Desmosterol May Replace Cholesterol in Lipid Membranes

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ABSTRACT Recently, knockout mice entirely lacking cholesterol have been described as showing only a mild phenotype. For these animals, synthesis of cholesterol was interrupted at the level of its immediate precursor, desmosterol. Since cholesterol is a major and essential constituent of mammalian cellular membranes, we asked whether cholesterol with its specific impact on membrane properties might be replaced by desmosterol. By employing various approaches of NMR, fluorescence, and EPR spectroscopy, we found that the properties of phospholipid membranes like lipid packing in the presence of cholesterol or desmosterol are very similar. However, for lanosterol, a more distant precursor of cholesterol synthesis, we found significant differences in comparison with cholesterol and desmosterol. Our results show that, from the point of view of membrane biophysics, cholesterol and desmosterol behave identically and, therefore, replacement of cholesterol by desmosterol may not impact organism homeostasis.

INTRODUCTION

Cholesterol is a major constituent of mammalian cellular membranes with various functions, e.g., its biophysical properties are essential for membrane domain formation (Simons and Ikonen, 1997; Anderson and Jacobson, 2002), protein-lipid interaction (Ayala-Sanmartin, 2001), and for functioning of membrane proteins (Mitchell et al., 1990; Albert et al., 1996). The cholesterol content of a cell is very tightly controlled. For instance, the cholesterol content of disc membranes in retinal rod outer segments is related to the spatial distribution and age of those membranes (Boesze-Battaglia et al., 1990). Even slight changes of the cholesterol content of (biological) membranes cause significant intolerable alterations of membrane properties and may severely affect various biological functions essential for cell homeostasis.

Recently, a very challenging report in *Science* described knockout mice entirely lacking cholesterol (Wechsler et al., 2003). In these animals, the biosynthetic reduction of desmosterol to cholesterol was blocked. Surprisingly, those mice showed only a mild phenotype, suggesting that the properties of desmosterol determining membrane structure, dynamics, and function should be very similar to those of cholesterol, thus, desmosterol may entirely replace cholesterol. Indeed, compared with cholesterol, desmosterol varies only by a single double bond at carbon 24 (Fig. 1). However, it has been shown for other cholesterol analogs that even small modifications of the sterol structure lead to significant alterations of the membrane properties (Endress et al., 2002; Scheidt et al., 2003). Those studies investigated the correlation between sterol structure and i), the influence of sterols on permeability; ii), ordering effects in lipid membranes; and iii), the formation and stabilization of ordered lipid domains (Demel et al., 1972; Yeagle et al., 1977; Butler and Smith, 1978; Rogers et al., 1979; Bloch, 1983; Urbina et al., 1995; Xu and London, 2000; Serfis et al., 2001; Martinez et al., 2004; Wang et al., 2004). However, only little is known about the physicochemical properties of desmosterol containing bilayer membranes. For cholesterol, a very specific condensation effect is well known. The sterol orders phospholipid chains by favorable van der Waals interactions thus reducing the area per lipid molecule and thereby influencing the fluidity of the entire membrane (Oldfield et al., 1978).

To address whether desmosterol may replace cholesterol in mammalian cell membranes we compared the biophysical properties of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) lipid membranes in the presence of these sterols by ^{2}H nuclear magnetic resonance (NMR), electron paramagnetic resonance (EPR), and fluorescence spectroscopy, which are well-suited tools to study membrane properties of lipids (Scheidt et al., 2003). For comparison, we also investigated lanosterol, a more distant precursor in the cholesterol synthesis. The chemical structures of these steroids are given in Fig. 1. Since cholesterol preferentially interacts with sphingomyelin (SPM), we also investigated the interaction between these steroids and SPM.

MATERIALS AND METHODS

Materials

The lipids POPC, 1-palmitoyl-d_{31}-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC-d_{31}), and spin-labeled lipids 1-palmitoyl-2-(5-doxylstearoyl)-*sn*-glycero-3-phosphocholine (C5-SL-PC) and 1-palmitoyl-2-(16-doxylstearoyl)-*sn*-glycero-3-phosphocholine (C16-SL-PC) were purchased from Avanti Polar Lipids (Alabaster, AL). SPM from chicken egg yolk was purchased

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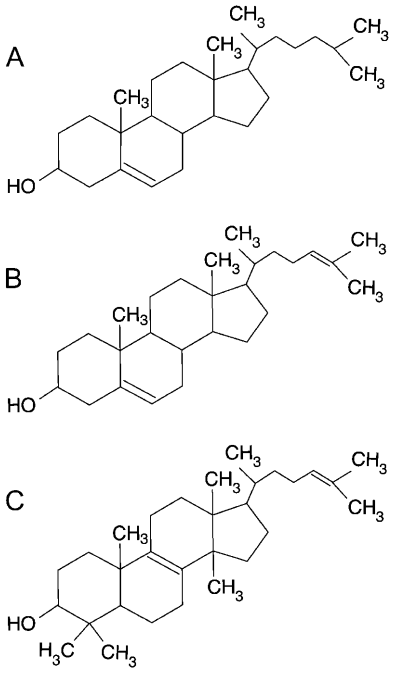

FIGURE 1 Chemical structure of cholesterol (*A*), desmosterol (*B*), and lanosterol (*C*).

from Sigma-Aldrich (Deisenhofen, Germany). All lipids were used without further purification. 25-doxyl-cholesterol (SL-Chol) was synthesized according to the protocol of Maurin et al. (1987); 6-lauroyl-2-(*N*,*N*-dimethylamino)naphthalene (Laurdan) was purchased from Molecular Probes (Eugene, OR).

Vesicle preparation

For ^{2}H-NMR measurements, mixtures of phospholipids and sterols were prepared in chloroform. After evaporating the chloroform under a stream of nitrogen, the samples were redissolved in cyclohexane and lyophilized. Samples were hydrated to 40 wt% deuterium depleted H_2O and equilibrated by freeze-thaw cycles and gentle centrifugation. Samples were transferred into 5-mm glass vials for static ^{2}H-NMR experiments.

For fluorescence and EPR measurements, lipids and Laurdan or spin-labeled lipids dissolved in organic solvent were combined in a glass tube to give the desired composition and concentration. The mixture was dried under nitrogen. Hepes buffered saline (150 mM NaCl, 5 mM Hepes, pH 7.4) was added to give a final lipid concentration of 5 mM (lipids) and 25 μM (Laurdan) or 50 μM (C5-SL-PC, C16-SL-PC, SL-Chol). Lipids were hydrated by vigorous vortexing, and large unilamellar vesicles (LUV) were prepared by extrusion (Lipex Biomembranes Inc., Vancouver, Canada) with five freeze-thaw-cycles and filtration through 0.1 mm pores (10 cycles) at 40°C (Mayer et al., 1985).

NMR measurements

^{2}H-NMR spectra were recorded on a Bruker Avance 400 NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) at a resonance frequency of 61.5 MHz for ^{2}H using a solids probe with a 5 mm solenoid coil. The ^{2}H-NMR spectra were accumulated using the quadrupolar echo sequence (Davis et al., 1976) and a relaxation delay of 500 ms. The two 3-μs $\pi/2$ pulses were separated by a 60-μs delay. ^{2}H-NMR spectra were depaked (McCabe and Wassall, 1995) and order parameters for each methylene group in the chain were determined as described in detail in (Huster et al., 1998). Average order parameters were calculated by adding all chain order parameters and dividing them by the number of methylene and methyl groups in the chain. ^{2}H-NMR spectra were acquired at temperatures of 4°C, 20°C, and 30°C.

EPR measurements

EPR spectra of LUV labeled with C5-SL-PC, C16-SL-PC or SL-Chol were recorded at 20°C and 30°C on a Bruker ECS 106 spectrometer (Bruker, Karlsruhe, Germany) with the following parameters: modulation amplitude 2.0 G, power 20 mW, scan width 100 G, accumulation nine times. From the spectra of C5-SL-PC and C16-SL-PC an order parameter (S) using the magnetic parameters $A_{xx} = 5.75$ G, $A_{yy} = 5.75$ G, $A_{zz} = 33.50$ G (Griffith and Jost, 1976; Ge et al., 2003) and a rotational correlation time (τ), respectively, was estimated (Keith et al., 1970; Morse, 1977).

Fluorescence measurements

Fluorescence spectra of Laurdan were recorded (Aminco Bowman spectrometer series 2, Rochester, USA) between 390 nm and 550 nm (excitation and emission slit widths of 4 nm) at 20°C and 30°C (POPC/steroid-LUV) or at 40°C (SPM/steroid-LUV). For quantifying the differences in fluorescence emission we calculated a generalized polarization (GP):

$$\mathrm{GP} = I_\mathrm{B} - I_\mathrm{R}/I_\mathrm{B} + I_\mathrm{R}, \quad (1)$$

where I_B and I_R correspond to the emission intensities measured at 437 nm and 482 nm, respectively (Parasassi et al., 1990; Parasassi et al., 1991).

Miscelleanous

For statistical comparison of data, the *t*-test was performed at a level of $P = 0.1$.

RESULTS

First, we compared the influence of cholesterol, desmosterol and lanosterol on phospholipid chain order. Fig. 2, *A* and *B*, shows the *sn*-1 chain order parameters of POPC-d_{31} in the presence of these sterols measured at 30°C. The continuous order decrease from the glycerol backbone toward the end of the chain indicated an increase in the motional amplitudes of the chain segments (Seelig, 1977). As is well known for cholesterol, a significant increase of lipid chain order was observed with increasing cholesterol concentration (condensation) (Oldfield et al., 1978; Ipsen et al., 1990). If desmosterol replaced cholesterol in the membrane, almost identical chain order parameters were observed (*solid symbols* in Fig. 2 *A*). In contrast, if cholesterol was replaced by lanosterol, significant alterations in the POPC-d_{31} order parameters occurred. Lanosterol still ordered the *sn*-1 chain of the lipid. However, the magnitude of the effect was clearly attenuated (*solid symbols* in Fig. 2 *B*). However, at the midplane of the bilayer the influence of the steroids on lipid motion was very similar.

The effects of chain ordering are best displayed if the average order parameter is plotted as a function of the sterol content. Such a plot is shown in Fig. 2 *C*. Each sterol

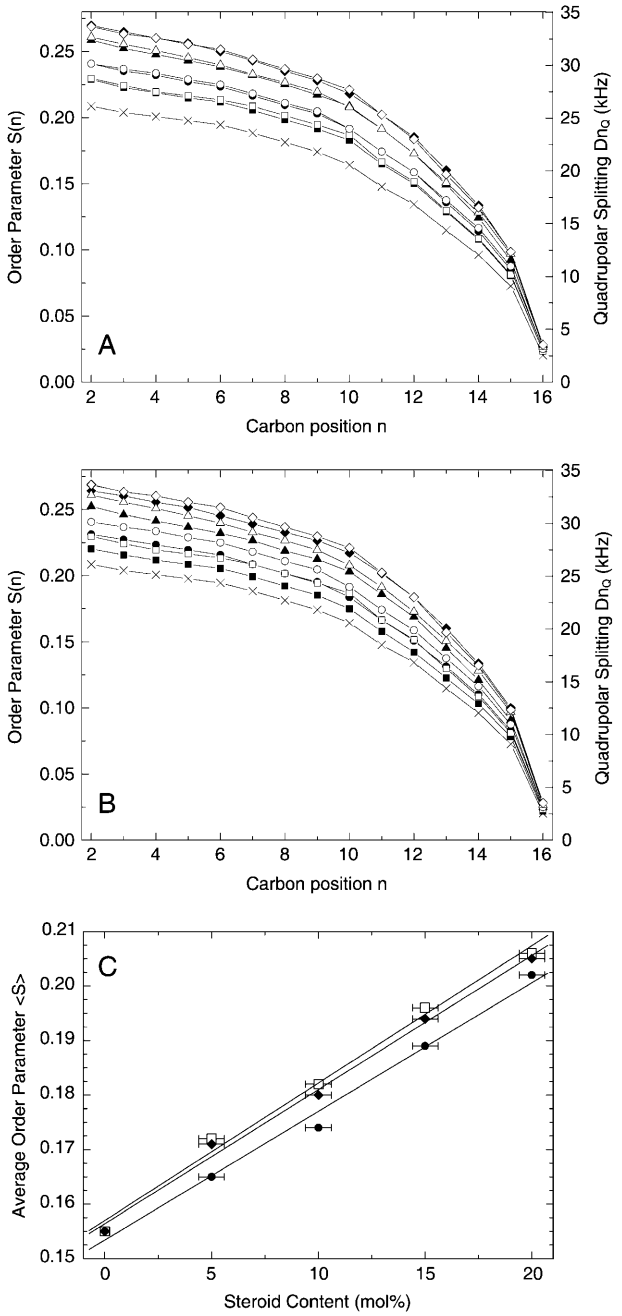

FIGURE 2 Smoothed ^{2}H-NMR order parameter profiles of POPC-d_{31} membranes in the presence of varying sterol concentrations at a temperature of 30°C. Order parameters are shown for desmosterol (*solid symbols* in *A*) and for lanosterol (*solid symbols* in *B*). In comparison, the values of pure POPC-d_{31} (×) and cholesterol (*open symbols*) are shown. The steroid concentrations were 5 mol % (*squares*), 10 mol % (*circles*), 15 mol % (*triangles*), and 20 mol % (*diamonds*). In *C*, the average order parameter of the palmitoyl acyl chain of POPC-d_{31} is given at varying concentration of (□) cholesterol, (♦) desmosterol, and (●) lanosterol at 30°C. The typical error of the order parameters is on the order of ±0.005 and thus covered by the symbol size in the plot.

increased the phospholipid chain order linearly as a function of the sterol content. Within experimental error, the POPC order parameters in the presence of cholesterol or desmosterol were indistinguishable, whereas significantly smaller order parameters were observed if lanosterol replaced cholesterol.

Next we measured the effect of steroids on the mobility of spin-labeled lipids. EPR spectra of POPC/steroid-LUV labeled with PC analogs (C5-SL-PC or C16-SL-PC) or with a cholesterol analog (SL-Chol) were recorded at 30°C and compared with those of pure POPC-LUV. In the presence of steroids (30 mol %) the motional order of C5-SL-PC and C16-SL-PC was reduced as revealed from an increase of the outer hyperfine splitting (Fig. 3, *A* and *C*, see *arrows*) as well as from the increase in order parameter and rotational correlation time of C5-SL-PC and C16-SL-PC, respectively (Fig. 3, *B* and *D*). The increase of the order parameter of C5-SL-PC was significantly higher for cholesterol and desmosterol in comparison to lanosterol (Fig. 3 *B*). For C16-SL-PC, the rotational correlation times indicated a similar effect of the steroids on membranes (Fig. 3 *D*). Like C16-SL-PC, SL-Chol has the spin label moiety close to the midplane of the bilayer. Upon addition of steroids (30 mol %) to POPC, the spectra of SL-Chol also reflected a decreased mobility of the analogs as seen from an increase of the outer hyperfine splitting (spectra not shown). Comparing the spectra of SL-Chol in membranes containing cholesterol, desmosterol, or lanosterol revealed that the analog detects a similar immobilization (spectra not shown).

In a third approach we employed the fluorescence emission of the lipid probe Laurdan, which is sensitive to lipid packing (Parasassi et al., 1997). It exhibits a 50 nm blue shift as membranes undergo phase transition from fluid to gel, due to altered water penetration. Indeed, when cholesterol or desmosterol (10 and 30 mol %) were added to POPC bilayers, we observed such a large emission shift with no difference between both sterols (Fig. 4 *A*, only shown for 30 mol % steroid). As indicated by the increase of the generalized polarisation (GP) of Laurdan (see Materials and Methods), the presence of both cholesterol and desmosterol lead to a similarly strong condensing of membrane lipids (Fig. 4 *B*, only shown for 30 mol % steroid). Compared with cholesterol and desmosterol, lanosterol had a significantly lower effect on lipid packing as deduced from its lower GP value (see Fig. 4 *B*).

In biological membranes cholesterol specifically interacts with SPM which may lead to the formation of lateral membrane domains in multilamellar vesicles (Simons and Ikonen, 1997; Samsonov et al., 2001; Veiga et al., 2001). To assess this property, the fluorescence of Laurdan was recorded in SPM vesicles containing 30 mol % of the respective steroid and the GP values were calculated (Fig. 4 *C*). The strongly increased GP values in the presence of steroids compared with pure SPM vesicles reflect an enhanced lipid packing. However, compared to POPC-LUV the increase in

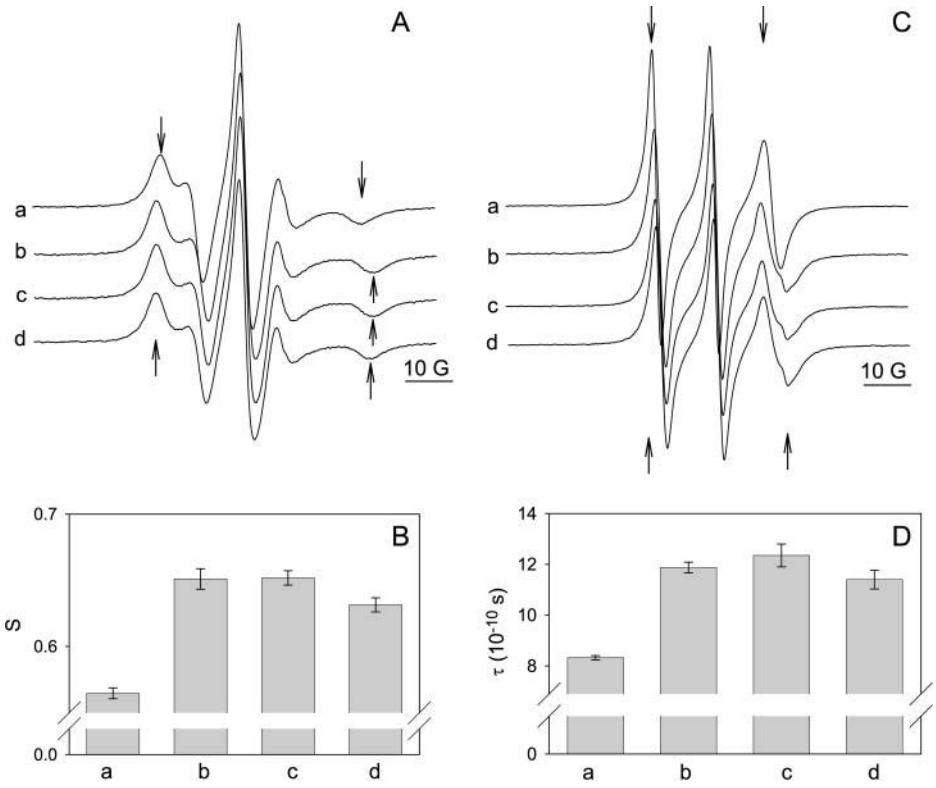

FIGURE 3 EPR spectra of spin-labeled phosphatidylcholine analogs in large unilamellar vesicles. Spectra of C5-SL-PC (*A*) and C16-SL-PC (*C*) were recorded in LUV consisting of POPC (*a*), POPC/cholesterol (70:30) (*b*), POPC/desmosterol (70:30) (*c*), and POPC/lanosterol (70:30) (*d*) at 30°C as described in Materials and Methods. The arrows show the outer hyperfine splitting (*A*), which is increased in the presence of steroids. Note that for C16-SL-PC, an additional component (see *high field peak* of the spectra) may indicate that a small part of this lipid is organized in a different ordered domain within the membrane. From the spectra of C5-SL-PC and C16-SL-PC, an order parameter (*S*, panel *B*) and a correlation time of rotation (τ, panel *D*), respectively, was calculated. Data are the average ± SE of estimate of 4–5 independent measurements.

GP upon addition of steroids was larger in SPM membranes suggesting a stronger lipid condensation by steroids in SPM membranes. Comparing the effect of steroids on SPM membranes, the GP values were similar in the presence of cholesterol and desmosterol and significantly lower for lanosterol (Fig. 4 *C*).

DISCUSSION

The background of this study is a recently published article describing the generation of ''cholesterol-free'' mice which were viable and had a relatively mild phenotype (Wechsler et al., 2003). Owing to an enzymatic blockade, in these animals the final reduction of desmosterol to cholesterol was abolished. Therefore, cholesterol was replaced by its metabolic precursor desmosterol. Those studies are linked to the question whether life is possible without cholesterol. To address this, at least two aspects have to be considered. One concerns cholesterol as a membrane constituent and the other one cholesterol as part of the metabolism of an organism, e.g., as a precursor of hormones. In our study devoted to the first aspect we investigated whether cholesterol might be substituted in lipid membranes by steroids of the cholesterol synthesis pathway using a comprehensive biophysical approach. Besides desmosterol we also employed lanosterol, which is a more distant precursor in the cholesterol synthesis, thereby, having a more different chemical structure (see Fig. 1).

Cholesterol as an important component of mammalian plasma membranes determines and modulates the structural characteristics of membranes in that, e.g., it causes a specific condensation of phospholipids (Oldfield et al., 1978). This characteristic effect of cholesterol has also been observed in our experiments: compared to pure POPC membranes, in POPC/cholesterol vesicles i), the order parameters measured by ^{2}H-NMR or EPR (C5-SL-PC); ii), the rotational correlation time (C16-SL-PC) and the outer hyperfine splitting (SL-Chol); and iii), the GP values calculated from Laurdan fluorescence were significantly increased.

We found that the influence of desmosterol on lipid order was very similar to that of cholesterol. This conclusion was supported by all experimental approaches employed, i.e., ^{2}H-NMR, EPR using C5-SL-PC, C16-SL-PC and SL-Chol as well as fluorescence spectroscopy using Laurdan. For example, the dependence of the order parameter measured by ^{2}H-NMR on the steroid content was similar for cholesterol and desmosterol.

Since in (biological) membranes a specific interaction between cholesterol and SPM has been described (Samsonov et al., 2001; Veiga et al., 2001), we also compared the effect of cholesterol and desmosterol on SPM membranes. Using Laurdan fluorescence, both steroids had a similar influence on lipid packing in SPM membranes. Although with the Laurdan approach a specific steroid-SPM interaction cannot be investigated, our results suggest that desmosterol is also able to interact with SPM. Further studies should clarify whether desmosterol, like cholesterol, is able to support the

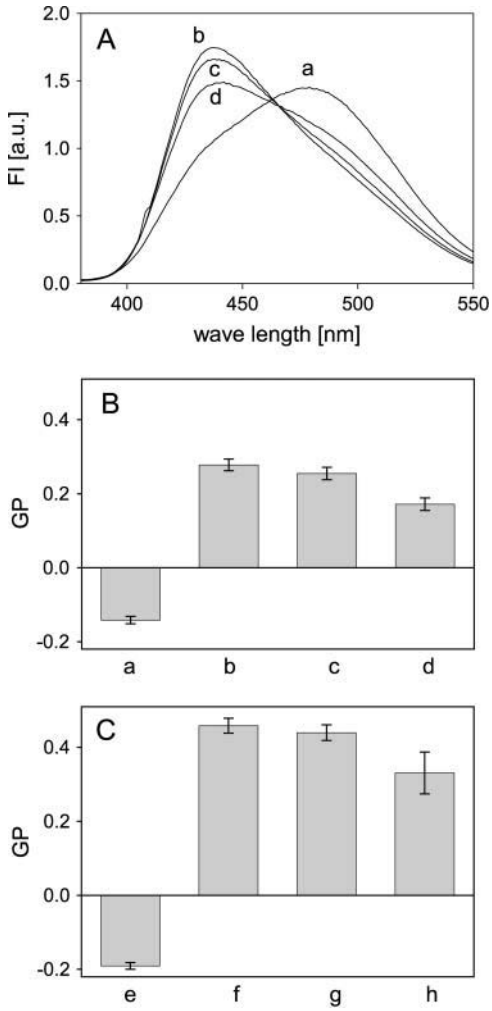

FIGURE 4 Fluorescence spectra of Laurdan in large unilamellar vesicles consisting of POPC (*a*), POPC/cholesterol (70:30) (*b*), POPC/desmosterol (70:30) (*c*), and POPC/lanosterol (70:30) (*d*) were recorded at 30°C as described in Materials and Methods (*A*). Fluorescence intensities were normalized to intensities measured after disruption of vesicles by Triton X-100 (0.5%). From these spectra, the generalized polarization of emission intensities $GP = (I_B - I_R)/(I_B + I_R)$ (I_B and I_R: intensities at 437 nm and 482 nm) was calculated (average ± SE of estimate of at least six independent measurements) (*B*). Fluorescence spectra of Laurdan were recorded at 40°C and the GP values calculated for vesicles consisting of SPM (*e*), SPM/cholesterol (70:30) (*f*), SPM/desmosterol (70:30) (*g*), and SPM/lanosterol (70:30) (*h*) (*C*). (*C*) Data are the average ± SE of estimate of at least five independent measurements.

formation of lateral membrane domains, so-called rafts (Simons and Ikonen, 1997). Recently, it has been shown that another intermediate of sterol biosynthesis, lathosterol, is able to form rafts with dipalmitoylphosphatidylcholine (Wang et al., 2004). Lathosterol which has a 7–8 carbon double bond in place of the 5–6 one of cholesterol is a more distant metabolic precursor of cholesterol than desmosterol.

Physical membrane properties are very sensitive to the chemical structure of the steroid as underlined by the results from several groups and those of the current study obtained with lanosterol (Yeagle et al., 1977; Bloch, 1983; Xu and London, 2000; Miao et al., 2002; Martinez et al., 2004; Wang et al., 2004). Compared with desmosterol this molecule has three additional bulky methyl groups, two at C4 and one at C14 position, and the double bound in the steroid backbone at a different place (see Fig. 1). This results in a less planar α-face of lanosterol and thus less favorable van der Waals interactions with the lipid chains. In general, we found that phospholipid chain order was less affected by lanosterol in comparison to cholesterol or desmosterol. Similar differences in the effect of lanosterol and cholesterol on lipid membranes have been described in other studies (Yeagle et al., 1977; Bloch, 1983; Urbina et al., 1995; Miao et al., 2002; Martinez et al., 2004). As shown by the different techniques of this study, the effect of lanosterol varies from that of both cholesterol and desmosterol. Those approaches which probe the upper part of the lipid bilayer indicated a significantly lower ordering effect of lanosterol compared with that of cholesterol or desmosterol. This is underlined by the smaller order parameters calculated from ^{2}H-NMR spectra of lower carbon positions as well as from EPR measurements of C5-SL-PC. Moreover, the altered lipid packing in this region causing a different water penetration is reflected by the GP values of Laurdan fluorescence, which were lower in POPC membranes in the presence of lanosterol than of the other two steroids. On the other hand, for the midplane of the bilayer no differences between lanosterol and cholesterol/desmosterol were observed. The order parameters derived from ^{2}H-NMR for higher carbon positions as well as the EPR spectra of C16-SL-PC and SL-Chol show that lanosterol affects lipid motion in this region similar to cholesterol and desmosterol. Very likely, the two additional methyl groups of lanosterol at C4 position are the major reason for the different effect of lanosterol on lipid motion compared with cholesterol and desmosterol (Urbina et al., 1995; Miao et al., 2002). These two methyl groups will modify the interaction of lanosterol with lipids especially in the upper region of the membrane bilayer. Notably, the mobility of spin-labeled lipid analogs as well as the fluorescence of Laurdan were similarly dependent on the steroids in LUV even at a rather unphysiological temperature of 20°C (data not shown). Also, very similar order parameter differences for POPC in the presence of the respective sterols have been observed at 20°C (data not shown).

The different GP values of Laurdan in SPM membranes containing lanosterol or cholesterol indicate that the differences in the structure of lanosterol also affect its interaction with SPM. From these results it could be surmized that lanosterol, compared with cholesterol has a different ability to support the formation of lateral membrane domains. Indeed, it has been recently shown that cholesterol has a stronger ability than lanosterol to promote domain formation in membranes (Xu and London, 2000).

In conclusion, our results obtained from lipid membranes suggest that lipid-lipid interactions determining properties of

biological membranes are very similar for both cholesterol and desmosterol. The double bond in the desmosterol chain region occupies additional free volume, which is available in the lower acyl chain region of the membrane. Thus, desmosterol can condense lipid bilayers as well as cholesterol in contrast to other sterols with modifications in the sterol backbone of the molecule (Scheidt et al., 2003). Indeed, for lanosterol, which is a more distant metabolic precursor of cholesterol synthesis a different influence of this steroid on membrane properties is found. However, this study also shows that the influence of steroids on lipid motion varies along the bilayer normal. Therefore, the effect of steroids has to be investigated, e.g., in various depth of the membrane.

Our results support that life may be possible without cholesterol as demonstrated for mice (Wechsler et al., 2003). Nevertheless, cell membranes need to contain sterols that condense lipids, form lipid domains as rafts (Simons and Ikonen, 1997), and provide the basis for a proper organization and function of membranes. From the point of view of membrane biophysics, cholesterol and desmosterol are identical sterols. However, in living organisms desmosterol may not serve for all functions of cholesterol. For example, cholesterol is an essential component of crucial metabolic pathways, e.g., in the synthesis of hormones. Therefore, a total replacement of cholesterol by desmosterol in the organism might affect these pathways resulting in the impairement of the respective biological function, e.g., the fertility (Wechsler et al., 2003).

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