Cholesterol

DOI: 10.1002/anie.201306753

Cholesterol's Aliphatic Side Chain Modulates Membrane Properties**

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Dedicated to Professor Jörg Kärger on the occasion of his 70th birthday

The composition and chemical structures of membrane-forming molecules determine all essential properties of nature's most important interface. Cellular membranes are subject to a hierarchical domain organization on the mesoscopic length scale.[1] This is triggered by preferential interactions between the major constituents of the membrane, for example, phospholipids, cholesterol (Chol), glycolipids, and membrane proteins.[2] In particular, the attractive interactions between Chol and sphingolipids or saturated phospholipids and/or membrane proteins have been identified as the driving force for membrane domain formation.[3] Key to the lipid phase separation phenomena is the preferential interaction of Chol with saturated lipid chains.[4] Numerous examples of the specific interaction between Chol and saturated phospholipids or sphingomyelin have been reported.[5] The interaction between Chol and saturated lipid chains is thought to originate from attractive van der Waals interactions between Chol's planar, fused ring system (particularly the smooth α-face) and the saturated fatty acyl chains.[6] This leads to a stiffening of the lipid chains, as indicated by an increase in order, which is referred to as area condensation.[7] This increased packing density decreases membrane permeability.[8] Surprisingly, the influence of the aliphatic Chol side chain on lipid condensation and domain formation in membranes is still not fully understood. Therefore, we have systematically studied the influence of the methyl-branched side chain (iso series) of Chol with 5 to 14 carbon atoms[9] (Figure 1 A) on important membrane properties.

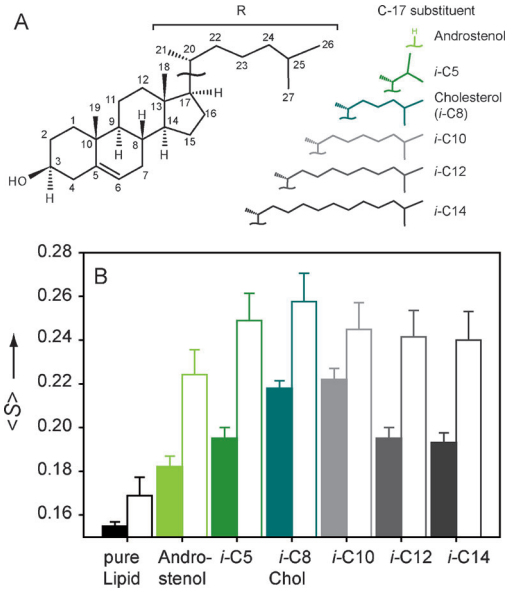

Figure 1. A) Structures of Chol and the sterol variants investigated in this study featuring different lengths of the branched side chain. B) Mean order parameter ⟨*S*⟩ of [D_{31}]POPC membranes (colored bars) and [D_{62}]DPPC membranes (white bars) in the presence of various sterol variants (20 mol%).

To evaluate the influence of the Chol side chain on the area condensation effect, ^{2}H NMR order parameters[4] were measured for mixtures of either fully saturated [D_{62}]DPPC or mono-unsaturated [D_{31}]POPC with 20 mol% of the respective sterol analogue (DPPC = dipalmitoylphosphatidylcholine; POPC = 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine). ^{2}H NMR spectra and order parameter plots are shown in Figures S1–S4 in the Supporting Information. Pure lipid membranes are used as a reference and feature the lowest chain order. DPPC, which is saturated, shows higher order parameters than the mono-unsaturated POPC. All sterols studied induce an increase in lipid chain order, that is, condensation, but the degree of condensation varies as a function of the length of the branched side chain. These differences are best displayed in the plot of the average order parameters ⟨*S*⟩ of the phospholipid in the presence of the sterols having a side chain of varying length (Figure 1 B). There is a gradual increase in the POPC chain order from androst-5-en-3-β-ol (androstenol), which lacks a side chain, to *i*-C5-sterol, native Chol, and *i*-C10-sterol, while the longer chain *i*-C12- and *i*-C14-sterol analogues lead to decreasing condensation again. Interestingly, the sterol ring system of Chol is responsible for only about 40% of the lipid

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[**] This work was supported in part by the NIH (grant HL-083187 to R.B.) and by the Deutsche Forschungsgemeinschaft (DFG; SFB 1052, grant B6 to D.H.).
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201306753.

condensation demonstrated for androstenol, while the *i*-C8 side chain of Chol accounts for roughly 60% of the condensation. Furthermore, it is remarkable that native Chol induces a slightly lower (6%) lipid condensation than *i*-C10-sterol.

For DPPC mixtures, the highest chain order is observed in the presence of Chol, and the attenuation of the ordering effect induced by the longer side-chain sterol analogues is somewhat attenuated. In contrast to the mono-unsaturated mixtures, the *i*-C8 side chain of Chol only accounts for about 40% of the condensation in DPPC.

Changes in lipid chain order mostly affect the upper chain part, where an increase in order between 40% (POPC) and 50% (DPPC) due to Chol is observed. This can be evaluated from the difference order parameter plots shown in Figures S2 and S4. The increase in the molecular order of the lower half of the chain depends greatly on the length of the side chain of the sterol analogue and is optimal only for cholesterol and *i*-C10-sterol. Order increase leads to an increase in lipid chain length in the presence of each sterol analogue, which is reported in Table S1.

Next, we investigated the influence of the sterol molecules on basic lipid membrane properties. First, the main phase transition temperature of DPPC in the presence of each sterol analogue was determined by differential scanning calorimetry (Figures S5 and S6). In the presence of 20 mol% Chol, the phase transition temperature of DPPC increases from 40.9°C to 41.5°C. In the presence of the sterol analogues, the phase transition temperature decreased to well below 40°C for all sterols and for androstenol it dropped to 30.5°C (Figure S6).

Furthermore, the lateral diffusion of DPPC and the sterols was measured using ^{1}H MAS PFG NMR spectroscopy (MAS = magic angle spinning, PFG = pulsed field gradient; Figure S7).[10] While lipid diffusion rates can also be measured by fluorescence correlation spectroscopy,[11] the NMR technique measures the diffusion rates of both phospholipid and the sterol in the same mixture without the need for fluorescence labels that may influence lipid diffusion rates. Diffusion coefficients determined for DPPC and the sterols are shown in Figure 2. Within experimental error, DPPC and the various sterol molecules show approximately the same diffusion coefficients (colored bars represent DPPC, white bars the sterol). The diffusion coefficients of *i*-C5-, *i*-C8-, and *i*-C10-sterols are rather similar and so are the diffusion coefficients for DPPC in the presence of these sterols. However, much larger diffusion coefficients are found for androstenol as well as for *i*-C12- and *i*-C14-sterols; these coefficients are even larger than that for DPPC in the absence of Chol.

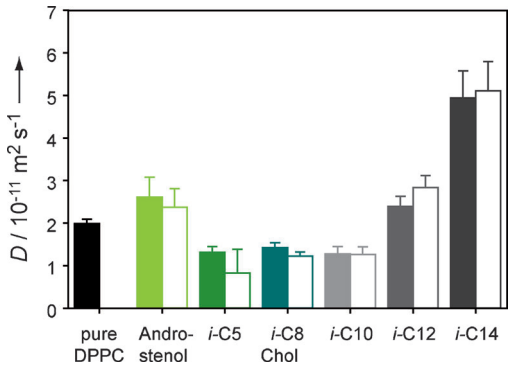

Figure 2. Lateral diffusion coefficients *D* in DPPC bilayers in mixture with the sterol analogues. Diffusion coefficients are reported for DPPC (colored bars) and the respective sterol (white bars) in the mixture at a molar ratio of 50:50 at 45°C.

We also determined the diffusion coefficients of POPC in mixtures with the sterol analogues. Since POPC is not available in the chain-perdeuterated form, signal superposition in the aliphatic region prevented the determination of the diffusion of the sterol and only POPC diffusion coefficients are reported in Figure S8. POPC showed the largest diffusion coefficients. In the presence of androstenol and *i*-C5- and *i*-C8-sterol, POPC diffusion is slower with relatively similar diffusion coefficients, while the diffusion coefficients of POPC in the presence of *i*-C10-, *i*-C12-, and *i*-C14-sterol decreased again significantly.

We also assessed the permeability of POPC and DPPC membranes in the presence of the respective sterols. A simple fluorescence assay was used that detects the permeation of dithionite ion across membranes by measuring the reduction of NBD-PE fluorescence in lipid vesicles (NBD-PE = N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl phosphatidylethanolamine)).[12] The reduction kinetics showed two components: a very fast decay due to the reduction of the NBD-PE in the outer leaflet of the bilayer and a slower decay, which reflects the reduction of the NBD-PE in the inner leaflet upon permeation of dithionite ion (Figure S9). Figure S10 shows the rate constants for dithionite ion permeation across the POPC and DPPC bilayers, which are obtained by fitting the curves to a biexponential equation. DPPC shows highest permeability for dithionite ion, at a temperature which is relatively close to the phase transition temperature, where the permeability is generally high.[13] Permeability is lower in the presence of all sterol analogues; the lowest permeation rate is measured with Chol, indicating that its structure is optimal for decreasing the membrane permeability. In the presence of a sterol with a longer or shorter branched side chain or with androstenol, as well as in the absence of any sterol, these permeation rates are clearly higher.

Finally, we studied the potential of sterols with different side-chain lengths to induce phase separation and the formation of liquid ordered (l_o) and liquid disordered (l_d) domains in mixtures of DOPC, *N*-stearoylsphingomyelin (SSM), and the respective sterol. Figure S11 shows confocal fluorescence images[14] of giant unilamellar vesicles (GUVs) composed of DOPC, SSM, and the respective sterol molecule at a 1:1:1 molar ratio. All sterols induced the formation of large lipid domains irrespective of the length of the side chain, as reported by the fluorescence of N-Rh-DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl), a well-established marker for the l_d phase of GUVs made of lipid raft mixtures. Therefore, this phase appears in red in the images, while the l_o phase, which is depleted of N-Rh-DOPE, appears in dark.[15]

Although all sterols induced phase separation, the partitioning of the fluorophore varies as a function of the length of

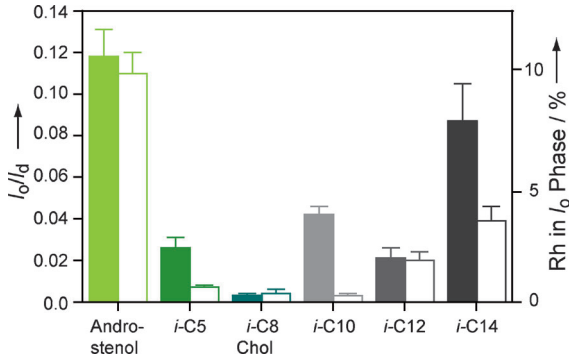

Figure 3. Ratio of the average fluorescence intensity of N-Rh-DOPE (colored bars) and the Rh-labeled transmembrane peptide (TMD, white bars) in the l_o domain and the l_d domain of the canonical raft mixture of DOPC/SSM/Chol or sterol variants (1:1:1 molar ratio). Error bars represent the standard error of the mean. Between 27 and 102 GUVs were analyzed per raft mixture.

the sterol side chain. To quantify this distribution ratio, we calculated the intensity ratio of N-Rh-DOPE in l_o and l_d phases[16] (Figure 3, filled bars). Almost exclusive partitioning of N-Rh-DOPE into the l_d domain was found for raft mixtures composed of native Chol (0.2% in the l_o phase), while partitioning was less pronounced for the *i*-C5- and *i*-C12-sterols (2.5% and 2.1% in the l_o phase, respectively). For *i*-C10-sterol, the l_o domain partition increases again to 4% and is highest in raft mixtures in the presence of *i*-C14-sterol (8%) and androstenol (10.6%). Interestingly, the average domains covered approximately one-half of the line shown at the particular cut made at the equator of the vesicle for all Chol analogues except for GUVs containing androstenol. Here the l_o domain is only about 20% of the GUV. The only literature data available on sterols with longer or shorter side chains report that *i*-C4-sterol fails and *i*-C10-sterol promotes domain formation.[17]

Finally, a rhodamine-labeled peptide (Rh-TMD), representing the transmembrane domain of Influenza A virus hemagglutinin, was incorporated at 1 mol% into the GUVs of the same mixtures.[18] The l_o/l_d distribution of the peptide is shown in Figure 3 (white bars). In the ternary mixtures composed of native Chol and *i*-C5- or *i*-C10-sterol, Rh-TMD partitioned almost exclusively into the l_d domains (i.e., only 0.4%, 0.7%, and 0.3% of the peptide were found in the l_o domain, respectively). In contrast, in mixtures containing *i*-C12- to *i*-C14-sterol, the amount of Rh-TMD found in the l_o domain increased to 2.6% and 3.7%, respectively. The partition equilibrium of the peptide was severely disturbed for mixtures containing androstenol, where 10% of the peptide was found in the l_o domain, which is 25 times more than in the l_o domain of GUVs prepared with Chol.

Sterols are typical lipids of eukaryotic cells. While Chol represents the sterol in the plasma membranes of all animal cells, a number of different phytosterols are representative for plants, and ergosterol is the sterol in fungi and yeast. All these molecules feature a side chain at C-17 that consists of 20 to 27 carbon atoms (see Figure 1A). Phytosterols often have a methyl or ethyl branch at C-24, and the number and position of double bonds in the side chain varies. However, the overall length of the branched side chain remains identical in the different sterols. In contrast to these differences in the sterols, the fatty acid composition of the cell membranes of plants, fungi, yeasts, and animals is rather similar. Typically, the lipids in most of these membranes (mostly phospho- and sphingolipids, but also glycolipids) contain 80–90% lipid chains of 16 or 18 carbons that are either saturated or contain one to three *cis* double bonds.[19] Thus the majority of cellular membranes have a thickness that matches well with the molecular dimensions of a sterol with a branched side chain consisting of eight carbon atoms.

Several studies have demonstrated that even small changes in Chol's tetracyclic structure can have a profound impact on the lipid condensation effect[6b,20] and membrane domain formation.[17,21] However, contrary to the assumption that the interaction of Chol with membrane phospholipids is based on interactions between the tetracyclic ring system of the former and the lipid chains of the latter molecules, we found that a major part of the condensation effect and the lateral organization of the lipids in raft mixtures can be attributed to the Chol side chain. The side chain of Chol contributes roughly 60% of the condensation in POPC membranes and about 40% in DPPC membranes. This suggests that the Chol side chain is crucial, especially for the interaction with unsaturated phospholipids, which represent the majority in biological membranes. Also, the lateral lipid diffusion and membrane permeability appears to be optimized for Chol with its *i*-C8 side chain. Our biophysical data suggest that the Chol side chain has been fine-tuned in evolution for an optimal interaction with phospholipids in lipid bilayers.

Sterols were introduced at a relatively late stage in evoluation because of the lack of molecular oxygen for biosynthesis.[8a] The evolutionary advantage of adding Chol to eukaryotic cellular membranes was to modulate and refine membrane properties. In particular, the barrier function of the membrane is markedly improved.[22] In that regard, it was not sufficient to develop a sterol lacking a side chain such as androstenol. As our study shows, only 40% of the total ordering of mono-unsaturated phospholipid membranes (and concomitant denser packing) is induced by the rigid tetracyclic carbon skeleton, and an important contribution comes from the aliphatic side chain. The additional van der Waals attraction between the Chol side chain and the lower part of the lipid chains leads to an optimal increase in packing density of the lipids and thus the improved barrier function of the membrane (Figure S2). Membrane permeation is most restricted for native Chol, and even small alterations in the side-chain length lead to a significantly impaired barrier function. Although *i*-C10-sterol induced a higher lipid condensation in POPC and an increased lipid chain ordering particularly in the lower half of the chain (Figure S2), the membrane permeation for dithionite ion is much larger for the sterol with the longer side chain.

The basis for the length of the Chol side chain is set in the biosynthesis of the molecule from dimethylallyl pyrophosphate and isopentenyl pyrophosphate in the isoprenoid metabolic pathway yielding squalene.[23] This is the precursor for Chol and phytosterols and explains the general similarity

between these molecules and in particular their aliphatic side chains. From squalene, lanosterol evolved, which features a relatively rough surface due to three additional methyl groups on the tetracyclic ring system (4-α-CH_3, 4-β-CH_3, and 14-CH_3), not present in Chol. This suggests that in the last steps of Chol evolution, the smoothness of the α-face was optimized so that the sterol-induced ordering of the lipid chains was increased: in the presence of 20 mol% lanosterol, the average chain order of POPC membranes is 0.202, while in the presence of 20 mol% Chol it is 0.206.[24] This final evolutionary step resulted in a 2% increase in lipid chain order. However, as our study on the length of the Chol side chain suggests, a very important evolutionary step was the development of the general architecture of the sterol structure featuring the branched octyl side chain of the molecule.

In summary, our work emphasizes the crucial role of the Chol side chain in modifying a number of basic membrane properties as well as the lateral organization of the membrane lipids and proteins. By a systematic analysis of sterols with varying branched aliphatic chains, we found that the side chain of Chol has a profound impact on all of these properties. Typically, the characteristic properties of Chol cannot be reproduced by molecules harboring a longer or shorter side chain. This highlights the importance of the allegedly disordered Chol side chain for intermolecular interactions and membrane properties with significant consequences for the cell-biological role of the sterol.

Received: August 1, 2013

Keywords: diffusion · domain formation · lipids · membranes · permeability

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