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Effect of the Acyl Chain Length of Phosphatidylcholines on Their Dynamic States and Emulsion Stability

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The relationship between the emulsion stability of phosphatidylcholines (PCs) and their headgroup motion of varied acyl chain lengths was investigated by ^{31}P and ^{13}C NMR. The acyl chains of PCs at glycerol *sn*-2 were shortened by the acylation of lysophosphatidylcholine (LPC) with C_2–C_{10} acids, and PCs with short glycerol *sn*-1 and *sn*-2 chains were also synthesized by the acylation of glycerophosphorylcholine (GPC) with C_6–C_{10} acids. Their physical properties and emulsion stability were compared with those of an equimolar mixture of egg PC and LPC, and similarities of the headgroup motion and emulsion stability were found between some short-*sn*-2-chained PCs and the mixture of PC and LPC. The results suggested that the headgroup motion correlated with the interfacial absorptivities of PCs and with their emulsion stability.

Phospholipids are widespread in nature as an important component of biomembranes. Phosphatidylcholine (PC) is one of common phospholipids, and it is also important as a naturally occurring emulsifier. In addition, lysophosphatidylchloline (LPC), although it exists as a minor component, has diverse functions such as the interaction with PC (Howell et al., 1973; Mandersloot et al., 1975; Inoue, 1977; Morris et al., 1980) and formation of a stable emulsion. The fatty acid composition and configuration of PC varies on its origin (Kuksis and Marai, 1967), and we are interested in the composition of fatty acid moieties in PC and their behavior in emulsion and in membranes. A number of reports have appeared on the carbon relaxation time measurements and thermal analysis of PC concerned with its constitution or length of acyl chains (Mason et al., 1981a; Burns et al., 1983). On the other hand, the line width of ^{31}P NMR has been employed to characterize the headgroup motion of phospholipids (Berden et al., 1974), and a marked difference has been found between the signals of PC and LPC (Wu et al., 1984). Moreover, the phosphorus signals are influenced by the states of phospholipids such as lamella, liposome, and emulsion (Smith and Ekiel, 1984).

Previously, we investigated the headgroup motion of emulsified PC and LPC by ^{31}P NMR and T_2^* relaxation times of the glycerol and choline carbons and found that the emulsion stability correlated well with the headgroup motional properties of phospholipids (Chiba and Tada, 1989). In a simple emulsion system composed of water, *n*-decane, and egg PC, a significant broadened signal of phosphorus was observed and the emulsion was unstable. It was suggested that PC was prone to aggregate to form lamella in emulsion. LPC formed a stable emulsion in comparison with PC; however, emulsion breaking occurred when the headgroup motion of LPC had much motional freedom. On the other hand, an equimolar mixture of PC and LPC had adequate freedom in emulsion, and the emulsion became more stable. As the acyl chain at glycerol *sn*-2 is replaced by a proton, the headgroup of LPC can rotate rapidly. Consequently, in the mixture of PC and LPC, it is suggested that the headgroup of PC also has motional freedom by the interaction of LPC and that they formed a stable emulsion.

Thus, we expected that the headgroup motion of PC also increased by replacement of acyl chain at glycerol *sn*-2 with short-chained one and that a stable emulsion could be obtained by using the short-*sn*-2-chained PC as an emulsifier. In this paper, we discuss a relationship between headgroup motion and emulsion stability comparing physical properties of short-chained PC at glyc-

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erol *sn*-2 (short-*sn*-2-chained PC) and at glycerol *sn*-1 and *sn*-2 (short-*sn*-1,2-chained PC) and a mixture of PC and LPC.

MATERIALS AND METHODS

Preparation of PC and LPC. PC of more than 95% purity was prepared from egg lecithin, and LPC was also separated by silica gel column chromatography after phospholipase A_2 treatment of PC to a purity of more than 95% (Chiba and Tada, 1988). The fatty acid composition of LPC at glycerol *sn*-1 was analyzed by GLC after transmethylation. The results were as follows: $C_{16:0}$, 56.7%; $C_{16:1}$, 1.3%; $C_{18:0}$, 24.8%; $C_{18:1}$, 14.1%; $C_{18:2}$, 2.0%; unidentified, 1.1%.

Synthesis of Short-*sn*-2-Chained PCs. The hydroxide of LPC at glycerol *sn*-2 was acylated by acetic, butanoic, hexanoic, octanoic, or decanoic acid anhydride in the presence of 4-(dimethylamino)pyridine in dry $CHCl_3$ (Regen et al., 1982), and the desired PCs were obtained in 60–80% yields. The resulting short-*sn*-2-chained PC is then purified by elution from silica gel, employing a gradient of chloroform and methanol. During the acylation of LPC, migration of the acyl chains can occur, resulting in contamination of the mixed-chain PC by positional isomer of the desired product (Mason et al., 1981b). The amount of this contamination is determined by HPLC after phospholipase A_2 treatment. The isomeric impurity, which was determined by the amount of short-chained LPC, was less than 7 mol %. The fatty acid composition at glycerol *sn*-1 was confirmed by GLC to be approximately same as that of LPC. The prepared PCs were abbreviated as follows: 2(C_6)PC indicates that the acyl chain of egg PC at glycerol *sn*-2 was substituted by hexanoic acid. The prepared PCs possess the acyl chain composition of LPC at glycerol *sn*-1.

Synthesis of Short-*sn*-1,2-Chained PC. Glycerophosphorylcholine (GPC) was obtained by the hydrolysis of PC with tetrabutylammonium hydroxide in ether (Regen et al., 1982), and the purified GPC was acylated by hexanoic, octanoic, or decanoic acid anhydride by the same procedure of short-*sn*-2-chained PC, and desired PCs were purified by silica gel column chromatography to a purity of more than 98%. The prepared PCs were abbreviated as follows: 1,2(C_6)PC indicates that the acyl chain of PC at glycerol *sn*-1 and *sn*-2 was substituted by hexanoic acid.

Measurements of NMR Spectra. ^{13}C and ^{31}P NMR spectra were recorded on a JEOL GX-270 spectrometer. T_2* relaxation times of the glycerol and choline carbons were calculated approximately from the line width of the signals ($\nu_{1/2}$), $T_2^* = 1/(\pi\nu_{1/2})$.

Emulsification and Evaluation of Stability. The short-chained PCs or an equimolar mixture of egg PC and LPC was dispersed in deionized water. Viscosity of their 1% aqueous dispersion was determined at 25 °C on a viscometer (Type B8L, Toki Sangyo Co., Ltd.) fitted with spindle No. 2 at 12 rpm. Emulsification was achieved by addition of *n*-decane (5 mL) dropwise to the aqueous dispersion of 64 mM phospholipids (5 mL) with agitation by a disperser (physcotron equipped with generator shaft NS-10) at 20 000 rpm for 2 min. Deionized water and 0.01 M HCl solution were used as an aqueous phase. The emulsion stability was evaluated by measuring the ratio of the separated aqueous layer to the total emulsion volume after the mixture was allowed to stand for 24 h at 25 °C and by the change of mean diameters of the emulsion droplets. The particle size was measured by multichannel particle counter (Coulter counter, Model T_{AII} fitted with apertures for 50 and 140 μm) calculated by the volumetric percentage.

RESULTS

Figures 1 and 2 show ^{13}C NMR spectra of aqueous dispersions and emulsions of short-*sn*-2-chained PCs and short-*sn*-1,2-chained PCs. The choline and glycerol carbons of PCs have been assigned as shown (Birdsall et al., 1972; Ramsammy et al., 1983). Relative heights and line widths of glycerol signals were influenced by the acyl chain length, and they also changed after emulsification. In the aqueous dispersion, the line widths of phosphorus

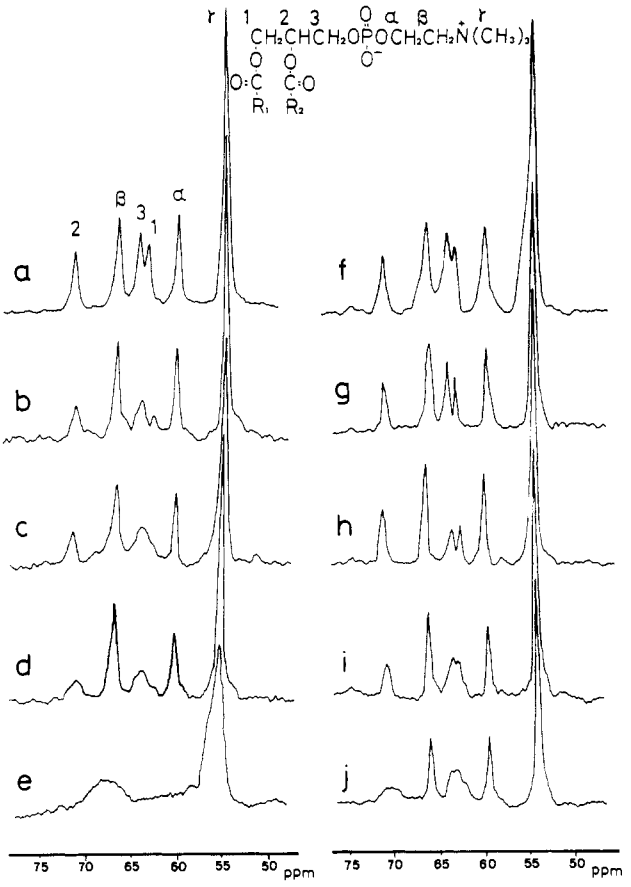

Figure 1. ^{13}C NMR spectra of short-*sn*-2-chained PCs in the region of the choline and glycerol carbons. Aqueous dispersion of (a) 2(C_2)PC, (b) 2(C_4)PC, (c) 2(C_6)PC, (d) 2(C_8)PC, and (e) 2(C_{10})PC and emulsified states of (f) 2(C_2)PC, (g) 2(C_4)PC, (h) 2(C_6)PC, (i) 2(C_8)PC, and (j) 2(C_{10})PC in *n*-decane and deionized water.

singnals of 2(C_2)PC, 1,2(C_6)PC, and 1,2(C_8)PC were narrower than 100 Hz (Figure 3) and T_2* values of glycerol *sn*-2 carbons were larger than 6 ms. These values were similar to those of LPC in globular micelles; consequently, it indicated that 2(C_2)PC, 1,2(C_6)PC, and 1,2(C_8)PC had headgroup motional freedom and were highly dispersive in the globular micelles. Their line widths of phosphorus and glycerol *sn*-2 carbons broadened by emulsification (Figure 4), indicating that their headgroup motion was restricted on the emulsion droplets in comparison with that in the aqueous dispersion.

The phosphorus and canbon signals of 2(C_4)PC, 2(C_6)PC, and 2(C_8)PC became broader and the viscosity of their aqeuous dispersion became higher (Table I) with increasing *sn*-2 chain length. On the other hand, mixtures of PC and LPC have been known to form diverse micelles by the interaction of LPC with PC; furthermore, the viscosity of the aqueous dispersion was also higher than 50 cP and the line width of phosphorus signal was around 250 Hz. It was interesting that the physical properties of short-*sn*-2-chained PC agreed with those of the mixture of egg PC and LPC. The results suggested that the intermolecular hydrophobic interaction of 2(C_4)PC, 2(C_6)PC, or 2(C_8)PC in the aqueous dispersion was similar to that of the mixture of egg PC and LPC, and these phospholipids arrayed continuously so as to increase the viscosity of the aqueous phase.

The aqueous dispersion of 2(C_{10})PC and 1,2(C_{10})PC gave broad phosphorus signals of more than 2000 Hz, indicating that their headgroup motion was tightly restricted. These phosphorus and carbon signals resem-

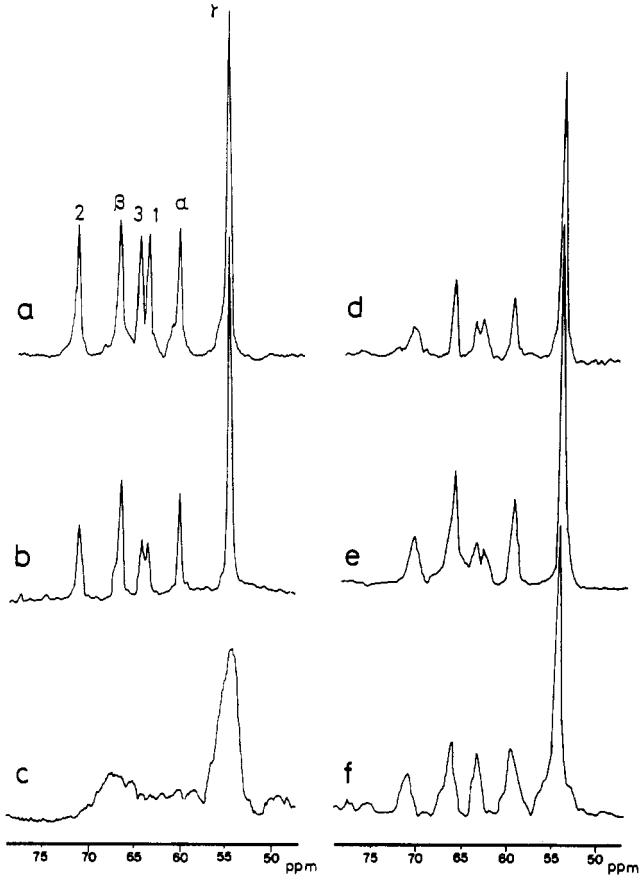

Figure 2. ^{13}C NMR spectra of short-*sn*-1,2-chained PCs in the region of the choline and glycerol carbons. Aqueous dispersion of (a) 1,2(C_6)PC, (b) 1,2(C_8)PC, and (c) 1,2(C_{10})PC and emulsified states of (d) 1,2(C_6)PC, (e) 1,2(C_8)PC, and (f) 1,2(C_{10})PC in *n*-decane and deionized water.

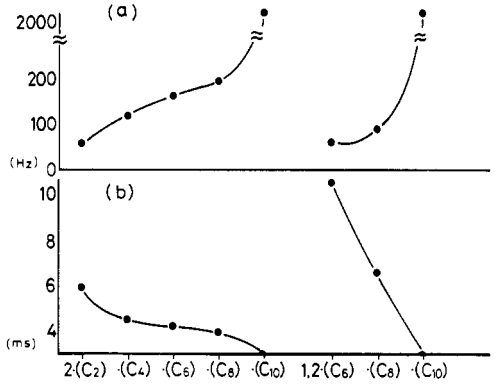

Figure 3. (a) Line widths of phosphorus NMR and (b) T_2* values of the glycerol *sn*-2 carbon of PCs in the aqueous dispersion (64 mM).

bled the signals of egg PC, and the widely broadened phosphorus signal has been regarded as the typical bilayer structure (Wu et al., 1984). It is therefore presumed that the viscosity of their aqueous dispersion became lower than 50 cP by forming multilamellar vesicles. On the other hand, the headgroup motional freedom of 2(C_4)PC, 2(C_6)PC, 2(C_8)PC, 2(C_{10})PC, and 1,2(C_{10})PC increased by emulsification with *n*-decane, and the phosphorus signal of multilamellar vesicle was not observed in the emulsion of 2(C_{10})PC and 1,2(C_{10})PC. These results indicated that the intermolecular interaction of PCs in the aqueous dispersion decreased after their reconstitution on the interface of the emulsion.

The emulsion stability varied with the phosphorus line widths and T_2* values of *sn*-2 carbon of PCs, which correlated with their acyl chain composition. Parts a and b of Figure 5 show the change of the mean diameters of the emulsion droplets and the ratio of separated aqueous layer during 24-h standing. Stable emulsions were obtained with 2(C_6)PC and 2(C_8)PC, and the stability almost agreed with the emulsion of the equimolar mixture of egg PC and LPC. Although the emulsions of 2(C_2)PC, 2(C_4)PC, 2(C_{10})PC, and short-*sn*-1,2 chained PCs were unstable in comparison with those of 2(C_6)PC or 2(C_8)PC, they became stable by acidifying the aqueous phases, except for the emulsion of 2(C_{10})PC in which a phase inversion was observed. In the stable emulsion, the phosphorus line widths were about 100 Hz and T_2* values of *sn*-2 carbon were approximately in the range 6–7 ms. The result suggested that an appropriate headgroup motion was required for PCs on the interface to form a stable emulsion.

The correlation of the emulsion stability with the line widths of phosphorus and glycerol *sn*-2 carbons suggested that the motional freedom and the radius of the headgroup rotation changed with the conformation of PCs

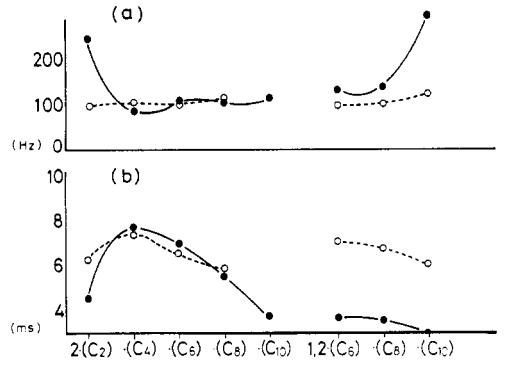

Figure 4. (a) Line widths of phosphorus NMR and (b) T_2* values of the glycerol *sn*-2 carbon of PCs in emulsion. Key: ●, deionized water as an aqueous phase; ○, 0.01 M HCl as an aqueous phase.

Table I. Viscosity (cP) of 1% Aqueous Dispersions of PCs Measured by a Viscometer at 25 °C

2(C_2)PC	<50
2(C_4)PC	70
2(C_6)PC	120
2(C_8)PC	140
2(C_{10})PC	<50
1,2(C_6)PC	<50
1,2(C_8)PC	<50
1,2(C_{10})PC	<50
PC + LPC	85

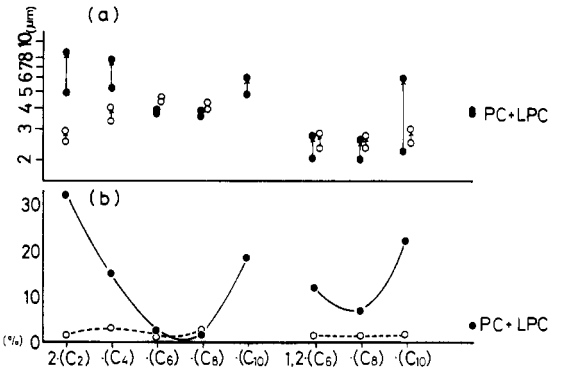

Figure 5. (a) Change of mean diameters of the emulsion droplets during 24-h standing at 25 °C. (b) Ratios of the separated aqueous layer of emulsions after 24-h standing at 25 °C. Key: ●, deionized water as an aqueous phase; ○, 0.01 M HCl as an aqueous phase.

and with the interaction between their headgroups and oil phase; consequently, the motional properties was thought to reflect the interfacial absorptivity of PCs.

DISCUSSION

The headgroup motional properties of PCs in the aqueous dispersion and emulsion were evaluated by ^{31}P and ^{13}C NMR spectra. In aqueous dispersion, the headgroup of LPC had motional freedom in comparison with that of egg PC. As the *sn*-2 acyl chain of LPC is replaced by a proton, the headgroup can rotate rapidly around *sn*-1 carbon; however, the headgroup of egg PC is almost restricted to rotate around *sn*-1 carbon (Wu et al., 1985). The difference of the headgroup motion between egg PC and LPC can be observed by NMR signals, and it has also been affected by the length of *sn*-2 chains. In the aqueous dispersion of short-*sn*-2-chained and short-*sn*-1,2-chained PCs, the phosphorus signals broadened with decreasing T_2^* values of the *sn*-2 carbon. Thus, the motional freedom of the glycerol *sn*-2 carbon correlated well with the rotational magnitude and the motional freedom of the phosphate residue. Namely, the motional freedom of the glycerol *sn*-2 carbon allowed the rapid rotation of the headgroup around the *sn*-1 carbon to form an anticone conformation. On the other hand, the radius was presumed to be shorter with restriction of the *sn*-2 carbon. Accordingly, the decreased T_2^* values of the *sn*-2 carbon of 2(C_{10})PC and 1,2(C_{10})PC indicated that they changed to a cylindrical conformation with restriction of the headgroup motion and were prone to aggregate in lamella.

In addition to the rotational radius controlled by the *sn*-2 chain length, interaction of the headgroup with the oil phase has been expected to occur on the interface of the emulsion. The narrow phosphorus and *sn*-2 carbon signals of the stable emulsion such as 2(C_6)PC and 2(C_8)PC indicated that their headgroup had motional freedom on the interface of the emulsion, whereas broad signals such as 2(C_{10})PC and 1,2(C_{10})PC in the emulsion suggested that they had less interfacial absorption force and were prone to aggregate. In the acidic condition, their interfacial absorption force was thought to increase as they become more hydrophobic by the protonation of the phosphate residue. In the emulsion of 2(C_{10})PC, the phase inversion was presumed to occur with higher hydrophobicity. It was presumed that the dynamic structure and the interfacial absorptivity of PCs correlated well with the headgroup motional properties evaluated by the signal of *sn*-2 carbon. That is, the liberation and the aggregation of PCs on the interface caused the association of oil particles to make the emulsion unstable; consequently, the headgroup of PCs in the stable emulsion was required to have an apparent magnitude and form the anticone conformation to fit on the interface of a globular particle.

CONCLUSION

Egg PC possesses mixed fatty acids of varying chain lengths and degrees of unsaturation, and most of the saturated chains exist at glycerol *sn*-1 and unsaturated chains at glycerol *sn*-2. Unsaturated acyl chain at glycerol *sn*-2 is presumed to increase the motional freedom of the headgroup compared with the saturated one; however, egg PC is still prone to aggregate to form lamella in emulsion, and the emulsion is not sufficiently stable. With the addition of LPC, an aqueous dispersion of phospholipids increased the viscosity and the headgroup of PC had motional freedom; therefore, the emulsion of PC and LPC became stable. By the shortening of the glycerol *sn*-2 chain, the PCs increased their headgroup motional freedom to form a stable emulsion, and the physical properties of 2(C_6)PC and 2(C_8)PC were similar to that of the equimolar mixture of PC and LPC.

It is interesting that the similarity of physical properties has been found between short-*sn*-2-chained PCs and the mixture of PC and LPC. It suggested that, in nature, the diverse functions of phospholipids were closely correlated with the headgroup motional properties, and they have been controlled by the interaction between phospholipids.

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Registry No. 1,2(C_6)PC, 34506-67-7; 1,2(C_8)PC, 19191-91-4; 1,2(C_{10})PC, 3436-44-0; glycerophosphorylcholine, 28319-77-9.