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Differential interactions of two local anesthetics with phospholipid membrane and nonerythroid spectrin: Localization in presence of cholesterol and ganglioside, GM_1

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ABSTRACT

Interactions of two local anesthetics, dibucaine and tetracaine have been studied with phospholipid vesicles containing cholesterol and/or monosialogangliosides (GM_1) using fluorescence spectroscopy. The fluorescence intensity of tetracaine showed a marked increase with the increasing molar ratio of the phospholipid to tetracaine, while that of dibucaine showed opposite effects. Steady state anisotropy and the wavelength of maximum emission (λ_{max}) decreased with the increasing phospholipids to tetracaine ratio. The extent of such changes in anisotropy and λ_{max} in the presence and absence of two important components of neuronal membranes, cholesterol and GM_1 indicated differential membrane localization of the two local anesthetics. To understand the intercellular mode of action of local anesthetics, we have also studied the interactions of dibucaine and tetracaine with brain spectrin which indicate differential spectrin interactions with similar binding strength. Thermodynamic parameters associated with such binding reveal that binding is favored by entropy. Tetracaine brings about distinct structural changes in spectrin compared to dibucaine, as reflected in the tryptophan mean lifetime and far-UV CD spectra. Tetracaine also exhibits a detergent-like property inducing concentration dependent decrease in spectrin anisotropy, further indicating structural changes in brain spectrin with probable implications in its anesthetic potential.

1. Introduction

Local anesthetics are known to produce loss of sensation in certain areas of the body by blocking nerve transmissions via state dependent binding of voltage-gated Na^+ channels [1,2]. They have the potential to cause serious harm if used without caution. Use of high doses of local anesthetics may cause cardiovascular collapse (etidocaine, bupivacaine, lidocaine) and irreversible nerve injury (lidocaine, tetracaine) due to its surfactant-like properties. The details of molecular mechanisms by which local anesthetics block impulses in peripheral nerves are well established. However, the overall mechanism for spinal and epidural anesthesia and drug induced toxicity is still an unsolved question [3–5]. Anesthetic action of local anesthetics in the peripheral nervous system is caused by blocking the propagating action potential through inhibition of voltage-gated sodium ion channels. Such inhibition results from the binding of local anesthetics within the cytoplasmic domain of the channel protein. Local anesthetics bind to the pore region S5–S6 transmembrane segments from all four pseudorepeated domains of channel protein [6,7]. Binding of local anesthetics to these channels depends on their conformation with the drug, generally having a higher affinity for the open and inactivated state of the channels, induced by membrane depolarization. In contrast with anesthesia in the peripheral nervous system, the overall mechanism for spinal and epidural anesthesia is more complex than simply blocking the impulses in nerve roots, involving both pre- and post-synaptic receptors as well as intercellular pathways. Delivery of local anesthetics to the spinal chord as well as to spinal roots allows the possibility of altering both synaptic activity and impulse conduction affecting the responses within the spinal cord. Local anesthetics can also interact with membrane lipids, thereby affecting the conformation of the drugs for presentation to a variety of neuronal membrane channels and receptors, leading to clinical analgesia. The local anesthetics membrane interactions can thus modulate sodium channels, favoring the inactivated state of the channel that is essential for their anesthetic action governing clinical effectiveness.

Cholesterol is an essential component of eukaryotic cells and is non-randomly distributed among the biological membranes [8,9]. Cholesterol is the most representative sterol enriched in plasma membrane.

Abbreviations: CD, circular dichroism; DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoyl-phosphatidylcholine; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; GM_1, monosialogangliosides; MOPS, 3-[-N-morpholino] propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PMSF, phenyl methylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SUVs, small unilamellar vesicles; Trp, tryptophan; λ_{max}, wavelength of maximum emission; SEM, standard error of mean

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The major function of cholesterol is to condense and order the polar lipids, thereby thickening, stiffening and strengthening the bilayer [10,11]. Cholesterol also maintains the lateral heterogeneity of lipid and protein distribution in the plasma membrane and forms clusters with other lipid components of membranes such as sphingolipids to form rafts, playing a crucial role in signal transduction [12,13]. Gangliosides, the most complex of glycosphingolipids, are abundant in the plasma membrane of the nerve cells (making up 5–10% of the local lipid mass) and show immense structural variations [14]. Gangliosides are sialic acid containing glycolipid, formed by a hydrophobic ceramide and a hydrophilic oligosaccharide chain. Nerve cell membranes are particularly rich in gangliosides which play an important role in signal transduction and brain pathology through cell–cell interaction [15,16]. Gangliosides are also involved in many important events occurring at the cell surface including binding of various antibodies, pericellular adhesive protein, bacterial toxins and viruses to plasma membrane. Gangliosides have been considered as an important component of lipid microdomains or raft that mediated protein sorting, transport and signal transduction [12,17,18].

The effects of local anesthetics on the organization of phospholipid bilayers and the motional constraints on the anesthetic molecule upon binding to lipid membranes have been studied through several spectroscopic techniques [19–26]. Earlier studies have shown that the potency of an anesthetic is proportional to its amphiphilic side chain [27–29]. Furthermore, it has been reported that the potency of an anesthetic is not regulated by its hydrophobicity alone. Polar interactions and other steric parameters also determine a specific or preferential localization of each anesthetic molecule inside the membrane [30,31]. In addition, DSC study indicates that local anesthetics can lower the phase transition temperature and increase electrical conductance of the phospholipid membrane by interacting with its hydrophilic head group region of lipid bilayers [32–34]. One of the most studied tertiary amine local anesthetics, tetracaine (2-[diethylamino] ethyl-4-[butylamino]benzoate) and dibucaine (2-butoxy-N-[2-diethylamino]-4-quinoline-carboxamide) can assume several forms such as neutral base, hydrogen bonded, monoprotonated, diprotonated species, depending upon the pH of the solvent and the nature of the microenvironment. Tetracaine and dibucaine are monoprotonated in water, in the range of pH 3–14. In hydrophobic environments, the deprotonated species prevails. Boulanger and coworkers have shown, using ^{2}H NMR, that charged tetracaine behaves like an inorganic ion and weakly bound charged anesthetics reside in the membrane water interface. On the other hand, neutral anesthetic molecules bind strongly to the membrane and penetrate deeply into the core of the bilayer [20]. NMR spectroscopic studies supported by molecular dynamics calculation indicate that tetracaine and dibucaine assume more than two conformations and exist as dimers in phosphatidylcholine (PC) vesicles [35]. In an earlier NMR study performed in pure PC vesicles using deuterated anesthetics, multiple binding sites for tetracaine and procaine were observed in phospholipid bilayers [22]. These studies also indicated two states of binding for tetracaine: a weakly bounded state at a low pH of 5.5 and a "strongly bound" state at a high pH of 9.5, signifying the presence of two differently charged or aggregated species of the local anesthetics. NMR and fluorescence studies indicated dibucaine binding in the vicinity of the phospholipid glycerol moiety [20,36]. Studies on depth profiling by fluorescence quenching and NMR investigation in different membrane systems indicated the location of dibucaine and tetracaine to be at a shallow position in the phospholipid bilayer [36–38]. NMR investigations have also been performed to study the interaction of tetracaine with a PC-based membrane containing cholesterol [39].

Local anesthetics have also been shown to interact with many membrane associated proteins other than the primary targets of Na^{+} channel. Those include the acetylcholine receptor [40], cytochrome oxidase and the F_1-ATPase [41,42] and band-3 erythrocytes [43]. In addition, it has been shown that dibucaine and tetracaine can alter the denaturation temperature of Ca^{+2}-ATPase of sarcoplasmic reticulum [44]. Dibucaine and tetracaine inhibit the activation of mitogen-activated protein kinase mediated calcium channels [45] and also cause inhibitions of dog kidney Na^{+}, K^{+}-ATPase activity [46] and G-protein couple receptor signaling [47]. Local anesthetics were also found to affect phospholipase D activity in differentiated human leukemic cells [48], brain microtubule assembly and were capable of binding membrane skeletal protein spectrin [50].

Brain spectrin, a protein homologous to erythroid spectrin, forms filamentous networks in the cytoplasmic face of the nerve cell membrane. To establish its planer structure, it interacts with a large number of proteins such as actin, adducin, ankyrin and band 4.1. Brain spectrin remains most stable in its tetrameric state, formed by the side by side association of the two heterodimers [51,52]. Like erythroid spectrin, it also binds phospholipids [53].

In spite of so many studies discussed above, few attempts have been made to study interactions of local anesthetics with other membrane components e.g. cholesterol and ganglioside, GM_1. Earlier we have studied, using fluorescence spectroscopy, the interaction of the protonated quinoline-based local anesthetic, dibucaine, with small unilamellar vesicles of dimyristoylphosphatidylcholine (DMPC) and dioleoyl phosphatidylcholine (DOPC) containing different mole percents of cholesterol and ganglioside [54,55]. We have extended the previous work using another benzene-based, fluorescent, tertiary amine local anesthetic, tetracaine with small unilamellar vesicles (SUVs) of DMPC and DOPC with or without cholesterol or the monosialoganglioside, GM_1. As a part of our broad objective towards building a working model to understand the mechanism of local anesthesia in the intercellular context we also studied interactions of the protonated local anesthetics with brain spectrin. In the present study, we show that, as tetracaine is known to be less toxic and more potent than dibucaine, it interacts differentially with the phospholipid membrane and the neuronal membrane skeletal protein. This is reflected in differential membrane localization in the presence and absence of cholesterol and GM_1 and in the thermodynamic parameters associated with binding to brain spectrin.

2. Materials & methods

Tetracaine hydrochloride, dibucaine hydrochloride, L-DMPC, L-DOPC, cholesterol, diethylamine, MOPS, Tris, KCl, phenyl methylsulfonyl fluoride (PMSF), dithiothreitol (DTT), EDTA, EGTA, Imidazole, $MgCl_2$, and NaCl were purchased from Sigma (St. Louis, MO). Deionized water from Milli-Q (Millipore Corporation, USA) was used for the preparation of buffer and all other solutions. Phospholipid concentrations were estimated after digestion with perchloric acid following published protocol [56]. Stock solution of dibucaine and tetracaine was prepared in ethanol and their concentrations were determined by absorbance measurement on a Cary Bio-50 UV spectrophotometer using molar extinction coefficient of 23,400 M^{-1} cm^{-1} at 310 nm for tetracaine and of 4400 M^{-1} cm^{-1} at 326 nm for dibucaine respectively [20,50].

Gangliosides were isolated from ovine brain following the extraction method elaborated in our previous work [55]. GM_1 was prepared from the ganglioside mixture by the treatment of neuraminidase at 37 °C for 30 min to obtain the monosialogangliosides following the method describe earlier [55,57]. GM_1 was purified on DEAE Sephadex column and the purity was checked by thin layer chromatography [55].

To prepare small unilamellar phospholipid vesicles (SUV) of DMPC and DOPC, solutions of the phospholipids in chloroform were first evaporated under a thin stream of nitrogen. The resulting lipid films were then dried overnight in a vacuum desiccator. Lipids were swollen by adding the buffer (10 mM MOPS, pH 6.5, containing 50 mM NaCl) to the films and the mixture of the lipid was dispersed using vortex. The dispersion was then sonicated for 10 min (in bursts of 1 min while being cooled in ice) using a Soniprep 150 sonicator from MSE, U.K. The sonicated samples were centrifuged at 10,000 rpm for 15 min to remove titanium particles. Cholesterol and GM_1 containing vesicles were prepared by co-solubilizing appropriate quantities of cholesterol and GM_1 with phospholipids in chloroform/methanol (2:1 v/v). SUVs of

phospholipids with and without cholesterol or GM_1 were characterized by electron microscopy showing average diameters within 250–350 Å. Cholesterol and GM_1 content in the membrane are expressed as mole percent with respect to the bulk phospholipid.

Brain spectrin in its tetrameric form was purified from ovine brain, in 10 mM Tris and 0.6 M KCl containing 20 μg/ml PMSF at pH = 8.0 following the published procedure elaborated in our previous work [58].

2.1. Steady state fluorescence measurement

Small aliquots of an ethanolic stock solution of dibucaine and tetracaine hydrochloride were added to the standard buffer to obtain the typical final concentration of about 10 μM and 5 μM respectively for fluorescence measurements. Steady state fluorescence measurements of dibucaine and tetracaine were performed using Cary eclipse spectrofluorometer at excitations of 325 nm and 311 nm with silts of 5 nm bandpass both for excitation and emission channels respectively. The tertiary amine-free vesicles were used as reference banks in absorption and emission measurements to cancel any contributions due to solvent Raman peak and other scattering artifacts. The spectral shifts obtained from different sets of sample were identical in most cases, otherwise, the values within ±1 nm were reported.

Steady state anisotropy (r) measurements were performed using Cary eclipse polarization accessory. Anisotropy values were calculated from the following equation — [59]

$$r = I_{VV} - GI_{VH}/I_{VV} + 2GI_{VH} \quad (1)$$

where I_{VV} and I_{VH} are the measured fluorescence intensities with excitation polarizer oriented vertically and horizontally respectively. G is the grating factor and is the ratio of the efficiencies of the detection system for vertically and horizontally polarized light and is equal to I_{HV}/I_{HH}. The temperature dependence study of λ_{max} of tetracaine in membrane environments was carried out using a NESLAB RTE-110 circulating bath.

The apparent partition coefficient (so-called because electrostatic effects were not taken account) of dibucaine and tetracaine between the membrane and the aqueous phases (K_p) was taken as the molar ratio of lipid at which anisotropy Vs [L]/[D] or [L]/[T] plots changes over from steeply increasing to saturation behavior [54,55].

2.2. Binding of dibucaine and tetracaine to brain spectrin

Small aliquots of an aqueous stock solution of dibucaine and tetracaine were added to 0.1 μM solution of non-erythroid spectrin upon excitation at 295 nm in a buffer of 10 mM Tris and 20 mM KCl at pH 8.0 for fluorescence measurement. Protein free buffer containing different concentration of anesthetic was used as blank in all fluorescence measurements. Fluorescence intensities were corrected for the inner filter effect, if any, due to absorption of the protein and the local anesthetics, when absorbance, at both excitation and emission wavelengths, of the samples exceeded 0.05 [50]. Changes in the fluorescence emission intensity of proteins upon progressive addition of anesthetics were measured to evaluate binding constants. Results from fluorometric titration were analyzed by two different methods. The apparent dissociation constant ($K_d = 1/K_{app}$) of protein to anesthetic was determined using non-linear curve fitting analysis based on the equation [50,58].

$$P + LA = P{-}LA \quad (2)$$

where P represents protein and LA represents local anesthetic. All experimental points for binding isotherms were fitted by list square analysis.

$$K_d = [C_s - (\Delta F/\Delta F_{max}) \cdot C_s \cdot C_{LA} - (\Delta F/\Delta F_{max}) \cdot C_s/(\Delta F/\Delta F_{max}) \cdot C_s \quad (3)$$

$$C_s \cdot (\Delta F/\Delta F_{max})^2 - (C_s + C_{LA} + K_d) \cdot (\Delta F/\Delta F_{max}) + C_{LA} = 0 \quad (4)$$

ΔF is the change in fluorescence intensity at 340 nm for each point of titration curves. ΔF_{max} is the same when the ligand molecules are completely bound to spectrin. C_{LA} is the concentration of local anesthetic; C_s is the concentration of the protein and K_d is the apparent dissociation constant. The value of ΔF_{max} was determined from a double reciprocal plot.

$$1/\Delta F = 1/\Delta F_{max} + 1/[K_{app} \cdot \Delta F_{max} \cdot (C_{LA} - C_S) \quad (5)$$

The linear double reciprocal plot of $1/\Delta F$ against $1/(C_{LA} - C_S)$ is extrapolated to ordinate to obtain value of ΔF_{max}. For monitoring the anesthetics induced conformational changes of spectrin anisotropy (r) were measured upon excitation at 295 nm with the emission monitored at 340 nm, as a function of increasing concentrations of the local anesthetics.

All the measurements with membranes were performed at 35 °C unless mentioned otherwise, with multiple sets of measurements. For 3 independent measurements, the average values are given. Error bars for all spectrin binding experiments were given as standard errors of mean (SEM) of 5 independent experiments.

2.3. Time-resolved fluorescence measurements

Fluorescence lifetime was measured from the decay profile of time resolved fluorescence intensity using Fluromax-3 spectrophotometer (Horiba Jobin Yvon, Edison, NJ) with Data Station software in the time correlated single photon counting mode using Nano-LEDs as source. Lamp profiles were measured at the excitation wavelength using powdered milk as the scattering control. To optimize the signal to noise ratio, 5000 photon counts were collected at the peak channel. All the experiments were performed in a buffer containing 10 mM Tris and 20 mM KCl, pH 8.0 using excitation and emission slits with a nominal bandpass of 4 nm or less. The excitation and emission wavelengths were fixed at 295 nm and 340 nm respectively for determining the tryptophan lifetime of brain spectrin. Fluorescence intensities decay curve so obtained was deconvoluted with instrument response function and analyzed as a sum of exponential terms:

$$F(t) = \Sigma_i \alpha_i \exp(-t/\tau_i) \quad (6)$$

where $F(t)$ is the fluorescence intensity at time t and α is the pre-exponential factor representing the fractional contribution to the decay of the lifetime component such that $\Sigma_i \alpha_i = 1$. The goodness-of-the-fit to the data of a given set was evaluated by the χ^2 ratio. A fit was considered acceptable when plot of weighted residue and autocorrelation function showed random deviation about zero with a minimum χ^2 value not more than 1.20. Mean (average) lifetime (τ) for triexponential fluorescence decays were calculated from decay times and pre-exponential factor using the following equation [59].

$$\tau_0 = \Sigma \alpha_i \tau_i^{\,2} / \Sigma \alpha_i \tau_i \quad (7)$$

2.4. Evaluation of thermodynamic parameters

The thermodynamic parameters for anesthetic binding with brain spectrin have been determined from the van't Hoff equation:

$$\ln\ K_a = -(\Delta H/RT) + \Delta S/R \quad (8)$$

where K_a is the binding affinity constant at temperature T and R is the gas constant. The equation gives the standard enthalpy change (ΔH°)

and standard entropy change (ΔS°) on binding. The free energy change (ΔG°) has been estimated from the following relationship:

$$\Delta G = \Delta H - T\ \Delta S. \quad (9)$$

2.5. Circular dichroism measurements

CD measurements were carried out at room temperature in a Biologic science spectropolarimeter. The spectra were scanned in a quartz optical cell with path length of 0.1 cm. For monitoring changes in secondary structure, all spectra were recorded with an increment in wavelengths of 0.5 nm in the far UV region from 200 nm to 250 nm. Each spectrum is an average of five continuous scans, corrected by subtraction of appropriate blank without spectrin. Spectrin (0.2 mg/ml) was incubated in a buffer of 10 mM 20 mM KCl, pH 8.0 for 1 h with different concentration of dibucaine/tetracaine before the spectra were recorded. All spectra were smoothened making sure that the overall shape of the spectra remains unaltered, expressed as observed value in millidegree. The CD results have been analyzed in terms of mean residue ellipticity (MRE) in $deg \cdot cm^2 \cdot dmol^{-1}$ according to the following equation

$$[\theta] = [\theta]_{obs}(mrw)/10.c.l \quad (10)$$

where c is the concentration of brain spectrin in g/ml, θ is observed rotation in degree, l is the path length in cm, and μ is the mean residual molecular weight of spectrin. Quantitative assessment of the percentage of α-helix in brain spectrin in the presence and absence of local anesthetic could be estimated by the relation [60]

$$\%\ \text{of}\ \alpha-\text{helix} = [\theta]_{222} + 2340/-30300 \quad (11)$$

where $[\theta]_{222}$ are the observed MRE value at 222 nm. In Eq. (11), 2340 is the MRE of the β-form and random coil conformation cross at 222 nm and 30,000 is the pure helix at 222 nm.

3. Results

3.1. Tetracaine and dibucaine in phospholipid membrane containing cholesterol

In aqueous environment (10 mM MOPS, 50 mM NaCl, pH 6.5) protonated dibucaine ($pK_a = 8.3$) and tetracaine ($pK_a = 8.5$) show emission maxima at 412 nm and 372 nm respectively, whereas its emission progressively blue shifted in more hydrophobic environments. Fig. 1 shows the changes in fluorescence intensity of tetracaine and dibucaine at the respective λ_{max}, as a function of [Lipid/Anesthetic]

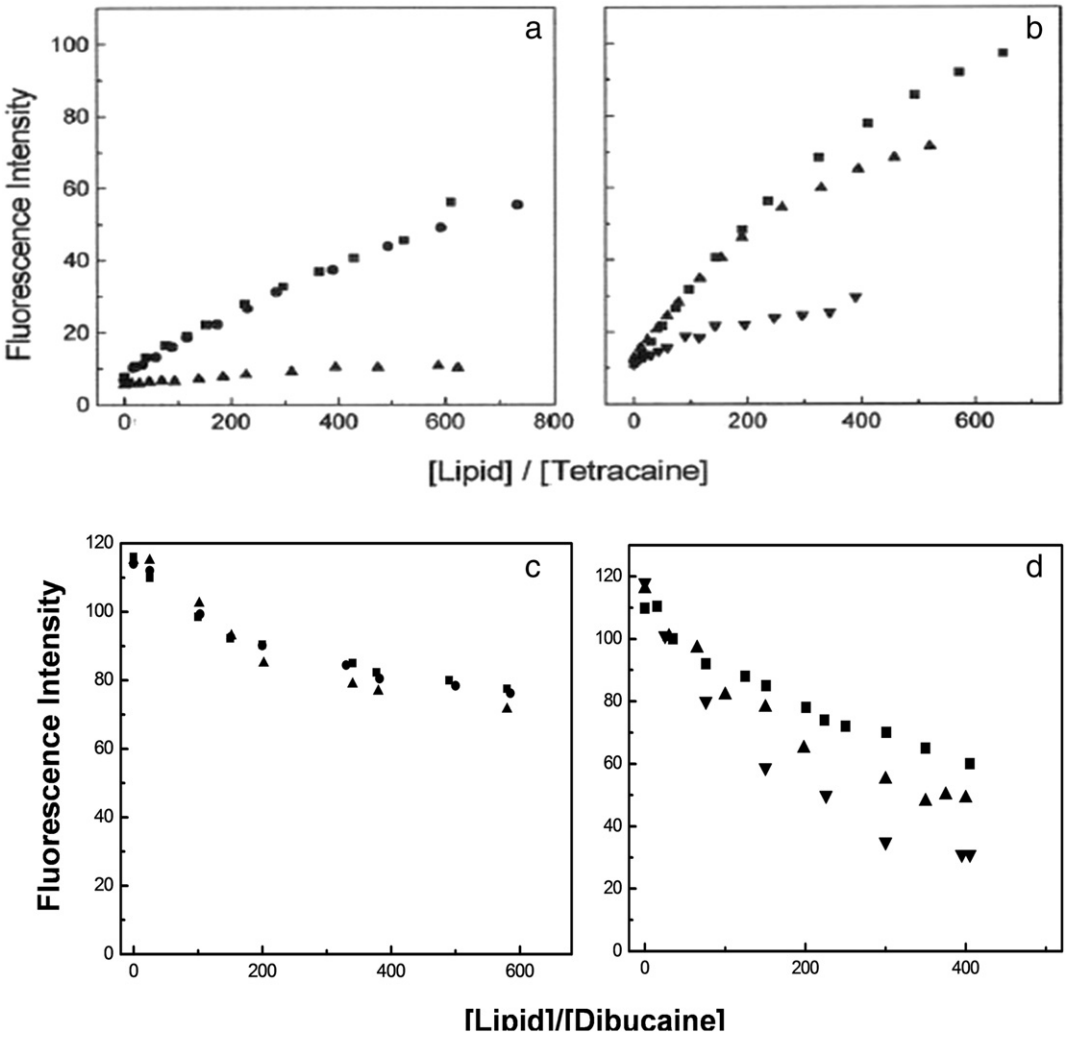

Fig. 1. Change of intensity at emission maxima of tetracaine at 35 °C with phospholipid to tetracaine molar ratio ([L/T]) in SUVs composed of (a) pure DMPC (■); DMPC with 10% (•) and 20% (▲) Cholesterol and (b) pure (DOPC) (■); DOPC with 20% (▲) and 40% (▼) Cholesterol. Change of intensity at emission maxima of dibucaine at 35 °C with phospholipid to dibucaine molar ratio ([L/D]) in SUVs composed of (c) pure DMPC (■); DMPC with 10%(●) 20% (▲) Cholesterol and (d) pure (DOPC) (■); DOPC with 20% (▲) and 40% (▼) Cholesterol. See Materials & methods for other details.

molar ratio in both DMPC and DOPC SUVs at 35 °C, well above the phase transition temperature of both the membranes, with increase in cholesterol. Tetracaine and dibucaine show opposite effect. The fluorescence intensity increased with increasing [L/T] for tetracaine in the absence of cholesterol (Fig. 1a & b). Dibucaine, on the other hand showed decrease in the intensity with increasing [L/D] both in the presence and absence cholesterol (Fig. 1c & d), in DMPC and DOPC SUVs.

Fig. 2 shows the change in λ_{max} as a function of lipid to tetracaine molar ratio in DMPC (Fig. 2a) and DOPC SUVs (Fig. 2b) containing different amounts of cholesterol at 35 °C. In pure DMPC vesicles λ_{max} of dibucaine appeared at 394 nm [54], whereas λ_{max} for tetracaine appeared at 354 nm from 372 nm indicating a blue shift of 18 nm. Incorporation of increasing amounts cholesterol in the DMPC vesicles had the effect of progressively decreasing the extent of blue shift, with λ_{max} appearing at 401 nm and 368 nm for dibucaine and tetracaine respectively, in the presence of 20% cholesterol ([L/A] > 500). Increasing the cholesterol content from 0 to 40 mol% produced a significant decrease of the blue shift in λ_{max} approaching the same observed in aqueous buffer. We had shown earlier that the λ_{max} of quinoline based dibucaine is blue shifted up to 30 °C beyond which it remained constant with further increase in temperature [54]. In case of tetracaine, the λ_{max} was red-shifted with increasing temperature up to 60 °C as shown in the inset of Fig. 2b. Difference in the temperature dependence indicates deeper incorporation of dibucaine than tetracaine at 25 °C in the liquid phase, where tetracaine remains closer to the aqueous phase near the lipid headgroups.

Fluorescence anisotropy probes the rigidity of the microenvironment of the local anesthetic molecules through monitoring of the rotational dynamics of the fluorophore. In our previous work we showed that in aqueous solution protonated dibucaine exhibited a small anisotropy (0.008 ± 0.02), implying unhindered rotation. Upon incorporation in DMPC or DOPC SUVs large increase in anisotropy is observed in dibucaine. The maximum increase was found in the absence of cholesterol, the presence of which decreased the value of anisotropy [54]. In contrast, the anisotropy value of protonated tetracaine was very high (0.26) in bulk water which decreased to (0.21 ± 0.01) at 35 °C in both DMPC (Fig. 3a) and DOPC (Fig. 3b) SUVs with or without cholesterol. The high anisotropy value of tetracaine in aqueous solution indicates that the absorption and emission dipoles are almost parallel, also observed in widely used molecules like diphenylhexatriene and perylene-bis-amide [59]. The mean lifetime of tetracaine in aqueous buffer yielded a very short lifetime of <50 ps compared to that of dibucaine (2.7 ns).

The apparent partition coefficient of dibucaine and tetracaine between the membrane and the aqueous phase (K_p) was taken as the molar ratio of lipid to anesthetics at which the anisotropy or the λ_{max} versus [L]/[D/T] plots changes over from steeply increasing or decreasing to saturation (Figs. 2 & 3), as described in our previous work [54,55]. K_p of dibucaine was estimated to be about 90, 75 and 55 in DMPC and DOPC SUVs containing 0, 10 and 20% cholesterol respectively (Table 1) at 35 °C [54,55]. K_p of tetracaine was found to be 2 to 3 times higher than that of dibucaine (Table 1). The partition coefficients for tetracaine were also measured according to the methods described by Huang and Haugland [61]. In DOPC membranes K_p was estimated to be 5.6×10^4 and 6×10^4 in absence and presence of 20% cholesterol,

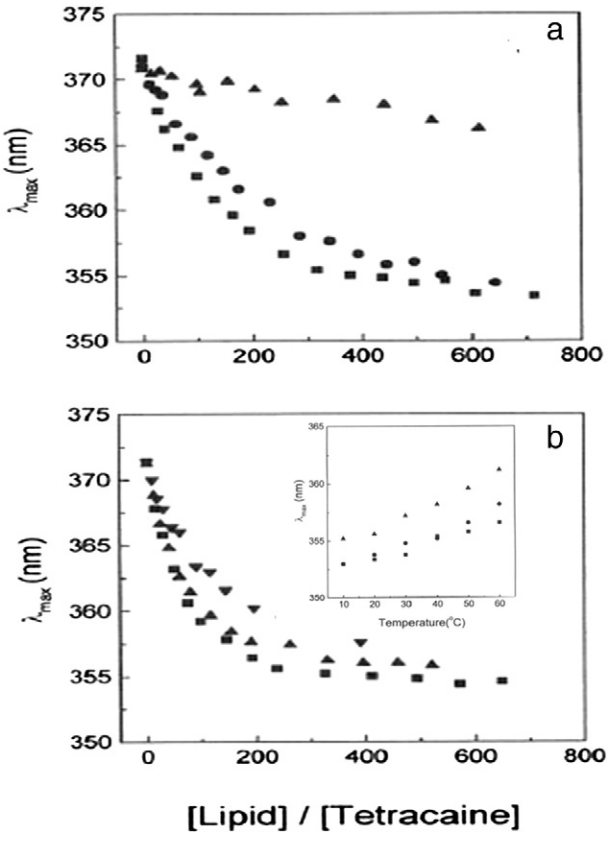

Fig. 2. Variation of Wavelength at emission maxima (λ_{max}) of tetracaine at 35 °C with phospholipid to – tetracaine molar ratio ([L/T]) in SUVs composed of (a) pure DMPC (■); DMPC with 10% (•) and 20% (▲) Cholesterol and (b) pure (DOPC) (■); DOPC with 20% (▲) and 40% (▼) Cholesterol. Inset show temperature dependence of fluorescence emission maxima of tetracaine in SUVs composed of pure DOPC (■, [L]/[T] = 400]) DOPC with 20% (•, [L/T] = 300) and 20% (▲, [L]/[T] = 200) Cholesterol. See Materials & methods for other details.

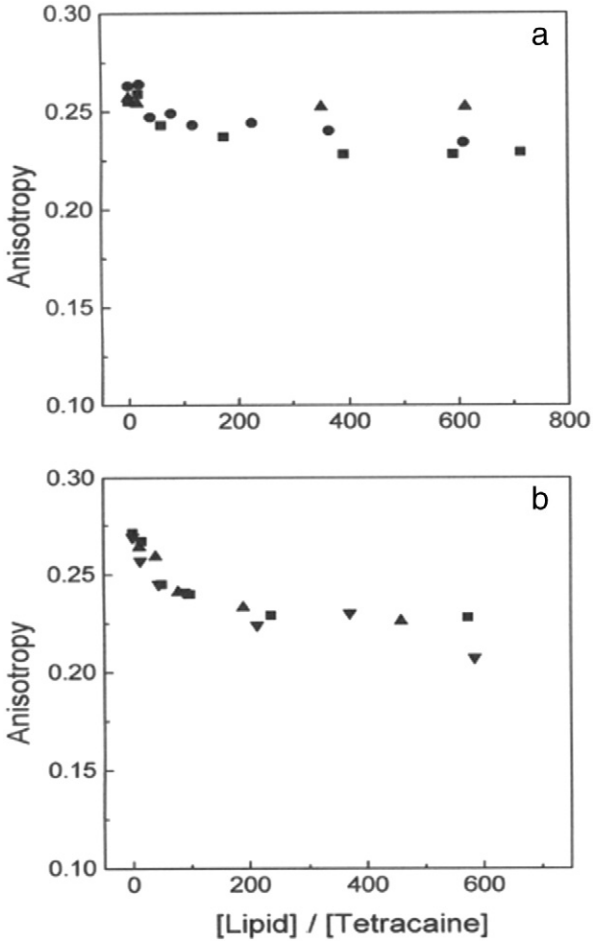

Fig. 3. Variation of steady state anisotropy of tetracaine at 35 °C with phospholipid to – tetracaine molar ratio ([L/T]) in SUVs composed of (a) pure DMPC (■); DMPC with 10% (•) and 20% (▲) Cholesterol and (b) pure (DOPC) (■); DOPC with 20% (▲) and 40% (▼) Cholesterol. See Materials & methods for other details.

Table 1
Estimates of apparent partition coefficient (K_p) of the two local anesthetics in different membranes with and without cholesterol or gangliosides.

Medium	Dibucaine	Tetracaine
DMPC	90	160
DMPC + 10% cholesterol	75	160
DMPC + 20% cholesterol	55	155
DMPC + 10% GM_1	65	160
DOPC	125	180
DOPC + 20% cholesterol	110	175
DOPC + 40% cholesterol	75	175
DOPC + 10% GM_1	80	175

respectively (Supporting information Table 1). The same in DMPC SUVs were 4.2×10^4 in absence and presence of 10% cholesterol. Estimation of partition coefficients by two independent methods indicated that incorporation of cholesterol did not alter the K_p of tetracaine or dibucaine. Although cholesterol prevents entry of both tetracaine and dibucaine deep into the hydrophobic core of the lipid bilayer, it only affects the localization and not the partition coefficient of the drugs, also with a possible effect on the anesthetic potential of the molecules.

3.2. Tetracaine and dibucaine in phospholipid membrane containing ganglioside

Nerve cell membranes are primary targets of local anesthetics and these membranes contain 5 to 10% ganglioside of the total lipid mass. We've carried out experiments on localization of protonated local anesthetics in phospholipid membranes with and without the ganglioside, GM_1 at 35 °C as described earlier [55]. Change in the λ_{max} and fluorescence anisotropy of tetracaine as a function of lipid to drug molar ratio in both DMPC and DOPC SUVs with and without 10% GM_1 is shown in Fig. 4a, b, c, and d respectively. Incorporation of increasing amounts of GM_1 led to progressive increase in the extent of blue shift of the λ_{max} of dibucaine [55]. The λ_{max} of tetracaine appeared at 354 nm in the absence and at 352 nm in the presence of 10% GM_1 from that of 372 nm in aqueous buffer. Further blue shift in λ_{max} of both dibucaine and tetracaine indicates a deeper localization of the local anesthetics in the presence of GM_1 to more hydrophobic interior of the membranes. Fig. 4c and d shows the variation of steady state anisotropy with increasing [L/T]. In the presence of 10% GM_1 the anisotropy further increased from 0.09 in the absence to 0.116 [55]. However, the anisotropy value of tetracaine remained unaltered in the presence and absence of GM_1 at 0.22. The apparent partition coefficient also remained same at 160 in the presence and absence of GM_1.

3.3. Binding of dibucaine and tetracaine to tetrameric brain spectrin

Brain spectrin shows the λ_{max} at 338 nm upon excitation at 295 nm and the addition of protonated dibucaine and tetracaine leads to progressive quenching of tryptophan fluorescence indicating formation of spectrin-anesthetic complex (Supporting information Fig. 1). We have estimated the apparent binding dissociation constant for such association of brain spectrin and the local anesthetics following methods elaborated in our earlier work [50,58]. Two representative binding isotherms for both the anesthetics are shown in Fig. 5. The insets in Fig. 5 show the representative double reciprocal plot of $1/\Delta F$ against $1/(C_{LA} - C_S)$ from which the binding constants were evaluated. The binding dissociation constants and stoichiometry show the binding

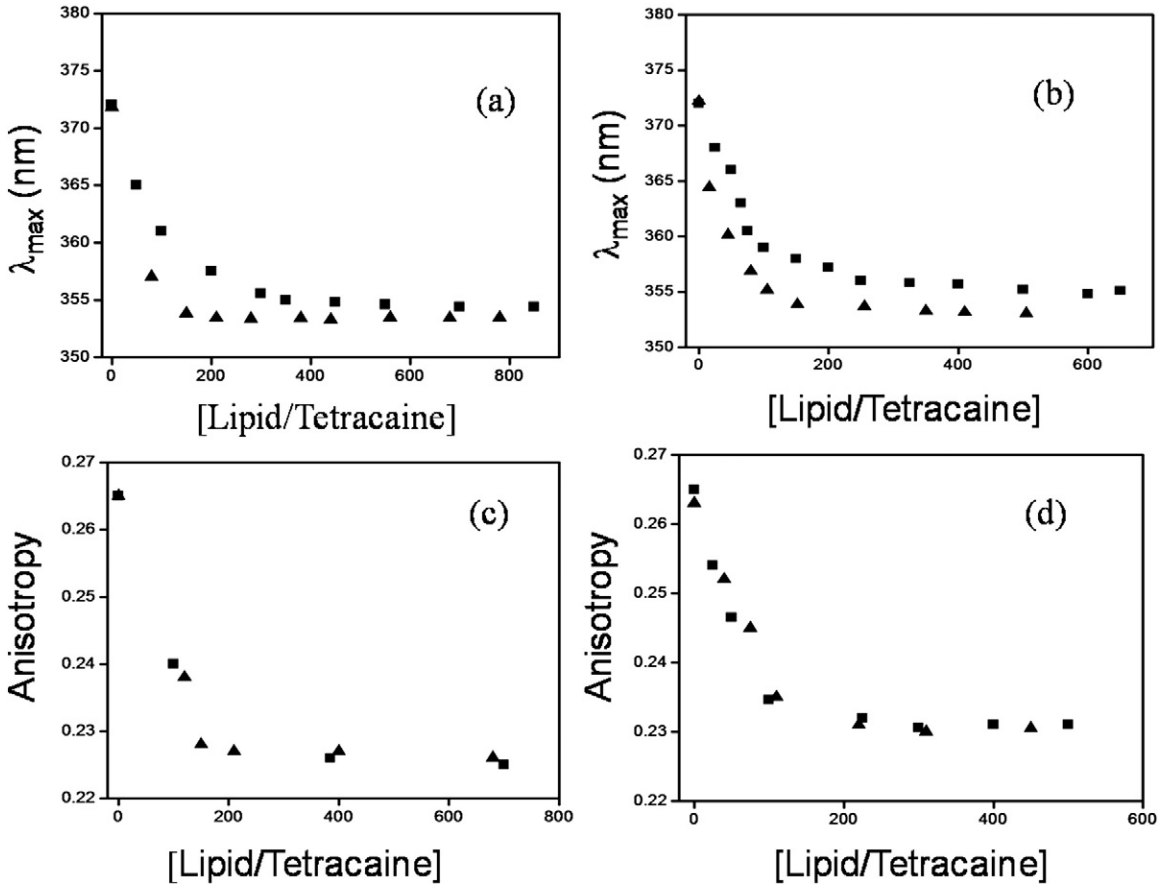

Fig. 4. Variation of wavelength at emission maxima (λ_{max}) of tetracaine at 35 °C with phospholipid to tetracaine molar ratio ([L/T]) in SUVs composed of (a) pure DMPC (■); DMPC with 10% (▲) ganglioside and (b) pure DOPC (■); DOPC with 10% (▲) ganglioside. Variation of steady state anisotropy of tetracaine at 35 °C with phospholipid to tetracaine molar ratio ([L/T]) in SUVs composed of (c) pure DMPC (■); DMPC with 10% (▲) ganglioside and (d) pure DOPC (■); DOPC with 10% (▲) ganglioside. See Materials & methods for other details.

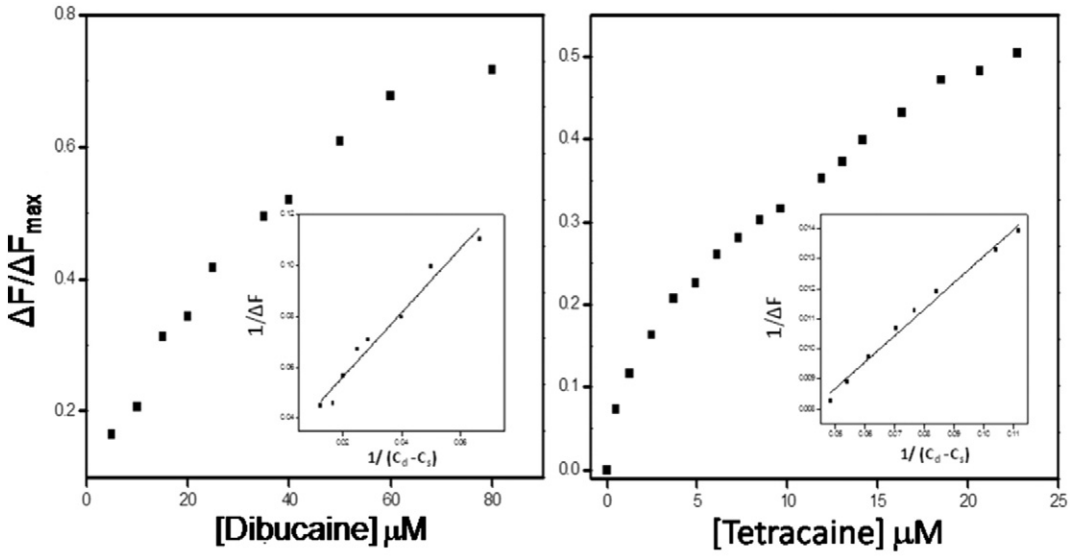

Fig. 5. Plot of the extent of anesthetic induced quenching vs. concentration of dibucaine/tetracaine for the evaluation of dissociation constant of the association of dibucaine/tetracaine with brain spectrin. See Materials & methods for other details.

affinities to be comparable but the binding stoichiometry is higher for the bulkier dibucaine than tetracaine, as summarized in Table 2.

3.4. Evaluation of thermodynamic parameters for the association of brain spectrin with local anesthetics

Thermodynamic parameters associated with the binding of dibucaine and tetracaine with spectrin e.g. changes in the ΔG, ΔH and ΔS were determined from the van't Hoff plots of $\ln K_a$ versus 1/T, as shown in Fig. 6 and summarized in Table 3. The ΔG value is negative indicating the process to be spontaneous. Formation of the anesthetic–spectrin complex was found to be exothermic with large positive changes in ΔH and ΔS suggesting hydrophobic interactions to play a key role in the molecular reorganization process.

3.5. Differential effects of dibucaine and tetracaine on brain spectrin

Further evidences of the binding of the local anesthetics with brain spectrin were obtained by measurements of anisotropy of tryptophan fluorescence and excited state lifetime. Steady state anisotropy of tetrameric brain spectrin is 0.13 ± 0.2 implying the localization of tryptophan residues in conserved region which remained more or less unchanged in the presence of increasing concentrations of protonated dibucaine, as shown in Fig. 7. In contrast, the anisotropy of the protein sharply decreased with increasing concentrations of protonated tetracaine which might be attributed to the fact that binding of tetracaine induces structural changes in brain spectrin causing release in rotational motion of the Trps. To further validate this result, we have done time-resolved fluorescence measurements with brain spectrin in the presence and absence of the two local anesthetics (Supporting information Fig. 2). Here again the mean lifetime of spectrin changed only marginally from 3.80 ns to 3.95 ns in the presence of dibucaine. On the other hand, the mean lifetime decreased substantially to 3.20 ns in the presence of tetracaine (Table 4).

Table 2
Binding dissociation constant for the interaction of dibucaine and tetracaine with brain spectrin in 10 mM Tris and 20 mM KCl at pH = 8.0 at 25 °C.

Ligand	Binding dissociation constant (μM)	Binding stoichiometry (n)
Dibucaine	0.246 ± 0.12	180 ± 20
Tetracaine	0.4705 ± 0.18	100 ± 20

3.6. Effects of local anesthetic on conformational changes of brain spectrin monitored by CD spectroscopy

CD spectra of brain spectrin showed two negative bands in the far UV region at around 208 nm and 222 nm, characteristic of a typical α-helical protein (Fig. 8). Both bands are attributed to n − π* transition for the peptide bond of the α-helical protein [62]. The CD spectrum of brain spectrin remains unaltered in the presence of protonated dibucaine. However, in the presence of protonated tetracaine the CD spectrum changes significantly without any shift in the peak positions indicating partial changes in the secondary structure of spectrin upon binding to tetracaine. Our results show that free spectrin contains about 89% α-helical structure which increased to 95% at molar ratio of tetracaine to brain spectrin of 200:1. The increase in the α-helical structure of a protein upon binding to drug molecule has also been reported previously for diomestin-HSA adducts [63].

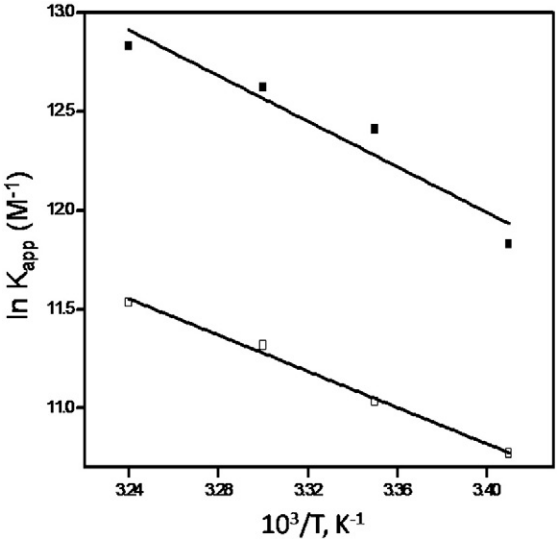

Fig. 6. Van't Hoff plot of for the interaction of brain spectrin (0.1 μM) with (■) dibucaine and (□) tetracaine in 10 mM Tris 20 mM KCl pH = 8.0. See Materials & methods for other details.

Table 3
Thermodynamics parameters for the interaction of dibucaine and tetracaine with brain spectrin in 10 mM Tris and 20 mM KCl at pH = 8.0.

Ligand	ΔH (k-cal mole^{-1})	ΔS (e.u.)	ΔG (k-cal mole^{-1})
Dibucaine	11.42	63.06	−7.253
Tetracaine	9.45	53.09	−6.482

Table 4
Fluorescence lifetime components of brain spectrin in the absence and presence of local anesthetic.

Sample	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	A_1	A_2	A_3	τ (ns)	χ^2
Brain spectrin	1.27	4.08	0.230	0.272	0.676	0.05	3.80	1.06
Brain spectrin + dibucaine	1.25	4.156	0.367	0.176	0.784	0.03	3.95	1.00
Brain spectrin + tetracaine	0.731	3.44	0.09	0.369	0.592	0.03	3.20	1.05

4. Discussion

Artificial membranes are widely used as a model system for the elucidation of biological processes that occur in the biological membrane along with different applications in biotechnology [64–66]. In pharmaceutical research, biological membranes act as drug delivering vehicles and interact with membrane associated protein such as ion channel, receptor etc. to exert their function [67]. Local anesthetics, such as dibucaine, tetracaine and other amphipathic amines could interact with lipids and modulate states of the sodium channel, favoring the inactivated form, partitioning within and permeate through the lipid bilayer [3,4]. Thus it is important to understand intrinsic interactions between the drug molecules and lipid membranes in controlling the rate limiting step for dissociation of the drug into the target protein.

Dibucaine and tetracaine along with other tertiary amine local anesthetics have been extensively studied previously using various spectroscopic and calorimetric methods [19–26,28–33]. However, the mechanism of local anesthesia and membrane localization of these anesthetics is still not well-known. In this paper we have studied the membrane localization of dibucaine (pK_a = 8.3) and tetracaine (pK_a = 8.5) the two well-known protonated monocationic tertiary amine local anesthetics in the presence and absence of two important membrane components cholesterol and GM_1 in phospholipid membranes using fluorescence spectroscopy. We have also considered the role, if any, of membrane skeletal proteins e.g. brain spectrin, in the overall mechanism of anesthesia upon translocation across the membrane bilayer, facilitating the entry of the cationic drug into the cell. Several studies have been done to explore the mechanism of membrane interactions of the local anesthetics. In a few studies high concentrations of local anesthetics have been used which caused membrane disruption and irreversible nerve injury [5,68]. Concentrations of charged dibucaine and tetracaine (<20 μM) used in this study are close to the concentration used as ion channel blocker. High concentration of local anesthetics changes phase transition temperature and bulk fluidity of formation of anesthetic-lipid mixed micelles [69,70]. On the other hand, at local anesthetics concentration between 1 and 10 μM, used in this study, there would be only 1 local anesthetic molecule per 500–10,000 lipids and the effect of local anesthetic on the phase behavior of the bulk bilayer properties at this stoichiometry would be insignificant.

The increase in the fluorescence intensity of protonated tetracaine and decrease of cationic dibucaine in the presence of increasing phospholipids indicated differential partitioning of both dibucaine and tetracaine from the aqueous to the membrane phase. This could be attributed to the difference in the hydrophobic clusters of their primary emitting chromospheres — quinoline for dibucaine and para-amino benzoate for tetracaine. Above the main phase transition temperature of DMPC (T_m at 24 °C) and DOPC (T_m at −18 °C) the partition coefficient did not change significantly up to 20% cholesterol, which is in general agreement with our earlier work [54]. The partition coefficients for tetracaine were about 6×10^4 in DOPC SUVs containing 0% and 20% cholesterol and 4×10^4 in DMPC vesicles containing 0% and 10% cholesterol. Similar changes in apparent partition coefficient of both protonated dibucaine and tetracaine in GM_1 containing membranes were also observed [54,55]. This trend of change of apparent partition coefficients of both the local anesthetics indicates that inclusion of cholesterol or ganglioside brings about conformational rearrangements of dibucaine and tetracaine inside the core of the bilayer without affecting the partition equilibrium.

The neutral form of dibucaine emits from its lowest singlet n, Π* state when protonated; the relative position of its n, Π* and Π, Π* states are interchanged [71]. In aqueous buffer, emission of monocationic dibucaine is thus due to the Π, Π* single transition, whereas in phospholipid membranes the equilibrium shifts towards neutral dibucaine, and emission thus takes place due to n, Π* transition. Since inter-system crossing probability is lower for Π, Π* than for n, Π* transitions, the emission quantum yield is higher in the former. No such state reversal is expected to occur for

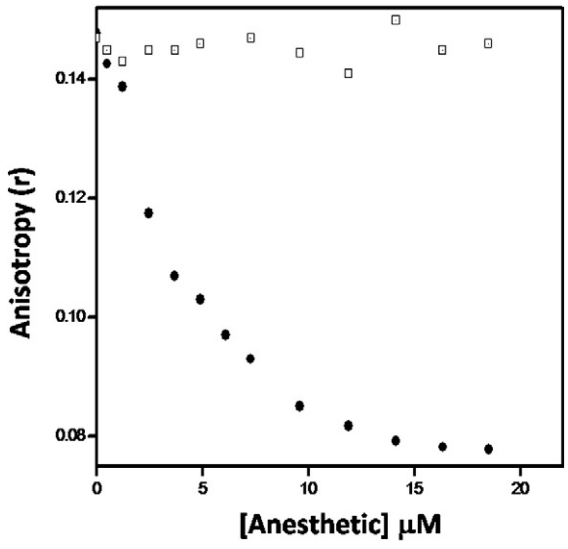

Fig. 7. Variation of steady state anisotropy of brain spectrin tryptophan with increasing concentration of dibucaine (□) and (●) tetracaine. See Materials & methods for other details.

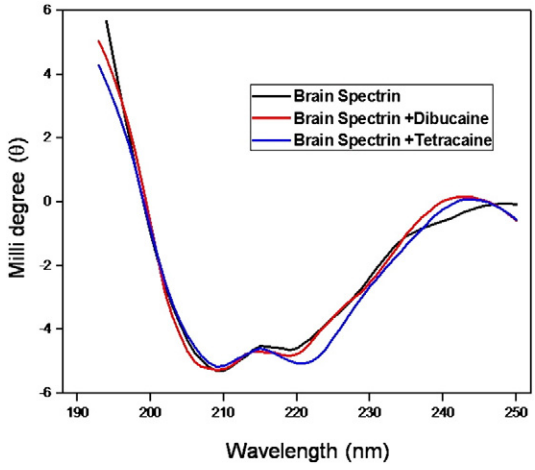

Fig. 8. Far UV CD spectra of brain spectrin alone and in the presence of 25 μM dibucaine and tetracaine respectively. See Materials & methods for other details.

tetracaine, for which the emitting chromophore is the para-amino benzoate and emission occurs from the lowest Π, Π* singlet states.

Earlier NMR and fluorescence spectroscopic studies have shown that dibucaine binds in the vicinity of the phospholipid glycerol moiety [21,36–38]. This has been confirmed by recent solid state NMR studies, placing the quinoline ring near the glycerol motif of phospholipids [72,73]. Using deuterated phospholipids Boulanger and coworkers showed that tetracaine resides near the polar head group of phospholipids causing an ordering of the head group, an observation also confirmed by Watts and Poile using quadruple splitting of methylenes group of the PC head group [20,24]. NMR studies on the interaction of tetracaine with DMPC membranes containing 30% cholesterol together with our previous fluorescence work showed that both tetracaine and dibucaine localize close to the aqueous interphase in the presence of cholesterol [39,54]. A relatively recent study indicates that both charged and neutral tetracaine localize at the same place in phosphatidylcholine based membranes both in the absence and presence of cholesterol [74] although previous NMR studies of Boulanger and coworkers proposed that two ionization states of tetracaine occupy distinct locations in the bilayer [20]. The changes in the extent of blue-shift in the λ_{max} from 372 nm to 354 nm for protonated tetracaine and from 412 nm to 394 nm for protonated dibucaine in both DMPC and DOPC membranes reveal that both the species reside in the hydrophobic interior of the membranes. The presence of cholesterol brings about similar effects for tetracaine at 35 °C as that for dibucaine, leading to displacement from the interior of the bilayers, towards the interface [54]. In other words, the presence of cholesterol inhibits the penetration of the local anesthetic molecules deep into the bilayer. The results are consistent with the NMR studies on localization of tetracaine that suggest deeper partitioning in the pure DMPC bilayer while the presence of 30% cholesterol squeezed it closer to the aqueous interface of the bilayer [54,39]. Further changes in the extent of blue shift of λ_{max} of tetracaine in the presence of GM_1, on the other hand, point to localization in the hydrophobic core of the phospholipid bilayers as observed in dibucaine [55].

An important aspect of the results reported here is the marked difference in fluorescence properties of protonated dibucaine and tetracaine in membranes with and without cholesterol. For example, the quantum yield of dibucaine in the DMPC membrane is ~40% lower than in water whereas that of tetracaine is enhanced by ~5 fold. The steady state anisotropy of dibucaine is expectedly higher in the interior of membranes than in bulk water; however, that of tetracaine is somewhat lower in membranes. We also found that the anisotropy of dibucaine was further increased in the presence of GM_1 when that of tetracaine remained almost the same in DMPC membrane with GM_1. Binding and partitioning of local anesthetics into the phospholipid membranes are controlled by both hydrophobic and ionic interactions. Hydrophobic forces are the major factor for partitioning of the local anesthetics in the core of the phospholipid bilayer. However, the interfacial ionic components of the membrane head groups also play a crucial role for such partitioning. NMR studies have demonstrated that dibucaine and tetracaine are located closer to the head group than to the center of the hydrocarbon chains and the charged form of tetracaine is effective in changing the conformation of the phospholipid head groups [38,75,76]. The magnitude of this latter effect depends on the phase behavior of the phospholipids and the charge of the anesthetics. Thus incorporation of local anesthetics in model membrane causes alteration of interfacial charge density leading to conformational rearrangements of phospholipid head groups. This results in the decrease in the rotational mobility which is reflected in the increase in fluorescence anisotropy. Upon interaction of local anesthetics with the hydrocarbon region of the membrane, a rearrangement of the intramolecular hydrogen bonding network between the phospholipid molecules takes place, associated with the liberation of water molecules from membranes. Fluorescence studies of Yun and coworkers also showed that this kind of dual effect could explain the decrease in rotational mobility at the membrane surface and the increase in the rotational mobility in the interior hydrocarbon core of the bilayer, in both model and native membrane systems [27–29]. Fraceto and coworkers proposed that perturbing efficiency of a local anesthetic does not correlate well with hydrophobicity alone. Using ESR, NMR and fluorescence spectroscopy they have shown that lidocaine, a lesser hydrophobic molecule, led to marked decrease in membrane organization compared to more hydrophobic local anesthetics indicating polar interactions, steric parameters and the free volume generated by the hydrophobic mismatch between the local anesthetic and phospholipids which together determine membrane structural defects [30,31]. Our fluorescence data suggest that dibucaine penetrates the lipid bilayer and remains in the vicinity of the glycerol backbone region of the phospholipid molecule, acting as a spacer. Dibucaine is a spherical molecule and the presence of two aromatic rings creates steric hindrance for its insertion between lipids allowing the charged anesthetic molecule to take part in electrostatic interaction between the positive charge on choline nitrogen and the phosphate groups of the adjacent lipids. An extra volume of dibucaine creates extra space in between lipids and alters interfacial charge density leading to conformational rearrangements of phospholipid head groups and changes in the hydration patterns. This creates a more hydrophobic environment within which lipids are less associated to water interacting more strongly to dibucaine, restricting its rotational motion and thus leading to higher anisotropy. On the other hand, tetracaine is an amino-ester local anesthetic that does not have cyclic aromatic moiety, assuming a cylindrical shape with a long amino butyl tail in the *para* position of the benzoic acids ring. Due to smaller size and higher charge density, tetracaine behaves like an inorganic ion affecting the head group conformation. The physical insertion of charged tetracaine molecules between the lipids and electrostatic interaction between phospholipids and the anesthetic dimethylamino moieties could together be responsible for altering the existing intra and intermolecular interactions. This is reflected in the emission maxima when measured at different temperatures, suggesting that the charged portion of tetracaine is located close to the polar head group region of the bilayer with the akyl chain descending in the acyl chain region. Such residence of tetracaine causes the ordering of the phospholipid head groups altering the inner hydrogen bonding network between phospholipids. This leads to an increase of gauche rotamers, further increasing the disordering in the membrane surface area causing a decrease in the fluorescence anisotropy.

The interactions between cholesterol and phospholipids have been extensively studied and are known to affect the phase transition between the liquid crystalline and the ripple gel phase of phospholipid membranes [18,77]. From the temperature dependence study, we have seen a progressive red-shift of the λ_{max} with increasing temperature, opposite to that of dibucaine [54]. The presence of cholesterol causes an expansion between the acyl tails of phospholipids and perturb of dipole–dipole interaction between the charged anesthetics and the phospholipids in the interface region. Perturbation of dipole interactions in the interfacial region is smaller compared to the same in the acyl tail region, preventing anesthetic molecules from penetrating deep into the inner core of the hydrophobic region. This is also consistent with our earlier observations with dibucaine localizing closer to the phospholipid head groups in the presence of cholesterol [54]. The membrane localization of protonated dibucaine and tetracaine is almost similar, in the presence and absence of cholesterol. However, the difference in localization, reflected in the changes in fluorescence anisotropy, and in the temperature-dependent variation of λ_{max} only depended on the change in membrane fluidity induced by the two local anesthetics. Similar differential interactions are also feasible with target proteins such as the sodium channel, giving rise to difference in anesthetic potencies between the benzene based and the quinoline based compounds.

Brain spectrin is a homologue of erythroid spectrin that remains mainly in its tetrameric from. It has a large number of tryptophan residue distributed along the entire length of the protein [50,53]. The most interesting structural feature is that although it has a large number

of tryptophan residue these residues are localized in the same position of each domain making them convenient intrinsic fluorescence reporter groups for monitoring conformational changes in the protein that contributes to its elastic deformability exhibited under physiological conditions. The fluorescence lifetime decay profiles of brain spectrin, in the presence and absence of both protonated dibucaine and tetracaine are shown in Supporting information Fig. 2. The lifetime components and the fitting parameters are summarized in Table 3. The three individual lifetime components of the tryptophan of the native spectrin were 1.27, 4.08 and 0.23 ns respectively with mean lifetime of 3.80 ns. In the presence of tetracaine the longer lifetime component decreases from 4.1 ns to 3.4 ns and the mean lifetime also decreases from 3.8 ns to 3.2 ns.

Fluorescence and CD studies indicate binding of protonated dibucaine and tetracaine to tetrameric brain spectrin. Binding dissociation constants are similar and independent of the size of the ligands. An estimate of the binding stoichiometry indicates about 200 molecules of dibucaine and 100 molecules of tetracaine to be associated with one tetrameric brain spectrin. Binding sites of both the ligands are near tryptophan residues in each repeat domain and resonance energy transfer between the local anesthetics and the tryptophan residues are indicated to be one of the most possible mechanisms through which the quenching takes place. Although the binding affinity of the protein with both the anesthetics is ($10^4\ M^{-1}$) not strong enough, the normal prevalence of brain spectrin is high enough to permit such conjugation. Knowledge of the thermodynamic parameters such as changes in free energy, enthalpy and entropy helps in the understanding of the molecular basis of the interactions between brain spectrin and the local anesthetics. Ross and Subramanian have characterized the sign and magnitude of thermodynamic parameters in various types of interactions associated with protein–ligand interactions [78,79]. From the thermodynamic point of view positive changes of both ΔH and ΔS indicate strong hydrophobic interactions whereas negative changes of both indicate the predominance of hydrogen bonding or van der Walls force. For the electrostatic interactions ΔH is often negative and ΔS is positive. The present case of local anesthetics binding to brain spectrin is associated with positive ΔH and ΔS suggesting that hydrophobic interactions play a key role. Favorable positive entropic contribution due to release of protein bound water molecules favors the association when both the ligands bind to brain spectrin. In physiological pH both the ligands remain in charged and uncharged forms, the charged and uncharged parts of the anesthetic molecule binds to charged and neutral side chain of the amino acid residues in brain spectrin without affecting the helices. This leads to the release of the protein-bound water molecules and provides the necessary entropic source of the binding.

CD spectra of brain spectrin showed two negative bands in the far UV region at around 208 nm and 222 nm, characteristic of a typical α-helical protein and both attributed to $n - \pi^*$ transfer for the peptide bond of α-helical protein [60,62]. Change in absorbance in the CD spectrum of brain spectrin in the 190–250 nm region in the presence of both the protonated local anesthetics suggests that dibucaine association does not perturb the helical backbone structure of spectrin. But the ellipticity at 222 nm increased significantly in the presence of tetracaine indicating structural perturbations in the secondary structure of spectrin. More attention needs to be directed to the fact that proteins usually serve as vectors for almost all endogenous and exogenous ligands to ship the ligands to their target where it elicits its functions. These features of the interaction imply that interaction of local anesthetics with brain spectrin together with the membrane localization could be taken into account for an understanding of the mode of action of the local anesthetics in vivo.

One interesting aspect of the present work is that the tryptophan anisotropy decreases in brain spectrin upon increasing concentrations of protonated tetracaine and the mean lifetime is also lowered upon binding tetracaine. Both anisotropy and mean lifetime remain unchanged upon spectrin binding of protonated dibucaine. This differential behavior further indicates that the benzene-based local anesthetic could induce large conformational changes in spectrin. Similar results were earlier reported with other benzene-based local anesthetics, lidocaine and procaine, inducing such conformational changes in cytoskeletal proteins [80,81]. Using light scattering techniques on the model membrane Kitagawa et al. reported that local anesthetics display detergent-like properties and can disrupt the model membrane. Such detergent-like properties are also the cause of irreversible injury caused by lidocaine and tetracaine when used in higher concentrations [5]. Studies of the effects of procaine, tetracaine and dibucaine on the polymerization–depolymerization behavior of microtubules indicate similar differential behavior of benzene based procaine and tetracaine as that of quinoline based dibucaine. While procaine and tetracaine were found to increase the rates of tubulin polymerization at 24 °C and of microtubule depolymerization at 4 °C as a linear function of the concentration of the local anesthetics, dibucaine, on the other hand, showed a linear decrease in the tubulin polymerization and microtubule depolymerization rates [49].

Koudra and co-workers proposed that the aromatic ring in the local anesthetic not only confers lipophilicity allowing its passage through the membrane, but also for Π-stacked binding with the phenyl group of the phenylalanine residue of the Na^+ channel inactivating peptides MP-1 and MP-2 [2]. They have proposed the molecular mechanism of local anesthesia using dibucaine, indicating that it locates at the polar head region of the boundary lipids in the vicinity of the Na^+ channel, interacts through Π-stacking face to face, also interacts with the phenylalanine residue allowing its protonated quaternary nitrogen to interact electrostatically with negatively charged amino acid residue of the peptide. Their activity depends on the optimal distance between the aromatic ring and the tertiary amine nitrogen. Taken together, our results also indicate that both charged dibucaine and tetracaine remain in the vicinity of the polar head region of the phospholipids to interact with the Na^+ channel in its inactive state. Previously Paula and his co-workers demonstrated that steric factor, location and orientation of anesthetics in the membrane play a crucial role in the mechanism of anesthesia [30,31], since they can modulate local anesthetic-Na^+ channel binding by directing the molecule to access the proper site of the channel. Our results also indicate factors like shape, size, orientation and location of the local anesthetics in the phospholipid membrane and steric effects arising out of a mismatch between the anesthetics and phospholipids. These may be the origins of differential anesthetic potential between dibucaine and tetracaine in terms of interactions with components of neuronal membrane lipids and the skeletal protein, spectrin.

5. Conclusion

Earlier NMR and fluorescence studies indicate that both dibucaine and tetracaine bind in the vicinity of the glycerol backbone of phospholipids [19–26,35–38]. Recent fluorescence studies show that neutral anesthetic tetracaine partitioning into the membranes more strongly than the cationic form of the anesthetic but the location of both forms remains the same [74]. At physiological pH the local anesthetics remains as a mixture of both cationic and neutral forms. The extent of partitioning of neutral form of tetracaine is about 6 times more than its cationic form whereas in case of dibucaine the extent of partitioning of both forms is almost similar. NMR and ESR studies indicate that the penetration of dibucaine into the lipid membrane is influenced by the steric factor [30,31]. Moreover electrophysiological data shows the importance of uncharged and smaller local anesthetic molecules e.g. tetracaine and lidocaine in permeating from extracellular space to the cytoplasmic compartment, to finally block the sodium ion channel by its inactive state [3,4]. The present fluorescence study indicates that both protonated dibucaine and tetracaine exist in the membrane interface. Tetracaine localizes closer to the aqueous phase and dibucaine near the glycerol backbone of phospholipids, also shown by ^1H MAS NMR

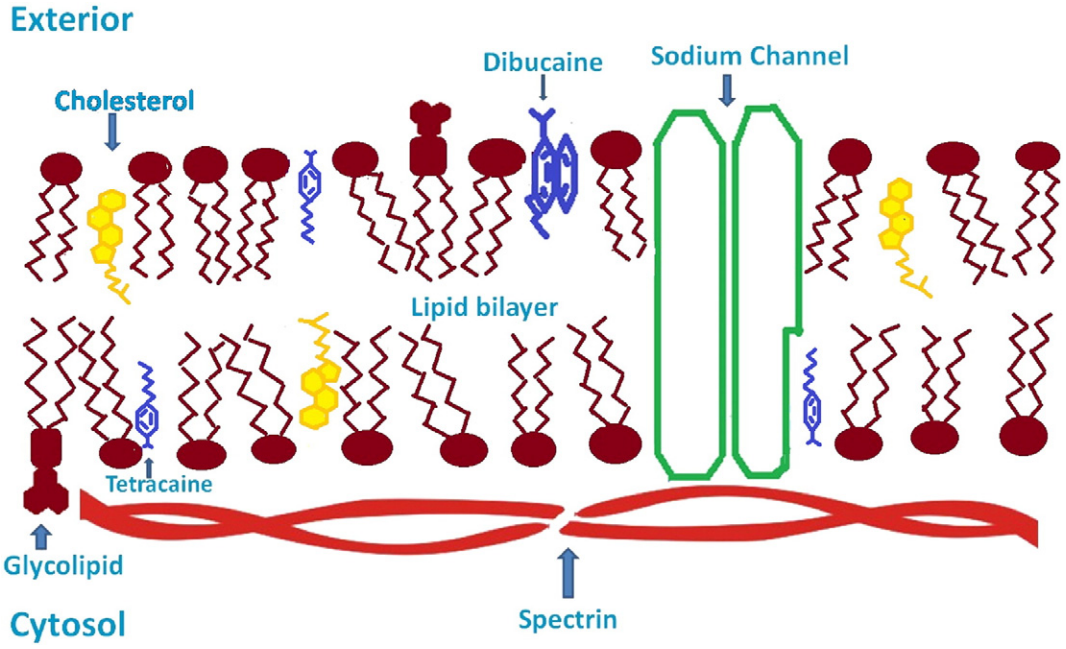

Fig. 9. Cartoon diagram showing possible localization of the local anesthetics in phospholipid bilayer.

spectroscopy [72]. Presence of cholesterol does not alter the partitioning of both the local anesthetics, however, the presence of GM_1 enhances the penetration of the drugs into the hydrophobic core of the lipid bilayer. Considering all these observations, one could conclude that in physiological pH the cationic tetracaine binds strongly to the negative phosphate groups of the membrane phospholipids whereas dibucaine binds more weakly than tetracaine and enters into the more hydrophobic glycerol backbone of the phospholipids. Due to its smaller size and stronger interaction with phosphate groups, tetracaine induces conformational changes at membrane interface accompanied by disordering of the fatty acyl chain region. This in turn increases the area of membrane cross section and allows entry of neutral tetracaine from the extracellular face to the cytoplasmic compartment of the cell in assistance with membrane skeletal proteins and promotes Na^+ ion channels to remain in an inactive state induced by membrane depolarization, shown in Fig. 9.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.11.022.

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