# Cholesterol Does Not Induce Segregation of Liquid-Ordered Domains in Bilayers Modeling the Inner Leaflet of the Plasma Membrane

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ABSTRACT A fluorescence-quenching method has been used to assess the potential formation of segregated liquid-ordered domains in lipid bilayers combining cholesterol with mixtures of amino and choline phospholipids like those found in the cytoplasmic leaflet of the mammalian cell plasma membrane. When present in proportions >20–30 mol %, different saturated phospholipids show a strong proclivity to form segregated domains when combined with unsaturated phospholipids and cholesterol, in a manner that is only weakly affected by the nature of the phospholipid headgroups. By contrast, mixtures containing purely unsaturated phospholipids and cholesterol do not exhibit detectable segregation of domains, even in systems whose components differ in headgroup structure, mono- versus polyunsaturation and/or acyl chain heterogeneity. These results indicate that mixtures of phospholipids resembling those found in the inner leaflet of the plasma membrane do not spontaneously form segregated liquid-ordered domains. Instead, our findings suggest that factors extrinsic to the inner-monolayer lipids themselves (e.g., transbilayer penetration of long sphingolipid acyl chains) would be essential to confer a distinctive, more highly ordered organization to the cytoplasmic leaflet of "lipid raft" structures in animal cell membranes.

## INTRODUCTION

Recent evidence suggests that, in the plasma and possibly other membranes of many eukaryotic cells, a significant fraction of the lipids is organized as liquid-ordered ($l_o$) domains in structures such as lipid "rafts" and caveolae (Brown and London, 1998a; Kurzchalia and Parton, 1999; Simons and Ikonen, 1997). Studies using lipid model systems have demonstrated that mixtures combining phospho- and sphingolipids with cholesterol can form coexisting $l_o$ and liquid-disordered ($l_d$) domains at physiological temperatures (Brown, 1998; Brown and London, 1997, 1998a, b; Rietveld and Simons, 1998). These model systems have generally been prepared from lipids that resemble those found in the extracytoplasmic leaflet of the plasma membrane, i.e., mixtures combining saturated phospho- or sphingolipids with unsaturated or other low-melting phospholipids and cholesterol (Schroeder et al., 1994, 1998; Silvius et al., 1996; Ahmed et al., 1997; Wang and Silvius, 2000; Wang et al., 2000; Dietrich et al., 2001).

Although only lipid mixtures characteristic of those found in the outer leaflet of the plasma membrane have been demonstrated to date to form segregated $l_o$ domains, various observations suggest that lipid raft domains in biological membranes have a transmembrane character. Proteins anchored to the cytoplasmic face of the plasma membrane by multiple saturated N- and/or S-acyl chains, for example, can be identified in raft-enriched fractions isolated biochemically (Shenoy-Scaria et al., 1994; Robbins et al., 1995; Wolven et al., 1997; Arni et al., 1998; Melkonian et al., 1999), and some proteins of this type have been shown to colocalize with outer-monolayer raft markers when the latter are induced to patch or otherwise redistribute in the membrane plane (Harder et al., 1998; Janes et al., 1999; Sheets et al., 1999; Holowka et al., 2000). Such results have led to suggestions that the lipids in the cytoplasmic monolayer of a raft may also comprise an $l_o$ domain into which proteins bearing saturated lipid chains can partition (Simons and Ikonen, 1997; Brown and London, 1997, 1998b).

Despite this suggestive evidence, the precise organization of the lipids in the cytoplasmic leaflet of a membrane lipid raft remains unclear, as do the factors that dictate its organization. The inner leaflet of the plasma membrane bilayer seems to contain little sphingolipid but, instead, consists mainly of unsaturated phosphatidylethanolamines (PEs), phosphatidylcholines (PCs) and anionic phospholipids together with cholesterol (van Meer, 1988; Gallet et al., 1999). To date, relatively little is known about the potential of mixtures of these latter lipids to support the formation of segregated $l_o$ bilayer domains. Monounsaturated PCs have been reported to form phases with $l_o$ characteristics when combined with physiological proportions of cholesterol (Thewalt and Bloom, 1992; Mateo et al., 1995). Evidence has also been reported that in bilayers cholesterol can discriminate significantly among different phospholipids based on differences in headgroup as well as acyl chain structure (van Dijck et al., 1976; van Dijck, 1979; Demel et al., 1977; Lange et al., 1979; Nakagawa et al., 1979; Wattenberg and Silbert, 1983; Yeagle and Young, 1986; Keough et al., 1989; Vilcheze et al., 1996; McMullen and McElhaney, 1997; Epand et al., 2000; McMullen et al., 2000). Such findings raise the possibility that segregated $l_o$ and $l_d$ domains could form in mixtures combining cytoplasmic-leaflet phospholipids with cholesterol, as Keller et al. (1998) have reported under some conditions in monolayer systems

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at the air-water interface. However, this possibility has not yet been evaluated directly in a bilayer system.

In this study we have used a fluorescence-quenching approach to examine the abilities of a variety of saturated, monounsaturated, and polyunsaturated phospholipids to support lateral segregation of $l_o$ domains in bilayers containing physiological proportions of cholesterol. Whereas (in agreement with previous findings) formation of segregated $l_o$ domains is readily demonstrated in bilayers including saturated phospholipids, systems combining cholesterol with mixtures of unsaturated phospholipids like those found in the plasma membrane inner leaflet do not exhibit lateral segregation of domains with distinct compositions. Our results imply that spontaneous segregation of $l_o$ domains is not an intrinsic property of the lipids of the cytoplasmic leaflet of the plasma (and other) membranes, and that additional (e.g., transmembrane) influences must be required to confer distinctive physical properties to the lipids in the cytoplasmic leaflet of a raft domain.

## MATERIALS AND METHODS

### Materials

Bovine brain- and liver-derived phospholipids, dioleoyl PE and PS (DOPE, DOPS), 1-palmitoyl-2-linoleoyl-PE and -PS (PLnPE, PLnPS), cholesterol, 1-palmitoyl-2-oleoyl-PC (POPC), and 1-stearoyl-2-oleoyl PC (SOPC) were obtained from Avanti Polar Lipids (Alabaster, AL). Di-(16-bromopalmitoyl)- and di-(16-(indol-1-yl)palmitoyl)-PC were synthesized from the corresponding fatty acids (Silvius, 1992) by reacting 6 equivalents of the fatty acyl anhydrides with 1 equivalent of dried glycerophosphorylcholine cadmium chloride complex (Sigma, St. Louis, MO) in 3:2 (v/v) dry dimethylsulfoxide/chloroform (1 h, 37°C), using 4-pyrrolidinopyridine (0.15 equivalent) as catalyst. 1-(16-Bromopalmitoyl)-2-oleoyl-PC and 1-(16-bromopalmitoyl)-2-palmitoyl-PC (BrPOPC, BrDPPC), 1-(16-(indol-1-yl)palmitoyl)-2-stearoyl-PC (16Ind/18:0-PC), and 1-(16-(indol-1-yl)-palmitoyl)-2-linoleoyl-PC (16Ind/18:2-PC) were synthesized from the above PCs by removing the 2-position acyl chain with phospholipase $A_2$ and reacylating the isolated lysophosphatidylcholine with the appropriate acyl anhydride (Mason et al., 1981). 1-Palmitoyl-2-linoleoyl-PC (PLnPC) was synthesized by acylating 1-palmitoyl lysophosphatidylcholine (Avanti) with linoleic anhydride (Mason et al., 1981). Synthetic PCs were purified on columns of BioSil A (BioRad Laboratories, Mississauga, Ontario, Canada), eluting with a gradient of 25–50% methanol plus 1% water in methylene chloride.

Brominated or indolyl-labeled PEs were synthesized from the corresponding PCs by cabbage phospholipase D-mediated transphosphatidylation (Comfurius and Zwaal, 1977). Fluorescent or brominated phosphatidylserines were also prepared from the corresponding PCs by transphosphatidylation, in this case however using phospholipase D from *Streptomyces sp.* (Sigma). Lipid stock solutions were standardized by the method of Lowry and Tinsley (1974). In some cases, stocks of polyunsaturated phospholipid were combined with a small amount of the antioxidant butylated hydroxytoluene (1 mol per 250 mol phospholipid), with no detectable effect on the quenching curves obtained.

### Vesicle preparation and fluorescence measurements

Lipid samples (normally comprising 100 nmol of total phospholipid including 2 nmol fluorescent lipid) were mixed as stock solutions in $CH_2Cl_2$/methanol 2:1 (v/v), then dried down under nitrogen with warming to ~45°C. The samples were further dried for 4–12 h under high vacuum to remove residual traces of solvent. The dried lipids were initially dispersed by vortexing at 37°C in 0.5 ml of sample buffer (150 mM KCl, 10 mM potassium phosphate, 0.1 mM ethylenediaminetetraacetic acid, pH 7.2), then incubated at 45°C for 10 min, vortexed, heated to 65°C for 1 min, and rapidly cooled to 45°C. The samples were then cooled (at <0.5°C/min) to the final experimental temperature and further incubated for 2–16 h.

A 200-$\mu$l aliquot of each lipid sample was withdrawn and mixed with 2.8 ml of sample buffer, and the fluorescence was measured in a Perkin-Elmer LS-5 spectrofluorometer (Beaconsfield, Buckinghamshire, UK), using excitation and emission wavelengths of 281 nm and 321 nm, respectively (slit widths 10 nm/10 nm). The fluorescence was similarly determined for a parallel 200-$\mu$l aliquot of each sample mixed with 2.8 ml methanol, which eliminated quenching of the probe fluorescence by the brominated lipids. Using these two (blank-corrected) fluorescence readings, $F_{cor}$(buffer) and $F_{cor}$(methanol), we calculated the normalized fluorescence $F_N = (F_{cor}[\text{buffer}]/F_{cor}[\text{methanol}])$, which corrected for possible small variations in the content of fluorescent lipid in different vesicle samples. Most of the fluorescence data reported in this study are presented in the alternative scaled form $(F/F_o)_{cor}$ suggested by London and Feigenson (1981a):

$$(F/F_0)_{cor} = (F_N - F_{100\%Q})/(F_{0\%Q} - F_{100\%Q}) \qquad (1)$$

where $F_N$ is the normalized fluorescence measured for the fluorescent molecule in vesicles containing a given molar percentage of quencher lipid (%$Q$) in the phospholipid fraction, and $F_{0\%Q}$ and $F_{100\%Q}$ are the normalized fluorescence values measured in vesicles containing 0% or 100% quencher, respectively, in the phospholipid fraction. In the experiments presented here, normalized fluorescence values measured for probes in bilayers where all of the phospholipid was brominated were typically 10–15% of those measured for the same probes in quencher-free bilayers. Quenching curves shown are representative of results obtained in at least two independent experiments for each system examined. Although the results shown were normally obtained using samples equilibrated for 16 h at the final experimental temperature, similar results were obtained for samples incubated for as little as 2–4 h.

### Detergent-solubilization assays

Dried lipid mixtures comprising 800 nmol of phospholipid (5:4:1 molar proportions PC/PE/PS) plus 33 mol % cholesterol were prepared and dispersed in buffer above their transition temperature as described above. Samples dispersed in this manner were used directly as multilamellar vesicles (MLVs) or extruded through 0.1-$\mu$m pore size polycarbonate filters (MacDonald et al., 1991) to form large unilamellar vesicles (LUVs). Vesicle samples prepared in either manner were mixed at 0°C with Triton X-100 (to 1%) in buffer to a final volume of 2 ml. After incubation for 20 min at 0°C, the samples were diluted to 5 ml with 1% Triton-containing buffer and centrifuged at 200,000 x g for 14 h in a Beckmann SW55Ti ultracentrifuge rotor (Beckmann Instruments Inc., Fullerton, CA) at 4°C. The supernatant was carefully removed at 0°C and the pellet solubilized in 2:1:0.5 (v/v) $CH_2Cl_2/CH_3OH$/buffer, then the volume proportions of the solvents were adjusted to 2:1:1 $CH_2Cl_2/CH_3OH$/buffer to induce phase separation. The $CH_2Cl_2$ phase was dried down and the recovered lipids separated by thin-layer chromatography on silica gel 60, developing in 65/35/1/1 (v/v) $CH_2Cl_2/CH_3OH$/concentrated $NH_4OH/H_2O$. The resolved phospholipid spots were visualized by spraying with sulfuric/molybdic acid reagent, slightly moistened with distilled water, and scraped from the thin-layer plates. The recovered silica from each spot (and an appropriate blank spot) were digested with perchloric plus nitric acid and the released phosphate determined by the method of Lowry and Tinsley (1974). An identically prepared lipid mixture that was not subjected to detergent fractionation was extracted and analyzed in the same manner to

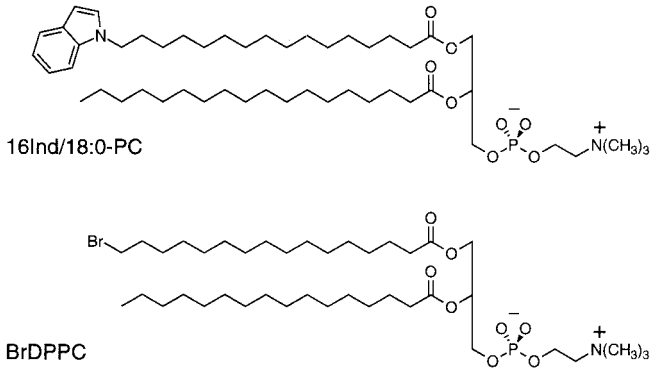


FIGURE 1 General structures of the brominated and indolyl-labeled phospholipids used in this study. The acyl chain at the 2-position and/or the polar headgroup were varied to generate the other quencher and fluorescent phospholipid species discussed in the text.

verify the uniform extraction and quantitation of the different phospholipid species.

## RESULTS

In Fig. 1 are shown representative structures of the indolyl-labeled and brominated lipids used as fluorescence probes and quencher species, respectively, in this study. Monobrominated quencher lipids like those used here offer the advantages that they can be made virtually isosteric with natural phospholipids, and that the relatively hydrophobic bromine substituent is not expected to perturb significantly the disposition of the bromine-substituted acyl chain within the bilayer (Wiener and White, 1991; Wiener et al., 1991). In agreement with this expectation, a monobrominated structural analog of dilauroyl PC has been shown to exhibit physical properties very similar to those of dilauroyl PC itself (Silvius et al., 1996).

The primary analytical tool used in this study is a quenching curve, determined by measuring the fluorescence intensity for a given probe as a function of the proportion of quencher lipid in bilayers combining the latter with one or more unlabeled lipid species (London and Feigenson, 1981a,b). In the BrPOPC/POPC system (Fig. 2 *A*), whose components should intermix nearly ideally, the quenching curve exhibits no abrupt changes in slope and closely matches the simple exponential form proposed by Chattopadhyay and London (1987) to describe quenching in bilayers where the lipids mix homogeneously in all proportions:

$$(F/F_0)_{cor} = A \cdot \exp(-K(\%Q)) + B \qquad (2)$$

where $A$ and $B$ are fitting constants, $(\%Q)$ is the molar percentage of quencher in the bilayer, and $K$ is a function of the critical distance for the quenching interaction. In contrast, when the system exhibits segregation of domains with distinct compositions, as in the BrDPPC/(4:1 DOPE + DOPS) system represented in Fig. 2 *B*, the quenching curve no longer follows the simple form given by Eq. 2 (London and Feigenson, 1981b; Huang et al., 1988; Silvius, 1992; Ahmed et al., 1997).

The usefulness of quenching curves to detect lateral segregation of lipid domains can be enhanced by comparing quenching curves obtained for different fluorescent probes that have closely related structures but strongly differing affinities for ordered versus disordered lipid domains (Wang et al., 2000). For the systems examined in this study, we compared quenching curves obtained using the fluorescent lipids 16Ind/18:0-PC and 16Ind/18:2-PC (and, in some systems, 16Ind/18:2-PE as well). As shown in Fig. 2 *A*, in the system BrPOPC/POPC the three fluorescent lipids give essentially identical quenching curves. This behavior is expected when all three probes experience the same, laterally homogeneous environment. In contrast, when laterally segregated domains with different properties and compositions are present in the bilayer, the different fluorescent lipids can distribute differently among the segregated domains and, hence, give distinct quenching curves. This behavior is illustrated in Fig. 2 *B*, where in the system BrDPPC/(4:1 DOPE + DOPS) the quenching curves measured for 16Ind/18:0-PC versus 16Ind/18:2-PC strongly diverge from roughly 35–100 mol % BrDPPC, where a BrDPPC-rich gel phase and a (DOPE + DOPS)-enriched fluid phase coexist.

Assuming that at 25°C the BrDPPC/(4:1 DOPE + DOPS) system represented in Fig. 2 *B* can be approximately treated as a pseudo-two–component lipid mixture, we can analyze the quenching data obtained over the region of phase separation to estimate the gel/fluid phase partition coefficient $K_p$ for the fluorescent lipid probes examined (Huang et al., 1988; Wang et al., 2000). From such an analysis (Fig. 2 *B*, *inset*) we estimate that in this system the 16Ind/18:0-PC probe exhibits a roughly 6-fold greater affinity for the gel over the liquid-crystalline phase ($K_p$ = 6.4 ± 0.5) whereas the 16Ind/18:2-PC probe prefers the liquid-crystalline to the gel phase by a similar factor ($K_p$ = 0.179 ± 0.006). The two probes exhibited similar extents of discrimination between the gel and liquid-crystalline phases in BrDPPC/DOPC bilayers at 25°C (results not shown). These results indicate that (1) the affinities of these probes for the more ordered gel phase are strongly influenced by the nature of the acyl group at the 2-position, and (2) the disaturated 16Ind/18:0-PC probe can partition efficiently into ordered regions of the bilayer, despite the presence of the indolyl substituent on the 1-position acyl chain.

### Domain formation by (saturated phospholipid/unsaturated phospholipid/cholesterol) mixtures

Segregation of $l_o$ from $l_d$ domains has been demonstrated previously in mixtures of saturated and unsaturated PCs (Schroeder et al., 1994; Ahmed et al., 1997). We therefore

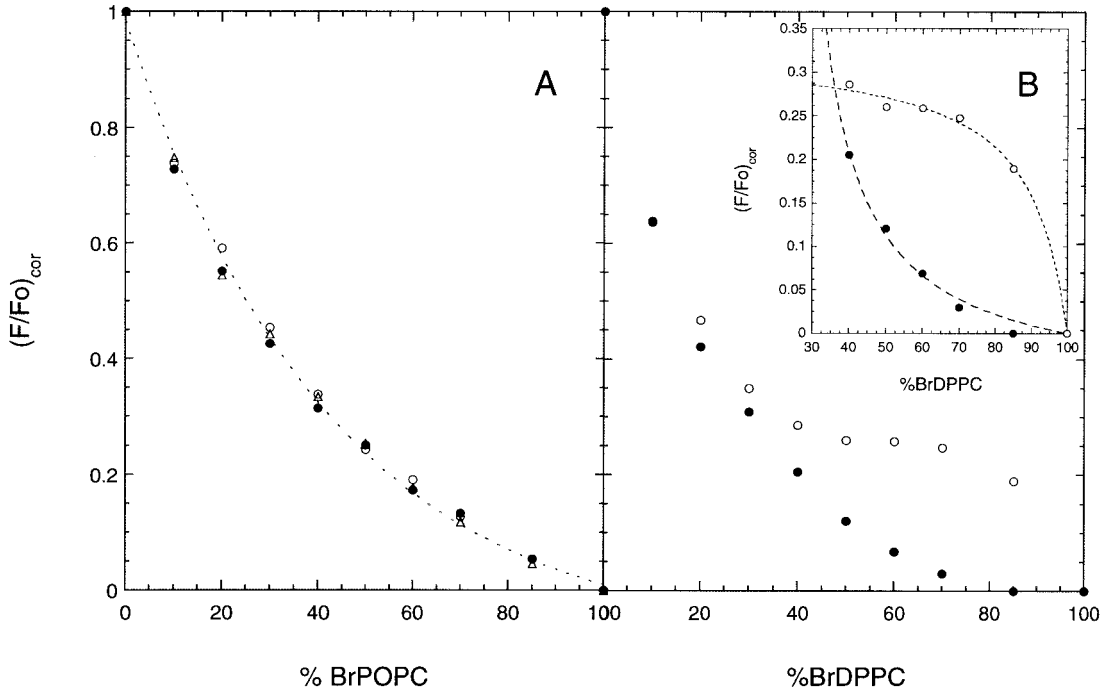


FIGURE 2 Quenching curves (plotted as scaled fluorescence $(F/F_o)_{cor}$), determined as described in Materials and Methods for (●) 16Ind/18:0-PC, (○) 16Ind/18:2-PC, and (Δ) 16Ind/18:2-PE in cholesterol-free bilayers at 25°C. (*A*) Quenching curves determined for POPC/BrPOPC bilayers incorporating the indicated molar percentages of BrPOPC (*dashed line*, fit of data to Eq. 1 in the text). (*B*) Quenching curves determined for bilayers combining the indicated molar percentages of BrDPPC with DOPE and DOPS, maintaining a constant 4:1 molar ratio of DOPE to DOPS. (*B inset*) Data shown in *B* for the composition range 40–100 mol % BrDPPC, fitted to the equation

$$(F/F_0)_{cor} = (F/F_0)_{cor}^{fluid} \cdot \left(\frac{\%Q_{gel} - \%Q}{K_p \cdot (\%Q - \%Q_{fluid}) + (\%Q_{gel} - \%Q)}\right) + (F/F_0)_{cor}^{gel} \cdot K_p \left(\frac{\%Q - \%Q_{fluid}}{K_p \cdot (\%Q - \%Q_{fluid}) + (\%Q_{gel} - \%Q)}\right)$$

where $\%Q$ is the molar percentage of quencher phospholipid in the total phospholipid fraction, $\%Q_{gel}$ and $\%Q_{fluid}$ are the values of $\%Q$ defining the boundaries of the region of gel/fluid phase separation, $(F/F_o)_{cor}$, $(F/F_o)_{cor}^{gel}$, and $(F/F_o)_{cor}^{fluid}$ represent the scaled fluorescence values measured for molar percentages of quencher equal to $\%Q$, $\%Q_{gel}$, and $\%Q_{fluid}$, and $K_p$ is the partition coefficient describing the relative affinity of the probe for the gel over the fluid phase.

first examined how saturated and unsaturated species intermix in systems combining cholesterol (33 mol %) with both amino and choline phospholipids.

The introduction of 33 mol % cholesterol into the system BrDPPC/(4:1 DOPE + DOPS) discussed above modifies, but does not abolish, the divergence in the quenching curves observed for 16Ind/18:0-PC versus 16Ind/18:2-PC, as shown in Fig. 3 *A*. A region of apparent domain segregation (as judged by the divergence of the quenching curves for the two fluorescent lipids) is still seen to extend from ~30–100 mol % BrDPPC in the phospholipid fraction. Very similar results were obtained in this system using an alternative pair of fluorescent lipids, 1-palmitoyl-2-(16-(indol-1-yl)palmitoyl)-PC and 1-oleoyl-2-(16-(indol-1-yl)palmitoyl)-PC (not shown). Quenching curves were routinely determined at 25°C for this and the other cholesterol-containing systems examined here to reduce the risk of lipid degradation during extended preincubation and to enhance the potential to detect segregation of domains in mixtures containing exclusively unsaturated lipid species. However, as indicated, key results were also tested at 37°C and, in some cases, at 10°C as well. Except where otherwise explicitly indicated, all systems discussed below incorporated cholesterol at 33 mol %.

The quenching curves determined for 16Ind/18:0-PC versus 16Ind/18:2-PC in the system BrDPPC/(4:1 PLnPE + PLnPS)/cholesterol (Fig. 3 *B*) diverge over a range of compositions similar to that observed in the system BrDPPC/(4:1 DOPE + DOPS)/cholesterol described above. The quenching curve measured for 16Ind/18:2-PE in the BrDPPC/(4:1 PLnPE + PLnPS)/cholesterol system closely resembles that measured for the corresponding fluorescent PC (Fig. 3 *B*), indicating that the unsaturated PE and PC probes partition similarly between quencher-rich and quencher-depleted domains. Segregation of domains was likewise detected, over a similar range of compositions, in BrDPPC/(4:1 PLnPE + PLnPS)/cholesterol bilayers at 37°C and in BrDPPC/(4:1 POPE + POPS)/cholesterol bilayers at 25°C (results not shown).

Extensive segregation of domains was also observed for mixtures combining cholesterol (33 mol %) and PLnPC

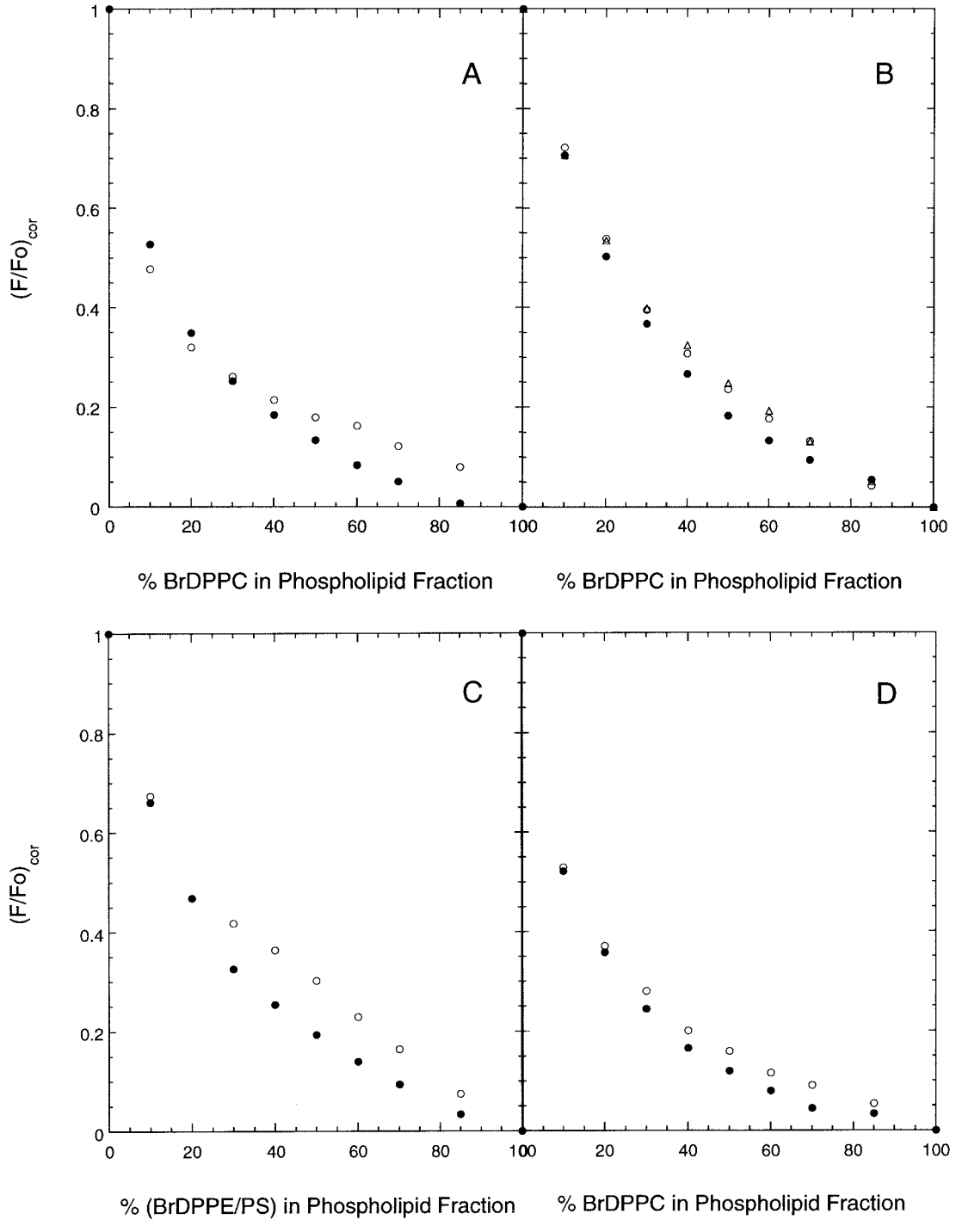


FIGURE 3 Quenching curves determined as described in Materials and Methods for (●) 16Ind/18:0-PC, (○) 16Ind/18:2-PC, and (Δ) 16Ind/18:2-PE at 25°C in bilayers combining saturated phospholipid quenchers with unsaturated phospholipids and cholesterol. (*A*) Bilayers combining BrDPPC with cholesterol (33 mol %) and varying proportions of a 4:1 mixture of DOPE and DOPS. (*B*) Bilayers combining BrDPPC with 33 mol % cholesterol and varying proportions of a 4:1 mixture of PLnPE and PLnPS. (*C*) Bilayers combining a 4:1 mixture of BrDPPE and BrDPPS with cholesterol (33 mol %) and varying proportions of PLnPC. (*D*) Bilayers combining BrDPPC with 33 mol % cholesterol and varying proportions of a 4:1 mixture of brain PE and brain PS.

with BrDPPE plus BrDPPS (the latter two species in a constant 4:1 molar ratio). The region of domain segregation appears to extend from ~15–100 mol % (BrDPPE + BrDPPS) in the phospholipid fraction (Fig. 3 *C*). Therefore, it appears that saturated aminophospholipids can form segregated ordered domains at least as readily as can saturated

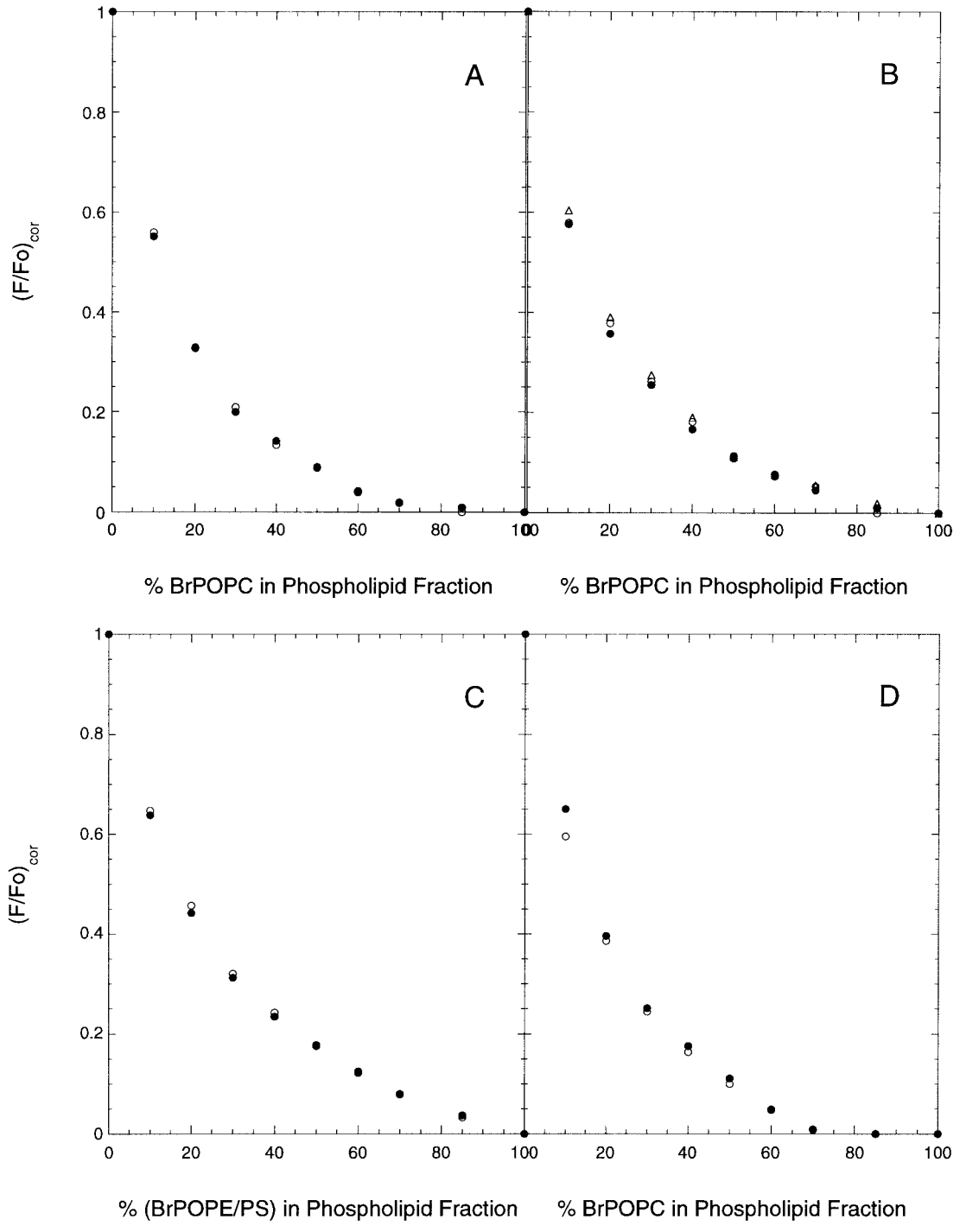


FIGURE 4 Quenching curves determined as described in Materials and Methods for (●) 16Ind/18:0-PC, (○) 16Ind/18:2-PC, and (Δ) 16Ind/18:2-PE in bilayers combining monounsaturated phospholipid quenchers with unsaturated phospholipids and cholesterol (temperature = 25°C). (*A*) Bilayers combining BrPOPC with cholesterol (33 mol %) and varying proportions of a 4:1 mixture of POPE and POPS. (*B*) Bilayers combining BrPOPC with 33 mol % cholesterol and varying proportions of a 4:1 mixture of PLnPE and PLnPS. (*C*) Bilayers combining a 4:1 mixture of BrPOPE and BrPOPS with cholesterol (33 mol %) and varying proportions of PLnPC. (*D*) Bilayers combining BrPOPC with cholesterol (33 mol %) and varying proportions of a 4:1 mixture of brain PE and brain PS.

choline lipids in the presence of cholesterol. Because the physical behavior of saturated phosphatidylserines is influenced by the presence of multivalent cations, the data shown in Fig. 3 *C* were obtained for lipid dispersions prepared in medium containing 0.5 mM $Mg^{2+}$ and 50 $\mu$M spermine, levels of these cations similar to those found in the cyto-

plasm of mammalian cells (Alvarez-Leefmans et al., 1984, 1986; Igarashi and Kashiwagi, 2000). Essentially identical quenching curves were however obtained when the same lipids were dispersed in a medium lacking divalent cations (not shown).

Natural membrane phospholipids are heterogeneous in their acyl chain composition. To determine whether such heterogeneity could affect the formation of segregated $l_o$ domains in lipid mixtures similar to those of interest here, we examined the mixing of lipids in the system BrDPPC/(4:1 brain PE + brain PS)/cholesterol. As shown in Fig. 3 *D*, in this system segregation of domains is observed from ~20–90 mol % BrDPPC in the phospholipid fraction. The behavior of this system is thus similar to that observed using comparable mixtures containing synthetic PLnPE/PLnPS in place of brain PE/PS (Fig. 3 *B*).

## Lateral organization of unsaturated phospholipid/cholesterol mixtures

Although small amounts of fully saturated phospholipids can occur in eukaryotic cell membranes, mono- and polyunsaturated phospholipids are much more abundant in the plasma membranes of both yeast (Schneiter et al., 1999) and at least some mammalian cells (Fridriksson et al., 1999). Mixtures containing purely unsaturated phospholipids could, in principle, form segregated $l_o$ and $l_d$ domains in the presence of cholesterol, based on differences in the headgroups and/or the degree of unsaturation of the phospholipids. To address the first possibility, we examined the mixing of the aminophospholipids POPE and POPS (in 4:1 molar proportions) with BrPOPC. As shown in Fig. 4 *A*, in the presence of 33 mol % cholesterol these species appear entirely miscible, as judged by the complete coincidence of the quenching curves for 16Ind/18:0PC and 16Ind/18:2PC. The quenching curves for these two fluorescent lipid species (and for 16Ind/18:2-PE) were still completely coincident when the experiment was repeated at 10°C (not shown). Thus, it seems that differences in headgroup structure alone cannot drive formation of segregated $l_o$ and $l_d$ domains in mixtures combining unsaturated choline and amino phospholipids with cholesterol, even at temperatures well below physiological.

We further examined whether simultaneous differences in phospholipid headgroup structure and degree of acyl chain unsaturation could drive segregation of $l_o$ domains in the presence of cholesterol. As shown in Fig. 4 *B*, at 25°C mixtures combining cholesterol (33 mol %) with the monounsaturated choline phospholipid BrPOPC and the polyunsaturated aminophospholipids PLnPE/PLnPS (the latter in a 4:1 molar ratio) again show complete miscibility of the lipid components at all proportions of the choline versus the aminophospholipid species. The quenching curve obtained for 16Ind/18:2-PE in this system also closely resembles that determined for the two fluorescent PC species, further supporting the conclusion that the lipid components mix homogeneously. Very similar results were obtained for this same system using an alternative pair of fluorescent lipid probes, 1-palmitoyl-2-(16-(indol-1-yl)palmitoyl)-PC and 1-oleoyl-2-(16-(indol-1-yl)palmitoyl)-PC (not shown). Essentially superimposable quenching curves were also obtained for 16Ind/18:0-PC versus 16Ind/18:2-PC in this system at 10°C and at 37°C, as well as in analogous phospholipid mixtures containing 20 mol % in place of 33 mol % cholesterol (results not shown). In a "reciprocal" experiment, using mixtures of cholesterol (33 mol %) with BrPOPE/BrPOPS (4:1 molar ratio) and PLnPC, the quenching curves for the two indolyl-PC probes were also essentially superimposable, again suggesting homogeneous intermixing of the lipid components (Fig. 4 *C*). The quenching curves shown in Fig. 4 *C* were obtained for lipid vesicles dispersed in medium containing roughly physiological levels of free $Mg^{2+}$ (0.5 mM) and spermine (50 $\mu$M). Essentially identical quenching curves were obtained for the same lipid mixtures prepared in medium without these multivalent cations (not shown).

To determine how the acyl chain heterogeneity characteristic of natural membrane phospholipids might affect their mixing properties in the presence of cholesterol, we examined how PE and anionic lipids derived from bovine brain and liver mixed with monounsaturated PCs in the presence of cholesterol. Mixtures combining cholesterol (33 mol %) and brain PE/PS with BrPOPC showed no evidence for domain separation, as judged by the complete superimposability of the quenching curves measured for 16Ind/18:0-PC and 16Ind/18:2-PC at 25°C (Fig. 4 *D*), at 37°C or at 10°C (not shown). Very similar results were obtained using bovine liver PE in place of brain PE, or bovine liver PI in place of brain PS, in parallel experiments (not shown).

Evidence has been reported in some systems that the phosphatidylserine component in the plasma membrane may be more saturated than the neutral phospholipid components present (Keenan and Morré, 1970; Schneiter et al., 1999). We therefore examined how saturated and monounsaturated phosphatidylserines intermix with more highly unsaturated neutral phospholipids in the presence of cholesterol. As shown in Fig. 5 *A*, at 25°C mixtures combining BrDPPS and PLnPC with 33 mol % cholesterol show evidence for $l_o/l_d$ domain segregation over a wide range of PS contents (from 15–20 mol % to ~100 mol % PS). However, under the same conditions, BrPOPS mixes homogeneously with PLnPC in the presence of cholesterol (Fig. 5 *B*). Comparable results were obtained in analogous experiments in which the PC component was replaced by equimolar mixtures of PLnPC and PLnPE, or when $Mg^{2+}$ (0.5 mM) and spermine (50 $\mu$M) were included in the buffer used for lipid dispersal (results not shown).

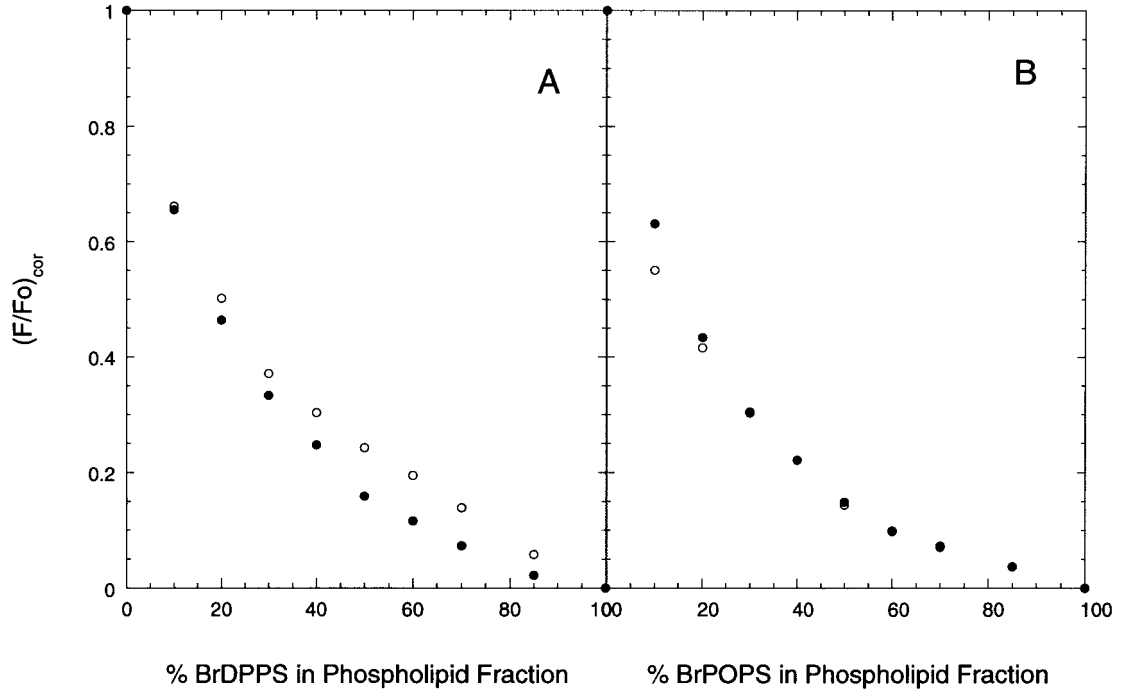


FIGURE 5 Quenching curves determined as described in Materials and Methods for (●) 16Ind/18:0-PC and (○) 16Ind/18:2-PC at 25°C in bilayers combining BrDPPS (*A*) or BrPOPS (*B*) with cholesterol (33 mol %) and varying proportions of PLnPC.

## Low-temperature detergent fractionation of lipid/cholesterol mixtures

In a final series of experiments, vesicles combining cholesterol with various combinations of PC, PE, and PS (5:4:1:5 molar proportions PC/PE/PS/cholesterol) were incubated with 1% Triton X-100 at 0–4°C, and the recovery of each lipid species in the detergent-insoluble fraction was then determined as outlined in Materials and Methods. This method is frequently used to isolate "lipid raft" fractions from biological membranes, and evidence has been reported that the presence of a low-temperature Triton-insoluble lipid fraction in artificial membranes can be correlated with the formation of $l_o$ domains (London and Brown, 2000).

As shown in Table 1, upon low-temperature detergent treatment of LUV combining DPPC with cholesterol and different unsaturated PE and PS species, the bulk of the DPPC and a minor fraction of the unsaturated lipids were recovered in the pellet fraction. In contrast, when similar vesicles were prepared incorporating POPC or SOPC in place of DPPC, the vesicle lipids were essentially completely detergent-soluble. Interestingly, MLVs of the same compositions gave significantly larger amounts of unsaturated species in the pellet fraction, even when the vesicles were composed purely of unsaturated lipid components. This result suggests that solubilization of lipids from MLVs may be influenced by kinetic or other factors that are specific to such vesicles and not directly related to the lateral organization of the bilayer lipids. The results obtained by low-temperature detergent fractionation of LUVs suggest that segregated detergent-resistant $l_o$ domains are not present in mixtures composed purely of unsaturated lipids.

**TABLE 1 Recovery of lipids in the detergent-insoluble fraction obtained after incubation of 50:40:10:50 PC/PE/PS/cholesterol vesicles with Triton X-100 at 0°C**

| Lipid composition | Type of vesicle | % in insoluble fraction | | |
|---|---|---|---|---|
| | | PC | PE | PS |
| DPPC/brain PE/brain PS | LUV | 75 ± 4 | 8 ± 3 | 9 ± 1 |
| DPPC/PLnPE/PLnPS | LUV | 68 ± 10 | 9 ± 2 | 11 ± 2 |
| DPPC/POPE/POPS | LUV | 50 ± 3 | 24 ± 3 | 18 ± 5 |
| POPC/brain PE/brain PS | LUV | 2 ± 2 | 1 ± 1 | 0 ± 0 |
| POPC/PLnPE/PLnPS | LUV | 2 ± 1 | 6 ± 1 | 5 ± 5 |
| SOPC/brain PE/brain PS | LUV | 1 ± 1 | 2 ± 1 | 6 ± 3 |
| SOPC/PLnPE/PLnPS | LUV | 1 ± 1 | 2 ± 1 | 9 ± 5 |
| DPPC/PLnPE/PLnPS | MLV | 64 ± 9 | 66 ± 5 | 64 ± 10 |
| POPC/brain PE/brain PS | MLV | 10 ± 1 | 11 ± 3 | 12 ± 3 |
| POPC/PLnPE/PLnPS | MLV | 24 ± 4 | 21 ± 3 | 21 ± 1 |

Lipid vesicles of the indicated compositions (LUVs, large unilamellar vesicles prepared by filter extrusion; MLVs, multilamellar vesicles prepared by vortexing) were incubated with 1% Triton X-100 at 0°C and then centrifuged for 4 h at 200,000 × *g*. Lipids recovered in the resulting pellet fraction were analyzed as described in Materials and Methods. Results are presented as the mean ± standard deviation of determinations from three independent experiments.

The above Triton-solubilization experiments were carried out at the standard temperature of 0–4°C, because at higher temperatures, Triton solubilizes $l_o$ domains more readily (Schroeder et al., 1994; London and Brown, 2000). As noted previously (Brown and London, 1997), results obtained using the low-temperature detergent solubilization assay may reflect the effects of the low assay temperature on bilayer organization. However, our observation that diverse mixtures of unsaturated phospholipids and cholesterol do not form Triton-resistant domains is consistent with the results of our fluorescence assay, which indicate that similar lipid mixtures do not exhibit segregation of $l_o$ and $l_d$ domains at temperatures down to at least 10°C, the lowest temperature at which (for technical reasons) the fluorescence experiments could be conducted.

## DISCUSSION

The fluorescence assay used in this study has previously been shown to be useful to detect gel/fluid phase separations (Huang et al., 1988) or segregation of $l_o$ from $l_d$ domains (Silvius, 1992; Silvius et al., 1996; Ahmed et al., 1997). Because the method relies on measurements of fluorescence quenching, a contact-dependent and therefore short-range phenomenon, it is well suited to detect even small-scale inhomogeneities in the lateral distributions of lipids within the bilayer. This capability is important because, at physiological temperatures, $l_o$ domains seem to be relatively small in both natural and model membranes (Pralle et al., 2000; Dietrich et al. 2001).

Our observations that mixtures combining saturated phospholipids with unsaturated phospholipids and cholesterol cam form coexisting $l_o$ and $l_d$ domains agree with previous findings using related lipid systems (Schroeder et al., 1994, 1998; Ahmed et al., 1997; Dietrich et al., 2001). Previous studies of this phenomenon have focused mainly on systems combining cholesterol with PC and sphingolipids. Our results indicate, however, that the segregation of unsaturated from saturated species in the presence of cholesterol is a fairly general phenomenon that is not strongly dependent on the structure of the phospholipid headgroups (cf. Figs. 3 *B*, 3 *C*, and 5 *A*). This conclusion is consistent with our previous finding that fluorescent PC and PE species with the same fatty acyl composition show only modest differences in their affinities for $l_o$ bilayer domains (Wang and Silvius, 2000), and with observations that sphingolipids and PCs with similar chain-melting transition temperatures show similar tendencies to form segregated $l_o$ domains in the presence of cholesterol (Schroeder et al., 1994; Ahmed et al., 1997; Dietrich et al., 2001).

In contrast to our findings for systems combining cholesterol with saturated and unsaturated phospholipids, we find no evidence that cholesterol can promote the lateral segregation of $l_o$ and $l_d$ domains when combined with mixtures of different unsaturated phospholipids. This result is obtained for a variety of systems, including bilayers incorporating mixtures of phospholipid species that differ markedly in both their degree of unsaturation and their headgroup structure, as well as bilayers incorporating highly heterogeneous lipid components from natural sources. Further, this basic result seems to be unaltered using experimental temperatures as low as 10°C, suggesting that at physiological temperatures, mixtures of unsaturated phospholipids and cholesterol such as those examined here do not exist close to a condition favoring domain segregation. It remains possible that cholesterol could promote at least some degree of lateral segregation in mixtures of unsaturated lipids showing extreme differences in their degree of unsaturation (e.g., a monounsaturated lipid and a lipid with two polyunsaturated acyl chains). However, the results of our experiments using phospholipids from natural sources suggest that such segregation does not occur in mixtures of phospholipids whose acyl-chain compositions are typical of those found in most mammalian cell membranes.

A potential qualification to the conclusion just noted would arise if the cytoplasmic leaflet of the plasma membrane contained sufficient proportions of saturated phospholipids to support the formation of segregated $l_o$ domains. Early analyses of membrane phospholipids in mammalian cells in fact suggested that the glycerophospholipids of the plasma membrane are more saturated than those found in intracellular membranes (Keenan and Morré, 1970). However, our findings with systems containing various saturated phospholipids suggest that substantial proportions of these species (~20 mol % or greater) are required to allow spontaneous segregation of $l_o$ domains in mixtures with unsaturated phospholipids and cholesterol. Recent mass-spectrometric analyses of both yeast and mammalian cells (Fridriksson et al., 1999; Schneiter et al., 1999) suggest that the proportion of fully saturated glycerophospholipids in the plasma membrane falls well below this level. Thus, it seems unlikely that the proportion of saturated phospholipids in the plasma membrane is sufficient to support spontaneous segregation of $l_o$ domains in the cytoplasmic leaflet.

The conclusions noted above have significant implications for understanding the organization of lipids at the cytoplasmic face of a lipid raft in the plasma or other membranes of animal cells. Although differences in the interactions of inner-leaflet phospholipids with one another and with cholesterol may be significant (Demel et al., 1977; Yeagle and Young, 1986; Keough et al., 1989; Kariel et al., 1991; Huster et al., 1998; Mitchell and Litman, 1998), they seem by themselves to be insufficient to drive the spontaneous segregation of $l_o$-domains within the cytoplasmic leaflet of the membrane. If the cytoplasmic leaflet of lipid rafts indeed exists in a $l_o$ state distinct from that of the surrounding lipids, it seems that this organization can be explained only by invoking factors extrinsic to the inner-monolayer lipids themselves. Two possibilities immediately suggest themselves in this regard. First, specific proteins

binding to the inner surface of a raft domain, e.g., annexins (Oliferenko et al., 1999) could locally order the lipids to which they bind. Although this possibility cannot be ruled out, the binding of such proteins to a given region of the membrane might also be expected to create steric hindrance to the binding of other proteins to the same region. It is thus not clear that local ordering of membrane lipids induced by this mechanism would by itself promote the binding of other raft-associating proteins (e.g., multiply N/S-acylated species) to the same region of the membrane's cytoplasmic surface.

Another class of molecules that could impart a distinctive ordered organization to the lipids at the cytoplasmic surface of a raft would comprise raft-associated molecules that penetrate from the outer membrane leaflet into the cytoplasmic monolayer. Transmembrane influences on raft organization have been suggested previously (Brown and London, 1997; Simons and Ikonen, 1997), and precedents indeed exist for transbilayer coupling of segregated lipid domains (Schmidt et al., 1978; Korlach et al., 1999; Dietrich et al., 2001). Although specific transmembrane proteins could in principle fulfill this role, no such candidate for such a function has yet been reported, and rafts in fact appear to be depleted in most transmembrane proteins (Brown and London, 1998b). As suggested previously, another attractive mechanism for transmembrane coupling would be transbilayer interdigitation of the very long saturated acyl chains carried by a significant fraction of membrane sphingolipids (Grant et al., 1987; Morrow et al., 1993, 1995; Rietveld and Simons, 1998). Further work will be required to assess such possibilities directly, either in asymmetric model bilayers or, more challenging still, in the native cellular membrane environment. However, we note that a requirement for transbilayer influences to confer a distinctive, more ordered organization on the lipids in the cytoplasmic leaflet of a raft would offer a particularly simple mechanism to ensure that formation of $l_o$ domains was tightly correlated between the inner and outer leaflets of the cell membrane.

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