Effect of the Structure of Lipids Favoring Disordered Domain Formation on the Stability of Cholesterol-Containing Ordered Domains (Lipid Rafts): Identification of Multiple Raft-Stabilization Mechanisms

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ABSTRACT Despite the importance of lipid rafts, commonly defined as liquid-ordered domains rich in cholesterol and in lipids with high gel-to-fluid melting temperatures (T_m), the rules for raft formation in membranes are not completely understood. Here, a fluorescence-quenching strategy was used to define how lipids with low T_m, which tend to form disordered fluid domains at physiological temperatures, can stabilize ordered domain formation by cholesterol and high-T_m lipids (either sphingomyelin or dipalmitoylphosphatidylcholine). In bilayers containing mixtures of low-T_m phosphatidylcholines, cholesterol, and high-T_m lipid, the thermal stability of ordered domains decreased with the acyl-chain structure of low-T_m lipids in the following order: diarachadonyl > diphytanoyl > 1-palmitoyl 2-docosahexenoyl = 1,2 dioleoyl = dimyristoleoyl = 1-palmitoyl, 2-oleoyl (PO). This shows that low-T_m lipids with two acyl chains having very poor tight-packing propensities can stabilize ordered domain formation by high-T_m lipids and cholesterol. The effect of headgroup structure was also studied. We found that even in the absence of high-T_m lipids, mixtures of cholesterol with PO phosphatidylethanolamine (POPE) and PO phosphatidylserine (POPS) or with brain PE and brain PS showed a (borderline) tendency to form ordered domains. Because these lipids are abundant in the inner (cytofacial) leaflet of mammalian membranes, this raises the possibility that PE and PS could participate in inner-leaflet raft formation or stabilization. In bilayers containing ternary mixtures of PO lipids, cholesterol, and high-T_m lipids, the thermal stability of ordered domains decreased with the polar headgroup structure of PO lipids in the order PE > PS > phosphatidylcholine (PC). Analogous experiments using diphytanoyl acyl chain lipids in place of PO acyl chain lipids showed that the stabilization of ordered lipid domains by acyl chain and headgroup structure was not additive. This implies that it is likely that there are two largely mutually exclusive mechanisms by which low-T_m lipids can stabilize ordered domain formation by high-T_m lipids and cholesterol: 1), by having structures resulting in immiscibility of low-T_m and high-T_m lipids, and 2), by having structures allowing them to pack tightly within ordered domains to a significant degree.

INTRODUCTION

Lipid rafts can be defined as sphingolipid and sterol-rich lipid bilayer domains that exist in the liquid-ordered (L_o) state. Within cell membranes, rafts are thought to coexist with liquid-disordered (L_d)-state domains rich in lipids with unsaturated acyl chains (1,2). L_o-state bilayers often form when cholesterol is mixed with a lipid which by itself forms bilayers with a high T_m, i.e., a lipid with a high gel (solid-like state)/L_d melting temperature (e.g., sphingomyelin). The L_o phase is an intermediate physical state that has tight lipid packing, similar to the gel phase, but which also exhibits high lipid lateral diffusion rates that are only slightly smaller than those of the L_d phase (3–5). The coexistence of raft and nonraft domains in the plasma membrane would allow for segregation of membrane constituents, and although still controversial, is thought to be involved in numerous cellular processes, including cell signaling, trafficking, and bacterial infection (2,6–9). For this reason, understanding the factors that control segregation of lipids and proteins is important to our understanding of raft function.

To define the principles of raft formation, we studied the effect of lipid composition upon raft formation in model membrane vesicles. Previous studies by our group and others have shown that the structure of both sterol and sphingolipid can have marked effects on raft formation (10–17), and that tight packing between sterol and sphingolipids is a major driving force for the formation of L_o domains (13,17). Sterols from fungi and plants share the tight packing properties of cholesterol, suggesting that the ability to form rafts is widespread (12). Studies showing that ceramide displaces cholesterol from rafts have demonstrated that raft composition can be influenced by the need to minimize exposure of lipid hydrophobic groups to the aqueous environment (18–21).

Recently, evidence has been presented, based on detergent insolubility data, in supporting the idea that the presence of lipids containing the polyunsaturated acyl chain docosahexaenoic acid can promote the separation/formation of sphingomyelin and cholesterol-containing ordered domains to a greater degree than less highly unsaturated lipids (22,23). It was proposed that the lesser miscibility of cholesterol in disordered domains rich in lipids with polyunsaturated chains is a driving force for this behavior (23). It has also been shown that cholera toxin, which associates with ordered domains, can induce the formation of disordered lipid domains in bilayers containing lipid mixtures that would otherwise

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form a homogeneous ordered state (24). Via an analogous mechanism, other studies have proposed that because Triton X-100 and sphingolipids are immiscible, Triton X-100 can promote L_o domain formation in membranes containing sphingolipid and unsaturated lipids, even when present in very modest amounts (25,26). (However, recent microscopy studies suggest that the action of Triton X-100 can be to physically cause fission of preexisting L_d and L_o domains (27).) The idea common to all these studies, that certain molecules may induce the formation of domains in which they do not locate, is not unexpected, because domain formation involves a lipid demixing phenomenon, energetically driven not only by the affinity of membrane components for one another but also by their immiscibility in domains formed by components with which they do not interact well.

For this reason, we studied the effect of low-T_m lipid structure on raft formation. In this study, we vary both the acyl chain and headgroup structure of phospholipids that prefer to form L_d domains at physiological temperatures (low-T_m lipids) and determine how they affect formation of ordered domains by high-T_m lipids and cholesterol. To do this, the thermal stability of ordered domains was measured using an approach in which the accessibility of the fluorescent membrane probe 1,6-diphenyl-1,3,5-hexatriene (DPH) to a membrane-bound quencher is measured. Most previous studies required that the quencher be covalently attached to one of the lipids used (11–14,17). In this study, we used the quencher 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO), which binds preferentially to disordered domains to allow variation of the structure of the low-T_m lipid. We find that low-T_m lipid structure can strongly modulate the tendency of high-T_m lipid and cholesterol to form ordered domains. Both low-T_m lipids with phosphatidylethanolamine (PE) and phosphatidylserine (PS) headgroups, and low-T_m lipids with especially poor acyl chain packing properties, can promote domain formation, but they do so in a conflicting manner that illustrates the operation of two distinct raft-formation principles. The implications of this behavior for ordered domain formation in natural membranes are discussed.

MATERIALS AND METHODS

Materials

1,2-Dipalmitoylphosphatidylcholine (DPPC), brain sphingomyelin (SM), 1,2-dioleoylphosphatidylcholine (DOPC), 1,2-dimyristoleoylphosphatidylcholine (DMoPC), 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE), 1-palmitoyl-2-oleoyl-phosphatidyl-L-serine (POPS), 1-palmitoyl-2-oleoyl-phosphatidic acid, 1-palmitoyl-2-oleoyl-phosphatidylglycerol, 1,2-diphytanoyl-phosphatidylcholine (DPhPC), 1,2-diphytanoylphosphatidylethanolamine (DPhPE), 1,2-diphytanoylphosphatidyl-L-serine (DPhPS), 1,2-diarachidonoylphosphatidylcholine (DArPC), 1-palmitoyl-2-docosahexaenoyl-phosphatidylcholine (PDoPC) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). The stable free radical TEMPO and DPH were purchased from Sigma-Aldrich (St. Louis, MO). Acetyl-$K_2W_2L_8AL_8W_2K_2$-amide (LW peptide) was purchased from Anaspec (San Jose, CA). All molecules were used without further purification. Oxidation of DArPC was not detected, as judged by diene absorbance at 233 nm (28). Lipids and probes were stored dissolved in ethanol at −20°C. Concentrations were determined by dry weight or, in the case of DPH, absorbance using $\varepsilon = 88{,}000\ cm^{-1}\ M^{-1}$ at 358 nm in ethanol. 22-diphenylhexatrienyldocosyl-trimethylammonium (LcTMADPH) was a gift of Denis Heissler and Guy Duportail (Université Louis Pasteur, Strasbourg, France).

Vesicle preparation

Multilamellar vesicles (MLV) and ethanol-dilution small unilamellar vesicles (SUV) were prepared as described previously (20,29). For SUV, the desired lipid mixtures dissolved in 15 μl ethanol were dispersed at 70°C in 980 μl of phosphate-buffered saline (PBS) (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, and 2.7 mM KCl, pH 7.4). For MLV samples, lipid mixtures were dried, redissolved in 20 μl chloroform, redried under nitrogen followed by high vacuum for at least 1 h, and then dispersed at 70°C in 1 ml of PBS. Final samples contained 50 μM lipid and 0.5 mol % DPH (for SUV) or 500 μM lipid and 0.05 mol % DPH (for MLV), unless otherwise noted. Background samples lacking fluorescent probe were also prepared when necessary (see below). Samples were cooled to room temperature before fluorescence measurements were made.

Fluorescence and absorbance measurements

Fluorescence was measured on a SPEX Fluorolog 3 (Jobin-Yvon, Edison, NJ) using quartz semi-micro cuvettes (excitation pathlength 10 mm, and emission pathlength 4 mm). Unless otherwise noted, DPH fluorescence was measured at an excitation wavelength of 358 nm and emission wavelength of 430 nm. For DPH fluorescence, slit-width bandwidths for fluorescence intensity measurements were generally set to 4.2 nm (2 mm physical size) for excitation, and 8.4 nm (4 mm) for emission. Trp fluorescence was measured at an excitation wavelength of 280 nm and emission wavelength of 340 nm. Slit-width bandwidths for Trp fluorescence intensity measurements were set to 8.4 nm for excitation and 10.5 nm (5 mm) for emission. The reported values have been corrected for background fluorescence when necessary. However, background intensities were not subtracted in most cases, because they exhibited negligible fluorescence (<2% of the DPH samples). Absorbance was measured on a Beckman 640 spectrophotometer (Beckman Instruments, Fullerton, CA).

Measurement of the temperature dependence of fluorescence quenching by TEMPO

Samples of SUV or MLV were prepared as described above. Two samples containing quencher (F samples) were prepared by adding TEMPO to a final concentration of 2 mM (from a 353-mM stock solution of TEMPO dissolved in ethanol). Two samples lacking quencher (Fo samples) were prepared by adding a volume of ethanol equal to that added to the F samples. F and Fo samples were then incubated for 10 min at room temperature, after which they were cooled to ~16°C and the fluorescence measurements initiated. After measuring cuvette temperature, fluorescence intensity was measured, and this was repeated as a function of increasing temperature (heating continuously, usually at a rate of ~1°C/min). Readings took only a few seconds per sample. The procedure is described in more detail elsewhere (29). The average ratio of fluorescence intensity in the presence of quencher to that in its absence (F/Fo) was then calculated. Background measurements in experiments in which temperature was varied were only taken at the lowest and highest temperatures, usually 16°C and 60°C, and background values at intermediate temperatures were estimated by linear interpolation. In experiments using DArPC, which has polyunsaturated acyl chains, minimizing oxidation by flushing samples with N_2 and then capping them had no effect on the F/Fo versus temperature curves.

Fluorescence anisotropy measurements

Fluorescence anisotropy measurements were made upon duplicate DPH-containing ethanol dilution samples prepared as described above, except that the samples contained 1 mol % DPH. Anisotropy measurements were made at room temperature (and in some controls as a function of temperature, modifying the protocol above by heating a few degrees, measuring anisotropy once temperature stabilized, and then repeating the process) using a SPEX automated Glan-Thompson polarizer accessory with slit-width bandwidths set to 8.4 nm (excitation) and 10.5 nm (emission). Anisotropy values were calculated from the fluorescence intensities, with polarizing filters set at all combinations of horizontal and vertical orientations, after subtraction of fluorescence intensity in background samples lacking fluorophore, when they were significant (>2% of sample fluorescence). The following equation, which corrects for the grating factor (G), was used to calculate anisotropy: $A = [((I_{vv} \times I_{hh})/(I_{vh} \times I_{hv})) - 1]/[((I_{vv} \times I_{hh})/(I_{vh} \times I_{hv})) + 2]$, where A is anisotropy, and I_{vv}, I_{hh}, I_{vh}, and I_{hv} are the fluorescence intensities with the excitation and emission polarization filters, respectively, in vertical (v) and horizontal (h) orientations (30).

It should be noted that absolute T_m values determined by TEMPO quenching were slightly lower (by a couple of degrees Centigrade) than those determined from the temperature dependence of fluorescence anisotropy (which exhibits a transition from high to low anisotropy upon melting of ordered domains (14)) (data not shown). T_m values were harder to determine by anisotropy than by quenching due to a dependence of anisotropy on temperature that was more gradual than that of quenching on temperature (data not shown), in agreement with previous studies (14). Whether the small difference in T_m values estimated by the two methods reflects a distortion due to the temperature dependence of anisotropy within a single phase, or to TEMPO, or to a combination of factors, is uncertain. The small amounts of ethanol used in these experiments appeared to have little effect upon T_m values (not shown). Since the relative stability of ordered domains in different mixtures was of much more importance than absolute T_m values, TEMPO quenching was chosen for most studies.

Measurement of FRET between membrane-bound peptide and a diphenylhexatriene derivative

To measure Förster resonance energy transfer (FRET) between the Trp of LW peptide and LcTMADPH, vesicle samples containing 3:3:2 mol/mol DPPC/low-T_m phosphatidylcholine (PC)/cholesterol with a total of 100 μM of lipid were dispersed in PBS as described above, except that they contained 2 mol % of LW peptide plus 1 mol % of LcTMADPH (F samples) or just 2 mol % LW peptide (Fo samples). Background samples lacking LW peptide were also prepared. Then the intensity of fluorescence was measured as a function of temperature, as described above. In one experiment, vesicles containing 100 μM lipid composed of 3:1 (mol/mol) DPPC/cholesterol, or containing 3:1 (mol/mol) DPPC/cholesterol and 1 mol % LcTMADPH, or containing 3:1 POPC/cholesterol with 2 mol % LW peptide, were prepared. In the F samples, equal aliquots of the LcTMADPH-containing vesicles and peptide-containing vesicles were then mixed, and in the Fo samples, equal aliquots of the DPPC/cholesterol vesicles lacking fluorophore and the peptide-containing vesicles were mixed. Background samples were also prepared as above. The level of FRET as a function of temperature (measured as described above) was then determined, calculating the fraction of unquenched LW peptide fluorescence (F/Fo) after subtraction of background values.

RESULTS

Assaying ordered-domain stability by fluorescence quenching using TEMPO

A fluorescence method that measures quenching of membrane-inserted DPH by the nitroxide-bearing molecule TEMPO was used to determine whether ordered domains/rafts were present in model membrane vesicles. TEMPO, which is water soluble, binds more strongly to disordered fluid-lipid-bilayer domains than to ordered lipid domains, and so preferentially quenches fluorescent molecules in disordered domains (20,31). To examine the thermal stability of ordered domains, two sets of model membrane vesicles are prepared. The first set contains TEMPO (F samples), and the second does not contain TEMPO (Fo samples). In vesicles consisting of lipids forming the ordered state, or containing coexisting ordered- and disordered-state domains, DPH molecules (which usually partition equally between ordered and disordered states (17,32,33)) residing in ordered-state regions are partially protected from contact with TEMPO, and so F/Fo is high. As temperature increases, ordered-state domains melt, allowing the concentration of TEMPO in the vicinity of DPH to increase, and thus F/Fo decreases. When samples exhibit a sigmoidal dependence of quenching upon temperature, an ordered-state T_m can be defined. The greater the stability of the ordered domains, the higher the T_m (12,13,34). It is important to note that for bilayers composed of lipid mixtures the T_m at which the ordered-state domains melt should not be confused with the gel-to-liquid-disordered T_m for bilayers composed of a single lipid. When the terms ''low-T_m'' and ''high-T_m'' lipid are used in this report, they simply refer to the gel-to-liquid-disordered T_m in vesicles composed of a single, pure lipid, with low T_m referring to lipids that have a $T_m < 25°C$, i.e., significantly lower than SM or DPPC.

Experiments were first performed to assess the performance of the TEMPO quenching method. Comparison of quenching in vesicles composed of 1:1 mol/mol DPPC/DOPC and various amounts of cholesterol showed that effective T_m values were more easily defined with 15–25 mol % cholesterol than with 33–40 mol % cholesterol (Fig. 1 *A*). The thermal melting transition was more gradual at the higher cholesterol levels (>25 mol %), presumably because domain size shrinks and melting becomes less cooperative. Thus, 25 mol % cholesterol was chosen for most studies. Quenching was also a function of TEMPO concentration, but leveled off somewhat above 1 mM TEMPO (Fig. 1 *B*). Measurement of F/Fo upon cooling showed that the melting process was reversible (data not shown).

Stability of ordered domains formed by various lipids in the presence of cholesterol: choline lipids

First, TEMPO quenching was used to rank different lipids in terms of their tendency to form ordered domains in the presence of cholesterol. To do this, the thermal stability of ordered-state formation was measured in bilayers composed of single phospholipids mixed with cholesterol (Fig. 2). Fig. 2 *A* compares the melting transitions for ordered states formed by various phosphocholine-headgroup lipids, i.e., SM or phosphatidylcholines (PC) with different acyl chains, in

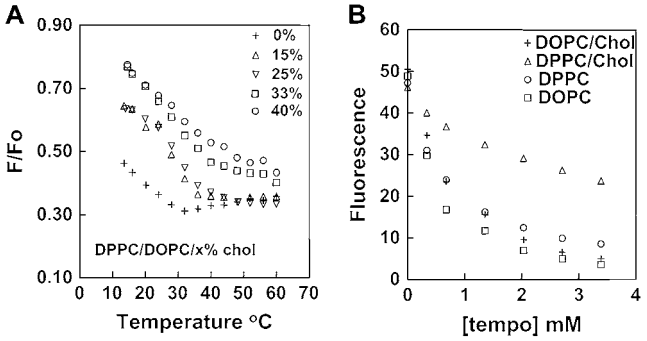

FIGURE 1 Effect of cholesterol and TEMPO concentration upon quenching of DPH fluorescence. (*A*) Effect of cholesterol concentration upon the temperature dependence of TEMPO-induced quenching. Samples contained a 1:1 (mol/mol) mixture of DPPC and DOPC plus 0 mol % (+), 15 mol % (△), 25 mol % (▽), 33 mol % (□), or 40 mol % (○) cholesterol. F samples contained 2 mM TEMPO, whereas Fo samples did not contain TEMPO. Unless otherwise noted, in this and all subsequent experiments, samples contained SUV with 50 μM total lipid plus 0.5 mol % DPH, and were dispersed in PBS at pH 7.4. In Figs. 1–7, average *F*/*Fo* is shown for at least duplicate samples, and ordered domain melting temperatures (defined by the point of maximum slope when a sigmoidal temperature-dependence was observed) did not vary by $>\pm 1.5$°C. (*B*) Effect of TEMPO concentration. Samples contained DOPC (□), 3:1 (mol/mol) DOPC/cholesterol (+), DPPC (○), or 3:1 (mol/mol) DPPC/cholesterol (△). At each successive point, 1-μl aliquots of 339 mM TEMPO dissolved in ethanol was added. Fluorescence (arbitrary units) reached final values immediately after each addition of TEMPO. Experiments were carried out at room temperature.

vesicles containing 25 mol % cholesterol. Mixtures containing high-T_m phospholipids with saturated acyl chains (SM or DPPC) clearly formed ordered states at lower temperature, as shown by the weak TEMPO quenching (high *F*/*Fo* values). These mixtures showed relatively high melting temperatures (35–45°C) as judged by the temperature dependence of quenching, and this is as expected based on previous studies (35,36). There was also clearly some formation of an ordered state at lower temperatures in mixtures of cholesterol with low-T_m PCs having one saturated and one monounsaturated acyl chain (POPC and 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC)), with the midpoint temperature of the melting transition appearing to occur at slightly below the starting temperature of 16°C (use of lower temperatures was prevented by low-temperature-dependent condensation on cuvette walls). Lipids with two monounsaturated acyl chains (DOPC, DMoPC), with one (PDoPC) or two polyunsaturated chains (DArPC), or with branched acyl chains (DPhPC) showed the least tendency to form an ordered state, forming, at most, a small amount of an ordered state at the lowest temperatures investigated.

The ranking of the tendency of different choline-headgroup lipids to form an ordered state in the presence of 40 mol % cholesterol (Fig. 2 *B*) was very similar to that observed in samples containing 25 mol % cholesterol. However, melting of the ordered state appeared to occur at a higher temperature than in the samples containing 25 mol % cholesterol. As noted above, the more gradual ''melting'' process makes determination of a clearly defined melting temperature difficult at this higher cholesterol concentration.

The lipid state present in choline phospholipid/cholesterol mixtures at room temperature was confirmed by measurements of steady-state DPH fluorescence anisotropy (Table 1). In the absence of cholesterol, SM and DPPC, which form the gel state at room temperature (35,36), exhibited high anisotropy values. The remaining lipids, which form the disordered liquid state at room temperature (36), exhibited low anisotropy at room temperature. Anisotropy increased significantly in the presence of cholesterol, especially for POPC. The ranking of anisotropy values in mixtures containing 25 mol % cholesterol followed the order SM = DPPC > POPC > other PC. (SOPC was not tested.) This is

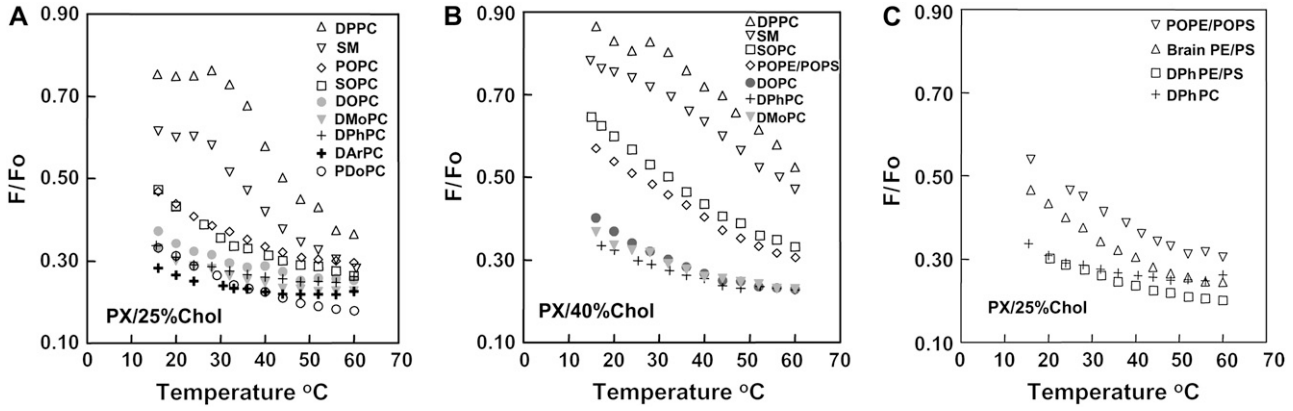

FIGURE 2 Melting curves for ordered domains in vesicles containing phospholipids mixed with cholesterol. Each sample contained 50 μM total lipid dispersed in PBS at pH 7.4. The ratio of DPH fluorescence in the presence and absence of TEMPO (*F*/*Fo*) was measured with increasing temperature. (*A*) Melting curves of binary mixtures of lipids with the PC headgroup and variable acyl chains. Phospholipids were SM (▽), DPPC (△), POPC (◇), SOPC (□), DOPC (●), DMoPC (▼), DPhPC (+), DArPC (+), or PDoPC (○). (*B*) Melting curves for mixtures of lipids with 40 mol % cholesterol. Symbols are the same as in *A*, with the addition of 1:1 (mol/mol) POPE/POPS (◇). (*C*) Melting curves of mixtures of lipids with varied headgroups. Phospholipids were 1:1 (mol/mol) POPE/POPS (▽), 1:1 (mol/mol) porcine brain PE/brain PS (△), DPhPC (+), and 1:1 (mol/mol) DPhPE/DPhPS (□).

TABLE 1 DPH anisotropy values for lipid mixtures containing 0, 25, or 40 mol % cholesterol

		DPH anisotropy			
Lipid	T_m (°C)*	0 mol % chol	25 mol % chol	40 mol % chol	$\Delta[A_{40\%} - A_{0\%}]$
DPPC (36)	41	0.292 ± 0.005	0.312 ± 0.003	0.302 ± 0.017	0.01
SM (35)	36	0.281 ± 0.010	0.308 ± 0.005	0.294 ± 0.011	0.013
POPC (36)	−3	0.100 ± 0.002	0.170 ± 0.008	0.237 ± 0.012	0.137
DOPC (36)	−18	0.090 ± 0.0	0.128 ± 0.006	0.174 ± 0.004	0.084
DMoPC (55)	<0	0.074 ± 0.0	0.115 ± 0.003	0.159 ± 0.014	0.085
PDoPC (36)	−3	0.072 ± 0.001	0.124 ± 0.005	0.167 ± 0.004	0.095
DPhPC (45)	<−120	0.137 ± 0.001	0.154 ± 0.020	0.173 ± 0.001	0.036
POPE (56)	24	0.127 ± 0.002	0.219 ± 0.007	0.235 ± 0.010	0.108
POPE/POPS 1:1	NA	0.126 ± 0.001	0.194 ± 0.010	0.218 ± 0.018	0.092
DPhPE/DPhPS 1:1	NA	0.138 ± 0.007	0.157 ± 0.004	0.166 ± 0.015	0.028

Anisotropy at room temperature (~23°C) was measured in vesicles with a total lipid concentration of 50 μM and containing 0.5 mol % DPH. Vesicles were dispersed in PBS, pH 7.4. Anisotropy values shown are the average and range for duplicate samples, except in the following cases, in which the average and standard deviation are given: DPPC ($N = 5$), SM ($N = 4$), POPC ($N = 5$), DOPC ($N = 5$), DPhPC ($N = 5$). Anisotropy measurements could not be made in DArPC-containing samples due to weak DPH fluorescence. $\Delta[A_{40\%} - A_{0\%}]$ is the increase in anisotropy in the presence of 40 mol % cholesterol relative to that in the absence of cholesterol. A reference citation identifies the study from which that lipid's T_m values were taken. In the case of DMoPC, T_m was estimated. NA, not applicable.

*T_m is the melting temperature for the gel-to-liquid-disordered transition state. For other lipids used in this study, T_m was −70°C for DArPC (36) and 14°C for POPS (41).

consistent with the quenching data above, as well as with previous studies showing that POPC has some tendency to form an ordered state in the presence of cholesterol (37). Again in agreement with the quenching data, higher anisotropies were observed at 40 mol % cholesterol (relative to 25 mol %) for all of the low-T_m lipid/cholesterol mixtures, and the ranking of anisotropy values versus choline phospholipid structure at 40 mol % cholesterol was similar to that at 25 mol % cholesterol. Combined with the quenching data, these results indicate that the differences in tendencies of the choline-headgroup lipids tested to form ordered states are maintained over a range of cholesterol concentrations.

The degree to which steady-state anisotropy increases in the presence of cholesterol (relative to that in the absence of cholesterol) was used to measure the tendency of cholesterol to promote the formation of tightly packed states by low-T_m lipids at 23°C. As shown in Table 1 (*right column*), the increase in anisotropy in the presence of 40 mol % cholesterol relative to that in its absence decreased in the order: POPC > PDoPC = DOPC = DMoPC > DPhPC. This suggests that a PC having one saturated chain and one monounsaturated chain (POPC) packs better with cholesterol than lipids with one saturated and one polyunsaturated chain (PDoPC) or with two monounsaturated chains (DOPC, DMoPC). It also indicates that a PC with two phytanoyl chains packs very poorly with cholesterol. There was no significant change in anisotropy for high-T_m lipids (DPPC, SM) in the presence of cholesterol, but this does not provide information about interaction of these lipids with cholesterol, because DPPC and SM vesicles are already in a tightly packed state at 23°C in the absence of cholesterol.

It was also noteworthy that although samples containing DPhPC showed very little increase in anisotropy in the presence of cholesterol, they did show slightly higher DPH anisotropy than the other low-T_m PCs in the absence of cholesterol (Table 1). It is possible that the unusual acyl chains of these lipids restrict DPH motion slightly when the lipids are in the disordered fluid state.

Stability of ordered domains in the presence of cholesterol: comparison of PC, PE, and PS

The effect of headgroup structure upon the tendency of lipids to form ordered domains with cholesterol was examined next. Fig. 2 *C* compares the temperature dependence of fluorescence quenching in mixtures of 25 mol % cholesterol and POPC to that in mixtures with 1:1 PE/PS (mol/mol). The behavior of PE and PS is of interest, because these lipids are abundant in the inner (cytofacial) leaflet of mammalian plasma membranes. As in the case of mixtures of cholesterol with POPC, at lower temperatures, e.g., 23°C, samples containing mixtures of cholesterol with 1:1 POPE/POPS showed an intermediate level of quenching, indicating some degree of ordered-state formation, but not as much as that observed for DPPC or SM (see Fig. 2 *A*). Comparison of Fig. 2, *A* and *C*, also shows that ordered-domain formation in mixtures containing 25 mol % cholesterol and 1:1 POPE/POPS (Fig. 2 *C*) appeared to occur to a slightly greater extent than in similar mixtures containing POPC or SOPC (Fig. 2 *A*). This is consistent with the behavior of these lipids without cholesterol: POPE bilayers and POPS bilayers have significantly higher T_m values than POPC or SOPC bilayers (T_m in the absence of cholesterol is −3°C for POPC, 4°C for SOPC, 14°C for POPS, and 24°C for POPE (36,38–41)).

It is also noteworthy that, as judged by weak quenching, vesicles composed of PO lipids and cholesterol or SOPC and cholesterol consistently showed formation of some ordered state at temperatures significantly above T_m values of the

pure phospholipids without cholesterol (Fig. 2, *A–C*). This is most obvious in mixtures containing 40 mol % cholesterol. This suggests that these lipids have a borderline tendency to form ordered domains at near-physiological temperatures.

Mixtures containing 25 mol % cholesterol and 1:1 brain PE/brain PS, natural lipids which contain a significant fraction of unsaturated acyl chains (at least 30–35% monounsaturated and 10–20% polyunsaturated as reported by the manufacturer), still showed some tendency to form ordered-state bilayers as judged by fluorescence quenching, although to a lesser degree than 1:1 POPE/POPS (Fig. 2 *C*). In contrast, similar to the behavior of vesicles with mixtures of DPhPC and cholesterol, vesicles with 1:1 DPhPE/DPhPS showed little or no ordered-state formation in the presence of cholesterol, consistent with the poor packing abilities of the diphytanoyl chain lipids, noted above.

Steady-state anisotropy measurements at room temperature for PE and PS (Table 1) were again consistent with the fluorescence-quenching experiments. Vesicles containing mixtures of POPE and cholesterol or containing 1:1 POPE/POPS and cholesterol exhibited intermediate anisotropy levels, consistent with the formation of a considerable amount of ordering, athough less than that formed by DPPC or SM. Also in agreement with quenching, anisotropy indicated formation of an ordered state in mixtures of 25 mol % cholesterol and 1:1 POPE/POPS to a slightly greater degree than in analogous vesicles containing cholesterol and POPC. Vesicles containing 1:1 DPhPE/DPhPS showed anisotropy behavior similar to that of vesicles containing DPhPC, in both the absence and presence of cholesterol, indicating very little formation of an ordered state. This shows that DPhPE and DPhPS packing behavior with and without cholesterol is similar to that of DPhPC, and is also in agreement with the quenching results.

Behavior of ternary mixtures of low-T_m lipid, high-T_m lipid, and cholesterol: effect of low-T_m PC acyl-chain structure

We next ascertained how low-T_m lipid structure would affect ordered-domain formation in ternary or more complex mixtures containing low-T_m lipid, high-T_m lipid (either DPPC or SM), and 25 mol % cholesterol. In such mixtures, ordered and fluid-disordered domains can coexist, with the ordered domains composed mainly of high-T_m lipid and cholesterol (34,42). Fig. 3 *A* illustrates the temperature dependence of fluorescence-quenching in mixtures containing various low-T_m PCs and containing DPPC as the high-T_m lipid. In most cases, the thermal stability of the ordered domains in such mixtures was less than that observed in DPPC-cholesterol vesicles lacking a low-T_m lipid (Fig. 2 *A*), melting at about a 10°C lower temperature. This is commonly expected phase behavior. Interestingly, in most cases the thermal stability of ordered domains was not appreciably affected by the identity of the low-T_m PC. However, ordered domains were more stable when the low-T_m lipid was DPhPC, and most stable when the low-T_m lipid was DArPC, the two low-T_m lipids with the least ability to form ordered domains (see Discussion). The presence of these low-T_m lipids elevated the ordered domain melting temperature by ~10–20°C relative to the other low-T_m PCs (Fig. 3 *A*). The increased melting temperature in the presence of DPhPC and DArPC was not related to vesicle curvature, as we observed similar results using MLV (see below, and data not shown). We hypothesize that the observation that the low-T_m lipids with the least tendency to pack tightly stabilize ordered domains can be explained by low miscibility of such low-T_m lipids in ordered domains (see Discussion).

The fluorescence quenching data in Fig. 3 *B* shows that the effect of low-T_m PC structure upon the thermal stability of

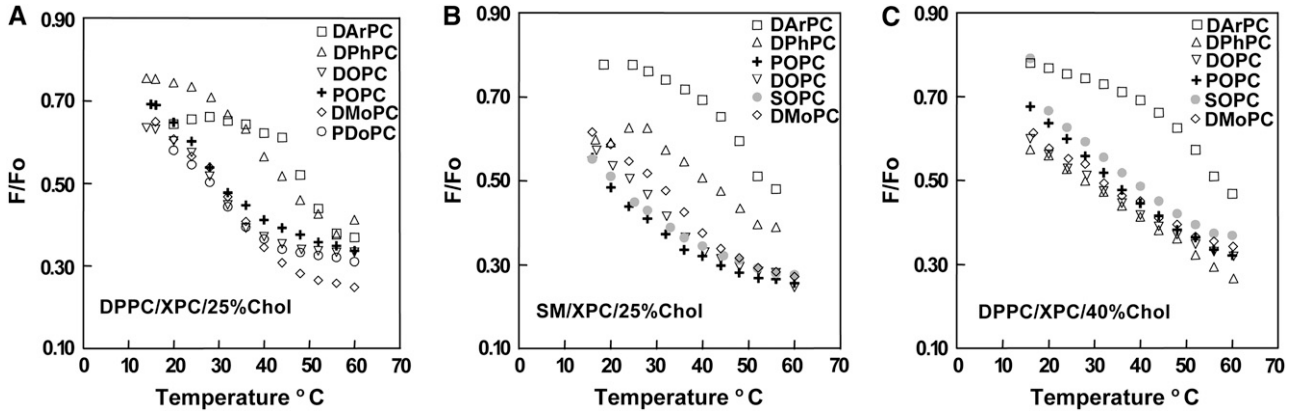

FIGURE 3 Melting curves for ordered domains in vesicles containing 1:1 (mol/mol) mixtures of high-T_m lipid/low-T_m PC plus cholesterol. Each sample contained 50 μM total lipid dispersed in PBS at pH 7.4. The ratio of DPH fluorescence in the presence and absence of TEMPO (*F*/*Fo*) was measured with increasing temperature. (*A*) Melting curves of lipid mixtures containing 25 mol % cholesterol and DPPC as the high-T_m lipid, and DOPC (▽), POPC (+), DPhPC (△), DMoPC (◇), DArPC (□), or PDoPC (○) as the low-T_m lipid. (*B*) Melting curves of lipid mixtures containing 25 mol % cholesterol and SM as the high-T_m lipid, and low-T_m lipids as in *A*, except when low-T_m lipid was SOPC (●). (*C*) Melting curves in mixtures containing 40 mol % cholesterol, DPPC as the high-T_m lipid, and low-T_m lipids as in *B*.

ordered domains in ternary lipid mixtures with SM and cholesterol was similar to that in vesicles containing DPPC and cholesterol.

The fluorescence quenching data in Fig. 3 *C* illustrates the thermal stability of ordered domains in ternary mixtures of low-T_m PC with DPPC and 40 mol % cholesterol. The effect of low-T_m lipid structure in the presence of 40 mol % cholesterol was similar to that at 25 mol % cholesterol. However, at 40 mol % cholesterol, DPhPC no longer stabilized ordered domains relative to the other low-T_m PCs. Also, the ordered domains in ternary mixtures with 40 mol % cholesterol appeared to be more thermally stable than in those with 25 mol % cholesterol, analogous to what was observed in binary lipid mixtures with 40 mol % cholesterol (Fig. 2, *A* and *B*).

FRET assay of ternary lipid mixtures containing DPPC, low-T_m PC, and cholesterol

The unusual behavior of the ternary mixtures containing DArPC and DPhPC prompted us to try to confirm that the greater thermal stability of ordered domains in such samples was not an artifact. In particular, ordered domains would be obviously more stable in mixtures of DPPC and cholesterol with DArPC (or DPhPC) if during sample preparation lipids did not mix so that samples contained separate DArPC (or DPhPC) vesicles and DPPC/cholesterol vesicles. This seemed unlikely, but to eliminate this possibility, control experiments were carried out in which FRET between a helical transmembrane-type peptide that partitions into disordered domains (LW peptide) (43) and a derivative of DPH that strongly partitions into ordered domains (LcTMADPH) (44) was measured. When these probe molecules are incorporated into vesicles, their spectral overlap is such that the Trp residues of the LW peptide act as a FRET donor, and the DPH group of LcTMADPH as a FRET acceptor.

When LW peptide/LcTMADPH FRET was measured in samples containing mixtures of DPPC, low-T_m PC, and 25 mol % cholesterol, FRET levels (as judged by quenching of donor fluorescence) exhibited the temperature dependence expected if the DPPC and low-T_m PC were in the same vesicles (Fig. 4). To be specific, weak FRET (high *F*/*Fo* values) was measured at low temperatures, at which the LcTMADPH, which resides in ordered domains, should be segregated from LW peptide, which locates in disordered domains, whereas at higher temperatures, i.e., above the temperature at which the ordered domains melt and segregation should be lost, FRET was stronger (low *F*/*Fo* values). When the low-T_m lipid structure was varied (i.e., for POPC, DArPC, and DPhPC), the temperatures at which the segregation of LW peptide and LcTMADPH was abolished mirrored the melting temperatures of the ordered domains as defined by TEMPO quenching. FRET increases occurred at the lowest temperatures in mixtures containing POPC, higher temperatures in samples containing DPhPC, and highest temperatures in samples containing DArPC.

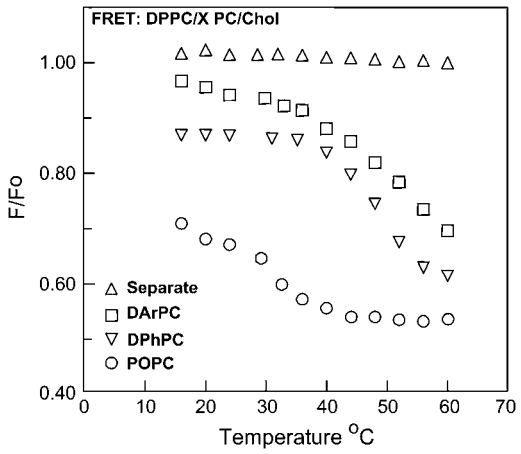

FIGURE 4 FRET assay of ordered-domain stability in mixtures of 25 mol % cholesterol with 1:1 (mol/mol) DPPC/low-T_m PC. Each sample contained 100 μM total lipid dispersed in PBS at pH 7.4. Unless otherwise noted, sample composition was as in Fig. 3, but also containing FRET donor (2 mol % LW peptide), and, when desired, FRET acceptor (1 mol % LcTMADPH). Mixtures with the low-T_m lipids DArPC (□), DPhPC (▽), or POPC (○) are shown. The open triangle represents 1:1 (mol/mol) mixture of vesicles containing DPPC, 25 mol % cholesterol, and 1 mol % LcTMADPH with vesicles containing POPC, 25 mol % cholesterol, and 2 mol % LW peptide.

Fig. 4 also shows that, in contrast, when LW peptide was incorporated into POPC-cholesterol vesicles and LcTMADPH was incorporated into DPPC-cholesterol vesicles, no FRET was observed at any temperature. This demonstrates that the increase in FRET at higher temperatures cannot be explained by a temperature-dependent migration of LcTMADPH or LW peptide between vesicles of different lipid composition. We conclude that the TEMPO quenching data cannot be explained by formation of separate vesicles such that the low-T_m lipids are in one set of vesicles, whereas high-T_m lipids and cholesterol are in another.

Behavior of mixtures of low-T_m lipid, high-T_m lipid, and cholesterol: the effect of low-T_m lipid headgroup structure for 1-palmitoyl 2-oleoyl low-T_m lipids

We next examined the effect of low-T_m phospholipids with different polar headgroups upon the stability of ordered domains in ternary and more complex mixtures. As shown in Fig. 5 *A*, in mixtures containing 1:1 DPPC/PO acyl chain lipid together with 25 mol % cholesterol, the thermal stability of ordered domains decreased in the order POPE > 1:1 POPE/POPS > POPC. Similar behavior was observed in 1:1 mixtures of PO lipids and SM with 25 mol % cholesterol (Fig. 5 *B*).

There was an interesting difference between varying headgroups and acyl chains in terms of their effect upon the stability of ordered domains formed by DPPC or SM and cholesterol. When headgroup structure of PO lipids was varied, those low-T_m PO lipids having a greater tendency to form tightly packed states, as judged by the data in Fig. 2 and

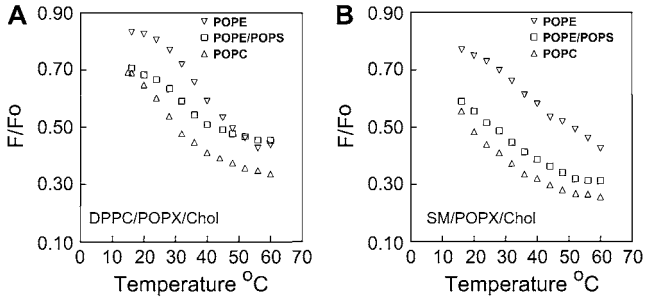

FIGURE 5 Melting curves of ordered domains in mixtures of cholesterol (25 mol %), high-T_m lipid (37.5 mol %), and various low-T_m PO lipids (37.5 mol %). Each sample contained 50 μM SUV dispersed in PBS at pH 7.4. The ratio of DPH fluorescence in the presence and absence of TEMPO (F/Fo) was measured with increasing temperature. (*A*) Melting curves of lipid mixtures containing DPPC as the high-T_m lipid. (*B*) Melting curves of lipid mixtures containing SM as the high-T_m lipid. Mixtures contained POPC (△), POPE (▽), or 1:1 (mol/mol) POPE/POPS (□) as low-T_m lipid. Mixtures containing 1-palmitoyl-2-oleoyl-phosphatidic acid and 1-palmitoyl-2-oleoyl-phosphatidylglycerol exhibited quenching profiles similar to that for mixtures containing POPE/POPS (data not shown).

Table 1 and by the T_m for the gel-to-fluid transition in bilayers composed of pure PO lipids, most strongly stabilized ordered domains. A likely explanation is that PO lipid packing within ordered domains affects ordered-domain stability. The better the PO lipids pack by themselves the better they pack within ordered domains formed by SM or DPPC and cholesterol (see Discussion). In contrast, as described earlier, when acyl chain structure was varied, it was the low-T_m lipids that had the least tendency to form tightly packed states that stabilized ordered domains to the greatest degree. These results suggest that ordered domains rich in high-T_m lipid and cholesterol are stabilized by low-T_m lipids via two different mechanisms.

Comparison of the effect of various diphytanoyl and PO lipids upon the stability of ordered domains formed by high-T_m lipids and cholesterol: evidence for multiple mechanisms of ordered domain stabilization

To test the hypothesis that ordered domains rich in high-T_m lipid and cholesterol are stabilized by low-T_m lipids via two different mechanisms, the behavior of samples containing PC, PE, or PS with PO chains mixed with DPPC and cholesterol was compared to samples containing PC, PE, or PS with diphytanoyl chains mixed with DPPC and cholesterol. The fluorescence quenching data in Fig. 6 *A* compares the thermal stability of ordered domains in mixtures of POPC, POPE, or POPS with DPPC and 25 mol % cholesterol, whereas the quenching data in Fig. 6 *B* compares the thermal stability of ordered domains in mixtures of DPhPC, DPhPE, or DPhPS with DPPC and 25 mol % cholesterol.

If there was just a single mechanism by which low-T_m lipids stabilize ordered domains, one might have expected an additive effect of the domain-stabilizing properties of

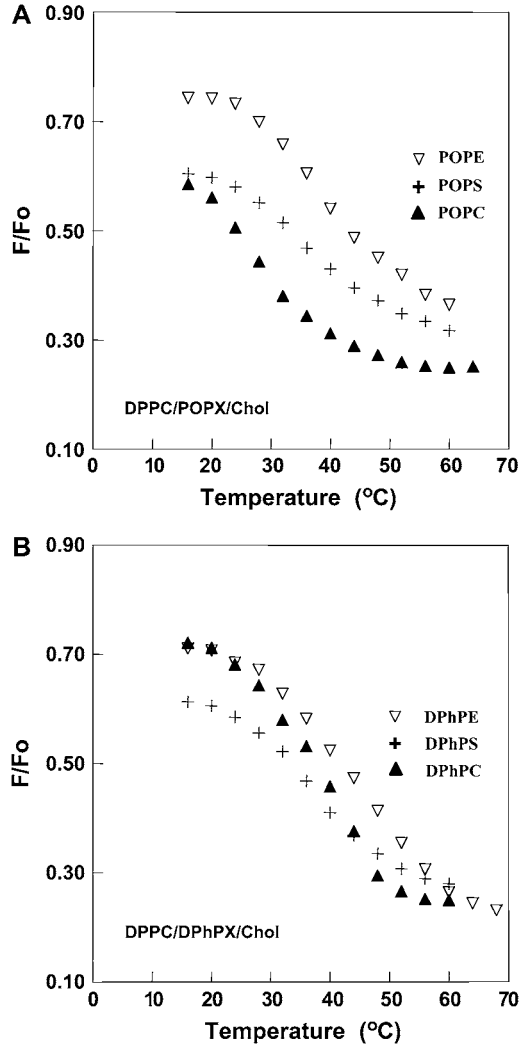

FIGURE 6 Melting curves of ordered domains in mixtures of cholesterol (25 mol %), high-T_m lipid (37.5 mol %), and various low-T_m lipids (37.5 mol %). Each sample contained 50 μM SUV dispersed in PBS at pH 7.4. The ratio of DPH fluorescence in the presence and absence of TEMPO (F/Fo) was measured with increasing temperature. (*A*) Melting curves of lipid mixtures with DPPC as the high-T_m lipid and PO lipids as the low-T_m component. Mixtures contained POPC (▲), POPE (▽), or POPS (+) as the low-T_m lipid. (*B*) Melting curves of lipid mixtures with DPPC as the high-T_m lipid and DPh acyl-chained lipids as the low-T_m component. Mixtures contained DPhPC (▲), DPhPE (▽), or DPhPS (+) as the low-T_m lipid.

diphytanoyl chains (relative to PO chains), as seen with DPhPC in Fig. 3 *A*, and the domain-stabilizing properties of PE or PS headgroups (relative to PC), as seen with the PO lipids (Fig. 5 *A*). However, comparison of Fig. 6, *A* and *B* (and of T_m values for these curves, shown in Table 2) indicates that the effects of low-T_m lipid acyl chain and headgroup structure upon the thermal stability of ordered domains are not additive. This is illustrated by the observation that the greater thermal stability of ordered domains formed by DPPC and cholesterol in samples containing POPE or POPS relative to samples containing POPC is not paralleled in samples containing diphytanoyl lipids. When

TABLE 2 Melting midpoint temperatures for ordered domains in vesicles containing low-T_m lipid components with different polar headgroups

	T_m (°C)	
Low-T_m lipid component	SUV	MLV
POPC	27.5	34.2
POPE	39.3	40.8
POPS	36.4	35.5
DPhPC	38.3	42.1
DPhPE	44.0	42.8
DPhPS	37.4	36.3

Table shows how low-T_m lipid acyl chain structure influences the effect of polar-headgroup structure. Samples were composed of 3:3:2 (mol/mol) DPPC/low-T_m lipid/cholesterol. Fluorescence data for the SUV samples is shown in Fig. 6. T_m was defined as the point of maximum slope of a sigmoidal fit of *F*/*Fo* data (Slidewrite Program, Advanced Graphics Software, Encinitas, CA). The average T_m from duplicate samples is shown. T_m values were reproducible to $\sim\pm1$°C.

attached to diphytanoyl chains, PE and PS do not stabilize ordered domains formed by DPPC and cholesterol much more than PC attached to diphytanoyl chains. If the effects were additive, the difference between ordered-domain thermal stability in samples containing PE, PS, and PC should have been the same with PO and diphytanoyl acyl chains. Stated another way, whereas DPhPC stabilizes ordered-domain formation by DPPC much more than POPC, DPhPE does so only moderately more than POPE, and DPhPS no more than POPS. This behavior implies that two mutually exclusive mechanisms influence ordered-domain stabilization by low-T_m lipid (see Discussion). It should be noted that similar results were obtained using MLV (Table 2).

It was of interest to see whether behavior analogous to that of mixtures of various DPh lipids with DPPC and cholesterol would be observed with analogous mixtures containing various DAr lipids with different headgroups. The temperature dependence of DPH fluorescence in the presence of TEMPO indicated that the domain melting temperatures of mixtures with DArPE was not higher than that of mixtures containing DArPC (data not shown). This indicated a lack of domain stabilization by PE, analogous to what was observed with DPh lipids. However, meaningful fluorescence quenching levels could not be determined, because there was anomalously low DPH fluorescence in DArPE-containing samples that lacked quencher at high temperatures. It appears that DPH fluorescence is unusually sensitive to the physical structure of DArPE-rich domains, for reasons that we do not understand.

DISCUSSION

How lipids that tend to form L_d domains can stabilize ordered domains formed by high-T_m lipids and cholesterol

This study aimed to fill a gap in our knowledge about the behavior of cholesterol-containing lipid mixtures by studying the ordered-state stability in a variety of binary and ternary lipid mixtures in which lipid acyl chain and headgroup structure was varied. We found that the structure of the low-T_m lipids can greatly influence the formation of ordered domains by high-T_m lipids and cholesterol. The low-T_m lipids that promote ordered-domain formation by high-T_m lipids and cholesterol most strongly, DArPC and DPhPC, have very low T_m values (36,45), an indication that they pack very poorly in bilayers. The poor packing properties of these two lipids are not surprising: arachidonoyl acyl chains have four *cis* double bonds, which result in extensive kinking, and phytanoyl acyl chains have an unusual multibranched structure, with four methyl groups that protrude from the main fatty acyl chain. Their very poor tight-packing abilities should result in their being much less miscible in tightly packed ordered domains than low-T_m lipids with less extreme poor packing properties, i.e., DArPC and DPhPC should be present at very low concentrations within the ordered domains. This could explain increased ordered-domain stability. The less low-T_m lipid in ordered domains, the more closely the ordered domains should exhibit the high thermal stability of DPPC/cholesterol mixtures. An additional stabilizing factor could be cholesterol concentrating within the ordered domains, such that its local concentration within ordered domains is higher than its average concentration in the bilayer. Consistent with this hypothesis, the thermal stability of ordered domains in the ternary mixtures composed of 25 mol % cholesterol, DPPC, and DArPC were similar to mixtures of DPPC with 40 mol % cholesterol, and more thermally stable than those formed just by DPPC mixed with 25 mol % cholesterol (see Results). Indeed, immiscibility of cholesterol and low-T_m lipids with one or two DHA chains has been proposed to explain the relative stability of ordered domains in the mixtures containing PE with DHA chains and SM (22,46).

In this regard, it should be pointed out that this combination of factors being responsible for stabilizing ordered domains is consistent with the tentative phase diagram that has been proposed for ternary mixtures of DPPC, DPhPC, and cholesterol, which indicates that the ordered domains in such mixtures are enriched in both DPPC and cholesterol (47). Future studies mapping out the full phase diagrams for additional lipid mixtures are needed to more precisely define lipid-lipid miscibility.

In contrast, in ternary mixtures containing various low-T_m lipids with more modest poor packing properties, differences in lipid acyl chain structure had little (if any) effect on the stability of ordered domains. Specifically, we could not observe differences between ordered-domain stability in the presence of low-T_m lipids with two monounsaturated acyl chains having different acyl chain lengths or between such lipids and a lipid with one saturated acyl chain and one (mono or poly) unsaturated acyl chain. This may have important implications for cell membranes (see below).

Headgroup structure of the low-T_m lipid also affected the stability of ordered domains formed by high-T_m lipids

and cholesterol. The behavior of PO lipids with different headgroups was particularly interesting. As noted above, PO lipids, which have higher gel-to-liquid-disordered T_m values than lipids with two unsaturated acyl chains or lipids with one saturated acyl chain and one polyunsaturated chain (36), should have a significant ability to pack together tightly relative to other low-T_m lipids. Combined with the observation that ordered-domain stability in ternary mixtures in which PO lipids were the low-T_m component decreased in the same order as PO lipid T_m values: PE > PS > PC (36,38–41), this suggests that the greater the tendency of a PO lipid to pack tightly, the greater the stability of the ordered domains in lipid mixtures in which PO lipid is the low-T_m lipid. Thus, the packing of PO lipids within ordered domains could explain the dependence of ordered-domain stability upon PO lipid headgroup structure. This mechanism of ordered-domain stabilization would be clearly distinct from that described above for low-T_m lipids with different acyl chains. As noted in Results, the nonadditive effects of low-T_m lipid acyl chain and headgroup structure on ordered-domain thermal stability strongly supports the notion that two distinct mechanisms are involved. Fig. 7 schematically illustrates the difference in these mechanisms. The alternate possibility, that POPE and POPS lipids are very insoluble in ordered domains, seems unlikely given their relative propensity to form ordered domains.

Relationship between the thermal stability of ordered states formed by single phospholipids with and without cholesterol

It is noteworthy that for the phospholipids studied in this report, the ranking of the tendency to form ordered states in binary mixtures with cholesterol largely mimicked the gel-to-liquid-disordered T_m values for bilayers composed of the pure phospholipids (36,38–41). This suggests that the interphospholipid interactions that stabilize ordered-domain formation are maintained to a significant degree in bilayers containing moderate to high amounts of cholesterol. This is remarkable, because a significant fraction of the interphospholipid interactions should be replaced by phospholipid-cholesterol interactions in such mixtures. We speculate that there is some arrangement of phospholipids and cholesterol that results in maintenance of a significant number of key phospholipid-phospholipid interactions that are largely unchanged from those in pure phospholipid bilayers.

Implications of the behavior of lipid mixtures for raft formation in natural membranes and in the cytofacial leaflet

The results of this report may also have implications for ordered-domain formation in cellular membranes. Only low-T_m PC with two acyl chains that pack very poorly was able to stabilize ordered domains to any significant degree. Because of the rarity of lipids having two polyunsaturated chains, and the nonphysiological structure of diphytanoyl lipids, it seems likely that in the PC-rich outer leaflet of cells the formation of ordered domains in cells would be controlled largely by the interactions between high-T_m lipids and cholesterol, with the details of the structure of low-T_m PC having little effect. If this is true, then in cells, small changes in the acyl chain composition of low-T_m PC under different physiological conditions may not greatly influence raft stability.

However, this conclusion must be qualified by the fact that we concentrated upon studies varying the acyl chain composition of PC molecules, and the effect of acyl chain structure could be headgroup-dependent. It is possible that in the inner leaflet of the plasma membrane of mammalian cells, in which PE and PS are abundant, the acyl chain composition of low-T_m lipids could have a greater impact on ordered-domain formation.

We previously speculated that PE might support raft formation in the inner leaflet (48). The possibility that PE and PS form ordered domains in the inner leaflet is supported by the observations that POPE and POPS have a greater tendency to stabilize ordered-domain formation than does POPC, and that cholesterol increases orderedness in PE/PS mixtures, as judged by fluorescence anisotropy, despite the fact that PE does not interact as well with cholesterol as does

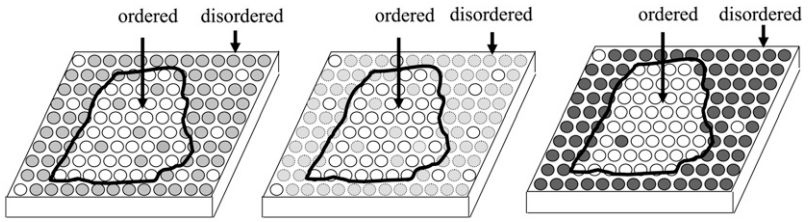

FIGURE 7 Schematic illustration of the effect of interactions between loosely (low-T_m) and tightly (high-T_m) packing lipids upon the stability of the ordered phases. Surface view of a portion of a lipid bilayer is shown. Heavy line represents boundary between disordered and ordered phases. The locations of individual lipids that prefer to form ordered phase (*open circles*) or those that prefer to form disordered phase (*shaded circles*) is also shown. The darker the shading of circles representing the disordered-phase-preferring lipids, the poorer their ability to pack tightly. Notice that the ordered phase is predominantly composed of lipids that prefer to form ordered phase but also contains some disordered-phase-preferring lipid, whereas the disordered phase is predominantly composed of lipids that prefer to form disordered phase but also contains some ordered-phase-preferring lipid. Ordered-phase stability is greater when disordered-phase-preferring lipid has a moderate ability to pack tightly (*center*) than when disordered-phase-preferring lipid has a poor ability to pack tightly (*left*). Ordered-phase stability is also greater when disordered-phase-preferring lipids pack so poorly that they do not dissolve well in the ordered phase (*right*).

PC (49). In fact, we found that brain PE and brain PS, which contain a natural mixture of acyl chains, also showed some tendency to form ordered domains in the presence of cholesterol as judged by quenching. The important finding of Wang and Silvius that POPE and POPS did not form ordered domains that separated from POPC in the presence of cholesterol (50), does not rule out the possibility that in the presence of other raft-forming components PE/PS/cholesterol-rich ordered domains might form. One possibility is that SM, which may be present in the inner leaflet of the plasma membrane to some degree, or ceramide, which might be generated by SM degradation and then flip-flop rapidly to the inner leaflet (51,52), might trigger ordered domain formation by PE and PS. However, we only were able to stabilize ordered-domain formation by PE/PS very modestly by addition of small amounts of SM or ceramide (data not shown). Another possible driving force for the formation of inner-leaflet domains could be interaction with ordered domains in the outer leaflet. The fact that under some conditions inner- and outer-leaflet ordered domains are found in register with each other suggests that cross-leaflet stabilization of ordered domains can occur (53). An exciting, very recent study by Kiessling et al. using supported bilayers supports this possibility (54). It was found that SM/cholesterol-rich ordered domains in one leaflet induced domain formation in the opposite leaflet, which contained a PC/PE/PS mixture mimicking the composition of a mammalian inner leaflet. However, the exact composition of the lipids forming the ordered domains in the PC/PE/PS-containing leaflet and their exact physical state were not determined. Further studies with asymmetric membranes, and others examining the effects of proteins, will be important for more fully defining the principles of raft formation.

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REFERENCES

1. Brown, D. A., and E. London. 1997. Structure of detergent-resistant membrane domains: does phase separation occur in biological membranes? *Biochem. Biophys. Res. Commun.* 240:1–7.
2. Brown, D. A., and E. London. 1998. Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol.* 14:111–136.
3. Almeida, P. F., W. L. Vaz, and T. E. Thompson. 1992. Lateral diffusion in the liquid phases of dimyristoylphosphatidylcholine/cholesterol lipid bilayers: a free volume analysis. *Biochemistry.* 31:6739–6747.
4. London, E. 2005. How principles of domain formation in model membranes may explain ambiguities concerning lipid raft formation in cells. *Biochim. Biophys. Acta.* 1746:203–220.
5. Rubenstein, J. L., B. A. Smith, and H. M. McConnell. 1979. Lateral diffusion in binary mixtures of cholesterol and phosphatidylcholines. *Proc. Natl. Acad. Sci. USA.* 76:15–18.
6. Simons, K., and R. Ehehalt. 2002. Cholesterol, lipid rafts, and disease. *J. Clin. Invest.* 110:597–603.
7. Lafont, F., L. Abrami, and F. G. van der Goot. 2004. Bacterial subversion of lipid rafts. *Curr. Opin. Microbiol.* 7:4–10.
8. Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. *Nature.* 387:569–572.
9. Simons, K., and D. Toomre. 2000. Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* 1:31–39 (Erratum in *Nat. Rev. Mol. Cell Biol.* 2:216).
10. Nyholm, T. K., M. Nylund, and J. P. Slotte. 2003. A calorimetric study of binary mixtures of dihydrosphingomyelin and sterols, sphingomyelin, or phosphatidylcholine. *Biophys. J.* 84:3138–3146.
11. Wang, J., Megha, and E. London. 2004. Relationship between sterol/steroid structure and participation in ordered lipid domains (lipid rafts): implications for lipid raft structure and function. *Biochemistry.* 43: 1010–1018.
12. Xu, X., R. Bittman, G. Duportail, D. Heissler, C. Vilcheze, and E. London. 2001. Effect of the structure of natural sterols and sphingolipids on the formation of ordered sphingolipid/sterol domains (rafts). Comparison of cholesterol to plant, fungal, and disease-associated sterols and comparison of sphingomyelin, cerebrosides, and ceramide. *J. Biol. Chem.* 276:33540–33546.
13. Xu, X., and E. London. 2000. The effect of sterol structure on membrane lipid domains reveals how cholesterol can induce lipid domain formation. *Biochemistry.* 39:843–849.
14. Wenz, J. J., and F. J. Barrantes. 2003. Steroid structural requirements for stabilizing or disrupting lipid domains. *Biochemistry.* 42:14267–14276.
15. Li, X. M., M. M. Momsen, H. L. Brockman, and R. E. Brown. 2003. Sterol structure and sphingomyelin acyl chain length modulate lateral packing elasticity and detergent solubility in model membranes. *Biophys. J.* 85:3788–3801.
16. Ramstedt, B., and J. P. Slotte. 2006. Sphingolipids and the formation of sterol-enriched ordered membrane domains. *Biochim Biophys Acta.*
17. Ahmed, S. N., D. A. Brown, and E. London. 1997. On the origin of sphingolipid/cholesterol-rich detergent-insoluble cell membranes: physiological concentrations of cholesterol and sphingolipid induce formation of a detergent-insoluble, liquid-ordered lipid phase in model membranes. *Biochemistry.* 36:10944–10953.
18. Nybond, S., Y. J. Bjorkqvist, B. Ramstedt, and J. P. Slotte. 2005. Acyl chain length affects ceramide action on sterol/sphingomyelin-rich domains. *Biochim. Biophys. Acta.* 1718:61–66.
19. Bjorkqvist, Y. J., T. K. Nyholm, J. P. Slotte, and B. Ramstedt. 2005. Domain formation and stability in complex lipid bilayers as reported by cholestatrienol. *Biophys. J.* 88:4054–4063.
20. Megha, O. Bakht, and E. London. 2006. Cholesterol precursors stabilize ordinary and ceramide-rich ordered lipid domains (lipid rafts) to different degrees. Implications for the Bloch hypothesis and sterol biosynthesis disorders. *J. Biol. Chem.* 281:21903–21913.
21. Megha and E. London. 2004. Ceramide selectively displaces cholesterol from ordered lipid domains (rafts): implications for lipid raft structure and function. *J. Biol. Chem.* 279:9997–10004.
22. Shaikh, S. R., A. C. Dumaual, D. LoCassio, R. A. Siddiqui, and W. Stillwell. 2003. Acyl chain unsaturation in PEs modulates phase separation from lipid raft molecules. *Biochem. Biophys. Res. Commun.* 311:793–796.
23. Wassall, S. R., M. R. Brzustowicz, S. R. Shaikh, V. Cherezov, M. Caffrey, and W. Stillwell. 2004. Order from disorder, corralling cholesterol with chaotic lipids. The role of polyunsaturated lipids in membrane raft formation. *Chem. Phys. Lipids.* 132:79–88.
24. Hammond, A. T., F. A. Heberle, T. Baumgart, D. Holowka, B. Baird, and G. W. Feigenson. 2005. Crosslinking a lipid raft component triggers liquid ordered-liquid disordered phase separation in model plasma membranes. *Proc. Natl. Acad. Sci. USA.* 102:6320–6325.
25. Heerklotz, H., H. Szadkowska, T. Anderson, and J. Seelig. 2003. The sensitivity of lipid domains to small perturbations demonstrated by the effect of Triton. *J. Mol. Biol.* 329:793–799.
26. Heerklotz, H. 2002. Triton promotes domain formation in lipid raft mixtures. *Biophys. J.* 83:2693–2701.
27. Staneva, G., M. Seigneuret, K. Koumanov, G. Trugnan, and M. I. Angelova. 2005. Detergents induce raft-like domains budding and fission from giant unilamellar heterogeneous vesicles: a direct microscopy observation. *Chem. Phys. Lipids.* 136:55–66.

28. Klein, R. A. 1970. The detection of oxidation in liposome preparations. *Biochim. Biophys. Acta.* 210:486–489.

29. Bakht, O., and E. London. 2007. Detection of ordered domain formation (lipid rafts) in model membranes using Tempo. *In* Lipid Rafts. T. J. McIntosh, editor. Humana Press, Totowa, NJ. 29–40.

30. Lacowicz, J. 1999. Priniciples of Fluorescence Spectroscopy, 2nd ed. Klewer Academic/Plenum, New York.

31. Kleemann, W., and H. M. McConnell. 1976. Interactions of proteins and cholesterol with lipids in bilayer membranes. *Biochim. Biophys. Acta.* 419:206–222.

32. Lentz, B. R., Y. Barenholz, and T. E. Thompson. 1976. Fluorescence depolarization studies of phase transitions and fluidity in phospholipid bilayers. 1. Single component phosphatidylcholine liposomes. *Biochemistry.* 15:4521–4528.

33. London, E., and G. W. Feigenson. 1981. Fluorescence quenching in model membranes: an analysis of the local phospholipid environments of diphenylhexatriene and gramicidin A'. *Biochim. Biophys. Acta.* 649: 89–97.

34. London, E., D. A. Brown, and X. Xu. 2000. Fluorescence quenching assay of sphingolipid/phospholipid phase separation in model membranes. *Methods Enzymol.* 312:272–290.

35. Koynova, R., and M. Caffrey. 1995. Phases and phase transitions of the sphingolipids. *Biochim. Biophys. Acta.* 1255:213–236.

36. Koynova, R., and M. Caffrey. 1998. Phases and phase transitions of the phosphatidylcholines. *Biochim. Biophys. Acta.* 1376:91–145.

37. Mateo, C. R., A. U. Acuna, and J. C. Brochon. 1995. Liquid-crystalline phases of cholesterol/lipid bilayers as revealed by the fluorescence of trans-parinaric acid. *Biophys. J.* 68:978–987.

38. Davis, P. J., B. D. Fleming, K. P. Coolbear, and K. M. Keough. 1981. Gel to liquid-crystalline transition temperatures of water dispersions of two pairs of positional isomers of unsaturated mixed-acid phosphatidylcholines. *Biochemistry.* 20:3633–3636.

39. Sanderson, P. W., L. J. Lis, P. J. Quinn, and W. P. Williams. 1991. The Hofmeister effect in relation to membrane lipid phase stability. *Biochim. Biophys. Acta.* 1067:43–50.

40. Anderle, G., and R. Mendelsohn. 1986. Fourier-transform infrared studies of CaATPase/phospholipid interaction: survey of lipid classes. *Biochemistry.* 25:2174–2179.

41. Mattai, J., H. Hauser, R. A. Demel, and G. G. Shipley. 1989. Interactions of metal ions with phosphatidylserine bilayer membranes: effect of hydrocarbon chain unsaturation. *Biochemistry.* 28:2322–2330.

42. Schroeder, R., E. London, and D. Brown. 1994. Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior. *Proc. Natl. Acad. Sci. USA.* 91:12130–12134.

43. Fastenberg, M. E., H. Shogomori, X. Xu, D. A. Brown, and E. London. 2003. Exclusion of a transmembrane-type peptide from ordered-lipid domains (rafts) detected by fluorescence quenching: extension of quenching analysis to account for the effects of domain size and domain boundaries. *Biochemistry.* 42:12376–12390.

44. Beck, A., D. Heissler, and G. Duportail. 1993. Influence of the length of the spacer on the partitioning properties of amphiphilic fluorescent membrane probes. *Chem. Phys. Lipids.* 66:135–142.

45. Lindsey, H., N. O. Petersen, and S. I. Chan. 1979. Physicochemical characterization of 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine in model membrane systems. *Biochim. Biophys. Acta.* 555:147–167.

46. Shaikh, S. R., V. Cherezov, M. Caffrey, W. Stillwell, and S. R. Wassall. 2003. Interaction of cholesterol with a docosahexaenoic acid-containing phosphatidylethanolamine: trigger for microdomain/raft formation? *Biochemistry.* 42:12028–12037.

47. Veatch, S. L., K. Gawrisch, and S. L. Keller. 2006. Closed-loop miscibility gap and quantitative tie-lines in ternary membranes containing diphytanoyl PC. *Biophys. J.* 90:4428–4436.

48. Brown, D. A., and E. London. 2000. Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J. Biol. Chem.* 275: 17221–17224.

49. Ohvo-Rekila, H., B. Ramstedt, P. Leppimaki, and J. P. Slotte. 2002. Cholesterol interactions with phospholipids in membranes. *Prog. Lipid Res.* 41:66–97.

50. Wang, T. Y., and J. R. Silvius. 2001. Cholesterol does not induce segregation of liquid-ordered domains in bilayers modeling the inner leaflet of the plasma membrane. *Biophys. J.* 81:2762–2773.

51. Contreras, F. X., A. V. Villar, A. Alonso, R. N. Kolesnick, and F. M. Goni. 2003. Sphingomyelinase activity causes transbilayer lipid translocation in model and cell membranes. *J. Biol. Chem.* 278:37169–37174.

52. Lopez-Montero, I., N. Rodriguez, S. Cribier, A. Pohl, M. Velez, and P. F. Devaux. 2005. Rapid transbilayer movement of ceramides in phospholipid vesicles and in human erythrocytes. *J. Biol. Chem.* 280: 25811–25819.

53. Korlach, J., P. Schwille, W. W. Webb, and G. W. Feigenson. 1999. Characterization of lipid bilayer phases by confocal microscopy and fluorescence correlation spectroscopy. *Proc. Natl. Acad. Sci. USA.* 96:8461–8466.

54. Kiessling, V., J. M. Crane, and L. K. Tamm. 2006. Transbilayer effects of raft-like lipid domains in asymmetric planar bilayers measured by single molecule tracking. *Biophys. J.* 91:3313–3326.

55. Koan, M. M., and G. J. Blanchard. 2006. Gauging the effect of impurities on lipid bilayer phase transition temperature. *J. Phys. Chem. B.* 110:16584–16590.

56. Koynova, R., and M. Caffrey. 1994. Phases and phase transitions of the hydrated phosphatidylethanolamines. *Chem. Phys. Lipids.* 69:1–34.