again. The efficiency of RET from tryptophan to Py-3-Py was calculated from the equation:

$$\text{RET} = (I_d - I_{da}) / I_d \quad \text{..... equation 4}$$

where I_d and I_{da} represent the fluorescence intensities of donor (in this case, endogenous tryptophan) in the absence and presence respectively of acceptor (in this case, Py-3-Py). The wavelengths of excitation and emission of tryptophan were 286 and 335 nm, respectively.

Effect of Mepivacaine·HCl on the Rotational Mobility of Bulk SPMVs

The intensities of the components of fluorescence parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the direction of the vertically polarized excitation light were determined by measuring the light emitted through polarizers oriented vertically and horizontally. The polarization (P) was obtained from the intensity measurements using $P = (I_{\parallel} - GI_{\perp}) / (I_{\parallel} + GI_{\perp})$ where G is a grating correction factor for the transmission efficiency of the monochromatic for vertically and horizontally polarized light. It 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 [$r = 2P/(3-P)$] (equation 5).

Effect of Mepivacaine·HCl on the Separate Monolayers of SPMVs: Selective Quenching of DPH

The experimental determination of the separate monolayer structure in SPMVs is based on a method by the method developed for tumor cell plasma membranes by Schroeder [32]. And a method previously established for LM fibroblast plasma membrane [33], for synaptic plasma membrane [21,34-36], for synaptosomal plasma membrane vesicles [19,25-27] for the plasma membrane vesicles of Chinese hamster ovary cells [23], for Mar 18.5 hybridoma cells [24] and for the myeloma cell line Sp2/0-Ag14 [20]. It does not simply provide a theoretically calculated or average value: instead it is based on the assumption that the system is composed of fluorescing compartments of different accessibility to TNBS. If fluorescence intensity, F, and anisotropy, r, are measured simultaneously, then

$$r = \sum Fjrj \quad \text{.....equation 6}$$

where Fj is the fraction of fluorescence intensity in compartment j. For a binary system composed of the outer and inner monolayers of the SPMVs, this leads to

$$r = \frac{Fi}{F} ri + \frac{F - Fi}{F} ro \quad \text{..... equation 7}$$

where F and Fi are the DPH fluorescence obtained for SPMVs incubated with buffer A and buffer A plus 2 mM TNBS for 40 min at 4°C (pH 8.5) (non-penetrating conditions), respectively. The values of fluorophore concentration-independent parameter anisotropies, r (anisotropy for both monolayers) and ri (inner monolayer anisotropy), were also determined for DPH in SPMVs incubated with buffer A and buffer A plus TNBS at 4ºC, respectively. The equation was then solved for ro (outer monolayer anisotropy).

Results

The Purity of SPMVs

We assessed the purity of SPMVs by enzymatic and morphological criteria. The specific activities of NaK-ATPase, acetylcholinesterase and 5'-nucleotidase were enriched about 4-, 2.5- and 3-fold, respectively, in the plasma membrane fraction with respect to crude homogenates. The transmission electron microscopic examination of the SPMVs indicated 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.

Effect of Mepivacaine·HCl on the Rate and Range of Lateral Mobility in Bulk Bilayer SPMVs

The I'/I value in intact SPMVs (mepivacaine·HCl-untreated) was 0.412 ± 0.007 (at 37°C, pH 7.4). Incubation with mepivacaine·HCl increased the range and rate of lateral mobility of bulk (inner + outer monolayer) SPMVs at concentrations as low as 0.1 mM (n = 5, P < 0.05), and above as demonstrated in (Table 1, Fig. 1).

Membrane	I'/I [a]	Anisotropy (r) [b]	Annular lipid fluidity	Protein clustering
Inner + Outer	0.412 ± 0.007	0.202 ± 0.004	0.245 ± 0.006	0.295 ± 0.003
Inner	0.377 ± 0.008	0.220 ± 0.003	—	—
Outer	0.445 ± 0.013	0.186 ± 0.005	—	—

[a] Synaptosomes were treated ± 4 mM 2,4,6-trinitrobenzenesulfonic acid (TNBS), pH 8.5, at 4°C for 80 min (I'/I), [b] synaptosomes were treated ± 2 mM TNBS, pH 8.5, at 4°C for 40 min (anisotropy), and the plasma membrane vesicles were isolated. 1,6-Diphenyl-1,3,5-hexatriene (or 1,3-di(1-pyrenyl) propane) was incorporated, and fluorescence measurements were performed at 37°C (pH 7.4). Values from TNBS-treated membrane represent the inner monolayer; values for the outer monolayer was calculated as described in Materials and Methods. Values are represented as the mean ± SEM of five determinations

Table 1: Structural parameters of intact Synaptosomal Plasma Membrane Vesicles (SPMVs) isolated from bovine cerebral cortex.

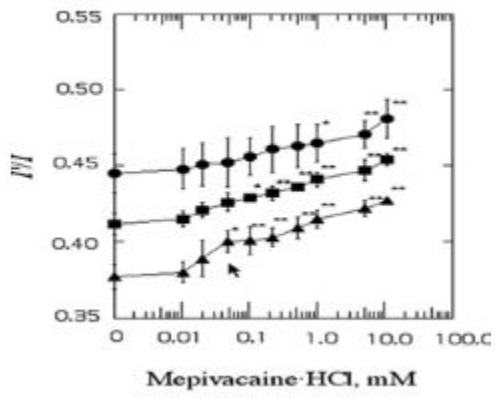

Figure: 1 The effect of mepivacaine·HCl on excimer to monomer fluorescence intensity ratio (*I'/I*) of Py-3-Py in SPMV. The excitation wavelength of Py-3-Py was 330 nm and the *I'/I* values were calculated from the 480 nm to 379 nm signal ratio. SPMVs was treated ± 4mM TNBS, pH 8.5, at 4˚C for 80 min. Py-3-Py was incorporated into SPMVs and fluorescence measurements were performed at 37˚C (pH 7.4). Untreated (inner and outer monolayers, ■); TNBS treated (inner monolayer, ▲); calculated for outer monolayer (●) by eq. 3 as described in Materials and Methods. Each point represents the mean ± SEM of 5 determinations. An asterisk and double asterisks signify $P<0.05$ and $P<0.01$, respectively, compared to control by Student's t-test.

The *I'/I* value of Py-3-Py in bulk SPMVs incubated with 1 mM mepivacaine·HCl was $0.441 \pm 0.005^{**}$ ($n = 5$, $P < 0.01$), and the change in *I'/I* value before and after adding the mepivacaine·HCl was 0.029. The *I'/I* values of Py-3-Py in the bilayer were 0.412 ± 0.007 (n = 5) and 0.356 ± 0.006 (n = 5) at 37 and 25˚C (pH 7.4), respectively. Hence the effect of 1 mM mepivacaine·HCl was equivalent to that produced by a temperature increase of approximate 6.2˚C.

Effect of Mepivacaine·HCl on the Rate and Range of Transbilayer Asymmetric Lateral Mobility of SPMVs Monolayers

The effect of increasing concentrations of mepivacaine·HCl on the *I'/I* values in the individual SPMVs monolayers is shown in Fig. 1. Mepivacaine·HCl increased the rate and range of lateral mobility of the inner monolayer to a significant extent (0.400 ± 0.007, $P < 0.05$, $n = 5$) at 0.05 mM mepivacaine·HCl (Fig. 1). It had a greater increasing effect on the lateral mobility of the inner monolayer (Fig. 1, filled triangles) than that of the outer monolayer (Fig. 1, filled circles). Since the changes in *I'/I* values is due primarily from the effect on the inner monolayer, we studied the selective effect of mepivacaine·HCl on the rate and range of mobility of the probe. The *I'/I* value in intact SPMVs inner monolayer (mepivacaine·HCl untreated) was 0.377 ± 0.008 (at 37˚C, pH 7.4). Incubation with mepivacaine·HCl increased the range and rate of lateral mobility of SPMVs inner monolayer at concentrations 0.05 mM ($n = 5$, $P < 0.05$) and above as demonstrated figure 1. The *I'/I* value of Py-3-Py in SPMVs inner monolayer incubated with 1 mM mepivacaine·HCl was 0.415 ± 0.006 ($n = 5$, $P < 0.01$), and the change in *I'/I* value before and after adding the mepivacaine·HCl was 0.038. The values of Py-3-Py in the bilayer were 0.377 ± 0.008 (n=5) and 0.326 ± 0.005 (n=5) at 37 and 25˚C (pH 7.4), respectively. Thus the effect of 1 mM mepivacaine·HCl was equivalent to that produced by a temperature increase of approximate 8.9˚C. To the best of our knowledge, the results for asymmetric lateral mobility presented here are the first to demonstrate that the Sheetz-Singer hypothesis [37] is valid in neuronal membranes.

Effect of Mepivacaine·HCl on Annular Lipid Fluidity in the SPMVs Lipid Bilayer

I'/I measurements showed that the annular lipid fluidity in SPMVs (intact membrane) was 0.245 ± 0.006 (37˚C, pH 7.4), and that this increased in response to concentration of 0.05 mM mepivacaine·HCl and above (Table 1, Figure 2).

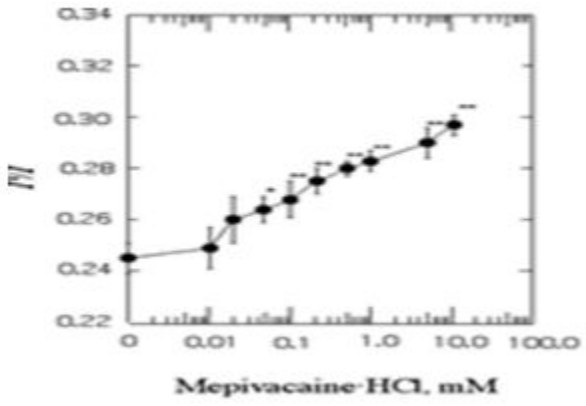

Figure: 2 The effect of mepivacaine·HCl on annular lipid fluidity in SPMVs. Py-3-Py was excited through RET from tryptophan (excitation wavelength, 286 nm) and the excimer to monomer fluorescence intensity ratio (*I'/I*) was calculated from the 480 nm to 379 nm signal ratio. Fluorescence measurements were performed at 37˚C (pH 7.4). Each point represents the mean ± SEM of 5 determinations. An asterisk and double asterisks signify $P<0.05$ and $P<0.01$, respectively, compared to control by Student's t-test.

The *I'/I* values of Py-3-Py in the bilayer are 0.245 ± 0.006 (n=5) and 0.199 ± 0.002 (n=5) at 37˚C and 25˚C, respectively. Thus, the effect by 1.0 mM mepivacaine·HCl was the same as that produced by a temperature increase of approximate 9.9˚C. The important finding is that those increasing effects were far greater in annular lipid fluidity than on the lateral and rotational mobilities.

Effect of Mepivacaine·HCl on Protein Distribution in SPMVs

We evaluated protein distribution by RET from tryptophan to Py-3-Py. The RET value of untreated SPMVs was 0.295 ± 0.003 (37°C, pH 7.4), (Table 1, Fig. 3) and it was lowered by concentrations of mepivacaine·HCl of 0.1 mM or more (Fig. 3). Protein clustering is probably caused by interaction among phospholipids, especially annular lipids, whose mobility is increased by mepivacaine·HCl and proteins around them.

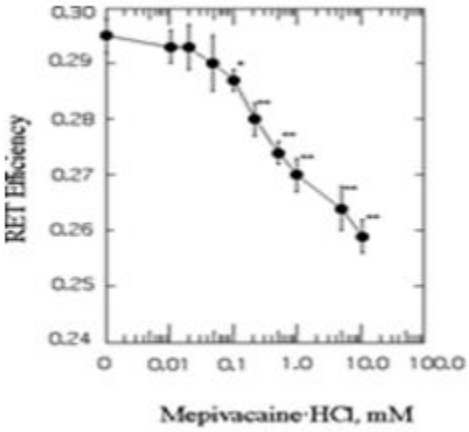

Figure: 3 The effect of mepivacaine·HCl on protein distribution in SPMVs. Efficiency of RET from tryptophan to Py-3-Py was taken as a measure of protein clustering and calculated by eq. 4. Fluorescence measurements were performed at 37°C (pH 7.4). Each point represents the mean ± SEM of 5 determinations. An asterisk and double asterisks signify $P<0.05$ and $P<0.01$, respectively, compared to control by Student's t-test.

Effect of Mepivacaine·HCl on the Range of Rotational Mobility of Bulk Bilayer SPMVs

The anisotropy (*r*) of DPH in the intact SPMVs was 0.202 ± 0.004 (at 37°C, pH 7.4) (Table1). Mepivacaine·HCl increased rotational mobility, with a significant decrease in anisotropy (*r*) even at 0.1 mM and above (Fig. 4). The difference in anisotropy of the bulk SPMVs lipid bilayer before and after adding 1 mM mepivacaine·HCl was 0.031. This can be evaluated by comparison with the effect of temperature on this parameter. The anisotropy of DPH in the bilayer was 0.202 ± 0.004 (n = 5) at 37°C and 0.257 ± 0.002 (n = 5) at 25°C (pH 7.4). The effect of 1 mM mepivacaine·HCl was thus the same as that of a temperature increase of approximate 6.8°C.

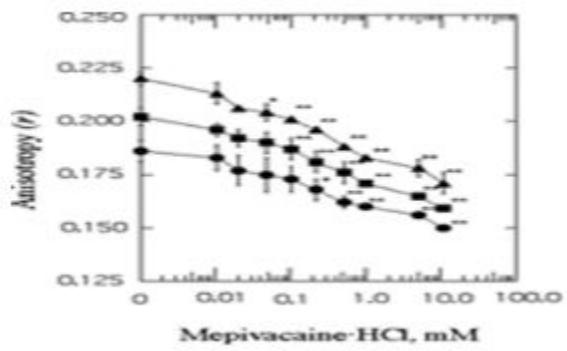

Figure: 4 The effect of mepivacaine·HCl on the anisotropy (*r*) of DPH in SPMVs. The excitation wavelength for DPH was 362 nm and fluorescence emission was read at 424 nm. SPMVs was treated ± 2 mM TNBS, pH 8.5, at 4°C for 40 min. DPH was incorporated into SPMVs and fluorescence measurements were performed at 37°C (pH 7.4). Untreated (inner and outer monolayers, ■); TNBS treated (inner monolayer, ▲); calculated for outer monolayer (●) by eq. 7 as described in Materials and Methods. Each point represents the mean ± SEM of 5 determinations. An asterisk and double asterisks signify $P<0.05$ and $P<0.01$, respectively, compared to control by Student's t-test.

Effect of Mepivacaine·HCl on the Range of Transbilayer Asymmetric Rotational Mobility of SPMVs Monolayers

The structures of the intact SPMVs (inner plus outer monolayers), and the outer (extracellular) and inner (intracellular) monolayers separately, were evaluated with DPH as a fluorescent reporter and trinitrophenyl groups as quenching agent. Trinitro phenylation of the intact synaptosomal at 4°C (non-penetrating conditions) results in covalent attachment of trinitrophenylated quenching agent to the outer monolayers. Approximately half of the DPH fluorescence was quenched in the treated SPMVs outer monolayer. When TNBS labeling was conducted in penetrating conditions (37°C), greater than 80% of the fluorescence of the DPH was quenched. Values of fluorescence parameters in intact SPMVs (both monolayers) and in TNBS-treated SPMVs (inner monolayer) are listed in Table 1. The anisotropy of DPH in the inner monolayer was 0.034, significantly greater than that calculated for the outer monolayer, as demonstrated in Table 1.

Fig. 4 shows that the anisotropy (r) of DPH in the TNBS untreated membrane (inner plus outer monolayers) decreased gradually (fluidization) with increasing mepivacaine· HCl concentrations (Fig.4, filled squares). There was a similar, but more gradual, decrease in the calculated anisotropy of the inner monolayer at any

mepivacaine· HCl concentration (Fig.4, filled triangles). However, there was no statistically significant decrease in the anisotropy (the rotational mobility range) of the outer monolayer at 0.01 ~ 0.1 mM of the mepivacaine·HCl concentrations used. These results suggest that the fluidizing effect (range of rotational mobility) of mepivacaine·HCl is selective.

Discussion

We used Py-3-Py, a pyrene derivative that has been used to quantify lateral mobility within native and model membranes [20,23-27,38,39], to determine the rate and range of lateral mobility in the SPMVs. With this probe one monitors emission of both the monomer (I) and the excimer (I´) components in such a way that a ratio can be derived and used as a measure of lateral mobility [20,23-27,38,39]. As the probe mobility increases emission from the excimer predominates, since formation of the intramolecular excimer is dependent upon lateral movement of its two components. Therefore, an increase in the *I´/I* ratio indicates increased lateral mobility of the probe within the membranes. The excimer fluorescence technique using Py-3-Py has the advantage over its counterpart basednon intermolecular excimerization that very low probe concentrations can be used ($<10^{-7}$ M) and perturbation of the SPMV by the probe molecule is minimized.

The covalently linked trinitrophenylated group has a broad absorbance range with a maximum near 420 nm. This peak has a large overlap with the fluorescence emission of Py-3-Py. This overlap is responsible in part for the high transfer (quenching) efficiency of the probe. Approximately half of the Py-3-Py fluorescence was quenched in the trinitrophenylated SPMVs. When TNBS labeling was conducted under penetrating conditions (37°C), nearly 80% of the fluorescence of the Py-3-Py was quenched. Values of the excimer to monomer fluorescence intensity ratio (*I'/I*) of Py-3-Py in intact SPMV (both monolayers) and in TNBS-treated SPMVs (inner monolayer) are listed in Table 1. The *I´/I* of Py-3-Py in the outer monolayer was 0.068, greater than that calculated for the inner monolayer. This means that the rate and range of lateral mobility of the outer monolayer is greater than that of the inner monolayer.

Although many researchers have reported that the inner and outer monolayers of native and model membranes differ in fluidity, all previous studies of asymmetric bilayer fluidity have examined the rotational range but not the rate and range of lateral mobility. In this study, using the selective quenching of Py-3-Py and DPH fluorescence by trinitrophenylated groups, we examined trans bilayer asymmetric fluidity.

The TNBS labeling reaction must be carefully monitored in order to ensure that the reagent does not penetrate into the synaptosomes and label both sides of the plasma membrane. For this purpose, three control procedures are routinely used. First, as an "internal control", mitochondria and microsomes are isolated from the synaptosomes from which the trinitrophenylated plasma membranes are isolated. If any significant degree of penetration of TNBS into the synaptosome occurs, these intracellular organelles also become trinitrophenylated. Only 1.8 ± 0.2% and 2.1 ± 0.4% of microsomal and mitochondrial phosphatidylethanolamine were trinitrophenylated by our treatment [19]. In contrast, when the TNBS treatment is performed under penetrating conditions (37°C), 60-80% of the phosphatidylethanolamine in microsomes or mitochondria is trinitrophenylated [19]. Second, approximately half of the Py-3-Py fluorescence was quenched in the trinitrophenylated SPMVs. Third, the trinitrophenylation of the synaptosomal may alter membrane enzyme activities. Unlike the results obtained under penetrating conditions (37°C), the activity of neither NaK-ATPase nor 5'-nucleotidase was significantly altered by the TNBS reaction under non- penetrating conditions [19].

It is important to note that the term "membrane fluidity" is often misused. It arose from a combination of spectroscopic studies, the realization that membranes can be regarded as two- dimensional fluids, and the desire to obtain a simple single physical parameter that would describe their properties. The difficulty with the membrane fluidity concept is that any physical parameter chosen will be a function of the spectroscopic method employed, specifically its particular time window, and the properties of the probe (shape, charge, location etc). The membrane fluidity concept also depends on the assumption that the hydrophobic region of cell membranes is structurally and dynamically homogeneous, an assumption that is now under serious challenge. Thus, while it may be true to say that the bulk or average spectroscopic properties of cell membranes may not be useful in building a hypothesis for the pharmacological action(s) of drug(s), local properties pertaining to domains or the immediate environment of a membrane protein may be very relevant.

As already pointed out, membrane bilayer mobility is one of the important factors controlling membrane micro viscosity or fluidity. Membrane bilayer mobility includes lateral mobility, rotational mobility and flip-flop and it is well known that the most important of these is lateral mobility. We are pleased to have been able to develop and describe, for the first time, a fluorescence quenching technique that can measure membrane transbilayer lateral mobility. I therefore believe that this study will make a contribution to the study of drug-membrane physical interactions. The clear mechanism of action of the drug on the increasing effects of annular lipid fluidity of the SPMV is unknown. However, the mechanism through which mepivacaine·HCl increases the annular lipid fluidity in the SPMVs lipid bilayer can be assumed as follows.

Annular lipids are known to surround proteins with or without being physically associated with them. Mepivacaine·HCl may alter the stereo structure or dynamics of these proteins by combin-

ing with the lipids, especially with the annular lipids, increasing their mobility and indirectly affecting the dynamic behavior of the proteins. Because biological membranes are of highly complex composition, it has not been feasible to monitor changes in the local lipid environment and at the same time to determine their effect on membrane protein function. Nevertheless, is likely that the observed effects are not only due to the influence of mepivacaine·HCl on lipids, but are magnified by the interactions between lipids and proteins.

Mepivacaine·HCl thus affects the lateral and rotational mobility of SPMVs mainly via an effect on the inner monolayer of the SPMVs. This is the first demonstration that mepivacaine·HCl has a differential effect on the transbilayer lateral and rotational mobility of the inner and outer monolayers of neuronal membranes. It seems that native membranes, specifically inner monolayer, are much more sensitive to the fluidizing effects of mepivacaine·HCl, and this finding can be extended to the transbilayer asymmetric fluidity of neuronal membranes.

It was confirmed that while the local anesthetic mepivacaine·HCl used in this study did not generate significant increasing effect of lateral and rotational mobilities on the outer monolayer as well as the bulk bilayer of the neuronal membrane at a concentration of 0.05 mM, it did generate significant increasing effect of the two kinds of mobilities on the inner monolayer at the concentration of 0.05 mM. However, it was confirmed that while the mobility in the inner monolayer is significantly increased at the concentration level of 0.05 mM, the mobility in bulk SPMVs showed a significant increase at the concentration level of 0.1 mM, which is twice as high as the concentration for the inner monolayer and the mobility in the outer monolayer did not show any significant increase until the concentration level reached 1.0 mM, which is 20 times higher than the concentration for the inner monolayer. Thus mepivacaine·HCl has a selective fluidizing effect within the transbilayer domains of the SPMVs.

From the results of our study, it is without a doubt that mepivacaine·HCl increased lateral and rotational mobilities of the neuronal membrane lipid bilayer and it may expand membrane. Mepivacaine·HCl which increase rotational and lateral mobility of the neuronal lipid bilayer mostly increased the mobility of inner monolayer, thereby expanding membranes. These effects are not solely due to the influence of mepivacaine·HCl on lipids, but they are magnified by the interaction between lipids and proteins. This conclusion can be drawn because we confirmed that the magnetite of increasing effect on annular lipid fluidity in SPMVs lipid bilayer induced by mepivacaine·HCl was significantly far greater than the magnetite of increasing effect of the drug on the lateral and rotational moblities of bulk SPMVs lipid bilayer.

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. 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 suggest that local anesthetics, in addition to its direct interaction with sodium channels, concurrently interact with membrane lipids, affecting fluidity of the neuronal membrane.

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