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Differential scanning calorimetry and ^{1}H-NMR study of aqueous dispersions of the mixtures of dipalmitoylphosphatidilcholine and phosphatidylinositol-4,5-bis(phosphate)[1]

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Abstract

Thermal phase transitions of aqueous dispersions of phospholipid mixtures of phosphatidylinositol-4,5-bis(phosphate) (PIP_2) and dipalmitoyl-phosphatidylcholine (DPPC) were studied by using differential scanning calorimetry (DSC) and proton nuclear magnetic resonance spectroscopy (^{1}H-NMR). The DSC curves are classified into three different types in their endothermic profiles according to the molar ratio of PIP_2/DPPC. From the results of DSC and ^{1}H-NMR, three different types of structural regions are distinguished in the aqueous dispersions of mixtures of DPPC and PIP_2; (I) Region of DPPC with some defects produced by insertion of a small amount of PIP_2, (II) region of DPPC–PIP_2 complexes and (III) pure domain of PIP_2. © 1998 Elsevier Science B.V.

Keywords: DPPC; DSC; Mixtures; PIP_2; ^{1}H-NMR

1. Introduction

Phoshatidylinositol-4,5-bis(phosphate) (PIP_2) is an acidic phospholipid, easily soluble in water with the exception of phospholipid; it is present at high levels in the cell membranes of brains and kidneys [1]. Biochemists first studied PIP_2 in connection with nerve excitation [2,3]. Today they concentrate their attention on PIP_2 as the source of inositol-diphosphate (IP_2), the secondary messenger molecule in cell signaling [4].

Previously, we studied thermotropic behaviors of PIP_2–water systems and reported a new type of phase transition in phospholipid–water system that might be important to understand the function of PIP_2 in membranes [5,6].

However, PIP_2 is a minor component in the total phospholipids, in consequent, the study of the structure and properties of mixtures of PIP_2 with other major phospholipids such as phosphatidylcholine is necessary to elucidate the function of PIP_2 in the biomembranes. In this study, we carried out DSC and NMR measurements of the aqueous dispersions of the mixtures of PIP_2 and dipalmitoylphosphatidylcholine (DPPC), typical phospholipid whose physicochemical properties have been studied extensively.

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2. Material and methods

PIP_2 was obtained by Folch fraction of bovine brain [7]. The crude extract of PIP_2 was purified further by diethylaminoethylcellulose chromatography [8]. The preparations of ammonium salts of PIP_2 were free from divalent cations. The prepared samples gave a single spot on a thin layer chromatography plate. The solution of PIP_2–NH_4 was dialyzed against distilled water and lyophilized powder of PIP_2 was obtained.

The DPPC samples used in this experiment were L-α-Lecithin-dipalmitoyl from Calbiochem-Behring and from Avanti polar Lipids. The 1-stearoyl-2-arachidonoyl phosphatidylcholine (1S-2A-PC) has the same type of hydrocarbon chains with PIP_2, and we used 1S-2A-PC from Avanti polar Lipids as a substitute for DPPC on trial.

The mixed samples of PC and PIP_2 were prepared by dissolving them in the monophasic solution of chloroform–methanol–water (2/2/0.6 (v/v)), and evaporating the solvents under nitrogen gas glow. The solvents were further removed by evacuation at 10^{-4} mbar for more than 5 h. The mixed dry samples were dispersed in phosphate buffer, or in distilled water. The dispersions were sonicated and used in DSC and ^{1}H-NMR measurements. For comparison, we tried another method of preparation of mixed solutions; the lyophilized powders of DPPC and PIP_2 were dissolved together directly in distilled water or buffer solutions and sonicated. We used a heat conduction scanning micro calorimeter that utilized Melcor's semiconductor thermopiles as sensitive detectors for the heat flow. Silver sample cells with net volumes of 70 μl were sealed to prevent evaporation of moisture by pressing down and squeezing tapered silver covers. Usually, the concentration of the mixed lipid was about 10 wt%. Heating rates were 0.5 and 1°C/min. Special caution was taken not to heat the sample above 65°C, because chemical changes may occur at the higher temperature.

The ^{1}H-NMR measurements were carried out using a JNM-FX90Q FT NMR spectrometer operating at 89.6 MHz with a standard technique of induction decay Fourier transform. The probe was tuned carefully according to the instruction of the spectrometer. The data were taken at a spectral range of 1000 Hz with sampling points of 4096. The acquisition time was determined automatically at 4.096 s under the above conditions, and the repetition time of 90 pulse (width, 26 μs) was taken to be greater than the acquisition time. The delay time for starting the acquisition was also automatically set in 1 ms, that is enough time to avoid dead time of the spectrometer.

3. Results

3.1. Differential scanning calorimetry

Fig. 1 shows DSC curves of dispersions of DPPC and PIP_2 mixtures in the 1/30 M phosphate buffer solution at pH 7. The dry mixtures were prepared from organic solutions as previously described. The DSC curve are arranged in the increasing order of the molar ratios of PIP_2/DPPC from bottom to up in the Fig. 1.

The aqueous dispersion of pure DPPC shows the typical pre- and main endothermic transitions. Remarkable changes in the DSC curve are observed by the additions of relatively little amounts of PIP_2 as shown in Fig. 1; both of the pre- and main transitions peaks become broad and shift to the lower temperature, and the pre-transition peak finally disappears at the molar ratio of 0.16 of PIP_2/DPPC.

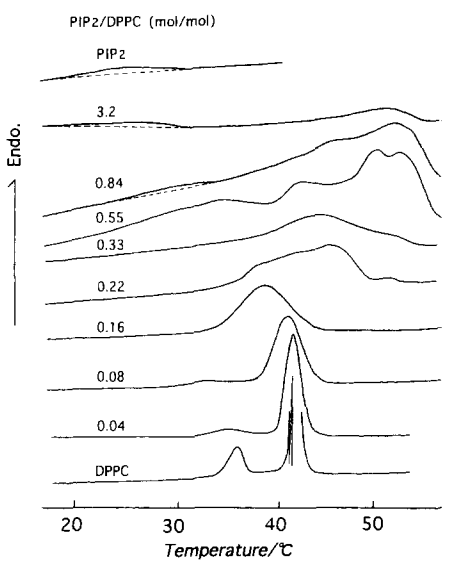

Fig. 1. DSC curves of aqueous dispersions of the mixtures of PIP_2 and DPPC (Calbiochem.) in 1/30 M phosphate buffer at pH 7. Molar ratios of PIP_2/DPCC are indicated in the figure. Scanning rate, 1°C/min.

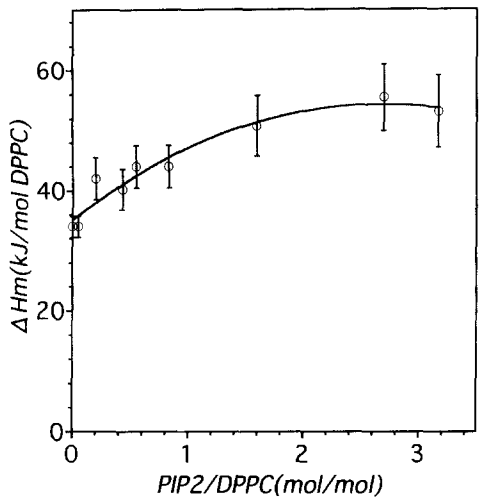

Fig. 2. Main transition enthalpies of DPPC in the aqueous dispersions of the mixtures of PIP_2 and DPPC (Calbiochem.) in 1/30 M phosphate buffer at pH 7.

The drastic changes occur above the molar ratio of 0.22; the main peak position shifts to the higher temperatures in this case, and the transition peaks become very broad and seem to be composed of multi-transition peaks.

At the molar ratio of 0.84, a small endothermic peak appears at around 30°C, besides the large endothermic peak above 50°C. The endothermic peak around 25°C is more evident at the molar ratio of 3.2. The pure PIP_2 dispersion in the phosphate buffer at pH 7 has a broad endothermic peak with a maximum around 25°C.

The molar enthalpies of the main transition of DPPC are estimated from the peak areas and plotted as a function of molar ratio of PIP_2/DPPC in Fig. 2. The transition enthalpy obtained for the dispersion of pure DPPC is nearly the same with the standard value, but those for mixed samples increase up to 50 kJ/mol DPPC with the increase of the ratio of PIP_2/DPPC.

DSC curves of the dispersions of DPPC–PIP_2 mixtures in distilled water are shown in Fig. 3. The characteristic profile of each transition curve resembles to the corresponding transition in Fig. 1. However, remarkable hysteresis curves in the DSC are observed for the samples with the molar ratio of 0.1 and 0.38; a broad endothermic peak with maximum at above 40°C is observed at the ratio of 0.1, at the first scanning; then the corresponding peak is observed above 50°C at the second scanning.

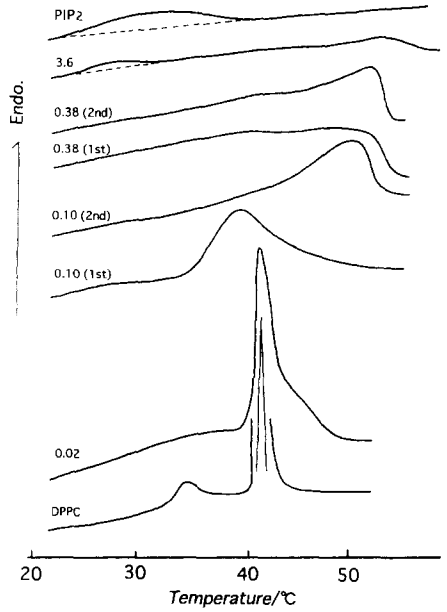

Fig. 3. DSC curves of aqueous dispersions of the mixtures of PIP_2 and DPPC in distilled water. Molar ratios of PIP_2/DPPC (Calbiochem.) and the sequences of the scanning are indicated in the figure. Scanning rate, 0.5°C/min.

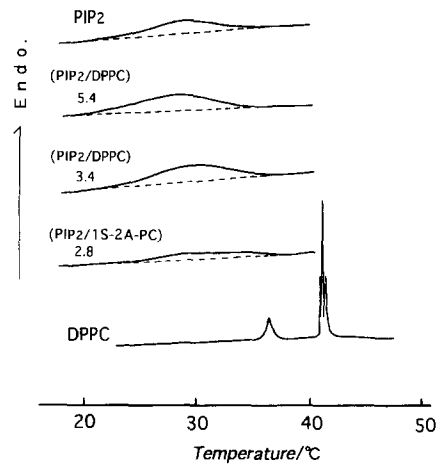

Fig. 4. DSC curve of dispersions of the mixtures of PIP_2 and DPPC (Avanti) in distilled water in PIP_2 rich regions. Molar ratios of PIP_2/PC are indicated in the figure. Scanning rate, 0.5°C/min.

Fig. 4 shows DSC curves of DPPC–PIP_2 mixtures in distilled water in PIP_2 rich regions obtained by using another series of mixed samples. The endothermic peaks at 20–35°C are observed for the dispersions

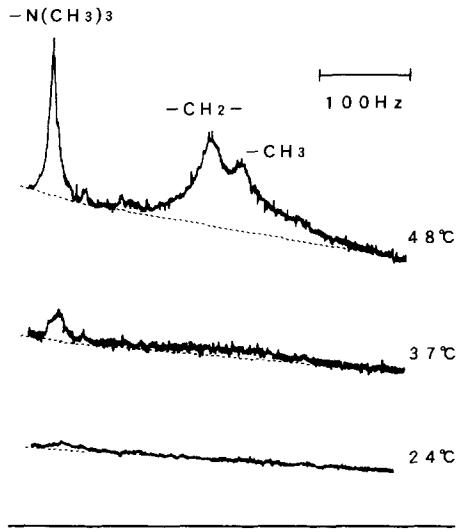

Fig. 5. Temperature dependence of ^{1}H-NMR signals of dispersions of a DPPC (Calbiochem.) sample in distilled water used in this experiment. The scale of the horizontal axis and temperature of measurements are indicated in the figure. Accumulation number, 100.

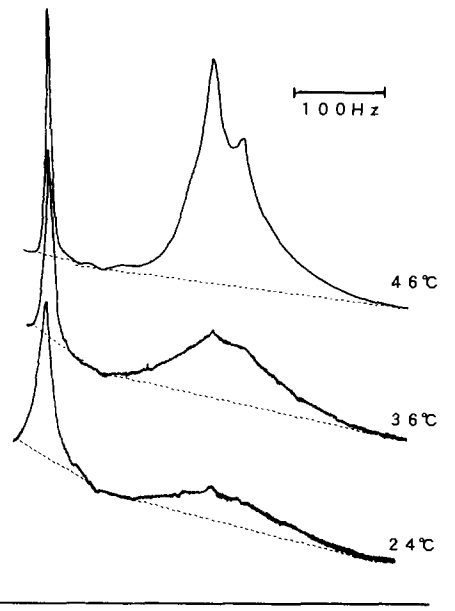

Fig. 6. Temperature dependence of ^{1}H-NMR signals of dispersions of the mixtures of PIP_2/DPPC=0.04 (mol/mol) in 1/30 M phosphate buffer at pH 7. The scale of the horizontal axis and temperature of measurements are indicated in the figure. Accumulation number, 300.

of pure PIP_2 and also for the mixed samples with the molar ratio of 3.4 and 5.4. The DSC curve for dispersion of mixed lipid of 1S-2A-PC and PIP_2 shows also a broad endothermic peak around 30°C. The aqueous dispersion of pure 1S-2A-PC has no transition in the temperature region studied in our experiment. A DSC curve for dispersion of pure DPPC is shown at the bottom of the Fig. 5.

3.2. *Proton nuclear magnetic resonance spectroscopy*

The results of NMR measurements are shown in Figs. 5–7. The numbers of the accumulation are indicated in each figure caption. The NMR spectra are not represented in the same noise level, because the noise levels of the signals are determined automatically according to the most intense signal in a spectrum of each sample; the signal from residual ^{1}H of water in the D_2O solutions in our samples.

Fig. 5 shows temperature dependence of ^{1}H-NMR signals of aqueous dispersion of DPPC sample used in this experiment. Signals from choline groups and from hydrocarbon chains appear at a temperature above the main transition temperature. However, signals from

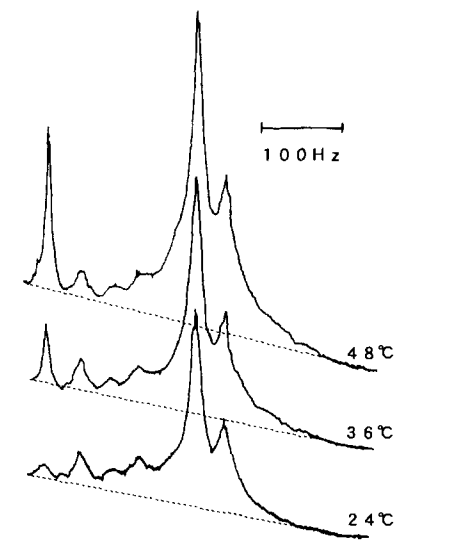

Fig. 7. Temperature dependence of ^{1}H-NMR signals of dispersions of the mixtures of PIP_2/DPPC=0.3 (mol/mol) in 1/30 M phosphate buffer at pH 7. The scale of the horizontal axis and temperature of measurements are indicated in the figure. Accumulation number, 300.

hydrocarbon chains are not observed at all at 24°C, and just a very weak signal from choline is observed.

The signal from choline groups in the dispersion of DPPC with a little amount of PIP_2 is clearly observed even at 24°C as shown in Fig. 6, and the broad signals from hydrocarbon chains are also observed in this case. Both of the signals from choline and hydrocarbon groups above transition temperature in Fig. 6 are sharper than those in Fig. 5, which represents the molecular motions of DPPC are faster in the mixed dispersion of DPPC–PIP_2 than in the dispersion of pure DPPC.

The molecular motion of choline groups at 24°C is suppressed again, when the ratio of PIP_2 increases up to 0.30, though the sharp signals from hydrocarbon chains are clearly observed at the temperature, as shown in Fig. 7. Here, we should notice that NMR signals from the hydrocarbon chains of PIP_2 are also included in the signals.

In our NMR measurement, we did not use internal references, because of the fear of unknown effect of those chemicals, and comparisons of the absolute intensities of NMR signals at different conditions are not possible. Then, we tried to compare the signal intensity ratios from hydrocarbon chain to those from choline group. The intensities of the signals are evaluated with the area under the signals. The base lines of the signals as taken for the measurements of the area are shown with dotted lines in the figures. Though some ambiguity is left in the evaluation of the signal intensity ratio due to the base line drawing, we obtained a result as shown in Fig. 8. Horizontal line drawn at 7.3 in the longitudinal axis indicates the ratio of the numbers of protons in the hydrocarbon chains to those in the choline group. The intensity ratio for pure DPPC is less than half level of the horizontal line even at a temperature above the main transition, which means that the molecular motions of more than half of all protons in the hydrocarbon chains of DPPC are not enough to be observed as the high resolution NMR signal.

The intensity ratio for the mixture of DPPC with a little amount of PIP_2 (PIP_2/DPPC=0.026) increases rapidly with the increase of temperature reaching to 7.3, implying both the molecular motions of choline and hydrocarbon groups are quite free at the higher temperature. For the sample of mixture of PIP_2/DPPC=0.30, the intensity ratio at 24°C is about 50,

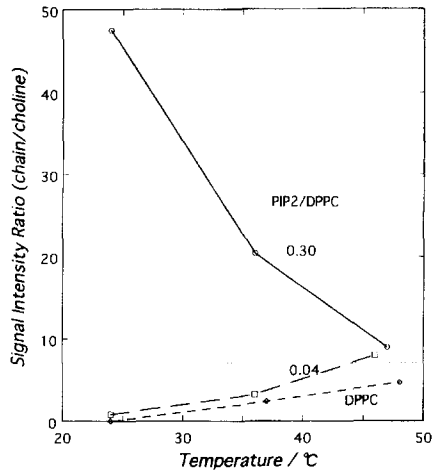

Fig. 8. ^{1}H-NMR signal intensity ratios of chain/choline as a function of temperature. Effect of addition of PIP_2 are shown. Molar ratios of PIP_2/DPPC are indicated in the figure. Horizontal dotted line indicates the ratio of number of protons in hydrocarbon chains to those in choline groups of DPPC.

reflecting that the signal from choline groups is very small as shown in the Fig. 7. However, it decreases rapidly to 9.0 at 47°C as the result of drastic increase of the intensity of choline signal.

4. Discussion

Interaction of phospholipids with water is complex even in the equilibrium state; hexagonal, lamellar, micellar or some other phases of the phospholipids appear according to the parameters such as concentration and temperature of the aqueous dispersions. In our study of the structure and properties of the mixed phospholipids in the aqueous state, the most important factor was the choice of the preparation method of the mixed systems.

We adopted the method of preparation of the mixed sample of PIP_2/DPPC as previously mentioned. The reason is as follows. The solvent can dissolve both PIP_2 and DPPC. Macroscopic phase separation to PIP_2 and DPPC does not occur through the drying process, and aqueous dispersions of the mixed sample of DPPC and PIP_2 seem in a stable state. We tried another method of preparation of mixed samples; lyophilized powders of PIP_2 and DPPC were directly dispersed in

aqueous solutions by sonication. However, the mixing was poor by this direct method; DSC curves for the samples obtained by this method are different with one other according to the delicate changes of the conditions in the mixing such as bath temperature and sonication. To the contrary, DSC curves of the aqueous dispersions of DPPC and PIP_2 prepared from the mixed dry phospholipids from mixed solvent gave the same thermograms, so far as the conditions of the scanning were not changed, even if the condition in the preparative process of the dispersions changed.

DSC curves and NMR signals for the mixed samples with a little amount of PIP_2 differ remarkably from those for pure DPPC. The transition peaks in the DSC curves broaden and shift to lower temperature. This phenomenon is explained as contamination effect of PIP_2 for the phase transition of DPPC. The molecular motion of choline groups in DPPC is much enhanced by the addition of impurity of a little amount of PIP_2, as shown in the Fig. 6.

When the addition of PIP_2 increase to the molar ratio of PIP_2/DPPC=0.22, the peak temperature shifts to higher than the transition temperature of the dispersion of pure DPPC. This means that the molecular motion of the hydrocarbon chains of DPPC is suppressed by formation of the molecular complexes with PIP_2. Multi-transition peaks in the curves of PIP_2/DPPC=0.22~0.55 in Fig. 1 suggest that several kinds of molecular complexes exist at those mixing ratios. The increase of the transition enthalpy with the increase of the ratio of PIP_2/DPPC tells us also that the molecular complexes are stable to heat than pure DPPC. The NMR signal from the choline groups of DPPC is suppressed by the addition of much amount of PIP_2 as shown in the spectrum at 24°C in Fig. 7. This confirms the formation of the thermally stable molecular complexes of PIP_2 and DPPC.

Next, we compare the DSC curves of the dispersions in the 1/30 M phosphate buffer solution at pH 7 with those in distilled water. The remarkable hysteresis curves are observed in distilled water at the molar ratios of 0.10 and 0.38 as described previously. In those cases, PIP_2 acts as an impurity for DPPC at the first scanning in DSC. However, the molecular complexes are formed at higher temperature regions at the first scanning, and because of that, the endothermic peaks shift to higher temperature at the second scanning.

Such a hysteresis was not observed in the DSC curves of the aqueous dispersions of DPPC and PIP_2 mixtures in the 1/30 M phosphate buffer solutions at pH 7. This experimental difference suggests that the state of water environment is important for the formation of the stable molecular complexes.

DSC curves of the mixed samples rich in PIP_2 show endothermic transition peaks due to the transition of pure PIP_2 as shown in Figs. 1, 3, 4. An endothermic peak is also shown for the dispersion of the mixture of PIP_2 and 1S-2A-PC in Fig. 4. The transition originates evidently in the region of PIP_2, because the dispersion of 1S-2A-PC has no transition at the temperature region studied in this experiment. The nature of the endothermic transition in PIP_2–water system was discussed in the previous papers as the thermal dehydration of the internally hydrated PIP_2 [5,6].

DSC thermograms for the dispersions at pH 7 are summarized into the next three characteristic types. (a) 0<PIP_2/DPPC<0.16: PIP_2 behaves as contamination for DPPC. The transition peak in DSC curve becomes broad, and the peak temperature shifts to lower than the transition temperature of the aqueous dispersion of pure DPPC. (b) 0.2<PIP_2/DPPC<1: The main transition peak broadens further, and the peak temperature shifts to higher, oppositely to the preceding case. (c) 1<PIP_2/DPCC: Endothermic peaks or shoulders characteristic to PIP_2 appear.

The DSC curves of the dispersions in distilled water are classified also into the three types, though the ranges of the mixing ratios of PIP_2/DPPC are more or less different from those for the dispersions at pH 7.

From the experimental results discussed above, we conclude that the following structural regions exist in the PIP_2–DPPC–water system.

Region I: Structured region of DPPC with some defects produced by the insertion of a small amount of PIP_2. Molecular motion of choline group is large notwithstanding below the transition temperature. This region appears at the lower mixing ratio of PIP_2/DPPC.

Region II: Structured region of DPPC–PIP_2 complexes: Region in which several kind of the specific interactions of DPPC and PIP_2 are formed.

Region III: Pure domain of PIP_2: This domain appears at relatively higher mixing ratio of PIP_2/DPPC.

The stable domain of pure PIP_2 can play the physiological role in the biological membranes especially in the nerve cells, as we discussed in the previous papers [5,6].

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