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Effects of Local Anesthetics on the Fluidity of Synaptosomal Plasma Membrane Vesicles Isolated from Bovine Brain

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ABSTRACT

To elucidate the mechanism of action of local anesthetics, the effects of local anethetics on the microenvironment of the lipid bilayers of synaptosomal plasma membrane vesicles (SPMV) isolated from bovine brain and dimyristoylphosphatidylcholine (DMPC) multilamellar liposomes were investigated employing the intermolecular excimer fluorescence technique and differential scanning calorimetry (DSC). The relative intensities of excimer and monomer fluorescence of pyrene are a simple linear function of the viscosity of a homologous series of solvents. The microviscosity(η)of the hydrocarbon region of SPMV was measured by this method and the value was 57.3 ± 5.3 cP at 37℃. In the presence of lidocaine-HCl and procaine-HCl, the values decreased to 46.5 ± 5.1 cP and 54.7 ± 4.8 cP, respectvely. The differential scanning thermograms of DMPC multilamellar liposomes showed that local anesthetics significantly lowered the phase transition temperature, broadened the thermogram peaks, and reduced the size of the cooperative unit. These results indicate that local anesthetics have significant fluidizing effects on biomembranes and perturbation of membrane lipids may produce some, but not all, of their pharmacological actions.

Key Words: Local anesthetics, Membrane fluidity, Pyrene excimer fluorescence, Differential scanning calorimetry

INTRODUCTION

Local anesthetics are drugs that reversibly block the generation and the conduction of the nerve impulse when applied locally to nerve tissue in appropriate concentrations. Their main site of action is the cell membrane, and they block conduction by decreasing or preventing the large transient increase in the permeability of the membrane to sodium ions that is produced by a slight depolarization of the membrane (Strichartz, 1981).

Theories of the molecular mechanism of the block of sodium conductance have not yet been satisfactorily established. There are several proposals for the action of local anesthetics. One and the most popular proposal is the specific receptor theory which states that local anesthetics bind to a specific receptor site within the sodium channel, hence physically block it (Ritchie, 1975; Hille, 1980). The second proposal is that local anesthetics induce the protein perturbation in the biomembrane, in which interaction between local anesthetics and the sodium channel causes conformational changes resulting in a block of conductance (Boggs *et al.*, 1976). The third proposal is the membrane expansion theory which proposes that anesthetics interact with the lipid component of the membrane to cause an expansion of the membrane and thus block the sodium channel (Seeman, 1972; Singer, 1977). The fourth proposal is the annular transition model in which the sodium channel is postulated to be surrounded by an

annulus of lipid in the gel state (Lee, 1976). Local anesthetics, which reduce the temperature of lipid phase transitions, trigger a change of the annular lipid from the gel to the liquid-crystalline state, with a consequent relaxation of the sodium channel to an inactive configuration, in which the sodium current is reduced or blocked.

Among the above proposals, we have been interested in the membrane expansion theory or the annular transition model which can explain the mechanism of action of a wide variety of different compounds acting as local anesthetics, including neutral and both positively and negatively charged molecules. In the present study, tempting to further understand the molecular mechanism of action of local anesthetics, we investigated the effects of local anesthetics on the miroviscosity of the hydrocarbon region of synaptosomal plasma membrane vesicles (SPMV) isolated from fresh bovine cerebral cortex by pyrene excimer fluorescence, and on thermotropic phase transitions of dimyristoylphosphatidylcholine (DMPC) multilamellar liposomes by differential scanning calorimetry (DSC).

MATERIALS AND METHODS

Materials

DMPC, pyrene, cyclohexane, 2-propanol, 1-octanol, 1-dodecanol, 1,2-propanediol, and 1,3-butanediol were purchased from Sigma Chemical Co.(St. Louis, MO). Dibucaine·HCl, tetracaine·HCl, lidocaine·HCl, and procaine·HCl were obtained from Shinjun Pharmaceutical Co. (Seoul, Korea). All other reagents were of the highest quality available. Water was double-distilled.

Preparation of synaptosomal plasma membrane vesicles

Synaptosomes were isolated by Hajös (1975) and a preparation of membrane vesicles was driven from Kanner (1978). All steps, subsequent to decapitation and removal of the cortex, were carried out at 0-4℃ as follows. Fresh bovine cerebral cortex was homogenized in 0.3 M sucrose at 7-10 %(w/v) with an all-glass Thomas tissue grinder (six up and down strokes), and centrifuged at 1,500×g for 10 min in a Sorvall SS-34 rotor. The supernatant was recentrifuged at 9,000×g for 20 min, and the pellet was dispersed in 5 mL 0.3 M sucrose. The suspension was layered over 20 mL 0.8 M sucrose, and centrifuged at 9,000×g for 25 min. The particles dispersed in 0.8 M sucrose were saved, and diluted with water. After centrifugation, 20 min at 27,000×g, the pellet (synaptosomal fraction) was lysed by resuspension in a minimal volume of 5 mM Tris-HCl/1 mM K-EDTA (pH 7.4), followed by homogenization (3 strokes). After dilution in the same low osmolarity buffer, lysis was completed by stirring for 45 min. Finally the lysate was centrifuged again at 27,000×g for 20 min, and the pellet was resuspended in 0.32 M sucrose, containing 5 mM Tris-SO_4, 1 mM $MgSO_4$, 0.5 mM EDTA (pH 7.4), at a protein concentration of 5-10 mg/mL. For the fluorescence measurements, the final pellet was resuspended in 0.1 M KCl/10 mM Tris-HCl (pH 7.4). Each membrane suspension was either used immediately or frozen in liquid nitrogen and stored at -70℃ for no longer than a month. Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Electron microscopic examination showed that the membranes were in vesicular form. The purity of SPMV was also confirmed by monitoring the specific activities of Na, K-ATPase (Adam-Vizi *et al.*, 1984) and acetylcholinesterase (Ellman *et al.*, 1961).

Fluorescence measurements

The SPMV were suspendend in 0.1 M KCl/10 mM Tris-HCl (pH 7.4) to a concentration of 1 mg protein/mL. The incorporation and distribution of pyrene into SPMV were accomplished by adding aliquots of methanolic solution of the probe (2.5×10^{-3}M) to the membrane and by gentle stirring for two hours below 4℃. These were made possible due to the fact that the fluorescence intensity of pyrene reached a steady state approximately two hours afterward. Concentrated solutions of the local anesthetics were prepared in 0.1 M KCl/10 mM Tris-HCl (pH 7.4), and added at desired concentrations to SPMV previously labeled with pyrene. The fluorescence spectra were taken on either JASCO spectrofluorometer or Shimadzu RF 510 spectrofluorometer, equipped with a thermostated cell holder. Blanks, always prepared under identical conditions, served as controls for the fluorometric measurement. Before the fluorescence spectra were obtained, all samples were degassed by bubbling dry nitrogen through the solution for at least 30 min. The excitation wavelength was 337 nm (10 nm excitation and 10 nm emission slits). The excimer to monomer fluorescence intensity

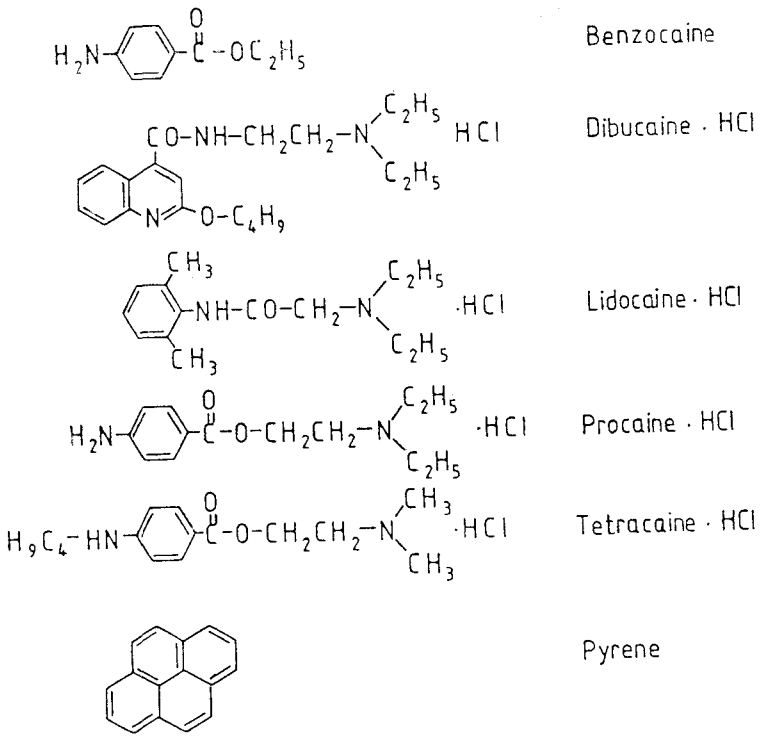

Fig. 1. Chemical structure of local anesthetics and the fluorescent probe.

ratio was calculated from the 460 nm to 394 nm signal ratio. All measurements were performed at 37±0.1℃.

Viscosity determinations

Viscosities of reference solvents were determined with Schott Geräte AVS 400 viscometer (Ubbelhode type) in a thermostated bath at 37℃ and densities with Westphal balance at 37℃.

Differential scanning calorimetry

The chloroform solution containing DMPC was transferred to a glass tube, which had been flushed with nitrogen. The chloroform was then evaporated under vacuum. To obtain multilamellar liposomes, the dried lipids were suspended in 0.1 M KCl/10 mM Tris/0.1 mM EDTA buffer (pH 6.9) at a temperature 5℃ higher than the phase transition temperature of the lipid with a vortexer for 10 min. The calorimetric measurements were performed with a Shimadzu differential scanning calorimeter, SC-30. Liposomal dispersions were incubated with desired concentrations of local anesthetics for 1 hr above the phase transition temperature, and then 15 μL of the dispersion was transferred to a volatile sample pan. The reference pan contained an equivalent volume of buffer. For each sample, several scans were performed between 20 and 40℃ with heating rates of 2℃/min in the sensitivity range of 5 mJ/sec. Transition enthalpy was determined from the ratio of the area under the endothermic curve of the sample to that of stearic acid as calibrant(Tm=69.5℃, ΔH=16.4 Kcal/mole).

INTERPRETATION OF FLUORESCENCE DATA

Pyrene forms excimers in a diffusion-controlled reaction, and can undergo the processes given below (Pownall *et al.*, 1973):

	Rate	Process
$P+h\nu \longrightarrow P^*$	I_a	Absorption, where I_a is

		the intensity of absorbed light
$P^* \longrightarrow P + h\nu'$	$k_f P^*$	Monomer fluorescence
$P^* \longrightarrow P$	$k_{rl} P^*$	Monomer radiationless transition
$P^* + P \longrightarrow P_2^*$	$k_a(P)(P^*)$	Excimer formation
$P_2^* \longrightarrow P^* + P$	$k_d(P_2^*)$	Excimer decomposition
$P_2^* \longrightarrow P_2 + h\nu''$	$k'_f(P_2^*)$	Excimer fluorescence
$P_2^* \longrightarrow P_2$	$k'_{rl}(P_2^*)$	Excimer radiationless transition
$P_2 \longrightarrow 2P$	$k'_d(P_2)$	Dimer decomposition

According to this reaction scheme the concentration dependencies of the fluorescence quantum efficienceis φ_M and φ_E for monomer and excimer fluorescence, respectively, have been shown to be

$$\varphi_M = \varphi_M^{max} (1 + C/C_h)^{-1} \quad (1)$$

$$\varphi_E = \varphi_E^{max} (1 + C_h/C)^{-1} \quad (2)$$

Where C_h respresent the concentration at which half the fluorescence intensity appears in both the monomer and excimer fluorescence spectra, and C is the concentration at which the quantum efficies φ_M and φ_E are observed; φ_M^{max} and φ_E^{max} represent the maximum intrinsic fluorescence of each specie. The rato of the quantum yield is

$$\frac{\varphi_E}{\varphi_M} = \frac{\varphi_E^{max}}{\varphi_M^{max}} \frac{C}{C_h} \quad (3)$$

Since the relative quantum yields are proportional to the relative fluorescence intensities of each specie, equivalent expressions utilizing the fluorescence intensity of each component can also be developed in which I_M^{max} and I_E^{max} represent the maximum intrinsic fluorescence intensity.

$$I_M = I_M^{max} (1 + C/C_h)^{-1} \quad (4)$$

$$I_E = I_E^{max} (1 + C_h/C)^{-1} \quad (5)$$

The ratio of the fluorescence intensities is

$$\frac{I_E}{I_M} = \frac{I_E^{max}}{I_M^{max}} \frac{C}{C_h} = \frac{C}{C'_h} \quad (6)$$

where $C'_h = (I_M^{max}/I_E^{max})\, C_h$. Provided the rate of excimer dissociation is negligible ($k_d \ll k'_f + k'_{rl}$), eq 7 and 8 are valid so that

$$\varphi_M^{max} = k_f (k_{rl} + k_f)^{-1} \quad (7)$$

$$\varphi_E^{max} = k'_f (k'_f + k'_{rl})^{-1} \quad (8)$$

by appropriate substitution, in eq 3 and 6, the following relationships are obtained.

$$C_h = (k_f + k_{rl})\, k_a^{-1} \quad (9)$$

$$C'_h = (k_f + k_{rl})\, k_a^{-1} (I_M^{max}/I_E^{max}) \quad (10)$$

In this expression the half-intensity concentration, C'_h, is a function of the rate of excimer formation, k_a, which has been shown to be diffusion controlled.

The Einstein-Schmoluchowski disffusion theory relates the rate of diffusion to the viscosity of the medium by the expression

$$k_a = 8\,(RT/3000\,\eta) \quad (11)$$

The relationship of half-intensity concentration, C'_h to the viscosity is apparent in the following equation.

$$C'_h = (k_f + k_{rl})\,(3000/8RT)\,(I_M^{max}/I_E^{max}) \quad (12)$$

This equation shows that the C'_h value can be utilized as an index of viscosity.

RESULTS

Viscosity of pure solvents

The total fluorescence spectrum of pyrene in

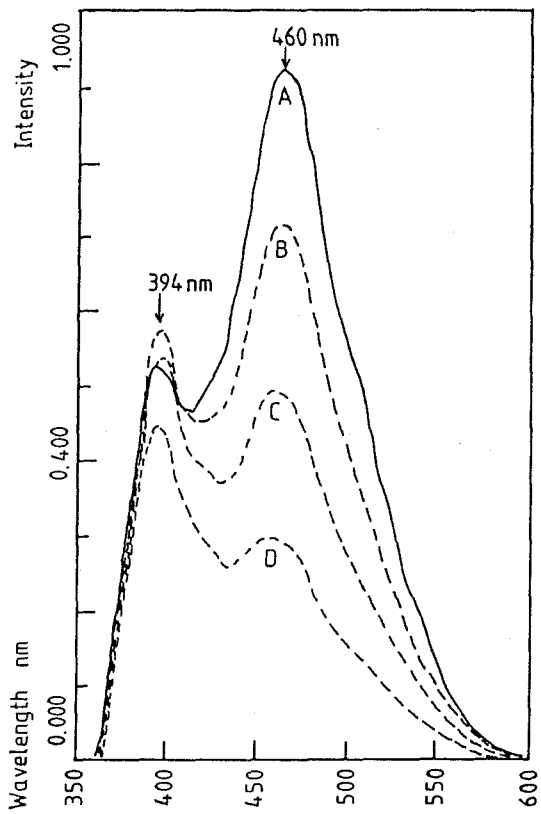

Fig. 2. Typical fluorescence spectra of pyrene in cyclohexane at 37°C are shown as a function of concentration. Pyrene concentrations are : (A) 0.8 mM ; (B) 0.6 mM ; (C) 0.4 mM ; (D) 0.3 mM.

cyclohexane at various concentrations is shown in Fig. 2. This spectrum is representative of those obtained in other solvents although the concentration range of pyrene in each solvent was different. A plot of I_E/I_M vs. pyrene concentration in cyclohexane at 37℃ is shown in Fig. 3. The good linearity obtained with all solvents. The C_h' value is obtained from the reciprocal of the slope of these plots. In Table 1, the C_h' values for a variety of solvents at 37℃ are compiled with the corresponding kinematic viscosities. As shown in Fig. 4, a linear relationship existed between these C_h' values and solvent viscosity. The reciprocal of the slope of this line, 1265.8, is equal to the quantity $1/[(k_f+k_{r1})\,I_M^{max}/I_E^{max}](3000/8RT)$. From this calibration curve, the viscosity of a solvent containing the pyrene probe can be determined by measurement at C_h' and evaluation with the expression $\eta = 1265.8C_h'$.

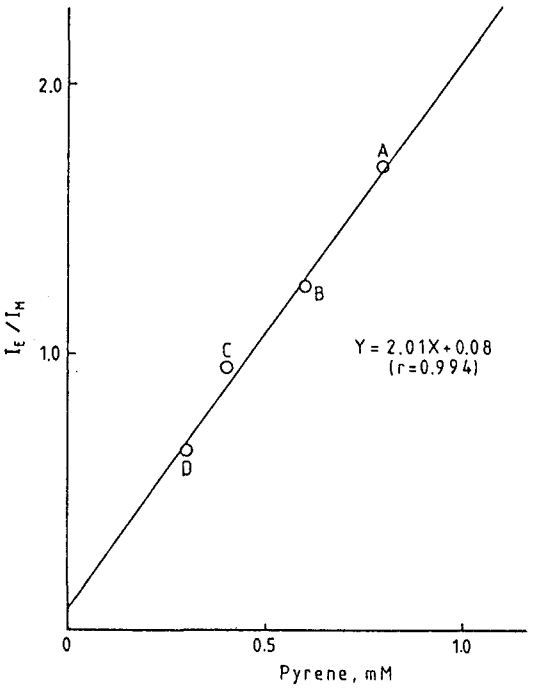

Fig. 3. A plot of I_E/I_M vs. concentration of pyrene in cyclohexane at 37°C. Pyrene concentrations are : (A) 0.8 mM ; (B) 0.6 mM ; (C) 0.4 mM ; (D) 0.3 mM.

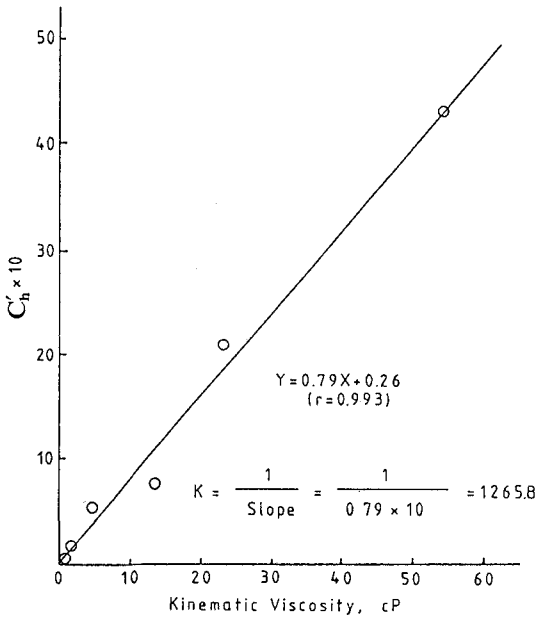

Fig. 4. A plot of C_h' vs. kinematic viscosity. In order of increasing viscosity the solvents are ; cyclohexane, 2-propanol, 1-octanol, 1-dodecanol, 1,2-propanediol and 1, 3-butanediol.

Table 1. Kinematic viscosity and half-intensity concentration values (C_h') at 37°C

Solvent	Kinematic viscosity, cP	$C_h' \times 10^3$, M	Lit. value[a], cP	
Cyclohexane	0.79	0.5	0.980	(20°C)
2-propanol	1.55	1.6	2.860	(15°C)
1-octanol	4.58	5.5	10.60	(15°C)
1-dodecanol	13.55	7.6	17.40	(25°C)
1,2-propanediol	23.31	21.1	56.00	(20°C)
1,3-butanediol	54.44	43.1	130.00	(20°C)

[a]H. J. Pownall and L. C. Smith, J. A. C. S., 95, 3136 (1973)

Microviscosity of SPMV in the absence and presence of local anesthetics

In pyrene-labeled SPMV, I_E/I_M is also plotted against microscopic concentration of pyrene in SPMV in the absence and presence of 1 mM local anesthetics (Fig. 5). The C_h' value was obtained from the reciprocal of the slope of this plot as previously, from which a microscopic viscosity was calculated. As shown in Table 2, the microviscosity value of SPMV, which was 57.3±5.3 cP at 37°C, decreased in the presence of local anesthetics. Procaine·HCl and lidocaine·HCl decreased the microviscosity of the hydrocarbon region of SPMV 4.5% and 18.8%, respectively.

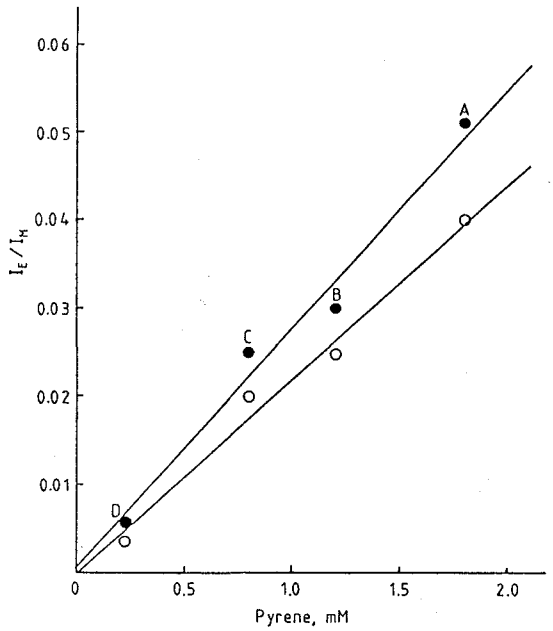

Fig. 5. A plot of I_E/I_M vs. microscopic concentration of pyrene in SPMV at 37°C in the absence (○) and in the presence (●) of 1 mM lidocaine HCl. Pyrene concentrations are : (A) 1.8 mM ; (B), 1.2 mM ; (C) 0.8 mM ; (D) 0.2 mM.

Effect of local anesthetics on the thermotropic phase transition of DMPC multilamellar liposomes

In order to validate the results of intermolecular excimer fluorescence of pyrene, the effects of local anesthetics such as procaine·HCl, lidocaine·HCl, tetracaine·HCl and dibucaine·HCl on the thermotropic phase transition of DMPC multilamellar liposomes were investigated by the use of DSC.

Fig. 6 shows the DSC scans of DMPC liposomes in the absence and presence of 1 mM local anesthetics. Local anesthetics lowered the main transition temperature (T_m) and broadened the thermogram peaks. As the concentration of the drug is increased, the half-height width of the transition temperature increases and the transition of the bilayer begins to appear at lower temperature than in untreated liposome (Table 3). The relative ability of local anesthetics to perturb the bilayer is in the order: dibucaine·HCl > tetracaine·HCl > lidocaine·HCl > procaine·HCl.

Broadening of the peaks in the thermograms in the presence of local anesthetics indicate that they may significantly reduce the size of the cooperative unit, which is the number of lipid molecules in the domain of simultaneous transition. The values were calculated by the method of Hinz *et al.* (1972) and listed in Table 4. Local anesthetics significantly reduced the size of the cooperative unit and this means that the lipid bilayer is fluidized.

Table 2. Summary of slope, relation coefficient (r), half-intensity concentration values (C_h'), and microviscosity values (η) of SPMV in the absence and presence of 1 mM local anesthetics

Local anesthetics (1 mM)	Slope	r (n=4)	$C_h' \times 10^3$ M	η' cP
None	0.022	0.994	45.5	57.3
Procaine · HCl	0.023	0.985	43.5	54.7
Lidocaine · HCl	0.027	0.990	37.0	46.5

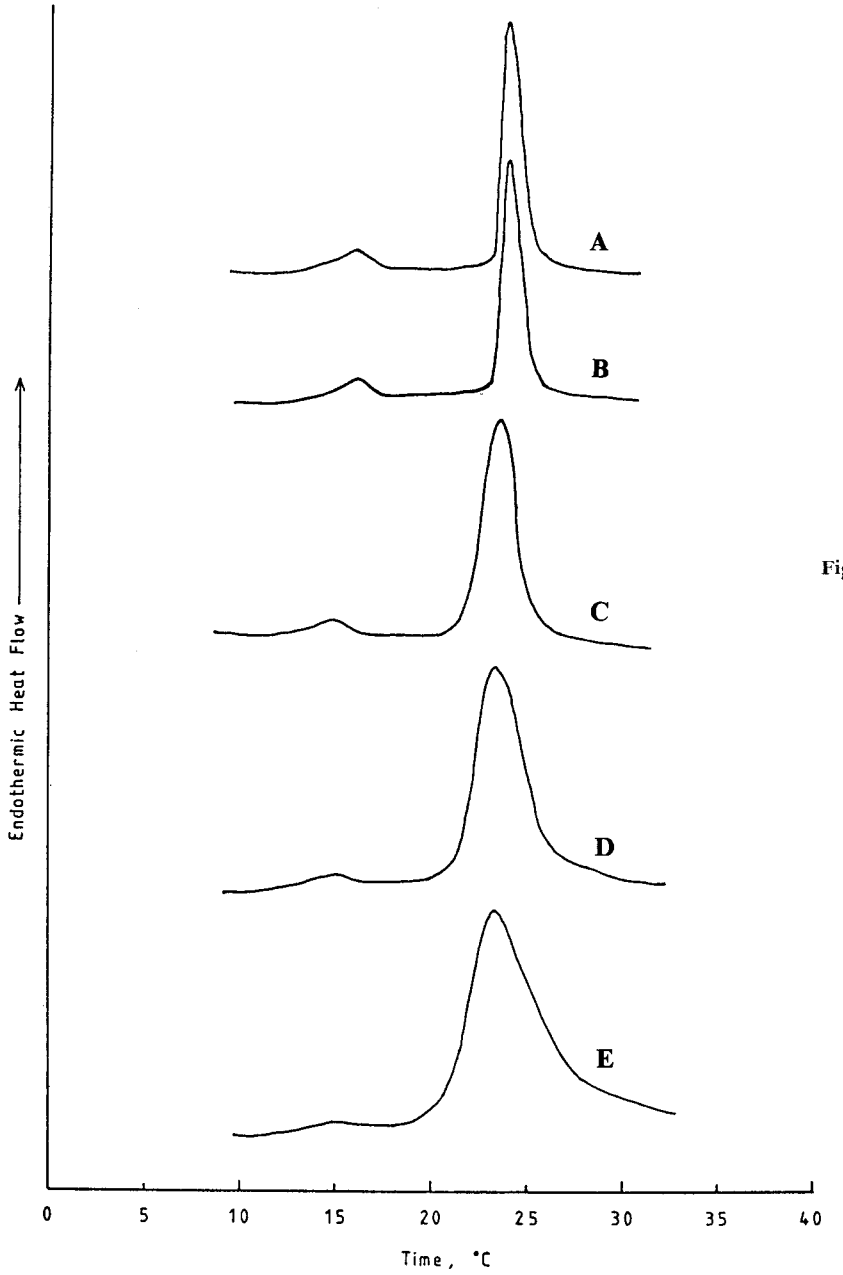

Fig. 6. Thermograms of DMPC liposome (A) and DMPC liposomes incorporated with 1 mM Procaine HCl (B), Lidocaine HCl (C), Tetracaine HCl (D) and Dibucaine HCl (E).

DISCUSSION

The concept of membrane fluidity lacks a precise definition, but it generally means a combination of different types of mobility of membrane components (Schachter, 1984). These include the following: flexibility of phospholipid acyl chains, lateral diffusion of molecules in the plane of the membrane, transverse diffusion of molecules from one monolayer to the other, and

Table 3. Thermotropic transition temperature of DMPC liposomal bilayer in the absence and presence of local anesthetics[a]

Local anesthetics	Conc, mM	Transition temp, °C	
		T_m	T_{onset}
None		24	23
Procaine · HCl	1.0	24	23
	0.5	24	23
Lidocaine · HCl	1.0	23.7	22.5
	0.5	24	23
Tetraceaine · HCl	1.0	23.4	21
	0.5	24	22
Dibucaine · HCl	1.0	23.4	21
	0.5	23.8	22

[a] T_m, main transition temperature ; T_{onset}, onset temperature

Table 4. The size of cooperative unit of DMPC liposomal bilayer in the absence and presence of local anesthetics

Local anesthetics	Size of cooperative unit	
	0.5 mM	1.0 mM
None	890	890
Procaine · HCl	890	890
Lidocaine · HCl	890	815
Tetracaine · HCl	806	610
Dibucaine · HCl	720	485

phase transitions leading to lateral phase separations.

Among numerous techniques which have been developed to quantitate membrane fluidity, fluorescence spectroscopy promises to be especially useful due to great sensitivity, versatitility, and simplicity of instrumentation. This technique includes the estimation of steady-state fluorescence polarization and excimer fluorescence intensity. Pyrene was chosen for this study since (a) polarizer was not available in our laboratory, (b) it is the most comprehensively studied of the excimer forming dyes, (c) it is totally insoluble in water, so that interference from unbound pyrene is negligible, and (d) it has a high quantum yield ($\varphi_f=0.6$) which permits the use of very small quantities.

The availability of pyrene as a microviscometric probe of the hydrocarbon interiors of micelles (Pownall *et al.*, 1973), phospholipid vesicles (Soutar *et al.*, 1974; Vanderkooi *et al.*, 1974), and biological membranes (Vanderkooi*et al.*, 1974; Dembo *et al.*, 1979) has been well demonstrated. Fig. 2-5, Table 1 and Table 2 show that the microviscosity of SPMV can be also determined rapidly and simply by pyrene excimer fluorescence. The measurement of microviscosity by the determination of C_h' values appears to be an attractive method for obtaining this somewhat inaccessible information.

The microviscosity value of the hydrocarbon region of SPMV determined by this method was 57.3 ± 5.3 cP at 37℃. However, the value decreased to 46.5 ± 5.1 cP and 54.7 ± 4.8 cP in the presence of lidocaine·HCl and proaine·HCl, respectively. This suggests that local anesthetics might increase the fluidity of the hydrocarbon region of SPMV according to their pharmacological activity. Dibucaine·HCl, tetracaine·HCl and benzocaine·HCl were excluded from this experiment because they tended to either quench or shift the fluorescence emission spectra of pyrene.

It may be impossible to find a homogeneous solvent that can truthfully represent the nature of the local environment in intrinsically inhomogeneous and anisotropic systems such as biological membranes. This exposes the limits inherent in the use of probe molecules, which moreover have molecular dimension that cannot be ignored and which always more-or-less perturb their environment. Probe molecules often find their optimal application not so much in experiments designed to give absolute values for some physical property, but rather in investigations of differences between structurally related media or of changes taking place in a particular system. As pointed out by Zachariasse *et al.* (1982), it seems reasonable that the results of a variety of different probe molecules will have to be collated and combined with the results of other methods of investigation.

In conjunction with the results of the intermolecular excimer fluorescence of pyrene in SPMV isolated from bovine brain, we obtained similar results in model membrane systems using DSC. DSC has been the technique of choice for obtaining basic thermodynamic data on the phase behavior of lipid molecules. As shown in Fig. 6, Table 3 and Table 4, local anesthetics significiantly lowered the phase transition temperature, broadened the thermogram peaks, and reduced the size of the cooperative unit in DMPC multilamellar liposomes. This means that the lipid bilayer is significantly fluidized. Further, this ability appears

to be correlated with their pharmacological activity. Our results obtained from both SPMV and model membranes are consistent with the disordered lipid hypothesis.

The relatively nonspecific nature of anesthetic action has long been recognized. Extensive studies have shown that the anesthetic potency is closely related to the partition coefficient of the anesthetic (Seeman, 1972; Miller *et al.*, 1972). In addition, the only obvious similarity between compounds with local anesthetic action is their hydrophobicity. However, there is evidence that local anesthetics can interact electrostatically with membrane proteins as well as membrane lipids (Chan *et al.*, 1984). Some investigators believe that the major mechanism of action of local anesthetics involves their combination with a specific receptor site within the sodium channel, hence physically blocking it (Ritchie, 1975; Hille, 1980). But the receptor would have to be surprisingly unselective, since a wide variety of different compounds act as local anesthetics.

The physical state or fluidity of membrane lipids has been shown to influence membrane enzymes (Dipple *et al.*, 1978; Chong *et al.*, 1985), membrane transport processes (Balcar *et al.*, 1980; Carriere *et al.*, 1986), and membrane permeability (Green *et al.*, 1980). Recently, Harris *et al.* (1985) have shown that the function of sodium channels is correlated with lipid order in the hydrophobic core of the membrane and the inhibitory effects of intoxicant-anesthetic drugs on neuronal sodium fluxes may be the result of their capacity to disorder these lipids.

In summary, although direct interaction of anesthetics with membrane proteins or lipoproteins cannot be ruled out, the results of the present study, together with others (Jain *et al.*, 1975; Singer *et al.*, 1980), indicate that local anesthetics have significant fluidizing effects on biomembranes and perturbation of membrane lipids may produce some, but not all, of their pharmacological actions.

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═국문초록═

국소마취제가 Synaptosomal Plasma Membrane Vesicles의 유동성에 미치는 영향

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국소마취제의 약리학적 작용기전을 탐구키 위하여 소의 신선한 대뇌피질로부터 synaptosomal plasma membrane vesicles(SPMV)를 분리한 후 그 소수성 중심부의 microviscosity에 미치는 국소마취제의 영향을 pyrene 형광 probe 법으로 측정한 결과 lidocaine·HCl과 procaine·HCl이 microviscosity를 낮춘다는 것을 알았고 그 정도는 procaine·HCl에 비하여 lidocaine·HCl이 더욱 컸다.

또 국소마취제가 dimyristoylphosphatidylcholine (DMPC) multilamellar liposomes의 상전이온도를 낮추며 cooperative unit 크기를 감소시킨다는 것도 시차 열량분석법으로 알게 되었다. 상전이온도와 cooperative unit 크기의 감소 정도는 dibucaine·HCl>tetracaine·HCl>lidocaine·HCl>procaine·HCl의 순이었다.