Cellular and Molecular Life Sciences (2018) 75:2137–2151
https://doi.org/10.1007/s00018-018-2789-9

REVIEW

Challenges and approaches to understand cholesterol-binding impact on membrane protein function: an NMR view

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Received: 23 October 2017 / Revised: 16 February 2018 / Accepted: 27 February 2018 / Published online: 8 March 2018
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Abstract

Experimental evidence for a direct role of lipids in determining the structure, dynamics, and function of membrane proteins leads to the term 'functional lipids'. In particular, the sterol molecule cholesterol modulates the activity of many membrane proteins. The precise nature of cholesterol-binding sites and the consequences of modulation of local membrane microviscosity by cholesterol, however, is often unknown. Here, we review the current knowledge of the interaction of cholesterol with transmembrane proteins, with a special focus on structural aspects of the interaction derived from nuclear magnetic resonance approaches. We highlight examples of the importance of cholesterol modulation of membrane protein function, discuss the specificity of cholesterol binding, and review the proposed binding motifs from a molecular perspective. We conclude with a short perspective on what could be future trends in research efforts targeted towards a better understanding of cholesterol/membrane protein interactions.

Keywords Membrane protein · Lipid · Cholesterol · Function · NMR spectroscopy · Structure

Introduction: transmembrane proteins and their functional lipids

The biological membrane plays a vital role in a variety of biological processes, including transport, cellular recognition, adhesion, energy production, and signaling cascades. The membrane properties, which influence these processes, are a result of a complex interplay between the protein and the lipid components [1]. While it is nowadays well accepted that lipid–protein interactions are essential for the aforementioned cellular processes, only little is known about their physicochemical nature as well as their actual role in these processes [1, 2]. This is because lipids and proteins can influence one another in multiple ways [3–5]. Membrane proteins influence the structure and dynamics of lipids, such as acyl chain order, membrane thickness and elasticity, permeability, lipid-domain formation, lipid headgroup orientation, and acyl chain dynamics [6]. In addition, in turn, the physicochemical nature of the membrane profoundly impacts the structure and dynamics of membrane-embedded proteins and peptides [7], and thereby modulates their function [6, 8, 9].

According to the fluid mosaic model, cellular membranes were originally thought to primarily serve as solvents for membrane proteins [10]. This view changed when the functional raft hypothesis was developed [11], and was further strengthened through the discovery of lipid molecules that act as secondary messengers [12, 13]. Nowadays, there is strong evidence that lipids play a direct role in determining the structure, dynamics, and function of membrane proteins [14], leading to the formulation of the term "functional lipids".

The activity of a variety of ion channels, including the members of all major ion channel families, is affected by changes in membrane cholesterol levels [15]. Consistent with a functional importance of cholesterol and other lipids, crystal structures of ion channels and different membrane

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protein classes revealed bound lipids [16]. For example, nonan-1-ol and diacylglycerol molecules were observed in complex with the K^+ channel KcsA [17]. In addition, phosphatidylglycerol and cardiolipin influence oligomerization of the voltage-dependent anion channel (VDAC) [18], which is the major ATP gate in the outer mitochondrial membrane and plays an important role in apoptosis [19]. The membrane-lipid composition also modulates the interaction of VDAC with tubulin [20], as well as the gating properties of the VDAC channel [21].

A very important lipid is cholesterol, because it influences the function of many membrane proteins. Robust and accurate experimental information on the precise nature of cholesterol-binding sites in membrane protein structures, however, is often lacking. Molecular flexibility of both protein structures and cholesterol molecules adds to the complexity of studying the interaction of cholesterol with transmembrane proteins. In addition, technical difficulties have to be overcome, such as the insolubility of cholesterol in polar environments.

Cholesterol–protein interaction motifs

X-ray crystallography [22, 23], electron spin resonance [24–27], cryo-electron microscopy [28–30], binding assays employing site-directed mutagenesis [31–33], docking and molecular dynamics simulations [34–36], and nuclear magnetic resonance (NMR) [37] spectroscopy have been used to characterize the interaction of cholesterol with transmembrane proteins (Fig. 1). Based on these studies, different cholesterol-binding motifs in transmembrane proteins have been proposed. These include the cholesterol recognition amino acid consensus (CRAC) motif and its inverted variant, the CARC motif, the cholesterol consensus motif (CCM) motif, and so-called "tilted peptides" [38, 39]. In addition, other specific or non-universal motifs were suggested [26, 30, 34, 39–41]. The interaction of cholesterol with soluble proteins has also been studied in detail and was summarized in a recent review based on the analysis of crystal structures of proteins containing cholesterol molecules [42]. The analysis showed that the hydroxyl group of cholesterol preferentially interacts with Asn, Gln, and/or Tyr residues, whereas the hydrophobic part of cholesterol makes contacts with Leu, Ile, Val, and Phe residues. Moreover, the hydroxyl group likes to form hydrogen bonds with residues in protein α-helices, while cholesterol's hydrophobic core often interacts with residues in β-strands or regions that do not fold into regular secondary structure [42]. As we summarize below, there appear to be commonalities with respect to protein/cholesterol interactions between soluble and transmembrane proteins, that is similar residue types and protein secondary structure elements contribute to binding to the polar and hydrophobic parts of the cholesterol molecule.

An often discussed cholesterol-binding motif is CRAC [32, 38, 39], which follows the sequence composition (from N- to C-terminus): apolar leucine or valine residue, followed by one to five residues of any type, then a mandatory aromatic residue (tyrosine), followed by another segment of one to five residues, and then "capped" by a final basic lysine or arginine residue, i.e., (L/V)-*X1–5*-(Y)-*X1–5*-(K/R). Since cholesterol does not display any structural and chemical variations, the looseness in the definition of the CRAC motif is surprising and raised some skepticism about its predictive value [43, 44]. Sequence analysis identified CRAC motifs in a number of transmembrane proteins, which are known to bind cholesterol, including G-protein-coupled receptors [45–47] and the ion-channel large-conductance Ca^{2+}-sensitive voltage-gated K^+ channels (BK), nicotinic acetylcholine receptor (nAChR), and Kir2.1 [39], as well as the translocator protein TSPO [32, 48]. Consistent with the importance of the CRAC motif, single mutations within this sequence motif, such as substitution of the central tyrosine residue, attenuated cholesterol binding [44, 49, 50]. In addition, it was suggested that binding of cholesterol to the CRAC motif is energetically favorable if the motif is

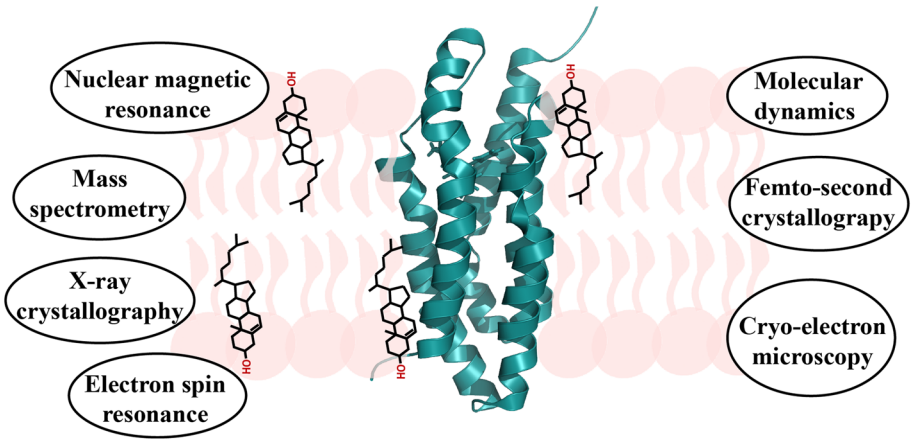

Fig. 1 Illustration of the complex interplay of cholesterol (black) with membrane environment (pink) and proteins (green). Selected techniques used to study protein–lipid interactions are listed

located within the transmembrane region, whereas binding to CRAC motifs outside of the membrane would be energetically unfavorable [38]. For example, measurements of cholesterol-dependent channel activity in combination with computational studies suggested that the membrane-adjacent CRAC motif, V444—Y450—K453, from a total of seven CRAC motifs that are present in the cytosolic domain of the BK channel, contributes to the sensitivity of the channel to cholesterol [51]. In addition, CRAC motifs of the ion channels nAChR and Kir2.1, which are located outside of the membrane bilayer, were suggested to be energetically less favorable for cholesterol binding [41, 51]. Experimental confirmation of cholesterol binding to CRAC motifs of transmembrane proteins was, however, obtained in only a few cases [31].

Very similar to the CRAC motif is the sequence motif called CARC. CARC represents an "inverted CRAC" motif with the amino acid composition (K/R)-*X1–5*-(Y/F)-*X1–5*-(L/V) [52]. The definition of CARC is even less restrictive than that of CRAC by including either tyrosine or phenylalanine in the central position. Due to the "snorkeling" effect [53], which is attributed to the burial of the side-chain of lysine (or arginine) in the hydrophobic part of the membrane and emergence of its cationic group at the membrane surface [52], the presence of the basic residue allows location of the CARC motif at the polar–apolar interface of a transmembrane domain. CARC motifs are present in the transmembrane domains of transient receptor potential vanilloid 1 channels (TRPV1) [31], nAChR [38], and Kir2.1 [41]. In TRPV1, the sequence motif R579—F582—L585 is located in transmembrane helix 5. Mutations of these three characteristic CARC residues affected the sensitivity of TRPV1 to cholesterol in measurements of capsaicin-induced currents [31]. AChR has sequence stretches, which fit to the CARC definition, in transmembrane helices 1, 3 and 4 [38]. Kir2.1 contains CARC motifs in both the cytosolic and transmembrane domain, with two of them located at the interface between the transmembrane and cytosolic region [41]. Substitution of V77 by isoleucine in the CARC motif R67—F73—V77 abolished Kir2.1's sensitivity towards cholesterol, while mutation of R67 and F73 resulted in non-functional channels. Conversely, mutation of all three residues within a second potential CARC motif, R82—F88—L90, did not affect the cholesterol-sensitivity of Kir2.1 [41]. Experimental structural data for cholesterol binding to the proposed CARC sequence motif are currently lacking.

The third cholesterol-binding motif, the so-called cholesterol consensus motif (CCM), was proposed on the basis of the crystal structure of cholesterol bound to the β_2-adrenergic receptor [22]. The CCM motif is predominantly found in GPCRs (Fig. 2) [22]. Unlike the proposed CRAC and CARC motifs, which include residues from one continuous segment of the protein, the CCM is a three-dimensional,

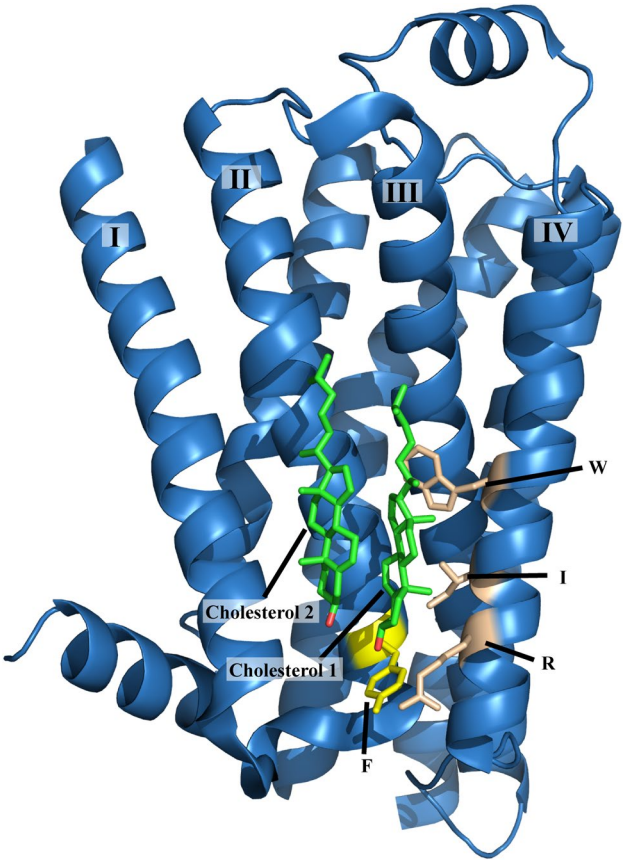

Fig. 2 Cholesterol-binding motif (CCM) of the β_2-adrenergic receptor (PDB code: 3D4S). Phenylalanine from one helix, and arginine, isoleucine, and tryptophan from another transmembrane helix contribute to cholesterol (green) binding

experimentally validated binding motif that includes residues from adjacent helices, i.e., (W/Y)-(I/V/L)-(K/R) on one helix, and (F/Y/R) on a second helix. Notably, the residue types contributing to the CCM are similar to those in the CRAC and CARC motifs.

Peptides that insert in membranes or snorkel at the membrane surface have also been suggested to interact with cholesterol. Characteristic features of these peptides are their folding into helical structure upon membrane interaction and perturbation of membrane organization [38]. The distribution of hydrophobic residues within these proposed cholesterol-binding peptides is asymmetric and induces a 45° tilt with respect to the membrane plane [54]. One potential example of a tilted peptide is the Alzheimer's β-amyloid peptide, of which residues ^{22}EDVGSNKGAIIGLM^{35} bind with high affinity to cholesterol [55]. Peptide sequences with asymmetric distributions of hydrophobic residues are also present in viral fusion proteins, which require cholesterol for membrane insertion [56], suggesting that tilted peptides evolved to acquire cholesterol-binding properties to facilitate biological functions [38]. Indirect support for tilted peptide

cholesterol-binding sites was furthermore provided by lipid-mixing assays and leakage of T-cell-like liposomes, and was consistent with molecular modeling predictions [57]. In addition, the protein α-synuclein, which plays an important role in Parkinson's disease, potentially through formation of oligomeric pores in neuronal membranes [58–60], has been discussed in the context of tilted peptides. An isolated peptide corresponding to the α-synuclein sequence ^{67}GGAVVTGVTAVA^{78} is toxic for cultured neurons [56] and has been suggested to bind cholesterol in a tilted orientation [61].

Besides the four cholesterol-binding motifs discussed above, transmembrane proteins might have developed other ways to interact with cholesterol. Examples thereof are the two putative non-annular-binding sites in the ion channel Kir2.1 [39, 41], as well as cholesterol interaction sites in VDAC [34, 40]. In case of Kir2.1, a combination of docking studies, all-atom molecular dynamics simulations, and functional assays on channels with site-directed mutagenesis lead to the proposition of two binding sites. The two proposed binding sites are located in the hydrophobic center of the membrane. In addition, a weaker cholesterol-binding site is potentially present at the interface between the cytosolic and the transmembrane domain of Kir2.1. Binding of cholesterol to Kir2.1 was suggested to depend on van der Waals interactions with atoms located in-between-helices [41].

The description above highlights that direct and conclusive evidence for a given cholesterol-binding site is lacking in many cases and the proposed cholesterol-binding motifs can currently at best act as a general guide to help in the design of further experiments. The strongest experimental evidence for cholesterol/transmembrane protein interactions is currently available from X-ray crystallography and cryo-electron microscopy. However, even for these very powerful techniques, it is often not possible to work with lipid environments that faithfully mimic a biological membrane. Indeed, many membrane-mimetic environments, which are used for structure determination of membrane proteins, can differ substantially in their physico-chemical properties from those of native membranes, such as hydrophobicity, monomeric concentrations of amphiphiles, dielectric properties, water concentration, and lateral pressure profile [62]. These differences can compromise the tertiary structure of transmembrane proteins, whereas the structural stability of individual transmembrane helices might be enhanced [62]. In addition, crystallization can potentially change protein and cholesterol conformations, crystal-lattice effects might affect cholesterol binding and crystallization does not capture the dynamic nature of cholesterol/transmembrane protein interactions. To mimic the natural environment of a membrane, NMR and EPR studies focusing on cholesterol/transmembrane protein interactions are best done in liposomes or small unilamellar vesicles, increasing the challenge of obtaining high-quality data. Docking and molecular dynamics simulations still lack exact force fields or suffer from other computational limitations. Functional or biophysical studies, on the other hand, are indirect and by themselves only provide circumstantial evidence.

Annular and non-annular sites for cholesterol interaction

A further classification of cholesterol-binding sites is based on their accessibility: (i) annular sites that are located directly at the transmembrane surface of the protein, and (ii) non-annular sites that are located between transmembrane helices and occluded from membrane phospholipids [39].

Annular lipids are lipids that are in direct contact with the hydrophobic surface of a membrane protein [16]. The interaction between annular lipids and transmembrane proteins occurs through hydrogen bonds, π–π and cation–π interactions, electrostatic, and van der Waals forces [63] and results in transient immobilization of the lipid. Annular-binding sites have been observed for various lipids of the cellular membrane [2]. For example, Marsh and Barrantes detected a population of immobilized lipids in the form of a two-component electron spin resonance spectrum in the vicinity of nAChR [24]. The immobilized lipids included fatty acids, steroids, and several kinds of phospholipids, suggesting that transient lipid binding was not specific [24, 64]. Subsequently, a combination of complementary surface pressure measurements suggested that nAChR interacts preferentially with sterols [65, 66]. Functional studies further revealed that increasing cholesterol levels enhance nAChR-mediated ion flux and functional activity of nAChR [67–69]. In case of the receptor serotonin1A, coarse-grained molecular dynamics simulations pointed to multiple 'hot-spots' for transient cholesterol binding [47]. High-occupancy sites, but with a high rate of exchange of cholesterol, were designated as annular-binding sites and were observed in both the extracellular and the intracellular side of the receptor [47, 70]. In addition, atomistic molecular dynamics simulations pointed to a large number of transient annular and non-annular-binding sites in the A2A adenosine receptor [71] and the β2-adrenergic receptor [72, 73]. Notably, cholesterol binding was not only found to be transient in these studies, but also the cholesterol molecules remained mobile in the bound state, stressing the dynamic nature of cholesterol/protein interactions.

The concept of non-annular-binding sites was developed on the basis of the observation that cholesterol does not displace annular phospholipids in nAChR [74]. In addition, electron spin resonance experiments suggested that sterols are immobilized between the transmembrane helices of nAChR [24, 74]. Non-annular cholesterol-binding regions were also proposed for the Ca2 + -ATPase of sarcoplasmic reticulum [75] and the β-adrenergic GPCR

[22, 76]. Moreover, a cholesterol molecule is positioned within a groove formed by three transmembrane helices (Ia, V, and VII) of the dopamine receptor [77]. A cholesterol-binding site was furthermore identified in the 5-HT_{2B}ERG receptor [78]. In case of the human A2A adenosine receptor, three cholesterol-binding sites were observed in its crystal structure [79].

Non-annular cholesterol-binding sites were revealed by X-ray crystallography in several G-protein coupled receptors (GPCRs) (Fig. 3) [80]. For example, two cholesterol molecules were observed between the transmembrane helices of two adjacent molecules (helices I and VII in one molecule and helix I in the adjacent molecule) of the proton pumping rhodopsin ARII [81]. In addition, a cholesterol molecule was also seen in between the transmembrane helices of two μ-opiod receptors [82], and two putative non-annular cholesterol-binding regions were proposed in Kir2.1 channels [41]. Cholesterol-binding sites have also been identified in TSPO [32, 37, 48] and VDAC [34, 40]. Out of the 103 protein data bank (PDB) entries, which contain bound cholesterol molecules, 30 entries with a sequence similarity of less than 90% are listed in Table 1. Some of these proteins have cholesterol close to CRAC and CARC motifs. Many others, however, contain CRAC and/or CARC motifs, but cholesterol is bound to a different site. In addition, several proteins do not contain CRAC and/or CARC motifs although they are able to accommodate cholesterol in their structure (Table 1).

Importance of cholesterol–protein interactions

Direct binding to protein versus modulation of membrane properties

Cholesterol is known to regulate the activity of ion channels and other transmembrane proteins [39, 83]. From a mechanistic point of view, two scenarios for cholesterol—membrane protein interaction have been proposed. In the first model, cholesterol directly binds to the protein as a ligand and thus influences membrane protein function. In contrast, in the second scenario, cholesterol does not specifically bind to the protein but regulates its activity by altering the physical properties of the surrounding membrane [39, 84]. Notably, a direct interaction does not rule out the possibility that membrane properties have also been altered due to hydrophobic mismatch or change in bilayer thickness [39]. Since the physical properties of membrane bilayers can also be affected by the presence of other sterols, it is difficult to identify the specificity of cholesterol-mediated effects in such cases. The existing studies are, therefore, going into two directions, assessing whether (i) the regulatory effect of cholesterol is specific and (ii) whether cholesterol directly binds to the channel. The former typically involves the use of enantiomers of cholesterol, whereas the latter is based on direct binding essays using radiolabeled cholesterol.

Direct binding of cholesterol has been shown for the transmembrane receptor nAChR, although binding might be unspecific [85]. nAChR–cholesterol interactions stabilize the channel in the resting state, while cholesterol-induced

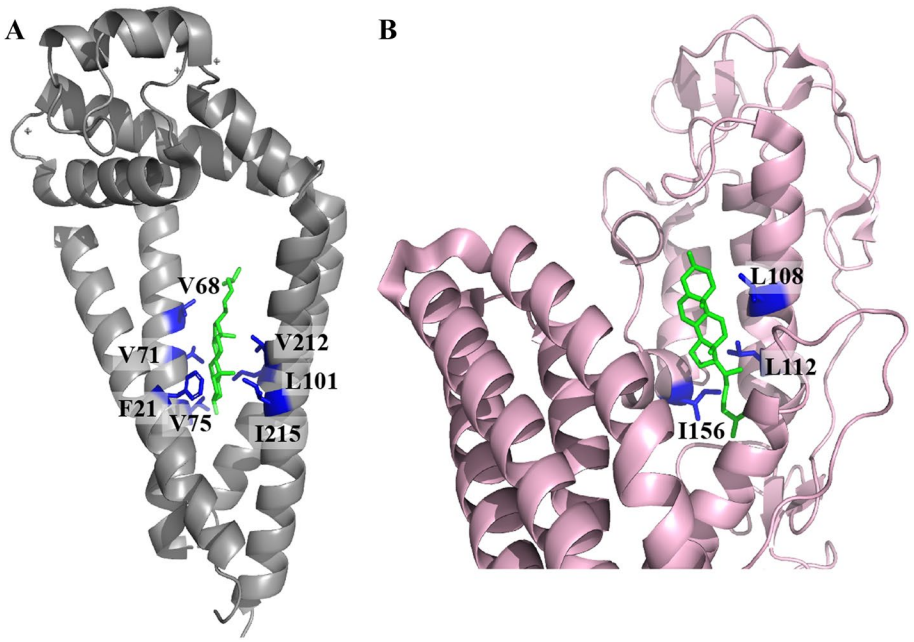

Fig. 3 Structural representations of non-annular cholesterol-binding sites in two transmembrane proteins. The cholesterol molecule is shown in green and a few residues in close proximity to cholesterol are shown in blue. PDB codes are: **a** 5TCX (human tetraspanin) and **b** selected region of 5L7D (human smoothened protein)

Table 1 Proteins with cholesterol (PDB code: CLR) bound to their structure

PDB ID	Protein/lipid/detergent used during purification/crystallization	Bound[a]	CRAC[b] motifs	CARC motifs	Binding CRAC/CARC[c]
X-ray diffraction					
1LRI	Fungal elicitor cryptogein	1	None	None	No
5XRA	CB1/ monoolein	1	None	None	No
2RH1	β-2 adrenergic receptor/ monoolein	3	None	None	No
	Na^+/K^+ ATPase/PC				
2ZXE	α subunit	1	5	12	No
	β subunit		3	7	Yes
3WGU	α subunit	6	4	14	No
	β subunit		5	9	Yes
4XT1	Human chemokine CX3CL1/1-oleoyl-R-glycerol	2	3	3	No
	μ-opioid receptor/monoolein				
4DKL		1	None	None	No
5C1M		1	3	2	No
4XP9	Dopamine transporter/DDM, PE	1	4	11	No
1N83	Nuclear receptor ROR-alpha	1	1	2	Likely
5LWE	Chemokine receptor type 9/DDM	1	3	6	Yes
5I6X	Human serotonin transporter/PC, PE, PG	1	6	5	No
5L7D	Human smoothened protein/DDM	1	None	None	No
3N9Y	Human CYP11A1	2	7	6	No
5JQH	β-2 adrenoceptor/ monoacylglycerol	2	None	None	No
4XNV	P2Y purinoceptor 1/DDM	1	None	None	No
5IU4	A2A adenosine receptor	4	None	None	No
5X93	Endothelin B receptor/ LMNG	1	None	None	No
5WVR	OSH1 OSBP-related domain	1	8	8	Yes
1ZHY	Oxysterol binding protein Osh4	1	3	11	Yes
5TCX	Human tetraspanin CD8/monoolein	1	1	5	No
3GKI	N-terminal domain of Niemann-Pick C1 protein	1	None	4	No
4BOE	Tick lipocalin japanin	1	4	4	Yes
4OR2	Metabotropic glutamate receptor 1/monoolein	6	None	None	No
4IB4	Chimera of 5-HT2B-BRIL/monoolein	1	None	None	No
3AM6	Proton pumping rhodopsin AR2/monoolein	8	2	3	No
4PXZ	P2Y12 receptor/monoolein	1	None	None	No
Electron microscopy					
5SY1	STRA6 receptor/ lauryl maltose neopentyl glycol	2	3	10	No
3JD8	Niemann-Pick C1 protein	1	6	18	No
Small angle neutron scattering					
3K2S	High density lipoprotein/POPC	20	5	4	No

Included proteins have less than 90% sequence similarity (date of analysis: 29th September 2017). Cholesterol-like molecules have not been included into the search

PC 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, *PE* 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, *PG* 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol, *DDM n*-dodecyl-β-D-maltopyranoside, *LMNG* lauryl maltose neopentyl glycol

[a]Total number of cholesterol molecules bound per structure

[b]CRAC motifs per chain

[c]Cholesterol motif present within 7 Å radius of one of the three defining residues of a CRAC or CARC motif

changes in membrane thickness facilitate transitions between the uncoupled and coupled states of nAChR [86]. In addition, cholesterol was found to be required for agonist-induced opening of the $GABA_A$ receptor, up-regulating it both specifically and non-specifically [87]. Conversely, the volume-regulated anion channel (VRAC) was

shown to be non-specifically down-regulated by cholesterol [88], presumably by the effects on lipid packing around the channel [89]. Cholesterol also down-regulates the activity of inwardly rectifying K^+-channels (KIR), by a direct and non-stereospecific interaction with the receptor [90, 91]. Several studies furthermore revealed an inhibitory effect of cholesterol on the activity of BK channels via specific protein–sterol interactions [39, 92–94]. An inhibitory effect of cholesterol as a result of stereoselective binding was also observed for TRPV1 [31]. Notably, direct evidence for cholesterol binding through biochemical methods has so far only been obtained for a small number of channels [95–98], making it often difficult to distinguish between a direct and indirect effect of cholesterol on transmembrane protein activity.

Cholesterol and mitochondrial membrane proteins

Disorders in lipid metabolism and transport play an important role in human disease [99]. Changes in the lipid profile of a cell drastically affect the cellular metabolism and signal transduction [99, 100]. In the particular case of cancer, an up-regulation of lipid metabolism is often observed during early stages of neoplasia and is a recognized hallmark of many types of cancer [100]. Changes in the mitochondrial phospholipid membrane composition, especially in cholesterol, can be triggered by external interventions (e.g., diet) and by a range of biological events (apoptosis, disease, and aging) [101].

Cholesterol is the sole precursor of steroids, whose synthesis at the inner mitochondrial membrane (IMM) requires translocation of cholesterol from the outer mitochondrial membrane (OMM) to the IMM [102]. Improper storage and targeting of cholesterol can be toxic for cells [102]. Cholesterol translocation involves various proteins. One of the first identified cholesterol-binding proteins was the sterol carrier protein-2 (SCP-2). SCP-2 plays a role in intracellular transfer of cholesterol, including the pathway from lysosomal to mitochondrial membranes [103]. Cholesterol, successfully imported via the plasma membrane or accessed through lipid droplets, is transported to the OMM, where it remains segregated until translocation to the IMM. The latter step is rate-limiting for steroidogenesis, and is thought to involve the mitochondrial translocator protein TSPO, previously known as the peripheral-type benzodiazepine receptor [102, 104]. A study employing TSPO ligands demonstrated that ligand binding to TSPO influences cholesterol translocation from the OMM to the IMM [105]. Subsequently, the role of TSPO in cholesterol translocation was supported by experiments using a bacterial TSPO-expression system [106]. In addition, cholesterol was associated with allosteric conformational changes in TSPO [37]. Because TSPO is preferentially located at mitochondrial contact sites, it was furthermore suggested that TSPO does not function alone, but TSPO function is modulated through interactions with other proteins [102]. One of these proteins is VDAC, which binds cholesterol in vivo [107, 108] and in vitro [109], and influences cholesterol distribution in mitochondria [110]. Cholesterol is also important for VDAC gating [109], enhances the structural integrity of isolated VDAC, aids channel insertion into membranes [107, 111, 112], and promotes uniformly open channel conductance [107, 112]. In addition, cholesterol might affect VDAC's interaction with other proteins [113]. VDAC function could thus be modulated during cancer, as well as aging and disease [101], when the content of cholesterol in the OMM increases [114, 115].

Insights into cholesterol–protein interactions through NMR spectroscopy

X-ray crystallography, NMR and electron spin resonance, and more recently cryo-electron microscopy [116], Fourier transform infrared spectroscopy [117], and femtosecond crystallography [118] have been used to study membrane protein structures and their interactions with lipids. In addition, mass spectrometry-based chemical photoaffinity labeling [119] and molecular dynamics simulations [120] were employed to characterize cholesterol binding and the impact of cholesterol on membrane protein activity.

Progress in sample preparation techniques, and methodological and technological advances have established NMR as an excellent technique to probe the interaction of two interacting species and provide atomic details of biomolecular interactions [121]. Based on these advances, detailed studies of the orientation of cholesterol and its derivatives in membrane environments have been performed by NMR [122]. NMR spectroscopy nowadays provides an important tool to study the impact of cholesterol on membrane protein structure and function.

Solution-state NMR studies of protein–cholesterol interactions

GPCRs form an important class of membrane proteins reported to bind cholesterol. Several GPCRs have been co-crystalized with cholesterol and structurally characterized by X-ray crystallography [80]. Cholesterol is known to impact the function of the GPCR β_2AR, both by direct binding and indirectly by influencing the lateral pressure and order of the bilayer [123]. To obtain insight into the interaction of cholesterol with β_2AR, Gater et al. performed saturation transfer difference NMR experiments on lipid cubic phase samples containing cholesterol and β_2AR [124]. In addition, NMR spectroscopy supported the finding that micelle-solubilized cannabinoid receptor CB2 is structurally stabilized by cholesterol hemisuccinate, a derivative of cholesterol [125].

To gain insight into the interaction of cholesterol with the C-terminal domain of the amyloid precursor protein (C99), Barrett et al. performed solution-state NMR titrations in combination with alanine scanning mutagenesis for C99 solubilized in DHPC/DMPC bicelles [126]. To this end, C99 residues ranging from 690 to 710 were replaced by alanine, followed by titration with increasing concentrations of cholesterol to detect chemical shift changes in 2D ^{1}H–^{15}N correlation spectra. Chemical shift perturbations indicated that cholesterol binds to the GXXXG motif of C99, which is known for its ability to promote homodimerization of transmembrane helices [127].

Hiller and coworkers investigated the interaction of cholesterol with VDAC using solution-state NMR spectroscopy [109]. VDAC, which was solubilized in lauryldimethylamine oxide micelles, showed chemical shift changes in the presence of cholesterol for nine backbone amide residues belonging to β-strands 7, 8, and 11 [109]. In addition, docking and molecular dynamics simulations suggested a total of five possible binding sites for cholesterol in VDAC, with cholesterol molecules primarily located in grooves defined by ridges of hydrophobic and sometimes aromatic residues [34]. Melissa and coworkers furthermore investigated the interaction of VDAC with cholesterol using photoaffinity labeling in combination with mass spectrometry [119]. Based on these studies, it was suggested that the cholesterol-binding pocket of VDAC is localized at the functionally relevant E73 [128] and T83 residues [119].

Solid-state NMR of membrane proteins embedded into cholesterol-containing membrane environments

Solid-state NMR spectroscopy is a powerful method to study the interaction between lipids and membrane-embedded proteins. Luo and coworkers used solid-state NMR to probe the effect of membrane environment on the dynamic properties of the Influenza A M2 peptide [129]. By recording ^{1}H–^{15}N, ^{1}H–^{13}C, and ^{13}C–^{15}N dipolar couplings and ^{2}H quadrupolar couplings, they showed that uniaxial diffusion of this transmembrane peptide is slowed down by two orders of magnitude ($< 10^{3}$ s^{-1}) in cholesterol-rich virus envelope-mimetic membranes. In addition, the structure and dynamics of full-length influenza A M2 proteins' were shown to be membrane-dependent [130]. Two-dimensional carbon–carbon correlation spectra of DMPC-embedded full-length AM2 showed β-strand chemical shifts for its serine, alanine, and leucine residues. In contrast, chemical shifts characteristic for α-helical secondary structure were observed in a cholesterol-rich membrane environment [130]. In agreement with the influence of cholesterol on the structural properties of transmembrane peptides and proteins [131], residues 7–11 of the human immunodeficiency virus fusion peptide convert from α-helical to β-strand conformation upon interaction with cholesterol [132].

In a recent elegant study, cholesterol binding to influenza M2 has been directly observed by high-resolution solid-state NMR using chain-fluorinated and sterol-deuterated cholesterol [133]. ^{13}C to ^{19}F distance measurements showed that two cholesterol molecules bind to each M2 tetramer [133]. In addition, deuterium NMR spectra of the sterol-deuterated cholesterol provided information about the orientation of cholesterol in the lipid bilayer and were used together with protein–cholesterol distances to derive a structural model of the protein/cholesterol complex [133]. This approach is particularly important, because it overcomes the problem, that chemical shift differences in the absence and presence of cholesterol can come from either direct interaction with cholesterol or cholesterol-induced changes in transmembrane protein structure.

Solid-state NMR of membrane-embedded TSPO

The translocator protein TSPO is preferentially expressed in tissues that synthesize steroids [134] and has been suggested to play an important role in cholesterol translocation from the OMM to the IMM [102]. In vitro, liposome-embedded TSPO binds cholesterol with nanomolar affinity [49]. It is, therefore, of high interest to understand how TSPO interacts with cholesterol, to which site in TSPO cholesterol binds and how cholesterol binding influences the structure and dynamics of TSPO. In addition, an open question is if and how TSPO contributes to translocation of cholesterol from the OMM to the IMM and how synthetic TSPO-specific ligands [135], which are used for diagnostics [135] or therapy [136], influence cholesterol binding.

To gain insight into TSPO/ligand interactions, we determined the three-dimensional structure of TSPO from mouse using solution-state NMR [48, 137, 138]. TSPO was solubilized in the detergent fos-choline-12 and structurally stabilized through binding of the radioligand (R)-1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide ((R)-PK1115). The three-dimensional structure of the TSPO/PK1115 complex revealed a tight bundle of five transmembrane helices. PK1115 is bound to a hydrophobic pocket, which is located in the interior of the structure on the cytosolic side of the lipid bilayer. The C-terminal half of transmembrane helix 5 contains a CRAC motif (residues A147–S159) and mutation of the central tyrosine in the CRAC motif abolishes cholesterol binding in vitro [49]. Side-chains of CRAC-defining residues are pointing away from the TSPO core, suggesting that these side-chains are accessible for binding to membrane-embedded cholesterol in an annular manner [48].

To obtain insight into the TSPO/cholesterol interplay, we reconstituted the protein into

1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) liposomes [139]. Similar to mouse TSPO in fos-choline-12 micelles [48], a radioligand—in this case *N*-(2,5-dimethoxybenzyl)-*N*-(5-fluoro-2-phenoxyphenyl)acetamide (DAA1106)—increased the structural stability of the protein. A large number of high-resolution solid-state NMR experiments in combination with site-directed mutagenesis demonstrated that mouse TSPO populates a concentration-dependent monomer–dimer equilibrium in DMPC liposomes (Fig. 4). Dimer formation at high protein concentrations is mediated by ^{83}GXXXG^{87} in transmembrane helix 3—a common motif for dimerization of transmembrane helices [140].

To study the interaction of TSPO with cholesterol, additional NMR samples were prepared, in which the DMPC liposomes contained cholesterol. Because of the known ability of cholesterol to cluster [141], i.e., to make sure that there are a sufficient number of cholesterol molecules, which are able to interact with TSPO, a tenfold excess of cholesterol over protein was used. Moreover, DAA1106 was used to stabilize the TSPO fold. Comparison of solid-state NMR spectra in the absence and presence of cholesterol showed chemical shift changes in different parts of the protein. The affected residues belong to the CRAC motif in helix 5, its neighboring helix 2, as well as the dimerization interface in transmembrane helix 3 (Fig. 5). In addition, quantification of monomer/dimer species by NMR signal intensities indicated that the presence of cholesterol favors TSPO monomerization [37].

To further support a potential cross-talk between the CRAC motif and the dimerization interface in transmembrane helix 3, residue Y152 within the CRAC motif was mutated to serine. This mutation resulted in an overall decrease of NMR spectral quality. Yet, unlike wild-type TSPO, Y152S-TSPO showed no evidence for protein dimerization. Vice versa, mutation of G87V in the dimerization interface caused chemical shift changes for residues in the CRAC motif, further strengthening the communication between the CRAC motif and the

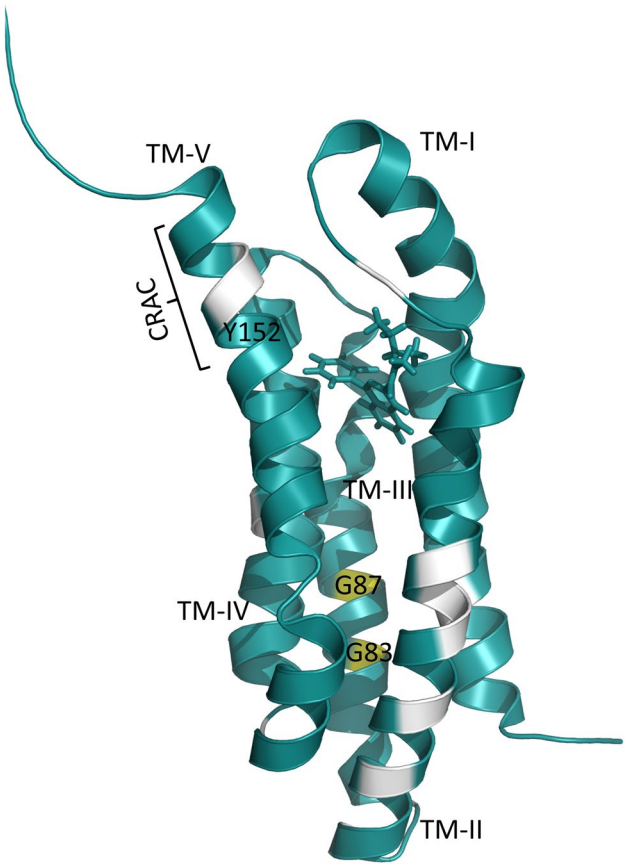

Fig. 5 Residues that experience chemical shift perturbation upon addition of cholesterol are colored white. TM-II undergoes major chemical shift perturbation

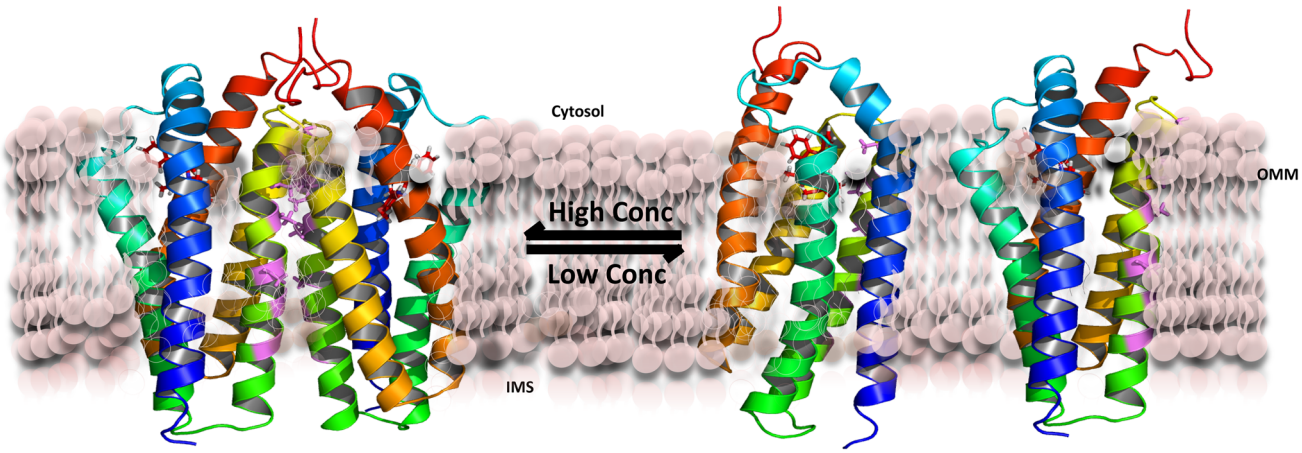

Fig. 4 Schematic representation of the concentration-dependent monomer–dimer equilibrium of TSPO in DMPC liposomes. The presence of cholesterol favors TSPO monomers

dimerization interface of TSPO and suggesting allosteric effects due to cholesterol-binding [37].

Outlook

Through a combination of different experimental techniques, the interaction of cholesterol with transmembrane proteins has been characterized at increasing resolution in recent years. Cholesterol can interact with membrane proteins either at annular or non-annular sites in a specific as well as non-specific manner. In addition, cholesterol changes the physical properties of the membrane and thus is able to indirectly—without specific binding to the protein—modulate the function of membrane proteins. Important aspects of both these mechanisms are cholesterol-mediated changes in the oligomerization of membrane proteins.

The variety of reported cholesterol/protein interactions and the influence of cholesterol on membrane proteins, however, suggest that general rules with respect to protein/cholesterol interactions and their functional consequences are difficult to define. Instead, detailed studies are required that identify cholesterol-binding sites at high resolution and connect it to changes in the structure and dynamics of individual membrane proteins and functional assays with cholesterol. An important aspect of these studies could be the direct and indirect effect of cholesterol on the interactions between different membrane proteins. We believe that NMR spectroscopy, in particular solid-state NMR, can play an important role in this endeavor, because it can simultaneously probe the direct binding of cholesterol and cholesterol-induced changes in the structure and dynamics of peptides and proteins embedded in near native-like membrane environments. In addition, lipid-protein nanodiscs offer a new possibility to structural biologists to study lipid–protein interactions by NMR [142, 143]. An understanding of cholesterol/protein interactions on an atomic-scale level would not only provide more profound insight into physiological and pathophysiological signaling processes related to health and disease, but could also turn out to be valuable for the identification of new treatment strategies.

Acknowledgements M.Z. was supported by the Deutsche Forschungsgemeinschaft Collaborative Research Center 803 (Project A11) and the European Research Council (Grant agreement number 282008).

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