Chapter 3

Structural Stringency of Cholesterol for Membrane Protein Function Utilizing Stereoisomers as Novel Tools: A Review

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Abstract

Cholesterol is an important lipid in the context of membrane protein function. The function of a number of membrane proteins, including G protein-coupled receptors (GPCRs) and ion channels, has been shown to be dependent on membrane cholesterol. However, the molecular mechanism underlying such regulation is still being explored. In some cases, specific interaction between cholesterol and the protein has been implicated. In other cases, the effect of cholesterol on the membrane properties has been attributed for the regulation of protein function. In this article, we have provided an overview of experimental approaches that are useful for determining the degree of structural stringency of cholesterol for membrane protein function. In the process, we have highlighted the role of immediate precursors in cholesterol biosynthetic pathway in the function of membrane proteins. Special emphasis has been given to the application of stereoisomers of cholesterol in deciphering the structural stringency required for regulation of membrane protein function. A comprehensive examination of these processes would help in understanding the molecular basis of cholesterol regulation of membrane proteins in subtle details.

Key words Cholesterol, Cholesterol-binding motif, *ent*-Cholesterol, *epi*-Cholesterol, GPCRs, Ion channels, Stereoisomers, Stereospecificity

1 Introduction

Biological membranes exhibit a vast degree of functional and compositional heterogeneity and provide an ideal environment for the function of a variety of membrane lipids and proteins. A comprehensive understanding of diverse membrane functions requires deciphering molecular details of interactions between membrane components. Work from a number of groups has led to our current understanding of the requirement of specific lipids in the function of membrane proteins [1]. An important membrane lipid in this context is cholesterol, which exhibits heterogeneous (nonrandom) distribution in membranes and has been shown to modulate functions of several membrane proteins [1–8]. In this context, two important classes of membrane proteins studied are seven transmembrane domain G protein-coupled receptors (GPCRs) and ion

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channels. GPCRs constitute an important superfamily of proteins that mediate a variety of physiological processes and serve as major drug targets in all clinical areas [9] (*see* below). Ion channels, on the other hand, are transmembrane proteins that regulate ionic permeability across cell membranes.

Although the cholesterol-dependent function for several proteins and peptides has been reported, the molecular details and specificity of their interaction are still emerging. Recent technical advancements, and ready availability of multiple agents for modulation of membrane cholesterol and close structural analogs of cholesterol, have made it possible to delineate the structural stringency associated with the interaction of cholesterol with membrane proteins and receptors. In this article, we provide an overview of the approaches, particularly utilizing structural analogs of cholesterol, for addressing structural stringency of cholesterol for the function of membrane proteins, with special emphasis on stereoisomers of cholesterol.

2 Requirement of Cholesterol for the Function of Membrane Proteins

The detailed mechanism underlying the modulation of the structure and function of membrane proteins and receptors by membrane cholesterol is not completely understood and appears to be complex [5, 10, 11]. It has been proposed that cholesterol could modulate the function of membrane receptors by a direct (specific) interaction, which could induce conformational change(s) in the receptor, or by altering the physical properties of the membrane in which the receptor is embedded. Yet another possibility could be a combination of both. Importantly, the concept of "nonannular"-binding sites of lipids in membrane proteins has been proposed as specific interaction sites [11, 12]. These sites are characterized by lack of accessibility to the annular lipids, i.e., annular lipids cannot compete and displace the lipids at these sites [13, 14].

Work from our laboratory and others has comprehensively demonstrated the role of membrane cholesterol in the organization, dynamics, function, and stability of GPCRs (reviewed in refs. [2–7, 9]). For example, cholesterol has been shown to play an important role in the function and stability of the serotonin$_{1A}$ receptor [15–17], β_2-adrenergic receptor [18–20], cholecystokinin receptor [21], serotonin$_{7a}$ receptor [22], oxytocin receptor [23, 24], and human type-1 cannabinoid receptor [25]. In addition, cholesterol has been shown to play a crucial role in the function and organization of several ion channels [8]. For example, the specific role of cholesterol in the activation, trafficking, and desensitization of the nicotinic acetylcholine receptor has been previously reported [26–31]. Cholesterol has been shown to modulate the agonist effectiveness of $GABA_A$ receptors and an optimal

requirement of cholesterol for the channel function has been reported [32–35]. In addition, membrane cholesterol has been shown to modulate the function of multiple types of K^+ channels (reviewed in refs. [8, 36], *see* below), the channel opening probability (lifetime), and the rate of desensitization of NMDA receptors [37].

As mentioned above, previous work from our laboratory has shown an absolute requirement of membrane cholesterol in the function of the serotonin$_{1A}$ receptor (reviewed in refs. [3, 5, 7]). We employed several approaches to explore the specific role of membrane cholesterol in the organization, dynamics, and function of the serotonin$_{1A}$ receptor. These approaches include: (1) acute modulation of membrane cholesterol using MβCD; (2) complexation of membrane cholesterol (without physical depletion) by agents such as nystatin and digitonin; (3) chemical modification of cholesterol to cholestenone using cholesterol oxidase; and (4) use of metabolic inhibitors of cholesterol biosynthesis such as statins and AY 9944. Interestingly, we utilized the loss in membrane cholesterol associated with receptor solubilization [38, 39] as an effective strategy to explore specific cholesterol effects on receptor function. We will discuss some of these approaches in detail later in the review.

Several structural features of proteins believed to assist preferential association with cholesterol have been recently reported [5, 7, 40, 41]. Prominent sites among them are CRAC (cholesterol recognition/interaction amino acid consensus) motif [41–44], CCM (cholesterol consensus motif) [45], SSD (sterol-sensing domain) [46, 47], and CARC (inverse CRAC) motif [41, 48, 49]. These cholesterol-binding sequences or motifs have been proposed to contain an aromatic amino acid that could interact with the near planar ring structure of cholesterol [45, 50], and a positively charged residue capable of participating in electrostatic interactions with the 3β-hydroxyl group of cholesterol [43, 50, 51]. In this context, it is important to note that the proposed "nonannular"-binding sites of lipids in membrane proteins could be considered specific interaction sites [11, 12] with possible locations at inter or intramolecular (interhelical) protein interfaces. Detailed analysis of the role of individual amino acids in these putative cholesterol interaction sites could help us understand the specific requirement of cholesterol observed for the function, organization, dynamics, and signaling of membrane proteins.

3 Approaches for Altering the Content and Availability of Membrane Cholesterol

A convenient way of exploring the structural stringency of lipids for the function of integral membrane proteins is to replace or modifty the lipid of interest to close structural analogs and examine the protein function. It therefore becomes important to look for specific tools to modulate or exchange the lipid of interest with its

close structural analogs. In many instances, enzymes that modify specific sites of lipids have been utilized for this purpose. The role of membrane cholesterol in the function of membrane proteins has been studied by a number of groups using a variety of agents to modulate the availability of membrane cholesterol. These include inhibitors of cholesterol biosynthesis (e.g., statins, triparanol, AY9944), cholesterol oxidase that oxidizes membrane cholesterol, agents physically modulating the cholesterol content (e.g., methyl-β-cyclodextrin (MβCD)), and cholesterol sequestering compounds (e.g., amphotericin B, digitonin, nystatin, filipin). We discuss some of these approaches in detail below.

3.1 Specific Carriers of Membrane Cholesterol

Acute and specific depletion of membrane cholesterol is possible due to the development of cyclodextrins that act as effective catalysts of cholesterol efflux from membranes [52]. Among a variety of cyclodextrins available with broad specificity for membrane lipids, the oligomer with seven methylated-glucose residues (MβCD) displays higher specificity for cholesterol relative to phospholipids (*see* Fig. 1a). The polar nature and small size of cyclodextrins compared to other lipid carriers, allow them to come close to the membrane without partitioning and favor efficient efflux of cholesterol. MβCD has therefore been extensively utilized and has evolved as a convenient tool to selectively and efficiently modulate membrane cholesterol by incorporating it in a central nonpolar cavity [53–56]. The stoichiometry of 1:2 (mol/mol) has been reported for such cholesterol-cyclodextrin complexes [56–58].

3.2 Cholesterol Complexing Agents

Complexation of membrane cholesterol, which effectively reduces the availability of cholesterol without physical depletion, represents a strategy to minimize any nonspecific effects associated with cholesterol depletion from membranes. When used at appropriate concentrations, cholesterol complexing agents partition into membranes and sequester cholesterol. These agents include digitonin, filipin, nystatin, and amphotericin B. Digitonin is a plant glycoalkaloid saponin detergent known to form water-insoluble 1:1 complex with cholesterol [59–61]. Nystatin [55, 62–65] and amphotericin B [62, 63, 66–70] are sterol-binding antifungal polyene antibiotics that are known to sequester membrane cholesterol (*see* Fig. 1). They effectively partition into membranes and sequester cholesterol (1:1 (mol/mol) complex) and form channels in the membrane. On the other hand, filipin is a fluorescent sterol-binding antifungal polyene antibiotic, often utilized to stain free cholesterol in fixed cells [54, 63]. These agents reduce the availability of cholesterol for its interaction with membrane receptors.

3.3 Inhibitors of Cholesterol Biosynthesis

A chronic and more physiological way of reducing membrane cholesterol content is by inhibiting cholesterol biosynthesis. A number of cholesterol biosynthesis inhibitors have been used for reducing

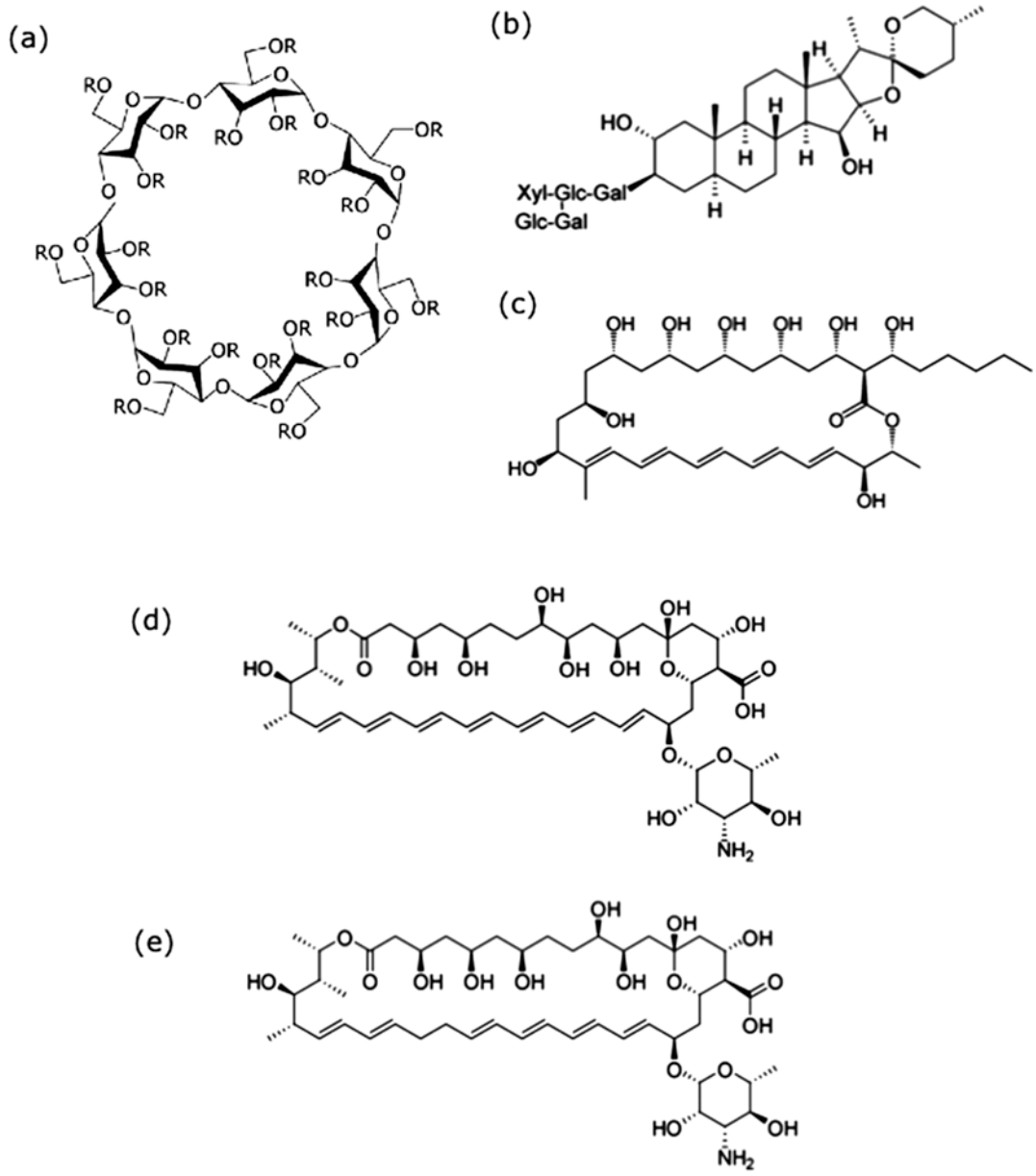

Fig. 1 Compounds that modulate availability of membrane cholesterol. (**a**) The chemical structure of β-cyclodextrin (containing seven glucose residues). Cyclodextrins can solubilize a variety of hydrophobic compounds by trapping them in their inner cavity. The oligomer with seven methylated-glucose residues (MβCD, where R denotes a methyl group) displays higher specificity for cholesterol relative to phospholipids. The stoichiometry of 1:2 (mol/mol) has been reported for such cholesterol-cyclodextrin complex. The chemical structures of cholesterol complexing agents such as (**b**) digitonin, (**c**) filipin, (**d**) amphotericin B, and (**e**) nystatin. Digitonin is a plant glycoalkaloid saponin detergent, while filipin, amphotericin B, and nystatin belong to the group of sterol-binding antifungal polyene antibiotics. Complexation of membrane cholesterol, which effectively reduces the availability of cholesterol without physical depletion, has been utilized as a strategy to minimize any nonspecific effects associated with use of MβCD to remove membrane cholesterol. Cholesterol complexing agents partition into membranes and sequester cholesterol. See text for more details

membrane cholesterol in metabolically active cells. For example, statins are a group of globally best selling drugs that are widely used for reducing membrane cholesterol. They act as competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a key rate-limiting enzyme in early cholesterol biosynthesis [71–73]. In addition, several distal inhibitors of cholesterol biosynthesis have been utilized. For example, AY9944 and BM15766 inhibit 7-dehydrocholesterol reductase (7-DHCR), an enzyme that catalyzes the last step in the Kandutsch-Russell pathway [74], and results in the accumulation of 7-dehydrocholesterol (7-DHC). This mimics one of the most serious autosomal recessive disease conditions called Smith-Lemli-Opitz Syndrome (SLOS) [75–79]. On the other hand, triparanol, another distal inhibitor of cholesterol biosynthesis, acts on 24-dehydrocholesterol reductase (24-DHCR), which catalyzes the last step in the Bloch pathway of cholesterol biosynthesis [80]. This results in accumulation of desmosterol which mimics another autosomal recessive disorder called desmosterolosis [78, 79, 81–84]. The use of these distal cholesterol biosynthesis inhibitors (AY9944, BM15766 and triparanol) has been limited because of severe effects resulting from accumulation of cholesterol precursors [85].

3.4 Enzymatic Oxidation of Cholesterol

Oxidation of membrane cholesterol by the enzyme cholesterol oxidase is yet another approach to modify the chemistry of cholesterol within the membrane without physical depletion. Cholesterol oxidase is a water-soluble enzyme that catalyzes the oxidation of cholesterol to cholestenone (cholest-4-en-3-one) at the membrane interface [86, 87]. The impact of oxidation of hydroxyl group of cholesterol appears to be relatively mild on membrane physical properties, and thereby is thought to minimize the nonspecific effects of cholesterol modulation.

4 Structural Analogs Utilized for Deciphering Stringency of Membrane Cholesterol in Protein Function

An efficient and quick way to explore structural stringency of cholesterol for a given process is to replace cholesterol with its close structural analogs. This is often conveniently achieved by depleting cholesterol using MβCD or metabolic inhibitors, and replacing it with its structural analogs either by utilizing a preformed sterol-MβCD complex, or by supplementation in reconstituted LDL particles in the culture medium of cells. Yet another convenient approach to explore the structural stringency of cholesterol for protein function is membrane solubilization using appropriate detergents [88, 89]. Membrane solubilization is often associated with delipidation (loss of lipids), and results in differential extents of lipid solubilization [38, 39]. Since membrane lipids play an

important role in maintaining the function of membrane proteins and receptors, such delipidation upon solubilization often results in loss of protein function. This phenomenon has been effectively utilized to explore molecular details of specific lipid requirements for the function of membrane proteins [90, 91] and has been recently reviewed [89].

As mentioned above, work from our laboratory and others has shown the crucial role of membrane cholesterol in the organization, dynamics, function, and stability of GPCRs [2–7, 9]. Availability of the above-mentioned agents and structural analogs of cholesterol (*see* sections 4.1 and 4.2) has made it possible to examine the structural stringency of cholesterol necessary for the function of several membrane proteins and peptides. These include ion channels, GPCRs, model peptides such as gramicidin and toxins such as *Vibrio cholerae* cytolysin and streptococcal streptolysin O. We discuss below some of the close structural analogs of cholesterol that have been utilized for exploring the stringent requirement of cholesterol in the function of membrane proteins and peptides.

4.1 Biosynthetic Precursors of Cholesterol: 7-DHC and Desmosterol

7-DHC and desmosterol are two close structural analogs of cholesterol, which differ with cholesterol merely in an additional double bond at the 7th position in the sterol ring and the 24th position in alkyl side chain, respectively (*see* Fig. 2b, c). 7-DHC and desmosterol are immediate biosynthetic precursors of cholesterol in the Kandutsch-Russell and Bloch pathways, respectively. Malfunctioning of enzymes that catalyze the conversion of 7-DHC and desmosterol to cholesterol (7-DHCR and 24-DHCR) results in low levels of serum cholesterol and accumulation (high levels) of the respective immediate precursors. This leads to fatal neurological disorders such as the Smith-Lemli-Opitz Syndrome (SLOS) and desmosterolosis [78, 79]. Availability of these structural analogs of cholesterol in relatively pure form has been useful to address the underlying mechanism of malfunctioning of proteins under such disease conditions.

Work from our laboratory and others has utilized these structural analogs to explore the function of important membrane proteins such as ion channels and GPCRs. For example, previous work from our laboratory has explored whether 7-DHC or desmosterol could replace cholesterol in supporting the function of the serotonin$_{1A}$ receptor, an important neurotransmitter receptor [92, 93]. An interesting aspect of our results is that the requirement of cholesterol for the function of the serotonin$_{1A}$ receptor was shown to be considerably stringent. Our results showed that while desmosterol could support the receptor function [84], 7-DHC could not [77, 94, 95]. In addition, cholesterol has been shown to inhibit the activity of a prokaryotic Kir (KirBac1.1) channel, while replacement with desmosterol has been reported to enhance channel activity [96]. In contrast, it has been shown that replacement of cholesterol

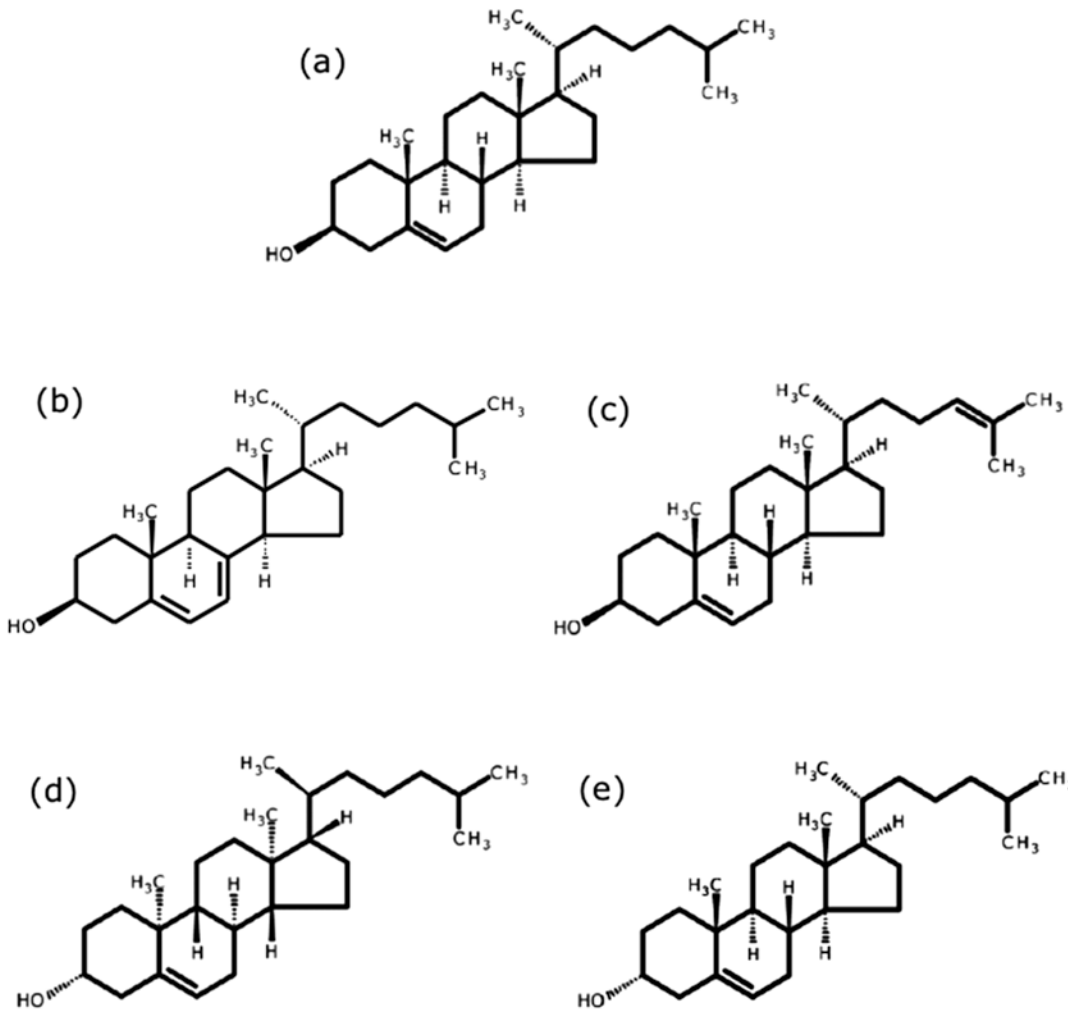

Fig. 2 Chemical structures of (**a**) cholesterol, and its structural analogs; (**b**) 7-dehydrocholesterol (7-DHC) and (**c**) desmosterol are immediate biosynthetic precursors of cholesterol in the Kandutsch-Russell and Bloch pathways, respectively, which differ with cholesterol merely in an additional double bond at the 7th position in the sterol ring and the 24th position in the alkyl side chain; (**d**) *ent*-cholesterol and (**e**) *epi*-cholesterol are stereoisomers of cholesterol. The enantiomer of cholesterol (*ent*-cholesterol) is the nonsuperimposable mirror image of natural cholesterol and exhibits similar physicochemical properties. *epi*-Cholesterol, on the other hand, is a diastereomer of cholesterol, that differs with cholesterol only in the orientation of the hydroxyl group at carbon-3, which is inverted relative to natural cholesterol. Adapted from ref. 89. See text for more details

with 7-DHC or desmosterol has relatively mild effect on the function of two structurally related peptide receptors, the oxytocin receptor and the cholecystokinin receptor [23].

4.2 Stereoisomers of Cholesterol

Stereoisomers of cholesterol such as enantiomer of cholesterol (*ent*-cholesterol) and *epi*-cholesterol (a diastereomer of cholesterol) have been developed as novel tools to differentiate the specific and

general effect of cholesterol in protein function. *ent*-Cholesterol is the nonsuperimposable mirror image of natural cholesterol (*see* Fig. 2d) and exhibits similar biophysical properties in the membrane (such as compressibility, phase behavior, and dipole potential) as natural cholesterol [97–99]. In addition, *ent*-cholesterol has been shown to support normal growth of a mutant mammalian cell line similar to its natural counterpart [100]. *epi*-Cholesterol, on the other hand, is a diastereomer of cholesterol that differs with cholesterol only in the orientation of the hydroxyl group at carbon-3, which is inverted relative to natural cholesterol (Fig. 2e). *epi*-Cholesterol has been shown to exhibit differences in membrane biophysical properties (such as condensing ability, tilt angles, and phase transition) relative to natural cholesterol (reviewed in refs. [97, 98]). *ent*-Cholesterol is often utilized to distinguish whether the effect of cholesterol observed is due to specific interaction with membrane components such as proteins and peptides, or due to general membrane (nonspecific) effects [97–103]. The selectivity of natural cholesterol and its enantiomer on the function of several peptides and proteins has been studied in detail. We discuss some of these examples below.

4.2.1 G Protein-Coupled Receptors

G protein-coupled receptors (GPCRs) are important superfamily of transmembrane proteins that primarily transduce signals from outside the cell to the cellular interior [104–106]. GPCRs mediate a vast variety of physiological processes and therefore serve as major drug targets in all clinical areas [9, 107–109]. Recent work from our laboratory has addressed the stereospecific requirement of cholesterol utilizing *ent*-cholesterol and *epi*-cholesterol for the function of the serotonin$_{1A}$ receptor. In order to determine the structural stringency of cholesterol, we replenished solubilized membranes (which contain significantly less cholesterol compared to native membranes [38, 39]) with *ent*-cholesterol or *epi*-cholesterol and examined if they could support receptor function. Our results showed that *ent*-cholesterol behaved similarly to native cholesterol in supporting the function of the serotonin$_{1A}$ receptor, although *epi*-cholesterol could not support receptor function [110] (*see* Fig. 3). Our results therefore point out the requirement of membrane cholesterol for the serotonin$_{1A}$ receptor function to be diastereospecific, yet not enantiospecific. These results also highlighted the *equatorial* configuration of the 3-hydroxyl group of cholesterol as a key structural feature for its ability to support the serotonin$_{1A}$ receptor function. These results, along with our previous observations with other close structural analogs of cholesterol [77, 84, 94, 95], extended our understanding of the degree of specificity of interaction of membrane cholesterol with the serotonin$_{1A}$ receptor. In an earlier study, it has been shown that *epi*-cholesterol could not support the specific ligand binding to the oxytocin receptor (a peptide binding GPCR for which the specific

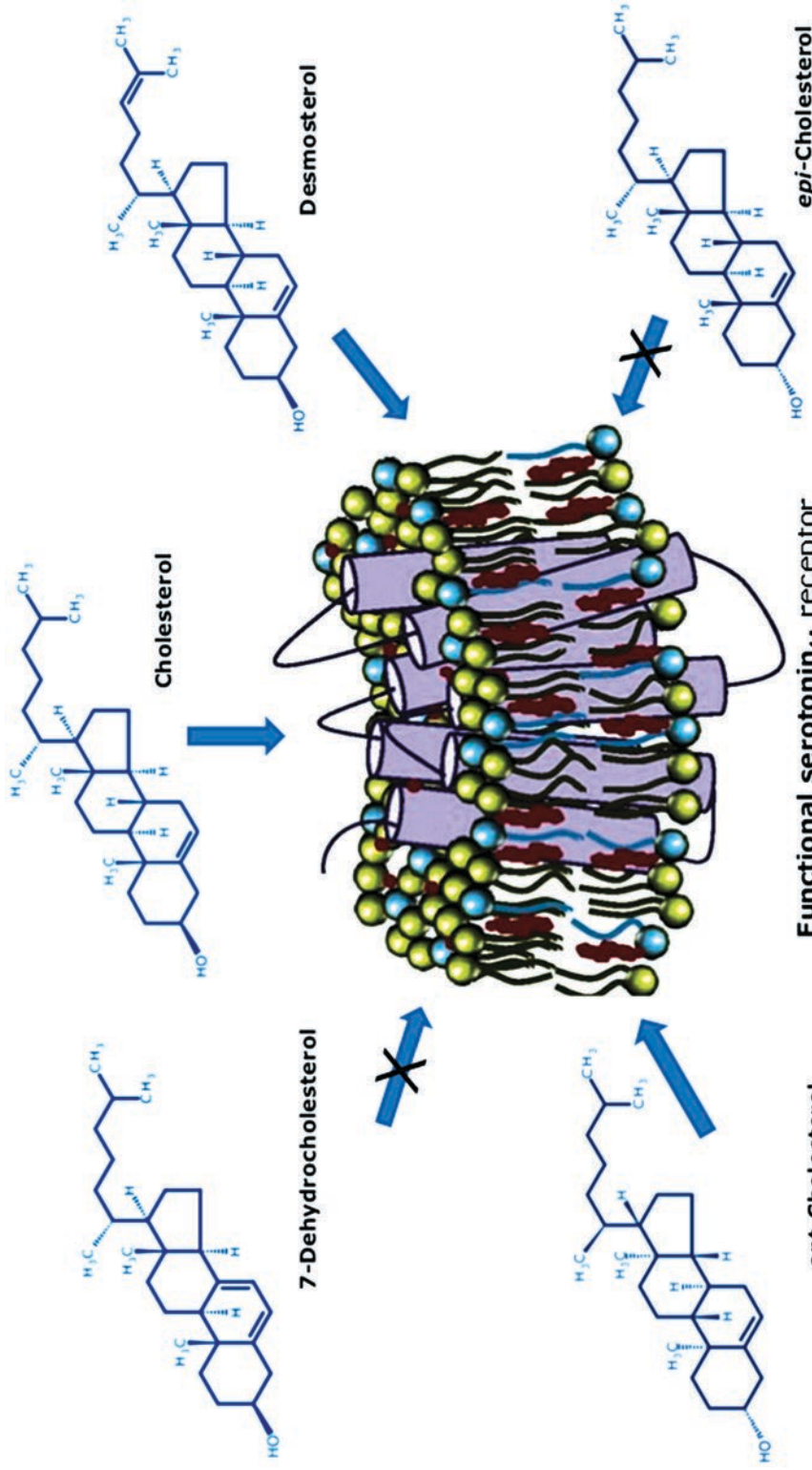

Fig. 3 Exploring the structural stringency of cholesterol required for the function of a representative GPCR, the serotonin$_{1A}$ receptor. Replenishment of solubilized hippocampal membranes containing the serotonin$_{1A}$ receptor with cholesterol and its close structural analogs allows us to effectively explore the stringency of cholesterol structure in receptor function. The schematic shows that cholesterol, desmosterol, and *ent*-cholesterol could support the function of the serotonin$_{1A}$ receptor, whereas 7-dehydrocholesterol and *epi*-cholesterol could not. The serotonin$_{1A}$ receptor is shown in purple, and the replenished sterol molecules are shown in maroon. The underlying message here is that even subtle changes in sterol structure may be crucial for receptor function, thereby reinforcing the stringency associated with receptor-cholesterol interaction. Adapted from ref. 89. See text for more details

requirement of membrane cholesterol for its function has been demonstrated [23]). Taken together, these results demonstrate the stringent requirement of cholesterol structure for the function of GPCRs.

4.2.2 Ion Channels

Ion channels are transmembrane proteins that regulate ionic permeability across cell membranes and are crucial for normal functioning of cells. Malfunctioning of ion channels has been implicated in a number of diseases collectively known as "channelopathies" [111]. Membrane cholesterol has been shown to modulate the function of several ion channels, such as multiple types of K^+ channels, including inwardly rectifying, Ca^{2+}-sensitive and voltage-gated K^+ channels, voltage-gated Na^+ and Ca^{2+} channels, volume-regulated anion channels (reviewed in ref. 8). In many cases, cholesterol inhibited the channel function either by decreasing the channel opening probability (lifetime) or the number of active channels. In contrast, cholesterol is observed to be essential for the function of the nicotinic acetylcholine receptor (nAChR) [27, 30] and $GABA_A$ receptors [32–34]. Although cholesterol has been shown to modulate the function of a number of ion channels, the structural stringency of cholesterol (stereospecificity in particular) and details of molecular interaction have been explored only in a few cases. For example, the enantioselectivity of cholesterol for the function of inward rectifier K^+ channels from bacteria (KirBac1.1 and KirBac3.1) and human (Kir2.1) has been studied. While natural cholesterol is known to inhibit these channels, its enantiomer, *ent*-cholesterol, does not inhibit the channel function. It was therefore concluded that the regulation of channel function by the membrane cholesterol is through possible direct channel-cholesterol interaction [102]. In addition, the stereoselectivity of cholesterol in the function of inward rectifier K^+ channels has been previously explored utilizing the diastereomer of cholesterol (*epi*-cholesterol) [112]. Similarly, *epi*-cholesterol has been shown to be significantly less efficient than natural cholesterol in inhibiting the activity of prokaryotic Kir (KirBac1.1) channels [96]. These results show an absolute requirement of cholesterol for maintaining channel function with possible direct interaction with the protein.

In contrast, the cholesterol dependence of agonist stimulated channel conductance of the nicotinic acetylcholine receptor has been shown to be supported by both *ent*-cholesterol and *epi*-cholesterol [113]. In yet another study, channel formation of gramicidin in the presence of stereoisomers of cholesterol was studied. Gramicidin is a 15-residue linear antimicrobial peptide that forms prototypical ion channels specific for monovalent cations and serves as an excellent model for studying the organization, dynamics, and function of membrane-spanning channels [114–116]. Both natural and *ent*-cholesterol were observed to support the formation of identical gramicidin ion channels [101]. The results with the nicotinic

acetylcholine receptor and gramicidin channels were therefore attributed to a nonspecific mode of regulation of protein function by membrane cholesterol (i.e., through influence on membrane physical properties).

4.2.3 Regulators of Cholesterol Homeostasis

Cholesterol homeostasis in cells is stringently maintained through interaction of key proteins that sense membrane cholesterol levels. Among the proteins involved, sterol regulatory element-binding protein 2 (SREBP-2) and SREBP cleavage-activating protein (Scap) play important roles in cholesterol homeostasis. Cellular cholesterol regulates its own synthesis by modulating the activation of SREBP-2 transcription factors [117]. When in excess, cholesterol in the endoplasmic reticulum (ER) is sensed by Scap which upon conformational change assists binding of Insig, a protein that tethers the SREBP-Scap complex at ER in inactive form [118–120]. When cholesterol levels fall below a certain threshold, SREBP-2 is transported to the Golgi by Scap and is activated upon proteolytic cleavage. The activated (cleaved) fragment gets translocated to nucleus that induces expression of proteins involved in biosynthesis and uptake of cholesterol.

In a recent study, cholesterol enantioselecivity for proteins involved in cholesterol homeostasis was explored [103]. This study showed that activation of SREBP-2, the master transcriptional regulator of cholesterol metabolism, is suppressed by *ent*-cholesterol with similar efficiency as natural cholesterol. In agreement with this, the expression of target genes of SREBP-2 such as *LDLR* (LDL receptor), *HMGCR* (HMG-CoA reductase), and *SQLE* (Squalene epoxidase/monooxygenase) is suppressed by *ent*-cholesterol, similar to natural cholesterol. Importantly, *ent*-cholesterol induced the conformational change in the cholesterol-sensing protein Scap like its natural counterpart, which would result in retention of SREBP-2 in ER. Taken together, these results show that *ent*-cholesterol exhibits similarly homeostatic responses as natural cholesterol. On this basis, it has been suggested that cholesterol could also maintain its homeostasis through alterations in membrane properties beyond those specific cholesterol–protein interactions currently recognized [103].

4.2.4 Enzymes

Enantioselectivity of some of the enzymes involved in cholesterol metabolism has been previously examined. Cholesterol oxidase that catalyzes oxidation of cholesterol is one of the well-studied and extensively utilized enzymes. Cholesterol oxidase is a water-soluble enzyme that catalyzes the oxidation of cholesterol to cholestenone (cholest-4-en-3-one) at the membrane interface [86]. The stereospecificity of cholesterol recognition by cholesterol oxidase has been explored earlier [121]. Results showed that while *ent*-cholesterol serves as a substrate for cholesterol oxidase, the kinetics of oxidation is slower and oxidation was incomplete as

compared to its natural analog. In another study, acyl CoA cholesterol acyltransferase (ACAT), an ER resident enzyme that catalyzes cholesterol esterification, has been shown to be enantioselective for cholesterol, with *ent*-cholesterol being a poor substrate [122]. In contrast, proteasomal degradation of squalene monooxygenase, a key enzyme in cholesterol biosynthesis, has been shown to be accelerated by *ent*-cholesterol similarly to natural cholesterol, although to a lesser extent [103]. While enzyme substrate interaction is thought to be very stringent, studies with close structural analogs, especially the stereoisomers help broaden our understanding of stringency of their interaction.

4.2.5 Bacterial Toxins

The mechanism of action of several pore-forming toxins to selectively permeabilize host membranes is explained by their specific interaction with sterols in eukaryotic membranes, and cholesterol in particular, in higher eukaryotes. The requirement of cholesterol for the activity of bacterial pore-forming toxins such as *Vibrio cholerae* cytolysin [123] and streptococcal streptolysin O [124] has been reported earlier. In the case of *Vibrio cholerae* cytolysin, cholesterol has been shown to be required for membrane permeabilization, and cytolysin could not permeabilize membranes when cholesterol was replaced with *ent*-cholesterol [125]. These results highlight the enantioselectivity of cholesterol for its function. On the other hand, cholesterol has been shown to be essential for the membrane binding of streptococcal streptolysin O, which exhibited permeabilization of membranes in the presence of *ent*-cholesterol, albeit with less potency [125]. Bacterial toxins such as *Staphylococcus aureus* α-hemolysin and *Streptococcus agalactiae* CAMP factor, whose erythrocyte lysis is dependent on membrane cholesterol, did not exhibit enantioselectivity [126]. These results suggest a lower degree of structural specificity in toxin-sterol interactions, and the change in cholesterol-dependent membrane properties, but not direct interaction, could affect the function of these bacterial toxins.

It is important to mention here that in all the above-mentioned examples where the stereospecificity of cholesterol has been explored, *ent*-cholesterol has been particularly utilized to differentiate the specific and general role of cholesterol in protein function. The crucial assumption in these studies is that the specific cholesterol binding site would be geometrically stringent enough that it could differentiate the enantiomers. While such stringency would require more than two specific interactions between the ligand and the receptor, at least four geometrical constraints are proposed to be required to distinguish the enantiomers [97, 98]. However, in a protein that is non-rigid, defining such geometrical constraints would be difficult. Interestingly, a possibility of a non-enantioselective pattern of binding in a non-geometrically constrained protein cleft (such as a non-annular lipid binding site, as discussed above) has been earlier

proposed [97, 98]. It is therefore important to keep this caveat in mind when interpreting a finding of lack of enantioselectivity.

5 Conclusion and Future Perspectives

Advances in techniques to modulate the accessibility of membrane cholesterol, along with the availability of close structural analogs of cholesterol, have made it possible to delineate the structural stringency of cholesterol required for maintaining the optimum function of several membrane proteins such as GPCRs and ion channels. In particular, the stereoisomers of cholesterol have been useful in examining the specific effect of cholesterol from its general effects on membrane properties. Taken together, these approaches have helped us address the molecular details of regulation of membrane protein function by cholesterol. Insights from such studies could help us understand details of functioning of important membrane proteins in healthy and diseased conditions with impaired cholesterol metabolism.

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