Ripple Phase in Asymmetric Unilamellar Bilayers with Saturated and Unsaturated Phospholipids†

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ABSTRACT: In a solution of phosphate-buffered saline (PBS), unilamellar bilayers with saturated phosphatidylcholines in one leaflet and negatively charged, unsaturated phospholipids in the other leaflet were observed in the ripple phase at room temperature using atomic force microscopy (AFM). This is the first observation of the ripple phase in asymmetric bilayers. Sodium and phosphate, components of PBS, were found to be necessary for the formation of the ripple structure in the asymmetric bilayers composed of dipalmitoylphosphatitdylcholine (DPPC) and 1-palmitoyl-2-oleoylphosphatidyglycerol (POPG), demonstrating a dependency for specific ions for this phase. These results indicate that the two leaflets of a bilayer are closely coupled to give rise to such a long range and complicated morphology.

The ripple, or $P_{\beta'}$, phase is characterized by large-scale, periodic corrugations in an otherwise planar, phospholipid bilayer. Thought to exist only for temperatures above the pretransition and below the main transition, this phase has been well studied, primarily using differential scanning calorimetry (Mabrey & Sturtevant, 1978; Lewis et al., 1987), X-ray diffraction (Tardieu et al., 1973; Rand et al., 1975; Janiak et al., 1976; Stamatoff et al., 1982), and freeze-fracture electron microscopy (Ververgaert et al., 1973; Luna & McConnell, 1977; Rüppel & Sackmann, 1983; Tillack et al., 1982; Tsuchida et al., 1987). Most of these studies have been with multilamellar bilayers of saturated phosphatidylcholines, even though the $P_{\beta'}$ phase was also observed in bilayers of saturated lipids with other headgroups, such as phosphatidylglycerol (Watts et al., 1978), anionic phosphatidylethanolamine (Stümpel et al., 1980), and doubly anionic phosphatidic acid (Harlos et al., 1979) as well as in bilayers with unsaturated phosphatidylcholines (Ververgaert et al., 1973). Despite the considerable body of literature on the subject, it is the least understood of the phospholipid phases: most of the models suggest that the size of the hydrated headgroup (Parsegian, 1983; Cevc, 1991), the degree of the acyl chain tilt (Doniach, 1979; Lubensky & MacKintosh, 1993), the extent of the chain movement (Schneider et al., 1983; Georgallas & Zuckermann, 1986; Tsuchida & Hatta, 1988), and the strength of intraleaflet interactions (Scott & McCullough, 1991) are significant for its formation. These models describe the ripple phase as a property of the lipids alone, with no ionic requirement, which is consistent with what is observed in vesicles of phosphatidylcholine (Cevc, 1991) but is in contrast with the observations of vesicles of phosphatidic acid (Harlos et al., 1979).

One aspect that has not been studied yet is the extent to which leaflets of different composition can form a common ripple structure. To date, all of the studies of the $P_{\beta'}$ phase have been of membranes with identical leaflets (symmetric bilayers), though methods have been described to prepare bilayers with different leaflets (asymmetric bilayers) (Frey & Tamm, 1991; Mou et al., 1995).

In this paper, we report the first observation of the ripple phase in unilamellar, asymmetric bilayers, using atomic force microscopy (AFM).[1] We found that in a solution of phosphate-buffered saline (PBS) a bilayer with dipalmitoylphosphatidylcholine (DPPC) or distearoylphosphatidylcholine (DSPC) in one leaflet and 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG), 1-stearoyl-2-oleoylphosphatidylglycerol (SOPG), or 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE) (at high pH) in the other leaflet will form, reversibly, the ripple phase at room temperature. We also determined that two components of PBS, sodium and phosphate, were specifically required for the formation of the ripple structure in the DPPC/POPG bilayers. The significance of this work is not only that it directly shows the ripple phase in asymmetric bilayers but also that it suggests there is a more local, interleaflet coupling, which may be relevant to biological membranes.

MATERIALS AND METHODS

Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), dipalmitoylphosphatidylethanolamine (DPPE), distearoylphosphatidylcholine (DSPC), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG), 1-palmitoyl-2-oleoylphos-

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[1] Abbreviations: AFM, atomic force microscopy; PBS, phosphate-buffered saline; DMPC, dipalmitoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DPPG, dipalmitoylphosphatidylglycerol; DPPE, dipalmitoylphosphatidylethanolamine; DSPC, distearoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine: POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; SOPG, 1-stearoyl-2-oleoylphosphatidylglycerol; DOPG, dioleoylphosphatidylglycerol.

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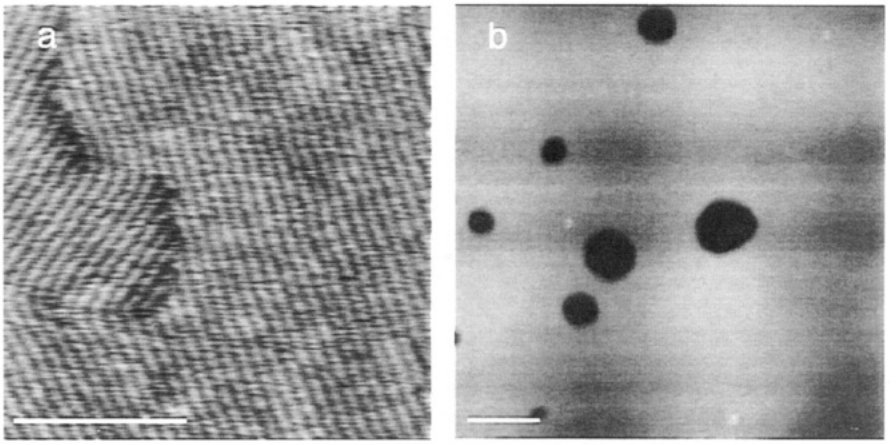

FIGURE 1: Supported, unilamellar bilayers of DPPC/POPG (leaflet 1/leaflet 2) imaged by AFM. In panel a, the bilayer is in PBS and at room temperature (22 ± 2 °C). The ripple structure has a periodicity of 32 nm and an amplitude of 2 nm. In panel b, the DPPC/POPG bilayer is in 0.5 mM KCl, 10 mM NaCl, and 1 mM HEPES, pH 7.5, and at room temperature. The dark regions are defects in the bilayer, and the small (20−100 nm) bright dots are likely adsorbed vesicles. The thickness, as measured from the edge of the defect, is 5.2 ± 0.3 nm, consistent with previous measurements (Mou et al., 1994). Scale bar, 400 nm.

phatidylethanolamine (POPE), 1-palmitoyl-2-oleoylphosphatidylserine (POPS), 1-stearoyl-2-oleoylphosphatidylglycerol (SOPG), and dioleoylphosphatidylglycerol (DOPG) were obtained from Avanti Polar Lipids (Alabaster, AL) and were used without further purification (better than 99% pure, according to the supplier).

A Langmuir trough (J. L. Automaton) was used to prepare the asymmetric bilayer on a freshly cleaved mica surface. The lipids were, first, dissolved in hexane/ethanol (9:1) or chloroform to 1 mg/mL and were applied to the air/water interface of the trough. After waiting for several minutes for the organic solvent to evaporate, the monolayer was compressed to a surface pressure of 45 mN/m. The fragment of mica, initially submerged in the subphase (deionized water), was slowly raised through the interface, depositing the first leaflet (leaflet 1) onto the substrate. After replacing the subphase, the lipids for the second leaflet (leaflet 2) were applied to the interface and compressed to the same surface pressure as the first, after a delay of several minutes for evaporation of the solvent. The monolayer-coated mica fragment was then lowered through the interface, forming the supported bilayer. Bilayers that were prepared from surface pressures between 35 and 45 mN/m formed the ripple phase, but there were fewer defects in those prepared from 45 mN/m.

Although a nonzero flip-flop rate could in principle symmetrize the asymmetrically prepared bilayers, the half-life of transbilayer lipid movement is about 10 h for fluid phase lipids and over 100 h for gel phase lipids, based on studies in vesicles (Kornberg & McConnell, 1971; Wimley & Thompson, 1990). Therefore, assuming that the movement of the gel phase lipid is rate-limiting, the amount of flip-flop should be less than 13% for our experimental period (less than 20 h). The negatively charged mica surface should further reduce this value because of the repulsive electrostatic interactions with the anionic lipids.

The samples were washed thoroughly with the solutions (see Table 1) before being imaged by AFM. For solutions without inorganic phosphate, 1 mM HEPES was used as the buffer. All solutions were maintained at pH 7.5.

The samples were imaged in a Nanoscope II AFM (Digital Instruments, Santa Barbara, CA), in solution. The images were taken in the contact mode, at a force of about 1 nN, with Si_3N_4 tips of spring constant 0.06 N/m. The typical scan speed was 5 Hz, and all images contained 400 × 400 pixels.

RESULTS

Figure 1a shows an image of the ripple structure in a supported bilayer of DPPC/POPG (leaflet 1/leaflet 2), in a solution of phosphate buffered saline (PBS, pH 7.4), at room temperature (22 ± 2 °C). The progression of the bilayer to this phase was rather slow: after 2 h of incubation in PBS at room temperature, the bilayer surface appeared frustrated with irregular bumps; after 4 h of incubation, domains of well ordered ripples began to appear throughout the membrane; and after 12 h of incubation, the ripple phase covered the entire bilayer. A DPPC/POPG bilayer in 0.5 mM KCl, 10 mM NaCl, and 1 mM HEPES, pH 7.5, did not form the ripple structure, even after 20 h (Figure 1b) of incubation.

The periodicity of the ripples varied somewhat from one sample to another, but generally it fell into three distinct ranges (Figure 2a−c): 20 ± 3 nm (n = 30) (the "small" population), 37 ± 5 nm (n = 32) (the "medium" population), and 57 ± 10 nm (n = 27) (the "large" population). In some bilayers, two of the populations of ripples were observed within the same region (Figure 3a,b), but samples that had been incubated in PBS at 55 °C for 0.5 h prior to imaging at room temperature were noticed to have periodicities associated only with the first two groups (data not shown).

The amplitudes of the different populations of ripple structures increased with periodicity: 0.5 ± 0.1 nm (n = 30), 3 ± 1 nm (n = 32), and 7 ± 2 nm (n = 27), for the small, medium, and large populations, respectively. These should be considered as lower limits for the actual amplitude, since the tip may not reach the bilayer surface in the trough of the ripple.

Since PBS was required for the ripple to form, bilayers of DPPC/POPG were imaged in different concentrations of the constituents of PBS (K^+, Na^+, Cl^-, PO_4^{2-}) to determine which were essential to induce the ripple phase. The results are summarized in Table 1. There was a specific requirement for sodium and phosphate for the ripple structure to develop, though in the presence of 0.5 mM potassium, the threshold for sodium was lowered by an order of magnitude.

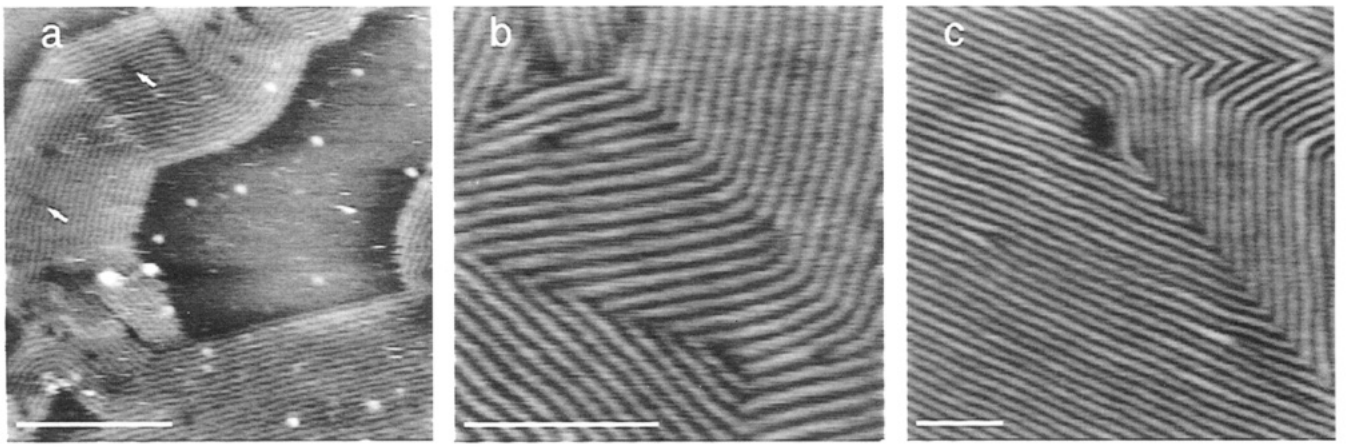

FIGURE 2: Three different types of the ripple structure in unilamellar bilayers of DPPC/POPG in PBS and at room temperature. In panel a, the periodicity is 18 nm and the amplitude is 0.4 nm. The arrows point to defects in the ripple pattern that are not expected for asymmetric ripples (see Discussion). In panel b, the periodicity and the amplitude are 36 and 2 nm, respectively, whereas in panel c, these are 55 and 7 nm, respectively. Scale bar, 400 nm.

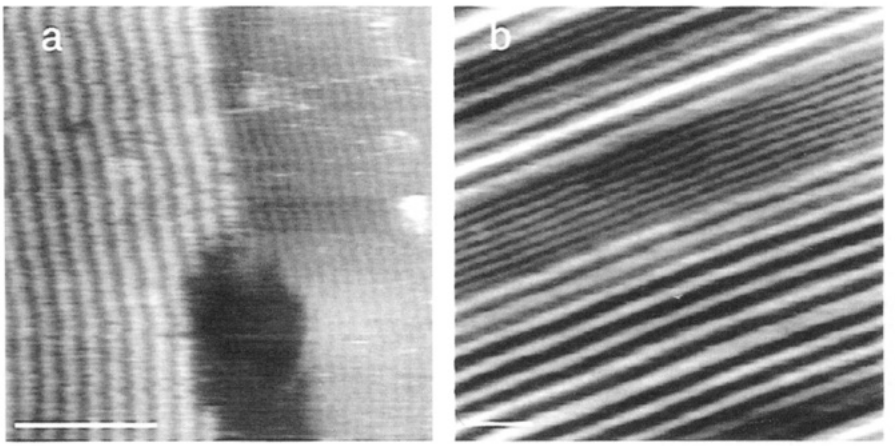

FIGURE 3: Two different types of the ripple structure within the same region of bilayers of DPPC/POPG in PBS and at room temperature. In panel a, the periodicity and amplitude are 17 and 0.5 nm for the "small" ripple structure, and 32 and 2 nm for the "medium" ripple structure. In panel b, the periodicity and amplitude are 32 and 2 nm for the "medium" ripple structure, and 70 and 7 nm for the "large" ripple structure. Scale bar, 200 nm.

Table 1: Dependence of Ripple Formation on Ionic Composition[a]

K^+	Na^+	Cl^-	PO_4^{2-}	ripple	periodicity (nm)
2			1	no	
202			100	no	
2		1		no	
101		100		no	
43		5	20	no	
	17		10	no	
	101	100	0.5	no	
	120	101	10	yes	35 ± 1
5	10	15		no	
50	50	100		no	
5	10	5	10	yes	33 ± 2
0.5	17	0.5	10	yes	36 ± 3
0.5	11	10	0.5	no	
4	136	123	10	yes[b]	$20 \pm 3, 37 \pm 5, 57 \pm 10$

[a] Supported bilayers were imaged at room temperature. Concentrations are in mM. All solutions were at pH 7.5, buffered with 1 mM HEPES when necessary. [b] PBS.

Table 2 : Dependence of Ripple Formation on Lipid Composition[a]

leaflet 1	leaflet 2	ripple	periodicity (nm)
DPPC	DPPC	no	
	DPPG	no	
	POPC	no	
	POPS	no	
	POPG	yes	$20 \pm 3, 37 \pm 5, 57 \pm 10$
	POPE	no	
	POPE[b]	yes	17 ± 1
	SOPG	yes	17 ± 2
	DOPG	no	
DSPC	POPG	yes	48 ± 8
POPC	POPG	no	
DPPE	POPG	no	
POPE	POPG	no	

[a] Supported bilayers were imaged in PBS at pH 7.5 at room temperature, except where indicated. [b] Imaged in PBS with 10 mM CAPS at pH 11.0 at room temperature.

Finally, we examined the composition of each leaflet to determine the lipid specificity for the formation of the ripple phase under these conditions, and the results are presented in Table 2. In general, bilayers formed the ripple phase if leaflet 1 was composed of saturated phosphatidylcholine and if leaflet 2 was composed of mixed chain lipids (*sn*-1, saturated; *sn*-2, unsaturated) with small, negatively charged headgroups [phosphatidylglycerol at pH 7.5 and phosphatidylethanolamine at pH 11.0 (Tsui et al., 1986)]. The progression of these bilayers to the ripple phase was similar to that observed in DPPC/POPG, except for bilayers with DSPC in leaflet 1, which progressed much slower (data not shown). The ripple phase was never observed for symmetric bilayers of DPPC under the same conditions.

DISCUSSION

Significance of Asymmetry. According to previous studies with symmetric bilayers (Marsh, 1990), different phospholipids form the ripple phase for different ranges of temperature. From those studies, none of the lipids used in this work are supposed to exist in the ripple phase at the same temperature, and, moreover, none are expected to be in the ripple phase at room temperature. Symmetric bilayers of DPPC or DSPC are in the gel state at room temperature, whereas those of POPG or SOPG are in the liquid crystalline state at room temperature. Indeed, in supported bilayers of DPPC/DPPC, we did not observe the ripple phase in PBS at room temperature (see Table 2). Although we were unable to prepare supported bilayers of POPG (because of the negatively charged mica), it is unlikely that symmetric bilayers of POPG would form the ripple phase at room temperature, since symmetric bilayers of POPC (which have the same main transition temperature, −2 °C, as POPG) form the ripple phase at −10 °C (Ververgaert et al., 1973). Thus, not only can asymmetric bilayers form the ripple phase, but they do so under a different set of conditions than the symmetric bilayers, implying that interleaflet coupling is significant for the ripple pattern.

Since a pretransition for these asymmetric bilayers cannot be directly inferred from the phase diagrams of the symmetric bilayers, the relationship between the pretransition and the ripple phase is not clear in this case. In fact, the main transition may also be changed because of the cross-bilayer interactions of this morphology, suggesting that the entire phase diagram of some asymmetric bilayers might be different than what the corresponding symmetric bilayers would suggest.

Effects of Ions. The ripple phase is typically considered an intrinsic property of the phospholipids in a bilayer, and the study by Cevc (1991) of symmetric bilayers of saturated PC lipids explicitly concluded that specific ions were not required to form the ripple phase in these membranes. Though we did not conduct a thorough survey of ions beyond the constituents of PBS, the specificity of sodium over potassium and of phosphate over chloride was apparent. Notwithstanding the role that the substrate could have in this specificity, this observation may indicate that one of the lipids required particular, extrinsic factors to form the ripple phase, similar to what was noticed in bilayers of phosphatidic acid (Harlos et al., 1979). In fact, sodium has been observed to interact specifically with PG headgroups in previous studies (Eklund et al., 1987; Pascher et al., 1987). In one, Pascher et al. (1987) crystallized dimyristoylphosphatidylglycerol (DMPG) in the presence of sodium, and they noticed that sodium filled a position in the headgroup region of DMPG, similar to that filled by the positively charged choline moiety in crystals of dimyristoylphosphatidylphosphatidylcholine (DMPC) (linking the phosphate groups into an almost linear array). This coincidence is particularly interesting, given the lipid specificity reported here.

Comparison with Other Ripple Structures. The types of ripples we observed are similar to those observed in symmetric bilayers (unilamellar or multilamellar vesicles). The range of periodicities was rather large, yet a similar range has been noted in studies of symmetric bilayers of DPPC (Hicks et al., 1987). In addition, the lipids that were observed to form the ripple structure in this study had the same headgroups as those that had been observed in the ripple phase in symmetric bilayers. This implies that there might be a set of lipids, defined by the headgroups, that can form the ripple phase only with the other lipids in this set.

The groups we designate as "small" and "medium" are similar in periodicity and amplitude to those referred to as $\lambda/2$ and λ, respectively, in studies of symmetric bilayers (Tillack et al., 1982; Sackmann, 1983; Hicks et al., 1987, Mou et al., 1994). The "large" group varied greatly in periodicity, but the image in Figure 3b suggests that this group may comprise a third ripple type, say 2λ, that is nearly double the periodicity of the λ ripple. The absence of this third type in samples that were incubated above the transition temperature of DPPC suggests that it could be an intermediate state with a free energy higher than the other two.

There is possibly one discrepancy between the prior observations and our own. In symmetric bilayers, there is evidence that the shape of the $\lambda/2$ ripple is asymmetric (one slope longer than the other) and that of the λ ripple is symmetric (Rüppel & Sackmann, 1983; Zasadzinski et al., 1988). Rüppel and Sackmann have argued that the types of defects present in a ripple structure are particular to the symmetry of the ripple. Although we could not determine the shape of the ripples in the AFM images directly, we were able to compare the defects in the ripples we observed with those expected for ripples of different symmetry. In most images, the types of defects in the "small" and "medium" ripple populations conform to those associated with the asymmetric ($\lambda/2$) and symmetric (λ) ripples, respectively. However, the arrows in Figure 2a are directed to defects that, according to Rüppel and Sackmann, should not be present in asymmetric ripples, though they are observed in the "small" ripple population.

Since all theories concerning the ripple phase do not explicitly take account of the interleaflet coupling or of the interaction between the bilayer and specific, extrinsic factors, it is not clear if the results presented here can be explained by these theories. However, since some of the theories are phenomenological, it might be possible to modify the definition of the parameters to accommodate these results.

To conclude, the results presented here demonstrate that the ripple phase is not solely a property of symmetric bilayers. The role of interleaflet interactions in this phase had not been explicitly addressed before, but it is clear from these observations that the nature of this coupling is important to the ripple formation. One of the previous considerations of the relationship between interleaflet coupling and lipid asymmetry focused on the pressure one leaflet might exert on the other and the influence of this pressure on the fusion of vesicles (Devaux et al., 1993). The findings of the present study suggest that a more local and lipid-specific interleaflet coupling may also be present in the asymmetric bilayers. The biological consequences of this more local coupling could be a mechanically induced, cross-bilayer transmission of information that would not be primarily mediated by proteins.

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