Chemistry and Physics of Lipids 198 (2016) 21–27

Contents lists available at ScienceDirect

Chemistry and Physics of Lipids

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

# Effect of local anesthetics on the organization and dynamics in membranes of varying phase: A fluorescence approach

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ARTICLE INFO

*Article history:*
Received 5 April 2016
Accepted 29 April 2016
Available online 3 May 2016

*Keywords:*
DPH
TMA-DPH
Phenylethanol
Membrane phase
Local anesthetics

ABSTRACT

The molecular mechanism underlying the action of local anesthetics remains elusive. Phenylethanol (PEtOH) is an ingredient of essential oils with a rose-like odor and has been used as a local anesthetic. In this work, we explored the effect of PEtOH on organization and dynamics in membranes representing various biologically relevant phases using differentially localized fluorescent membrane probes, DPH and TMA-DPH. We show here that PEtOH induces disorder in membranes of all phases (gel/fluid/liquid-ordered). However, the extent of membrane disorder varies in a phase-specific manner. Maximum membrane disordering was observed in gel phase, followed by liquid-ordered membranes. The disordering was minimal in fluid phase membranes. Interestingly, our results show that the disordering effect of PEtOH in gel phase is sufficiently large to induce phase change at higher PEtOH concentrations. Our results are relevant in the context of natural membranes and could be useful in understanding the role of anesthetics in membrane protein function.



## 1. Introduction

Local anesthetics are a class of compounds that suppress sensation in a limited area of application in the body by reversibly blocking the action potential responsible for neuronal transmission, thereby relieving pain. The molecular mechanism underlying the action of local anesthetics is not completely understood. The question of whether anesthesia results from an indirect anesthetic-lipid environment effect (Arias, 1999) or a specific anesthetic-protein interaction (Rehberg et al., 1995) remains elusive.

Phenylethanol (PEtOH) (see Fig. 1a) is an ingredient of essential oils with a rose-like odor which has been used as a local anesthetic (Anbazhagan et al., 2010; Gray et al., 2013). PEtOH is one of the most important contributors of aroma in several fresh fruits such as tomato (Tieman et al., 2006). PEtOH has been reported to alter membrane order by altering lipid packing (Anbazhagan et al., 2010; Jordi et al., 1990; Killian et al., 1992). In addition, PEtOH has been shown to induce translocation of the mitochondrial precursor protein apocytochrome *c* (Jordi et al., 1990), and influence oligomerization of membrane proteins by altering helix-helix interaction (Anbazhagan et al., 2010).

In this paper, we have utilized two depth-dependent fluorescent membrane probes DPH and TMA-DPH (see Fig. 1b) to comprehensively explore the interaction between PEtOH and membranes of varying phases. Since the membrane bilayer represents a two-dimensional anisotropic fluid, any possible change in membrane order may not be uniform and restricted to a unique location in the membrane. It is therefore important to monitor the change in membrane order at more than one location. We have previously shown that stress such as heat shock can induce anisotropic changes in membrane order, *i.e.*, the change in membrane order could vary when monitored in different regions of the membrane (Revathi et al., 1994). In addition, such depth-dependent modulation of membrane order induced by alcohols (Kitagawa and Hirata, 1992) has been previously reported. DPH is a rod-like molecule and partitions into the interior of the bilayer. TMA-DPH is a derivative of DPH with a cationic moiety attached to the *para* position of one of the phenyl rings (Prendergast et al., 1981). While DPH is known to be located in the hydrophobic region of the membrane, the amphipathic TMA-DPH is localized in the shallower region of the bilayer with its positive charge anchored at the membrane interface. Its DPH moiety is located at ~11 Å from the center of the bilayer and provides information from the membrane interface (Kaiser and London, 1998). In contrast to this,

*Abbreviations:* DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; LUV, large unilamellar vesicle; PEtOH, phenylethanol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; TMA-DPH, 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene.

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http://dx.doi.org/10.1016/j.chemphyslip.2016.04.009
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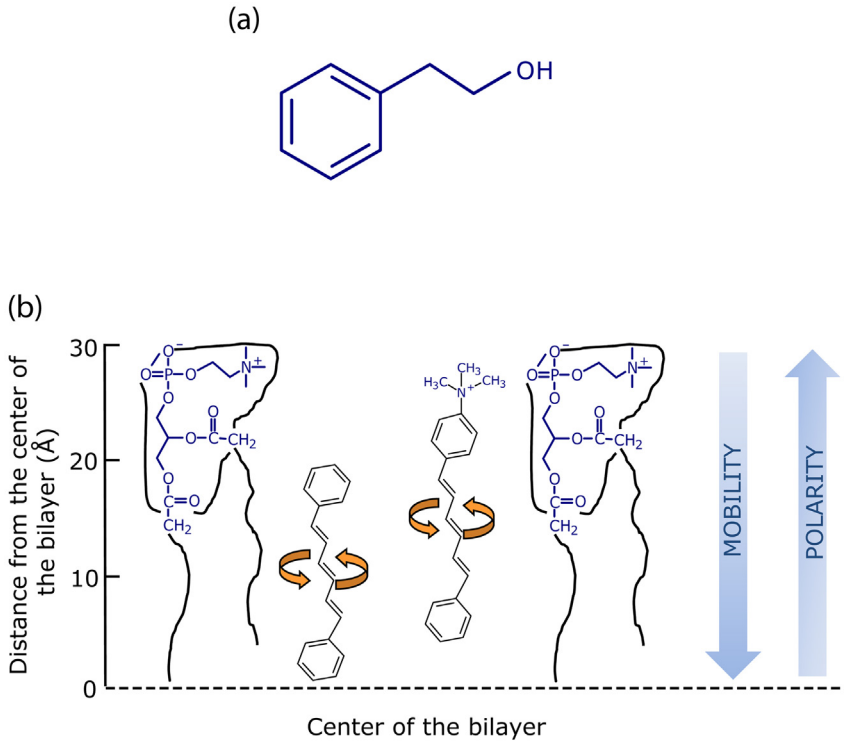


**Fig. 1.** (a) The chemical structure of PEtOH. (b) A schematic representation of a leaflet of the membrane bilayer showing the chemical structures and locations of the fluorescent probes DPH and TMA-DPH. The membrane location of the DPH group in these compounds are shown according to Kaiser and London (1998). The mobility and polarity gradients along the bilayer normal are also shown (see text for more details). The horizontal line at the bottom indicates the center of the bilayer.

the average depth of DPH alone has been shown to be ~8 Å from the bilayer center (Kaiser and London, 1998).

Keeping in mind the relevance of membrane phase in physical properties of membranes (van Meer et al., 2008), we explored the effect of PEtOH on the organization of membranes of varying phase. Our results show that PEtOH induces membrane fluidization (acyl chain disordering) in each of the three different membrane phases as shown by the reduction in apparent rotational correlation time of DPH and TMA-DPH with increasing concentration of PEtOH. In addition, our results suggest that local anesthetics such as PEtOH modulates membrane physical properties to the extent of even being able to induce phase separation in gel (ordered) phase membranes.

## 2. Materials and methods

### 2.1. Materials

Cholesterol, DMPC and PEtOH were purchased from Sigma Chemical Co. (St. Louis, MO). DPPC and POPC were obtained from Avanti Polar Lipids (Alabaster, AL). DPH and TMA-DPH were from Molecular Probes/Invitrogen (Eugene, OR). Phospholipids were checked for purity by thin layer chromatography on silica gel precoated plates (Sigma) in chloroform/methanol/water (65:35:5, v/v/v) and were found to provide single spot for all lipids with a phosphate-sensitive spray followed by charring (Baron and Coburn, 1984). Concentration of phospholipids were estimated by phosphate assay subsequent to complete oxidation by perchloric acid (McClare, 1971). DMPC was used as an internal standard to evaluate lipid digestion. The concentrations of stock solutions of DPH and TMA-DPH in methanol were estimated from their molar extinction coefficient (ε) of 88,000 $M^{-1}$ $cm^{-1}$ at 350 nm (Arora et al., 2004). All other chemicals used were of the highest purity available. Solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

### 2.2. Sample preparation

All experiments were performed using large unilamellar vesicles (LUVs) of 100 nm diameter of POPC, DPPC, or DPPC/40 mol% cholesterol with increasing concentration of PEtOH and 1 mol% probe (DPH or TMA-DPH). In general, 320 nmol of total lipid and 3.2 nmol of DPH (or TMA-DPH) were mixed well and dried under a stream of nitrogen while being warmed gently (~35 °C). After further drying under a high vacuum for at least 3 h, samples were hydrated (swelled) by addition of 1 ml of buffer (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) containing increasing amount of PEtOH (0–2%, v/v). Each sample was vortexed for 3 min to uniformly disperse the lipids and form homogeneous multilamellar vesicles. The buffer was always maintained at a temperature well above the phase transition temperature of the phospholipid used as the vesicles were made. For this reason, lipids were swelled at a temperature of ~40 °C for POPC and ~60 °C for DPPC or DPPC/cholesterol samples. LUVs of 100 nm diameter were prepared by the extrusion technique using an Avestin Liposofast Extruder (Ottawa, Ontario, Canada) as previously described (MacDonald et al., 1991; Mukherjee and Chattopadhyay, 2005). Briefly, the multilamellar vesicles were freeze-thawed five times using liquid nitrogen to ensure solute equilibration between trapped and bulk solutions and then extruded through polycarbonate filters (pore diameter of 100 nm) mounted in an extruder fitted with Hamilton syringes (Hamilton Company, Reno, NV). The samples were subjected to 11 passes through the polycarbonate filters to give the final LUV suspension. Background samples were prepared in the same way except that DPH (or TMA-DPH) was not added to them. The optical density of the samples measured at 358 nm was less than 0.15 in all cases, which rules out any