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The Effect of Sterol Structure on Membrane Lipid Domains Reveals How Cholesterol Can Induce Lipid Domain Formation†

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ABSTRACT: Detergent-insoluble membrane domains, enriched in saturated lipids and cholesterol, have been implicated in numerous biological functions. To understand how cholesterol promotes domain formation, the effect of various sterols and sterol derivatives on domain formation in mixtures of the saturated lipid dipalmitoylphosphatidylcholine (DPPC) and a fluorescence quenching analogue of an unsaturated lipid was compared. Quenching measurements demonstrated that several sterols (cholesterol, dihydrocholesterol, epicholesterol, and 25-hydroxycholesterol) promote formation of DPPC-enriched domains. Other sterols and sterol derivatives had little effect on domain formation (cholestane and lanosterol) or, surprisingly, strongly inhibit it (coprostanol, androstenol, cholesterol sulfate, and 4-cholestenone). The effect of sterols on domain formation was closely correlated with their effects on DPPC insolubility. Those sterols that promoted domain formation increased DPPC insolubility, whereas those sterols that inhibit domain formation decreased DPPC insolubility. The effects of sterols on the fluorescence polarization of diphenylhexatriene incorporated into DPPC-containing vesicles were also correlated with sterol structure. These experiments indicate that the effect of sterol on the ability of saturated lipids to form a tightly packed (i.e., tight in the sense that the lipids are closely packed with one another) and ordered state is the key to their effect on domain formation. Those sterols that promote tight packing of saturated lipids promote domain formation, while those sterols that inhibited tight packing of saturated lipids inhibited domain formation. The ability of some sterols to inhibit domain formation (i.e., act as "anti-cholesterols") should be a valuable tool for examining domain formation and properties in cells.

Many studies now suggest a model of eukaryotic plasma membrane structure in which there exist both Triton X-100-insoluble domains (rafts) rich in cholesterol and saturated lipids (i.e., sphingolipids) and Triton X-100-soluble domains enriched in unsaturated phospholipids (*1*, *2*). The sphingolipid- and cholesterol-rich domains are believed to have a distinct protein composition (*3*−*5*) and have been implicated in numerous biological processes, including signal transduction events (especially in the immune system), membrane component sorting, viral budding, toxin entry into cells, prion action, and amyloid formation (*5*−*16*). Thus, the origin of, and the interactions controlling, lipid domain formation are of great interest. We have shown that sphingolipid and cholesterol domains are likely to exist in the liquid-ordered (L_o) state, while the phospholipid-enriched domains are more

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likely to be in the more familiar fluid liquid crystalline L_α phase (*17*−*19*). The L_o state is characteristic of mixtures of cholesterol with highly saturated lipids (*20*, *21*), and is unlike the L_α state in that it contains lipids in a tightly packed, relatively ordered state that is detergent-insoluble. Cholesterol can promote separation of lipid mixtures in the L_α phase into coexisting L_α and (detergent-insoluble) L_o domains (*18*, *19*, *22*). Cholesterol appears to promote domain formation in cells, and cholesterol levels can influence both the domain association and biological activity of proteins that co-isolate with detergent-resistant membranes (*23*−*25*).

Such studies raise two important questions. Why does cholesterol induce domain formation? Is it linked to the ability to pack tightly with some lipids (*1*, *18*, *26*)? To investigate these questions, variations in sterol structure were correlated with their effect on lipid domain formation. The results indicate that the ability of cholesterol to participate in strong, close packing with saturated lipids is the critical feature required for its promotion of lipid domain formation. Sterols that lack the ability to pack well with saturated lipids do not promote domain formation. Furthermore, some of these sterols are found to have effects that are the opposite of those of cholesterol, loosening lipid packing and inhibiting lipid domain formation.

EXPERIMENTAL PROCEDURES

Materials. DPPC (dipalmitoylphosphatidylcholine), DOPC (dioleoylphosphatidylcholine), 12SLPC [1-palmitoyl-2-(12-doxyl)stearoylphosphatidylcholine], and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Crude lanosterol, epicholesterol, 4-cholesten-3β-one (cholestenone), cholesterol sulfate, androstenol, and 25-hydroxycholesterol were purchased from Steraloids, Inc. (Newport, RI), and lanosterol, dihydrocholesterol (cholestanol), and cholestane were from Sigma Chemical (St. Louis, MO). Coprostanol was purchased from Applied Science Laboratories. The purity was confirmed by thin-layer chromatography on silica gel plates, using as the solvent system 65:25:4 (v/v) chloroform/methanol/water for the phospholipids and either 15:10 or 22:3 (v/v) hexane/ethyl acetate for the sterols and sterol derivatives. After the plates had been sprayed with sulfuric acid and charred, only one spot was observed. For the sterol and sterol derivatives, melting points agreed with the values reported by the Steraloids, Inc., catalog.

Fluorescence Measurements. Fluorescence emission spectra were measured on a Spex 212 Fluorolog fluorimeter using, unless otherwise noted, a 10 mm excitation path length, 4 mm emission path length semi-micro quartz cuvette. 1,6-Diphenylhexatriene (Aldrich Chemical Co., Milwaukee, WI) fluorescence was measured at an excitation wavelength of 358 nm and an emission wavelength of 427 nm. Narrow excitation and emission slits (1.25 mm) were used.

Fluorescence Quenching Experiments. Multilamellar vesicles containing 50 μM total lipid and 1 mol % DPH were prepared in 10 mM sodium phosphate and 150 mM NaCl (pH 7) (PBS) as described previously except at 23 °C (*18*). The sample volume was 900 μL. Samples with quencher (*F* samples) contained a 1:1 mixture of DPPC and 12SLPC, or DOPC and 12SLPC, with or without 15 mol % sterol or sterol derivative. Corresponding samples without quencher (F_o samples) contained 1:1 DPPC/DOPC or DOPC, respectively, with or without 15 mol % sterol or sterol derivative. Background samples were prepared identically, except without DPH. Fluorescence in *F* samples was measured at a series of increasing temperatures. The temperature was monitored with an electronic thermometer, and fluorescence was read 2 min after the samples reached the desired temperature. For most samples, after being heated to the final temperature, samples were cooled to 23 °C, and fluorescence was remeasured. Fluorescence in the F_o samples and background samples was measured only at 23 °C and the highest temperature that was used, and the values at intermediate temperatures were estimated by linear interpolation. Control experiments showed this interpolation did not cause a significant error. Background values were subtracted before F/F_o values were calculated.

Percent Solubilization Experiments. Multilamellar lipid vesicles containing 500 nmol of total lipid were prepared in PBS as they were for the quenching experiments, except that the lipid was dispersed in buffer at around 80 °C, and then cooled to room temperature so more uniform dispersions could be obtained at the higher lipid concentrations that were used. The sample volume was 950 μL. For measuring solubilization by the loss of light scattering, the optical density was measured at 400 nm (using a Beckman 650 spectrophotometer). Then 50 μL of 10% (w/v) Triton X-100/PBS was added. After mixing and incubation at 23 °C overnight (about 19 h), the optical density was remeasured. %OD is the ratio of optical density after Triton X-100 incubation to that before the addition of Triton X-100 (not corrected for dilution with the Triton X-100 solution). Similar results were obtained after incubation for 2 h with detergent. To test solubilization by phosphate analysis, samples were prepared as described above, except in 10 mM Tris-HCl and 150 mM NaCl (pH 7). One set was treated with Triton X-100 as described above, and the control set was not. The insoluble lipid in the samples and controls was collected by centrifugation for 10−15 min at medium speed (2600 rpm) in a Sorvall GLC-3 centrifuge. The pellets were washed twice with 1 mL of the Tris/NaCl buffer, recollecting the pellets by centrifugation. The pellets were then subjected to chemical phosphate analysis, measuring the phosphate concentration by absorbance at 820 nm (*27*). $\%A_{820}$ was calculated from the ratio of the amount of phosphate in the pellet after Triton X-100 treatment to that in the absence of Triton X-100 treatment. Duplicate samples were measured, and in each case only showed a variation of a few percent.

Fluorescence Polarization. Samples for polarization measurements were prepared as they were for the quenching experiments except that each sample contained DPPC or DOPC as the sole phospholipid and the sterol concentration was 33 mol %. The total lipid concentration was 100 μM, and the DPH concentration was 0.5 μM. Fluorescence was measured with the polarization filters in the appropriate series of positions, after warming samples to the desired temperature. After background values had been subtracted, the extent of fluorescence polarization was calculated as described previously (*17*).

RESULTS AND DISCUSSION

To detect domain formation in lipid mixtures, the fluorescence of 1,6-diphenylhexatriene (DPH) was measured in

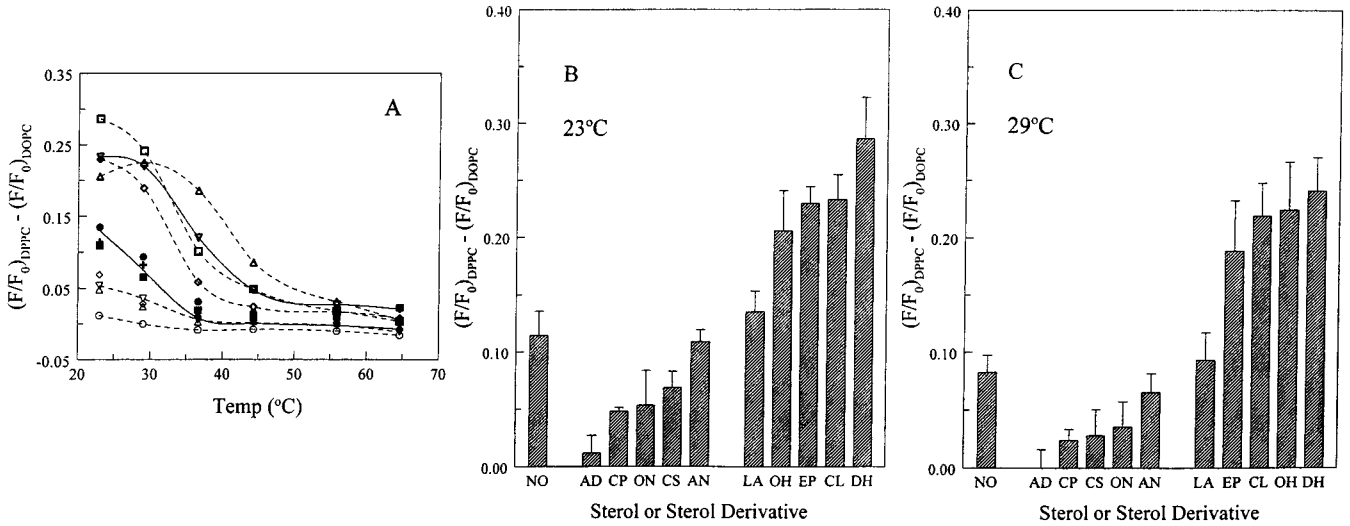

FIGURE 1: Effect of temperature on domain formation as assayed by the quenching in 12SLPC-containing lipid mixtures with and without sterol or sterol derivatives. Samples contained multilamellar vesicles with 50 μM total lipid and 1 mol % DPH dispersed in PBS. (A) Effect of temperature on the difference between the fluorescence of DPH in samples containing DPPC/12SLPC mixtures [$F_{(DPPC/12SLPC)}$] normalized to that in a mixture in which DOPC replaces quencher (F_o) (with and without 15% sterol or sterol derivative), and the normalized fluorescence in DOPC/12SLPC mixtures [$F_{(DOPC/12SLPC)}/F_o$] (with and without 15% sterol or sterol derivative). The $F_{(DOPC/12SLPC)}/F_o$ average was about 0.06. Each experiment was performed three times: (+) no sterol, (○) androstenol, (△) coprostanol, (▽) 4-cholesten-3β-one (cholestenone), (◇) cholesterol sulfate, (■) cholestane, (●) lanosterol, (bold △) 25-OH cholesterol, (bold ◊) epicholesterol, (bold □) dihydrocholesterol (cholestanol), and (bold ▽) cholesterol. (B and C) Bar graph representation of the data in panel A at 23 and 29 °C, respectively, with standard deviations shown. Values with sterol or sterol derivative are ordered from lowest to highest values, grouping the five molecules giving the lowest values and the five giving the highest values together. Abbreviations: NO, no sterol; CL, cholesterol; AD, androstenol; AN, cholestane; CS, cholesterol sulfate; DH, dihydrocholesterol; OH, 25-hydroxycholesterol; EP, epicholesterol; CP, coprostanol; ON, cholestenone; LA, lanosterol. The lanosterol used in this experiment and the experiments described below was a roughly 2:1 mixture of lanosterol and dihydrolanosterol. Similar results were obtained with pure lanosterol (not shown).

model membrane vesicles containing both the saturated lipid dipalmitoylphosphatidylcholine (DPPC) and the fluorescence quenching lipid 1-palmitoyl-2-(12-doxyl)stearoylphosphatidylcholine (12SLPC) (*28*). In addition to its quenching properties, the bulky doxyl group of 12SLPC disrupts tight lipid packing in a manner similar to that of the double bonds of unsaturated lipids, and exhibits phase behavior similar to that of an unsaturated lipid (*18*). The amount of quenching of DPH fluorescence by 12SLPC is given by F/F_o, the ratio of fluorescence intensity in a 12SLPC-containing mixture (F) normalized relative to the intensity (F_o) in a mixture in which the quencher is replaced with an ordinary unsaturated lipid with similar properties, dioleoylphosphatidylcholine (DOPC) (*18*). When DPPC/12SLPC mixtures separate into DPPC-rich and 12SLPC-rich domains, there is an increase in DPH fluorescence, i.e., an increase in F/F_o, due to the movement of DPH molecules into the quencher-depleted DPPC-rich lipid domains. The degree of DPPC-rich domain formation is given by the difference between the level of quenching of DPH in the DPPC/12SLPC mixture and that in a DOPC/12SLPC mixture [$F/F_{o(DPPC/12SLPC)} - F/F_{o(DOPC/12SLPC)}$]. The DOPC/12SLPC mixture forms a single L_a phase (i.e., does not form domains) at ≥23 °C (*18*).

Figure 1A illustrates the temperature dependence of the difference between fluorescence quenching in model membrane vesicles containing 1:1 (molar ratio) DPPC/12SLPC and that in DOPC/12SLPC, both in the absence and in the presence of 15 mol % sterol or sterol derivative. These compositions were chosen because they exhibit the greatest changes in quenching upon domain formation (ref *18* and data not shown). As shown in Figure 1A, there is domain formation at 23 °C in the absence of sterol, as shown by the significantly stronger fluorescence (weaker quenching) in a 1:1 DPPC/12SLPC mixture relative to that in the 1:1 DOPC/12SLPC mixture [i.e., a high value of $F/F_{o(DPPC/12SLPC)} - F/F_{o(DOPC/12SLPC)}$]. The degree of domain formation decreases as the temperature is increased, and is abolished by 37 °C, at which point the value of $F/F_{o(DPPC/12SLPC)} - F/F_{o(DOPC/12SLPC)}$ becomes approximately zero.

The temperature dependence of fluorescence quenching for DPPC/12SLPC model membranes with a variety of 15 mol % sterols and sterol derivatives (hereafter all termed sterols) can be quite distinct from that in bilayers lacking sterol. The effect of sterol on domain formation can be assessed both by the change in the temperature range over which domain formation occurs (Figure 1A) and (at a fixed temperature) by the difference in quenching relative to that with no sterol (Figure 1B,C). Domain formation is enhanced by the presence of cholesterol (Figure 1). There is clearly a greater degree of domain formation at 23 and 29 °C than in the absence of cholesterol (Figure 1B,C). In addition, domain formation persisted to significantly higher temperatures than in the absence of sterol, disappearing only above 45 °C in the presence of cholesterol (Figure 1A).[1] Dihydrocholesterol (cholestanol), 25-hydroxycholesterol, and epicholesterol appear to promote domain formation to a degree similar to that of cholesterol at 23 and 29 °C. However, the temperature dependence of quenching suggests that 25-hydroxycholesterol stabilizes domain formation somewhat more than cholesterol, as exemplified by somewhat more domain formation in the 37−45 °C range, whereas epicholesterol stabilizes domain formation somewhat less than cholesterol (Figure 1A).

In contrast, lanosterol and cholestane induce little change in domain formation relative to that in the absence of sterol. Even more striking and unanticipated is the observation that

some sterols, i.e., coprostanol, androstenol, cholesterol sulfate, and 4-cholesten-3β-one, inhibit domain formation. They all decrease the level of domain formation at 23 and 29 °C, and decrease the temperature at which domain formation is abolished relative to that in the absence of sterol. Thus, the effect of a sterol on lipid domain formation is strikingly dependent on the sterol used.

It should also be noted that, both with and without sterol, any domain formation was not only abolished at high temperatures (Figure 1A) but also fully restored upon cooling to 23 °C (data not shown). This indicates that domains are stable at equilibrium at 23 °C, and are not an artifact of sample preparation techniques. In addition, in samples containing 15% cholesterol, the thermal dependence of quenching during cooling was found to be similar to that upon heating (not shown), suggesting the extent of domain formation observed during heating is close to the equilibrium value over the entire temperature range. In one other experiment, the effect of cholesterol and coprostanol on domain formation was compared over a wide range of DPPC/12SLPC compositions. At all compositions in which cholesterol induced domain formation, coprostanol tended to suppress domain formation relative to cholesterol or no sterol (data not shown). Thus, suppression of domain formation is not restricted to 1:1 DPPC/12SLPC mixtures.

Because the formation of DPPC- and cholesterol-rich domains is closely associated with the appearance of lipid insolubility in the detergent Triton X-100 (*17*−*19*), we also examined the effect of sterols on the sensitivity of lipid mixtures to detergent solubilization. Figure 2A shows the effect of sterol structure on Triton X-100 solubilization of vesicles composed of 1:1 (molar ratio) DPPC/DOPC mixtures with and without 15 mol % sterol. [These experiments used more lipid, and since the use of large amounts of 12SLPC is impractical DOPC, which exhibits phase behavior very similar to that of 12SLPC (*18*), was used instead.] Solubilization was monitored by the loss in optical density, which reflects the decrease in light scattering upon detergent solubilization. Figure 2A shows there are significant differences in the ability of different sterols to affect insolubility. Samples containing cholesterol, dihydrocholesterol, 25-hydroxycholesterol, epicholesterol, and lanosterol give the least solubilization at 23 °C. Solubilization is virtually complete with the other sterols. Comparison of Figures 1 and 2A shows a strong correlation between those sterols that promote insolubility most strongly and those that induce domain formation. Because the degree of insolubility was relatively small, these experiments were repeated for samples with 33 mol % sterol. Except for smaller decreases in optical density (i.e., greater insolubility), solubilization results at 33 mol % sterol were very similar to those at 15 mol % (Figure 2B).

These results illustrate a close connection between detergent insolubility and the degree of sterol-induced domain formation. However, the correlation between insolubility and domain formation is not exact. For example, DPPC/DOPC mixtures lacking sterol are known to separate into fluid and solid-like gel domains (*29*) but are fully Triton X-100-soluble (Figure 2A). This is not a surprise, and reflects the fact that at 23 °C the DPPC-enriched gel phase, although ordered, is not sufficiently stable to resist solubilization.

Thus, the detergent insolubility observed in panels A and B of Figure 2 is indicative of the formation of domains in an ordered state that is stable to the extent that its lipid−lipid interactions are stronger than lipid−Triton X-100 interactions. Because DOPC/cholesterol mixtures are soluble in Triton X-100 (*17*), it is most likely that the degree of insolubility reflects interactions between DPPC and sterol. To confirm that stable lipid−lipid interactions specifically involve interactions between DPPC and sterol, detergent insolubility was re-examined in sterol/DPPC mixtures without DOPC. As shown in panels C and D of Figure 2, sterol structure has a strong effect on DPPC insolubility in Triton X-100. DPPC by itself is in the gel state but, as shown in panels C and D of Figure 2, is only marginally stable to Triton X-100 solubization at 23 °C. At both 15 and 33 mol %, those sterols that are most efficacious at promoting domain formation are most efficacious at promoting DPPC insolubility. At 15% sterol, the pattern of insolubility is very similar to that in the DPPC/DOPC mixture. As might be expected, those sterols that increase insolubility at 15% tend to have a larger effect at 33%.[2] Panels C and D of Figure 2 also show that those sterols that inhibit domain formation actually promote solubilization of DPPC by Triton X-100. Therefore, there is a close connection not only between an increased level of domain formation and stronger lipid−lipid interactions in DPPC/sterol mixtures but also between a decreased level of domain formation and weaker lipid−lipid interactions in DPPC/sterol mixtures.

One concern with the optical density assay is that it is only a crude measure of solubilization, and does not identify which molecule(s) are insoluble. It is conceivable that only the sterol molecules, rather than both sterol and DPPC, are Triton X-100-insoluble. To investigate this possibility, the detergent-insoluble fraction was collected by centrifugation and phospholipid content tested by phosphate analysis. As shown in panels E and F of Figure 2, the values for insolubility are quite similar whether assayed by optical density or phosphate analysis, showing that OD reflects phospholipid insolubility. The relatively small differences between the two methods may involve a small differential solubilization of phospholipid and sterol, or be an artifact of either the effici-

[1] It should be pointed out that by itself, a change in fluorescence quenching at a single lipid composition does not distinguish between a change in the absolute amount of domains present, and a second case in which domains are present at a fixed amount, but there is a change in the enrichment of the DPPC concentration in one set of domains relative to the other. However, previous experiments in which composition is varied strongly suggest that changes in quenching mainly reflect a change in the total amount of DPPC-rich domains as the temperature is increased (*18*). It should be noted there is no ambiguity when the difference in quenching between the DPPC/12SLPC and DOPC/12SLPC samples is zero. This corresponds to no domain formation. Therefore, differences in the temperature at which domain formation disappears with different sterols are a slightly more unambiguous measure of the tendency to form domains than a comparison of quenching levels at a fixed temperature. Nevertheless, both parameters give a similar rank order for the effect of sterol structure on domain formation (Figure 1).

[2] It is interesting to note that the interactions probed by insolubility do not show the exact same sterol concentration dependence in all cases. In contrast to the behavior of cholesterol, lanosterol exhibited a more extreme dependence on sterol concentration, while an increase in sterol concentration did not significantly increase insolubility of 25-hydroxycholesterol.

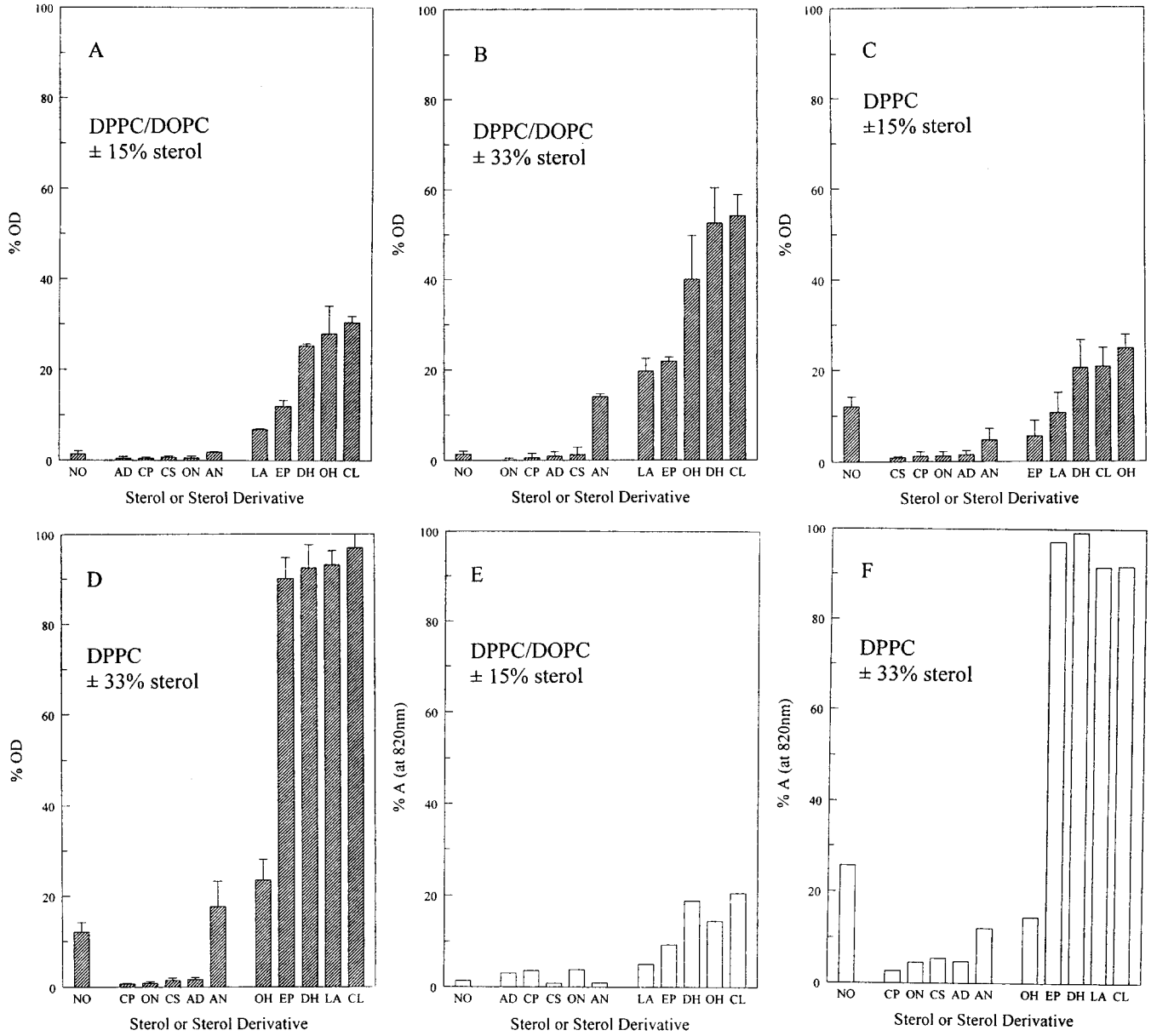

FIGURE 2: Solubilization of lipid mixtures at 23 °C by Triton X-100 as detected by optical density (%OD) or phosphate analysis (%A_{820}): (A) 1:1 DPPC/DOPC with or without 15 mol % sterol or sterol derivative, (B) 1:1 DPPC/DOPC with or without 33 mol % sterol or sterol derivative, (C) DPPC with or without 15 mol % sterol or sterol derivative, and (D) DPPC with or without 33 mol % sterol or sterol derivative. The partly off-scale error bar for cholesterol was about the same size as for dihydrocholesterol. Average values and standard deviation for three samples are shown, except for DPPC with no sterol or 15 mol % cholestane, dihydrocholesterol, lanosterol, epicholesterol, 25-hydroxycholesterol, and cholesterol, for which there were five or six samples. (E and F) Same as panels A and D, respectively, except assayed by phosphate analysis. The average values from duplicate samples are shown.

ency of vesicle recovery or a small nonlinearity between optical density and the amount of lipid that has been solubilized.

The link between domain formation (or inhibition) and strengthened (or weakened) lipid−lipid interactions between DPPC and sterol could be due to an increase or decrease in the degree to which the lipid forms an ordered, closely packed state (and the resulting increase or decrease in the strength of van der Waals interactions). To investigate this possibility, the degree of steady-state DPH fluorescence polarization was measured in samples containing a small amount of DPH incorporated into mixtures of DPPC with the various sterols at 33 mol %. The degree of DPH fluorescence polarization reflects the degree to which a DPH molecule reorients while in the excited state. This degree of reorientation decreases (and thus that of polarization increases) in an environment in which motion is restricted, i.e., a closely (tightly) packed, ordered lipid environment (*30*). Table 1 shows the effect of various sterols on DPH polarization. At 36 °C, where DPPC by itself is in the tightly packed gel state ($P = 0.41$), those sterols that decrease the extent of domain formation and decrease DPPC insolubility induce significant decreases in the degree of polarization ($P = 0.30-0.35$), i.e., loosen packing to a significant degree, while the effects of sterols that promote domain formation and DPPC insolubility are relatively small ($P = 0.38-0.40$). At 52 °C, where DPPC by itself is in the loosely packed L_α state ($P = 0.12$), those sterols that promote domain formation and insolubility induce significant increases in the degree

Table 1: DPH Fluorescence Polarization in DPPC (and DOPC) with or without 33 mol % Sterol or Sterol Derivative[a]

sterol or sterol derivative	*P* with DPPC at 36 °C	*P* with DPPC at 52 °C	*P* with DOPC at 36 °C
none	0.41 ± 0.04	0.12 ± 0.07	0.095 ± 0.03
androstenol	0.35 ± 0.01	0.22 ± 0.03	0.11 ± 0.02
coprostanol	0.32 ± 0.03	0.22 ± 0.02	0.13 ± 0.01
cholesterol sulfate	0.33 ± 0.02	0.20 ± 0.04	0.17 ± 0.02
cholestenone	0.30 ± 0.02	0.19 ± 0.02	0.17 ± 0.01
cholestane	0.33 ± 0.01	0.16 ± 0.02	0.13 ± 0.02
lanosterol	0.40 ± 0.01	0.30 ± 0.05	0.13 ± 0.01
25-hydroxycholesterol	0.40 ± 0.01	0.31 ± 0.03	0.15 ± 0.01
epicholesterol	0.39 ± 0.02	0.31 ± 0.02	0.16 ± 0.03
dihydrocholesterol	0.40 ± 0.01	0.30 ± 0.04	0.19 ± 0.03
cholesterol	0.38 ± 0.01	0.32 ± 0.01	0.17 ± 0.04

[a] Samples contained DPPC or DOPC, with or without 33 mol % sterol, dispersed in PBS. The total lipid concentration was 100 μM, and the DPH concentration was 0.5 μM. In each case, average and standard deviations are shown for three independent experiments. P = polarization.

of polarization ($P = 0.30-0.32$), i.e., induce significantly tighter packing. In this case, those sterols that inhibit domain formation have much weaker effects on packing ($P = 0.16-0.22$). The polarization results are consistent with previous studies on the relationship between sterol structure and two other measures of tight packing, the surface area occupied by DPPC in sterol-containing monolayers, and on membrane permeability in DPPC/sterol mixtures (*31*, *32*). Therefore, the effects of sterols on DPPC packing are closely linked to their effects on insolubility and domain formation. Interestingly, Table 1 also shows the effects of sterols on the polarization of samples containing DOPC are generally small, with little difference between the effects of sterols that promoted domain formation ($P = 0.13-0.19$) and those that inhibited it ($P = 0.11-0.17$). Thus, under the conditions examined here, sterols affect DPPC packing much more than DOPC packing, indicating that the former appears to be linked to domain formation. This inference is supported by the observation that DOPC remains in a loosely packed state and is fully detergent-soluble both in the absence and in the presence of 33% cholesterol (*17*).

Combined, these experiments strongly support the model in which it is the ability of cholesterol and similar sterols to pack tightly (or loosely) with lipids, and the *selectivity* of this tight packing for saturated lipids, that together are critical for domain formation. It appears likely that tight sterol packing with DPPC increases the likelihood of DPPC self-associating and forming separate DPPC and sterol-rich domains. Those sterols that inhibit domain separation appear to act by inhibiting tight packing between DPPC molecules, so that the likelihood of DPPC self-associating and forming separate domains is reduced.

It is interesting to compare the details of sterol structures to their effects on domain formation. The tight packing interaction of cholesterol with saturated lipids can be attributed to several factors, including its planar structure, its overall dimensions, and the properties of its small polar 3-OH group. The small polar group appears to favor packing close enough to allow the polar headgroup of a neighboring lipid to prevent exposure of sterol hydrocarbon to water (*33*). In addition, the size and hydrogen bonding capabilities of the OH group may help position the sterol at the proper depth in the bilayer to allow tight packing with saturated lipids. Those sterols that are most like cholesterol in terms of structure and planarity (dihydrocholesterol, epicholesterol, and 25-hydroxycholesterol) would be expected to have packing properties closest to those of cholesterol, and experimentally show effects on domain formation most similar to those of cholesterol. In contrast, those molecules that have no effect on domain formation, or inhibit it, either have structures that should interfere with tight packing (coprostanol, which has a large bend between the A and B rings, androstenol, which is totally lacking the hydrocarbon tail attached to the D ring, and lanosterol, which has several extra methyl groups) or have a substitution at the position of the critical 3-OH group (cholestane, cholestenone, and cholesterol sulfate). The lack of a 3-OH group in cholestane and its replacement by a keto group in cholestenone may disrupt the ability to pack tightly with DPPC by affecting their orientation or depth in the bilayer. Cholesterol sulfate packing with DPPC may also be disrupted due to a shallow depth arising from the charged sulfate group (*34*, *35*). Alternately, steric effects associated with the relatively large sulfate moiety may be involved. The observation that epicholesterol, which has an a 3-OH in place of the β 3-OH of cholesterol, promotes domain formation to *some* degree suggests that it is unlikely that there is an *absolute* requirement for a precise orientation of the 3-OH group for tight packing with DPPC to occur.

DPPC was used for these studies because its interactions with cholesterol have been extensively characterized. However, the results obtained with DPPC should be relevant to membranes with a natural eukaryotic lipid composition (i.e., in which the most saturated lipids are sphingolipids). DPPC has properties very similar to those of sphingomyelin (SM), which can be the predominant sphingolipid in plasma membranes. For example, SM exhibits phase behavior similar to that of DPPC both by itself and when mixed with cholesterol (*21*). It also shows a tendency for domain formation and detergent insolubility in the presence of cholesterol that is similar to that of DPPC (*18*). Preliminary experiments show domain formation in SM/12SLPC mixtures show a dependence on sterol structure similar to that of the DPPC/12SLPC mixtures described in this report (data not shown). Finally, it should be noted that a recent study compared the effect of cholesterol, cholestane, and androstenol on the detergent insolubility of SM (*36*). At least for these molecules, a dependence of lipid solubility in detergent upon sterol structure was found that is similar to that described in this report for DPPC.

It should be noted that these experiments do not identify the nature of the DPPC-rich domains that form at low temperatures with 15% cholesterol. A fully liquid-ordered state exists above 30% cholesterol (*21*). At 15% cholesterol, they could be either in the form of a single state with properties between those of the gel and liquid ordered phase or in the form of multiple coexisting DPPC-rich, 12SLPC-depleted domains.

In summary, this report shows that cholesterol has the ability to promote the formation of tightly packed lipid domains. In addition, the results strengthen the link between tightly packed lipid domains and the detergent-resistant membranes that can be isolated from cells. However, how the exact fractional composition of cholesterol and other lipids in natural membranes affect domain formation and

behavior must still be determined before the degree to which the effects observed in this report can be extrapolated to domain formation in cells can be ascertained, and before any detailed picture of lipid domain behavior in natural membranes can be proposed. For example, the degree of lipid unsaturation in unsaturated lipids is likely to significantly influence interaction with cholesterol, little is known about the effect of sphingolipid structure on domain formation, and the cholesterol concentration can have profound effects on domain formation (*1*, *22*, *37*, *38*). The difference in the lipid compositions in the inner leaflet and outer leaflet of plasma membranes is another important complication. Thus, a number of models for plasma membrane organization are possible (*1*). Nevertheless, given the accumulating evidence that cholesterol promotes some sort of membrane domain formation in cells, it is likely that the ability of cholesterol to form tightly packed domains with saturated lipids is important in vivo.

Finally, it is noteworthy that some sterols can act as "anticholesterols" in the sense that they abolish the formation of tightly packed lipid domains. Because it is possible to carry out sterol substitution in cells, this could provide a new tool for investigating the role of sterols in cell membrane structure and function.

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