Biochimica et Biophysica Acta 1848 (2015) 821–832

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

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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ARTICLE INFO

Article history:
Received 5 July 2014
Received in revised form 18 November 2014
Accepted 19 November 2014
Available online 5 December 2014

Keywords:
Local anesthetic
Phospholipid vesicle
Cholesterol
Ganglioside
Brain spectrin

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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http://dx.doi.org/10.1016/j.bbamem.2014.11.022
0005-2736/

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