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BRIEF COMMUNICATION

Effect of Local Anesthetics on Dipole Potential of Different Phase Membranes: A Fluorescence Study

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Abstract

The molecular mechanism behind the action of local anesthetics is not well understood. Phenylethanol (PEtOH) is an ingredient of essential oils with a rose-like odor, and it has previously been used as a local anesthetic. In this work, we explored the effect of PEtOH on dipole potential in membranes representing biologically relevant phases, employing the dual-wavelength ratiometric method utilizing the potential-sensitive probe di-8-ANEPPS. Our results show that PEtOH reduces membrane dipole potential in membranes of all biologically relevant phases (gel, liquid-ordered, and fluid) in a concentration-dependent manner. To the best of our knowledge, these results constitute one of the early reports describing reduction of membrane dipole potential induced by local anesthetics, irrespective of membrane phase.

Keywords Di-8-ANEPPS · Dipole potential · Dipolar reorganization · Local anesthetics · Membrane phase · Phenylethanol

Abbreviations

DMPC	1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine
Di-8-ANEPPS	4-(2-(6-(Dioctylamino)-2-naphthalenyl) ethenyl)-1-(3-sulfopropyl)-pyridinium inner salt
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

Introduction

Local anesthetics represent a class of amphiphilic compounds that repress sensation in a specific area of the body by reversibly inhibiting the action potential responsible for neuronal transmission, thereby reducing pain in that area. However, the precise molecular mechanism for the action of local anesthetics is not very well understood. There are two hypotheses proposed to understand the mechanism of action of local anesthetics. The "protein hypothesis" suggests specific interaction of anesthetics with membrane proteins, modulating the function of membrane proteins (Arias 1999), whereas the "lipid hypothesis" attributes anesthesia to an indirect anesthetic-induced change in membrane lipid properties, which could modulate the function of membrane proteins (Rehberg et al. 1995). Another possible mechanism could be a combination of the above two mechanisms, since it is not trivial to dissect lipid and protein effects in the cooperative membrane milieu. Regardless of the mechanism of action, an important prerequisite for the action of local anesthetics is their partitioning into the membrane. Insight into the interaction of local anesthetics with membranes is therefore crucial in understanding anesthetic action.

Phenylethanol (PEtOH) (see Fig. 1a) is an important component present in essential oils, which has a pleasant rose-like aroma. Importantly, it is used as a local anesthetic (Anbazhagan et al. 2010; Gray et al. 2013) and exhibits antibacterial activity (Corre et al. 1990). PEtOH is the main contributor of aroma in various fresh fruits, such as tomato (Tieman et al. 2006). Notably, PEtOH has been shown to modulate membrane order by perturbing lipid acyl chain packing (Jordi et al. 1990; Killian et al. 1992; Anbazhagan et al. 2010; Shrivastava et al. 2016; Reddy et al. 2018). In addition, PEtOH has been reported to facilitate translocation of apocytochrome c, which is a mitochondrial precursor protein (Jordi et al. 1990) and alter helix–helix interaction

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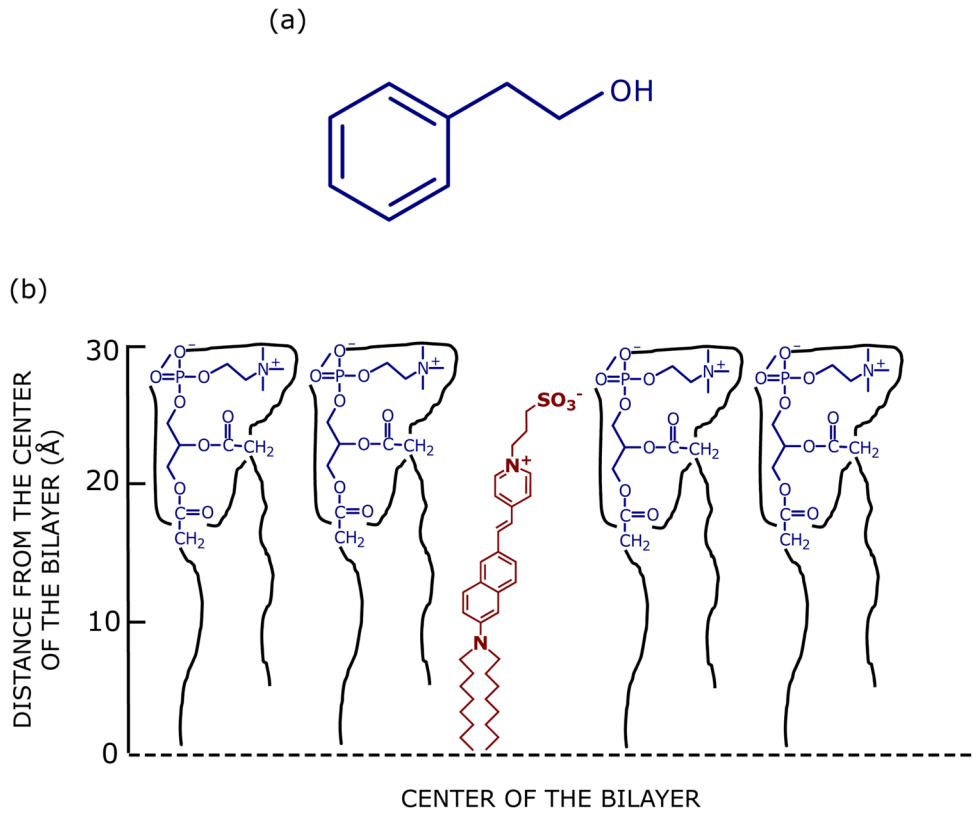

Fig. 1 **a** The chemical structure of PEtOH. **b** A schematic representation showing a leaflet of the membrane bilayer and the chemical structure and location of the fluorescent probe di-8-ANEPPS. The membrane location of the fluorescent styrylpyridinium group in di-8-ANEPPS is shown according to Haldar et al. (2012). The horizontal-dotted line at the bottom indicates the center of the bilayer

of membrane proteins, which affects their oligomerization (Anbazhagan et al. 2010).

Dipole potential represents an important electrostatic property of biological and model membranes and is attributed to the potential difference within the membrane due to the non-random organization of amphiphile dipoles and water molecules at the interfacial region of the membrane (Clarke 2001; O'Shea 2005; Wang 2012). It has been reported that membrane dipole potential could be an important determinant of the activity of membrane proteins and peptides (Duffin et al. 2003; Starke-Peterkovic et al. 2005; Singh et al. 2013; Richens et al. 2015). Membrane dipole potential is sensitive to interaction of proteins with membranes (Cladera and O'Shea 1998; Chaudhuri and Chattopadhyay 2014; Thombare et al. 2018). Interestingly, cholesterol has been reported to increase membrane dipole potential in both model and natural membranes (Starke-Peterkovic et al. 2006; Haldar et al. 2012; Singh et al. 2013) in a stereo-specific manner (Bandari et al. 2014).

In this work, we have monitored the effect of PEtOH on membrane dipole potential measured by the dual-wavelength ratiometric method utilizing the potential-sensitive probe di-8-ANEPPS (see Fig. 1b). In view of the fact that membrane phase is an important factor in defining membrane properties, domain organization (van Meer et al. 2008; Brown and London 1998), and function of membrane proteins (Fong and McNamee 1986, 1987; Pal et al. 2016; Rao et al. 2016), we measured membrane dipole potential with increasing concentrations of PEtOH in varying membrane phases. Our results show that PEtOH reduces membrane dipole potential in membranes of all phases (gel, liquid-ordered, and fluid). To the best of our knowledge, these results constitute one of the first reports describing reduction of membrane dipole potential induced by local anesthetics, irrespective of membrane phase.

Materials and Methods

Materials

Cholesterol, DMPC, and PEtOH were from Sigma Chemical Co. (St. Louis, MO). DPPC and POPC were obtained from Avanti Polar Lipids (Alabaster, AL). Di-8-ANEPPS was from Molecular Probes/Invitrogen (Eugene, OR). The concentration of stock solution of di-8-ANEPPS was measured using its molar absorption coefficient (ε) of 37,000 M^{-1} cm^{-1} at 498 nm (Le Goff et al. 2007) [See Supplementary Information (Section S1) for more details].

Sample Preparation

All experiments were performed utilizing large unilamellar vesicles (LUVs) of POPC or DPPC/40 mol% cholesterol

or DPPC, with appropriate concentration of PEtOH and 1 mol% di-8-ANEPPS (background samples lacked di-8-ANEPPS). In particular, 320 nmol of total lipid (POPC or DPPC/40 mol% cholesterol or DPPC) and 3.2 nmol of fluorescent probe (di-8-ANEPPS) were thoroughly mixed and dried under a stream of nitrogen while being warmed gently (~ 35 °C). LUVs were made as described previously (MacDonald et al. 1991; Mukherjee and Chattopadhyay 2005) [see Supplementary Information (Section S2) for more details].

Steady-State Fluorescence Measurements

Steady-state fluorescence measurements were carried out using a quartz cuvette of 1-cm path length in a Hitachi F-7000 spectrofluorometer (Tokyo, Japan). Fluorescence excitation spectra of di-8-ANEPPS was recorded by keeping the emission wavelength fixed at 670 nm. All experiments were performed with excitation and emission slits with bandpass of 5 nm. The fluorescence intensity ratio (R), defined as the ratio of fluorescence intensities at an excitation wavelength of 420 nm to that at 510 nm (keeping the emission wavelength fixed at 670 nm), which provides an estimate of membrane dipole potential, was calculated (Starke-Peterkovic et al. 2006; Haldar et al. 2012; Singh et al. 2013) [see Supplementary Information (Section S3) for more details]. Details of fluorescence anisotropy measurements are provided in Supplementary Information (Section S3).

Statistical Analysis

Statistical significance of data was analyzed using Student's two-tailed unpaired t test using GraphPad Prism software, version 4.0 (San Diego, CA).

Results and Discussion

Effect of PEtOH on Dipole Potential of Varying Phase Membranes

Dipole potential is defined as the potential difference that exists within the membrane, which results due to the non-random arrangement of lipid dipoles and water molecules at the interfacial region of the membrane (Clarke 2001; Brockman 1994; O'Shea 2005; Wang 2012). In this work, we explored the effect of PEtOH on membrane dipole potential, estimated by the dual-wavelength ratiometric approach using the fluorescent probe di-8-ANEPPS. We have previously shown that the average depth of penetration of the fluorescent styrylpyridinium moiety in di-8-ANEPPS is ~ 12 Å from the center of the bilayer (see Fig. 1b, Haldar et al. 2012), suggesting interfacial localization of the fluorophore in di-8-ANEPPS in the membrane. The dual-wavelength ratiometric approach utilizing the voltage-sensitive fluorescent probe di-8-ANEPPS constitutes a convenient method to measure membrane dipole potential (Gross et al. 1994; Haldar et al. 2012; Sarkar and Chattopadhyay 2017; Sarkar et al. 2017). The relevant parameter estimated using this approach is the fluorescence intensity ratio (R), which is defined as the ratio of fluorescence intensities at an excitation wavelength of 420 nm to that at 510 nm, when the emission wavelength is kept constant at 670 nm. In response to dipolar reorganization occurring in the area where the probe is located, the ratio (R) changes, independent of specific molecular interactions and membrane fluidity (Gross et al. 1994; Robinson et al. 2011). The observed shift in the excitation spectrum of di-8-ANEPPS is associated with the local electric field strength, the underlying mechanism being electrochromic in nature (Haldar et al. 2012; Loew et al. 1979). An important advantage of this method is that it is not dependent on the concentration of the fluorescent probe.

Membrane phase plays an important role in the organization of membrane domains and membrane function (Brown and London 1998; van Meer et al. 2008). The lipid acyl chains are ordered and extended in all *trans* conformation in the gel (ordered) phase, whereas they are fluid and disordered (due to the *gauche* conformations of the lipid acyl chains) in the liquid-disordered (fluid) phase. On the other hand, the liquid-ordered phase, represents a phase characterized by extended and ordered lipid acyl chain packing (as observed in gel phase), but displays lateral mobility similar to the liquid-disordered phase (Mouritsen 2010). For binary lipid mixtures, liquid-ordered phase is formed above a threshold level of cholesterol (Mouritsen 2010).

In this work, we used LUVs of POPC, DPPC/cholesterol and DPPC as these vesicles represent liquid-disordered (fluid), liquid-ordered, and gel (ordered) phase membranes, respectively (Brown and London 1998). Figure 2 shows R of di-8-ANEPPS in POPC (fluid), DPPC/cholesterol (liquid-ordered), and DPPC (gel) membranes. The value of R was found to be ~ 2.8 in DPPC/cholesterol (liquid-ordered) phase membranes, whereas POPC (fluid) and DPPC (gel) membranes display almost similar fluorescence intensity ratio (R) of ~ 1.1 and ~ 1.05, respectively. These results suggest that presence of cholesterol is able to induce change in R. It has been previously shown that presence of cholesterol results in higher membrane dipole potential (Starke-Peterkovic et al. 2006; Haldar et al. 2012; Singh et al. 2013). Our results showing DPPC/cholesterol membranes in the liquid-ordered phase displaying higher R could be attributed to the presence of cholesterol. On the other hand, R was found to be similar in POPC (fluid) and DPPC (gel) phase membranes.

Figure 3 shows the change in R of di-8-ANEPPS in relation to membrane phase (i.e., in POPC (fluid), DPPC/

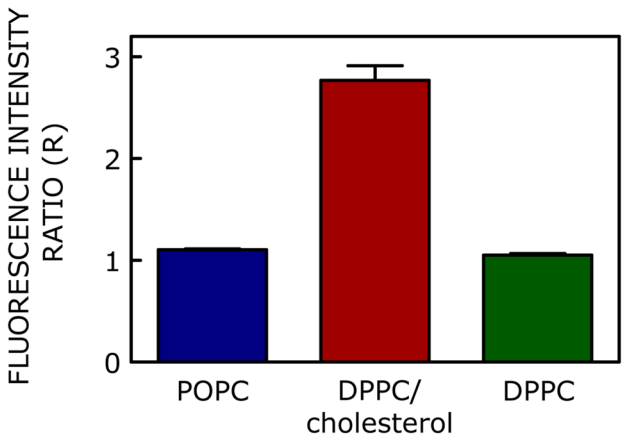

Fig. 2 Fluorescence intensity ratio (*R*) of the excitation spectra of di-8-ANEPPS in POPC (fluid phase, blue), DPPC/cholesterol (liquid-ordered phase, maroon), and DPPC (gel phase, green). R defined as the ratio of fluorescence intensities measured upon excitation at 420 nm to that at 510 nm (emission at 670 nm in all cases). The ratio of probe (di-8-ANEPPS) to lipid was 1:100 (mol/mol). All experiments were carried out at room temperature (~23 °C). Data points shown represent means ± SE of at least three independent measurements. See Materials and methods for more details

cholesterol (liquid-ordered), and DPPC (gel) membranes) with increasing concentrations of PEtOH. The figure shows a concentration-dependent progressive reduction in R with increasing concentrations of PEtOH in all membrane phases (panels a–c). The reduction in R was found to be ~ 51% in the liquid-disordered phase (POPC) at the maximum concentration of PEtOH used (see Fig. 3a and the corresponding inset). Membrane dipole potential in liquid-ordered phase (DPPC/40 mol% cholesterol) was found to be more due to the presence of cholesterol (Starke-Peterkovic et al. 2006; Haldar et al. 2012). The corresponding reduction in R at the maximum concentration of PEtOH was ~ 80% (Fig. 3b and the inset). The change in R at the highest concentration of PEtOH was found to be lowest (~ 47%) in case of gel-phase (DPPC) membranes (see Fig. 3c and the corresponding inset). Taken together, these results show that membrane dipole potential is reduced in presence of the local anesthetic PEtOH in a phase-independent manner, although the extent of reduction in dipole potential is phase dependent.

Membrane dipole potential is related to dielectric constant and dipole moment of the medium according to the Helmholtz equation (Haldar et al. 2012):

$$\Psi_d = \mu_\perp/(A\varepsilon_0\varepsilon), \quad (1)$$

where Ψ_d is the membrane dipole potential, $\mu_\perp$ is the perpendicular component of the dipole moment (μ) along the bilayer normal, ε_0 is the permittivity in vacuum, ε is the dielectric constant, and A is the area/lipid molecule. Interestingly, local anesthetics were previously reported to increase the area/lipid in membranes (Seelig 1987; Pasenkiewicz-Gierula et al. 2003). This could result in the observed reduction in membrane dipole potential in the presence of PEtOH in varying phase membranes.

Fluorescence Anisotropy of Di-8-ANEPPS in Membranes of Different Phases in Presence of PEtOH

Fluorescence anisotropy of a fluorescent probe provides information about the fluorophore's rotational mobility in the membrane environment (Lakowicz 2006) and is sensitive to the packing of lipid fatty acyl chains. This is because fluorescence anisotropy depends on the degree to which the probe is able to reorient after excitation, and probe reorientation is a function of local lipid packing. As seen from Fig. 4a, the maximum fluorescence anisotropy of di-8-ANEPPS was observed in gel-phase DPPC vesicles (~ 0.34) followed by liquid-ordered phase DPPC membranes containing 40 mol% cholesterol (~ 0.32) and minimum anisotropy was observed in liquid-disordered phase POPC vesicles (~ 0.30). These results are consistent with packing of acyl chains in the respective membrane phases. The acyl chains are extended and ordered in gel-phase membranes, whereas the liquid-ordered phase is characterized by acyl chains that are extended and ordered (such as in the gel phase), but display high lateral mobility similar to the liquid-disordered (fluid) phase (Mouritsen 2010). The minimum fluorescence anisotropy was observed in fluid-phase POPC vesicles, due to the relatively flexible packing in the liquid-disordered phase indicating a higher degree of freedom for the fluorophore to reorient.

To explore the effect of PEtOH on membrane organization, we monitored the fluorescence anisotropy of di-8-ANEPPS in POPC, DPPC/cholesterol, and DPPC membranes in presence of 2% PEtOH (v/v). Figure 4b shows that the fluorescence anisotropy of di-8-ANEPPS shows reduction in the presence of 2% PEtOH (v/v) in POPC (fluid), DPPC/cholesterol (liquid-ordered), and DPPC (gel) membranes. Fluorescence anisotropy of di-8-ANEPPS exhibited ~ 4% reduction in presence of 2% PEtOH (v/v) in liquid-disordered (fluid) phase (POPC) membranes. The corresponding reduction in fluorescence anisotropy of di-8-ANEPPS in liquid-ordered membranes (DPPC/cholesterol) was ~ 8%. The maximum reduction in anisotropy (~ 9%) was observed in gel-phase DPPC membranes in the presence of 2% PEtOH (v/v). These results indicate the disordering effect of PEtOH on varying phase membranes. Interestingly, the general trend in the extent of phase-dependent reduction in anisotropy using the commonly used fluorescent probe DPH previously reported by us (Shrivastava et al. 2016) is similar to the trend we report here. Taken together, these findings suggest that

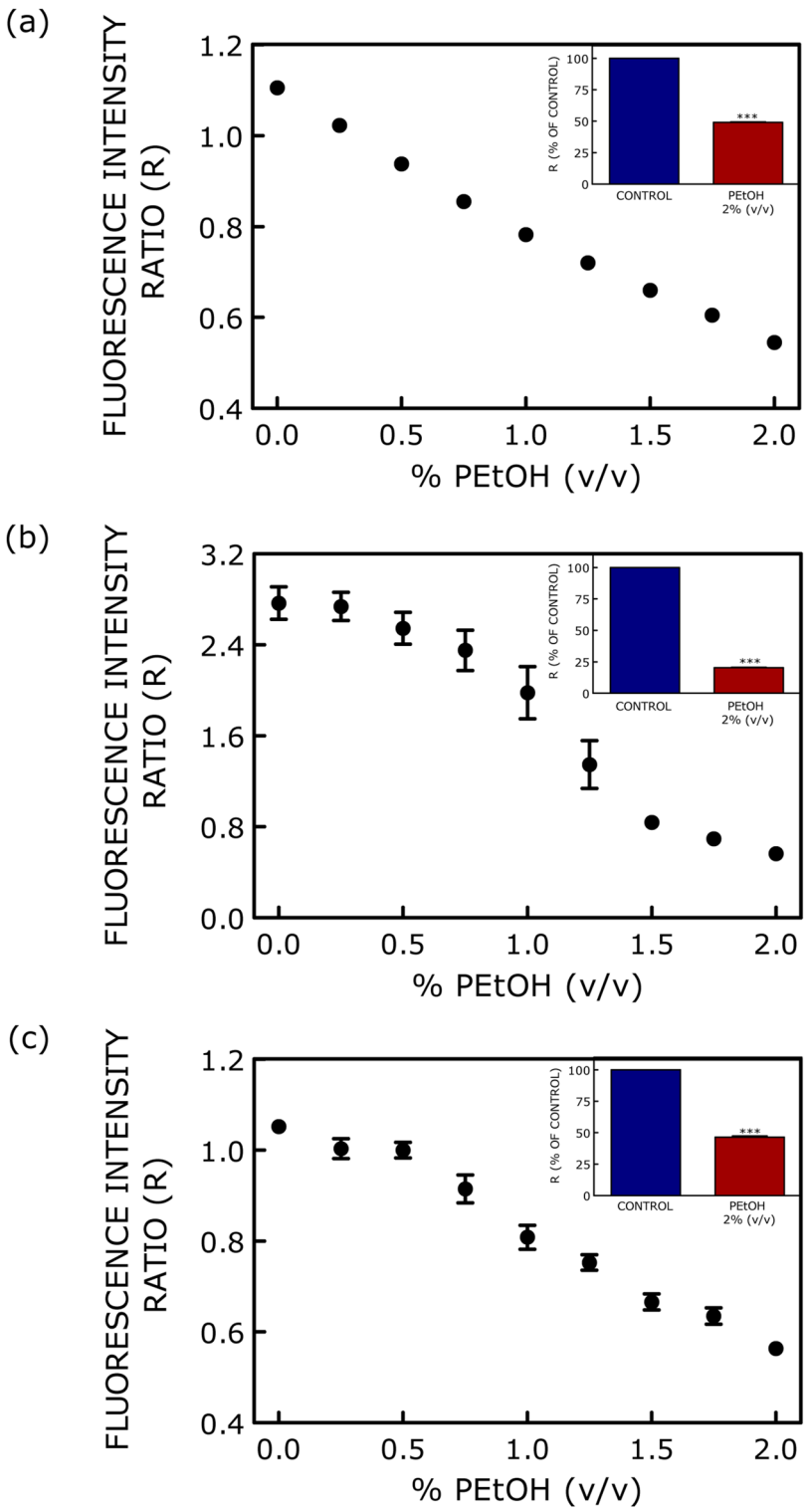

Fig. 3 Change in fluorescence ratio (R) of the excitation spectra of di-8-ANEPPS with increasing PEtOH concentration in varying phase membranes (**a** POPC (fluid phase), **b** DPPC/cholesterol (liquid-ordered phase), and **c** DPPC (gel phase)). All other conditions are as in Fig. 2. Data points represent means ± SE of at least three independent measurements. The insets in all panels show the percent change in R at the highest concentration of PEtOH (2% PEtOH (v/v); *** corresponds to significant ($p < 0.001$). See Materials and methods for more details

PEtOH modulates the organization of membrane bilayer in a phase-dependent manner.

We have previously reported that PEtOH induces disorder in membranes in a phase-dependent manner (Shrivastava et al. 2016). In another study, we reported that the presence of PEtOH reduces phase transition temperature and induces interdigitation above a certain concentration in gel-phase membranes (Reddy et al. 2018). In this work, we show that membrane dipole potential, as exhibited by the dual-wavelength ratiometric technique using di-8-ANEPPS, exhibits concentration-dependent decrease in the presence of PEtOH in all three biologically relevant phase

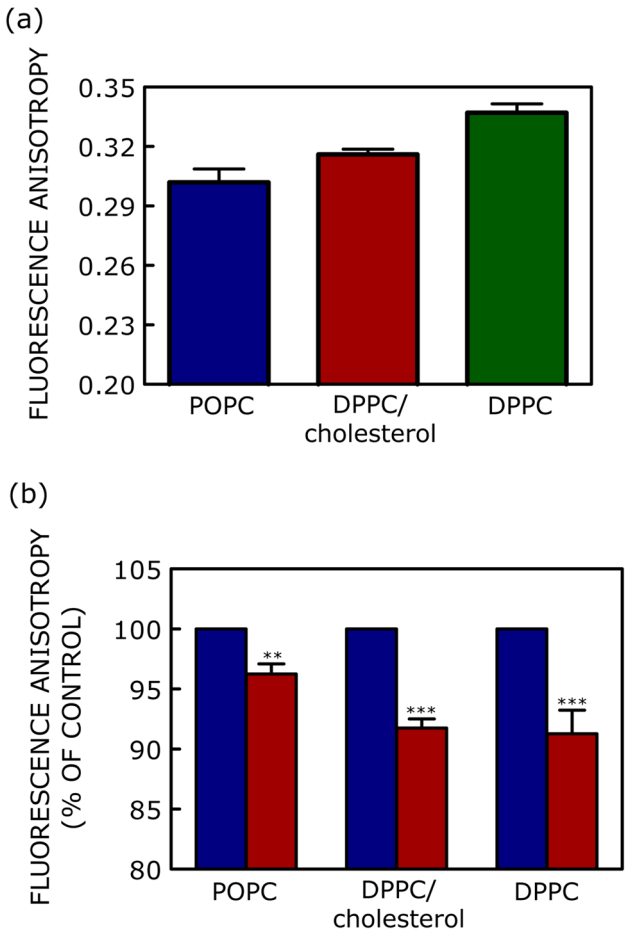

Fig. 4 Fluorescence anisotropy of di-8-ANEPPS in **a**. POPC (fluid phase, blue), DPPC/cholesterol (liquid-ordered phase, maroon), and DPPC (gel phase, green) membranes. **b** Change in fluorescence anisotropy of di-8-ANEPPS in POPC (fluid phase), DPPC/cholesterol (liquid-ordered phase), and DPPC (gel phase) membranes in the presence and absence of 2% PEtOH (v/v). Values are normalized to fluorescence anisotropy in different phase membranes in the absence of PEtOH. The ratio of probe (di-8-ANEPPS) to lipid was 1:100 (mol/mol). Data represent means ± SE of at least three independent measurements (*** and ** correspond to significant ($p < 0.001$ and $p < 0.01$, respectively) difference in fluorescence anisotropy upon addition of 2% PEtOH (v/v) (maroon bars) relative to control (blue bars)). See Materials and methods for more details

membranes. In addition, we recently reported that PEtOH could induce a reduction in dipole potential in hippocampal membranes (Rao et al. 2019). Although it has been previously reported that general anesthetics could modulate membrane dipole potential (Qin et al. 1995; Cafiso, 1998; Davis et al. 2017), our results represent one of the early reports showing reduction of membrane dipole potential induced by a local anesthetic in biologically relevant phase membranes.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00232-022-00240-2.

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Author Contributions AC and SS conceptualized the project and designed experiments; PR and SS performed the experiments; SS analyzed the data; SS and AC wrote the manuscript; AC edited the manuscript, organized access to research facilities and funding, and provided overall supervision and mentoring.

Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

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