Biochimica et Biophysica Acta, 1104 (1992) 261–268

BBAMEM 75540

The local anesthetic tetracaine destabilizes membrane structure by interaction with polar headgroups of phospholipids

Takashi Shimooka, Akira Shibata and Hiroshi Terada

Faculty of Pharmaceutical Sciences, University of Tokushima, Shomachi, Tokushima (Japan)

(Received 5 August 1991)

Key words: Local anesthetic; Tetracaine; Liposome; Membrane stability; Osmotic shrinkage; Zeta potential

The effect of the local anesthetic tetracaine at less than 10 mM on the water permeability of the phospholipid membrane was examined using liposomes composed of various molar ratios of negatively charged cardiolipin to electrically neutral phosphatidylcholine by monitoring their osmotic shrinkage in hypertonic glucose solution at 30°C. The concentration of tetracaine causing the maximum velocity of shrinkage of liposomes increased with increase in the molar ratio of cardiolipin. Tetracaine increased the ζ-potential of the negatively charged liposomal membrane toward the positive side due to the binding of its cationic form to the negatively charged polar headgroups in the membrane. The maximum velocity of water permeation induced by osmotic shock was observed at essentially the same tetracaine concentration giving a ζ-potential of the liposomal membrane of 0 mV. These concentrations were not affected by change in the sort of acyl-chain of phospholipids in the liposomes when their negative charges were the same. These results suggests that the membrane integrity is governed mainly by the electrical charge of phospholipid polar headgroups when phospholipid bilayers are in the highly fluid state, and that positively charged tetracaine molecules neutralize the negative surface charge, lowering the barrier for water permeation through phospholipid bilayers.

Introduction

Local anesthetics are known to exhibit diverse effects on various biomembranes [1–5] besides their anesthetic activities, although the mechanism(s) of the latter activities is still controversial [6–10]. Study on the interaction of local anesthetics with phospholipid membranes such as liposomes and bilayer phospholipid membranes (BLM) is important, because it provides useful information for understanding the mode(s) of action of these compounds in the phospholipid region of biomembranes. However, despite extensive studies [11–15], it is not yet fully understood which region of phospholipid bilayers interacts with local anesthetics, or what type of modification of the membrane structure is important for induction of their effects on the membranes. Studies on these problems are important not only per se, but also for understanding the factors that govern the stable structure of biomembranes.

We recently found that the local anesthetics bupivacaine and tetracaine caused a unique uncoupling of oxidative phosphorylation in mitochondria that was unlike the effect of weakly acidic uncouplers [16–18]. The uncoupling action of these anesthetics was greatly potentiated by addition of the amphipathic anion ANS [16,17]. This uncoupling action was suggested to be caused mainly by perturbation of the membrane structure in a way such that their uncoupling potencies depended on their degrees of membrane perturbation. Local anesthetics without ANS possibly interact with the membrane at the surface, or very close to the surface, whereas the nonpolar ion-pair complex between the local anesthetic cation and ANS anion moves to the inner part of the phospholipid bilayers, causing greater perturbation and hence greater uncoupling [16,17].

These results prompted us to study the action mechanism of local anesthetics on phospholipid membranes. In this study, we examined the effect of tetracaine on

Abbreviations: EyPC, egg yolk phosphatidylcholine; EyPG, egg yolk phosphatidylglycerol; BhCL, bovine heart cardiolipin; DCP, dicetyl phosphate; DMPC, dimyristoylphosphatidylcholine; LUV, large unilamellar vesicles; ANS, 1-anilino-8-naphthalene sulfonate; P_i, orthophosphate.

Correspondence: H. Terada, Faculty of Pharmaceutical Sciences, University of Tokushima Shomachi-1, Tokushima 770, Japan.

the barrier properties of phospholipid bilayers by measuring the water permeability induced by osmotic shock. We found that the tetracaine cation interacted with negatively charged phospholipid polar headgroups, and that this interaction loosened the tight arrangement of phospholipid molecules. The importance of the hydration layer at the membrane surface in stabilization of the membrane structure was suggested.

Materials and Methods

BhCL was isolated as its sodium salt from bovine heart according to the methods of Faure and Morelec-Coulon [19] and Pangborn [20]. EyPC and DMPC were purchased from Nichiyu Liposome Co. Ltd. (Tokyo), EyPG and DCP were obtained from Nippon Fine Chemicals Co. Ltd. (Osaka) and Sigma Chemicals Co. (St. Louis), respectively, and were used without further purification. They were stored as solutions in chloroform in sealed ampoules under an argon atmosphere at −20°C. Concentrations of phospholipids were determined in terms of phosphorus (P_i) according to the method of Ames [21]. Other reagents including tetracaine were commercial products.

LUV with a diameter of about 220 nm, determined with a particle sizer Nicomp 370 (Particle Sizing Systems Products, Santa Barbara), were prepared by reverse phase evaporation [22,23] in 10 mM Tris-HCl buffer (pH 7.3), and then filtrations through 0.4 μm and 0.2 μm filters (Nuclepore Co., Pleasanton) in an Amicon type-8010 ultrafiltration apparatus. Experiments on osmotic shrinkage were carried out essentially by the method of Blok et al. [24]. LUV with a P_i concentration of 0.5 mM were suspended in 10 mM Tris-HCl buffer (pH 7.3), in a total volume of 2.88 ml at 30°C. A typical example of the time course of absorbance change of liposomes is depicted in Fig. 1. On addition of a volume of 60 μl of tetracaine of a given concentration by microsyringe, the absorbance of the liposome suspension at 450 nm increased slightly. Then 60 μl of glucose solution (final concentration, 20 mM) was added rapidly under stirring, and the rapid change in absorbance of the liposome suspension with time was measured in a Shimadzu spectrophotometer, model UV-3000 at 450 nm. 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 least-squares method. A linear relationship was observed between the reciprocal of the absorbance change and the reciprocal of the concentration of glucose at least up to 50 mM, indicating that phospholipids in the liposomes were tightly arranged and the liposomes behaved as perfect osmo-meters.

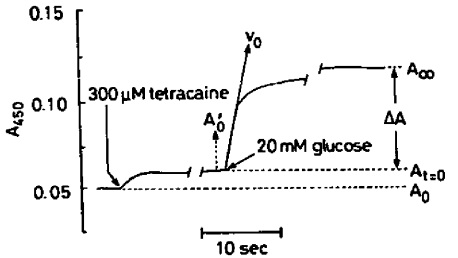

Fig. 1. Typical time-course of shrinkage of liposomes of BhCL/EyPC = 2:98 caused by addition of hypertonic glucose solution measured as absorbance increase at 450 nm. Liposomes (0.5 mM P_i) were suspended in an isotonic solution of 10 mM Tris-HCl buffer (pH 7.3) containing 300 μM tetracaine in a total volume of 2.94 ml, and incubated for 2 min at 30°C. Then glucose at a final concentration of 20 mM was added rapidly. Change in the absorbance was monitored, and values of A_0 (absorbance of the liposome suspension), A'_0 (absorbance after addition of tetracaine), $A_{t=0}$ (absorbance extrapolated to $t = 0$ just after addition of glucose), and A_∞ (absorbance at the plateau level after glucose addition) were read. v_0, initial velocity of absorbance change; ΔA, difference between A_∞ and $A_{t=0}$.

The fluidity of the liposomal membrane was measured as the fluorescence polarization of the probe perylene at 470 nm excited at 411 nm [25] in a Hitachi fluorophotometer, model 650 under similar experimental conditions to those in osmotic experiments: The suspension of 0.5 mM P_i liposomes was incubated with 500 nM perylene (molar ratio of liposomes to perylene, 1000) for 1 h at 30°C in the dark, and then the degree of fluorescence polarization P was determined by Eqn. 1.

$$P = (I_\parallel - I_\perp G)/(I_\parallel + I_\perp G) \quad (1)$$

where $I_\parallel$ and $I_\perp$ are the polarized intensities in the horizontal and vertical directions, respectively. The correction factor G for instrument polarization was determined as reported previously [26]. For examination of the effect of tetracaine on membrane fluidity, it was incubated with liposomes for 5 min before measurement of P.

The ζ-potential of liposomes was measured according to their electrophoretic mobility in a Laser-Zee, model 500 (Pen Kem Inc., New York) at 20°C.

Results

As shown in Fig. 1, addition of tetracaine caused slight increase in the optical absorbance at 450 nm (A'_0) due to slight aggregation of liposomes, and A'_0 became constant after about 2 min. This absorbance was greatly enhanced by hypertonic osmotic shock induced by addition of 20 mM glucose and the absorbance attained a certain constant level (A_∞) after less than 10 min. Increase in the absorbance (A) is associated with shrinkage and/or aggregation of liposomes. The initial velocity of liposome shrinkage (v_0)

reflects the barrier ability of the liposomes against water permeation (cf. Eqn. 3) [24,27–29], because the possible effect of liposome aggregation is unlikely in the initial stage of osmotic shock.

In this study we wanted to know the effect of tetracaine on water permeability through the liposomal membrane induced by osmotic shock as a key event for the anesthetic to exert its action in the membrane. Therefore, we used the v_0 value determined from Eqn. 2 as an index of the change in the membrane structure induced by tetracaine [24,27–29].

$$v_0 = (\mathrm{d}(1/A)/\mathrm{d}t)_{t=0}/(1/A_{t=0}) \quad (2)$$

where $A_{t=0}$ is the A'_0 extrapolated to the time of glucose injection ($t = 0$). As v_0 is proportional to the initial velocity of volume change ($\mathrm{d}V/\mathrm{d}t$) in liposomes, the following relationship holds [24,27–29]:

$$v_0 = k(\mathrm{d}V/\mathrm{d}t)_{t=0} = kP_w SRT\Delta C_{\mathrm{glucose}} \quad (3)$$

where P_w is the water permeability coefficients, S is the surface area of the membrane, R is the gas constant, T is the absolute temperature, $\Delta C_{\mathrm{glucose}}$ is the difference between the concentrations of glucose outside and inside the liposome membrane and k is a constant. Increase in v_0 is dependent on P_w and S, but S would not change greatly on binding of tetracaine under the present experimental conditions in which the concentrations of tetracaine were much lower than those generally used in studies of its effect on membrane lysis [30,31]. Thus, the initial velocity of the absorbance change can be regarded essentially to represent the velocity of water permeation.

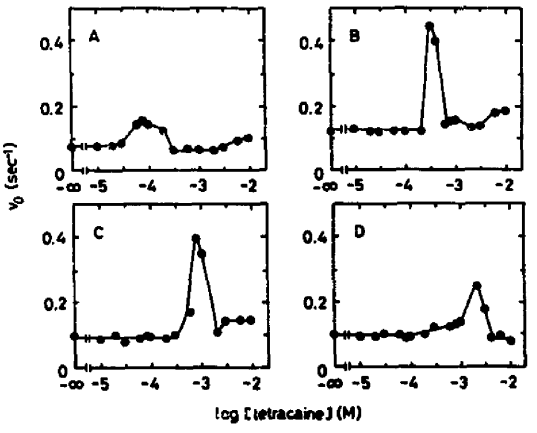

Fig. 2. Effect of tetracaine on the absorbance change of liposome suspension. The initial velocity of shrinkage, v_0, was plotted against the tetracaine concentration on a log scale. Experimental conditions were as for Fig. 1. The molar ratios of BhCL/EyPC in liposomes were: (A) 0:100; (B) 2:98; (C) 5:95; (D) 10:90. The value at $-\infty$ indicates that without tetracaine.

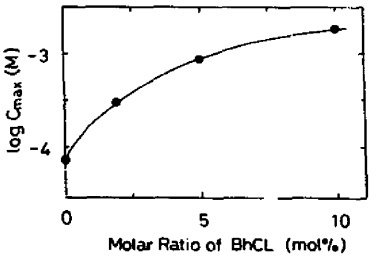

Fig. 3. Change in the tetracaine concentration required for induction of maximal water permeability, C_{max}, with the molar ratio of BhCL in BhCL/EyPC liposomes. Values were taken from the results in Fig. 2.

Fig. 2 shows the effects of tetracaine on the permeability of liposomal membranes to water, v_0, induced by osmotic shock on addition of 20 mM glucose at 30°C. In the experiments, liposomes composed of various molar ratios of negatively charged BhCL to electrically neutral EyPC (BhCL/EyPC liposomes) were used. With all liposomes carrying various negative surface charges, there was a distinct concentration of tetracaine that caused a sharp peak of maximum shrinkage of liposomes due to water efflux on hypertonic osmotic shock induced by injection of glucose into the medium. The concentration of tetracaine necessary for induction of maximal shrinkage is referred to as C_{max}. It is noteworthy that below and above C_{max}, the values of v_0 were almost the same. Thus at a certain concentration, tetracaine loosened the tight arrangement of phospholipid molecules, allowing water to penetrate rapidly through the phospholipid bilayer according to the osmotic pressure, but at higher concentrations than C_{max}, tetracaine restored the perturbed membrane structure to the level in the absence of tetracaine. The finding that A'_0 was almost the same with all the liposomes suggests that the binding of tetracaine did not affect the size of liposomes. The value of C_{max} increased with increase in the content of the negatively charged BhCL in the liposomes (Fig. 3).

As the effect of tetracaine on the water permeability of the liposomal membrane was dependent on the amount of BhCL in liposomes (Fig. 2), we examined the change in their ζ-potential with tetracaine concentration. In the absence of tetracaine, the ζ-potential became more negative with increase in the amount of BhCL (Fig. 4), and the ζ-potential changed exponentially with the molar ratio of BhCL to EyPC in the liposomes, as observed with phosphatidylserine/phosphatidylcholine liposomes [32]. This finding suggests that BhCL was distributed uniformly on the outer leaflet as well as the inner leaflet of the lipid bilayers. The reason why EyPC liposomes (BhCL/EyPC = 0:100 liposomes) showed a ζ-potential of -6.1 mV would be because the N^+ end of the headgroup is

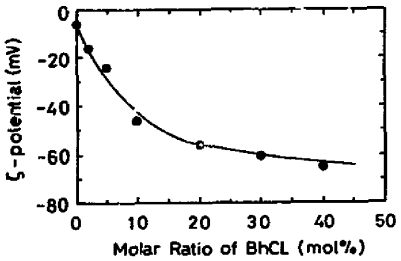

Fig. 4. Dependence of ζ-potential of liposomes on the molar ratio of BhCL in BhCL/EyPC liposomes suspended in 10 mM Tris-HCl buffer (pH 7.3).

oriented 'backwards' into the hydrocarbon environment, rather than into the water phase due to the low ionic strength of the background solution [33], i.e., 10 mM Tris-HCl buffer (pH 7.3). Thus, increase in the ionic strength in the bulk solution should diminish the electrostatic interaction in the headgroup region of EyPC. In fact, the ζ-potential of EyPC liposomes was about 0 mV in medium of higher ionic strength (50 mM KCl plus 10 mM Tris-HCl buffer (pH 7.3)) due to a charge screening effect as predicted by the Gouy-Chapman model.

Fig. 5 shows the change in the ζ-potential of the liposomal membrane with tetracaine concentration. The ζ-potential increased with increase in tetracaine concentration, and at above a certain concentration of tetracaine, the membrane surface became positively charged, indicative of an increase in the positive surface charge density of the liposomal membrane due to binding of tetracaine cation. From the results in Fig. 5 and the relationship between the ζ-potential and surface potential (Ψ_0), the amounts of tetracaine cation adsorbed on the membrane can be roughly estimated assuming that the cationic form of tetracaine is adsorbed on the surface of the membrane in a manner such that the positively charged tetracaine faces the polar-aqueous surface, and its insertion does not alter

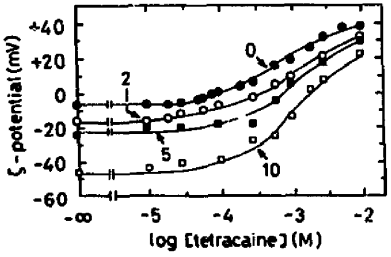

Fig. 5. Effect of tetracaine on the ζ-potential of liposomes composed of various molar ratios of BhCL/EyPC. Liposomes were suspended in 10 mM Tris-HCl buffer (pH 7.3). Figures adjacent to traces indicate the molar atios of BhCL in the BhCL/EyPC liposomes. Values at $-\infty$ are those without tetracaine.

TABLE I

Amount of binding of tetracaine cation to liposomal membranes at a concentration of tetracaine of 100 μM

	BhCL/EyPC liposomes (molar ratio)		
	2:98	5:95	10:90
ζ-potential [a] (mV)	−6.0	−17.8	−38.2
Ψ_0 [b] (mV)	−7.5	−22.6	−49.5
C_s [c] (μM)	135	247	724
$K_s C_s$ [d] $\times 10^4$ (Å^{-2})	4.1	7.4	21.7

[a] Taken from the results in Fig. 5.
[b] Surface potential of the membrane determined from the ζ-potential in Ref. 32.
[c] Concentration of tetracaine cation at the membrane/water interface calculated by Eqn. 4.
[d] Number of bound tetracaine cation molecules per unit area of membrane surface taking the surface partition K_s as 3.0 with EyPC liposomes from Ref. 34.

the surface area of the membrane. The amount of charged tetracaine bound can be estimated as the product of the surface partition coefficient (K_s) and the concentration of tetracaine cation at the membrane/water interface (C_s) [32]. C_s is expressed by Eqn. 4 as a function of the concentration of tetracaine cation in the bulk phase (C_b) and the surface potential at the membrane/water interface after binding with tetracaine (Ψ_0).

$$C_s = C_b \exp(-e\Psi_0/k_B T) \quad (4)$$

where e is the elementary electric charge, and k_B is the Boltzmann constant. In the calculation, we used a K_s value of 3.0, which was determined with EyPC liposomes [34]. Table I summarizes the amounts of bound tetracaine cation ($K_s C_s$) per Å^2 of membrane surface to liposomes with various BhCL/EyPC ratios at 100 μM tetracaine. It is evident that binding of tetracaine with BhCL/EyPC liposomes increased with increase in the molar ratio of BhCL in the liposomes due mainly to increase in the negative surface potential of the liposomal membranes.

There was a linear relationship between the concentration of tetracaine giving a ζ-potential of 0 mV, $C_{\zeta=0}$, and its concentration for maximum water permeability of the liposomes, C_{max}, as shown in Fig. 6. This linear relationship was expressed by Eqn. 5 with the very high correlation coefficient of 0.999.

$$\log C_{\zeta=0} = 0.98 \log C_{max} - 0.07 \quad (5)$$

The results that the coefficient (0.98) was close to unity and that the constant term (−0.07) was close to 0 indicate that lowering of the barrier ability caused by tetracaine is maximal at the concentration at which the local anesthetic neutralized the negative surface charge of the liposomes.

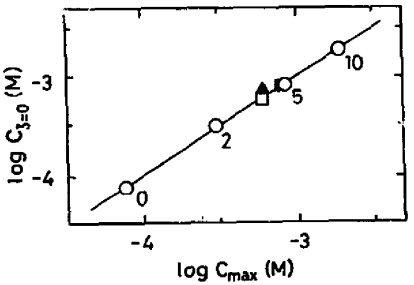

Fig. 6. Linear relationship between the concentration of tetracaine required for induction of maximal permeability to water by osmotic shock (C_{max}), and that for changing the ζ-potential to 0 mV ($C_{\zeta=0}$) with liposomes composed of various molar ratios of BhCL/EyPC. Values were taken from the results in Fig. 3 and Fig. 5. Numbers adjacent to data points are molar ratios of BhCL in the BhCL/EyPC liposomes. The open square, closed square and closed triangle are data with DCP/EyPC = 10:90, EyPG/EyPC = 10:90, and BhCL/DMPC = 5:95, respectively.

The dependencies of v_0 and the ζ-potential on the concentration of tetracaine were examined with liposomes of DCP/EyPC = 10:90 and EyPG/EyPC = 10:90. As shown in Fig. 7, the changes of v_0 with tetracaine concentration with both types of liposomes were similar to those with BhCL/EyPC liposomes (cf. Fig. 2), and the C_{max} values with DCP/EyPC = 10:90 liposomes, and EyPG/EyPC = 10:90 liposomes, were determined as 600 μM and 800 μM, respectively. These values were very similar to, and the same as, that of 800 μM with BhCL/EyPC = 5:95, which have the same apparent negative charge density as these two types of liposomes. Similarly, the values of $C_{\zeta=0}$ with DCP/EyPC = 10:90 liposomes (= 600 μM) and EyPG/EyPC = 10:90 liposomes (= 850 μM) were very similar to that with BhCL/EyPC = 5:95 liposomes (= 800 μM). Data with these liposomes are well accommodated by the relationship in Eqn. 5 (cf. Fig. 6).

Next, the fluidity of phospholipid bilayers was determined from the fluorescence polarization (P) of perylene with the BhCL/EyPC = 0:100 and 5:95 liposomes at 30°C. As summarized in Table II, the value of P with BhCL/EyPC = 0:100 liposomes was essentially the same as that with BhCL/EyPC = 5:95 liposomes, and did not change on addition of tetracaine at C_{max}.

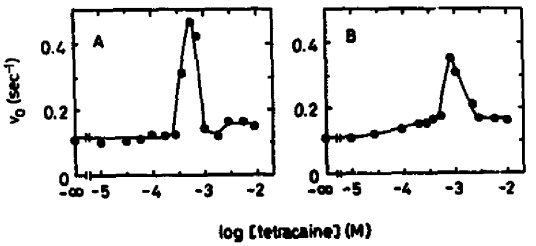

Fig. 7. Effect of tetracaine on the absorbance change of liposomes composed of DCP/EyPC = 10:90 (A) and EyPG/EyPC = 10:90 (B) at 30°C. The initial velocity of shrinkage, v_0, was plotted against the tetracaine concentration on a log scale. Experimental conditions were as for Fig. 1. The value at $-\infty$ indicates that without tetracaine.

TABLE II

Change in the fluorescence polarization of BhCL/EyPC liposomes labeled with perylene under various conditions

Additions	Fluorescence polarization [a]	
	BhCL/EyPC liposomes (molar ratio)	
	0:100 (EyPC liposomes)	5:95
A. None (liposomes alone)	0.057 ± 0.003	0.057 ± 0.001
B. A + tetracaine [b]	0.057 ± 0.002	0.056 ± 0.002

[a] Measured as the fluorescence polarization of perylene at 470 nm excited at 411 nm. Values are average for six runs (± standard deviation).

[b] Tetracaine corresponding to C_{max} was added: 80 μM with BhCL/EyPC = 0:100 liposomes, and 800 μM with BhCL/EyPC = 5:95 liposomes.

It was of interest to examine whether tetracaine affects the phase transition of phospholipid bilayers. As the phase transition temperature of EyPC is about −10°C [35], it is very difficult to study the effect of tetracaine on the phase transition using EyPC liposomes. Therefore, we used DMPC and BhCL/DMPC = 5:95 liposomes. As shown in Fig. 8A, DMPC liposomes (BhCL/DMPC = 0:100) showed distinct phase

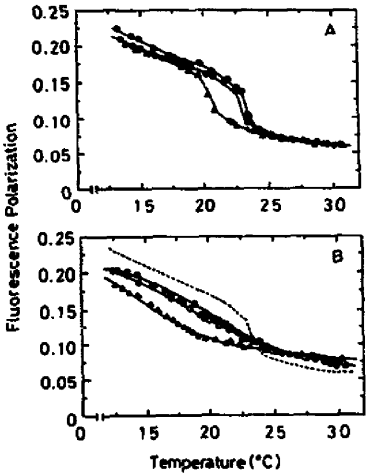

Fig. 8. Effect of tetracaine on the change with temperature of the fluorescence polarization of liposomes composed of DMPC (A) and BhCL/DMPC = 5:95 (B). (A) Closed circles, without tetracaine; open circles, with 80 μM tetracaine; closed triangles, with 1 mM tetracaine. (B) Closed circles, without tetracaine; open circles, 800 μM tetracaine, closed triangles, with 10 mM tetracaine; dotted curve, result with DMPC liposomes without tetracaine.

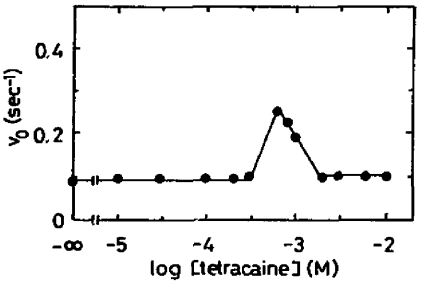

Fig. 9. Effect of tetracaine on the initial velocity of shrinkage (v_0) of liposomes composed of BhCL/DMPC = 5:95 at 30°C. Experimental conditions were as for Fig. 1. The value at $-\infty$ indicates that without tetracaine.

transition at 23.5°C, coinciding with the reported values of about 23°C [36], and they were completely in the liquid crystalline phase above 25°C. Tetracaine at 80 μM lowered the phase transition temperature slightly, and its effect was significant at 1 mM. In the case of BhCL/DMPC = 5:95 liposomes without tetracaine (Fig. 8B), change of the fluorescence polarization P with temperature was gradual, and a clear inflection point of the curve was not observed due to incorporation of the unsaturated acyl chain of BhCL. However, the curve was apparently shifted to a lower temperature than that of DMPC liposomes, showing that BhCL fluidized the bilayer structure of DMPC. As with DMPC liposomes, tetracaine at 800 μM had only a slight effect on the curve of temperature-dependent change of P, but at 10 mM it shifted the curve distinctly to lower temperature, showing that at high concentrations it fluidized the BhCL/DMPC mixed bilayers. However, at the experimental temperature for osmotic shock of 30°C, the fluidity of the acyl chains was not affected.

Fig. 9 shows the effect of tetracaine on the osmotic shock of BhCL/DMPC = 5:95 liposomes at 30°C. Distinct increase in v_0 was observed at 600 μM. This value of the C_{max} coincides well with those of other mixed liposomes carrying the same negative charge, especially with that of DCP/EyPC = 10:90 liposomes. The value of $C_{\zeta=0}$ of the BhCL/DMPC = 5:95 liposomes was determined to be 800 μM, being similar to its C_{max}. These values are shown in the plot of log $C_{\zeta=0}$ against log C_{max} in Fig. 6. Apparently, the results with BhCL/DMPC = 5:95 liposomes were well consistent with the relationship shown in Eqn. 5, indicating again that the surface charge of the liposomes is important for the barrier ability of their membrane to water.

Discussion

Injection of glucose into an isotonic suspension of liposomes results in efflux of water, due to hypertonic osmotic shock, and shrinkage of the liposomes reflected by increase in the absorbance of the liposomal suspension [37]. In this work we examined the effect of the local anesthetic tetracaine on the water permeability of liposomes composed of various molar ratios of negatively charged BhCL to neutral EyPC (BhCL/EyPC liposomes) with up to 10 mol% BhCL at 30°C, at which temperature the bilayer of the liposomal membrane was in the fluid liquid crystalline phase. With all liposomes tested, we found that tetracaine increased the rate of water permeation at a certain concentration, referred to as C_{max}, but had little effect below C_{max}, and that the enhanced permeability to water decreased to the original level above C_{max}. Thus, only a limited concentration range of tetracaine perturbed the tight membrane structure. As the value of C_{max} increased with increase in the molar ratio of BhCL, the negative polar head of phospholipids is concluded to be important for the barrier properties of the membrane against water permeation. This conclusion is consistent with reports showing the importance of the surface negative charge of liposomes and phospholipid monolayer for their stability [38–42]. The existence of the C_{max} with the neutral EyPC liposomes was due to their slightly negative surface charge. Furthermore, we found that tetracaine at higher concentrations than the C_{max} protected the phospholipid bilayer membrane against the perturbation caused by an osmotic difference.

Tetracaine was found to neutralize the negative surface charge of liposomes, and to induce a positive surface carge at concentraitons of above the C_{max}. As the value of pK_a of tetracaine is 8.46 [43], and its critical micelle concentration (cmc) is 65 mM [30], which is much higher than the concentrations of less than 10 mM used in this study, the effect of tetracaine on the liposomal membrane must be due to the cationic form of tetracaine monomer. When there is a negatively charged polar head of BhCL in the membrane, the electrostatic interaction between the cationic moiety of tetracaine and negative charge of BhCL will be dominant, leading to neutralization of the negative surface charge of the mixed BhCL/EyPC layer.

As the concentration of tetracaine that neutralized the surface charge of liposomes ($C_{\zeta=0}$) was approximately the same as C_{max}, the effect of tetracaine in decreasing the barrier ability of the mixed BhCL/EyPC bilayer membrane was concluded to be due mainly to its neutralization of the negatively charged polar headgroups of liposomes. This finding also indicates that the barrier properties of the phospholipid bilayer are governed by the surface charge of the membrane, when phospholipid bilayers are in the highly fluid state. This conclusion was supported by the findings that at above the C_{max}, tetracaine restabilized the membrane by imposing a positive charge on it (Fig. 2), that tetracaine at

about the C_{max} did not alter the motion of acyl chains of phospholipids of liposomes in the liquid crystalline phase (Table II and Fig. 8), and that liposomes with different acyl-chain lengths but carrying the same negative charge showed essentially the same values of C_{max} and $C_{\zeta=0}$ (Fig. 6).

The bilayer surface is known to form water bridges that can hold EyPC molecules together, and the charged headgroup of BhCL stabilizes networks of structured water molecules which are important for the stable arrangement of phospholipidmolecules in the membrane [42,44]. When the electrical charge of the polar headgroups of phospholipids on the membrane surface is neutralized by the tetracaine cation, the hydration layer becomes destabilized, resulting in decrease of the barrier ability of the membrane to water permeation. The interaction between acyl chains of phospholipids has usually been regarded to be responsible for the tight structure of the phospholipid membranes [24,37,45]. In fact tetracaine lowered the gel-liquid crystalline phase transition temperature of the DMPC liposomes. However, the polar headgroups of phospholipid molecules are important for the barrier properties of the membrane bilayer, and hence for stabilization of the membrane structure, when the phospholipid bilayer is in the fluid state. In fluid membranes, the effect of tetracaine on the hydrocarbon core of bilayer structure could be insignificant, because the motion of acyl chains is already so great due to thermal energy. The importance of the polar headgroups of phospholipids has been suggested in the effects of insulin [46] and lysophosphatidylcholine [47] on the stability of liposomes. Possibly tetracaine cation binds with phospholipid bilayers in a way such that its nonpolar portion is inserted into the bilayer region, and its cationic moiety faces the membrane/water interface, as suggested from the results of NMR spectroscopy [38,48]. Our results are in line with the report by Inoue et al. [28] that anesthetics such as 1-alkanols weaken the membrane structure by perturbation of the membrane/water interface.

In most studies, the effect of local anesthetics on the membrane structure has been studied under equilibrium conditions, such as their effects on phase transition [49], membrane expansion [2] and membrane stability [30,50], and hence, their effects are observed as changes in the overall membrane organization. In this study, we examined the effect of tetracaine at the initial stage of its action on liposome membranes in the fluid state. Thus, the present finding of the importance of the effect of tetracaine at the membrane/water interface could be a key event for the action of local anesthetics in various membranes, including membrane perturbation in the uncoupling of oxidative phosphorylation by local anesthetics [16,17]. The present results may be associated with the action mechanism of local anesthetics in induction of their anesthetic activities, but the C_{max} values in various liposome systems are a couple of orders higher than the effective concentration of tetracaine for its anesthetic action [1].

References

1 Skou, J.C. (1954) Acta Pharmacol. Toxicol. 10, 281–291.
2 Seeman, P. (1972) Pharmacol. Rev. 24, 583–655.
3 Feinstein, M.B., Fiekers, J. and Fraser, C. (1976) J. Pharmacol. Exp. Ther. 197, 215–228.
4 Garlid, K.D. and Nakashima, R.A. (1983) J. Biol. Chem. 258, 7974–7980.
5 Nishizawa, Y., Gusovsky, F. and Daly, J.W. (1988) Mol. Pharmacol. 34, 707–713.
6 Blaustein, M.P. and Goldman, D.E. (1966) Science 153, 429–432.
7 Lee, A.G. (1976) Nature 262, 545–548.
8 Strichartz, G. (1976) Anesthesiology 45, 421–441.
9 Hille, B. (1977) J. Gen. Physiol. 69, 497–515.
10 Kaminoh, Y., Kamaya, H. and Ueda, I. (1989) Biochim. Biophys. Acta 987 63–68.
11 Papahadjopoulos, D. (1972) Biochim. Biophys. Acta 265, 169–186.
12 Fernández, M.S. and Cerbón, J. (1976) Arch. Biochim. Biophys. 172, 721–725.
13 Davio, S.R. and Low, P.S. (1981) Biochim. Biophys. Acta 644, 157–164.
14 Gutierrez-Merino, C. and Macias, P. (1989) Biochem. Pharmacol. 38, 1407–1414.
15 Auger, M., Smith, I.C.P., Mantsch, H.H. and Wong, P.T.T. (1990) Biochemistry 29, 2008–2015.
16 Terada, H., Shima, O., Yoshida, K. and Shinohara, Y. (1990) J. Biol. Chem. 265, 7837–7842.
17 Yoshida, K., Shima, O. and Terada, H. (1990) Hiroshima J. Anesth. 26, 197–204.
18 Van Dam, K., Shinohara, Y., Unami, A., Yoshida, K. and Terada, H. (1990) FEBS Lett. 277, 131–133.
19 Faure, M. and Morelec-Coulon, M.J. (1958) Ann. Inst. Pasteur 95, 180–186.
20 Pangborn, M.C. (1942) J. Biol. Chem. 143, 247–256.
21 Ames, B.N. (1966) Methods Enzymol. 8, 115–118.
22 Szoka, F., Jr. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 9, 4194–4198.
23 Szoka, F., Olson, F., Heath, T., Vail, W., Meyhew, E. and Papahadjopoulos, D. (1980) Biochim. Biophys. Acta 601, 559–571.
24 Blok, M.C., Van Deenen, L.L.M. and De Gier, J. (1976) Biochim. Biophys. Acta 433, 1–12.
25 Shinitzky, M. and Barenholz, Y. (1974) J. Biol. Chem. 249, 2652–2657.
26 Zolese, G., Ambrosini, A., Bertoli, E., Curatola, G. and Tanfani, F. (1990) Chem. Phys. Lipids 56, 101–108.
27 Inoue, T., Kamaya, H. and Ueda, I. (1985) Biochim. Biophys. Acta 812, 393–401.
28 Inoue, T., Kamaya, H. and Ueda, I. (1985) Biochim. Biophys. Acta 815, 68–74.
29 Kedem, O. and Katchalsky, A. (1958) Biochim. Biophys. Acta 27, 229–246.
30 Fernández, M.S. (1980) Biochim. Biophys. Acta 597, 83–91.
31 Fernández, M.S. (1981) Biochim. Biophys. Acta 646, 27–30.
32 Ermakov, Y.A. (1990) Biochim. Biophys. Acta 1023, 91–97.
33 Dill, K.A. and Stigter, D. (1988) Biochemistry 27, 3446–3453.
34 Ohki, S. (1984) Biochim. Biophys. Acta 777, 56–66.
35 Helmkamp, G.M., Jr. (1983) in Membrane Fluidity in Biology (Aloia, R.C., ed.), Vol. 2, pp. 151–186, Academic Press, New York.

36 Van Dijck, P.W.M., De Kruijff, B., Van Deenen, L.L.M., De Gier, J. and Demel, R.A. (1976) Biochim. Biophys. Acta 455, 576–587.
37 Bittman, R. and Blau, L. (1972) Biochemistry 11, 4831–4839.
38 Boulanger, Y., Shirley, S. and Smith, I.C.P. (1981) Biochemistry 20, 6824–6830.
39 Brownig, J.L. (1983) Biochemistry 20, 7123–7133.
40 Borle, F. and Seelig, J. (1983) Biochemistry 22, 5536–5544.
41 Beyer, K. (1986) Biochim. Biophys. Acta 855, 365–374.
42 Cevc, G. (1987) Biochemistry 26, 6305–6310.
43 Kamaya, H., Hayers, J.J., Jr. and Ueda, I. (1983) Anesth. Analg. 62, 1025–1030.
44 Parsegian, V.A. and Rau, D.C. (1984) J. Cell. Biol. 99, 196–200.
45 Blok, M.C., Van Deenen, L.L.M. and De Gier, J. (1977) Biochim. Biophys. Acta 464, 509–518.
46 Senisterra, G.A., Gagliardino, J.J. and Disalvo, E.A. (1991) Biochim. Biophys. Acta 1064, 148–154.
47 Senisterra, G.A., Disalvo, E.A. and Gagliardino, J.J. (1988) Biochim. Biophys. Acta 941, 264–270.
48 Kelusky, E.C. and Smith, I.C.P. (1984) Can. J. Biochem. Cell. Biol. 62, 178–184.
49 Papahadjopoulos, D., Jacobson, K., Poste, G. and Shepherd, G. (1975) Biochim. Biophys. Acta 394, 504–519.
50 Ohki, S. (1970) Biochim. Biophys. Acta 219, 18–27.