氏名	菅原　恒		
学位の種類	博士(マテリアルサイエンス)		
学位記番号	博材第 434 号		
学位授与年月日	平成 29 年 9 月 22 日		
論文題目	Destabilization of raft-mimetic structure on liposomal membrane induced by local anesthetics （局所麻酔薬によるリポソーム膜中におけるラフト模倣構造の不安定化）		
論文審査委員	主査	高木　昌宏	北陸先端科学技術大学院大学　教授
		藤本　健造	同　教授
		筒井　秀和	同　准教授
		濱田　勉	同　准教授
		三浦　佳子	九州大学　教授

論文の内容の要旨

Introduction

Local anesthetics (LAs) are often used as pain killer among the surgical or dental operation. Despite of long time after development of LAs, mechanism of pain suppression by LAs have not been fully explained yet. Pain sensation is transmitted through neural membranes as the electric signal: the repetition of membrane potential inversion due to the cross-membrane permeation of cation [1–3]. LAs are known to suppress the function of the protein: voltage-dependent sodium channel which is controlling the influx of sodium ion into neural cell [4]. Until recently, LAs are thought to interact between channel proteins by direct bonding [5,6] (Fig.1, left). However, these studies have not been fully explained the interaction between LAs and whole ion channels which can be influenced by LAs. Recently, some studies indicated the correlation between efficacy and hydrophobicity of LAs [7,8] (Fig.1, right). These facts indicates the indirect interaction between LAs and channels due to the physical property change of biomembranes.

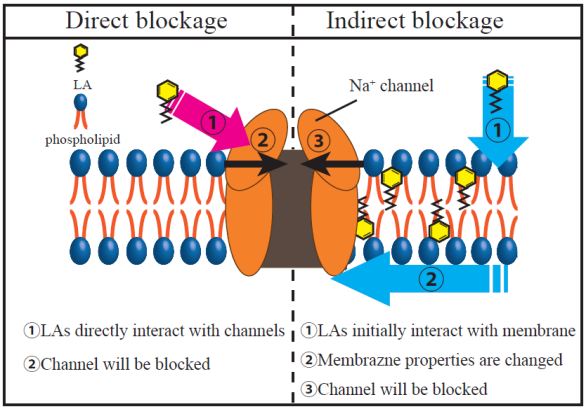

Fig.1 Direct (left) and indirect (right) mechanisms of

Biomembranes are composed of bilayer structure mainly contains phospholipids and cholesterol (Chol) [9–13]. Membrane molecules are oriented with weak attraction derives from hydrophobic interaction, and they are forming fluidic and flexible structure. On the surface of biomembranes, there are several of

integral molecules such as sugar chains and proteins are exist. Functions of these molecules can be influenced by the behaviors of biomembranes. Furthermore, recently, Ikonen *etal*. suggested the specific lipid region lipid raft [14–16]. Lipid raft is known as a region rich with saturated phospholipid and Chol, and various integral proteins such as ion channels are localized. Therefore, biomembranes and lipid raft on them are regarded as important platform, and their physical properties and behaviors should strongly influenced to the cross-cellular signal transduction. However, biomembranes are composed with diverse components such as lipid, sugar, and proteins. Moreover, behaviors of biomembranes are corresponds to the other organelle such as cytoskeleton. As above, to discuss the properties of biomembranes, we need consider plenty of factors.

In recent research, biomimetic membrane: liposome is widely used as a simplified experimental system excluding such unnecessary factors. Liposome is the aggregation of amphiphilic phospholipid which has spherically-closed bilayer structure. Furthermore, we can realize raft-mimetic structure in multi-components liposome as phase-separated structure. Therefore, many studies discuss the fundamental phenomena in biomembrane with analyzing property of liposomal membranes.

Research objective

Through our study, we investigated the effect of LAs on the stability of raft-mimetic structure; phase-separated structure in liposomal membrane composed with unsaturated phospholipid (DOPC), saturate phospholipid (DPPC), and cholesterol (Chol). To discuss the effects of LAs on membrane physical property more precisely, we measured membrane fluidity with GP value of Laurdan fluorescence, and line tension at boundary of 2-phase liposomes. We also performed DSC experiment to understand the affinity and localization of LAs in different phases.

Materials

Liposomal membranes were prepared with Natural swelling method (Fig.2). Lipids (DOPC, DPPC, Chol) and LAs (lidocaine, tetracaine), fluorescents (Rhod-DHPE, Laurdan) were dissolved in chloroform, and their concentrations were 2mM, 0.5mM, and 10□M, respectively. 10mM Glucose dissolved in methanol. These stock solutions were put into glass test tubes, and then mixed all of them. These solutions were dried under vacuum for least 3 hours to form thin lipid films. The films were then hydrated overnight with deionized water at 55 °C to produce unilamellar liposomes. The final concentrations were 0.2mM lipids and LAs, 0.6mM glucose, 0.01mM Rhod (10□M in case of GP value measurement), and 0.25□M Laurdan.

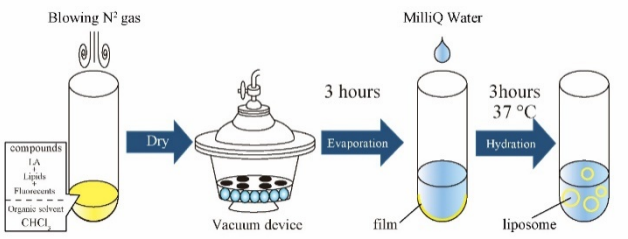

Fig.2 Schematic of liposomal preparation

Result and discussion

In chaptor 2, initially, we observed formation of phase separation on LAs presented DOPC/DPPC (solid ordered: S_o/liquid disordered: L_d), and DOPC/DPPC/Chol (liquid ordered: L_o/L_d) membranes. This experiment revealed that the presence of LAs suppressed L_o/L_d phase separation in DOPC/DPPC/Chol (Fig.3). On the other hand, S_o/L_d phase separation on DOPC/DPPC liposome was not influenced by LAs. We also clarified the fluidity decrease of DOPC-rich region (L_d) in DOPC/DPPC/Chol liposomes by presence of LAs (Fig.4). Fluidity decrease of L_d phase should make the fluidity difference between DOPC-rich phase and DPPC/Chol-rich phase. This action of LAs should decrease the line energy between Lo and L_d phases, and suppressed phase separation.

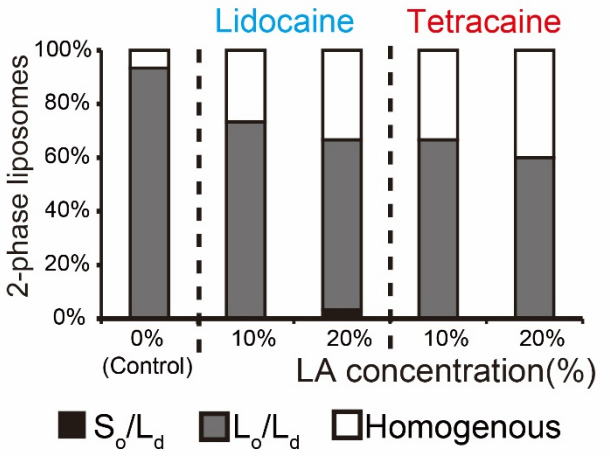

Fig.3 The ratio of heterogeneous LA containing DOPC/DPPC/Chol membranes in each different concentration of

In chapter 3, we observed decrease of miscibility temperature of L_o/L_d phase separation in DOPC/DPPC/Chol membrane. Furthermore, based on image analysis, we revealed the line tension decrease at L_o/L_d phase boundary of LA added DOPC/DPPC/Chol liposomes (Fig.5). On the other hand miscibility temperature of S_o/L_d phase separation in DOPC/DPPC liposomes was not influenced by LAs. Finally, we performed Differential Scanning Calorimetry (DSC) experiments with LA added DPPC and DPPC/Chol membrane to discuss the effect on membrane thermal-stability and localization of LAs in multi-lipid liposomes (Fig.6). Through the DSC experiment with DPPC membrane, addition of LAs decreased the transition temperature of both S_o and L_o membranes in concentration dependent manner. At the same time, in the experiment of DPPC (S_o) membrane with higher LA concentration (5~10 mol%), we observed the shoulder on the lower temperature side on the thermal peaks. On the other hand, we could not observe similar asymmetric shoulder of thermal peaks on DPPC/Chol (S_o/L_o coexist) membranes despite the presence of higher LAs concentration. This phenomena indicates relatively stronger affinity of LAs with L_o phase rather than with S_o phase. Therefore, we discussed localization LAs into DPPC-rich region was enhanced by the contribution of Chol. This localization change of LAs result into decrease of the line tension on

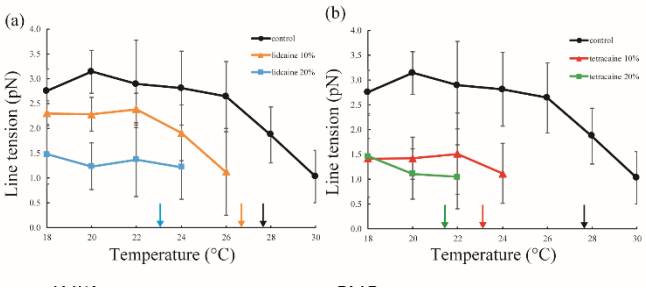

Fig.5 Line tension at L_o/L_d phase boundary in DOPC/DPPC/Chol/LAs mixtures as a function of temperature. Miscibility temperatures measured

Fig.4 Fluidity of different phases in LA containing

phase-boundary, and finally destabilized phase-separated structure.

Results of our study indicates correlation between LA function and phase behavior of biomembrane. Lacking of heterogeneity on membranes could change various membrane properties like membrane thickness, bending or expansion rigidities, spontaneous curvature, etc. These physical property changes can effect on gaiting or binding abilities of membrane proteins such as ion-channels. Our results are suggesting some correlation between raft destabilization and protein disordering by LAs

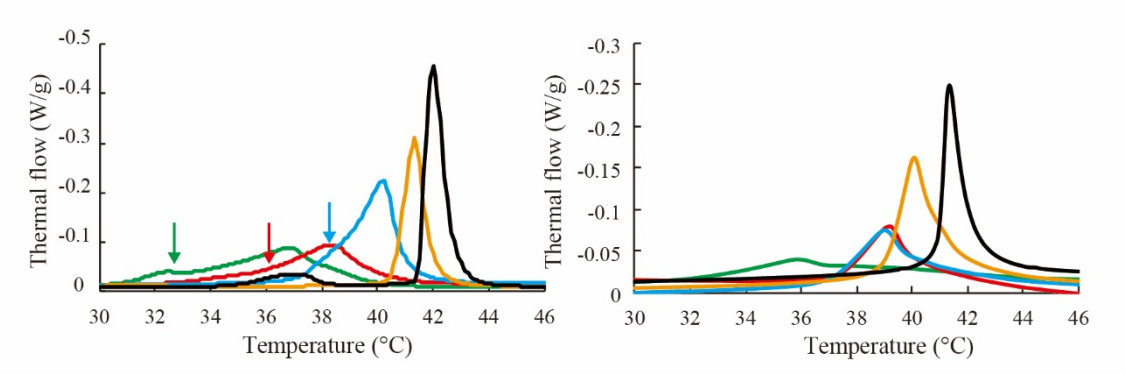

Fig.6 Thermograph of tetracaine containing DPPC (left), and DPPC/Chol (right) membranes. The color of lines are corresponds to different LA concentrations (LA 0% black, 2.5% yellow, 5% blue, 7.5% red, 10% green). Location of small shoulder derives from LA-rich phase in DPPC membranes are shown as arrows. The colors of arrows are corresponds to those of LA concentration.

References

1. Baumann TK, Chaudhary P, Martenson ME. Background potassium channel block and TRPV1 activation contribute to proton depolarization of sensory neurons from humans with neuropathic pain. *Eur J Neurosci*. 2004;19(5):1343-1351. doi:10.1111/j.1460-9568.2004.03097.x.
2. Okada M, Corzo G, Romero-Perez GA, Coronas F, Matsuda H, Possani LD. A pore forming peptide from spider Lachesana sp. venom induced neuronal depolarization and pain. *Biochim Biophys Acta - Gen Subj*. 2015;1850(4):657-666. doi:10.1016/j.bbagen.2014.11.022.
3. Witschi R, Punnakkal P, Paul J, et al. Presynaptic α2-GABAA receptors in primary afferent depolarization and spinal pain control. *J Neurosci*. 2011;31(22):8134-8142. doi:10.1523/JNEUROSCI.6328-10.2011.
4. Becker DE, Reed KL. Local anesthetics: review of pharmacological considerations. *Anesth Prog*. 2012;59(2):90-101-3. doi:10.2344/0003-3006-59.2.90.
5. Boiteux C, Vorobyov I, French RJ, French C, Yarov-Yarovoy V, Allen TW. Local anesthetic and antiepileptic drug access and binding to a bacterial voltage-gated sodium channel. *Proc Natl Acad Sci*. 2014;111(36):2-7. doi:10.1073/pnas.1408710111.
6. Catterall W a. From Ionic Currents to Molecular Mechanisms : The Structure and Function of Voltage-Gated Sodium Channels. *Neuron*. 2000;26:13-25. doi:10.1016/S0896-6273(00)81133-2.

7. Komai H, McDowell TS. Differential effects of bupivacaine and tetracaine on capsaicin-induced currents in dorsal root ganglion neurons. *Neurosci Lett*. 2005;380(1-2):21-25. doi:10.1016/j.neulet.2005.01.004.
8. Tsuchiya H, Mizogami M. Interaction of local anesthetics with biomembranes consisting of phospholipids and cholesterol: Mechanistic and clinical implications for anesthetic and cardiotoxic effects. *Anesthesiol Res Pract*. 2013;2013. doi:10.1155/2013/297141.
9. Armbruster BN, Li X, Pausch MH, et al. Molecular cell biology. *Science (80-)*. 2009;101(1):1-4. doi:10.1038/nrm2330.Membrane.
10. Head BP, Patel HH, Insel PA. Interaction of membrane/lipid rafts with the cytoskeleton: Impact on signaling and function: Membrane/lipid rafts, mediators of cytoskeletal arrangement and cell signaling. *Biochim Biophys Acta - Biomembr*. 2014;1838(2):532-545. doi:10.1016/j.bbamem.2013.07.018.
11. Gaber BP, Yager P, Peticolas WL. Interpretation of biomembrane structure by Raman difference spectroscopy. Nature of the endothermic transitions in phosphatidylcholines. *Biophys J*. 1978;21(2):161-176. doi:10.1016/S0006-3495(78)85516-7.
12. Goñi FM. The basic structure and dynamics of cell membranes: An update of the Singer-Nicolson model. *Biochim Biophys Acta - Biomembr*. 2014;1838(6):1467-1476. doi:10.1016/j.bbamem.2014.01.006.
13. Biomembranes_ Structural Organization and Basic Functions - Molecular Cell Biology - NCBI Bookshelf.pdf.
14. Simons K, Ikonen E. Functional rafts in cell membranes. *Nature*. 1997;387(6633):569–572. http://www.nature.com/articles/42408. Accessed March 25, 2015.
15. Simons K, Sampaio JL. Membrane Organization and Lipid Rafts. *Cold Spring Harb Perspect Biol*. 2011;3(10):a004697-a004697. doi:10.1101/cshperspect.a004697.
16. Simons K, Vaz WLC. Model systems, lipid rafts, and cell membranes. *Annu Rev Biophys Biomol Struct*. 2004;33:269-295. doi:10.1146/annurev.biophys.32.110601.141803.

論文審査の結果の要旨

博士論文の内容は、以下の通りである。

（Chapter 1: Introduction and Abstract）局所麻酔薬(以後 LA)は細胞膜上に存在する電位依存性チャネルに直接作用し、信号の伝達を阻害するとされるが、LA の親油性とその作用強度・毒性と関連が見られること等から、生体膜脂質との相互作用を介した間接的メカニズムが提唱されている。生体膜中のラフト構造は、凝集・分散などの動的変化を介して、生体内のシグナル伝

達における重要な場として機能している。本研究では、生体膜の物性やその中で起きる現象を深く理解するために、人工生体模倣膜（リポソーム）を用いた。リポソームは実際の生体膜と構造・組成が類似しているだけで無く、複数種の脂質で組成することで、ラフト模倣構造である脂質相分離構造を再現する事が出来る。本研究ではリポソーム膜相分離構造の安定性と、膜物性に、局所麻酔薬 lidocaine および tetracaine 添加が与える影響を解析した。

(Chapter 2) 飽和脂質 DPPC、不飽和脂質 DOPC、cholesterol（以後 Chol）からなるリポソーム膜中の相分離構造形成に、LA が与える影響を蛍光顕微鏡観察によって解析した。また相分離構造の形成に重要とされる脂質膜の流動性への影響を、蛍光分子 Laurdan の GP 値より解析した。本研究を通して、LA は脂質膜の DOPC リッチ相(L_d相)の流動性を低下させ、結果的に DOPC/DPPC/Chol 膜の相分離構造を不安定化している事が分かった。

(Chapter 3) 飽和脂質 DPPC、不飽和脂質 DOPC、cholesterol（以後 Chol）からなるリポソーム相分離構造の熱安定性に LA が与える影響を、蛍光顕微鏡観察によって解析した。また相境界に働く線張力を、顕微鏡画像解析によって測定した。更に、DPPC 膜および DPPC/Chol 膜を用いた DSC 測定実験を行った。結果、LA 分子は L_d 相だけでなく、Chol を含む DPPC 相(L_o相)にも分配され、線張力を低下させ、相分離構造の熱安定性を低下させている事が分かった。

(Chapter 4) (Conclusions) 本研究を通して、LA の添加が濃度依存的にリポソーム膜の物性に影響を与え、ラフト模倣構造である脂質相分離構造を不安定化している事が分かった。大変興味深いことに、全ての実験において、比較的強力な麻酔薬である tetracaine の方が、lidocaine よりも高い膜物性への関与を示した。この実験結果は LA 分子によるラフト構造の不安定化を示唆するものであり、ラフト中に存在するイオンチャネルタンパク質の機能への膜物性変化を介した関与を示唆するものである。

以上本論文は、局所麻酔薬の膜相分離構造に与える影響について、詳細に解析したものであり、学術的に貢献するところが大きい。よって博士（マテリアルサイエンス）の学位論文として十分価値あるものと認めた。