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Effect of Phenothiazine Derivatives on the Thermotropic Phase Transition of Liposomal Phospholipid Membrane

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Abstract□The effect of phenothiazine derivatives on the thermotropic transition of liposomal lipid bilayer made of dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidic acid was investigated with differential scanning calorimetry. The thermograms of the liposomal bilayer incorporated with levomepromazine, chlorpromazine, prochlorperazine, perphenazine and fluphenazine were obtained and the size of cooperative unit of the transition were calculated from the ratio of the van't Hoff enthalpy change to the calculated enthalpy change of the transition. The results showed that incorporation of phenothiazine derivatives into the liposomal bilayer reduced the transition temperature at which the transition from solid state to liquid-crystalline state occurs, and broadened the thermogram peaks. Phenothiazine derivatives also significantly reduced the size of cooperative unit of the transition. The effect of the drugs was proportional to the concentration of the drug in the bilayer. This means that phenothiazine derivatives might have significant fluidizing effects on the biomembrane. The sizes of cooperative unit were successfully correlated with pharmacological activities of the drugs and the surface pressure increases of lipid monolayer by these drugs. These correlations might be ascribed to a possible hydrophobic nature of interaction between the biomembrane and the drugs involved in their pharmacology.

Keywords□Phenothiazine derivatives, Thermotropic phase transition, Liposomal membrane, Differential scanning calorimetry, Size of cooperative unit.

Phenothiazine derivatives are a major tranquilizer[1], and have been widely employed as therapeutic agents of psychological disorders. They have been known to exert their neuroleptic and antipsychotic effects through interference with the transmitter function of dopamine[2,3]. However, the exact mechanism of action of these drugs have not been fully elucidated. An anesthetic is a drug which, when applied directly to the nerve cell, reversibly block the action potential without appreciably affecting the resting membrane potential. According to this definition, phenothiazine derivatives are also included in this category, and the mechanism of action of these drugs might be closely related to phenomenon in biomembrane[4]. A possibility has been suggested that local anesthetics induce the anesthesia through blockade of the sodium channel by increasing the fluidity of the membrane lipid bilayer[5-7]. It might be postulated that the effect of phenothiazine derivatives on the fluidity of biomembrane might also have some significance in their pharmacology.

It is proteins that are responsible for the specific transport properties and many of the enzymatic functions of the membrane. In many cases these activities have been found to be sensitive to the physical state of the membrane lipids. The fluidity of lipid surrounding integral proteins can not only affect their conformations, but it can also regulate their activities. Thus changes in the fluidity of a biological membrane, caused either by the cell itself or by external factors, can lead to dramatic effects on the membrane functions[8]. In spite of importance of the membrane fluidity in the membrane functions such as fusion, endocytosis, excitability, osmosis and all membrane-mediated processes, studies along this line have been on only its verging state due to restriction in the methodology in this field. However, recent development in membrane-mimetic chemistry enables this kind of researches feasible by employing phospholipid liposomes or other lipid bilayers as a model membrane[9].

In this research multilamellar liposome made of dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidic acid was employd as a model membrane system, and the effect of phenothiazine derivatives such as levomepromazine, perphenazine, prochlorperazine, chlorpromazine and fluphenazine on the thermotropic phase transition of the lipid bilayer was investigated by

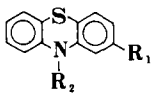
the differential scanning calorimetry. From the thermograms the size of cooperative unit, pharmacological activities of phenothiazine derivatives and the surface pressure increases by the drugs were explored. From the results the effects of the phenothiazine derivatives on the biomembrane properties and their possible role in the pharmacological activities were discussed.

EXPERIMENTAL METHODS

Materials

Dipalmitoyl L-α-phosphatidylcholine (DPPC), dipalmitoyl L-α-phosphatidic acid (DPPA) and stearic acid were purchased from Sigma Chemical Company, MO., USA., and used as received. Phenothiazine derivatives, levomepromazine maleate, chlorpromazine hydrochloride, prochlorperazine dimaleate, perphenazine dihydrochloride and fluphenazine dihydrochloride were obtained from National Institute of Health, Seoul, Korea. Their chemical structures are shown in Table I. Water was deionized and distilled. Other reagents were all reagent grades. The buffer used for all measurements was 0.1M KCl/0.01M Tris/0.1mM EDTA adjusted to pH 6.9.

Table I. Structures of Phenothiazine Derivatives



General Name	R_1	R_2
Levomepromazine	OCH_3	$CH_2CH(CH_3)CH_2N(CH_3)_2$
Chlorpromazine	Cl	$CH_2CH_2CH_2N(CH_3)_2$
Prochlorperazine	Cl	$CH_2CH_2CH_2$–N⟨⟩NCH_3
Perphenazine	Cl	$CH_2CH_2CH_2$N⟨⟩NCH_2CH_2OH
Fluphenazine	CF_3	$CH_2CH_2CH_2$N⟨⟩NCH_2CH_2OH

Preparation of Multilamellar Liposomes for Differential Scanning Calorimetry

The chloroform solution containing DPPC: DPPA (95:5 mol%) from a newly opened ampoule was transferred to a glass tube which had been flushed with high-purity nitrogen. The chloroform was then evaporated under vacuum. The dried lipid was suspended in the buffer above the phase transition temperature with a vortex mixer for 10 min. Phosphorous determination in the liposome was performed with Inductively Coupled Plasma Quantorecorder (ICPQ-1000, Shimadzu Plasma). The concentration of lipid in the liposomes was 20mg/ml.

Differential Scanning Calorimetry

The phase transition temperature of the phospholipid dispersions was determined with a Shimadzu differential scanning calorimeter, SC-30, according to the usual procedure[10,11]. Liposomal dispersions were incubated with a desired concentration of a phenothiazine derivative for 60 min above the phase transition temperature, and then 15 μl of the dispersion was transferred to a volatile sample pan. Reference was prepared with addition of the same amount of the buffer to a pan. Each sample was scanned between 30°C to 80°C at a rate of 2°C/min and a range of 5mJ/sec. Transition enthalpy was determined from the ratio of the area under the endothermic curve of the phospholipid sample to that of stearic acid as calibrant (T_m = 69.5°C, ΔH = 16.4 Kcal/mole).

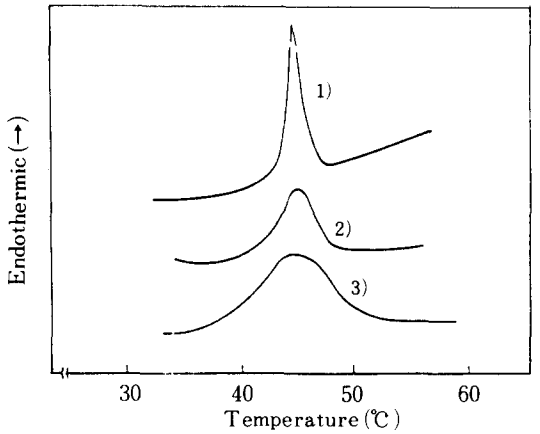


Fig. 1. Thermograms of phospholipid liposomes: 1) DPPC:DPPA (95:5 mol %), 2) and 3) DPPC: DPPA (95:5 mol %) incorporated with 0.25 mM and 0.5 mM chlorpromazine, respectively.

RESULTS AND DISCUSSION

The differential scanning thermograms of the multilamellar liposome of DPPC:DPPA (95:5 mol%) in the absence and in the presence of phenothiazine derivatives were obtained and some are illustrated in Fig. 1. The transition from crystalline state to liquid-crystalline state of the bilayer was endothermic. The midtransition temperature was taken as a main transition temperature (T_m), which is characteristic for a given phospholipid composition. The thermograms showed that the phase transition profile for pure DPPC and DPPA liposomal bilayer exhibited a sharp symmetrical transition. However, the incorporation of a phenothiazine derivative into the lipid bilayer lowered T_m and broadened the peak. That means that the size of cooperative unit is reduced in the presence of phenothiazine derivatives, and the DPPC:DPPA bilayer is fluidized and perturbed by the drugs. Table II lists the main transition temperatures, the onset temperatures and half-height widths of

Table II. Lipid Phase Transition Temperature of DPPC: DPPA(95 : 5 mol%) Liposome in the Absence and in the Presence of 0.25 mM Phenothiazine Derivatives

Phenothiazine	Transition Temperature (℃)		
	T_{onset}	T_m	$T_{1/2}$
None	44	45	1.0
Levomepromazine	43.4	45	1.4
Chlorpromazine	43	44	1.6
Prochlorperazine	42.6	44	1.9
Perphenazine	42.2	43.6	2.3
Fluphenazine	42	43.6	2.5

the transition temperature. As the concentration of tne drug was increased, the half-height width of the transition temperature increased and the transition of the bilayer from crystalline state to liquid-crystalline state began to appear at lower temperature than in untreated liposome. The relative ability of the phenothiazine derivatives to perturb the bilayer was decreased in the order: fluphenazine > perphenazine > prochlorperazine > chlorpromazine > levomepromazine.

The size of cooperative unit is the number of lipid molecules in the domain of simultaneous transition in such transition. In highly cooperative transition, the unit is extremely large, and the transition shows a sharp peak in the thermogram. However, when small molecules are incorporated into the lipid bilayer the cooperative unit should be reduced. In such case, the transition usually starts at lower temperature than the phase transition temperature, occurs progressively and shows broadening of the peak in the thermogram. The size of cooperative unit (ξ) can be calculated by the ratio of van't Hoff enthalpy (ΔH_{VH}) to calculated enthalpy (ΔH_{cal}) as follow[12-14];

$$\xi = \frac{\Delta H_{VH}}{\Delta H_{cal}} \qquad (1)$$

When the heats of transition of a quantity of lipid and a standard substance are measured under same condition, the enthalpy change of the phase transition can be calculated by the following equation[15,16];

$$\Delta H = \frac{\Delta H_S \times M_S}{A_S} \times \frac{A}{M} \text{ (Kcal/mole)} \qquad (2)$$

where ΔH is the heat for sample (Kcal/mole), ΔH_s is the heat of fusion of standard substance (Kcal/mole), M_s the quantity of standard substance (mole), M the quantity of sample (mole), A_s the peak area for standard substance and A the peak area for the sample.

The van't Hoff enthalpy (ΔH_{VH}) of liposome can be calculated as follows. If θ is the fraction of the lipid in the liquid-crystalline state, there assuming an equilibrium constant $K = \theta/1\text{-}\theta$, one obtains from $d\ln K/dT = \Delta H_{VH}/RT^2$,

$$\frac{d\theta}{dT} = \theta(1-\theta)\frac{\Delta H_{VH}}{RT^2} \qquad (3)$$

where R is the gas constant and ΔH_{VH} is van't Hoff enthalpy At the midpoint temperature T_m, $\theta = 1/2$ is substituted and the following equation is obtained;

$$\left(\frac{d\theta}{dT}\right)_{T_m} = \frac{\Delta H_{VH}}{4RT_m^2} \qquad (4)$$

Comparison of ΔH_{VH} with ΔH_{cal} provides important information on the cooperativity of the phase transition. For noncooperative processes $\Delta H_{VH} / \Delta H_{cal} = 1$: for cooperative processes $\Delta H_{VH} / \Delta H_{cal} \gg 1$. The number of molecules in the cooperative unit is operationally defined as the ratio of $\Delta H_{VH} / \Delta H_{cal}$.

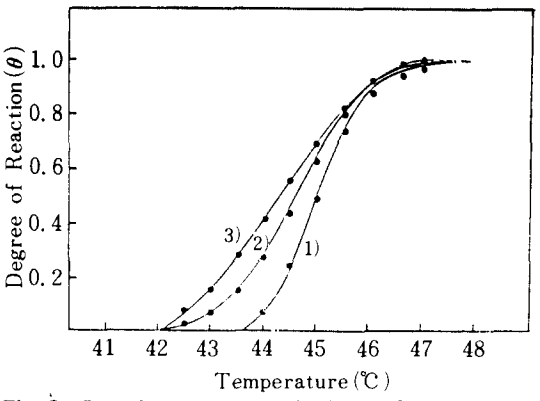


Fig. 2. Reaction degree of the gel to liquid-crystalline transition vs. the temperature of various phospholipid liposomes: 1) DPPC: DPPA (95:5 mol%), 2) and 3) DPPC:DPPA (95:5 mol %) incorporated with 0.25 mM and 0.5 mM chlorpromazine, respectively.

The plots of the reaction degree vs. temperature for DPPC:DPPA liposomal bilayer in the presence of phenothiazine derivatives are shown in Fig. 2. The reaction degree was calculated from the fraction of transition in the area under curve of the thermogram in Fig. 1. Since in this differential scanning calorimetry the temperature was scanned at a constant rate, the slope of the curve of this plot could be measure of the velocity of transition. The sizes of cooperative unit of the phase transition of DPPC:DPPA liposomal bilayer incorporated with phenothiazine derivatives were calculated employing equation (1). The data are listed in Table III which also includes the neuroleptic activities (NLA) and antiemetic activities (AEA) of the drugs and the surface pressure increases (ΔF) of lipid monolayer by these drugs quoted from references[17,18]. Here also, the relative ability of the drugs to reduce the size of cooperative unit

Table Ⅲ. Summary of the Size of Cooperative Unit, Pharmacological Activities and Surface Pressure Increases

Phenothiazine	Cooperativity[a] (ξ)	Pharmacological Activity, ED_{50} (mg/kg) NLA[b]	AEA[c]	ΔF[d] (dyne/cm)
None	95			
Levomepromazine	79	3.6	3.0	3.6
Chlorpromazine	72	2.3	1.08	7.0
Prochlorperazine	60	1.6	0.323	8.2
Perphenazine	50	0.16	0.009	8.6
Fluphenazine	45	0.072	0.0	8.8

[a] Size of cooperative unit of DPPC:DPPA (95:5 mol%) liposome in the absence and in the presence of 0.25mM phenothiazine derivatives
[b] Neuroleptic activity of phenothiazine derivatives
[c] Antiemetic activity of phenothiazine derivatives
[d] Surface pressure increase by the penetration of phenothiazine derivative into dipalmitoyl phosphatidylcholine monolayer

was in the same order as in their ability to fluidize the bilayer.

The sizes of cooperative unit of the transition of DPPC:DPPA liposomal bilayer in the presence of phenothiazine derivatives were correlated with the NLA and AEA values of the drugs and ΔF values by the drugs. The correlations are shown in Fig. 3, Fig. 4 and Fig. 5, respectively. They clearly showed that there are

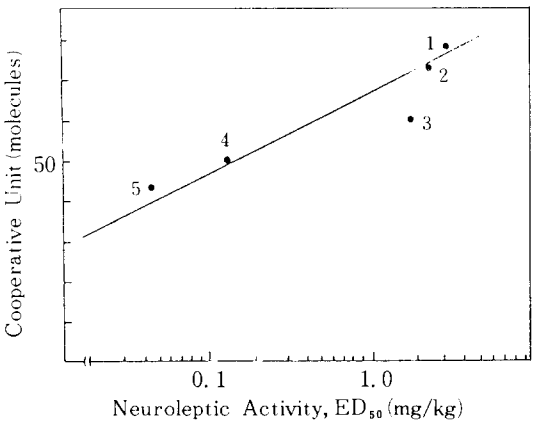


Fig. 3. Relationship between cooperative unit of DPPC:DPPA (95:5 mol %) liposomes in the presence of 0.25 mM phenothiazine derivatives and pharmacological activity of phenothiazine derivatives: 1) levomepromazine, 2) chlorpromazine, 3) prochlorperazine, 4) perphenazine and 5) fluphenazine (correlation coefficient, R=0.848).

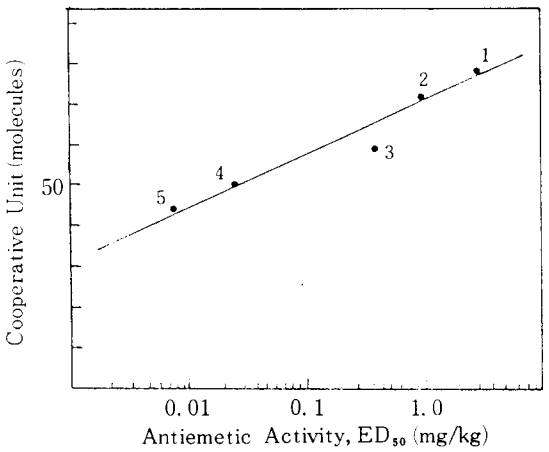


Fig. 4. Relationship between cooperative unit of DPPC:DPPA (95:5 mol %) liposomes in the presence of 0.25 mM phenothiazine derivatives and pharmacological activity of phenothiazine derivatives: 1) levomepromazine, 2) chlorpromazine, 3) prochlorperazine, 4) perphenazine and 5) fluphenazine (correlation coefficient, R=0.843).

linear relationships between these data. The correlation coefficients are highly significant. That means that the effect of phenothiazine derivatives on the order-disorder transition of the lipid bilayer of membrane might play a critical role or at least play some role in exerting their pharmacological activities. The successful correlations

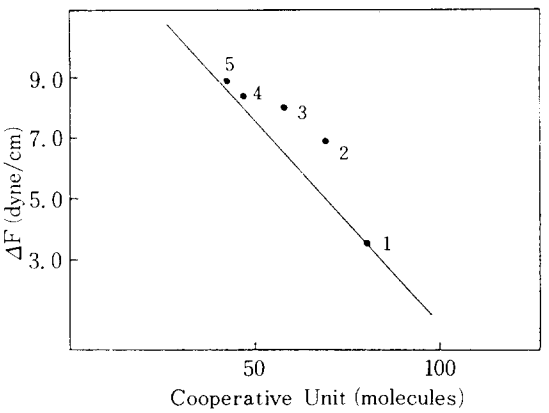


Fig. 5. Relationship between the surface pressure increase by the penetration of phenothiazine derivative into DPPC monolayer at 75A² molecule of DPPC and the size of cooperative unit of DDPC:DPPA (95:5 mol %) liposomes incorporated with 0.25 mM phenothiazine derivative : 1) levomepromazine, 2) chlorpromazine, 3) prochlorperazine, 4) perphenazine and 5) fluphenazine (correlation coefficient, R=0.883).

between these data suggest that the correlation should be due to hydrophobic nature of interaction involved in their pharmacology. The fluidization of biomembrane by these drugs might result in a drastic change in permeabilities of membrane to ions and small molecules, and also result in some change in the properties of enzyme, recognition site or receptor on the membrane. However, there might be some arguments that the concentration levels of phenothiazine derivatives by usual dosing are far below than the concentrations that could show some effects on the fluidity of the lipid bilayer. This suggests that the effect of phenothiazine derivatives on the fluidity of the liposomal bilayer might be insignificant or simply secondary in exerting their pharmacological activities. However, it has been known that there are many special domains in the biomembrane, and if phenothiazine derivatives or other local anesthetics preferentially bind to some area in biomembrane as suggested by Singer and Jain[19], they should locally induce significant effect on the fluidity of membrane and this fluidization might have some effects on the block of the sodium channel. This possibility should not be excluded in explanation of pharmacology of phenothiazine derivatives.

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