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Membrane Properties of Cholesterol Analogues with an Unbranched Aliphatic Side Chain

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Abstract

The interactions between cholesterol and other membrane molecules determine important membrane properties. It was shown that even small changes in the molecular structure of cholesterol have a crucial influence on these interactions. We recently reported that in addition to alterations in the tetracyclic ring structure, the *iso*-branched side chain of cholesterol also has a significant impact on membrane properties (Scheidt H. et al. 2013 Angew. Chem. Int. Ed. Engl. 52, 12848-12851). Here we used synthetic cholesterol analogs to investigate the influence of an unbranched aliphatic side chain of different length. The ^2H NMR order parameter of the phospholipid chains and therefore the molecular packing of the phospholipid molecules shows a significant dependence on the sterol's alkyl side chain length, while , membrane permeation studied by a dithionite ion permeation assay and lateral diffusion measured by ^1H MAS pulsed field gradient NMR are less influenced. To achieve the same molecular packing effect similar to that of an *iso*-branched aliphatic side chain, a longer unbranched side chain (*n*-dodecyl instead of *n*-octyl) at C17 of cholesterol is required. Obviously, sterols having a branched *iso*- alkyl chain with two terminal methyl groups exhibit altered cholesterol-phospholipid-interactions compared to analogous molecules with a straight unbranched chain.

Keywords

Cholesterol; Lipid membrane; Order parameter, Domain formation; Diffusion; Permeability

1. Introduction

The properties and therefore the functions of cellular membranes are determined by a multitude of physical interactions between many different molecules, which form nature's most important interface. Especially, the interactions between cholesterol and phospholipids and also membrane proteins have been the objective of an enormous number of studies, since cholesterol plays a major role in modifying membrane properties. In fungi and plants, other sterols such as ergosterol, campesterol, sitosterol, and stigmasterol fulfil the role of cholesterol. Special attention has been paid to the influence of various sterols on domain formation and the raft hypothesis (Xu et al., 2001; Leslie, 2011; Simons and Ikonen, 1997; Marsh, 2009). Specific interactions between cholesterol and phospholipid molecules, in particular with their saturated acyl chains, induce tight molecular packing by ordering the acyl chain of the phospholipids (Oldfield et al., 1978). The increased packing density changes essential membrane properties such as membrane permeability (Demel et al., 1972; Huster et al., 1997) and lateral lipid diffusion (Filippov et al., 2003; Scheidt et al., 2005). The membrane interactions of cholesterol are mostly described as attractive van der Waals forces between the saturated acyl chain of phospholipids and the ring system of cholesterol (Wang et al., 2004; Davies et al., 1990). Moreover, hydrogen bonds between the 3 β -hydroxyl group of cholesterol and the neighboring phospholipids play an important role (Soubias et al., 2004). Several studies have shown that even small changes in the cholesterol ring system disturb these interactions and change the membrane properties of cholesterol, sometimes dramatically (Demel et al., 1972; Xu and London, 2000; Wang et al., 2004; Scheidt et al., 2003; Huster et al., 2005; Endress et al., 2002; Milles et al., 2013; Shrivastava et al., 2008; Benesch and McElhaney, 2014).

Recently, we demonstrated that not only the tetracyclic ring system of cholesterol, but surprisingly, also the length of the *iso*-branched aliphatic side chain of cholesterol significantly affects membrane properties (Scheidt et al., 2013). This study indicated that the *iso*-branched side chain of cholesterol is responsible for 40 to 60% of the ordering effect of cholesterol. Therefore, the interactions between the alkyl side chain of cholesterol with the neighboring molecules should be considered in order to fully understand the impact of cholesterol on various properties of lipid membranes.

Here, we address the specificity of the *iso*-branched side chain of cholesterol. To this end, we investigated the influence of an unbranched aliphatic side chain on the membrane properties of cholesterol by using synthetic sterols bearing an unbranched side chain with 3 to 14 carbons attached to C17 of cholesterol (see Fig. 1). In particular, we investigated the influence of this cholesterol modification on the molecular packing of the membrane lipids, their lateral diffusion, and the membrane permeability of bilayers composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) or 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and the respective cholesterol analog. While membrane permeation and lateral diffusion are influenced only to a small extent by the sterol's alkyl side chain length, a significant dependence on the molecular packing of the phospholipid molecules was observed.

2. Materials and Methods

2.1. Materials.

The phospholipids and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Androst-5-en-3- β -ol (androstenol) was from Sigma-Aldrich (Steinheim, Germany). These chemicals were used without further purification. All other chemicals were from Sigma-Aldrich. The structures of the cholesterol analogs comprising varying lengths of the unbranched side chain are shown in Fig. 1; they were synthesized by modifications of previous procedures (Vilchze et al., 1996; Chia et al., 1993; Baek and Bittman, 2013)

Figure 1. Chemical structures of the sterol analogs used in this study.

2.2. Preparation of NMR samples.

For the NMR measurements, chloroform solutions of phospholipids and sterol analogs were mixed in a glass tube. After the solvent was evaporated, the samples were dissolved in cyclohexane and lyophilized overnight at a pressure of 0.2 mbar. The resulting fluffy powder was hydrated to 40 wt% with deuterium-depleted H₂O for ²H NMR or D₂O for ¹H PFG MAS NMR. After homogenization by ten freeze-thaw cycles and centrifugation steps, the samples were transferred to 5-mm glass vials for static ²H NMR or 4-mm MAS rotors for ¹H PFG MAS NMR experiments.

2.3. ^2H NMR experiments.

The ^2H NMR experiments were performed on a Bruker Avance 750 MHz NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) at a resonance frequency of 115.0 MHz for ^2H using a solids probe with a 5-mm solenoid coil. ^2H NMR spectra were acquired using a quadrupolar echo pulse sequence with a relaxation delay of 1 s. The two $\pi/2$ pulses with a typical length of around 3 μs were separated by a 60 μs delay. The spectral width was 500 kHz. ^2H NMR spectra were dePaked and smoothed order parameters were determined as described previously (Huster et al., 1998). From these order parameters the lipid chain extent as a measure of the lipid chain length was calculated according to the mean torque model (Petrache et al., 1999; Petrache et al., 2000).

2.4. ^1H PFG MAS NMR measurements.

^1H PFG MAS NMR experiments were carried out on a Bruker DRX 600 NMR spectrometer equipped with a 4 mm HR MAS probe with a gradient coil along the magic angle. Experiments were conducted at a spinning frequency of 6 kHz at 318 K. A stimulated echo sequence (Cotts et al., 1989) with sine-shaped bipolar gradient pulses with a length of 6 ms, an eddy current delay of 5 ms was used and typical ^1H $\pi/2$ pulse lengths were 11–13 μs . The relaxation delay was set to 5 s. The gradient strength, which was calibrated using a sample of pure water and the known diffusion coefficient of water, was varied in seven increments between 0.03 and 0.45 T/m. The diffusion time was varied in five increments between 25 and 300 ms. Apparent diffusion coefficients D_{app} were determined from a plot of peak intensity vs. gradient strength according to (Gaede and Gawrisch, 2003); furthermore, the diffusion coefficients were corrected for the radius of curvature of the vesicles using diffusion time dependent measurements (Gaede and Gawrisch, 2003; Scheidt et al., 2005).

2.5. Permeation assay.

The permeation of dithionite across lipid membranes was measured as described before (Pomorski et al., 1994; Scheidt et al., 2013). Briefly, large unilamellar vesicles (LUVs) containing POPC and 0.5 mol% 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) without and with 20 mol% of the respective sterol were prepared using the extrusion method (Mayer et al., 1986). The NBD fluorescence intensity of 20 μM LUVs was recorded in a cuvette at 540 nm ($\lambda_{\text{ex}} = 470$ nm, slit width for excitation and emission 4 nm) at 37°C using an Aminco Bowman Series 2 spectrofluorometer. After 30 s, sodium dithionite was added from a 1 M stock solution in 100 mM Tris (pH 10.0) to give a final concentration of 50 mM. Dithionite ion rapidly quenches the fluorescence of NBD-PE molecules localized in the outer leaflet, which is reflected by a rapid initial decrease of fluorescence intensity (kinetics not shown). Subsequently, the fluorescence intensity decreased slowly caused by a slow permeation of dithionite ion across the bilayer. By that process, dithionite reacted with the NBD-PE molecules in the inner leaflet. After 300 s, Triton X-100 (0.5% (w/v) final concentration) was added, enabling complete reaction of dithionite ion with NBD-PE, resulting in a complete loss of fluorescence. The curves were normalized to the fluorescence intensities before addition of dithionite and were fitted to a bi-exponential equation. From the fittings, the rate constants for the rapid fluorescence decrease (representing reduction of NBD-PE in the outer leaflet) and those for the slow decrease (representing permeation of dithionite ion across the bilayer) were determined. The latter ones were used as the parameter for membrane permeability.

3. Results

3.1. Influence of the unbranched chain length of the cholesterol analogs on the deuterium order parameter of POPC in lipid bilayers

The effect of the length of an unbranched sterol side chain on the molecular packing of the phospholipids in membranes was studied by measuring ^2H NMR order parameters of POPC- d_{31} in the presence of 20 mol% of the respective sterol analogs. It was previously shown that the phospholipid order parameter is significantly influenced by alterations in the structure of the tetracyclic ring system of cholesterol (Huster et al., 2005; Scheidt et al., 2003; Xu and London, 2000) as well as by the length of the *iso*-branched side chain of cholesterol (Scheidt et al., 2013). To assess the ability of sterols with an unbranched alkyl side chain of varying length on the POPC order parameter, we analyzed the ^2H NMR spectra of the respective samples to calculate the smoothed order parameters profiles shown in Fig. 2. In comparison to the order parameter plot of pure POPC- d_{31} , all sterol analogs cause an increase in the phospholipid chain order. However, there is a pronounced influence of the side chain length of the sterol analogs on the POPC order parameters. With the exception of the *n*-C14 cholesterol analog, which exhibits the lowest order parameters, all of the sterol analogs induced a lower order increase than native cholesterol but higher than androstenol. The *n*-C12 sterol analog gave order parameters that were closest to that of native cholesterol. However, in the presence of the *n*-C12 cholesterol analog the order parameters of the upper half of the *sn*-1 chain of POPC were slightly smaller, and the order parameter for the lower half slightly were higher than for native cholesterol.

Figure 2. ^2H NMR chain order parameter plots of POPC- d_{31} membranes containing 20 mol% of the sterol variants. For comparison, the order parameter plots of pure POPC- d_{31} and of POPC- d_{31} in the presence of 20 mol% androstenol and cholesterol are shown. Measurements were carried out 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.

The individual effect of each sterol analog on the POPC order parameter is best compared in a plot of the average chain order parameters as shown in Fig. 3A. Compared to the effect of androstenol, which lacks an alkyl side chain, the average chain order parameters of POPC gradually increase up to a side chain length of 12 carbons, which induces a similar increase of the mean order parameter as native cholesterol. A further increase of the sterol side chain length by one or two additional methylene groups (*n*-C13 and *n*-C14, respectively) leads to a very drastic decrease in lipid chain order parameters, which for *n*-C14 are even lower than that of androstenol. In DPPC-*d*₆₂ membranes similar results for the chain order parameters were achieved (Fig. 3B). The maximal chain order parameter value seems to be shifted to *n*-C10 cholesterol analog and the differences from *n*-C7 to *n*-C13 are smaller than in POPC membranes, but all these changes are very small and within the error of measurement.

These results are mirrored in the calculation of the lipid chain extent of the phospholipid hydrocarbon chains according to the mean torque model (Petrache et al., 1999; Petrache et al., 2000), which are listed in Table 1. For POPC membranes the same hydrocarbon chain length like in the presence of cholesterol is achieved for the *n*-C12 sterol analog.

Figure 3. Mean chain order parameters of (A) POPC- d_{31} membranes at 30°C and (B) DPPC- d_{62} membranes at 45°C containing 20 mol% of the sterol variants. For comparison, mean chain order parameters of pure phospholipids and of the lipid membranes in the presence of 20 mol% androstenol and cholesterol are shown. The bars start at a mean order parameter of 0.15. The error bars represent the error of measurements and errors of the molecular concentrations during sample preparation.

Table 1: Calculated chain extent (L_c^*) for the deuterated hydrocarbon chains of POPC- d_{31} at 30°C and DPPC- d_{62} at 45°C in the presence 20 mol% of the respective sterol.

$L_c^* / \text{\AA}$	POPC- d_{31}	DPPC- d_{62}
pure phospholipid	11.7	12.1
Cholesterol	13.2	14.2
Androstenol	12.3	13.4
n-C3	12.7	13.6
n-C4	12.9	13.5
n-C5	12.9	13.7
n-C7	13.0	14.2
n-C10	13.0	14.2
n-C12	13.2	14.1
n-C13	12.7	14.0
n-C14	12.1	12.5

3.2. Influence of the sterol's unbranched side chain length on membrane diffusion

Next, the influence of the sterol analogs with varying unbranched side chain lengths on the lateral membrane diffusion was investigated by using ^1H PFG MAS NMR. For this measurement, the sterols were incorporated into DPPC- d_{62} lipid bilayers. The spectral resolution and the fact that individual phospholipid and sterol analog signals can be separated allows the determination of the diffusion coefficients for both molecules simultaneously (Scheidt et al., 2005). The deuteration of only the hydrocarbon chains of DPPC- d_{62} allows the detection of the ^1H NMR signals of the sterol molecules in the region around 1 ppm. We used the signal at 0.8 ppm from the CH_3 -group at the C18 position of the sterols for the analysis of the sterol diffusion. On the other hand, the signal of the still protonated choline headgroup of DPPC- d_{62} at 3.2 ppm was used for the analysis of the phospholipid diffusion. ^1H NMR spectra of these membrane preparations are shown in Fig. 4.

Figure 4: 600 MHz ^1H MAS NMR spectra of pure DPPC- d_{62} bilayer membranes and of DPPC- d_{62} bilayer membranes in the presence of 50 mol% of the respective sterol. The signals used for the analysis of the diffusion of the sterols and DPPC- d_{62} are marked by arrows. ^1H MAS NMR measurements were carried out at 45°C.

The corrected diffusion coefficients determined from diffusion time-dependent measurements (Gaede and Gawrisch, 2003) are shown in Fig. 5. With the exception of the *n*-C10 sterol, where the phospholipid exhibited a lower diffusion coefficient than the sterol, DPPC- d_{62} and sterol analogs showed similar diffusion coefficients. For all measurements, the diffusion coefficients are smaller than those of pure DPPC- d_{62} and of DPPC- d_{62} in the presence of androstenol. Within experimental error, most of the diffusion coefficients determined for DPPC and for the respective sterol analog are similar to those for DPPC in the presence of native cholesterol. Fig. 5 also shows the following trend in the diffusion coefficients: the values decrease with increasing chain length up to *n*-C7, and increase with

a further increase of the chain length. Notably, for *n*-C14, which exhibits a very low lipid molecular packing effect, lower diffusion coefficients were measured.

Figure 5: Diffusion coefficients of DPPC-*d*₆₂ (open bars) and the respective sterol (filled bars) in lipid membranes containing 50 mol% of the respective sterol. For comparison, the data for pure DPPC-*d*₆₂ and in the presence of 50 mol% androstenol and cholesterol are shown. The error bars represent the errors of the fit of the data to the used diffusion models.

3.3. Influence of the sterol's unbranched side chain length on membrane permeability

For determining the influence of the sterols on membrane permeability, the permeation of dithionite ion across LUV bilayers was measured (Scheidt et al., 2013). The rate constants for dithionite ion permeation in the absence and in the presence of the respective sterols are shown in Fig. 6. A comparison of POPC vesicles in the absence and in the presence of cholesterol analogs reveals that the sterol caused the well-known decrease of permeability toward polar molecules. Androstenol caused an increase in the rate of dithionite permeation. For most of the *n*-Cx sterol analogs, no change or only a small decrease in dithionite permeation rate compared with that of pure POPC membranes was measured. For *n*-C13, a higher permeation rate was determined, which was similar to that of androstenol. Only for *n*-C7 a very low dithionite ion permeation rate was determined, which was even lower than that in the presence of cholesterol.

Figure 6: Rate constants for the permeation of dithionite ion across lipid membranes composed of POPC and the respective sterol (20 mol%). The rate constants were determined from the kinetics of the dithionite-mediated reduction of NBD-PE additionally localized in the LUV membrane at 37°C (see Materials and Methods). Data represent the mean $\pm$ standard error of mean (3 measurements).

4. Discussion

Sterols are a major component of eukaryotic cell membranes and play an important role in the regulation of the physico-chemical membrane properties. The sterol content of cellular membranes is used to regulate the membrane properties to meet their specific requirements. In particular, the degrees of phospholipid chain unsaturation and sterol content vary between the membranes from various cell species and organelles. It is assumed that evolution has adjusted the molecular structure of cholesterol to optimize its molecular interactions with other membrane components (Bittman, 1997). In particular, cholesterol's molecular architecture and side chain structure is optimal for the interaction with saturated lipid chains with a length of 16 to 18 carbons (Huster et al., 1998; Bunge et al., 2008; Tsamaloukas et al., 2006). A number of studies have shown that even minor changes in the molecular structure of cholesterol significantly modify/alter the interactions between cholesterol and other membrane constituents, and, therefore, influence the membrane properties (Demel et al., 1972; Xu and London, 2000; Wang et al., 2004; Scheidt et al., 2003; Huster et al., 2005; Endress et al., 2002; Milles et al., 2013; Shrivastava et al., 2008; Benesch and McElhaney, 2014). For a long time, research has focused on the role of the sterol's tetracyclic ring system since it was believed that the ring structure mainly determines the decisive influence of cholesterol on membrane properties. The *iso*-octyl

side chain of cholesterol was assumed to be of minor importance for the interactions of cholesterol with membrane lipids. For example, it was shown that desmosterol, which differs from cholesterol by having a double bond at C24, induces identical lipid ordering as native cholesterol (Huster et al., 2005).

However, we recently investigated the role of cholesterol's *iso*-branched side chain systematically and showed that the length of the side chain has a significant influence on various important membrane properties (Scheidt et al., 2013). For instance, the *i*-C8 side chain of native cholesterol accounts for ~60% of the increased molecular packing of the phospholipid molecules, while androstenol induces only 40% of the ordering effect of native cholesterol. Here, we show that the unbranched alkyl chain in the *n*-series of cholesterol analogs also causes an increased molecular packing, which increases with increasing length of the side chain (Figs. 2 and 3). However, to achieve a similar effect as native cholesterol on membrane properties, a longer chain (*n*-C12) is required. Interestingly, compared to native cholesterol, the lipid order parameters in the presence of *n*-C12 are somewhat lower in the upper membrane region (carbons 2-8 of the phospholipid fatty acyl chains) and increased in the lower membrane region (carbons 10-15). This suggests that there are significant differences in the influence to the membrane packing between a shorter branched side chain and a longer unbranched sterol side chain, since the latter would reach more deeply into the phospholipid bilayer. Presumably, the unbranched *n*-C12 side chain is less ordered than the *i*-C8 chain of native cholesterol. Obviously, the two methyl groups at the side chain terminus of cholesterol occupy more space, which has to be compensated by an increased alkyl chain length. Also, the van der Waals interactions between the fatty acyl chains of the phospholipids and the sterol will be influenced by these two methyl groups. However, a cholesterol analog with an *i*-C12 side chain induces much less lipid condensation than native cholesterol; only *i*-C10 shows a slightly higher ordering effect than native cholesterol (Scheidt et al., 2013). Clearly, a side chain that is too long fails to produce a cholesterol-like effect irrespective of its chemical nature. In accordance with this, the order parameters for *n*-C13 and *n*-C14 decrease, and for *n*-C14 even to values below that for androstenol. Similarly, low order parameters have been observed for *i*-C12 and *i*-C14 (Scheidt et al., 2013).

Interestingly, the dependence of the impact of *n*-Cx sterol side chain length on lipid chain order is not similarly reflected in the lipid diffusion or permeability data, as was observed for *iso*-branched cholesterol analogs (Scheidt et al., 2013). The lateral diffusion coefficients are, for most analogs, similar to that of native cholesterol with only a slight dependence on the chain length. The lowest diffusion coefficients are measured in the presence of *n*-C7 whereas the highest chain order was found for *n*-C12. For sterols with a longer alkyl chain than *n*-C7, the diffusion coefficients increase up to *n*-C13. The quite low diffusion rates in the presence of *n*-C14 may be unexpected (even this is the sterol with the largest molecular weight in this study), since *n*-C14 induces only order parameters clearly lower than for all the other investigated sterols. It is conceivable that the long *n*-C14 chain may hinder the lateral diffusion of this molecule in the membrane.

With regard to the influence of *n*-Cx sterols on membrane permeability, most of the sterols caused no change or only a small decrease in the dithionite ion permeation rates compared with that of pure POPC bilayers. Only the *n*-C7 sterol caused a significantly lower permeation rate, which was even smaller than that of native cholesterol. Notably, we found the lowest lipid diffusion coefficient in the presence of *n*-C7 and, besides *n*-C12, the largest

order parameter. Thus our data suggest that *n*-C7 is the straight-chain sterol that best emulates endogenous cholesterol with regard to its impact on membranes permeability. However, endogenous cholesterol, with its *iso*-branched side chain, seems to be optimally evolved for realizing its unique membrane properties.

5. Conclusion

In summary, we show that sterols bearing an unbranched alkyl side chain of varying length influence important properties of lipid membranes. However, the extent of this impact is lower compared with that measured for the respective branched *iso*-side chain sterols (Scheidt et al., 2013). It has to be assumed that a sterol bearing a branched *iso*-alkyl chains with its two terminal methyl groups exhibits significantly different cholesterol-phospholipid-interactions than a synthetic sterol having an unbranched aliphatic chain, which lead to larger changes in the membrane properties.

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