Increase in Water Permeability of Negatively Charged Liposomal Membrane by Local Anesthetics

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Effect of the local anesthetics dibucaine, tetracaine, lidocaine and procaine on the water permeability of phospholipid membrane was examined using liposomes composed of bovine heart cardiolipin and egg yolk phosphatidylcholine in a molar ratio of 2/98 by monitoring the osmotic shrinkage of liposomes in hypertonic glucose solution at pH 7.3 and 30 °C. These local anesthetics greatly accelerated the water permeability by destabilizing the membrane structure. The effect was found to be governed by the hydrophobicity of the anesthetics. There was also a significant correlation between the membrane destabilizing actions and the anesthetic activities.

Keywords local anesthetic; liposome; membrane destabilization; partition coefficient; anesthetic potency

Introduction

Local anesthetics are known to exert a wide variety of effects on the functions of biological membranes as well as anesthetic activity.[1] There is a view that local anesthetics first perturb the membrane bilayer structure, thereby modifying the state of the membrane proteins responsible for membrane functions. Studies on the interaction of local anesthetics with phospholipid membranes such as liposomes and bilayer phospholipid membranes (BLM) are useful for understanding the anesthetics' primal action mechanism at the membranes that triggers induction of the former's biological activities.

We recently found that tetracaine increases water permeability of negatively charged liposomes induced by hypertonic osmotic shock. This effect is due primarily to the neutralization of the negative surface charge by binding of the tetracaine cation, whereas tetracaine does not affect the bilayer structure of the liposomes when the membrane is in the fluid liquid crystalline phase.[2] It would thus be of interest to learn whether the effect is specific to tetracaine, or is common to all local anesthetics, and, if it is the latter, what structural property is responsible for their membrane destabilizing effects.

In this study, we examined the effects of various local anesthetics on the water permeability of negatively charged liposomes induced by hypertonic osmotic shock. We determined the initial rate constant of water permeability in the presence of various concentrations of local anesthetics to know their effects at the initial stage of their actions in the membranes. We found that all the local anesthetics tested accelerated water permeability to a degree depending on their hydrophobic nature.

Materials and Methods

Reagents Bovine heart cardiolipin (BhCL) was isolated according to the reported procedure as its sodium salt.[3] Egg yolk phosphatidylcholine (EyPC) was purchased from Nichiyu Liposome Co., Ltd. (Tokyo) and was used without further purification. These phospholipids were stored as solutions in chloroform in sealed ampules under an argon atmosphere at −20 °C. Concentrations of phospholipids were determined in terms of phosphorous (P_i).[4] The local anesthetics used in this study were the HCl salts of dibucaine (Wako Pure Chemical Industries Co., Ltd., Osaka), tetracaine (Wako), lidocaine (Sigma Chemicals Co., Ltd., St. Louis), and procaine (Sigma). Other chemicals were of the highest grade commercially available.

Preparation of Liposomes Large unilamellar vesicles (LUV) composed of BhCL/EyPC = 2/98 (molar ratio) with a diameter of about 220 nm, determined with a particle sizer Nicop 370 (Particle Sizing Systems Products, Santa Barbara), were prepared by the reversed phase evaporation method in 10 mM Tris–HCl buffer, pH 7.3.[5] The size of liposomes was adjusted by filtrations through 0.4 and 0.2 μm filters (Nuclepore Co., Pleasanton) in an Amicon type-8010 ultrafiltration apparatus.

Osmotic Shrinkage Experiments Experiments on osmotic shrinkage were carried out essentially by the method of Blok *et al.*[6] To the suspension of LUV at a P_i concentration of 0.5 mM in 10 mM Tris–HCl buffer, pH 7.3, in a total volume of 2.88 ml, 60 μl of a given concentration of local anesthetic was added. After about 2 min, 60 μl of the glucose solution (final concentration, 20 mM) was added rapidly under stirring. Change in the absorbance at 450 nm of the liposome suspension was monitored in a Shimadzu spectrophotometer, model UV-3000 at 30 °C. Output signals were stored in a microcomputer, NEC PC-9801, at a sampling rate of 80 ms, and were recorded on an X–Y plotter, Roland DXY-101. The initial velocity of shrinkage was determined by the linear least-squares method.

Results and Discussion

As shown in Fig. 1, addition of dibucaine (final concentration, 100 μM) caused a small increase in the optical absorbance at 450 nm (A), probably due to slight aggregation of liposomes, and A became constant after about 2 min. This absorbance was greatly enhanced by hypertonic osmotic shock induced by addition of the final concentration of 20 mM glucose. The absorbance attained a constant level (A_∞) after less than 10 min. Increase in A is associated with shrinkage and/or aggregation of liposomes.[6] The initial velocity of liposome shrinkage (v_0) reflects the barrier ability of the liposomes against water permeation (*cf.* Eqs. 1 and 2).[6] Similar changes were observed with the local anesthetics tetracaine, lidocaine and procaine.

In this study, we used the v_0 value determined from Eq. 1 as an index of the change in the membrane structure

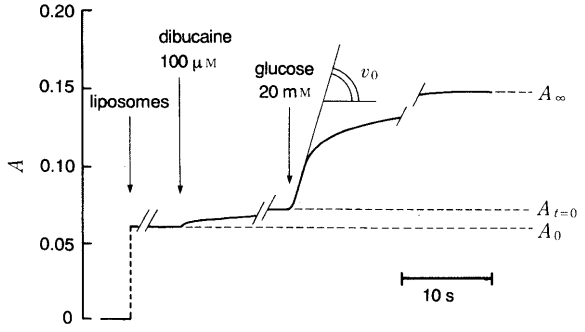

Fig. 1. Change in the Optical Absorbance at 450 nm (A) of Liposome Suspension Upon Injection of Hypertonic Glucose Solution in the Presence of Dibucaine at 30 °C

Liposome: BhCL/EyPC = 2/98 (0.5 mM P_i) suspended in 10 mM Tris–HCl buffer, pH 7.3, containing 100 μM dibucaine.

induced by local anesthetics.[6]

$$v_0 = (d(1/A)/dt)_{t=0}/(1/A_{t=0}) \quad (1)$$

where $A_{t=0}$ is the absorbance at 450 nm extrapolated to the time of glucose injection ($t=0$). As v_0 is proportional to the initial velocity of volume change $(dV/dt)_{t=0}$ in liposomes, the following relationship holds[6]:

$$v_0 = k(dV/dt)_{t=0} = kP_wSRT\Delta C_{glucose} \quad (2)$$

where P_w is the permeability coefficient of water, S is the surface area of the membrane, $\boldsymbol{R}$ is the gas constant, T is the absolute temperature, $\Delta C_{glucose}$ is the difference between the concentrations of glucose outside and inside the liposomal membrane, and k is a constant. Increase in v_0 is dependent on P_w and S, but S would not change greatly by binding of local anesthetics under present experimental conditions in which the concentrations of the anesthetics were much lower than those generally used in studies of their effects on membrane lysis[7] and their critical micelle concentrations (cmc).[8] Thus, the initial velocity of the absorbance change can be regarded to represent essentially the velocity of water permeation.[2,6]

Figure 2 shows the effects of dibucaine on the permeability, v_0, of the BhCL/EyPC=2/98 liposomal membranes to water induced by osmotic shock on addition of 20 mM glucose at 30 °C. v_0 was not affected by dibucaine at concentrations less than 50 μM. However, it increased above this concentration, the maximal effect being observed at 100 μM. Further increase in the dibucaine concentration resulted in a decrease of v_0, indicating that this local anesthetic stabilizes membrane structure at higher concentrations. The concentration at which the maximal v_0 was induced was referred to as C_{max}, and is the concentration of dibucaine required for induction of the maximal destabilization of the membrane structure of liposomes. Similarly, tetracaine, lidocaine and procaine showed distinct C_{max} values. Values of C_{max} are summarized in Table I.

We recently found that increase in v_0 induced by tetracaine is associated with increase in the ζ-potential of the BhCL/EyPC liposomes by its binding to the negatively charged polar head of BhCL, and the destabilizing effect of tetracaine on the membrane structure is maximal at the concentration at which tetracaine cation completely neutralizes the surface charge of the liposomal membrane.[2] Thus, at C_{max}'s induced by dibucaine, lidocaine and procaine the surface charge of the BhCL/EyPC liposomes is believed neutralized by local anesthetics, and this charge neutralization is directly associated with the destablization of the membrane structure as reflected by increase in the water permeability of the liposomal membranes.

Next, we examined the relationship between the effectiveness of local anesthetics to perturb membrane structure as represented by $\log(1/C_{max})$ and the hydrophobicity of local anesthetics represented by their partition coefficients between octanol and water, P_{oct}, as determined by Abe *et al.*[9] As both of the neutral and cationic forms of local anesthetics were present in the incubation medium, the partition coefficients of their neutral and cationic forms were referred to as P^0_{oct} and P^+_{oct}, respectively, and these values are listed in Table I.

Figure 3 shows the significant linear relationships between $\log(1/C_{max})$ and $\log P^0_{oct}$, and $\log P^+_{oct}$, and these are represented by Eqs. 3 and 4,

$$\log(1/C_{max}) = 0.311 \underset{(\pm 0.154)}{} + \underset{(\pm 0.049)}{0.861} \cdot \log P^0_{oct} \quad (3)$$
$$(n=4,\ r=0.997,\ s=0.112)$$

$$\log(1/C_{max}) = 1.982 \underset{(\pm 0.237)}{} + \underset{(\pm 0.168)}{0.891} \cdot \log P^+_{oct} \quad (4)$$
$$(n=4,\ r=0.966,\ s=0.356)$$

where the value in parentheses is the 95% confidence interval, n is the number of compounds tested, r is the correlation coefficient and s is the standard deviation.

In these relationships, the membrane destabilizing effect was shown to be linearly dependent on the hydrophobicity of both the neutral and cationic forms of local anesthetics. Values of P^+_{oct} were about 2 orders of magnitude less than those of P^0_{oct}, as observed with the partition coefficients of the anionic forms of 2,4-dinitrophenol and indomethacin.[10] Thus, similar correlations should hold between $\log(1/C_{max})$ and $\log P^0_{oct}$, and between $\log(1/C_{max})$ and $\log P^+_{oct}$. pK_a

TABLE I. Biological Activities and Physicochemical Properties of Local Anesthetics

Local anesthetic	C_{max} (mM)	$\log(1/C_{max})$ (M^{-1})	$\log(1/MBC)$[a] (M^{-1})	$\log P^{0}_{oct}$ [b]	$\log P^{+}_{oct}$ [b]	pK_a[c]
Procaine	20	1.70	4.67	1.74	−0.56	9.05
Lidocaine	10	2.00	4.96	1.83	0.49	7.91
Tetracaine	0.3	3.52	5.90	3.73	1.53	8.46
Dibucaine	0.1	4.00	7.20	4.29	2.24	8.72

a) From ref. 13. b) From ref. 9. c) From ref. 11.

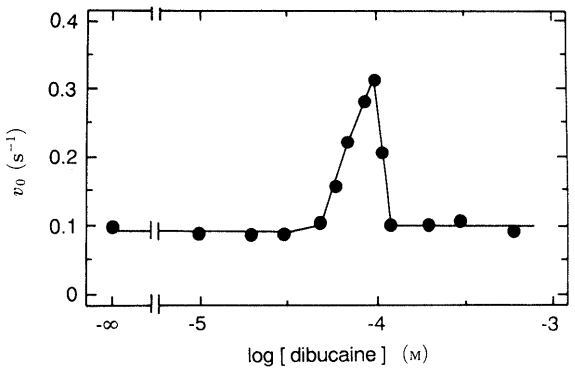

Fig. 2. Effect of Dibucaine on the v_0 Determined from the Absorbance Change of the BhCL/EyPC=2/98 Liposome Suspension

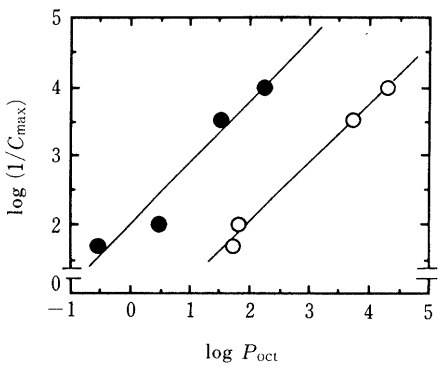

Fig. 3. Dependence of $\log(1/C_{max})$ on $\log P_{oct}$
Open circles, P^0_{oct}; closed circles, P^+_{oct}.

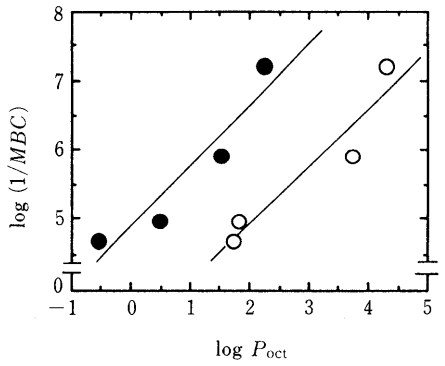

Fig. 4. Linear Relationship between $\log(1/MBC)$ and $\log P_{oct}$
Open circles, P^0_{oct}; closed circles, P^+_{oct}.

values of these local anesthetics are in a range between 7.91 and 9.05[11] so that local anesthetics were present mostly as cationic forms under the experimental conditions used. Further, as the destabilization of the negatively charged liposomal membrane is directly associated with neutralization of the surface charge of the membrane,[2] the membrane destabilizing effect of local anesthetics is believed responsible for their cationic forms. The cationic forms of the anesthetics may bind to the negatively charged polar head groups of BhCL in such a way that their cationic moieties face the membrane/water interface and their nonpolar portions are inserted into the bilayer region, as suggested by Ohki.[12] This could be one reason why C_{max} was governed by the hydrophobic nature of local anesthetics.

The anesthetic activities of these local anesthetics were reported by Agin *et al.*[13] as the minimum blocking concentration (*MBC*) of the nerve impulse. Values of the effectiveness of anesthetic potency, $\log(1/MBC)$, are summarized in Table I, and $\log(1/MBC)$ was found to be dependent on $\log P^0_{oct}$ and on $\log P^+_{oct}$, as shown in Fig. 4. These linear relationships are shown in Eqs. 5 and 6. Accordingly, $\log(1/MBC)$ was closely related to $\log(1/C_{max})$ with a very significant correlation, as shown in Eq. 7.

$$\log(1/MBC) = 3.279 + 0.830 \cdot \log P^0_{oct} \quad (5)$$
$$(\pm 0.603)\ (\pm 0.194)$$
$$(n=4,\ r=0.949,\ s=0.439)$$

$$\log(1/MBC) = 4.869 + 0.879 \cdot \log P^+_{oct} \quad (6)$$
$$(\pm 0.309)\ (\pm 0.219)$$
$$(n=4,\ r=0.943,\ s=0.465)$$

$$\log(1/MBC) = 2.981 + 0.963 \cdot \log C_{max} \quad (7)$$
$$(\pm 0.650)\ (\pm 0.219)$$
$$(n=4,\ r=0.952,\ s=0.427)$$

Values of the coefficients with $\log P^0_{oct}$ and $\log P^+_{oct}$ in Eqs. 5 and 6 are almost the same as those in Eqs. 3 and 4, and the value of the coefficient with $\log(1/C_{max})$ in Eq. 7 is close to unity; therefore, it is suggested that the anesthetic activity is based on the destabilizing effect of local anesthetics on nerve membrane. This cannot be confirmed, however, because values of C_{max} were a couple of orders higher than those of *MBC*. It is noteworthy that the susceptibility of membranes to the destabilization caused by local anesthetics is governed mainly by the negative surface charge of the membranes in the liquid crystalline phase, because the C_{max} of the local anesthetic tetracaine was greatly dependent on the negative surface charge of the BhCL/EyPC liposome membranes.[2] Thus, the activities of local anesthetics should first be discussed in terms of the negative surface charge of biomembranes and their model membranes.

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