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SHORT COMMUNICATION

Do local anesthetics interact preferentially with membrane lipid rafts? Comparative interactivities with raft-like membranes

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Abstract Membranous lipid bilayers have been reconsidered as the site of action of local anesthetics (LAs). Recent understanding of biomembranes indicates the existence of lipid raft microdomains enriched in cholesterol and sphingolipids as potential platforms for channels and receptors. Based on the hypothesis that LAs may interact preferentially with lipid rafts over non-raft membranes, we compared their effects on raft model membranes and cardiolipin-containing biomimetic membranes. Liposomes were prepared with phospholipids, sphingomyelin, cerebroside, and cholesterol to have compositions corresponding to lipid rafts and cardiomyocyte mitochondrial membranes. After reacting LAs (50–200 μM) with the membrane preparations, their interactivities were determined by measuring fluorescence polarization with 1,6-diphenyl-1,3,5-hexatriene. Although bupivacaine and lidocaine acted on different raft-like liquid-ordered membranes to reduce polarization values, their effects on biomimetic less ordered membranes were much greater. LAs interacted with biomimetic membranes with the potency being *R*(+)-bupivacaine > racemic bupivacaine > *S*(−)-bupivacaine > ropivacaine > lidocaine > prilocaine, which is consistent with the rank order of pharmacotoxicological potency. However, raft model membranes showed neither structure-dependence nor stereoselectivity. The relevance of membrane lipid rafts to LAs is questionable at least in their effects on raft-like liquid-ordered membranes.

Keywords Local anesthetics · Lipid rafts · Interaction · Raft-like membranes

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Although many publications have emphasized the interactions of anesthetics with functional proteins, for example ion channels and receptors, it is premature to exclude the possibility of interactions with membrane lipids from the mechanistic contribution to anesthesia [1]. General and local anesthetics (LAs) modify the physicochemical properties of phospholipids bilayers. A more sophisticated understanding is emerging about the structure of biomembranes. It has become clear that biomembranes are not a simple bilayer structure with uniformly distributed lipids, but rather include the microdomains known as lipid rafts [2]. In lipid rafts, cholesterol and sphingolipids are packed in a more highly ordered structure (liquid-ordered, Lo) distinct from the rest of plasma membranes (liquid-disordered, Ld). The lipid raft theory evoked a new perspective for the mode of drug action on lipid membranes. Accumulating evidence suggests that lipid rafts might be the target of general anesthetics [3, 4]. However, the interactions of LAs with lipid rafts are unclear.

Membrane lipid rafts play an important role as potential platforms concentrating specific proteins and spatially segregated molecules. Ion channels, β-adrenergic receptors, and signaling proteins have been discovered to be localized in lipid raft microdomains within the cardiovascular system [5, 6]. LAs affect the activities of channels and receptors by altering their lipid bilayer environments in

addition to directly binding to such functional proteins. As one of possible mechanisms underlying the pharmacotoxicological effects on cardiomyocytes, it is of interest to know whether LAs induce larger physicochemical changes in channel-localizing and receptor-localizing lipid rafts than in overall lipid bilayers. Lidocaine at 18.4 mM was recently found to disrupt the raft structures in erythrocyte membranes [7], suggesting the involvement of raft-related signal transduction in the anesthetic mechanism [8].

The objective of this study was to verify the possibility that LAs act on lipid raft microdomains. The raft property is reproducible in unilamellar vesicles formed from a defined molar mixture of phospholipid, cholesterol, and sphingomyelin [9]. On the basis of the hypothesis that LAs may interact preferentially with lipid rafts over non-raft membranous structures, we compared their effects on liposomal raft-like and biomimetic membranes.

Unilamellar vesicles (total lipids 0.14 mM) labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH; Molecular Probes, Eugene, OR, USA) were prepared as reported previously [10]. The lipids used, that is, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), sphingomyelin (SM), cerebroside (CB), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-myo-inositol) (POPI), cardiolipin (CL), and cholesterol, were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Raft-1 [11], raft-2 [12] and raft-3 [13] model membranes, and CL-containing biomimetic membranes as a model membrane for human cardiomyocyte mitochondria [10] were prepared to have the lipid compositions shown in Table 1. Bupivacaine (*S*(−), *R*(+), and racemic), lidocaine, ropivacaine, and prilocaine (50–200 μM for each) were reacted with the membrane preparations suspended in 10 mM TES (*N*-tris(hydroxylmethyl)methyl-2-aminoethanesulfonic acid) buffer of pH 7.4 containing 125 mM NaCl and 25 mM KCl. Bupivacaine and ropivacaine were supplied by Maruishi Pharmaceutical (Osaka, Japan) and AstraZeneca (Södertälje, Sweden), respectively. Lidocaine and prilocaine were obtained from Sigma–Aldrich (St Louis, MO, USA). After the reaction at 37°C for 30 min, membrane interactivities were determined by measuring DPH fluorescence polarization at 360 nm for excitation and at 430 nm for emission [14]. Compared with controls, a decrease of polarization is indicative of membrane disordering. Because the polarization values of drug-untreated (intact) membranes vary depending on their lipid compositions, the polarization changes (%) relative to control polarization values were used for comparing raft model membranes and biomimetic membranes. Results are expressed as mean ± SEM ($n = 6$). Data were statistically analyzed by a one-way analysis of variance (ANOVA), followed by a post hoc Fisher's protected least significant difference (PLSD) test. $P < 0.05$ was considered significant.

A fluorescent probe, DPH, penetrates into membrane lipid bilayers to align with lipid acyl chains. Because DPH is subject to the rotational restriction imparted by the lipid order, its rotation is reduced in highly structured or ordered membranes to produce a higher degree of fluorescence polarization. Disordered membranes allow more rotation of DPH to exhibit lower polarization. Drug-untreated membranes showed different DPH polarization for raft-1, raft-2, and raft-3 model membranes and biomimetic membranes (Table 1). The comparative polarization values indicate that raft-like membranes are liquid-ordered, whereas biomimetic membranes are less ordered. The ordering state of raft-2 model membranes was close to that of biomimetic membranes, which is attributed to the larger molar ratio of unsaturated phosphatidylcholine DOPC with the ability to reduce the order of membranes. The sphingolipid-rich and cholesterol-rich compositions make lipid rafts more rigid and less detergent-soluble than non-raft membranes, which is relevant to the raft property [5, 11].

Table 1 Membrane lipid compositions and DPH fluorescence polarization values of drug-untreated membranes

Membrane preparation	Lipid composition (mol%)									Polarization value
	DOPC	POPC	POPE	POPS	POPI	SM	CB	CL	Cholesterol	
Raft-1	16.7		16.7			16.7	16.7		33.3	0.2702 ± 0.0004
Raft-2	33.3					33.3			33.3	0.2191 ± 0.0004
Raft-3	5		5	10		40			40	0.3083 ± 0.0004
Biomimetic		25	16	3	3	3		10	40	0.2047 ± 0.0006

Raft-1, raft-2, and raft-3 model membranes and biomimetic membranes were prepared according to the lipid composition in Refs. [10–13], respectively

Molar percentages are rounded to one decimal place and polarization values are expressed as mean ± SEM ($n = 6$)

DOPC 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, *POPC* 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, *POPE* 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, *POPS* 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine], *POPI* 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-myo-inositol), *SM* sphingomyelin, *CB* cerebroside, *CL* cardiolipin, *DPH* 1,6-diphenyl-1,3,5-hexatriene

Bupivacaine and lidocaine interacted with all membrane preparations and reduced DPH polarization at 50–200 μM (Fig. 1). LAs increase the rotational mobility of membrane lipids. Their membrane interactions rearrange the intermolecular hydrogen-bonded network among phospholipid molecules and also change the orientation of the P–N dipole of phospholipid molecules, resulting in the disordering of lipid bilayers [15]. However, the effects of bupivacaine and lidocaine on raft-like membranes were less than those on biomimetic membranes.

No raft model membranes showed significant differences between LAs (Fig. 2), even though all drugs were

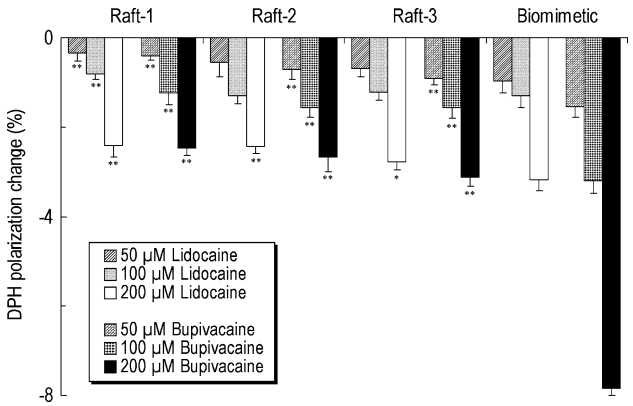

Fig. 1 Interactions of bupivacaine and lidocaine with raft-like membranes and biomimetic membranes. Drugs of 50–200 μM for each were reacted with raft-1, raft-2 and raft-3 model membranes and CL-containing biomimetic membranes. Their membrane interactivities were compared on the basis of DPH polarization changes. Each *bar* represents mean ± SEM ($n = 6$). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus biomimetic membranes

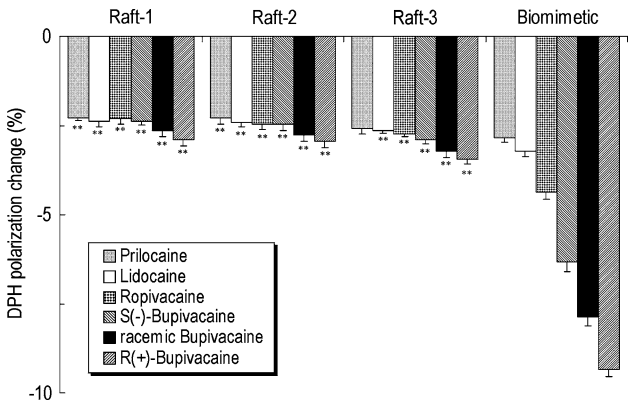

Fig. 2 Comparative interactivities of LAs with raft-like membranes and biomimetic membranes at the identical concentration of 200 μM. LAs were reacted with raft-1, raft-2, and raft-3 model membranes and CL-containing biomimetic membranes. Their membrane interactivities were compared on the basis of DPH polarization changes. Each *bar* represents mean ± SEM ($n = 6$). $^{**}P < 0.01$ versus biomimetic membranes

reacted at the pharmacotoxicologically active plasma concentration [16]. In contrast, biomimetic membranes produced different interactions with the potency being *R*(+)-bupivacaine > racemic bupivacaine > *S*(−)-bupivacaine > ropivacaine > lidocaine > prilocaine, which is consistent with the rank order of cardiac and anesthetic action [17]. Such structure-dependence and stereoselectivity are also found in LA-induced physicochemical changes of cardiomyocyte model membranes prepared with CL and cholesterol [10, 14, 18]. The DPH polarization changes in biomimetic membranes were 2.71, 2.44, 2.19, 1.59, 1.22, and 1.10 times larger than those in raft-3 model membranes for *R*(+)-bupivacaine, racemic bupivacaine, *S*(−)-bupivacaine, ropivacaine, lidocaine, and prilocaine, respectively.

All the tested raft-like membranes are more ordered than biomimetic membranes, as shown by larger DPH polarization values of drug-untreated membranes. Because it is difficult for membrane-disordering agents to affect the relatively ordered membranes, the membrane effects of LAs would be smaller on such membranes. Biomimetic membranes contain CL, but not raft model membranes. Anionic CL contributes to enhancing the interactivity with positively ionized molecules of LAs [10, 18]. Raft model membranes devoid of CL are disadvantageous for the intensive interaction with LAs. Cholesterol with several chiral carbons is responsible for discriminating the membrane effects between stereoisomeric LAs [14]. Although raft model membranes contain cholesterol, similarly to biomimetic membranes, they did not produce the discriminable interactivities of bupivacaine enantiomers. The more ordered structure and the absence of CL may be associated with the non-stereoselective interactions with raft model membranes. The ordered-liquid phase induced by cholesterol and sphingolipid in lipid rafts is also more resistant to the membrane perturbation by anesthetics [1].

All the tested LAs disordered biomimetic membranes more than raft-2 model membranes at all the tested concentrations even though both membranes show the almost comparable ordering state (Figs. 1, 2). Such different disordering is considered to be because of CL specifically contained in biomimetic membranes. The interactions of LAs with the phosphate moiety of CL are much stronger than those with the other phospholipids contained in raft model membranes [14, 15]. In this study, biomimetic membranes were prepared with CL to resemble human cardiomyocytes in mitochondrial membrane lipid composition. CL is preferentially localized in cardiomyocyte mitochondrial membranes, especially in the inner membranes, and it plays a crucial role in myocardial energy metabolism and membrane dynamics. LAs can rapidly diffuse intracellularly to mitochondria and can reduce or even collapse their transmembrane potential [19]. The

CL-dependent interactions of LAs with cardiomyocyte mitochondrial membranes could very well contribute to their cardiotoxic effects [10, 18].

In conclusion, the preferential interactions of LAs with lipid rafts are questionable at least in their effects on liquid-ordered membranes as a raft model. The membrane lipid raft microdomains may not be relevant to the pharmacotoxicological features of LAs.

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