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The Penetration Site of Local Anesthetics into Liposomal Membrane

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Abstract □ The distribution of local anesthetics between the hydrocarbon interior and surface area of the lipid bilayer of liposomal membrane was calculated employieg fluorescence probe technique. The quenching of fluorescence of 12-(9-anthroyl) stearic acid and N-octadecyl naphthyl-2-amine-6-sulfonic acid by the local anesthetics in liposomal system was used to calculate the distribution. The Stern-Volmer equation was modified and employed for this calculation. The results showed that procaine hydrochloride and benzocaine were mainly distributed on the surface area of the lipid bilayer of the liposoal membrane, while tetracaine hydrochloride penetrated effectively into the hydrocarbon interior and showed even distribution in the lipid bilayer.

Keywords □ Local anesthetics, Penetration site, Liposomal membrane, Fluorescence probe, Stern-Volmer equation.

Local anestheticas are drugs that block nerve conduction when applied locally to nerve tissue. Compounds which reversibly block the formation of an action potential, but have little effect on the resting potential of the target cell, induce anesthesia through blockade of the sodium channel.[1~4] A wide variety of chemically diverse compounds can act as local anesthetics, including neutral and both negatively and positively charged molecules.[5] This suggests that anesthesia is induced not through a specific reaction of these compounds with the target protein but because they initiate a common structural perturbation which is sensed by the target protein. A common feature in their chemical structure is that most of the useful local anesthetics contain both a hydrophilic and a hydrophobic moiety in their molecule and hence are surface active in nature.[6]

The molecular mechanism by which local anesthetics reversibly block nerve conduction is still not satisfactorily understood. Cell membranes are accepted as the site of local anesthetic action.[2] Considerable evidences also support the idea that local anesthetics partition into the membrane by interacting with the polar head group located at the polar-apolar interface of the lipid bilayer. The hydrophobic tail group appears to be situated in the hydrocarbon interior.[7,8,9] Since a wide variety of different compouds can act as local anesthetics, it is not clear whether theree is a single mechanism for local anesthesia, or whether each class of anesthetics act in a different way. Staiman and Seeman,[10] however, have shown that effects of anesthetic mixtures are close to being additive, suggesting a single mode of action. Among various local anesthetic comounds, clinically used agents are tertiary amine compounds. At physiological pH, tertiary amine local anestethics exist in both positively charged and neutral forms in proportion given by the Henderson-Hasselbach equation. The ability of certain local anesthetics to compete with calcium ion and interact with acidic phosphatidylcholine liposomes suggests that an elect-

rostatic interaction at the membrane surface is involved.[6] However, the findings that tertiary amine local anesthetics obey the Meyer-Overton rule of anesthesia and that the activity of these drugs parallels their octanol/water partition coefficient are highly indicative of hydrophobic interactions.[12] Skou's experiment[13] that the relative anesthetic potency exactly parallels their effectiveness in increasing the surface pressure of monolayer films of lipids reinforces the possibility of the hydrophobic interactions.

Despite the numerous studies concerning the interaction of local anesthetics with model membranes, little attention has been given to the effectiveness of these drugs in penetrating the lipid bilayer of biomembranes. Koblin *et al.*[17] investigated the degree of penetration of some local anesthetics into the lipid bilayer of ghost red-cell membrane, employing the fluorescent probe technique. In order to monitor the penetration site of the local anesthetics into the lipid bilayer, they employed two fluorescent probes, 12-(9-anthroyl) stearic acid and N-octadecyl naphthyl-2-amine-6-sulfonic acid. The analysis of preferential quenching of these probes by the local anesthetics enabled them to suggest qualitatively the relative accessibility of these drugs to the hydrocarbon region of the lipid bilayer. In this research, the same fluorescent probes were employed. However, the Stern-Volmer equation of fluorescent quenching was modified for the membrane system and employed to calculate the concentration gradient of local anesthetics between membrane's surface and interior area of hydrocarbon of the lipid bilayer of dipalmitoyl phosphatidylcholine liposome.

EXPERIMENTAL METHODS

Materials

Dipalmtoyl phosphatidylcholine (DPPC) and the fluorescent probe, 12-(9-anthroyl) stearic acid (AS) were purchased from Sigma Chemical Company, St. Louis, MO., USA, and the other fluorescent probe, N-octadecyl naphthyl-2-amine-6-sulfonic acid (ONS) was generously donated by Dr. H.H. Wang, University of California, Santa Cruz, Calif., USA. These were used without further purification. The local anesthetics, procaine hydrochloride, tetracaine hydrochloride, lidocaine hydrochloride, dibucaine hydrochloride and benzocaine were obtained from Shinjin Pharmaceutical Company, Seoul, Korea. All other reagents employed were of reagent grade. Water was double-distilled.

Preparation of Liposome

Thirty milligrams of DPPC was dissolved in 20ml of chloroform. The chloroform was removed under nitrogen and the lipid film was deposited on the wall of a conical flask by rotatory evaporation. Complete solvent removal was ensured by drying the thin film in a vacuum dessicator for several hours. Removal of the lipid film from the wall of the conical flask was accomplished by vigorous stirring with an appropriate amount of 0.01M Tris buffer (pH 7.4) with a magnetic stirrer. The dispersion was then sonicated for 30 minutes above 45°C with 20 Khz Lab-Line Sonicator, Model No. 9,100 at 50% full power. The dispersion was then centrifuged at 15,000rpm for 30 minutes. The supernatant was employed as unilamellar liposome.

Procedure

Stock solutions of AS in ethyl alcohol (10^{-3}M) and ONS in dimethyl sulfoxide (10^{-3}M) were made and kept in a cold dark place. To the unilamellar liposome, 0.01M of AS or 0.01M of ONS was added and stirred for 2 hours. During this procedure the hydrophobic fluorescent probe was incorporated into the lipid bilayer of the

liposome. Concentrated solutions of the local anesthetics were prepared in 0.01M Tris buffer (pH 7.4) and added to the labelled liposome to give the desired concentration of the anesthetic. In case of water-insolule benzocaine, a concentrated solution in alcohol was employed for this purpose. The fluorescence intensity of the liposome was measured on Spectrofluorometer, Shimdzu RF 510 with 386 nm excitation and 440 nm emission for AS and 360 nm excitation and 420 nm emission for ONS, respectively. Band width was 5 nm for excitation and 10nm for emission. Solution-quenching experiments were carried out in 1 : 1 mixture of dimethyl sulfoxide and ethyl alcohol. The temperature of the sample was kept at 25±0.1°C with Thermobath Circulator, Shimadzu TB 85 during measurement. All experiments were repeated at least three times and the values are the average of these experiments. The relative values did not differ by more than±5%.

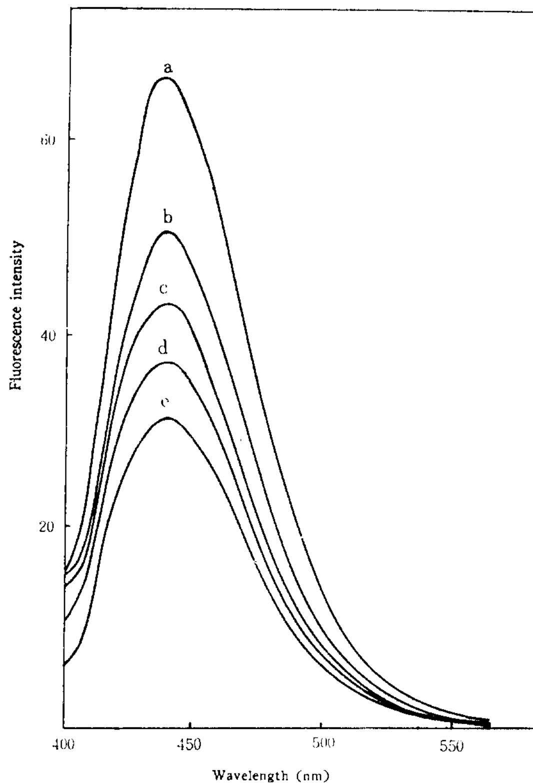

Fig. 2. Typical fluorescence spectra of 12-AS in the presence of various concentrations of tetracaine. HCl in DPPC liposome from 400nm to 600nm at the excitation wavelength 386nm. a: none, b: 0.4mM, c: 0.8mM, d: 1.2mM, e: 1.6mM

RESULTS AND DISCUSSION

The fluorescence spectra of AS and ONS in the presence of various concentrations of tetracaine hydrochloride in the liposomal system were shown in Fig 2 and Fig. 3, respectively. Tetracaine hydrochloride, benzocaine and procaine hydrochloride effectively reduced the fluorescence intensities. However, lidocaine hydrochloride and dibucaine hydrochloride did not quench the fluorescences as expected. It was ascertained that p-aminobenzene moiety is essential for quenching the fluorescence of these probes.

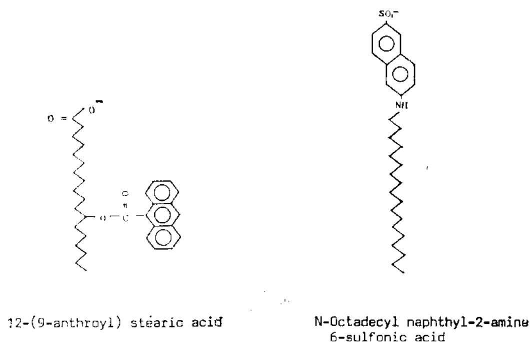

Fig. 1. Chemical structure of fluorescent probes.

Fluorescence quenching in homogeneous solution has been described by the Stern-Volmer equation:

$$F_0/F=1+k_q\mathcal{T}_0[Q]=1+K[Q] \cdots\cdots\cdots\cdots (1)$$

where F_0 aud F are the fluorescence intensities in the absence and presence of the quencher, Q, respectively. $\mathcal{T}_0$ is the fluorescence lifetime

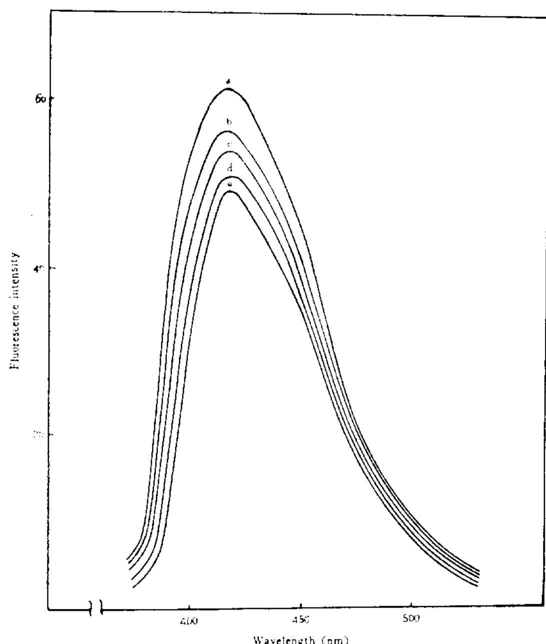

Fig. 3. Typical fluorescence spectra of ONS in the presence of various concentrations of tetracaine. HCl in DPPC liposome from 360nm to 600nm at the excitation wavelength 360nm. a: none, b: 0.4mM, c: 0.8mM, d: 1.2mM, e: 1.6mM

of the chromophore, k_q is the quenching rate constant, and $K=k_q \mathcal{T}_0$ is the Stern-Volmer constant. A plot of F_0/F vs $[Q]$ gives a line of slope K. The Stern-Volmer equation was modified for uneven distribution of the quencher in liposomal membrane system as following. Local anesthetics partition between aqueous phase and the lipid bilayer in the liposomal system. In case of water-soluble anesthetics, the concentration in the aqueous phase is excessively larger than that in the lipid bilayer at pH 7.4. Consequently, $[Q]_L \cong P[Q]_T$ and equation (1) can be modified:

$$F_0/F=1+fKP[Q]_T \quad \cdots\cdots (2)$$

where $[Q]_L$ and $[Q]_T$ are the concentration of the quencher in the lipd bilayer and the total concentration of the quencher in the system, respectively. P is the partition coefficient and f is a regional correction factor for uneven distribution of the quencher between the surface area and the interior area in the liposomal membrane. When AS or ONS is employed as a fluorescence probe, it has been known that the fluorophore of *AS* is incorporated in the interior area of the lipid bilayer and that of *ONS* on the surface area. Consequently, *AS* or *ONS* quenching in liposomal membrane system gives the following relations, respectively:

$$F_0/F=1+f_iK_{AS}P[Q]_T \quad \cdots\cdots (3)$$

$$F_0/F=1+f_sK_{ONS}\, P[Q]_T \quad \cdots\cdots (4)$$

where K_{AS} and K_{ONS} are the Stern-Volmer constants of the fluorescence quenching of *AS* and *ONS* by the quencher, respectively. f_i/f_s is the ratio of the regional correction factors in the interior area and the surface area of the lipid bilayer of the liposomal membrane, and becomes the concentration gradient of the quencher between these two areas. The plots F_0/F vs. $[Q]_T$ of equation (3) and (4) are shown in Fig. 4

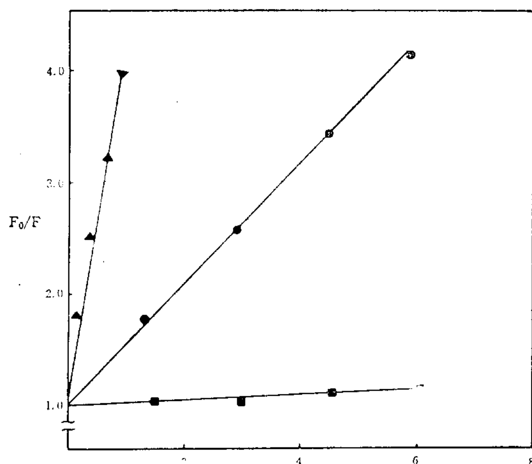

Fig. 4. Stern-Volmer plot of quenching of 12-AS fluorescence in DPPC liposome by tetracaine· HCl (▲), benzocaine (●) and procaine. HCl (■).

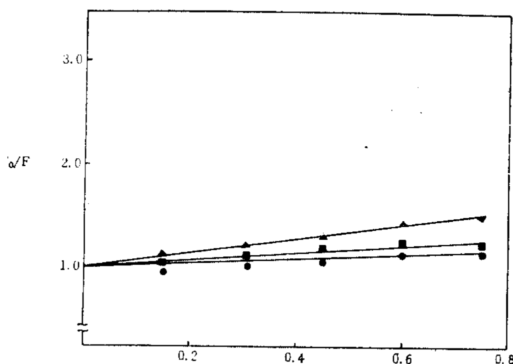

Fig. 5. Stern-Volmer plot of quenching of ONS fluorescence in DPPC liposome by tetracaine·HCl (▲), benzocaine (●) and procaine·HCl (■).

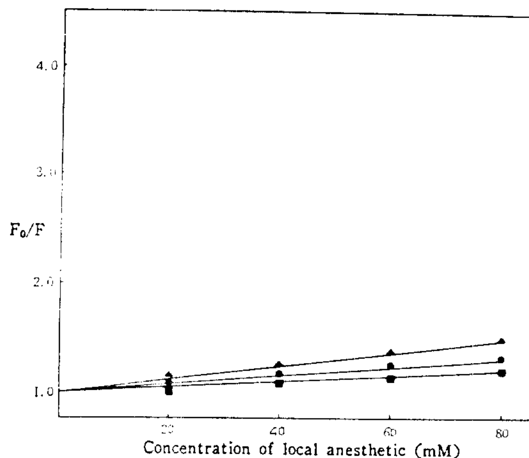

Fig. 7. Stern-Volmer plot of quenching of ONS fluorescence in DMSO and ethyl alcohol mixture (1:1) by tetracaine·HCl(▲), benzocaine (●) and procaine·HCl(■).

and Fig. 5. The ratio of the slopes of the plots give the following relation:

$$f_i/f_s = \frac{S_i\ K_{ONS}}{S_s\ K_{AS}} \quad \cdots\cdots(5)$$

where S_i and S_s are the slope of the plots of equation (3) and (4), respectively. When the quencher is a water-insoluble local anesthetics, $[Q]_L \cong [Q]_T$, and with only slight modification, equation (5) could be easily arrived. Quenching of *AS* and *ONS* fluorescence in 1 : 1 mixture of

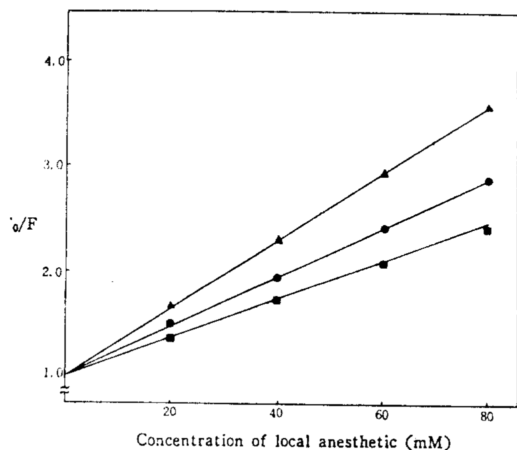

Fig. 6. Stern-Volmer plot of quenching of 12-AS fluorescence in DMSO and ethyl alcohol mixture (1 : 1) by tetracaine·HCl (▲), benzocaine (●) and procaine·HCl (■).

dimethyl sulfoxide and ethyl alcohol by local anesthetics are shown in Fig. 6 and Fig. 7. The slopes of these plots give the K_{AS} and K_{ONS} values. The Stern-Volmer constants of *AS* and *ONS* fluorescence quenching by local anesthetics obtained from the plots are listed in Table I. Assuming K_{AS} and K_{ONS} values in bulk solution are not much different from the values in liposomal membrane system, these values can be substitued into the equation (5) for the calculation. The result of the calculation of f_s/f_i, the ratio of the concentration of local anesthe-

Table I. Stern-Volmer Constant of Quenching of AS and ONS Fluorescence by Local Anesthetics in DMSO and Ethanol Mixture (1 : 1)

Local Anesthetics	Stern-Volmer Constant (M^{-1})	
	AS	ONS
Tetracaine·HCl	31.8	5.8
Benzocaine	23.3	1.5
Procaine·HCl	18.3	2.1

Table II. The Ratio of the Concentration of Local Anesthetics in Surface Area to the Concentration in the Hydrocarbon Interior of the Lipid Bilayer of DPPC Liposome.

Local Anesthetics	Ratio
Tetracaine·HCl	0.77
Benzocaine	11.9
Procaine·HCl	116.2

tics in the surface to the concentration in the interior area is listed in Table II. This result indicates that procaine hydrochloride is exclusively distributed on the surface area of the lipid bilayer of the liposomal membrane, while tetracaine hydrochloride has a moderate ability to penetrate into the hydrophobic lipid bilayer. The distribution of tetracaine hydrochloride is nearly even. The enhanced ability of tetracaine hydrochloride to penetrate into the interior should be ascribed to the increased hydrophobicity due to the N-substituted n-butyl moiety. However, in spite of the high hydrophobicity of benzocaine it could not effectively penetrate into the interior, and distributed mainly on the surface. This suggests that the hydrophobicity of the aromatic ring in the local anesthetics' moiety might be an important factor in determining the accessibility of the drug to the interior area of the lipid bilayer. Local anesthetics have been known to reduce the phase transition temperature of biomembrane and to increase the bilayer fluidity, and this ability is closely related to their activities. It could be deduced from this result that the more effective penetration into the bilayer could result in higher perturbation of bilayer fluidity and possibly higher anesthetic activity. In the design of more potent local anesthetics the introduction of more hydrophobic radical to the aromatic ring might be desirable.

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