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Sterol Structure Determines Miscibility versus Melting Transitions in Lipid Vesicles

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ABSTRACT Lipid bilayer membranes composed of DOPC, DPPC, and a series of sterols demix into coexisting liquid phases below a miscibility transition temperature. We use fluorescence microscopy to directly observe phase transitions in vesicles of 1:1:1 DOPC/DPPC/sterol within giant unilamellar vesicles. We show that vesicles containing the "promoter" sterols cholesterol, ergosterol, 25-hydroxycholesterol, epicholesterol, or dihydrocholesterol demix into coexisting liquid phases as temperature is lowered through the miscibility transition. In contrast, vesicles containing the "inhibitor" sterols androstenolone, coprostanol, cholestenone, or cholestane form coexisting gel (solid) and liquid phases. Vesicles containing lanosterol, a sterol found in the cholesterol and ergosterol synthesis pathways, do not exhibit coexisting phases over a wide range of temperatures and compositions. Although more detailed phase diagrams and precise distinctions between gel and liquid phases are required to fully define the phase behavior of these sterols in vesicles, we find that our classifications of promoter and inhibitor sterols are consistent with previous designations based on fluorescence quenching and detergent resistance. We find no trend in the liquid-liquid or gel-liquid transition temperatures of membranes with promoter or inhibitor sterols and measure the surface fraction of coexisting phases. We find that the vesicle phase behavior is related to the structure of the sterols. Promoter sterols have flat, fused rings, a hydroxyl headgroup, an alkyl tail, and a small molecular area, which are all attributes of "membrane active" sterols.

INTRODUCTION

All vertebrates synthesize cholesterol (1), which is found predominantly in the plasma membranes of cells (2). Cholesterol has at least two classes of important functions. First, it controls metabolic processes and is a specific precursor of D-vitamins, sex hormones, and bile acids (1). However, the amount of cholesterol in animal tissues is much higher than needed in these roles. Another function of cholesterol may be to alter the properties of lipids in membranes (1). Above the lipid melting temperature, the presence of cholesterol orders lipid acyl chains. Below the melting temperature, cholesterol prevents lipids from condensing into solid phases. Furthermore, the addition of cholesterol to lipid mixtures promotes the formation of two coexisting liquid phases, a familiar ''liquid crystalline'' (L_α) phase and a ''liquid-ordered'' (L_o) phase (3,4). The liquid-ordered phase has been distinguished from a solid phase by faster translational diffusion of the lipids (5) and greater mobility of the acyl chains (6). The separation of lipids into two immiscible liquid phases is often linked to the formation of ''rafts'' in cell membranes (7). These raft domains are characterized as rich in cholesterol and saturated lipids, and they have been implicated in a host of important cell functions such as membrane trafficking and signaling (8).

Although it is known that lipids and sterols interact in membranes through hydrogen bonding and van der Waals forces, little is known about how these interactions give rise to immiscible liquid phases in bilayers (7). To directly probe how sterol structure affects the miscibility phase behavior of lipids, we incorporated the sterols in Fig. 1 into giant unilamellar vesicles (GUVs) and utilized fluorescence microscopy to observe micron-scale domains of coexisting phases.

We chose some of the sterols because they are naturally related to cholesterol. For example, lanosterol is a precursor of both cholesterol, which is found primarily in animal tissue, and ergosterol, which is found primarily in yeast and fungi (1). The sterol 25-hydroxycholesterol is an inhibitor of HMG-CoA reductase and in turn prevents the synthesis of cholesterol (1). Cholesterol oxidase catalyzes the conversion of cholesterol to cholestenone (9). Finally, as its name suggests, coprostanol appears in feces and arises due to the conversion of cholesterol by bacteria (10).

In our experiments, we use fluorescence microscopy to directly observe micron-scale domains in membranes containing equimolar ratios of the saturated lipid DPPC (dipalmitoyl phosphatidylcholine), the unsaturated lipid DOPC (dioleoyl phosphatidylcholine), and a sterol. We classify sterols as promoters if vesicles contain immiscible liquid phases as temperature is lowered. Vesicles made with inhibitor sterols instead form coexisting solid and liquid phases as temperature is lowered. We measure the transition temperature (miscibility or melting) of vesicles containing different sterols and determine the area fraction of the more ordered phase. Our findings are consistent with previous work that classified the sterols used as either promoters or inhibitors of ordered membrane domains using fluorescence quenching and detergent resistance (4,11).

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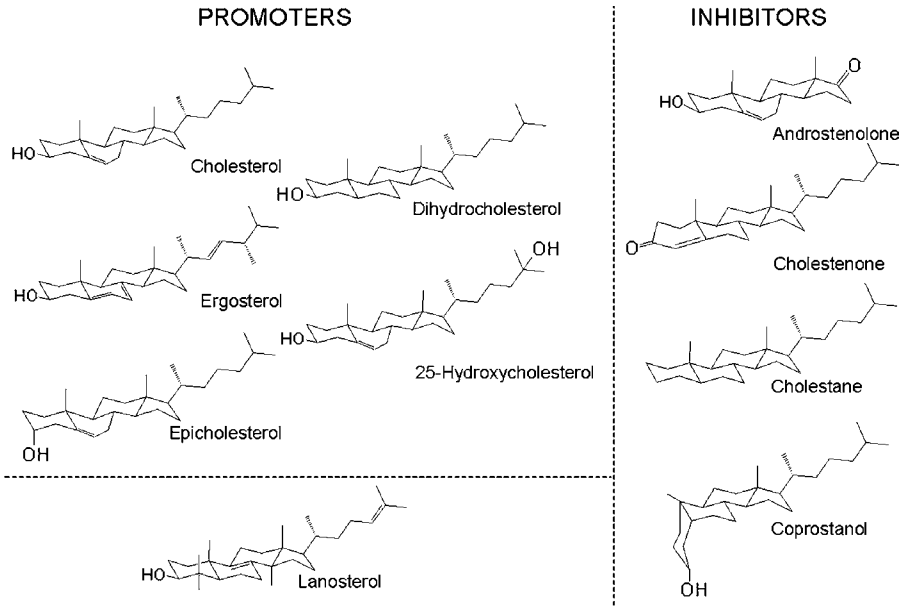

FIGURE 1 Sterol structures drawn to highlight assignment of hydrogen, hydroxy, and methyl groups to either the α-face (*bottom*) or β-face (*top*) of the sterol. Distortion of the rings due to double bonds is not shown for the sterols ergosterol and lanosterol.

MATERIALS AND METHODS

Transition temperatures

The lipids DOPC and DPPC have gel phase melting temperatures of −20°C and 41°C, respectively (12), and were obtained from Avanti Polar Lipids (Alabaster, AL). Androstenolone (5-androsten-3β-ol-17-one), coprostanol (5β-cholestan-3β-ol), cholestenone (4-cholesten-3-one), cholestane (5α-cholestane), and epicholesterol (5-cholesten-3α-ol) were obtained from Steraloids (Newport, RI). Cholesterol (5-cholesten-3β-ol), dihydrocholesterol (5α-cholestan-3β-ol), ergosterol, lanosterol, and 25-hydroxycholesterol were obtained from Sigma (St. Louis, MO). The dye Texas Red DPPE (TR-DPPE) was purchased from Molecular Probes (Eugene, OR) and was used for contrast between membrane domains. Vesicles were produced with 33 mol % sterol because vesicles of 1:1:1 DOPC/DPPC/cholesterol are comfortably far from the gel phase transition that appears at low sterol concentrations (13) and from the solubility limit of cholesterol in vesicles at high sterol concentrations (14). We have no data for solubility limits for other sterols, although evidence suggests that the solubility limit of epicholesterol may be below 33 mol % (15).

GUVs of 1:1:1 DPPC/DOPC/sterol and 0.8 mol % TR-DPPE were prepared by electroformation at 60°C in deionized water (18 MΩ-cm, Barnstead, Dubuque, IA) as described previously (16,17). The resultant vesicles were transferred between glass coverslips for examination by fluorescence microscopy on a temperature controlled microscope stage. The vesicles were free floating and not attached either to the glass slide or to other vesicles. The phase transition in a single vesicle is identified as the temperature at which domains of at least 1 μm appear (or disappear) in the membrane as temperature is lowered (or raised). Temperature was ramped at 0.2°C/s and the sample equilibrated for 30 s before viewing. The lowest temperature probed was 10°C. All transitions are fully reversible. Errors bars reported in transition temperature represent the full range of temperatures over which many vesicles pass through the transition in a single preparation of GUVs. The measured range of transition temperatures is reproducible when vesicles are prepared with the same initial lipid composition and experimental conditions (e.g., growth temperature). The spread in transition temperature between vesicles is due to variation in lipid composition and the location of that composition with respect to the nearest phase boundary. Previously we have noted that there is increased spread in measured transition temperatures near steep regions of the phase diagram, where small changes in lipid composition can lead to large changes in transition temperature (18). The midpoint of this range (*black points* in Fig. 2) roughly corresponds to the temperature at which 50% of vesicles contain coexisting phases. The spread in transition temperature is unusually large for membranes containing the sterol 25-OH-cholesterol (ΔT = 22°C). This sample contains some vesicles with a transition close to the growth temperature. Previously we have observed a large distribution in transition temperatures for vesicles of 1:1 DOPC/DPPC + 35% cholesterol when vesicles are grown at or below their miscibility transition temperature (19,25).

Area fractions

Vesicles were quenched from one uniform phase at 60°C to the temperature of the equilibrated microscope stage (either 23°C or 29°C). Images of each vesicle hemisphere were collected after an equilibration time of roughly 1 min. The area fraction of dark phase was evaluated as described previously (20); namely, boundaries between bright and dark phases were determined by hand, a threshold was applied to the images to result in only black and white pixels, and the number of pixels were added (without geometric correction) to yield an area fraction of dark phase. Area fractions were added for both hemispheres of the vesicle and then averaged over multiple vesicles for each sterol at each temperature. Error bars represent the standard deviation of the mean ($error = \sigma/\sqrt{N}$), and the number of vesicles analyzed (N) is shown in Table 1. Similar standard deviations in area fraction have been found in vesicles grown by electroformation on a wire and imaged by confocal microscopy, including geometric corrections (Vernita Gordon and Paul Beales, University of Edinburgh, personal communication, 2005). We estimate a systematic error of ≤20% between area fractions determined by individual researchers due to the qualitative identification of domain boundaries, although trends in area fraction are not affected (20).

Solid versus liquid

We characterize domains as either solid (gel) or liquid based on domain shape and the kinetics of domain coalescence. All domains identified as solid in this work are noncircular. Liquid domains are circular and usually coalesce quickly upon colliding, typically within seconds. All systems studied here except one (vesicles containing 25-hydroxycholesterol at 23°C) fall clearly into these two categories. In the case of 25-hydroxycholesterol, vesicles clearly exhibit liquid phases at 29°C. At 23°C, domains in these

same vesicles coalesced more slowly, within minutes rather than within seconds. Based on known diffusion constants (below) we reasoned that since two domains at 23°C coalesced completely within several minutes in vesicles containing 25-hydroxycholesterol, the domains probably contain a liquid phase.

Our characterization of liquid versus solid phases for the particular case of vesicles containing 25-hydroxycholesterol is based on lipid mobility and is consistent with previous measurements of lipid diffusion. Lipid diffusion constants (D) have been measured in liquid states (both L_o and L_α) by a variety of experimental methods to be ~1–20 $\mu m^2/s$ (5,21–23). Therefore, the time required for a lipid to traverse a micron across the membrane's surface ranges between seconds and hundredths of seconds. In contrast, lipids in S_o states diffuse significantly slower ($D \sim 10^{-2} - 10^{-8}$ $\mu m^2/s$) (5,22,24), leading to much longer timescales for μm-scale lipid motion, from hundreds of seconds to hundreds of days. Interestingly, binary membranes of saturated lipids and cholesterol with S_o-L_o coexistence also show slow diffusion ($D \sim 1–5 \times 10^{-2}$ $\mu m^2/s$) (5,23), and large-scale phase separation is not detected by fluorescence microscopy (16). For this reason, we are unable to distinguish pure S_o domains from domains containing both S_o and L_o phases in this study, although it is a focus of current work in our laboratory. We have discussed S_o-L_o coexistence and evidence for three phase S_o-L_o-L_α coexistence in greater detail in the context of mixtures containing sphingomyelin lipids and cholesterol (19,25).

RESULTS

To study the influence of different sterols on lipid phase behavior, we produced free-floating GUVs containing equimolar ratios of DOPC, DPPC, and each of the sterols shown in Fig. 1. The lipids DOPC and DPPC were chosen because they are readily available as pure, single components, and we have studied the ternary mixture of DOPC/DPPC/cholesterol extensively (16,20).

Membranes in the presence and absence of cholesterol

When vesicles are produced from an equimolar mixture of DOPC, DPPC, and cholesterol, coexisting liquid phases are observed below the miscibility transition temperature (Fig. 2). Liquid domains are large and circular when imaged well below the miscibility transition temperature (Fig. 3) and two domains coalesce quickly upon collision (typically <1 s). In vesicles containing cholesterol, liquid domains ripen through coalescence until vesicles contain only one bright domain and one dark domain (16). This ripening and completion of phase separation typically occurs within minutes. We have previously shown that the bright phase is rich in unsaturated lipid (DOPC) in a liquid-crystalline state, whereas the dark phase is rich in saturated lipid (DPPC) and cholesterol and is in a liquid-ordered state (20). Other lipid mixtures produce similar phase behavior. Coexisting liquid phases are observed in a wide variety of ternary mixtures of saturated lipids, unsaturated lipids, and cholesterol (16).

In the absence of cholesterol, vesicles of DOPC and DPPC contain coexisting solid and liquid phases below the melting transition temperature (Fig. 3 *f*) (16,26). A dark domain is clearly solid rather than liquid if it has a static, noncircular boundary. The dark solid phase is rich in saturated lipid, and the bright liquid phase is rich in unsaturated lipid and the fluorescent dye (16).

Membranes containing other sterols

It is argued that cholesterol is necessary to form the liquid-ordered phase in membranes. Specific interactions between cholesterol and saturated lipids lead to an average ordering of the acyl chains while maintaining the liquid state of the membrane (3). Here we demonstrate that other sterols besides cholesterol promote micron-scale coexisting liquid phases in lipid membranes.

Vesicles of DOPC, DPPC, and the various sterols in Fig. 1 exhibit either two coexisting liquid phases or coexisting solid and liquid phases at low temperatures. Above the transition temperatures shown in Fig. 2, vesicles are in one uniform liquid phase. Vesicles containing the sterols 25-hydroxycholesterol, cholesterol, dihydrocholesterol, epicholesterol, or ergosterol produce coexisting liquid phases below a miscibility transition (Fig. 3, *a–e*). We classify these sterols as promoters because they promote the formation of a second liquid phase as cholesterol does. In contrast, we observe a transition to coexisting solid and liquid phases in vesicles containing the sterols androstenolone, cholestane, cholestenone, or coprostanol (Fig. 3, *g–j*). We classify these sterols as inhibitors because they do not promote the formation of a second liquid phase. The remaining sterol, lanosterol, does

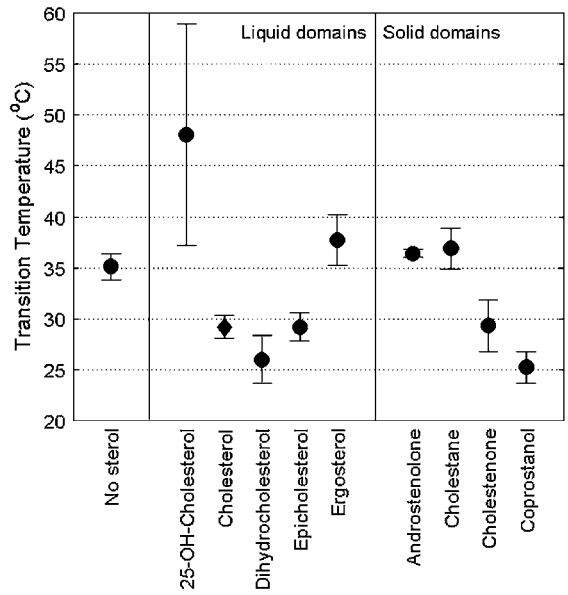

FIGURE 2 Transition temperatures of vesicles produced from 1:1:1 DOPC/DPPC/sterol with 0.8 mol % TR-DPPE. As temperature is decreased, a transition is observed from one uniform phase to either (*middle*) two coexisting liquid phases for vesicles containing promoter sterols or (*right*) a solid and liquid phase for vesicles containing inhibitor sterols. No correlation is seen between the type of transition and the transition temperature.

not produce coexisting liquid or coexisting solid and liquid phases over a wide range of temperatures (5°C–60°C) and lipid compositions (DOPC/DPPC/lanosterol with ratios 33/33/33, 35/35/30, 37/38/25, 22/45/33, and 24/49/27). Coexisting liquid domains can be induced in membranes containing lanosterol by exposure to light. Previously we noted that light can induce coexisting liquid domains in vesicles containing DOPC, DPPC, and cholesterol near the miscibility transition boundary (16).

We find no distinct correlation between transition temperatures and the phases produced, solid domains for the inhibitor sterols, and coexisting liquid phases for the promoters (Fig. 2). When a solid phase is formed, transition temperatures are lower than for pure DPPC (41°C), as expected from a colligative melting point depression due to the addition of DOPC and sterol to the membrane. Liquid miscibility transition temperatures are not constrained to be below the melting point of pure DPPC, although they generally fall below 41°C. Vesicles that exhibit liquid miscibility transitions may also have melting transitions to solid phases at lower temperatures, including coexistence of three phases containing a solid with two distinct liquids (19,25). We find no direct evidence for a gel phase in vesicles containing promoter sterols down to 23°C, except perhaps in membranes containing 25-hydroxycholesterol, in which the coalescence of two dark, circular domains at 23°C requires ~1 min (see partially coalesced domains in Fig. 3 *a* and the Materials and Methods section). It is useful to relate these results to membranes containing cholesterol in which slow domain coalescence is observed in regions of the phase diagram where there is evidence for coexisting S_o, L_o, and L_α phases (S. L. Veatch and S. L. Keller, unpublished results, and (25)).

Based on our previous results with membranes containing cholesterol (16), we conclude that the lipids in the dark domains in Fig. 3 are more ordered and contain more DPPC, whether those domains are solid or liquid. Area fractions of dark phases for vesicles containing each sterol are shown in Fig. 4. For the most part, vesicles containing solid domains with inhibitor sterols have smaller areas of dark phase than liquid domains with promoter sterols. This difference in area fraction is consistent with a smaller molecular area in solid phases than in liquid phases. In addition, solid phases should contain little sterol (27), whereas ordered liquid phases can incorporate significant fractions of sterol (20). By extension, inhibitor sterols are expected to have low solubility in DPPC below its melting temperature. The fraction of solid phase in vesicles with inhibitor sterols in Fig. 4 follows the trend in melting temperature in Fig. 2. This is simply explained by an increase in solid phase as temperature is lowered further below the melting temperature. Similarly, as temperature decreases from 29°C to 23°C, the fraction of dark area in vesicles either remains constant or increases (Fig. 4). This is consistent with our previous observations in vesicles of DOPC/DPPC/cholesterol (20).

We would like to emphasize that we measure transition temperatures and dark phase surface fractions at a single point in the ternary phase diagram for each sterol system and do not attempt to map entire miscibility phase boundaries. It is important not to draw too much significance from a single data point. Although higher transition temperatures could be due to an increased interaction energy between the sterol and saturated lipid DPPC, it could also indicate that the phase boundary is shifted to higher sterol concentrations or that <33% sterol partitions into the membrane. Similarly, a wide spread in transition temperatures could be due to the close proximity of a steep region of the phase diagram. A wide spread may also indicate that the sterol produces nonuniform films during preparation or that vesicles of that sterol should be grown at alternate temperatures (19). Clearly, studies over a range of lipid compositions are necessary to make direct comparisons between individual sterols. Our results provide a first step toward those broad studies.

We also investigated the effect of gradually substituting cholestenone for cholesterol in vesicles (Fig. 5). The activity of cholesterol oxidase to produce cholestenone has been used as a measure of lipid phase and stability of cholesterol in the membrane (9,28). We find that the addition of cholestenone has two effects on vesicles of DOPC, DPPC, and cholesterol. First, at low cholestenone concentrations (0–50% of total sterol), the miscibility transition temperature is depressed, although coexisting liquid phases are still observed below the transition. When cholestenone is 50% of total sterol, the liquid phase boundaries fluctuate, implying that line tension is small and that the transition temperature, as well as a critical point, are probably nearby. Second, at higher cholestenone fractions (>50–100% of total sterol), a solid phase is observed. Our results indicate that a gradual conversion of cholesterol to cholestenone by cholesterol oxidase can change the phase state of the membrane. At higher temperatures, two coexisting liquid phases become one uniform

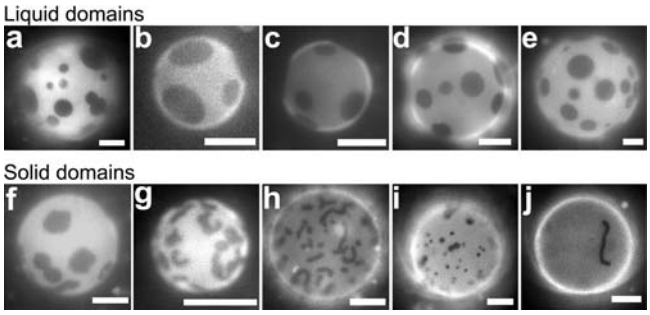

FIGURE 3 Fluorescence micrographs of vesicles produced from 1:1:1 DOPC/DPPC/sterol with 0.8 mol % TR-DPPE at 23°C. Vesicles contain (*a*) 25-hydroxycholesterol, (*b*) cholesterol, (*c*) dihydrocholesterol, (*d*) epicholesterol, (*e*) ergosterol, (*f*) no sterol (control), (*g*) androstenolone, (*h*) cholestane, (*i*) cholestenone, and (*j*) coprostanol. As temperature is lowered, phase coexistence is observed between either (*top row*) two liquid phases or (*bottom row*) a solid and liquid phase. With time, liquid phases coalesce to form one bright domain and one dark domain. Scale bars are 10 μm.

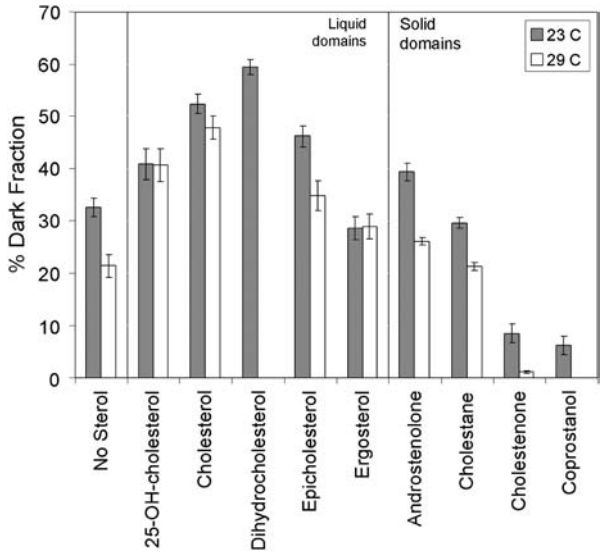

FIGURE 4 Area fraction of dark phase of vesicles produced from 1:1:1 DOPC/DPPC/sterol with 0.8 mol % TR-DPPE. In general, vesicles containing liquid domains have larger area fractions of dark domains. As temperature decreases, the fraction of dark area either remains constant (25-hydroxysterol and ergosterol) or decreases. Area fractions are not reported at 29°C for membranes containing dihydrocholesterol and coprostanol because membranes are in one uniform phase at this temperature.

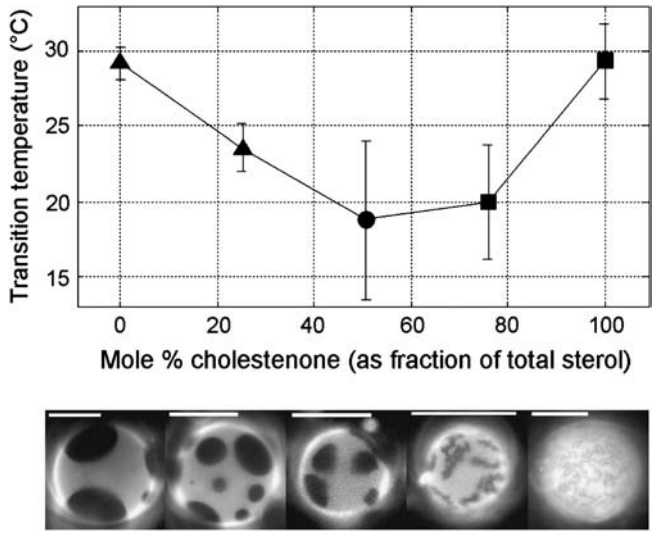

FIGURE 5 Transition temperatures (*top*) and vesicle micrographs (*bottom*) for vesicles of 1:1:$(1-x)$:x DOPC/DPPC/cholesterol/cholestenone with 0.8 mol % TR-DPPE. Coexisting liquid phases are observed below the miscibility transition temperature in membranes with more cholesterol (▲), and solid and liquid phases are observed below the melting transition in membranes with more cholestenone (■). In membranes with an equal ratio of cholesterol and cholestenone (●), fluctuations at domains boundaries relax slowly (~1–10 s) and dark domains may contain a solid phase. Vesicles contain $x = 0\%, 25\%, 50\%, 75\%$, and 100% cholestenone (from *left* to *right*) and are imaged at 16°C. All scale bars are 20 μm.

liquid when cholesterol converts to cholestenone. At lower temperatures, a solid phase can appear when cholesterol converts to cholestenone.

DISCUSSION

Promoters and Inhibitors

Our classification of promoter and inhibitor sterols in terms of their ability to produce coexisting liquid domains in vesicle membranes containing 1:1:1 DOPC/DPPC/sterol is consistent with their previous designation as either promoting or inhibiting the formation of DPPC-rich domains based on fluorescence quenching experiments (Table 1) (4,11). We find that the phases of DPPC-rich domains differ strongly between promoter and inhibitor sterols. Vesicles containing promoter sterols exhibit two coexisting liquid phases, whereas vesicles containing inhibitor sterols exhibit coexisting solid and liquid phases. On average, vesicles containing promoter sterols have larger area fractions of dark, ordered, DPPC-rich domains. Area fractions increase as temperature decreases, consistent with more DPPC-rich phases at lower temperatures (4). Values for the fraction of dark area do not fall in exactly the same sterol order as values for fluorescence quenching (Table 1). This result is not surprising since our vesicles contain the unsaturated lipid DOPC rather than the quencher 12doxyl-stearoylPC and incorporate more sterol (33% vs. 15%) (4).

Wenz and Barrantes have suggested that a sterol's ability to promote DPPC-rich phases in membranes may be correlated with higher sterol hydrophobicity. They compiled and calculated values for the log of the partition coefficient (LogP) of sterols between octanol and water (29). We find no clear correlation between this measure of hydrophobicity and either our own measurements of phase behavior and dark area fraction or published values of fluorescence quenching (Table 1).

Sterol requirements

We are particularly interested in the question of why sterols produce coexisting liquid domains in bilayer phospholipid membranes. What structural components are required for a sterol to mimic cholesterol's effect on lipid miscibility? Other researchers have previously asked what is required for a sterol to be ''functionally competent'' (1) or ''membrane active'' (30). We will discuss each below.

Bloch lists several attributes required for cholesterol's functional competence in membranes, among which are 1), a flat, rigid fused ring system, 2), the absence of methyl groups on the α-face, 3), an equatorial 3-hydroxy group, 4), bridgehead methyl groups at the C10 and C18 positions, and 5), an unmodified branched, isooctyl chain (1). Using our results above, we evaluate these criteria with respect to lipid miscibility. If part of cholesterol's ''functional competence'' involves the production of coexisting liquid phases, then we expect all sterols that follow Bloch's criteria to be promoters.

For criterion 1, all of our sterols possess rigid fused rings. However, coprostanol does not fulfill the requirement of flatness. Comparing coprostanol to dihydrocholesterol, the two sterols differ only in the linkage of the first two rings. Coprostanol is curved due to a *cis* fusion of the *A* and *B* rings (31), which likely prevents it from packing closely with neighboring phospholipids and explains why it is an inhibitor. Vesicles containing coprostanol exhibit coexisting solid and liquid phases, whereas vesicles containing dihydrocholesterol exhibit coexisting liquid phases. For criterion 2, lanosterol is our only sterol with methyl groups on the α-face, and it does not consistently produce coexisting liquid domains. Hence, within our set of sterols, criterion 2 is valid. The enhanced planarity of cholesterol with respect to lanosterol may explain its ability to alter the ordering and phase behavior of lipids (32,33). For criterion 3, three of our sterols do not possess equatorial hydroxyl groups at the 3 position: cholestane has a hydrogen, cholestenone has a ketone group, and epicholesterol has an axial hydroxyl group. Cholestane and cholestenone are inhibitors, yet the promoter epicholesterol behaves like cholesterol in its ability to produce coexisting liquid domains in vesicles, in its fraction of dark area, and in its miscibility transition temperature. Given our set of sterols, the criterion for a hydroxyl group is valid, but the criterion for the group to be equatorial is not important for miscibility phase behavior. For criterion 4, only androstenolone is missing the C10 methyl group, and it is an inhibitor sterol. However, androstenolone is not a sufficient test of criterion 4 since it also violates criterion 5. For criterion 5, several sterols do not possess unmodified isooctyl chains, including the promoter sterols of ergosterol and 25-hydroxycholesterol. Hence, the criterion for isooctyl chains is not sufficient to explain miscibility phase behavior. Overall, we conclude that Bloch's requirements for functionally competent sterols are more strict than necessary to predict the formation of liquid phases, particularly for the promoter sterol epicholesterol (without an equatorial hydroxyl group) and the promoter sterols ergosterol and 25-hydroxycholesterol (with modified isooctyl chains).

Barenholz presents less stringent requirements for ''membrane active sterols'', which usually predict our observed formation of liquid phases. A membrane active sterol is defined as affecting short- and long-range lipid order within membranes, minimizing volume, and decreasing membrane permeability. Specifically, the sterol should possess 1), a flat, fused ring system, 2), a hydroxyl or other small polar group at position 3, 3), a ''cholesterol-like'' tail, and 4), a small area per molecule (<40 Å^2 when assembled at the air/water interface at a surface pressure of 12 mN/m) (30). As an example of applying rule 4, cholestenone is an inhibitor. Although it satisfies rules 1–3, it violates rule 4 since it has an area per molecule of 52 Å^2 in a monolayer at 12 mN/m (34). The rules above correctly predict the observed formation of liquid phases except in the case of 25-hydroxycholesterol, which has an alkyl tail that terminates in a hydroxyl group that is not ''cholesterol-like''. We do not know how 25-hydroxycholesterol orients in the membrane or exactly why its miscibility transition temperature is high with a large spread in values (see Materials and Methods section).

TABLE 1 Summary of data for vesicles produced from 1:1:1 DOPC/DPPC/sterol with 0.8 mol % TR-DPPE

	Giant vesicles of 1:1:1 DOPC/DPPC/sterol					
	Transition temp. (°C)	% Dark area (23°C)	% Dark area (29°C)	ΔQuench (23°C)	ΔQuench (29°C)	LogP
LIQUID DOMAINS						
25-OH-cholesterol	48 ± 11	41 ± 3 ($N = 14$)	41 ± 3 ($N = 17$)	0.21 ± 0.04	0.23 ± 0.07	7.71
Cholesterol	29 ± 1	52 ± 2 ($N = 33$)	48 ± 2 ($N = 34$)	0.23 ± 0.03	0.22 ± 0.03	8.71
Dihydrocholesterol	26 ± 2	60 ± 1 ($N = 16$)	–	0.29 ± 0.04	0.24 ± 0.03	–
Epicholesterol	29 ± 1	46 ± 2 ($N = 20$)	35 ± 3 ($N = 14$)	0.23 ± 0.02	0.19 ± 0.01	8.71
Ergosterol	38 ± 3	29 ± 2 ($N = 23$)	29 ± 2 ($N = 24$)	0.44 ± 0.04	–	8.86
SOLID DOMAINS						
No sterol	35 ± 1	33 ± 2 ($N = 17$)	21 ± 2 ($N = 14$)	0.12 ± 0.02	0.08 ± 0.01	–
Androstenolone	36 ± 1	39 ± 2 ($N = 11$)	26 ± 1 ($N = 15$)	(0.01 ± 0.02)	(0.00 ± 0.0.2)	(2.77)
Cholestane	37 ± 2	30 ± 1 ($N = 20$)	21 ± 1 ($N = 18$)	0.07 ± 0.02	0.07 ± 0.02	10.31
Cholestenone	29 ± 3	9 ± 2 ($N = 3$)	1 ± 0.2 ($N = 3$)	0.05 ± 0.03	0.04 ± 0.02	8.54
Coprostanol	25 ± 2	6 ± 2 ($N = 5$)	–	0.05 ± 0.01	0.02 ± 0.01	–
NO DOMAINS						
Lanosterol	–	–	–	0.13 ± 0.02	0.10 ± 0.02	–

As temperature is decreased, a transition is observed from one uniform phase to either two coexisting liquid phases (''Liquid domains'') or a solid and liquid phase (''Solid domains''). To calculate the fraction of dark, ordered area at 23°C and 29°C, images of both hemispheres of *N* vesicles were captured. Xu and London (4) evaluated the fluorescence of a probe that partially partitions with DPPC in vesicles containing the quencher 12 doxyl-stearoyl PC and a variety of sterols. Demixing of the dye and quencher resulted in a reduction in quenching with respect to a uniformly mixed control. Hence, large changes in fluorescence quenching (''ΔQuench'') are attributed to enhanced formation of DPPC-enriched domains in vesicles by promoter sterols (4,11). LogP represents the log of the sterol partition coefficient in octanol/water as compiled and calculated by Wenz and Barrantes (29) and does not correlate with sterol phase. Quenching and logP results are not available in the literature for androstenolone but are instead quoted for androstenol and placed in parentheses.

Headgroups and alkyl chains

Several researchers have investigated the headgroup and alkyl chain moieties of sterols in depth. For example, the 3-hydroxy headgroup is equatorial in cholesterol and axial in epicholesterol. Cholesterol and epicholesterol are similar in that both produce comparable pressure-area isotherms (34). However, cholesterol and epicholesterol differ in the condensation of monolayers, the collapse pressure of monolayers, and the enthalpy of the 18:1-18:0 PC bilayer gel transition (15,34). Comparing to our own results, we find that the substitution of epicholesterol for cholesterol makes no significant difference in vesicle phase behavior, transition temperature, or area of dark phase.

Other studies have replaced the hydroxyl headgroup of cholesterol with a ketone, an acetate, or an ether and found that the compressibility of DPPC/sterol monolayers is lowest for the hydroxy-sterols (35). Similarly, replacing the hydroxyl headgroup of cholesterol with a ketone to produce 6-ketosterol renders the sterol less effective at reducing monolayer lateral elasticity (36). Returning to our own results on hydroxysterols versus ketosterols, we find that cholestenone is an inhibitor. The structural reason that cholestenone is an inhibitor is likely because its ketone headgroup lacks the ability of a hydroxyl group to directly hydrogen bond with the ester linkage of phosphatidylcholine lipid acyl chains (37).

With respect to the alkyl-chain structure of sterols, lipid vesicles are more permeable when they contain sterols with alkyl chains that are longer and more unsaturated than the alkyl chain of cholesterol (38). In a separate experiment, a group of cholesterol analogs with branched and unbranched chains containing 3–10 carbons all exhibited roughly equal area condensation in monolayers of DPPC or 18:0-18:1PC (28). Sterols with unbranched alkyl chains had smaller molecular areas and smaller collapse pressures than those with branched chains (28). Although unbranched cholesterol analogs fit the requirement of a smaller molecular area for a ''membrane active sterol'' (30), it is unclear if an unbranched tail qualifies as ''cholesterol-like''. In any case, it is clear that membrane active sterols require an alkyl chain. Androstenol (5-androsten-3β-ol) is similar to androstenolone, lacks an alkyl chain, has no significant condensing effect on monolayers (28), and has a much smaller effect on monolayer lateral elasticity than cholesterol (36).

Applying this information to our results, it is difficult to find correlations that consistently apply between the membrane phases we observe and previous measurements of membrane order and permeability. We find that membrane phase often, but not always, correlates with the general magnitude of area condensation of sterols with oleoyl-stearoyl PC (34), the solubilization of sterols in sonicated eggPC extracts, the decrease in vesicle permeability (10), or the equilibrium constants for lipid dimer exchange (31).

A similar conclusion applies to monolayers. A variety of cholesterol analogs condense lipid monolayers (DPPC or stearoyl-oleoylPC in this case) (28), but not all produce immiscible liquid phases in lipid monolayers (DPPC or palmitoyl-sphingomyelin in this case) (35,39). As another example, sterol molecular area in monolayers predicts the temperature of the inverted hexagonal phase transition in bilayers of lipids and many sterols, with the striking exception of androstenol (40).

Cholesterol, ergosterol, and lanosterol

Historically, there has been intense theoretical and experimental interest in the natural sterols of cholesterol, ergosterol, and lanosterol. From the theoretical side, molecular dynamics simulations for membranes of 8:1 DMPC/sterol find similar ordering, number of gauche defects, molecular area, and electron density for all three sterols of cholesterol, ergosterol, and lanosterol. At 1:1 DMPC/sterol, these simulations conclude that cholesterol has stronger condensing compared to lanosterol (41). Simulations and experiment find evidence of a liquid-liquid coexistence region for bilayers of cholesterol and palmitoyl-petroselinoylPC (PPetPC), but not for bilayers of lanosterol and PPetPC (33).

From the experimental side, the effects of lanosterol on lipid membranes are typically less pronounced than of cholesterol and ergosterol. For example, cholesterol reduces vesicle permeability more than lanosterol (42). Similarly, the microviscosity of eggPC/cholesterol membranes is greater than of eggPC/lanosterol membranes as measured by fluorescence microscopy (43). Nuclear magnetic resonance finds that sterols produce conformational ordering of saturated lipid chains of dimyristoylPC and PPetPC in the hierarchy ergosterol $>$ cholesterol $>$ lanosterol. For the mixed saturated and unsaturated chains of palmitoyl-oleoylPC, ordering occurs as cholesterol $>$ ergosterol $\sim$ lanosterol (33,44). For the unsaturated chains of DOPC, ordering occurs as cholesterol $>$ lanosterol (45). Cholesterol and ergosterol also produce more membrane order than lanosterol as measured by a spin probe incorporated in vesicles of bovine brain lipids (46). However, smaller concentrations of lanosterol (15%) than cholesterol (25%) are required to diminish the endothermic melting peak of PPetPC (33).

Implications

What can we conclude from the discussion above? We often think of cholesterol as uniquely suited to the task of altering membrane structure, as befitting a molecule at the end of a biochemical pathway and optimized through evolution. Cholesterol is certainly more effective in this role than lanosterol. However, in terms of cholesterol's ability to produce coexisting liquid phases in membranes of DOPC, DPPC, and sterol, it is not clearly more effective than

ergosterol or the other promoter sterols of dihydrocholesterol, epicholesterol, or 25-hydroxycholesterol. Although many promoter sterols are associated with low vesicle permeability, low molecular area, and high compressibility, none of these parameters individually predicts the phase behavior we observe in vesicles. Instead, we find that the conglomerate description of ''membrane active sterols'' (30) applies well to all but one of the promoter sterols in this study. All promoter sterols may sufficiently fulfill the role of altering membrane phase behavior, and the specific sterol utilized by different biological systems may depend further on specific biochemical processes of each system's cell cycle (47).

CONCLUSION

In summary, we find coexisting liquid phases in bilayer lipid membranes containing those sterols previously characterized as promoting the formation of DPPC-rich phases. Although detailed phase diagrams are required to completely compare the effectiveness of each promoter sterol in producing liquid phases, it seems likely that the following structural features that fulfill requirements of ''membrane active sterols'' play an important role: 1), flat, fused rings, 2), a hydroxyl or other small polar group at position 3, 3), a ''cholesterol-like'' tail, and 4), a small area per molecule (30).

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REFERENCES

1. Bloch, K. 1991. Cholesterol: evolution of structure and function. *In* Biochemistry of Lipids, Lipoproteins and Membranes. D. E. Vance and J. E. Vance, editors. Elsevier Science Publishers B. V., Amsterdam. 363–381 .
2. Lange, Y., M. H. Swaisgood, B. V. Ramos, and T. L. Steck. 1989. Plasma membranes contain half the phospholipid and 90% of the cholesterol and sphingomyelin in cultured human fibroblasts. *J. Biol. Chem.* 264:3786–3793.
3. Ipsen, J. H., G. Karlström, O. G. Mouritsen, H. Wennerström, and M. J. Zuckermann. 1987. Phase equilibria in the phosphatidylcholine-cholesterol system. *Biochim. Biophys. Acta.* 905:162–172.
4. 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.
5. Alecio, M. R., D. E. Golan, W. R. Veatch, and R. R. Rando. 1982. Use of a fluorescent cholesterol derivative to measure lateral mobility of cholesterol in membranes. *Proc. Natl. Acad. Sci. USA.* 79:5171–5174.
6. Vist, M. R., and J. H. Davis. 1990. Phase equilibria of cholesterol/dipalmitoylphosphatidylcholine mixtures: 2H nuclear magnetic resonance and differential scanning calorimetry. *Biochemistry.* 29:451–464.
7. Pralle, A., P. Keller, E.-L. Florin, K. Simons, and J. K. H. Hörber. 2000. Sphingolipid-cholesterol rafts diffuse as small entities in the plasma membrane of mammalian cells. *J. Cell Biol.* 148:997–1007.
8. Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. *Nature.* 387:569–572.
9. Ahn, K.-W., and N. S. Sampson. 2004. Cholesterol oxidase senses subtle changes in lipid bilayer structure. *Biochemistry.* 43:827–836.
10. Demel, R. A., K. R. Bruckdorfer, and L. L. M. Van Deenen. 1972. The effect of sterol structure on the permeability of lipomes to glucose, glycerol and Rb. *Biochim. Biophys. Acta.* 255:321–330.
11. Xu, X., R. Bittman, G. Duportail, D. Heissler, C. Vilcheze, and E. London. 2001. Comparison of cholesterol to plant, fungal, and disease-associated sterols and comparison of sphingomyelin, cerebrosides, and ceramide. *J. Biol. Chem.* 276:33540–33546.
12. Silvius, J. R. 1982. Thermotropic phase transitions of pure lipids in model membranes and their modifications by membrane proteins. *In* Lipid-Protein Interactions. P. C. Jost and O. H. Griffith, editors. John Wiley and Sons, New York.
13. Veatch, S. L., and S. L. Keller. 2002. Organization in lipid membranes containing cholesterol. *Phys. Rev. Lett.* 89:268101.
14. Huang, J., J. T. Buboltz, and G. W. Feigenson. 1999. Maximum solubility of cholesterol in phosphatidylcholine and phosphatidylethanolamine bilayers. *Biochim. Biophys. Acta.* 1417:89–100.
15. De Kruyff, B., R. A. Demel, and L. L. M. Van Deenen. 1972. The effect of cholesterol and epicholesterol incorporation on the permeability and on the phase transition of intact Acholeplasma Laidlawii cell membranes and derived liposomes. *Biochim. Biophys. Acta.* 255: 331–347.
16. Veatch, S. L., and S. L. Keller. 2003. Separation of liquid phases in giant vesicles of ternary mixtures of phospholipids and cholesterol. *Biophys. J.* 85:3074–3083.
17. Angelova, M. I., S. Soleau, P. Meleard, J. F. Faucon, and P. Bothorel. 1992. Preparation of giant vesicles by external AC electric fields. Kinetics and applications. *Prog. Colloid Polym. Sci.* 89:127–131.
18. Veatch, S. L. 2003. A closer look at the canonical 'raft mixture' in model membrane studies. *Biophys. J.* 84:725–726.
19. Veatch, S. L., and S. L. Keller. 2005. Seeing spots: complex phase behavior in simple membranes. *Biochim. Biophys. Acta.* In press.
20. Veatch, S. L., I. V. Polozov, K. Gawrisch, and S. L. Keller. 2004. Liquid domains in vesicles investigated by NMR and fluorescence microscopy. *Biophys. J.* 86:2910–2922.
21. Filippov, A., G. Oradd, and G. Lindblom. 2004. Lipid lateral diffusion in ordered and disordered phases in raft mixtures. *Biophys. J.* 86:891–896.
22. 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.
23. 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.
24. Schneider, M. B., W. K. Chan, and W. W. Webb. 1983. Fast diffusion along defects and corrugations in phospholipid P beta, liquid crystals. *Biophys. J.* 43:157–165.
25. Veatch, S. L., and S. L. Keller. 2005. Miscibility phase diagrams of giant vesicles containing sphingomyelin. *Phys. Rev. Lett.* 94:148101 1–4.
26. Parasassi, T., E. Gratton, W. M. Yu, P. Wilson, and M. Levi. 1997. Two-photon fluorescence microscopy of laurdan generalized polarization domains in model and natural membranes. *Biophys. J.* 72: 2413–2429.
27. Feigenson, G. W., and J. T. Buboltz. 2001. Ternary phase diagram of dipalmitoyl-PC/dilauroyl-PC/cholesterol: nanoscopic domain formation driven by cholesterol. *Biophys. J.* 80:2775–2788.
28. Slotte, J. P., M. Jungner, C. Vilcheze, and R. Bittman. 1994. Effect of sterol side-chain structure on sterol-phosphatidylcholine interactions in monolayers and small unilamellar vesicles. *Biochim. Biophys. Acta.* 1190:435–443.
29. Wenz, J. J., and F. J. Barrantes. 2003. Steroid structural requirements for stabilizing or disrupting lipid domains. *Biochemistry.* 42:14267–14276.

30. Barenholz, Y. 2002. Cholesterol and other membrane active sterols: from membrane evolution to ''rafts''. *Prog. Lipid Res.* 41:1–5.

31. Cao, H., N. Tokutake, and S. L. Regen. 2003. Unraveling the mystery surrounding cholesterol's condensing effect. *J. Am. Chem. Soc.* 125: 16182–16183.

32. Huang, C. 1977. Configurations of fatty acyl chain in egg phosphatidylcholine-cholesterol mixed bilayers. *Chem. Phys. Lipids.* 19:150–158.

33. Miao, L., M. Nielsen, J. Thewalt, J. H. Ipsen, M. Bloom, M. J. Zuckermann, and O. G. Mouritsen. 2002. From lanosterol to cholesterol: structural evolution and differential effects on lipid bilayers. *Biophys. J.* 82:1429–1444.

34. Demel, R. A., K. R. Bruckdorfer, and L. L. M. Van Deenen. 1972. Structural requirements of sterols for the interaction with lecithin at the air-water interface. *Biochim. Biophys. Acta.* 255:311–320.

35. Slotte, J. P. 1995. Effect of sterol structure on molecular interactions and lateral domain formation in monolayers containing dipalmitoyl phosphatidylcholine. *Biochim. Biophys. Acta.* 1237:127–134.

36. 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.

37. Ben-Yashar, V., and Y. Barenholz. 1989. The interaction of cholesterol and cholest-4-en-3-one with dipalmitoylphosphatidylcholine. Comparison based on the use of three fluorophores. *Biochim. Biophys. Acta.* 985:271–278.

38. Yamauchi, H., Y. Takao, M. Abe, and K. Ogino. 1993. Molecular interactions between lipid and some steroids in a monolayer and a bilayer. *Langmuir.* 9:300–304.

39. Mattjus, P., R. Bittman, C. Vilcheze, and J. P. Slotte. 1995. Lateral domain formation in cholesterol/phospholipid monolayers as affected by the sterol side chain conformation. *Biochim. Biophys. Acta.* 1240: 237–247.

40. Gallay, J., and B. De Kruyff. 1982. Correlation between molecular shape and hexagonal HII phase promoting ability of sterols. *FEBS Lett.* 143:133–136.

41. Smondyrev, A. M., and M. L. Berkowitz. 2001. Molecular dynamics simulation of the structure of dimyristoylphosphatidylcholine bilayers with cholesterol, ergosterol, and lanosterol. *Biophys. J.* 80:1649–1658.

42. Bloch, K. 1983. Sterol structure and membrane function. *Crit. Rev. Biochem.* 14:47–92.

43. Dahl, C. E., J. S. Dahl, and K. Bloch. 1980. Effect of alkyl-substituted precursors of cholesterol on artificial and natural membranes and on the viability of mycoplasma capricolum. *Biochemistry.* 19:1462–1467.

44. Urbina, J. A., S. Pekerar, H.-B. Le, J. Patterson, B. Montez, and E. Oldfield. 1995. Molecular order and dynamics of phosphatidylcholine bilayer membranes in the presence of cholesterol, ergosterol and lanosterol: a comparative study using 2H-, 13C- and 31P-NMR spectroscopy. *Biochim. Biophys. Acta.* 1238:163–176.

45. Yeagle, P. L. 1985. Lanosterol and cholesterol have different ordering effects on phospholipid acyl chain ordering. *Biochim. Biophys. Acta.* 815:33–36.

46. Butler, K. W., I. C. P. Smith, and H. Schneider. 1970. Sterol structure and ordering effects in spin-labelled phospholipid multibilayer structures. *Biochim. Biophys. Acta.* 219:514–517.

47. Suarez, Y., C. Fernandez, B. Ledo, A. J. Ferruelo, M. Martin, M. A. Vega, D. Gomez-Coronado, and M. A. Lasuncion. 2002. Differential effects of ergosterol and cholesterol on Cdk1 activation and SRE-driven transcription. *Eur. J. Biochem.* 269:1761–1771.