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Persistence at low temperature of the $P_{\beta'}$ ripple in dipalmitoylphosphatidylcholine multilamellar vesicles containing either glycosphingolipids or cholesterol

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The disappearance and reappearance of the $P_{\beta'}$ ripple in multilamellar liposomes of dipalmitoylphosphatidylcholine (DPPC) has been examined by freeze-etch electron microscopy. The presence of less than 10 mol% of various glycosphingolipids or cholesterol in the liposomes markedly increases the time required for ripple disappearance when the vesicles are cooled from 38°C to 30°C, as compared to the pure phospholipid. Once the ripples have begun to disappear in the two-component vesicles, they do not uniformly reappear until the system is heated above the main transition of DPPC and allowed to cool into the pretransition region. These results suggest that the long time for ripple disappearance in the two-component systems reflects a slow molecular reorganization process which occurs when the systems are forced to change from the $P_{\beta'}$ gel to the $L_{\beta'}$ gel by a temperature downshift.

Introduction

Phosphatidylcholines exhibit well characterized temperature-dependent phase transitions. Dispersions of this class of phospholipid in excess water, examined by a variety of methods, have been shown to exist at low temperatures as lamellar solid phases which are crystalline or gel. Upon heating they undergo a variable number of phase changes ultimately forming the fluid L_α liquid-crystalline phase. The number of phase states and their transition temperatures are dependent on the chain length and degree of unsaturation of the esterified fatty acids. Disaturated phosphatidylcholines exhibit a phase change from a $L_{\beta'}$ gel to a $P_{\beta'}$ gel at the so-called pretransition. The $P_{\beta'}$ gel in turn gives way to an L_α liquid-crystalline phase at the temperature of the main transition. In the electron microscope, freeze-fracture replicas of multilamellar liposomes formed from such phosphatidylcholine classes, quenched in the $P_{\beta'}$ phase, are seen to have a long ridge system or 'ripple' in the bilayer with a uniform period of approximately 150 Å [1].

Several models have been proposed to explain this structure. Larsson [2] has suggested that rippling or folding of the vesicle surface is a way of accommodating the mismatch in cross-sectional area of the headgroup and the acyl chains while maximizing Van der Waals interactions. Marder et al. [3] propose alternating regions of fluid-like and gel-like phospholipid. Recently, Georgallas and Zuckerman [4] have defined a statistical model that correlates the occurrence of one or two *gauche* isomers per acyl chain with the periodic vertical displacement of phospholipid molecules.

Tsuchida and co-workers [5] have examined by electron microscopy the disappearance and reappearance of the ripple structure in multilamellar liposomes of dipalmitoylphosphatidylcholine (DPPC) when the temperature is changed. This phosphatidylcholine calorimetrically exhibits a pretransition at 32.5°C and a main transition (T_m) at 41.5°C [6]. When liposomal dispersions are rapidly shifted from 38°C to 30°C these authors reported that the ripple disappears in two stages. The first is a widening of ripple spacing, which is maximal in about 3 min. The second is a much slower decrease in ripple amplitude, with the ripples vanishing in about 3 h.

In the course of our freeze-fracture electron microscopic studies on the organization of glycosphingolipids in various phospholipid matrices, we observed that liposomes composed of DPPC with relatively low mole fractions of various glycosphingolipids exhibit the characteristic $P_{\beta'}$ ripple when held for periods of time much

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in excess of three hours at temperatures well below that of the pretransition. A preliminary report of portions of this work has appeared previously [7].

Materials and Methods

Lipids. DPPC was obtained from Avanti Polar Lipids and was used without further purification. Asialo-G_{M1} was purified from mixed bovine brain gangliosides as previously described [8]. G_{M1} was isolated as previously described [9]. Three times recrystallized cholesterol was a gift from L. Bar, the Forssman glycolipid was obtained from the mucosa of dog small intestine as reported by Sung et al. [10]. The purity of all lipids was confirmed by the single band that appeared following H_2SO_4 charring of an HPTLC plate developed in chloroform/methanol/water (60:35:8, v/v). As previously reported, Forssman glycolipid migrated as two bands that did not resolve well, an upper representing approximately 80% of the material and a smaller lower band. The two bands may represent different molecular species varying in the length of the amide-linked acyl chains [11].

Vesicles. Multilamellar liposomes were prepared by mixing the desired amounts of the lipids together as chloroform/methanol (2:1, v/v) solutions and evaporating to dryness with N_2. They were then resuspended in a small amount of chloroform/methanol (2:1, v/v) and transferred to a specially designed conical flask. Solvents were removed by rapid rotary evaporation at reduced pressure and a temperature of 55°C to minimize unmixing of the lipids. Lipids were resuspended in phosphate-buffered saline (150 mM sodium phosphate, 0.9% NaCl, pH 7.4) with 0.02% sodium azide at 55°C with gentle vortexing, typically to a concentration of 5 mM. Glycolipid concentrations were determined by the fluorescamine assay of Higgins [12] and phospholipid concentrations by the method of Marshall-Stewart [13]. The presence of glycosphingolipids in the bilayer was confirmed by visualization of specific anti-glycolipid antibodies bound to the surface of the vesicles using the technique of freeze-etch electron microscopy (Rock and Tillack, unpublished observation).

Temperature shift experiments. Vesicle suspensions were initially raised to 45–50°C, a temperature above the T_m of DPPC, for at least 30 min and then allowed to cool to 38°C in a water bath. Specimens for electron microscopy were quenched from 38°C or from 30°C, after being held for various times at this temperature, by plunging into liquid freon cooled by liquid nitrogen and then transferred to liquid nitrogen. Alternatively, specimens were quenched from 28°C to be certain that the vesicles were sufficiently below the pretransition. The results were the same regardless of a 28°C or 30°C quench temperature. The specimens were freeze-fractured in a Balzers freeze-etching device and replicas prepared by platinum/carbon shadowing. Replicas were floated onto distilled water, cleaned in chloroform/methanol (2:1, v/v) to remove traces of lipid, picked up on 200 mesh grids and observed in an Hitachi HU 12A electron microscope [8].

Results

Ripple behavior in pure DPPC

We also observed the disappearance of ripples, as previously described [5], to be a two stage process. The first, an increase in ripple spacing, occurs over a time scale of several minutes, when the vesicles are cooled from 38°C to 30°C. The second, a slow loss of ripple amplitude is virtually complete within three hours after the temperature downshift. The reappearance of ripples is a rapid process when the temperature is raised from 23°C to 35°C. Ripples begin to appear within minutes and the regular ripple pattern is essentially established within 10 minutes.

Two differences between our observations and the previous findings should be noted. Unlike Tsuchida et al. [5], we frequently observed during the first stage of ripple disappearance a ripple which terminates between two continuous ripples (Fig. 1). This suggests that the increase in ripple spacing in the first stage may be due to a loss of ripples rather than, or in addition to, the actual physical movement of ripples away from each other as suggested by Tsuchida et al. [5]. Also, in contrast with these authors is our observation that infrequent areas containing widely spaced ripples are still apparent many days after the temperature downshift.

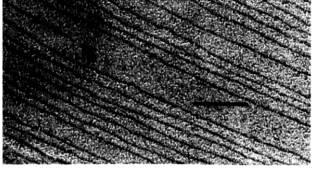

Fig. 1. Replica of a pure DPPC vesicle held for 0.5 min at 30°C after a 38°C incubation, as described in Materials and Methods. Arrow indicates a ripple which has terminated between two continuous ripples. Bar represents 100 nm. The magnification is the same in all figures.

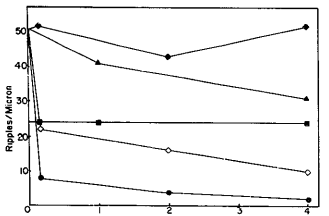

Fig. 2. Graph of ripple density vs. time held at 30°C for DPPC vesicles containing: ●, no additions; ◇, 0.5 mol% Forssman; ◆, 7 mol% Forssman; ▲, 7 mol% asialo-G_{M1}; and ■, 7 mol% cholesterol.

Effect of a second lipid component on ripple disappearance

The presence of 7 mol% of either asialo-G_{M1} or Forssman glycolipid markedly increases the time required to detect an increase in ripple spacing when the vesicles were cooled from 38°C to 30°C. These results are summarized in Fig. 2. The asialo-G_{M1}-containing vesicles begin to show some increase in spacing in about one hour. The changes in ripple spacing are, however, not uniformly distributed over the vesicle surface. Areas on vesicle surfaces that have widely spaced ripples are juxtaposed to areas that continue to have the high ripple density characteristic of the initial 38°C ripple spacing. Two weeks after the shift, there is little change in spacing from the one hour time point (Fig. 3). The Forssman containing vesicles exhibit an even greater tendency for ripple persistence. Changes in ripple spacing are scarcely detectable even after two weeks at 30°C (Fig. 4).

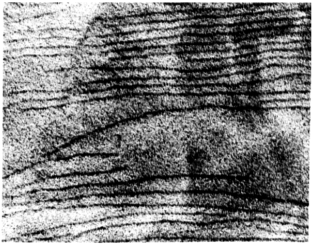

Fig. 3. Replica of a DPPC vesicle containing 7 mol% asialo-G_{M1} held for 15 days at 30°C after a 38°C incubation. Areas of widely spaced ripples coexist with areas of high ripple density.

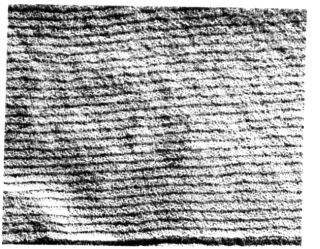

Fig. 4. Replica of a DPPC vesicle containing 7 mol% Forssman held for 15 days at 30°C after a 38°C incubation. No change from the initial 38°C spacing is detected.

Fig. 5. Replica of a DPPC vesicle containing 0.5 mol% Forssman held for 10 min at 30°C after a 38°C incubation. Significant widening of ripples is apparent.

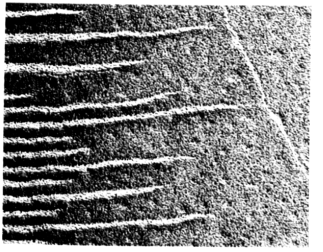

Fig. 6. Replica of a DPPC vesicle containing 7 mol% Forssman allowed to slow-cool from 38°C to 30°C.

A series of time points was obtained with 0.5 mol% Forssman-containing vesicles. Increases in ripple spacing are detectable within minutes of the temperature change, however the ripples continue to persist at a higher density than with the pure phospholipid (Fig. 5).

The effect of a negatively charged glycosphingolipid, ganglioside G_{M1}, on ripple persistence was also examined. The presence of 7 mol% G_{M1} causes the ripples to persist in much the same way as the neutral glycolipids. Two weeks after the downshift, very little change in ripple spacing can be discerned.

Cholesterol inclusion in DPPC liposomes has been shown to affect ripple spacing [14]. At cholesterol concentrations up to 20 mol% the ripple repeat distance increases as a function of cholesterol concentration [14]. Cholesterol at 7 mol% increases the ripple repeat distance to approximately 300 Å and decreases the ripple amplitude. Liposomes containing 7 mol% cholesterol show no change in ripple spacing even after two weeks at 30°C.

In the experiments described above, the cooling rate in going from 38°C to 30°C was greater than 8 C°/min. Experiments were also carried out using a much slower cooling rate of about 0.008 C°/min. Aliquots of the slow-cooled vesicle suspensions were fast-frozen and replicas made as described previously. Although ripples continue to persist, all the vesicles containing a second component have significantly more ripple free regions when compared to the rapidly cooled vesicles. The ripples that do remain are more widely spaced (Fig. 6).

Ripple reappearance

As previously reported by Tsuchida et al. [5] the $P_{\beta'}$ ripples reappear in DPPC vesicles when the temperature is increased from 23°C to 35°C. Preliminary experiments with various concentrations of Forssman glycolipid in DPPC vesicles have revealed that one the ripples have widened and/or begun to disappear, they do not reappear with a regular ripple spacing unless and until the temperature is raised above the main transition (42°C for DPPC) for several hours and then allowed to cool back into the $P_{\beta'}$ region (between 32°C and 42°C).

Discussion

The experiments described in this paper confirm most of the results on pure DPPC liposomes reported by Tsuchida and co-workers [5]. The novel finding which we report is the observation that, in DPPC liposomes containing low mole fractions of several glycosphingolipids or cholesterol, the ripple structure, characteristic of the $P_{\beta'}$ phase, persists for very long periods when the temperature is lowered below the pretransition temperature. When the cooling rate from 38°C to 30°C is slowed from 8 to 0.008 C°/min, the fraction of ripple-free surfaces significantly increases. This observation suggests that the persistence of the ripples caused by the second lipid component is a kinetic phenomenon. That is, the second component serves to markedly increase the relaxation time for the disappearance of the ripple structure at low temperatures. It seems possible that this increase in relaxation time is due to a difference in organization of the two lipid components in the $P_{\beta'}$ rippled phase and in the $L_{\beta'}$ phase which is the equilibrium form at 30°C and below. In pure DPPC, since all molecules are the same, reorganization requires only cooperative conformational changes of individual molecules. However, in two component systems, in addition to these conformational changes, the different species of molecules must translate laterally and must cross grain boundaries to achieve molecular reorganization when the phase structure alters in response to a change in temperature. Such molecular redistributions would be expected to be very slow since both the $P_{\beta'}$ and $L_{\beta'}$ phases are gel states.

What then is known about the molecular organization of the molecules in the various phases adopted by the two-component systems examined? A linear localization for cholesterol between the $P_{\beta'}$ ridges of dimyristoylphosphatidylcholine (DMPC) has been inferred by Copeland and McConnell [14] based on the dependence of ripple spacing on cholesterol concentration. G_{M1} appears to adopt a dispersed organization throughout the plane of the bilayer in $P_{\beta'}$ phase DMPC vesicles [9]. Recent freeze-etch labelling studies employing liquid helium fast-freezing have similarly demonstrated a random distribution for Forssman and asialo-G_{M1} in DPPC vesicles quenched in the $P_{\beta'}$ phase (Tillack, Thompson and Rock, unpublished observation). Previous studies [8], which suggested a linear localization of asialo-G_{M1} between the $P_{\beta'}$ ridges of DMPC, are now believed to have been due to an artefactual redistribution of the label because of the much slower freezing process that was available at the time.

It is not known how the glycolipids are organized in the gel phase, since the presence of a glycosphingolipid increases so drastically the rate of formation of the $L_{\beta'}$ phase. However, the phase diagrams for asialo-G_{M1} in DPPC [15] and cholesterol in DPPC [16] suggest that two gel phases coexist in the $L_{\beta'}$ state in both these systems, in the concentration range of our studies. A similar situation appears to be true for Forssman antigen in DPPC (Rock, Biltonen and Thompson, unpublished observation). Calorimetric information available for G_{M1}/DPPC mixtures shows this system to be complex [17]. It thus seems reasonable that the long relaxation time for the ripple structure observed in the two component systems reflects a slow molecular reorganization process that occurs when the systems are forced to change by a temperature downshift from the $P_{\beta'}$ gel to the $L_{\beta'}$ gel. If this is indeed the case, then it seems in

general that relaxation times for structural reorganization in two or more component systems may be expected to be much longer than in one component systems even when liquid-crystalline phases are involved.

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References

1 Hicks, A., Dinda, M. and Singer, M.A. (1987) Biochim. Biophys. Acta 903, 177–185.
2 Larsson, K. (1977) Chem. Phys. Lipids 20, 225–228.
3 Marder, M., Frisch, H., Langer, J. and McConnell, H.M. (1984) Proc. Natl. Acad. Sci. USA 81, 6559–6561.
4 Georgallas, A. and Zuckerman, M. (1986) Eur. Biophys. J. 14, 53–61.
5 Tsuchida, K., Ohki, K., Sekiya, T., Nozawa, Y. and Hatta, I. (1987) Biochim. Biophys. Acta 898, 53–58.
6 Tsuchida, K., Hatta, I., Imaizumi, S., Ohki, K. and Nozawa, Y. (1985) Biochim. Biophys. Acta 812, 249–254.
7 Rock, P., Thompson, T.E., Tillack, T.W. and Young, W.W. (1988) Fed. Proc. 2, A1779.
8 Tillack, T.W., Wong, M., Allietta, M. and Thompson, T.E. (1982) Biochim. Biophys. Acta 691, 261–273.
9 Thompson, T.E., Allietta, M.A., Brown, R.E., Johnson, M.L. and Tillack, T.W. (1985) Biochim. Biophys. Acta 817, 229–237.
10 Sung, S.J., Esselman, W.J. and Sweeley, C.C. (1973) J. Biol. Chem. 248, 6528–6533.
11 Nichols, G.E., Lovejoy, J.C., Borgman, C.A., Sanders, J.M. and Young Jr., W.W. (1986) Biochim. Biophys. Acta 887, 1–12.
12 Higgins, T.J. (1984) J. Lipid Res. 25, 1007–1009.
13 Marshall-Stewart, J.C. (1980) Anal. Biochem. 104, 10–14.
14 Copeland, B.R. and McConnell, H.M. (1980) Biochim. Biophys. Acta 599, 95–109.
15 Maggio, B., Ariga, T., Sturtevant, J.M. and Yu, R.K. (1985) Biochim. Biophys. Acta 818, 1–12.
16 Ipsen, J.H., Karlstrom, G., Mouritsen, O.G., Wennerstrom, H. and Zuckerman, M.J. (1987) Biochim. Biophys. Acta 905, 162–172.
17 Kojima, H., Hanada-Yoshikawa, K., Katagiri, A. and Tamai, Y. (1988) J. Biochem. 103, 126–131.