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Tris(hydroxymethyl)aminomethane ($C_4H_{11}NO_3$) Induced a Ripple Phase in Supported Unilamellar Phospholipid Bilayers†

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ABSTRACT: A commonly used buffer compound, tris(hydroxymethyl)aminomethane ($C_4H_{11}NO_3$), was found to induce a ripple phase in supported unilamellar phospholipid bilayers at room temperature. The ripple structure showed various types of domains that could extend to several micrometers in length with many well-defined bendings of either 120° or 60°. Two different periodic ripples were found to coexist in 1,2-dipentadecanoyl-*sn*-glycero-3-phosphatidylcholine bilayers. Some intermediate states during the transition to the ripple phase were also observed.

The structure of the phospholipid membrane is an important part for many biological functions, because it provides not only the necessary compartmentalization in a cell but also the proper environment for membrane-associated macromolecules, such as ion-pumping proteins and various channels (Wallach, 1987; Yeagle, 1987; Kotyk et al., 1988; Glaser, 1993). Any structural changes in the membrane, therefore, could have profound influence on various cell functions and biological processes. As a model system, the morphology of phospholipid bilayers had been subjected to intensive study by various methods (Kotyk et al., 1988; Glaser, 1993). It was found that, below the first-order phase transition (main transition) temperature, there exists a so-called ripple phase when the temperature is above the pretransition temperature (Tardieu et al., 1973). So far, it has only been demonstrated in stacked-up bilayers (multilamellar bilayers) that, in the ripple phase, the membrane surface becomes wrinkled with certain periodicities, rather than flat as in the fluid phase ($L\alpha$) or the gel phase ($L\beta$) (Rand et al., 1975; Janiak et al., 1976; Luna & McConnell, 1977; Stamatoff et al., 1982; Parsegian, 1983; Zasadzinski et al., 1988). This ripple phase has only been characterized as a function of temperature and hydration and has not been demonstrated to exist in single bilayers. The induction of the "ripple" phase by other molecules has never been reported.

Most recently, atomic force microscopy (AFM) has been shown to be capable of obtaining high-resolution images of supported biological samples (Engel, 1991; Hoh & Hansma, 1992; Bustamante et al., 1993; Lyubchenko et al., 1993; Yang et al., 1993a,b; Hansma & Hoh, 1993). Therefore, it is particularly suited for the study of the morphology of supported membranes. In this paper, we demonstrate the direct observation of the ripple structure in supported single phospholipid bilayers by AFM under physiological conditions. It was found that a commonly used buffer compound, tris(hydroxymethyl)aminomethane ($C_4H_{11}NO_3$), can reversibly induce the ripple phase *in situ* in lecithin bilayers at room temperature, indicating that the interaction between these small molecules and the lipids can have significant influence on the morphology and the properties of the membrane. Our results also provide direct observation on the domain structures of the ripples, thus shedding some light on the formation and the interaction of the domains in a two-dimensional system.

MATERIALS AND METHODS

Supported phospholipid membranes were prepared by direct fusion of vesicles onto the mica surface (Brian & McConnell,

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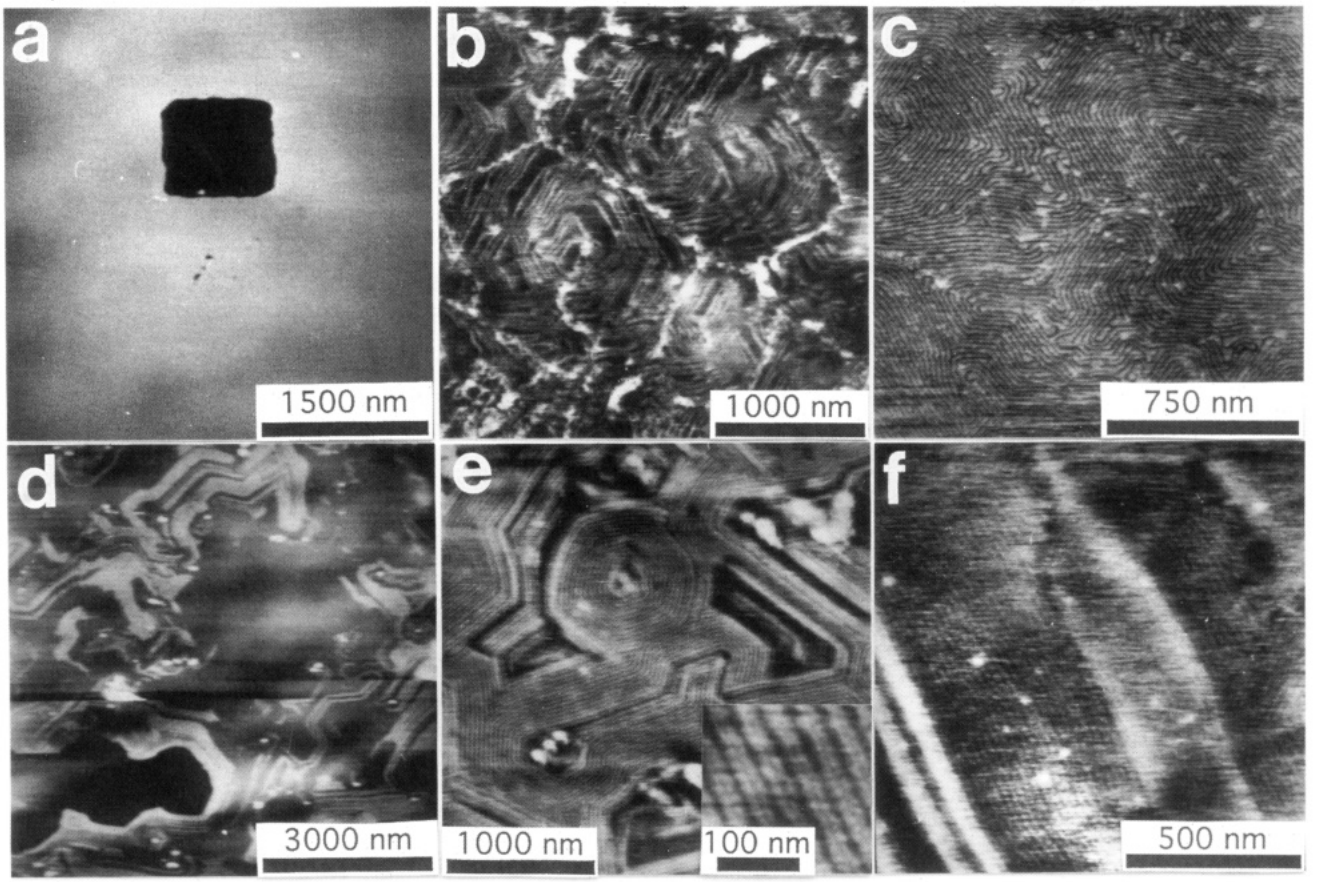

FIGURE 1: AFM images of various membrane surface morphologies at room temperature. diC15-PC was used for its moderate main transition temperature of 33 °C, which can be easily reached in a controlled manner. (a) diC15-PC bilayer on the mica surface as imaged by AFM in 20 mM NaCl. The thickness of the bilayer, as measured from the defect, is about 6 nm, indicating a single bilayer on the surface, consistent with the known value. The dark area is a defect, created by the AFM tip with high speed and large force scanning. Presumably, the bilayer was scraped away by the tip. (b) The same bilayer after 2-h incubation at 20 ± 2 °C in 20 mM Tris and 50 mM NaCl at pH 7.5. Some ripple-like structures have started to form. The membrane thickness is about 7 nm. (c) After 16-h incubation in 50 mM Tris and 20 mM NaCl, the ripple structure was observed. The periodicity of the ripple, though not perfectly ordered yet, is 18 ± 2 nm, with an amplitude of 0.3 nm. The membrane remained at the same thickness as in (b). (d) After the sample was heated above the main transition temperature (42–50 °C) for 0.5 h in 50 mM Tris and 20 mM NaCl, two distinct domains were observed: thicker domains and thinner domains, with the former about 2–3 nm higher than the latter. (e) High-resolution image of the thicker domain, showing beautifully formed ripple structures of (32 ± 2)-nm periodicity and 1.2-nm amplitude. At even higher resolution (inset), each ripple is seen as two ridges with a separation of 11 nm. Due to the longer period, we also called these domains the 2λ domains. (f) High-resolution image of the thinner domain, showing the (18 ± 2)-nm periodicity and the reduced amplitude (0.3 nm). These structures are called the λ domains.

1984; Pearce et al., 1992). The vesicles were prepared by suspending diC15-PC powder (Avanti Polar Lipids, Alabaster, AL) in 20 mM NaCl. Repeated sonification was used to make unilamellar vesicles (~25 nm in diameter) (Huang, 1969; Huang & Thompson, 1974). A droplet (~40 μL) of the vesicle solution at 1 mg/mL was applied to the freshly cleaved mica surface, followed by heating the droplet to above the main transition temperature for a short period. Normally, the mica surface was covered by a single bilayer after repeated washing (see Figure 1a). The bilayer was never exposed to air. To prepare the mixture, G_{M1} and diC15-PC were first mixed in chloroform and dried under nitrogen. The powder was resuspended in 20 mM NaCl and 1 mM EDTA, pH 7.5, at 1 mg/mL. Repeated sonification was used to form unilamellar vesicles. The specimen was heated to 50 °C in 50 mM Tris, 20 mM NaCl, and 1 mM EDTA. Since G_{M1} has a negative charge, 1 mM EDTA was used to eliminate the effect of any residual divalent cations. EDTA never induced the ripple phase under our conditions. All AFM images were obtained in the appropriate solution (see the legend for each figure) with a NanoScope III AFM, with Si_3N_4 tips having a spring constant of $k = 0.06$ N/m (Digital Instruments, Santa Barbara, CA). A homemade fluid cell retrofitted to the commercial AFM was used. Images shown were obtained at room temperature (20 ± 2 °C). The typical scanning speed was 5 Hz, with the probe force controlled at about 0.5 nN.

RESULTS AND DISCUSSION

Supported single bilayers and monolayers prepared by the Langmuir trough under various solutions have been directly observed by AFM at room temperature (20 ± 2 °C), without any intrusive treatment of the specimen (Hoh & Hansma, 1992; Yang et al., 1993a,b; Hansma & Hoh, 1993). We show here for the first time that the fusion of small unilamellar phospholipid vesicles (~25 nm in diameter) can produce stable single bilayers on mica for *in situ* AFM imaging. Figure 1a shows the 1,2-dipentadecanoyl-*sn*-glycero-3-phosphatidylcholine (diC15-PC; main transition temperature 33 °C) bilayer imaged by AFM in 20 mM NaCl. The thickness measured from the edge of defects was ~6 nm, indicating a single bilayer with a hydration layer between the lower leaflet and the mica surface (Tamm & McConnell, 1985). These bilayers were very stable and featureless, even after repeated temperature cycles between 20 and 50 °C. Upon the addition of 50 mM Tris, a physiologically relevant strength, the surface of the membrane immediately started to wrinkle, from very irregular bumps converging to elongated lines. Figure 1b shows the

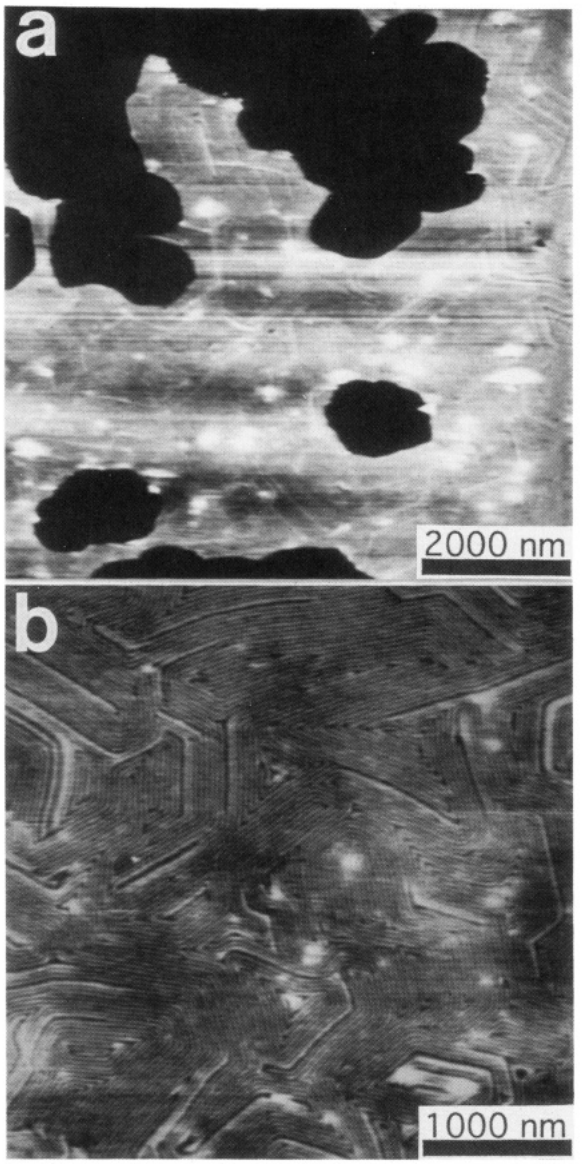

FIGURE 2: Ripple structure in a binary mixture of G_{M1}–diC15-PC (1:9) at room temperature. (a) The large-scale image of the bilayer surface of the mixture of diC15-PC with 10 mol % G_{M1}. Tris (50 mM) was used to induce the ripple structure. It is seen that one domain dominates the structure, which has the same thickness as the thicker domains in pure diC15-PC bilayers. Some domain boundaries could also be seen. (b) At higher resolution, the ripple structure was clearly observed with an even better order than the pure bilayer. The period (32 ± 2 nm) was the same as that of the 2λ domains. For some reason, the addition of GM1 favored the 2λ structure without affecting the periodicity. As in the pure bilayer case, these structures could be repeatedly induced by Tris.

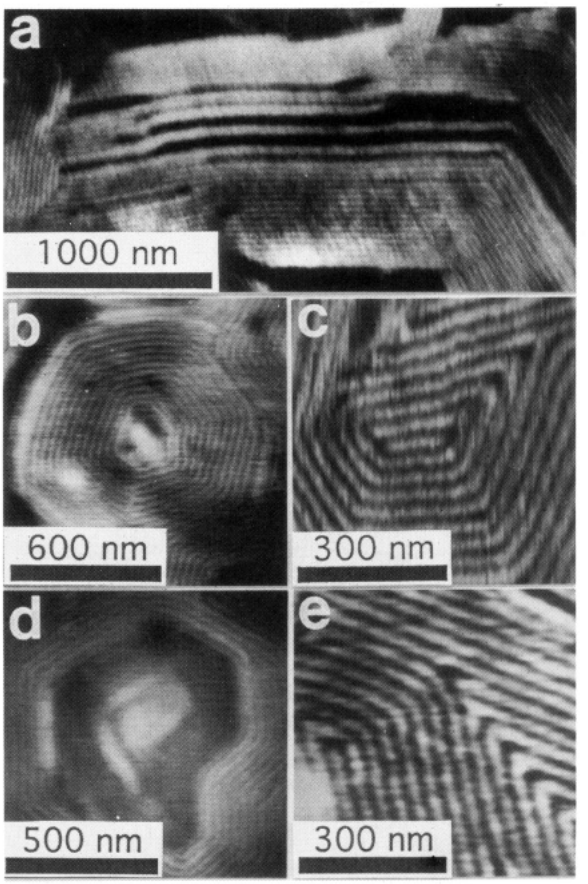

FIGURE 3: Variety of domains in the ripple structure. (a) A long stripe with 120° bends. It is seen that the domain extended to many times of its width. (b) A hexagon: many ripples appeared as closed rings. Notice the 120° bending angles at each corner. (c) This irregularly shaped self-enclosed domain has two 60° bendings and two 120° bendings at the corners. At the center, the ripples have the shape of a trapezoid. (d) A λ domain is seen enclosed in a 2λ structure, like an island. The orientation and the different periodicity of the ripples are clearly seen. Interconversion of these two structures was never observed at room temperature. (e) 60° bend in a stripe-like domain. It is not clear whether such sharp bends were the result of two growing domains or of one domain under the restriction of existing domains.

membrane morphology after 2-h incubation at room temperature (20 ± 2 °C, 50 mM Tris, 20 mM NaCl, pH 7.5). These structures continued to adjust themselves, and a regular pattern started to emerge after 6 h. Figure 1c shows the ripple structure after 16-h incubation at 20 °C in the same buffer. The dominant period is 18 ± 2 nm with an amplitude of ~0.3 nm. The apparent membrane thickness now became 7 nm. After the specimen was heated above the main transition temperature (33 °C) and cooled to room temperature, the membrane morphology was drastically changed. Now two types of domains could be seen (Figure 1d): thicker ones and thinner ones. Measurements showed that the thinner domains had the same bilayer thickness as before, but the thicker domains were about 2–3 nm thicker. At higher resolution, very regular and interesting ripple structures appeared in the thicker domains (Figure 1e) with a periodicity of 32 ± 2 nm, considerably larger than those observed before, and with an average amplitude of 1.2 nm. At even higher resolution, this single ripple was revealed as two ridges with a separation of ~11 nm (Figure 1e, inset). We call these thicker domains 2λ domains, because the periodicity was nearly twice that of the thinner domains (the λ domains) where the periodicity of the ripple structure was 18 ± 2 nm at a lower amplitude (~0.3 nm) with less regularity (Figure 1f). Interconversion between these two domains at room temperature was not observed. Such mixed periodicity was also observed in multilamellar bilayers (Tillack et al., 1982; Sackmann, 1983). There is no explanation why the 2λ domains were 2–3 nm thicker than the λ domains. The molecular packing cannot be inferred directly from these observations. The measured amplitude of the ripple could be smaller than the actual value, because the finite dimension of the tip may prevent the tip from reaching the lowest point of the ripple, and some vertical compression may also be possible, although such compression was never apparent with gel phase membranes. Therefore, it is highly possible that the actual amplitude in the 2λ domain is close to 2–3 nm, as measured from the edge of the domain. Upon removal of Tris through repeated flushing with 20 mM NaCl solution, the ripple structure immediately started to

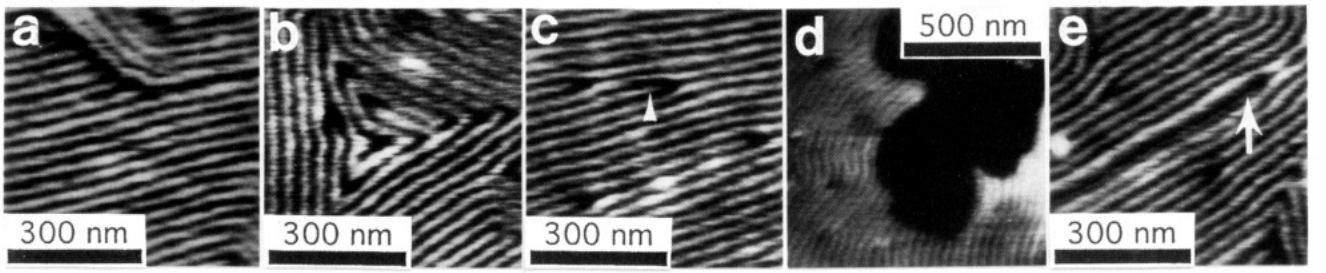

FIGURE 4: Boundary structures of the ripple domains. (a) Two stripe-like domains. One of the domains completely stopped without connecting with or crossing the other. (b) The spirally grown ripple filled the triangular area left by three stripe-like domains. All bending angles were 60°, due to the strong boundary restriction. (c) A new ripple could suddenly appear in a perfectly ordered domain (see arrow) without affecting the structure. These points appear to be the initiation points of the "new ripples". (d) At the edge of the natural bilayer defect, the ripple structure remained nearly flawless in both orientations—perpendicular and parallel to the edge. Therefore, the ripple structure was exceptionally stable and insensitive to defects. (e) When two parallel domains grew to meet, a narrow space was often left that was insufficient for a complete ripple (see arrow). But a weak line in such space can often be seen.

fade. The 2λ domains were converted into λ domains, as the amplitude of the ripple started to decrease. The entire process took about 6 h. On the same specimen, the ripple structure could be repeatedly induced by adding Tris to the buffer. Such processes never damaged the bilayer, showing excellent repeatability and reversibility. Such a phenomenon was not confined to diC15-PC bilayers. In fact, the same treatment also induced the ripple structure in 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) bilayers at room temperature, which is about 15 °C below the pretransition temperature (Rand et al., 1975; Janiak et al., 1976; Luna & McConnell, 1977). The periodicity of the ripples in the DPPC bilayer was 25 nm, about twice those observed in multilamellar vesicles (Tardieu et al., 1973; Rand et al., 1975; Janiak et al., 1976; Luna & McConnell, 1977; Stamatoff et al., 1982; Parsegian, 1983). We ruled out the possibility that a change in ionic strength induced the ripple phase in these membranes by subjecting the diC15-PC bilayers to identical treatment with up to 170 mM NaCl solutions alone, which never induced ripple structure. Therefore, the presence of Tris was necessary for the formation of the ripple structure at room temperature. These results unequivocally demonstrate that the ripple phase can indeed exist in single phospholipid bilayers and that the interlayer interaction is not required.

The same ripple phase was also induced by Tris in the diC15-PC bilayer mixed with 10 mol % ganglioside G_{M1}. It is interesting to note that, at low resolution, the surface appeared rather smooth, with only some domain boundaries visible (Figure 2a). The thickness measured from the edge of the defect was ~10 nm, consistent with the 2λ domains. At high resolution, the ripple structure appeared much better arranged, with many interesting domain shapes. The measured periodicity is 33 ± 2 nm, the same as the one measured in the pure diC15-PC bilayers, indicating the insensitivity of the ripple structure to molecular defects. For unknown reasons, the addition of G_{M1} favored the 2λ structure over the λ structure. It is worth mentioning that G_{M1} was previously found to increase the persistence of the ripple structure in DPPC bilayers without affecting the ripple periodicity (Rock et al., 1989). In all the specimens studied, the so-called "macro" ripple structure was never observed (Zasadzinski, 1988).

The domains of the ripple structure have a variety of interesting shapes, providing an opportunity to study their formation and interaction. Figure 3 shows the most commonly observed domains. The majority of the domains appeared as long stripes with their widths in the range of 0.2–0.6 μm, extending to many micrometers and perfectly ordered (Figure 3a), even at the bending regions. It is noted that the bending angle was primarily 120°, but occasionally 60° angles were observed (Figure 3c,e), due to the hexagonal packing of the lipid molecules, consistent with previous observations (Zasadzinski, 1988). Some of the domains could also form perfectly ordered and self-enclosed ripple structures (Figure 3b,c). In Figure 3b, the structure was rather symmetric, like a hexagon, and in Figure 3c it was trapezoidal-like. Occasionally, a λ domain appeared in 2λ domains (Figure 3d), and these λ domains were normally small but rather stable. At the edge of natural defects, only the 2λ structure was observed for unknown reasons.

Several intriguing features on the domain boundaries were also observed. When two domains grew to meet, the ripples never crossed each other. Often, they came to a sharp stop and remained in perfect order (Figure 4a). Only occasionally could a T-type branching be observed. When three domains grew to fill a space, a triangular area was often left, so that the ripples from one domain could start to grow and fill the area in a sort of spiral fashion with some isolated ripples (Figure 4b). In a growing domain, a new ripple could suddenly appear to fill up the available space (Figure 4c) without affecting the existing order of the domain. At the edge of natural bilayer defects, the ripple structure remained well ordered, either when the ripple was perpendicular or parallel to the boundary (Figure 4d), indicating the exceptional stability of the ripple structure and its insensitivity to geometric defects. When two parallel domains grew to meet, occasionally a narrow gap was left unfilled (Figure 4e), but a very weak line could sometimes be observed in the gap. Due to the very long time required for the ripple structure to form (Figure 1a–c), one should be able to trace the formation of the various domains in "real time" with further refinement of the method, providing some interesting information on the dynamics of a complex two-dimensional system.

How the Tris molecule interacts with the lipid molecules in order to form the ripple structure is not entirely understood. However, the hydration level of the head group, in addition to the temperature, is known to play a critical role in the appearance of the ripple structure in multilamellar bilayers. If the Tris molecule, which was called a "hydrophobic ion" that is protonated at pH 8, would affect the hydration level of the head groups, one would expect some concentration-dependent effect. We tested this idea by varying the concentration of Tris and observed the induction of the ripple structure and its periodicity. To our surprise, beyond the threshold of about 10 mM, the concentration of Tris (up to 500 mM) had no effect on the ripple structure within our measurement accuracy. Below this, the ripple structure could no longer be induced at room temperature. We also found that, with 50 mM Tris even at 4 °C, the ripple structure

remained unchanged, within our observation period, with the same periodicity as measured at 20 °C. This indicates that the Tris molecules drastically extended the region in the phase diagram where the ripple phase exists. Since the charge on the Tris molecules depends on the pH of the solution, we also performed experiments at different pH values. Between pH 5 and pH 9, which changes the charge of the Tris molecules from positive to negative, the same ripple phase was induced in 50 mM Tris and 20 mM NaCl, with the same periodicity. Ripple structure was never induced with 170 mM NaCl with the pH adjusted from 5 to 9 by HCl or NaOH. However, below pH 4.5, the ripple phase was not induced by Tris. It was previously suggested that Tris could induce a fully interdigitated state in 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylglycerol (DPPG) multilamellar bilayers at room temperature (Wilkinson et al., 1987; Slater & Huang, 1988). If this would also happen in the PC bilayers, one should expect to see some reduction in bilayer thickness. This was never observed in these single PC bilayers. Rather, an increase in bilayer thickness was always observed. Therefore, the mechanism of Tris-induced ripple phase remains to be elucidated.

Since Tris could have such a strong effect on the membrane morphology of supported bilayers, even in the gel phase membrane, some effect might also be expected in the fluid phase membrane that is more physiologically relevant. The success of direct observation of the membrane morphology by AFM under physiological conditions also opens the possibility to the study of other drugs that directly act on the membrane, such as those used in anesthetics.

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