Korean J. of Pharmacology
Vol. 26, No. 1, pp. 77-82, 1990

The Effects of Calcium and Phenothiazine Derivatives on the Thermotropic Phase Transition of Acidic Phospholipid Bilayers

Nam Hong Kim and Sung Bae Roh*

College of Pharmacy, Pusan National University, Pusan 609-735, Korea

ABSTRACT

The effects of phenothiazine derivatives and calcium on the thermotropic phase transition of bilayers in dipalmitoyl phosphatidylcholine (DPPC) and dipalmitoyl phosphatidic acid (DPPA) liposomes were investigated with differential scanning calorimeter (DSC). Bilayers underwent an abrupt organizational changes at a characteristic temperature when heated. Such temperature-dependent transition was particularly striking and sharp in the bilayers prepared from pure phospholipids. The ability of phenothiazine derivatives to modify the phase transition of phospholipids liposomes was measured by a broadening of the phase transition profile, that transition began to appear at lower temperature than which occurs in untreated liposomes.

Calcium ion caused a large upward shift in the transition temperature of DPPC:DPPA (34:66mol%) liposomes. When the liposomes were first incubated with calcium ion followed by phenothiazine derivatives, disappearance of the broad curve centering at 73°C indicated displacement of calcium ion by phenothiazine derivatives at the anionic site. It is supposed that calcium ion and phenothiazine derivatives might compete with each other on the head group of acidic phospholipid.

Key Words: Phenothiazine derivatives, Phase transition, Differential scanning calorimeter (DSC), Phospholipids liposomes

INTRODUCTION

Phenothiazine derivatives are a major tranquilizer as well as, in a broad sense, a general anesthetics. They contain a hydrophobic moiety and also a hydrophilic moiety in a molecular or ionic form, and are expected to be surface active. Phenothiazine derivatives have been widely employed as therapeutic agents of psychological disorders such as schizophrenia, which are associated with overproduction of dopamine in the brain (Jain *et al.*, 1978; Forrest *et al.*, 1984). It has been proposed that the dopamin released by the interneuron during the period of stimulation combines with specific receptors on the postsynaptic membrane (Shibl *et al.*, 1984). Both binding and the catalytic sites are localized in the membrane. The complex with the dopamine binding site facing outward and the catalytic unit facing inward extends through the entire thickness of the plasma membrane.

Calcium ion has been known to play the important roles in releasing a transmitter from the synthetic membranes and in blocking the entry of sodium ions due to binding relatively strongly with proteins composing of sodium channels (Van Dijick *et al.*, 1975; Luterbacher *et al.*, 1983). To some extent the relative contributions of the various effects of calcium ion can be assessed by studying the Ca^{++} gate protein and chemically induced fusion (Van Dijick *et al.*, 1975). Calcium is known to bind stoichiometrically to acidic phospholipids, reducing their surface charges and area, thus increasing the phase transition temperature. This binding is influenced by surface charge, ionic strength and pH and is maximal when the lipids are in the close-packed crystalline state (Galla *et al.*, 1975; Chowdhry *et al.*, 1984).

Acidic phospholipids are particularly sensitive to

* To whom correspondence should be addressed.

changes in their environments. The fluxes of cations, protons or changes in membrane potential that surround many cellular events may well lead to alterations in membrane structure and function by perturbing acidic phospholipid species. This will change the composition of the fluid bulk lipid pool in which the integral proteins segregated. Depending upon the physical nature of the acidic phospholipids we can expect the fluidity of the fluid lipid pool either to increase or decrease. The phase transition of negatively charged phospholipids is markedly influenced by charges in pH, ionic strength and divalent cation concentration. In particular calcium ion can induce mixtures of neutral phospholipids with negatively charged phospholipids to separate the lipid phase. Phase separations are a common property of mixtures of phospholipids, and indicate both fluid and solid domains of lipid can co-exist over a range of temperatures. The composition of the fluid and solid domains will be different and influenced by changes in temperature. The wide variation of phospholipids found in biological membranes is likely to show non-ideal mixing. This will certainly be true of the solid state where the various headgroups exhibit different conformations and hence will not pack together in crystals but form various mixtures and solid solutions. It is also possible that non-ideal mixing will occur in the fluid state yielding fluid-fluid immiscibility. All of these phenomena may modulate the nature of the environment of protein species embedded in the membrane.

With all the importance of the effect of phenothiazine derivatives on the biomembrane properties, such studies have not yet been extensively carried out.

Differential scanning calorimetry is a valuable tool in determining physical changes of bilayers resulting from ion or drug interactions with liposomes, *e.g.*, gel-to-liquid phase transitions, phase separation, or membrane fusion (Madurai *et al.*, 1983). This study investigates the actions of calcium ion and phenothiazine derivatives on thermotropic properties of synthetic negatively charged and neutral liposomes with well-defined transition temperature.

MATERIALS AND METHODS

Materials

DPPC, DPPA and stearic acid were purchased from Sigma Chemical Company (U.S.A.) and used as received. The chemical structures of phospholipids are shown in Fig. 1. Phenothiazine derivatives as shown in table I such levomepromazine maleate, chlorpromazine hydrochloride, prochlorperazine

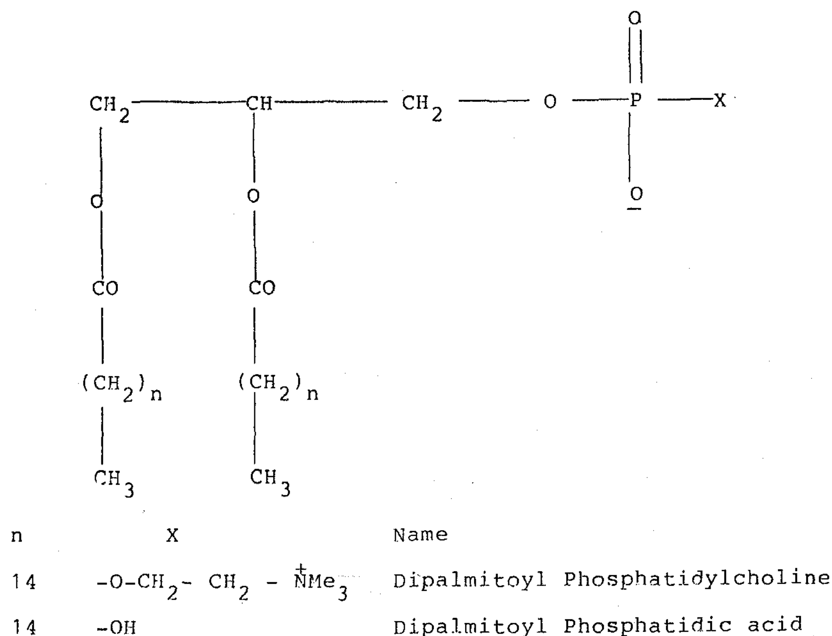

Fig. 1. Structures of phospholipids.

Table 1. 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	$CH_2CH_2CH_2N$ N CH_3
Perphenazine	Cl	$CH_2CH_2CH_2N$ N CH_2CH_2OH
Fluphenazine	CF_3	$CH_2CH_2CH_2N$ N CH_2CH_2OH

dimaleate, perphenazine dihydrochloride and fluphenazine dihydrochloride as tranquilizers were obtained from National Institute of Health (Korea) and their chemical structures are listed in Table I. Water was deionized distilled water. 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.

Preparation of multilamellar liposomes for differential scanning calorimetry

The chlorform solution containing purified lipid from a newly opened ampoule was transferred to a glass tube which had been flushed with nitrogen. The chlorform was then evaporated under vacuum (Bangham, A.D., 1982; Chowdhry *et al.*, 1984) The dried lipids were suspended in buffer adjusted to pH 6.9 above the phase transition temperature with a vortex mixer for 10 min and multilamellar liposomes were obtained. In this study on the effect of calcium ion on DPPC:DPPA (34:66mol%) liposomes, EDTA was depleted from the buffer, and the multilamellar liposomes were formed by vortexing the lipids at about 68°C for 30 min.

Differential scanning calorimetry (DSC)

The phase transition temperature of the phospholipid dispersions was determined with a Shimadzu differential scanning calorimeter, SC-30, accordingly to the usual methods (Hinz *et al.*, 1972). Phosphorous determination in the liposomes was performed with Inductively Coupled Plasma Quantorecorder (ICPQ-1000) (a Shimazu Plasma, collant gas 15*l*/min, plasma gas 1.0*l*/min, carrier gas 1.0*l*/min).

Liposomal dispersions were incubated with a desired concentration of phenothiazine derivative and/or 3mM $CaCl_2$ for 60 min above phase transition temperature. Liposomal dispersion 15μL was transferred to a volatile sample pans were prepared with the same amount of buffer. Inclusion of calcium ion and drugs or Ca-drug mixtures in the reference sample pan did not significantly affect the thermograms. Each sample was scanned between 30°C and 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 endothermic curve of phospholipid sample to that of stearic acid as calibrant ($Tm = 69.5°C$, $\Delta H = 16.4$ Kcal/mole). Phase transition (Tc) were determined as the intercept between the slope of the ascending endothermic peaks and the base line. The midpoint value of transition was estimated as the maximal endothermic peak. All experiments were repeated on at least three separate preparations.

RESULTS AND DISCUSSION

A small but significant fraction of the lipid pool of most biological membrane is acidic phospholipids imparting a net negative charge on the membrane surface. These acidic phospholipids have been known to play important role in forming specific structures on the membrane.

The ability of phospholipids to segregate into domains is of considerable physiological interest, especially in relation to the possible existence of some specific microenvironment in the vicinity of membrane proteins (Jain *et al.*, 1980; Guyton, C., 1981). A carrier-like transport peptide has been known to have an affinity for acidic lipid domain. Another possibility is that the components of the pore are disordered in an acidic lipid components and cocrystallize with the acidic domain to form conductive channels. Basic protein has been shown to bind acidic lipids preferentially when added to liposomes containing a random mixture of an acidic lipid and a neutral lipid (Jain *et al.*, 1980). Calcium ions have been known to bind stoichiometrically to acidic phospholipids, reducing their surface charges and areas, thus increasing the phase transition temperature.

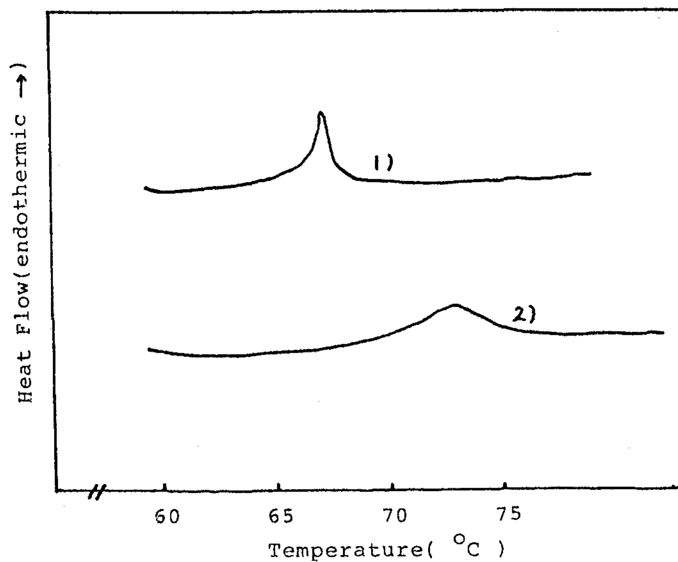

Fig. 2. Thermograms of DPPA liposomes: 1) without addition and 2) incubated with 3mM $CaCl_2$ at 68°C.

Table 2. Lipid phase transition temperature of some synthetic phospholipid bilayer incubated with 3mM $CaCl_2$

Lipid	Composition (mol%)	Transition Temperature (°C)		
		T_{onset}	T_{max}	upper temp
DPPC		41	42	
DPPC:DPPA	95:5	44	45	
DPPC:DPPA	34:66	58	62	73
DPPA		66	67	73

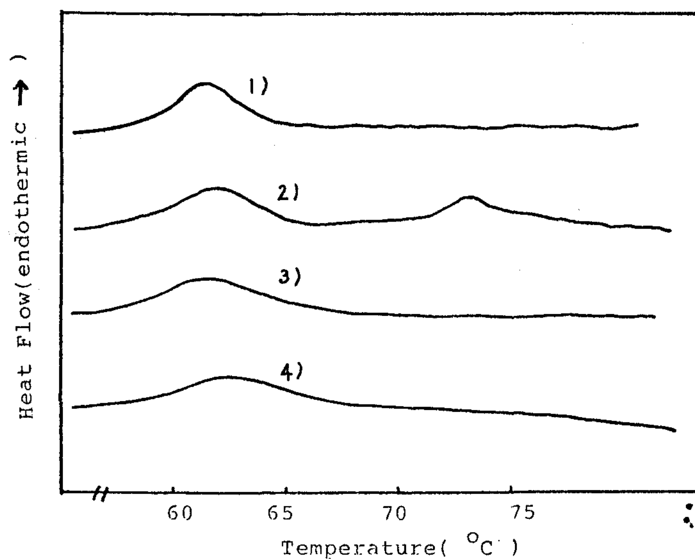

Fig. 3. Thermograms of DPPC:DPPA (34:66mol%) liposomes: 1) without addition, 2) incubated with 3mM $CaCl_2$ at 65°C, 3) incubated with 0.5mM chlorpromazine and 4) incubated with 3mM $CaCl_2$ followed by 0.5mM chlorpromazine at 65°C.

The effect of calcium ion on the thermotropic properties on the DPPA bilayer was investigated. As shown in Fig. 2. the presence of clacium ion has a pronounced effect on the thermotropic properties of the acidic phospholipids. Dipalmitoyl phosphatidic acid liposomes showed a relatively sharp peak at 67°C in its thermogram. However, when the lipid bilayer was incubated with calcium ion, the peak shifted to 73°C and appeared quite borad. Table II shows that calcium ion did not have any significant effect on the thermogram of DPPC liposomes and DPPC:DPPA (95:5mol%) liposomes but simply shifted the thermogram of DPPC:DPPA (34: 66mol%) to higher temperature. Furthermore, DPPC:DPPA (34:66mol%) liposomes showed the new peak at 73°C on addition of calcium ion.

The effect of phenothiazine derivatives on the thermotropic properties of acidic phopholipid bilayer incubated with calcium ion was examined. The thermograms in Fig. 3 showed that the mixed liposomes of DPPC:DPPA (34:66mol%) had a single broad centering at 62°C. However, when the mixture was incubated with 3mM $CaCl_2$, a new peak 73°C appeared in addition to the peak at 62°C. This new peak should be due to the Ca-DPPA or Ca-DPPA-DPPC complex formed. On addition of chlorpromazine hydrochloride to the mixed liposomes of DPPC:DPPA, the single peak at 62°C became broad, and the transition started at lower temperature than at which the untreated liposomes started, but again no significant effect on the neutral phospholipid and mixed lipids in the presence of small amount of DPPA as shown in table II. And addition of 0.5mM phenothiazine derivatives to mixed DPPC:DPPA (34:66mol%) liposomes, preincubated with calcium ion, produced disappearance of the single broad curve at 73°C. This result strongly indicates that there should be a competition between phenothiazine derivatives cation and calcium ion for the anionic site, and phenothiazine derivatives clearly displaced calcium from DPPC:DPPA (34:66mol%) liposomes. Charged drug such as phenothiazine derivatives, especially cationic species, by virtue of their abilities to interact specially with charged phospholipid species and also to displace membrane-bound calcium ion, might not only increase the fluidity of bilayer in membranes but could alter or induce lipid lateral segregation in biological membrane. This kind of competition might be significant for other kinds of cationic drugs, and play an important role in pharmacological activity.

REFERENCES

Bangham AD: *Preparation of liposomes and methods for measuring their permeabilities. Techniques in the Life Sci., Elsevior/North-Holland, Vol B4/11, B420/1-25, 1982*

Chowdhry BZ, Lipka G, Dalziel AW and Sturtevanat JM: *Effect of Lanthanum ions on the phase transitions of lecithin bilayers. Biophys J 45:633-635, 1984*

IBID: *Phase transition properties of 1,3-dipalmitoyl phosphatidyl-ethanolamine. J Phy Chem 88:5397-5400, 1984*

Forrest BJ, Linehan PTP and Mattai J: *Interaction of a phenothiazine tranquilizer with phosphatidylcholine and phosphatidylcholine-cholesterol membranes. Biochemistry 23:2288-2293, 1984*

Guyton C: *Nerve and muscle. Textbook of Medical Physiology, WB Saunders Co, 104-149, 1981*

Hinz JJ and Sturtevant JM: *Calorimetric investigation of the influence of cholesterol on the transition properties of bilayers formed synthetic L-lecithin in aqueous suspensions. J Bio Chem 247:3697-3705, 1972*

Luterbacher S and Cchatzmann HJ: *The site of action of La^{+++} in the reaction cycle of the human red cell membrane Ca^{++}-pump ATPase. Experimentia 39:311-312, 1983*

Madurai G Ganesan, Norman D Weiner and Jochen Schacht: *Effects of calcium and neomycin on phase behavior of phospholipid bilayers. J Pharm Sci 72(12):465-1467, 1983*

Shibl AM, Hammouda Y and AL-Sowaygh I: *Comparative effects of selected phenothiazine tranquilizers and antihistaminics on bacterial cells and possible interactions with antibiotics. J Pharm Sci 73:841-843, 1984*

Van Dijick PWM, Ververgaert PHJ Th, Verkleij AJ, Van Deenen LLM and De Gier J: *Influence of Ca^{++} and Mg^{++} on the thermotropic behavior and permeability properties of liposomes prepared from dipalmitoyl phosphatidylglycerol and mixtures of dimyristoyl phosphatidylglycerol and dimyristoyl phosphatidylcholine. Biochmi Biophys Acta 406:465-478, 1975*

=국문초록=

산성 인지질 이중층의 열적 상전이에 미치는 칼슘과 페노치아진 유도체의 영향

부산대학교 약학대학

김 남 홍 · 노 승 배

Dipalmitoyl phosphatidylcholine(DPPC)과 dipalmitoyl phosphatidic acid(DPPA) 리포좀내 이중층의 열적 상전이에 미치는 칼슘과 페노치아진 유도체의 영향을 시차 열량 분석계로 연구하였다. 인지질 이중층은 가열시 어느 특정온도에서 급격한 구조적 변화를 가져왔다. 이러한 온도에 의존한 변화는 순수 인지질의 경우 특이할 만큼 예민하였다. 순수 인지질에 페노치아진 유도체를 가하였을 때 순수 인지질의 상전이 온도보다 낮은 온도에서 일어 났으며 상전이 열그림을 넓적하게 만들었다.

칼슘 이온은 DPPC: DPPA(34: 66mol%)의 상전이 온도를 높였다.

그러나 위의 혼합 인지질에 페노치아진 유도체를 가하였을 때 73℃에서의 작고 넓적한 곡선 부분이 사라졌다. 이러한 현상은 아마도 칼슘과 페노치아진 유도체의 산성 인지질의 친수 부분에서 상호 경쟁적 작용에 기인된 것으로 추정되어진다.