BBA - Molecular and Cell Biology of Lipids 1865 (2020) 158706

Contents lists available at ScienceDirect

BBA - Molecular and Cell Biology of Lipids

journal homepage: www.elsevier.com/locate/bbalip

Review

Direct and indirect cholesterol effects on membrane proteins with special focus on potassium channels

Florina Zakany[a,b,1], Tamas Kovacs[a,1], Gyorgy Panyi[a], Zoltan Varga[a,*]

[a] Division of Biophysics, Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen, Egyetem ter 1, Debrecen H-4032, Hungary
[b] Doctoral School of Molecular Medicine, University of Debrecen, Egyetem ter 1, Debrecen H-4032, Hungary

ARTICLE INFO

Keywords:
Cholesterol
Direct cholesterol effects
Indirect cholesterol effects
Ion channels
Transporters
Receptors

ABSTRACT

As described in the literature the interaction between cholesterol and membrane proteins can occur via direct, ligand-like and indirect mechanisms, in which cholesterol effects are mediated by alterations in the biophysical properties or in the protein-organizing functions of the lipid membrane. Early studies emphasized the importance of indirect and raft-mediated effects, but improvements in computational and structural imaging techniques allowed the definition of a wide range of functionally active cholesterol binding domains and sites suggesting the relevance of direct cholesterol effects in various proteins. However, the intramolecular rearrangements induced by cholesterol leading to modulation of ion channel gating, membrane transport and receptor functions still have not been revealed. In this review we summarize the novel findings of the topic by focusing on recent studies about direct and indirect effects of cholesterol on potassium ion channels, and we extend the review to transporters and receptors with different domain structures to introduce the general mechanisms of cholesterol action among membrane proteins. We propose that rather than pure direct or indirect effects, cholesterol action on membrane proteins can be better described as a mixture of indirect and direct interactions with system-specific variability in their contributions, which can be explored by using a multi-level approach employing multiple experimental techniques.

1. Introduction

Cholesterol is a major factor in influencing the biophysical properties of biological membranes and establishing their lateral heterogeneity, thereby determining the functional diversity of membrane microdomains (see also in Section 2). Cholesterol modulates the function of a wide range of proteins embedded in the membrane via specific and non-specific mechanisms. Due to its physiological and pathophysiological importance, the interaction between cholesterol and transmembrane proteins including ion channels, transporters and receptors has been widely investigated. Technical advances in structural imaging (cryo-EM, super-resolution imaging) and computational techniques (molecular dynamics simulations) can provide valuable new data to the field of protein and cholesterol interaction research leading to the revival of this topic.

In general, the molecular rearrangements resulting in the gating of ion channels, the transporting processes of transporters and the operation of transmembrane receptors are mediated through the permission and cooperativity of the surrounding lipid bilayer, thus all of these functions can be modified by membrane cholesterol. Previous studies have distinguished two ways of interaction between cholesterol and transmembrane proteins: direct, ligand-like interactions [1–31] and a broad collection of indirect mechanisms, in which cholesterol acts through influencing the biophysical properties of the membrane (fluidity, rigidity, thickness, lateral pressure, lipid order and dipole potential), the microdomain structure of the membrane or the trafficking and expression level of the proteins [2,19,32–48] (see details in Section 3).

In the classical examples of direct interactions, the effect is mediated by specific cholesterol binding domains or sites. Along with other techniques, the growing body of X-ray crystallography, cryo-EM and molecular dynamics data provide more accurate insight into the detailed structures of cholesterol binding motifs, domains or sites in Kir2 [49,50], GIRK or Kir3 [51,52], BK [53,54], K_V7 [55], TRP [8,56] channels, nicotinic acetylcholine receptors [10,57,58], $GABA_A$ receptors [59,60], Na^+-K^+ ATPase [61–63], ABCB1 [64], ABCG2 [65–67], rhodopsin [21], β_2-adrenerg receptors [13,68], $adenosin_{2A}$ receptors [16], P2Y12 receptors [24], metabotropic glutamate

* Corresponding author.
E-mail address: veze@med.unideb.hu (Z. Varga).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.bbalip.2020.158706
Received 30 December 2019; Received in revised form 19 March 2020; Accepted 30 March 2020
Available online 01 April 2020

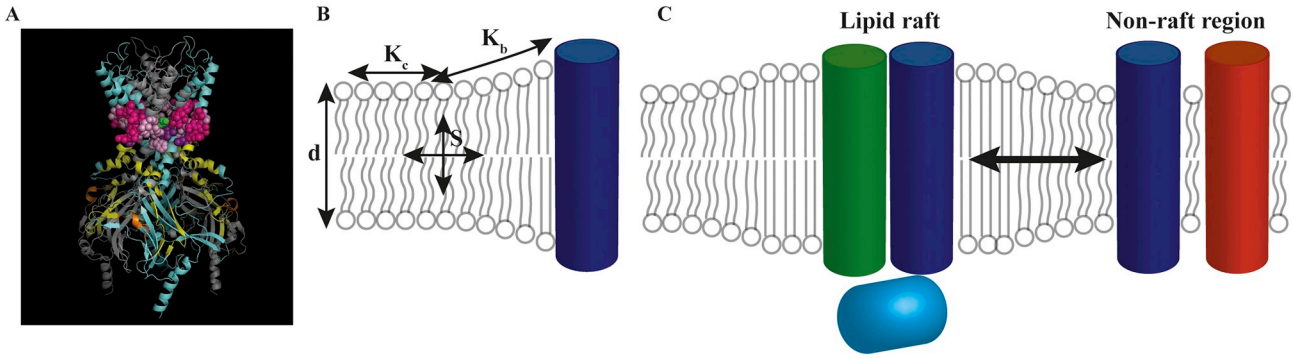

Fig. 1. Proposed mechanisms of cholesterol action on transmembrane proteins.
(**A**) As shown in the ribbon representation of a transmembrane protein (Kir2.2 ion channel, see details in Fig. 3), cholesterol can affect the structure and function through direct binding to cholesterol binding motifs, such as CRAC (*orange*), CARC motifs (*yellow*), or cholesterol binding regions and sites outside these motifs (*purple* and *pink*). (**B**) Alternatively, cholesterol could induce effects on transmembrane proteins (*dark blue*) via changing bulk membrane biophysical parameters, such as membrane order (*S*), thickness (*d*, as described by hydrophobic mismatch theory), or stiffness described by elastic compressibility (K_c) or bending modulus (K_b, according to curvature mismatch theory). (**C**) As the third level of action, cholesterol may change protein distribution between lipid rafts (thicker membrane) and non-raft microdomains of the cell membrane, thereby changing interaction efficiency with raft resident or associated molecules (*green* and *light blue*) and proteins excluded from rafts (*red*). In addition, protein expression levels may be regulated via the modulation of membrane trafficking.

receptors [22], CB1 endocannabinoid receptors [69], opioid receptors [14], serotonin receptors [17], Patched [25,26], Smoothened [27–29] and ErbB proteins [30,31]. In the last decade multiple cholesterol binding motifs were proposed including CRAC, CARC and CCM [70]. These have many practical limitations since the 3D structures of proteins are ignored in their definitions. Thus, their functional relevance may be highly overestimated [4,53,71]. On the other hand, these motifs may be just a subset of cholesterol binding regions, as cholesterol can also bind in a highly hydrophobic environment, fixed by the stacking interactions with aromatic residues and hydrogen bonding between its hydroxyl group and protein residues [7].

Many studies show that even in the absence of specific cholesterol binding domains the function of numerous proteins can be affected by membrane cholesterol loading, indicating the role of non-specific mechanisms as well [2,19,32–48]. In contrast to studying direct interactions, it is more challenging to distinguish experimentally between direct and indirect effects of cholesterol. Applying cholesterol chiral analogues that bind to ion channels without changing the biophysical properties of the membrane provides a suitable alternative to discriminate between the direct and indirect effects [2,49,54] (see details in Section 3.3). The intramolecular target of indirect effects has also been investigated; however, this should get more impact in the literature [9]. Changes in raft association have also been described for many membrane proteins, which can also be indirectly responsible for the complex cholesterol effects via placing the proteins into a new signaling environment, as shown for several voltage-gated cation channels [9,72–79], TRPM8 [80], nicotinic acetylcholine receptors [81], Na^+-K^+ ATPase [82], G-protein coupled receptors [83–89] and ErbB proteins [45,47,90,91].

Here, we wish to present a comprehensive view of cholesterol-membrane protein interactions and summarize the direct and indirect effects of cholesterol on major groups of potassium and other cation conducting ion channels, transporters and transmembrane receptors with distinct domain structures. Ion channels are suitable models to describe cholesterol effects, because the expansion of their transmembrane helices forming the ion conducting pore during their activation gating are mediated by the permission of the surrounding lipid bilayer. Changes in ionic current amplitudes, kinetics and sensitivity to voltage or agonists give easily quantifiable readouts of cholesterol induced changes on protein function. Interactions of cholesterol with potassium conducting ion channels (mainly K_V, Kir and BK channels), including direct and indirect effects, are extensively investigated, since their pore domains and voltage sensing domains (for K_V and BK) are also in contact with membrane lipids and undergo conformational changes, which are prone to cholesterol modulation. Therefore, these channels stand in the focus of interest of this review, but we also broaden our discussion to effects on other cation conducting ion channels, ATP-driven transporters and transmembrane receptors in the later sections of this work (see details in Sections 4 to 7) to introduce the possible general trends in the field of cholesterol-membrane protein interactions by analyzing and comparing cholesterol effects in membrane proteins with highly different transmembrane regions. We review the most significant results gained by electrophysiological, microscopic, computational, molecular dynamics simulations, spectroscopic and structural imaging techniques and summarize the diverse array of mechanisms suggested to be responsible for the observed effects. In most cases a sharp differentiation between direct and indirect effects seems arbitrary; instead a spectrum and mixture of these effects are likely to act in synchrony to influence membrane protein functions. Thus, we propose that wide-ranging multi-technique (functional, computational, imaging) approaches should be used to reveal the multiple levels of possible parallel direct, indirect and lipid raft-mediated cholesterol effects on a given protein, which may resolve the many contradictions currently found in studies mostly focusing on one level of cholesterol action (see also in Summary and conclusion). With this review we would like to provide motivation for further investigations in the field of cholesterol-membrane protein interactions, which offers numerous perspectives for structure-function and pharmacological studies, and we wish to emphasize the importance of investigating indirect cholesterol effects and the identification of their intramolecular targets. This knowledge can contribute to the deeper understanding of the pathomechanism of many diseases, in which one of the major alterations is the increased or altered cholesterol level of the cell membrane, as observed in various tumors, metabolic, neurodegenerative, immunological disorders or aging [92–99].

2. Cholesterol in membranes

2.1. Chemical structure of cholesterol

Cholesterol is an important structural component of vertebrate cellular membranes, which contains proteins performing a wide variety of functions, including ion channels, ATP driven pumps, ABC transporters, G protein coupled receptors or receptor tyrosine kinases, which can be subject to modulation by cholesterol. Its widespread biological effects can be attributed to the great abundance of interactions with lipids and proteins, which is made possible by its unique amphipathic chemical structure. While its small polar portion due to a single

hydroxyl group can form a hydrogen bond with a polar group of a membrane lipid or protein, its larger remaining apolar rigid tetracyclic ring system displays a planar α face that can bind to aromatic side chains of proteins through C-H-π interactions and a rough β surface with several aliphatic groups that may interact with side chains of branched amino acids of proteins through van der Waals interactions [70,100].

2.2. Biosynthesis and trafficking of cholesterol

The required amount of cholesterol is provided by de novo intracellular synthesis or uptake from circulating lipoproteins. Main steps of cellular biosynthesis include the conversion of acetyl-CoA into 3-hydroxy-3-methylglutaryl-CoA (HMGCoA) and subsequently into mevalonate, followed by formation of the parental steroid lanosterol, which is then converted into cholesterol via the Block or the Kandutsch-Russell pathway. Defects in main enzymes of these pathways result in human diseases (desmosterolosis and Smith-Lemli-Optiz syndrome, respectively). The HMGCoA reductase catalyzing the rate limiting step of cholesterol biosynthesis is the target of negative feedback regulation, since increases in cholesterol or oxysterol levels lead to its decreased activity through changes in rates of transcription and/or degradation of the enzyme. Consequently, HMGCoA reductase is the target of statins, the most commonly used drugs to lower cholesterol levels [92]. Since enzymes of the later steps are ER resident proteins, cholesterol is synthesized in the ER from where it is rapidly distributed to cellular membrane compartments by transport vesicles and non-vesicular mechanisms. Cells can also import cholesterol through receptor-mediated endocytosis of lipoproteins, followed by hydrolysis of cholesteryl esters in acidic endocytic compartments and subsequent export of the unesterified form into other organelles [92,93,101]. Cholesterol is distributed heterogeneously between cellular membranes with the highest abundance in the plasma membrane (typically 25–40% of total lipids, accounting for 40–90% of total cellular cholesterol) and high levels in the endocytic recycling compartment and the trans part of Golgi [94,101]. On the other hand, concentrations in the ER and mitochondria are low, yet these control cellular functions, such as protein transport, cell survival or regulation of cholesterol metabolism [94,101,102]. Imbalances in cholesterol trafficking and homeostasis are frequently observed in cancer, metabolic, neurodegenerative or immunological disorders [92–94].

2.3. Transbilayer distribution of cholesterol

Cholesterol is present in both the inner and outer leaflets of the cell membrane; however, its exact transbilayer distribution is controversial [103]. Although its high transbilayer diffusion (flip-flop) rate might suggest homogeneous vertical distribution [104], its interactions with lipids or proteins in either leaflet would result in asymmetrical cholesterol levels. While exofacial localization was proposed initially because of its preferential interaction with sphingomyelin that resides almost exclusively in the outer membrane leaflet [105], most of the recent studies demonstrated higher cholesterol levels in the cytoplasmic membrane layer [106,107]. Localization of cholesterol in the inner leaflet could be explained by its preference towards membrane regions with high curvature to reduce bending free energy penalty due to the presence of phosphatidylethanolamine [108]. In striking contrast, a recent study with tunable orthogonal cholesterol sensors showed 12-fold higher cholesterol levels in the exofacial membrane layer, which depended on the activity of ABC transporters [109]. However, the reliability of the study was questioned recently [110]. Alterations in the transbilayer distribution of cholesterol may cause disturbances in cellular functions, as suggested recently [109].

2.4. Lateral distribution of cholesterol and the lipid raft hypothesis

The lateral distribution of cholesterol is not homogeneous in the cell membrane, which results from its interaction with various membrane components. Transmembrane proteins are surrounded by a shell of lipids characterized by restricted mobility, often referred to as the lipid annulus. Depending on the relationship with the annulus, cholesterol can be localized in the nonannular areas (buried within a protein), annular regions (in the neighboring lipid shells on the surface of the protein) or in the bulk phase of the membrane outside lipid shells [111]. Due to its unique chemical structure, cholesterol has a strong preference to bind to sphingolipids and glycosphingolipids. When compared to glycerolipids, (glyco)sphingolipids are more saturated and capable of formation of hydrogen-bonded networks because of their amide and hydroxyl groups serving as both acceptors and donors for these bonds. As a result, (glyco)sphingolipids tend to associate with each other, however, the bulk of headgroups limits packing density [100]. According to the “umbrella model”, this is favorable for cholesterol, since it can localize into the area between sphingolipids, thus, the nonpolar part of cholesterol can be shielded by large headgroups to avoid the unfavorable free energy of cholesterol contact with water [112]. The interaction between cholesterol and (glyco)sphingolipids might be further augmented through formation of stoichiometric “condensed complexes”, facilitating formation of large clusters [113]. This preferential interaction between lipids was the basis of the initial lipid raft hypothesis, i.e. formation of thermodynamically unstable dynamic clusters of the size of 10–200 nm characterized by increased levels of cholesterol, phospholipids with saturated chains and (glyco) sphingolipids and higher packing density of lipids [114]. The lipid raft hypothesis was questioned from its inception, as reviewed recently [115], which eventually led to novel extended raft definitions emphasizing the active contribution of proteins, in particular the actin cytoskeleton to the formation of these microdomains. According to the most widely accepted current view, the association potential between sphingolipid and cholesterol molecules creates the basis for the core subcompartmentalization propensity that can be precisely and actively modulated by transmembrane proteins and the actin cytoskeleton to coalesce and form functionally active platforms through cooperation of membrane order and specific chemical interactions [97,116–118]. Molecules can dynamically move in and out of these microdomains, however, certain proteins show higher affinity to these microdomains due to the presence of a glycosylphosphatidylinositol (GPI)-anchor, palmitoylation and/or myristoylation, sterol conjugation, the features of the transmembrane domain or the presence of cholesterol binding motifs [119]. Rafts can form concentrating platforms for interacting molecules, while excluding others, which can efficiently modulate signaling pathways, regulate apoptosis, cell adhesion and migration, synaptic transmission, cytoskeletal organization, protein sorting, pathogen entry, formation of amyloid plaques or extracellular vesicles. Alterations in the distribution of proteins between raft and non-raft domains may result in functional changes contributing to the pathomechanism of cancer, immunological, metabolic or neurodegenerative disorders [95–99,120]. Consistently, functionally relevant raft localization was demonstrated in a great variety of transmembrane proteins, such as ion channels including $K_V1.3$ [9], $K_V1.4$ [72], $K_V2.1$ [73], $K_V4.2$ [72], $K_V7.1$ [74], $K_V10.1$ [9,75], $K_V11.1$ [76], $Na_V1.8$[79], $Na_V1.9$ [78] and TRPM8 [80], and in other transmembrane proteins as nicotinic acetylcholine receptors [81], Na^+-K^+ ATPase [82], β_2-adrenerg receptors [85,89], CXCR4 [86], metabotropic glutamate receptors [83,84], CB1 endocannabinoid receptors [87], opioid receptors [88] and ErbB proteins [45,47,90,91].

3. Basis of cholesterol and transmembrane protein interactions

3.1. Direct interactions between cholesterol and proteins: cholesterol binding motifs, sites, regions and domains

The first motif suggested to bind cholesterol in transmembrane proteins was the Cholesterol Recognition Amino Acid Consensus (CRAC) motif, which consists of a loosely defined sequence of amino acids: ((L/V)-X_{1-5}-(Y)-X_{1-5}-(K/R)), where X represents any amino acid [70,121–123]. Later the family of cholesterol binding motifs has been extended to the reverse sequence, named CARC ((K/R)-X_{1-5}-(Y/F)-X_{1-5}-(L/V)) [70,123,124], which seems to be more favorable energetically for cholesterol binding in ion channels [70]. In contrast to the previous two the third Cholesterol Consensus Motif (CCM) can be interpreted in the presence of neighboring subunits where (K/R)(I/L/V)(W/Y) is located on one helix and (F/Y/W) on an adjacent helix [68,70,123]. These motifs can be found in many proteins like K_V [1], BK [125–127], Kir [128], $Na_V1.9$ [78], TRPV1 [56], TRPV4 [129] channels, nAchR [124], ABCG1 [130], ABCG2 [12] and a great variety of G protein coupled receptors [131]. These motifs are located both in the transmembrane and/or cytoplasmic domains and in many cases they are the main determinants of cholesterol effects for the given protein (see later in specific sections). The common in all three motifs is that the amino acids can interact with cholesterol by hydrophobic interactions (alkyl group of cholesterol with isoleucine, leucine and valine in motif), hydrogen bonds (hydroxyl group of cholesterol with positively charged arginine, lysine) and stacking interactions (between the ring of cholesterol and aromatic hydrophobic amino acids in the motif) [7]. In the past few years several studies raised criticism against the domination of these motifs in the explanation of cholesterol effects, including the following arguments [53,71]. 1. CRAC and CARC are defined as a linear sequence of amino acids thus they have many limitations in the 3D structured proteins, they cannot account for intra- and intersubunit binding sites. 2. In some cases these motifs can be found in the cytosolic domains of ion channels as in K_V [1], Kir [49,128], BK [126], or P2X [132], which makes it unlikely that they mediate cholesterol effects since it is an integral membrane lipid. In spite of this, it has been shown for K_V [1] and BK [126] channels that these cytosolic motifs are crucial in sensing cholesterol because the removal of the cytosolic ends of these channels disrupts the well-known cholesterol effects. 3. A novel study applying computational and experimental analyses led to a hypothesis that these three motifs are just a subset of cholesterol binding protein regions responsible for cholesterol effects [7]. Thus, the definition of the general features for cholesterol binding has been expanded and emphasizes the presence of a highly hydrophobic environment where cholesterol can be stabilized by stacking interactions between its ring and hydrophobic aromatic residues in the protein and hydrogen bonding between the hydroxyl group of cholesterol and different amino acids. In that way the location of cholesterol binding amino acids is not limited to a linear sequence inside the protein, which eliminates one of the major limitations of CRAC and CARC motifs [7]. 4. It has been shown that in Kir2.1 the location of the cholesterol binding motifs and the plausible regions for cholesterol binding determined by MD simulation and docking studies do not overlap. Based on these studies two cholesterol sensing regions can be defined in Kir [128,133], in which different binding sites have been revealed using coarse grained simulations [50] (discussed in Section 4.2). The finding that numerous CRAC and CARC motifs are non-functional has led to the definition of cholesterol sensing protein regions in the case of more transmembrane proteins like for nAchR [134,135], GABAA [59], ABCG2 [66,67], GPCRs [136–142], which are independent from these well-known cholesterol binding motifs. Besides cholesterol sensing motifs, regions or sites, in some cases distinct domains are responsible for cholesterol binding, such as the sterol sensing domain of Patched protein [143].

3.2. Indirect interactions between cholesterol and proteins: the role of biophysical properties of the membrane

3.2.1. Membrane order and hydration

Besides direct interactions, cholesterol can affect protein function through alterations in bulk membrane biophysical parameters, which had been among the most popular topics of membrane research for a long time. Calorimetry based model calculations and NMR spectroscopy showed early that cholesterol exerts a dual effect on lipid order in model membranes by disturbing it in the solid phase, while promoting it with relatively small changes in lateral molecular motility in the liquid phase, which may result in the formation of “liquid-ordered” membrane regions [144,145]. MD simulations and wide-angle X-ray scattering measurements demonstrated that ordering is related to the stretching of acyl chains by increasing the relative amount of *trans* torsion angles in the chain at the expense of *gauche* conformers and/or changes in the average tilt of hydrocarbon chains, accompanied by decreases in the average cross-sectional area per lipid molecule [146–148]. The ordering effect of cholesterol (and lanosterol or epicholesterol but not coprostanol or cholestenone) and subsequent increases in the degree of motional constraints (decreases in membrane fluidity) were confirmed in model membranes by increases in the fluorescence anisotropy of the environment sensitive probe DPH [149]. In Langmuir isotherm measurements the enantiomeric form of cholesterol (ent-cholesterol) induced effects that were similar to those of native cholesterol [150], while desmosterol and 7DHC resulted in slightly reduced condensing [151], probably due to increased sterol tilt angles from the membrane normal [152]. The positive correlation between membrane order and cholesterol levels was also demonstrated in living cells using environment-sensitive fluorophores and emission ratiometric [153] or lifetime imaging [154] and determination of generalized polarization [155].

Along with ordering, cholesterol also changes membrane hydration, a strongly related property of bilayers by displacing water molecules from intermolecular void spaces under phospholipid head groups. This was demonstrated in model bilayers by ESR spin labeling [156] and spectrofluorometric determination of Laurdan generalized polarization [157], and in living cells by emission ratiometric imaging of hydration-sensitive probes [158], time-resolved generalized polarization analysis of Laurdan using FLIM [159] and superresolution microscopy [160].

3.2.2. Membrane thickness and hydrophobic mismatch

Consistent with induced stretching of lipid acyl chains, increasing cholesterol levels resulted in increased hydrophobic thickness of model bilayers, as observed with X-ray diffraction [161], calculations based on order parameters from NMR spectroscopy [162] and MD simulations [148]. Furthermore, bilayer thickness was shown to depend on the presence and particularly the hydrophobic length of transmembrane peptides [162,163]. In X-ray diffraction studies, ent-cholesterol exerted similar effects on bilayer thickness [164], while the effects of ergosterol were significantly different [165]. Consistently, solution X-ray scattering demonstrated that the thickness of membrane preparations from exocytic pathway components depended on cholesterol and mainly on transmembrane protein levels [166].

Differences between the hydrophobic lengths of phospholipids building liquid-disordered (non-raft) and liquid-ordered (raft) membrane domains may create significant interfacial energies termed line tension, at boundaries between membrane regions, which originates from the unfavorable exposure of hydrophobic parts to an aqueous environment. As shown with AFM, small-angle neutron scattering, NMR spectroscopy, time-resolved fluorescence spectroscopy and MD simulations, the magnitude of the difference between hydrophobic lengths and the resulting line tension substantially regulates the formation and

size of ordered microdomains in model membranes, and presumably those of lipid rafts in living cell membranes, in a manner significantly dependent on cholesterol levels [167–169]. Thus, cholesterol modified thickness mismatch between different lipid species may contribute to the formation of raft domains in the cell membrane.

Besides lipid-lipid interactions, lipid-protein interactions are also regulated by differences between the hydrophobic length of lipids and proteins, referred to as hydrophobic mismatch. The effect of mismatch was first described by the "mattress model", according to which the free energy of the lipid-protein system is the sum of a mixing entropy term and the lipid-protein interaction energy due to van der Waals interactions, the hydrophobic effect associated with mismatch and subsequent exposure of hydrophobic regions to water and the elastic deformation free energy of lipid chains [170]. The presence of rigid proteins causes perturbations in bilayer structure resulting from changes in packing and conformation of lipid tails and an interfacial component due to alterations in headgroup repulsion and hydrocarbon-water surface tension accompanying curvature deformations. Although the free energy term is positive even in cases of optimal hydrophobic matching due to conformational ordering of lipids, it is minimal, since conformational changes to accommodate proteins with hydrophobic mismatch lead to more positive values. Higher protein hydrophobic thickness (when compared to that of lipids, called positive mismatch) can lead to stretching of acyl chains with additional unfavorable decreases in entropy, while lower protein thickness (negative mismatch) may result in large increases in interfacial free energy components [111,171].

Thus, a difference in hydrophobic lengths of proteins and lipids induces adaptation mechanisms to decrease mismatch. These include changes in proteins as aggregation or oligomerization to minimize the exposed hydrophilic area, tilt of TM helices, adopting other conformations or slightly adjusting the hydrophobic length of proteins by changing the orientation of interfacial side chains; and alterations in lipid configurations as changing the stretching of acyl chains or aggregation into preferential assemblies as formation of membrane microdomains [111,172,173]. The presence of cholesterol is likely to influence matching mechanisms, thereby changing protein-lipid and protein-protein interactions. For example, lower membrane thickness in a cholesterol-poor lipid environment could favor protein conformations associated with smaller hydrophobic lengths of transmembrane domains. On the other hand, increased membrane thickness in a cholesterol-rich environment as observed in lipid rafts may induce conformational changes leading to conformations characterized by longer hydrophobic transmembrane regions or in cases of high degrees of mismatch alternative adaptation mechanisms may occur, such as protein aggregation, oligomerization or association with other proteins. Since different protein configurations are associated with different activities, cholesterol-induced changes in the relative distribution of various conformations can lead to significantly modified functional activities of transmembrane proteins [174,175].

Consistently, the functions of various membrane proteins are modulated by cholesterol-dependent changes of membrane thickness and the resulting altered degree of hydrophobic mismatch, as shown for BK channels [176], voltage gated sodium channels [177], nicotinic acetylcholine receptors [33], Na^+-K^+ ATPase [178], rhodopsin [42], metabotropic glutamate receptors [39], β_2-adrenergic receptors [41], serotonin receptors [19] and ErbB proteins [48].

3.2.3. Membrane curvature and elastic stress

Curvature of the membrane and the resulting curvature elastic energy is a property of lipid bilayers related to but distinct from hydrophobic matching. Attractive and repulsive forces (the lateral pressure profile) tend to be balanced between membrane lipids, resulting in an energetically favorable equilibrium distance between the molecules. At the level of headgroups attractive interactions include a hydrophobic effect (due to unfavorable contact of hydrocarbon chains with water) with hydrogen bonds between headgroups, while repulsion is due to hydration, steric and electrostatic effects. In the region of acyl chains, attraction originates from van der Waals interactions between methyl groups and repulsion is mainly derived from lateral pressure due to thermally activated dihedral angle isomerization. When the equilibrium separation distance is different in the two regions, spontaneous curvature may occur being negative when the equilibrium separation between headgroups is smaller, and positive when it is larger than that between chain regions. However, when spontaneous curvature is not fulfilled due to steric constraints (for example due to hydrophobic mismatch with proteins), at least one region is not at free energy minimum resulting in curvature elastic stress (frustration) and free energy. Depending on their conformation, proteins could decrease or increase curvature elastic stress of lipids, and, vice versa, the intrinsic curvature of lipids may influence the proteins as well. Thus, this elastic coupling between proteins and lipids may increase or decrease the stability of a given protein conformation or lead to aggregation, thereby influencing protein functions, as summarized in the flexible surface model [38,111,179,180].

In line with ordering effects, the presence of cholesterol (and to a lesser extent lanosterol and ergosterol) induces increases in the interfacial elastic area expansion/compressibility moduli of model membranes implying increased stiffness of the bilayer [181,182]. Increased cholesterol level induces significant redistributions in the lateral pressure profile (depth-dependent distribution of lateral stresses within the membrane mainly arising from repulsion between neighboring regions of lipids) [183,184]. Besides these in-plane effects, cholesterol was shown to largely increase the elastic bending modulus of bilayers, a parameter strongly related to membrane thickness and area expansion modulus [185]. Cholesterol precursors desmosterol and 7DHC induce qualitatively similar but reduced effects on lateral pressure redistribution and bending rigidity [186]. Furthermore, cholesterol is characterized by a spontaneous curvature much larger than that of most phospholipids [187], thus, it can subsequently change radii of the intrinsic curvature of membranes [188] and influence its own transbilayer distribution [189]. Consistently, cholesterol was shown to be accumulated in high curvature membrane regions of model vesicles [190], which can be further augmented by its caveolin induced clustering in these membrane areas [191]. The bending rigidity of membranes can be also modified in the presence of integral membrane proteins [192]. Altogether, cholesterol induced changes in these membrane biophysical parameters can substantially alter the mechanical work required for the conformational changes of the proteins thereby changing the stability of a certain protein configuration or affecting clusterization, especially when the shape of the transmembrane cross section of the protein differs between conformations [180,193]. This was shown in rhodopsin [38,43], serotonin receptors [40], ErbB proteins [48] and the recently identified mechanosensitive Piezo channels [194].

3.2.4. Membrane dipole potential

The least-known component of membrane potentials (besides transmembrane and surface potentials), the dipole potential (DP) originates from the preferential non-random alignment of molecular dipoles of carbonyl groups, cholesterol and water molecules at the membrane-water interface. The arrangement of dipoles generates a large positive (150–450 mV) potential that drops over a very short distance, resulting in an immensely strong intramembrane electric field, which is far stronger than that of the transmembrane potential (10^8–10^9 vs 2.5×10^7 V/m, respectively) [195–197]. The magnitude of DP is mainly determined by factors related to lipid composition of the membrane, such as lipid packing density and the chemical types of phospholipids [198,199]. However, cholesterol is the most important determinant, since it was found to largely increase the magnitude of DP (at least by 65–100 mV), which originates from its intrinsic dipole moment, increases in lipid packing order, changes in the value of the dielectric constant in the membrane or alterations in water penetration

into the membrane in response to cholesterol [200–202]. Ent-cholesterol was shown to induce similar changes in DP as native cholesterol, while the effects of epicholesterol were much smaller [203]. Furthermore, resulting from the lateral heterogeneity of lipid composition and cholesterol in particular, the value of DP is higher in raft microdomains than in the bulk phase of the cell membrane [204].

As a consequence of the enormous electric field generated by DP and the mostly non-uniform distribution of charges in proteins, DP is expected to play a role in modulating the conformational stability, thus the function of membrane proteins [195,205,206]. Consistently, the magnitude of DP can influence a multitude of membrane-coupled events, including the association and function of bacteria-derived ionophores [207], membrane binding of drugs [208] or β-amyloid [209], gating mechanisms of voltage-gated ion channels [210], the activity of Na^+/K^+-ATPase [34,211] and ABCB1 [35], ligand affinity of serotonin receptors [37,44] and ligand-induced clustering and activation of ErbB receptors [45].

3.3. Differentiation between direct and indirect cholesterol effects on membrane proteins

While the functional and electrophysiological effects of membrane cholesterol loading are well documented for many ion channels, transporters and transmembrane receptors, discrimination between the mechanisms of its action (direct and indirect cholesterol effects) is still complicated. It is difficult to separate the effects of direct binding from the simultaneous modifications caused in biophysical properties of the membrane by sterols.

Using cholesterol chiral analogues, epi- and ent-cholesterols, this problem can partially be resolved. Epicholesterol only differs from cholesterol in the 3α position of its hydroxyl group of C3 (3β for cholesterol), while ent-cholesterol is the mirror image of cholesterol with an opposite configuration at each of the eight stereocenters of cholesterol [2,149,212]. These differences establish major changes in their 3D structures compared to cholesterol, which can result in changes in their functional effects on proteins without altering basic membrane properties [2,36,49,149,212–214]. Stereospecific effects, when a chiral analogue has a different or opposite effect on protein function from cholesterol, have been described for Kir [215,216], TRPV1 [56], BK [217], $GABA_A$ [218] receptors, while for nAChR cholesterol regulation happens through a non-stereospecific manner, thus cholesterol and its isomers exert the same functional effects [135]. According to early studies, stereospecific (opposite or different) effects are mostly attributed to the direct binding of cholesterol to proteins, while non-stereospecific (similar) effects suggest the indirect mechanism of cholesterol action. Recent studies revealed many limitations of the application of chiral analogues. As described for nAchR, despite the same effects of cholesterol and its chiral analogues on protein function, a direct interaction has been proposed between nAchR and cholesterol/chiral analogues mediated by a “lax cholesterol binding site” [219,220] (see later in Section 5.4). In that way the lack of stereospecificity cannot exclude the existence of direct protein-cholesterol interactions as suggested before, thus the application of stereoisomers is more sensitive to discriminate between the direct and indirect interactions if cholesterol and its isomers have different or opposite effects on protein functions, which indicates a probable direct cholesterol-protein interaction [2,49]. Second, in Kir channels where cholesterol and its isomers have stereospecific effects (cholesterol: decrease in current, epicholesterol: increase in current, ent-cholesterol: no effect on current) it has been shown that the binding site is the same for these molecules, just the orientation of the bound molecules is different, which results in no or opposite effects when compared to cholesterol [2,49,50] (see later in Section 4.2).

Finally, due to the differences in the 3D structures of cholesterol and its isomers it has been shown that their vertical positions in the lipid bilayer are not identical. Due to its mirrored structure, the position of ent-cholesterol is almost identical to that of cholesterol, while epicholesterol is located in an upward shifted and tilted manner compared to them [2,221,222]. This results in moderate changes in membrane lipid order in the bilayer and can also alter the interactions with membrane phospholipids, which is modulated in a stereospecific way [213]. Thus, the impact of chiral analogues on membrane parameters requires further elucidation [2]. In spite of the disadvantages specified above, applying cholesterol chiral isomers is still one of the most feasible methods to distinguish direct and indirect cholesterol-protein interactions, because of their easy application and their compatibility with a wide range of membrane proteins.

The two-electrode voltage-clamp fluorometry (TEVCF) technique provides a unique combination of electrophysiological and fluorescence techniques. During its application, typically the voltage-sensor domain (VSD) of the voltage-gated channel is labeled with an MTS-conjugated fluorescent die via a cysteine introduced in the sequence, while the current through the channels is measured by electrodes, informing about the pore domain (PD). The rearrangements of these domains can be simultaneously monitored throughout the gating process, thus the major intramolecular target (VSD or PD) of cholesterol can be revealed [223]. Although TEVCF data cannot discriminate between direct and indirect interactions, it can yield important information about the intramolecular target of cholesterol at the functional domain level, even in the lack of knowledge about specific cholesterol binding motifs, sites or domains. This method was successfully applied in the case of investigating the mechanism of cholesterol action on $K_V1.3$ and $K_V10.1$ [9] and for determining the intramolecular targets of different PUFAs [224].

Recent improvements in cryo-EM and molecular dynamics simulation techniques have provided powerful data for identifying and exploring new cholesterol binding sites in proteins to expand the library of direct cholesterol-protein interactions. While these techniques can provide strong support for the direct interactions they cannot unequivocally prove them and still need experimental verification, for example by introducing mutations in the putative binding sites to perturb the interaction with cholesterol, as described for certain proteins below.

4. Effects of cholesterol on potassium conducting ion channels

4.1. Voltage-gated potassium channels (K_V)

Voltage-gated potassium channels (K_V) exhibit the classical structure of voltage-gated ion channels. Channels are built up by four alpha subunits held together by noncovalent bonds, where each subunit consists of six transmembrane helical segments connected by intra- and extracellular loops [225,226] (Fig. 2**A**). Because the general structural and functional phenotype is highly conserved within K_V channel families, they can form functional heterotetramers, which is the basis of their broad tissue distribution and specific roles in controlling a wide range of cellular processes. $K_V1.x$–$K_V4.x$, $K_V7.x$, $K_V10.x$–$K_V12.x$ subunits can form homotetramers, while for $K_V5.x$, $K_V6.x$, $K_V8.x$, and $K_V9.x$ the obligatory channel forming partner is $K_V2.x$ [227]. The function of K_V channels is also fine-tuned in different tissues by accessory proteins like $K_V\beta$, KCHIP (K^+ channel interacting protein), KCNE, or calmodulin, which are connected to the channels mostly via their N- or C-terminal domains [228]. Functionally each subunit can be divided to two parts, where the S1–S4 helices play a role in forming the voltage-sensor domain while the S5–S6 helices form the pore domain in K_V channels (Fig. 2**A** VSD: purple and yellow, PD: blue). As it is indicated in many crystallographic and cryo-EM structures, the PD is in the central part of the protein with the ion conducting pore in its axis, while the VSD is located in an outer ring surrounded by the annular lipid bilayer.

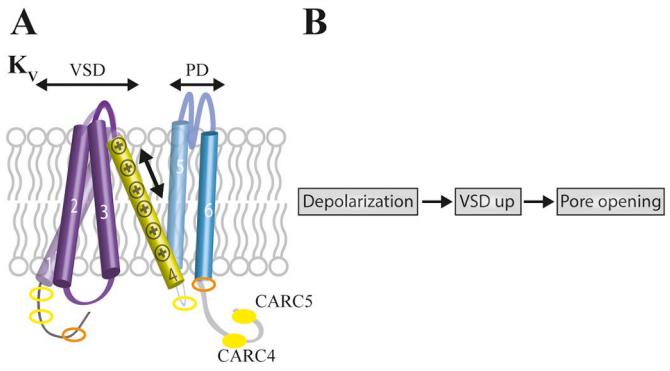

Fig. 2. Structure, gating and cholesterol binding motifs of K_v channels. (**A**) One alpha subunit of a K_v channel consists of six transmembrane segments, S1–S4 (*purple* and *yellow*) form the voltage-sensor domain (VSD) and S5–S6 (*blue*) form the pore domain (PD) of the channel. Panel A represents one subunit from a lateral view. The cholesterol binding CRAC and CARC motifs are represented by *orange* and *yellow* ovals, where experimentally confirmed, functionally active motifs are symbolized by filled ovals. (**B**) For K_V channels activation gate opening is determined by membrane depolarization (*black* arrow). The upward movement of the positively charged S4 segment is transmitted to the pore domain leading to activation gate opening.

The key structural elements of voltage-sensing in K_V channels are the positively charged amino acid residues in the S4 segments, which move upward in the plane of the membrane in response to membrane depolarization (Fig. 2**A**, yellow, Fig. 2**B**). This upward movement is transmitted to the PD and opens the activation gate formed by the intracellular ends of S6 segments [229]. Based on the coupling between the VSD and PD two mechanisms can be distinguished: the linear gating model where the communication between these two domains is tight [230], and a more complex allosteric model the connection is looser [231,232]. Long-term depolarization can induce N-type inactivation in certain K_V channels, occurring on a millisecond timescale, thus preventing the flow of ions through the pore [233]. Another mechanism, C-type inactivation, is structurally linked to the region of the selectivity filter [225,234], and occurs on a much slower time scale. Based on the phenotype of the generated currents K_V channels can be classified into two major groups: channels producing A-type currents, which quickly decay due to N-type inactivation, and the slowly or non-inactivating, inward rectifier channels. The first group mainly controls cellular excitability in neurons and muscle cells [235–237] while the second group is responsible for the repolarization phase of action potentials and for controlling essential processes in non-excitable cells (like antigen dependent activation of lymphocytes [238,239], or proliferation of cancer cells [240]).

The importance of cholesterol in K_V channel function was first proposed by studies describing the relationship between these channels and lipid rafts [241], as reviewed recently [120]. Preferential (or at least partial) raft localization was demonstrated for $K_V1.3$ [9], $K_V1.4$ [72], $K_V2.1$ [73], $K_V4.2$ [72], $K_V7.1$ [74], $K_V10.1$ [9,75] and $K_V11.1$ [76] channels, probably due to preferential matching of channel hydrophobic domains with raft-resident lipids, protein-lipid interactions or binding to raft-associated proteins, such as PDZ domain proteins [72,242] and caveolin [243] for $K_V1.3$ and $K_V1.4$ or accessory KCNE subunits for $K_V1.3$ [244] and $K_V7.1$ [74]. Raft localization has been suggested to be functionally relevant in $K_V1.3$ targeting to the immunological synapse [245] and subsequent Ca^{2+} signals of lymphocytes [246]. In general, raft localization was suggested to exert inhibitory roles on channel function [9,73,75,76]; however, facilitated clustering in these microdomains may promote non-canonical signaling roles of these channels [73,75].

The intimate and multiple connections between K_V channels and cholesterol-rich raft microdomains stimulated studies investigating interactions between cholesterol and K_V channels. In general, cholesterol effects are inhibitory for K_V channel functions. It has been shown that both cholesterol enrichment and depletion alters the voltage-dependent steady-state activation and inactivation kinetics of the channels, and activation and inactivation time constants for currents of $K_V1.3$, $K_V10.1$, $K_V1.5$ and $K_V2.1$ channels [9,241,247,248]. In addition, a decrease in current amplitudes was also described upon cholesterol loading but, in contrast to Kir and BK (see later), its reason was found to be the decrease in the unitary conductance rather than in the open probability of single channels in $K_V1.3$ and $K_V10.1$ as revealed by non-stationary current noise analysis [9]. Similarly to several other voltage-gated channels the G-V curves of the wild-type $K_V1.3$ and $K_V10.1$ channels shifted rightward in response to cholesterol loading (decreased channel activity). $K_V10.1$ demonstrates a pronounced Cole-Moore shift, which manifests as altered current activation kinetics when depolarization occurs from different holding potentials, which cause a different population of the closed states [249,250]. Recently it has been shown that only the opening transition is slowed by sterols, but the rate-limiting transitions among deep closed states are not [9]. Thus, despite the movement of the VSD across the lipid bilayer its kinetics is not significantly affected by sterols. In contrast to the K_V channels mentioned above the $K_V4.2$ channel is inhibited by cholesterol depletion without affecting its voltage-dependent properties [251]. In $K_V7.2/K_V7.3$ channels it has been proposed recently, that an optimal cholesterol level is essential for proper channel functions: not only cholesterol depletion but interestingly, cholesterol enrichment of the membrane decreased ionic currents as well [252]. All other K^+ conducting channels reported so far exhibit opposite changes in channel functions due to cholesterol depletion and enrichment, which may be explained by the direct perfusion of the cells with MβCD-cholesterol during the patch-clamping by the authors, rather than the typical long-term incubation. The resultant current decrease was described as a prompt effect of the treatment, but it is questionable whether a significant quantity of cholesterol can be incorporated into the membrane during such a short timescale.

Cholesterol effect on slowing the activation gating of $K_V1.3$ first was proposed to be the effect of the increased membrane viscosity due to membrane cholesterol enrichment, where the increased frictional force retards the conformational changes in the VSD upon channel activation [247]. Later, cholesterol effects on the gating parameters of K_V channels have been ascribed to direct interactions between cholesterol and channel proteins. The major mediators were thought to be the CRAC and CARC motifs because they generally appear in K_V channel sequences. The sequence of $K_V1.3$ contains a total of two CRAC and five CARC motifs, where the CRAC motifs and CARC1-3 are located in the N-terminal domain or in the transmembrane helices, and the last two CARCs are in the C-terminal end of the channels, located intracellularly [253] (Fig. 2**A** orange and yellow ovals). It has been shown that removal of the C-terminal end of the channels, including CARC4 and 5, does not have any significant effect on the gating parameters [242] while totally abolish cholesterol induced electrophysiological changes [253] (Fig. 2**A** yellow filled ovals). According to the proposed mechanism, these two intracellular CARC motifs can interact with cholesterol located in the membrane through their hydrophobic residues, which results in changes in the gating parameters of the channels. Although the same mechanism has been proposed in BK (see later) and P2X7 [254] channels, this hypothesis has not been verified experimentally in K_V channels.

Previously, we aimed to determine the intramolecular target of cholesterol in K_V channels. Simultaneously acquired signals from the voltage-sensing and pore domains by TEVCF during gating indicated that the main target of cholesterol is the PD itself, rather than the VSD both in $K_V1.3$ and $K_V10.1$ (linear and complex coupling models) [9]. We proposed a model where the main contributing factor in decreasing single channel conductance and slowing the activation kinetics of currents is the increase in lateral stress of the membrane due to the cholesterol loading, which then hinders the opening transition and reduces

the conductance by distorting the inner vestibule of the channels. Even though in our model we emphasized the role of indirect cholesterol effects on the family of K_V channels, the role of direct interactions cannot be ruled out from the global scheme of cholesterol action, because not all observed gating parameters have shifted in parallel, as would have been expected assuming a general indirect effect. However, in accord with other channels and proteins discussed later, the significance of CRAC and CARC domains in mediating direct cholesterol effects in K_V channels has been also questioned. The C-terminal end of the S4 helix includes a CARC sequence without any functional consequence on the VSD movements during channel activation following cholesterol loading. This is supported by the lack of changes in the voltage-dependence of fluorescence signals during TEVCF measurements.

4.2. Inward rectifier potassium channels (Kir)

Inward rectifier potassium channels (Kir) are built up by four subunits, but in contrast to K_V channels, each subunit consists of only two transmembrane helical segments (TM1 or outer helix, and TM2 or inner helix), which are linked by the potassium selective pore loop forming the ion conducting pore (Fig. 3**A**). Each subunit has intracellular N- and C-terminal domains, which are responsible for the fine tuning of channel gating [255,256]. In the lack of VSDs the gating of all eukaryotic Kir channels is determined by the binding of phosphatidylinositol-4,5-bisphosphate (PIP_2) located in the inner leaflet of the plasma membrane [257], which activates the channels and allows potassium conduction [256,258–260]. The mammalian Kir channels are classified into seven subfamilies (Kir1-7) based on their biophysical properties (like degree of current rectification, unitary conductance), organ distribution and different regulation of activation (ATP, G-proteins, pH, etc.) [255,261]. Due to high sequence similarities, Kir channels can form homo- and heterotetramers with each other, except for Kir5.1, which exists only as a heterotetramer [255,260]. Based on specific gating regulatory mechanisms, the seven subfamilies form functional subgroups like classical Kir channels (Kir2.x), G protein-gated Kir channels (Kir3.x or GIRK), ATP-sensitive K^+ channels (Kir6.x) and K^+ transport channels (Kir1.x, Kir4.x, Kir5.x, and Kir7.x) [255]. Their main function is to control cellular excitability in many tissues like brain, heart, kidney, skeletal and smooth muscle cells [260,262], regulating vascular smooth muscle [263] and regulation of insulin secretion [256,262]. Almost all members of the Kir family have been reported to colocalize with lipid rafts in the membrane [255,264–266].

A vast number of articles emphasize the effects of membrane cholesterol on the gating of Kir channels. Similarly to most potassium conducting ion channels, the effect of cholesterol on most of the Kir channels is suppressive, which means that cholesterol loading decreases channel activity, whereas membrane cholesterol depletion results in its increase. This relationship was observed for the homomeric Kir1.1, Kir2.1, Kir4.1, Kir3.1 F137S pore mutant and Kir6.2Δ36 [260,267–270] ion channels. Interestingly the opposite effect has been described for the Kir3.1/3.4 [271] and Kir3.1/3.3/3.4 [272,273] heterotetramers, and the homomeric Kir7.1, and Kir3.2, Kir3.4 pore mutants [267,274,275] despite the high sequence similarity with another group of Kir channels with suppressive cholesterol effects (~70% between Kir2.1 and Kir2.2, and between Kir3.2 and Kir3.4; while ~45% between Kir2.1 and Kir3.2 or Kir3.4) [260].

The major mechanism of current decrease induced by cholesterol loading in Kir channels has been shown to be the stabilization of channels in the closed states. This 'silent channel' hypothesis was supported by patch-clamp studies showing that the open probability of the channels was significantly decreased while there was no effect on the unitary conductance or on channel expression [216,268]. Cholesterol and the major activator PIP_2 were shown to regulate the function of Kir2 channels via distinct binding sites, which signals finally converge through the intramolecular transduction pathways. This way cholesterol depletion

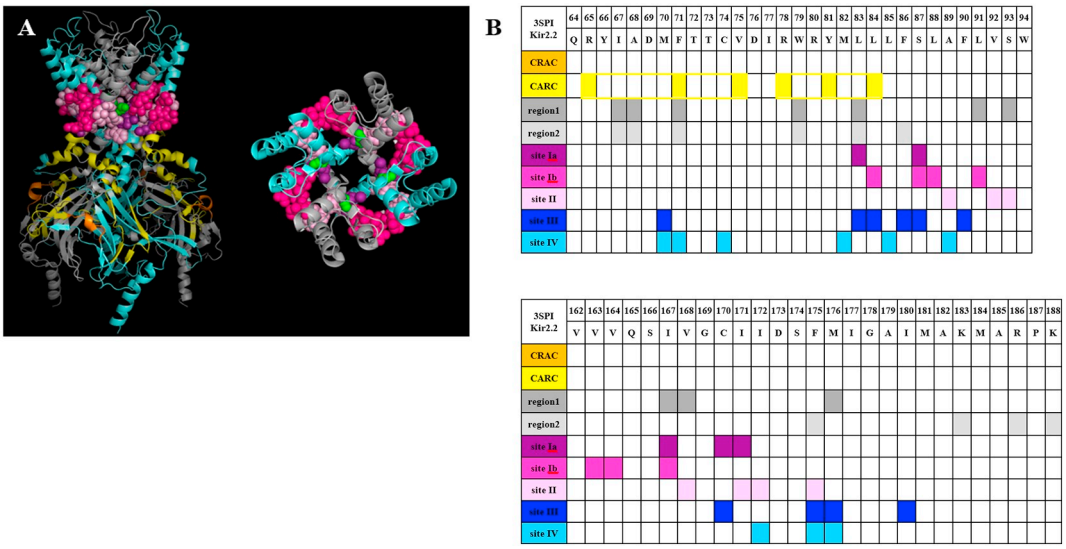

Fig. 3. Cholesterol binding motifs, regions and sites in Kir channels.
(**A**) Kir2.2 channels exhibit the general structure of the Kir family. The channel consists of four subunits, where each subunit contains two transmembrane helices (TM1 and TM2). Panel A is created by PyMol program based on the 3SPI PDB file (Kir2.2 in a PIP_2 bound (open) conformation [259]) and represents the channel from lateral (left) and top (right) views. The opposing subunits are indicated by *gray* and *cyan* helices. In panel A CRAC (*orange*), CARC (*yellow*) motifs, and of the five identified cholesterol binding sites Ia (*purple*), Ib (*magenta*), II (*light pink*) have been indicated (see text), since these can bind cholesterol in the open conformation of the channel. As it is indicated by panel A, all of the binding sites form a belt-like structure in the annular and non-annular regions of the channel. *Green* residues represent the location of the glycine-hinge in TM2 helices. (**B**) Panel B demonstrates possible relevant structural determinants for cholesterol- Kir2.2 direct interactions. Residues 78 to 94 belong to TM1, while residues 162 to 185 belong to TM2. Since Kir2.x channels contain CRAC (*orange*) and CARC (*yellow*) motifs their role in cholesterol binding was investigated, but molecular dynamics and electrophysiology studies revealed that none of these motifs bind cholesterol, and then the importance of functionally active cholesterol sensing regions distinct from these motifs (region 1: *black*, region 2: *gray*) was claimed. More recently, discrete cholesterol binding sites were described within these regions in the open (site Ia, Ib and II; *purple*, *magenta* and *light pink* respectively) and closed (site Ib, III and IV; *purple*, *blue* and *light blue* respectively) conformations with different affinities for cholesterol.

enhances the interaction between PIP_2 and Kir2 channels [276] thus triggering an increase in ionic currents. Interestingly for Kir3.1/3.4, which is the predominant potassium channel in atrial myocytes, contrary to its 'conventional' effects, cholesterol loading increases the conductance of these channels [274]. This effect is independent of but synergistic to PIP_2, the crosstalk is mediated by distinct cholesterol and PIP_2 binding sites, in which cholesterol binding forces the opening of the channel while PIP_2 stabilizes this open state [51,271].

A variety of applied techniques allowed a constant refinement of the proposed interaction of cholesterol with Kir channels. Early studies suggested that sterol interactions are highly stereospecific: cholesterol loading decreases, while epicholesterol increases current amplitudes of Kir2.1 [216] and ent-cholesterol has no effect on the ionic currents [215]. Thus, cholesterol interaction with Kir channels was assumed to be mainly a direct ligand-like mechanism (see also in Section 3.3). Although Kir2.1 contains CRAC and CARC cholesterol binding motifs (Fig. 3, orange and yellow) their significance in cholesterol sensing has been questioned, because molecular dynamics simulation and electrophysiological studies showed that in Kir2.1 and Kir2.2 these motifs do not bind cholesterol molecules [128,133]. Instead, these studies revealed the presence of two cholesterol sensing regions, which do not or only partially overlap with the CRAC or CARC motifs and importantly the functional relevance of which was supported by mutagenesis and electrophysiological experiments. The 'principal' (or region 1, Fig. 3**B** black contour), energetically more stable binding region is located in the center of the transmembrane domain close to the glycine hinge, while the second (or region 2, Fig. 3**B** gray contour), which seems to be a transient binding site, is between the transmembrane and the cytosolic domains [7,128,133]. After docking cholesterol, epi- and ent-cholesterol into these regions it has been shown that the stereoisomers can bind to the same regions of the channels with similar binding energies as cholesterol, therefore the binding of these isomers is not stereospecific, only their effects [2]. The structural background of this phenomenon may be the different orientation of the stereoisomers inside these binding regions, which may cause the different effects on Kir function compared to cholesterol [2,49]. With improvements in computational techniques binding sites could be even more accurately determined within these two cholesterol sensing regions using coarse grained simulations on Kir2.2 expressed in a POPC membrane [50]. Site Ia (purple), Ib (magenta) and II (light pink) have been proposed as potent cholesterol binding sites in the open channels, while the closed channels preferentially bind cholesterol at sites Ib (magenta), III (blue) and IV (light blue) (Fig. 3). Sites Ia, Ib and site III are formed between adjacent subunits, while sites II and IV are formed between the TM1 and TM2 helices of the same subunit. These sites bind cholesterol in a state dependent manner (open: sites Ia, Ib and II, closed: Ib, III and IV). The interactions between cholesterol and Kir can be divided into persistent contacts in the non-annular sites and temporary contacts with higher frequency at the interface of the membrane and the channel. Applying different cholesterol levels (between 30 and 15 mol%) revealed that lowering of the cholesterol level resulted in a decrease in the occupancy of sites Ib and II in the open state and site IV in the closed state by cholesterol.

For Kir channels distinct developmental stages in mapping protein regions responsible for direct cholesterol action can be identified, such as the demonstration of non-functional CARC motifs, the existence of cholesterol binding regions and inside them, exact cholesterol binding sites in different conformations of the channel. This way the Kir family is an excellent precedent on how direct cholesterol actions should be investigated and confirmed by the wide range of novel techniques.

Interestingly, as it is mentioned above, Kir channel complexes expressed in atrial myocytes (Kir3.1-Kir3.4 heterotetramers) [277] and hippocampal neurons (Kir3.1-Kir3.2) [272] exhibit an opposite response compared to Kir2.1 to cholesterol loading: instead of current decrease cholesterol loading lead to a significant increase in the amplitudes of ionic currents. Despite these different effects, computational and experimental data indicate that the putative cholesterol sensing regions are identical in Kir2.1, Kir3.2, and Kir3.4, except for minor differences [51,128,260,272]. The reason for the opposite cholesterol effect in these channels has been attributed to the differences in the position of the 'principal' binding region (region1) relative to the glycine hinge (Fig. 3 green spheres) located in the TM2 helix, which is believed to be an important determinant of the gating of Kir [278]. This binding region is adjacent to the glycine hinge in Kir2.1, while in Kir3.2, and Kir3.4 it is located past the glycine hinge in the extracellular direction, while it is shifted towards the outer helix. In that way in Kir2.1 cholesterol binding influences the hinging motion of the TM2 helix thus stabilizing the closed state of the channel. Recent studies also suggest that the cholesterol sensing region is not formed by exactly the same residues in Kir3.2, and Kir3.4 [52,260].

Cholesterol regulation of Kir channels has always been in the focus of interest since Kir channels act as key regulators in many biological processes in the heart, brain, vascular smooth muscle, etc. A great body of evidence suggests that in hyperlipidaemia the suppression of endothelial Kir2.1 by cholesterol is responsible for the changes in flow induced vasodilatation and triggers the development of atherosclerosis [279].

4.3. The voltage- and calcium activated large conductance potassium channel (BK)

The structure of the voltage- and calcium activated large conductance potassium channel (BK) represents the basic structure of K_V channels. The channel has a fourfold symmetry where the alpha subunits (named as slo1) are responsible for forming the VSD (Fig. 4**A**

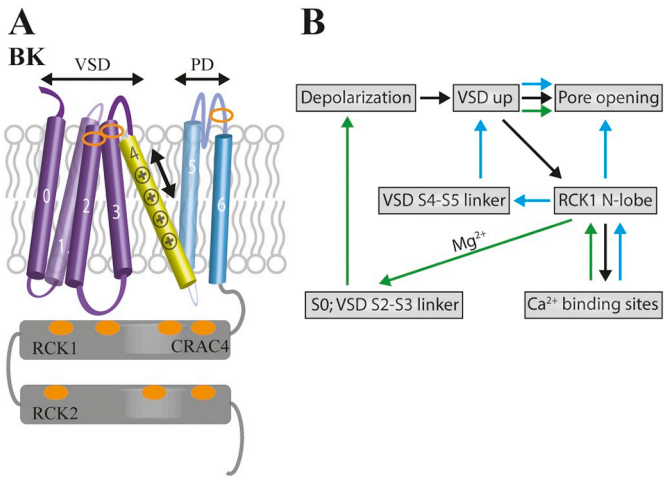

Fig. 4. Structure, gating and cholesterol binding motifs of the BK channel. (**A**) In contrast to K_v channels (Fig. 2**A**), one subunit of the BK channel consists of seven transmembrane segments, where S1–S4 helices (*purple* and *yellow*) form the voltage-sensor domain (VSD) and S5–S6 (*blue*) form the pore domain (PD) of the channel and the additional S0 segment places the N-terminus to the extracellular side of the membrane. Panel A presents one subunit from a lateral view. The cholesterol binding CRAC motifs are represented by *orange* ovals in panel A, where experimentally confirmed, functionally active motifs are symbolized by filled ovals. The functionally active motifs are found intracellularly out of the plane of the membrane. BK channels have two C-terminal intracellular domains, RCK1 and 2 (*gray*) where binding sites for Ca^{2+} and Mg^{2+} are present. (**B**) The presence of RCK1 and 2 forms the structural basis of the more complex gating of the BK channel compared to the gating of K_V channels (Fig. 2**B**). While in K_V channels opening is determined by membrane depolarization, in BK the voltage (*black* arrow)- and Ca^{2+} dependent (*blue* arrow) gating is coupled to each other. Ca^{2+}-binding to RCK domains also favors channel opening in cooperation with Mg^{2+} acting on the VSD (*green* arrow). The gating of both types of channels is affected by membrane cholesterol, as described in the text.

purple and yellow) and the PD (Fig. 4**A** blue) of the channel. The exact location of the activation gate in BK is still a question. In contrast to most K_V channels the VSDs are ordered in a non-domain swapped structure, thus a VSD only influences the PD of the same polypeptide chain [125]. The second major difference is the existence of an additional S0 segment, which precedes the voltage-sensor (S1–S4) and pore forming (S5–S6) helices, and accordingly places the N-terminus into the extracellular environment, thereby providing new interaction surfaces for accessory proteins [280,281] (Fig. 4**A**). In addition, the S0 segment while communicating with the VSD helps to fine-tune channel gating (see later) (Fig. 4**B**). Another structural signature is a large cytosolic tail domain (CTD), which contains two Regulator of Conductance for K^+ domains (RCK1,2) in each subunit (Fig. 4**A** gray). These domains form an octameric ring-like structure connected to the C-terminal end of S6 helix by a 16 amino-acid long linker. This gating-ring is liable for determining channel gating by sensing the intracellular Ca^{2+} level via two cooperatively acting high affinity Ca^{2+} binding sites [282,283]. According to the latest cryo-EM structure of BK described by the MacKinnon group, the Ca^{2+} bowl site is defined by the interface of the RCK domains of adjacent subunits, while the RCK1 site is located between the RCK1 N-lobe and the rest of the RCK1 domain from the same subunit [125]. In addition, the CTD contains sites for recognizing carbon monoxide [284], heme [285,286], phosphatidylinositol 4,5-bisphosphate [287], caveolin-1 [288] and cholesterol (four CRAC motifs, see later).

BK channels present many electrophysiological signature features like the large unitary conductance, high K^+ selectivity over other ions and the dual gating determined by the actual membrane potential and the intracellular Ca^{2+} level. The voltage dependence of the open probability of BK can be described by a sigmoid function, which shifts leftward due to the increase in the intracellular free Ca^{2+} level [125,282]. The molecular basis of the coupling between voltage- and calcium dependent gating is complex and has many structural determinants revealed by many experimental and cryo-EM data (Fig. 4**B**) [125]. According to this, due to the occupancy of the high affinity Ca^{2+} binding sites not only the activation gate is pulled out directly by the expanding gating ring, but via the non-covalent interactions between the RCK1 N-lobe and the S4-S5 linker, Ca^{2+} binding results in an outward displacement of the VSD (Fig. 4**B** blue arrows), which then facilitates pore opening via the conventional VSD pathway (Fig. 4**B** black arrows). Because the RCK domains align to the transmembrane helices on a domain swapped way, in contrast to VSDs, one RCK domain affects the pore opening in two neighboring subunits thereby creating the molecular basis of the inter-subunit cooperativity of Ca^{2+} dependent gating [125,283]. The pathway mediated by the RCK1 N-lobe and the S4-S5 linker is bi-directional, thus membrane depolarization also can facilitate Ca^{2+}-binding in the gating ring throughout the VSD [125] (Fig. 4**B** black arrows). The last interface, which couples Ca^{2+}- and voltage-dependent gating is between the RCK1 N-lobe and the S0 helix-S2-S3 linker complex supported by Mg^{2+}, which facilitates pore opening via the VSD [125,289,290] (Fig. 4**B** green arrows). As described below cholesterol can influence this complex gating process at numerous points, which still need to be elucidated by further research.

The gating mechanism of the alpha subunits can be modified by auxiliary beta and gamma subunits. Due to their highly tissue specific distribution they fine tune ionic currents, which control a large variety of biological and pathological processes [54,291,292]. Four types of beta (named beta1, 2, 3 and 4) and four gamma subunits (leucine-rich repeat-containing (LRRC)) have been described so far. Auxiliary beta subunits modify the voltage-dependence of gating, the kinetics, activator and inhibitor sensitivity of the current [292–294]. The sensitivity to cholesterol can also be modified by the accessory beta subunits, although beta subunit association is not necessary for the cholesterol modulation of BK currents [295,296]. In the presence of beta2 or beta4 subunits cholesterol cannot induce its typical electrophysiological alterations [297], while beta 1 does not influence cholesterol effects [296]. These studies have many limitations (experiments only performed at only one cholesterol concentration, etc.) so the topic requires further investigation [54,291].

In many types of cells BK channels have been found to preferentially co-localize with lipid rafts in the plasma membrane, which provides a rationale for the investigation of cholesterol-BK channel interactions [298–300]. In addition, BK has a caveolin-1 binding site located in the CTD [288], which promotes its plasma membrane localization [301]. The site also contributes to the effect of cholesterol depletion, which decreases the caveolin-1 level thus relocating BK channels to non-raft domains [298].

Cholesterol has a well-known, concentration-dependent inhibitory effect on BK channels similarly to the majority of K_V or Kir channels. The main component of this effect is the decrease in Po, while the unitary conductance is just slightly modified. The decrease in Po is generated by the decrease in mean open and the increase in mean closed times and leads to a decrease in ionic current amplitudes [54,295,302,303]. Interestingly, at the cellular level, cholesterol effects are much more complex, sometimes opposite to that seen at the protein level. For example, in rat uterine myocytes cholesterol depletion can reduce the function of BK by triggering channel internalization [304].

In contrast to Kir channels, early studies suggested that cholesterol affects BK channel function by indirect mechanisms. As discussed in Section 3.2, cholesterol modifies the biophysical properties of biological membranes, which can result in changes of channel parameters. The cholesterol effect on BK was initially attributed to the decrease in membrane fluidity [305] or in the increase in lateral stress [303]. These alterations are thought to favor the closed state of the channels resulting in a significant decrease in Po. The importance of indirect interactions in cholesterol effects was challenged by using epi- and ent-cholesterol sterol chiral analogues. As discussed in Section 3.3, despite the similarities to cholesterol regarding the biophysical effects on the lipid bilayer, these chiral analogues had no effects on BK currents. These results point to a highly stereospecific effect of sterols on BK channels, emphasizing the importance of direct protein-cholesterol interactions [2,306]. It is still a question whether the chiral analogues have no effects because of the lack of binding or just cannot induce the molecular rearrangements at the cholesterol binding site. This latter scenario is supported by the experiments using coprostanol, which has a different effect on lateral stress from cholesterol [149], but exerts the same reduction in BK current amplitudes [306].

The demonstration of stereospecific cholesterol effects on the BK channel stimulated high interest to find the location of functionally relevant cholesterol binding regions in the protein. As discussed in Section 3.1, CRAC motifs have been identified as plausible mediators of direct protein-cholesterol interactions (Fig. 4**A** orange empty ovals). The alpha subunit of BK contains ten CRAC motifs altogether, three of them located in the S0–S6 segments (top of the S2 and S3 segments, and inside the S5–S6 linker) and seven in the CTD according to the 3D structures of the channel [125,127]. The truncation of the CTD (directly after the S6 helix) totally eliminated the cholesterol inhibition of BK current, and it seems that CRAC4 (in the N-terminus of the CTD) plays a crucial role in cholesterol sensing. A single mutation at position 450 (Y450F) inside this motif reduces the inhibitory effect of cholesterol, while similar mutations in motifs upstream do not have any effect [126]. The importance of individual CRAC motifs in cholesterol sensing needs more elucidation but it seems that CRAC motifs inside the CTD interact with each other because sequential Y to F mutations from CRAC4 to CRAC10 gradually and additionally eliminated cholesterol effects [54] (Fig. 4**A** orange filled ovals).

Because BK channels play an important role in neurotransmitter and hormone release, smooth muscle and vascular myocyte coordination, and channel function is known to be affected in hypercholesterolaemia [291], clarifying the mechanism of cholesterol action on BK has high significance. As for other membrane proteins, cholesterol effects cannot be classified into purely direct or indirect effects, a mixture of the two

pathways must be considered, but further research is needed to elucidate the details of interactions.

5. Effects of cholesterol on sodium and calcium conducting ion channels and non-selective cation channels

5.1. Voltage-gated sodium and calcium channels

Voltage-gated sodium (Na_V) and calcium (Ca_V) channels are activated by the depolarization of the cell membrane and allow the influx of Na^+ and Ca^{2+} ions into the cell, respectively [307,308].

The nine members of the voltage-gated sodium channel family ($Na_V1.1$–1.9) are mostly expressed in excitable cells [307]. Their principal function is the generation of action potentials (AP). $Na_V1.4$ is the main sodium channel of skeletal muscles, while $Na_V1.5$ is that of cardiac myocytes, and the other seven members are predominantly expressed in neurons. Their fast gating kinetics enables the steep depolarization phase of APs, the shape of which controls the function of cells and organs. Since the timing and amplitude of currents contributing to APs is critical, changes induced in channel gating by mutations, drugs or environmental interactions may have serious consequences at the cellular or organ level, as exemplified by numerous Na_V channel related diseases [307].

There are ten members of Ca_V channels in three major families (Ca_V1–Ca_V3), of which members of the Ca_V1 family (L-type channels) are found, among others, in skeletal, smooth and cardiac muscle cells and have fundamental roles in muscle contraction. Other members are mostly expressed in neurons controlling neurotransmitter release, but also by a wide variety of non-excitable cells regulating gene expression, hormone release or steps of Ca^{2+} signaling. The pore forming subunits of Ca_V channels typically co-assemble with several accessory subunits that can modify virtually any aspect of the channel function [308].

Voltage-gated sodium and calcium channels have similar structures to voltage-gated K^+ channels, but their functional pore-forming entity is a continuous polypeptide chain consisting of four homologous domains (DI-DIV) of six TM helices (S1–S6) as opposed to the non-covalent association of four K^+ channel subunits [307,308]. Although the Na_V and Ca_V domains structurally resemble the K_V channel subunits, their functioning is less symmetrical, as individual domains are specialized for specific tasks. For example, in $Na_V1.5$ channels DIV is mostly involved in the development, while DIII in the recovery from inactivation [309]. Similarly to K_V channels the voltage-dependent gating of Na_V and Ca_V channels involves large scale conformational movements of the voltage sensor and then the activation gate, both of which interact with the membrane and therefore their rearrangements are prone to be sensitive to changes in lipid composition [310,311].

As for other membrane proteins, a variety of cholesterol-associated effects have been reported for Na_V and Ca_V channels and the earliest of these proposed indirect membrane effects. An extensive study using non-physiological amphiphiles (NPA), which reduced lipid bilayer stiffness, found that the voltage dependence of the steady-state inactivation of $Na_V1.4$ channels shifted towards hyperpolarized potentials, meaning fewer openable channels at a given holding potential [177]. As cholesterol depletion by MβCD caused a comparable negative shift in Na_V channel inactivation, which could be reversed by cholesterol enrichment, the authors attributed this effect to non-specific bilayer elastic properties regulating channel function. Earlier the same group found similar effects on N-type calcium channels, suggesting that the phenomenon is not channel specific [312]. The comparable changes in membrane elasticity and channel gating induced by NPAs of different structures imply that the underlying mechanism is unlikely to be specific binding. The authors also argue against the disruption of cholesterol-associated raft structure being responsible for the effects on sodium channel gating. Although such disruption could be induced by cholesterol depletion by MβCD, Triton X-100, on the other hand, enhances the formation of raft-like domains [313] and still causes a similar gating shift. Thus, the study concludes that via the hydrophobic matching between the bilayer and the embedded protein, the bilayer elastic properties are the major regulators of membrane protein function. However, some observations of the study were not explained by the suggested mechanism. For example, cholesterol enrichment over the control level did not shift the inactivation curve in the positive direction as would be expected, but similarly to other previously described voltage-gated channels, it shifted the voltage-dependence of the activation curve to depolarized potentials and significantly reduced peak currents [9]. In contrast, cholesterol depletion shifted the inactivation curve but did not affect the activation curve or the current amplitudes. These contradictions do not rule out the role of non-specific membrane effects on Na_V channel activation gating, but suggest that the elastic properties may influence multiple functional parts of the channel. The four Na_V channel VSDs have varying voltage-sensitivities, kinetics of movement and roles in channel opening, inactivation and recovery from inactivation, and thus are likely to be affected differently by cholesterol content [309,314]. In addition, the pore domain may also be a direct target of cholesterol enrichment leading to reduced current amplitudes, as has been described for other channels [9].

Searching for regulatory cholesterol binding sites, MD simulations using the crystal structure of the bacterial voltage-gated Na_VAb channels in the closed and inactivated states have found no evidence for channel modulation by direct cholesterol binding [315]. Only the pore domain was included in the simulations, which showed that cholesterol may enter near the pore laterally with very low probability in the inactivated state, and not at all in the closed state. In accord with other studies, cholesterol was found to increase lipid packing, restrict lipid movement and increase membrane thickness, thereby regulating channel function via indirect membrane effects.

In contrast to the indirect effects suggested by these studies, 17 CRAC, CARC and CCM cholesterol binding motifs have been identified in $Na_V1.9$ and the binding of cholesterol to at least three domains of the channel has been shown by surface plasmon resonance [78]. Liposomes with or without cholesterol were immobilized on a sensor chip and then perfused with peptides from different areas of the channel containing cholesterol binding motifs in a microfluidic system. Two peptides were located in the S4 segments of the VSDs in DI and DIII and one in the S1 helix of DIII. The study has shown the in vivo role of cholesterol in tuning the pain sensitivity of nociceptive neurons. Inflammation reduced the cholesterol content both in a sensory neuronal culture and mouse skin tissue and the depletion of membrane cholesterol induced hyperalgesia by potentiating $Na_V1.9$ channel activity. Inflammatory mediators were found to induce the partitioning of voltage-gated $Na_V1.9$ channels from cholesterol-rich lipid rafts into non-raft regions, resulting in augmented neuronal excitability. This was due to a hyperpolarizing shift in the voltage-dependence of activation of $Na_V1.9$ channels, an effect observed in several other voltage-gated channels in response to cholesterol depletion. Restoration of cholesterol reversed the observed effects and transcutaneous cholesterol application reduced inflammation-associated pain in animal models. Despite the identification of the binding sites, their role in the observed effects has not been proven. Since raft localization can affect channels indirectly by the altered membrane properties or changing signaling partners, the importance of direct cholesterol binding in $Na_V1.9$ is not conclusive.

Removal of $Na_V1.8$ channels from lipid rafts by MβCD showed an opposite outcome compared to $Na_V1.9$, as it impaired nociceptor excitability as assessed by the number of neurons capable of conducting depolarizations induced by mechanical and chemical stimuli [79]. In DRG neurons $Na_V1.8$ channels were originally found in clusters along the axons and associated with lipid rafts, which was disrupted by cholesterol depletion. Although the gating properties of $Na_V1.8$ were not investigated directly by electrophysiology, the authors suggest that perturbation of the rafts as signaling platforms interferes with the interaction of the channel with its signaling partners, especially with certain kinases, which modulates channel gating. Alternatively,

rejection from rafts may enhance the endocytosis of the channel, resulting in reduced surface expression and impaired impulse propagation. The fact that two closely related channels showed opposite responses to removal from rafts underlines the multiple and variable ways, in which rafts can modulate channel function.

Among voltage-gated Ca^{2+} channels, cholesterol effects have been investigated mostly on the L-type (Ca_V1 family) in various cellular systems. Cholesterol enrichment was found to increase the current in vascular smooth muscle cells [316], but decrease it in coronary arterial smooth muscle cells from miniature swine with hypercholesterolemia [317]. Although not investigated in detail, in both cases indirect effects on gating were suggested rather than changes in channel expression. Cholesterol depletion increased L-type currents both in cochlear hair cells [318] and ventricular myocytes [77], which has been observed for numerous other ion channels, and thus is likely to represent a general non-specific mechanism. However, in the hair cells the authors observed diverse responses of different ion channels in the same cells to MβCD application, so they concluded that cholesterol effects must be channel-specific and not acting via global indirect ways. In the myocytes, besides increasing the current cholesterol removal also suppressed the current increase induced by β-adrenergic stimulation. The authors attributed both effects to the perturbation of macromolecular signaling complexes, which are organized by lipid rafts. Even the basal activity of the L-type Ca^{2+} channel, $Ca_V1.2$, which plays a key role in cardiac excitation–contraction coupling, is regulated by phosphorylation, but the G-protein pathway conveying β-adrenergic stimulation especially relies on the balanced activity of kinases and phosphatases. It is therefore feasible that the altered cholesterol content restructures the membrane domains and disintegrates the molecular complexes inhibiting structural coupling among adjacent regulatory proteins thereby preventing efficient signaling. Interestingly, to our knowledge no studies so far have investigated the existence or role of direct cholesterol binding sites in Ca_V channels.

5.2. TRP channels

The structure of Transient Receptor Potential (TRP) channels is homologous to that of K_V channels, as four six-transmembrane subunits form a functional channel, but they also usually have large variable intracellular domains for multimodal modulation. TRP channels are cation selective ion channels with wide tissue distribution. They can be activated by a large variety of chemical compounds, and various members are also sensitive to temperature, voltage, mechanical stress and often a combination of these gating stimuli and many play important roles in the cells of sensory organs. Several TRP members are found in nociceptors to detect noxious stimuli of dangerous chemical compounds, low pH, extreme temperatures or mechanical interventions [319].

The seven TRP channel subfamilies are TRPA (ankyrin), TRPC (canonical), TRPM (melastatin-related), TRPML (mucolipin) and TRPN (no mechanoreceptor potential C), TRPP (polycystin) and TRPV (vanilloid). Several TRP channels are involved in temperature sensing, such as TRPA1, TRPM2–5, TRPM8 and TRPV1–4 [319]. Although the structure of TRP channels is similar to that of voltage-gated channels, their gating appears to be very different. Cryo-EM structures in various conformations suggest that TRP channels have two gates, one at either end of the pore, which are allosterically coupled [320]. The S1–S4 helices in TRPs corresponding to the voltage-sensor domains of voltage-gated channels do not significantly move during gating. As expected based on the multimodal activation and complex gating mechanisms TRP channels are subject to modulation by cholesterol by different modes of action.

In some TRP channels cholesterol modulation was ascribed to the general indirect membrane effects. For example, the activity of TRPV3 channels induced by activator compounds and temperature changes was found to be strongly potentiated in cholesterol enriched cells [321]. The authors concluded that the sensitizing effect of cholesterol was not due to the increased plasma membrane targeting of the channel, but suggested that the altered biophysical properties of the membrane facilitated the voltage-dependent gating of the channel.

Many observations indicate that being included in rafts is essential for the normal operation of several members of the TRP family. TRPC1 channels, for example, which provide store-operated Ca^{2+} entry (SOCE) in different cell types were found to localize to caveolae, and interact with caveolin-1. Cholesterol extraction by MβCD disrupted this interaction, impaired SOCE and the downstream cellular functions [322,323]. Thus, in the case of TRPC1, raft-regulated protein-protein interactions are the dominant factors for cholesterol action.

Similarly to TRPC1, the raft localization and co-localization of TRPV4 channels with caveolin-1 has also been shown. Cholesterol-dependent mobility of TRPV4 was suggested to regulate channel function. CRAC motifs have also been found in TRPV4 channels, and using blot-overlay experiments the binding of cholesterol, its precursors and some derivatives has been demonstrated to various segments of the channel [129]. However, the role of direct regulation via cholesterol binding sites remains to be determined.

Raft localization also seems important for the regulation of TRPM8 function, but the observations differ regarding the exact mechanism. TRPM8 channels exhibited potentiated responses to activating stimuli such as cold temperature and menthol following cholesterol extraction from the membrane by MβCD [80]. Raft localization of TRPM8 was shown both in sensory neurons and heterologous expression systems and that the disruption of rafts by cholesterol depletion sensitized the channel, shifting its temperature activation threshold to warmer temperatures. Both direct and indirect effects were proposed as explanations for the observations, but the mechanism was not investigated experimentally. An interesting way of TRPM8 channel regulation by cholesterol was described using single particle tracking by TIRF microscopy [324]. Vesicles containing TRPM8 channels were suggested to bind and fuse with the plasma membrane, but not immediately disassemble and release the channels. Vesicles were shown to “hop” from one membrane corral to the next, their residency time and consequent channel availability being controlled by cholesterol content. Removal of cholesterol by MβCD stabilized TRPM8 motion in the membrane and resulted in augmented current amplitudes. Although the data presented by the authors indicate slight increases in unitary conductance and open probability, they attribute the larger currents to the increase in channel number in the plasma membrane. Thus, rafts may control the expression level and gating parameters of TRPM8, but no binding sites or functional motifs have been identified on the channel so far.

Multiple modes of cholesterol regulation were shown for the capsaicin receptor TRPV1 as well. Reduced currents were detected in DRG neurons in response to MβCD treatment accompanied by a reduction of TRPV1 protein in membrane fractions [325]. The authors thus concluded that the regulation of TRPV1 activity by cholesterol occurs via modulating the membrane expression level. A completely different regulatory mechanism was shown in TRPV1-expressing HEK cells using current recordings from excised patches [56]. By not using whole-cell recordings the possible effects of cellular TRPV1 trafficking were excluded as regulators of the current amplitude. TRPV1 currents were unaffected by cholesterol depletion but cholesterol enrichment significantly reduced current amplitudes. The specificity of the cholesterol effect on capsaicin-induced TRPV1 currents was tested using its diastereoisomer epicholesterol, which proved completely ineffective. The authors identified a CRAC sequence in TRPV1, in which several residues face the lipid-exposed side of the S5 helix, and pinpointed residue 585 among them as a critical determinant of cholesterol binding to the channel. Mutation of this residue dramatically affected the ability of cholesterol to reduce the current, and interestingly species differences were found at this position of the sequence. The inhibitory effect of cholesterol enrichment could be reproduced by thermal or voltage activation in the absence of capsaicin as well. Noise analysis experiments revealed no significant decrease in the unitary conductance or the open

probability of the conducting channels, but indicated a decrease in the number of agonist activated channels. The authors concluded that cholesterol drives the channel into a non-conducting state, probably by preventing opening via specific binding.

The investigation of indirect membrane effects on TRP channels should get more attention, as many members are involved in temperature sensing, and membrane fluidity is strongly temperature- and cholesterol-dependent, so the gating of thermo-TRPs is likely to be modulated by this indirect pathway.

5.3. Piezo channels

The evolutionarily conserved Piezo channels belong to the family of mechanically activated channels [326] and were identified as large homo-multimerizing nonselective cation channels with a slight preference for Ca^{2+} permeability [327,328]. The two identified Piezo isoforms play important roles related to mechanical sensations such as blood vessel development, regulation of red blood cell volume, and blood pressure, proprioception, breathing, and nociception [329–332]. According to recent high-resolution cryo-EM structures, these channels are characterized by a unique large homotrimeric structure adopting a symmetric three-blade propeller shape with an EC cap at the top of the central axis. The unique structure of a protomer contains 38 TM helices with an inner and outer helix lining the pore intervened by the EC cap, and a series of nine four-TM helix bundles ("Piezo Repeats") build the highly curved blades of the propeller. The innermost Piezo Repeat A of one protomer is adjacent to the outer helix of a different protomer, producing a 'domain-swapped' arrangement. The intracellular C-terminal domain (ICTD) connects to the IC end of the inner helices, thus, the central pore is lined by the EC cap, inner TM helix and the ICTD. The Piezo Repeats are connected to an IC "beam" interacting with the inner TM helix and the ICTD, which contributes to the conformational coupling between the blades and the core of the channel. The gating process of Piezo channels was suggested to involve concerted movements of the inner helix, outer helix, ICTD and beam domains [333–335].

In general, two mechanisms were proposed to describe the opening of mechanically activated channels. According to the "tethered" model, auxiliary structures such as the cytoskeleton or a component of the extracellular matrix transmit the opening signal to the channel. In contrast, the "force from lipid" model proposes no requirement for proteins for gating. Although the latter was initially suggested to describe gating of prokaryotic channels like MscS or MscL, recent studies underlined their relevance in mammalian mechanosensitive channels like TREK-1, TREK-2 or TRAAK as well. However, the exact mechanism of lipid action is still unclear but generally thought to depend on hydrophobic mismatch, intrinsic curvature of lipids and membrane fluidity, with the active contribution of proteins as well [326,336,337].

Consistently, quantitative analysis of electrophysiological measurements revealed that membrane tension was the principal activating stimulus for rapidly-inactivating inward currents. Currents were evoked by both negative and positive pressure induced membrane curvatures, and sensitivity of Piezo1 channels depended on the magnitude of resting membrane tension, suggesting a "force from lipids" mechanism [338,339]. Piezo1 was shown to create a dome-shaped structure, the central EC cap located mostly inside the dome and the curved blades projecting approximately 30 degrees out of the plane defined by the pore. Due to this structure, membrane tension induced flattening of the semi-spherical dome can be associated with membrane plane expansion that pushes the pore-lining helices away from the center and open the pore [340]. Furthermore, membrane mechanical calculations showed that the Piezo dome can strongly curve the surrounding membrane, which depends on membrane lateral tension, elastic bending modulus and intrinsic curvature of lipids and could amplify the tension sensitivity of these channels with increasing tension strongly favoring the open conformation. This theory implies that lipid composition in general, and cholesterol in particular, could substantially affect gating of Piezo1 channels through alterations in membrane stiffness and lateral tension [194]. Furthermore, the blade and beam structures of the channel form a lever-like apparatus displaying uneven movement with large motion at the distal end while subtle movement at the proximal end. This lever is pivoted at the proximal end of the beam, which could lead to the amplification of the force acting on distal parts and propagation of the gating stimulus from the distal blade to the central pore mediated by interactions between the beam and the ICTD [341,342].

The functions of Piezo channels are widely investigated recently and the process of mechanosensitive gating can be extensively modified at various steps by cholesterol. However, cholesterol effects on Piezo are scarcely documented. Although Piezo channels were shown to be inherently mechanosensitive [343], their gating can also be modified by membrane proteins. The lipid raft resident and cholesterol binding STOML3 was shown to modulate Piezo channels through increased stiffness of the cell membrane [344], an effect that depended on its intact cholesterol binding site, since its mutant failed to sensitize Piezo. Consistently, cholesterol depletion with MβCD or deficiency of STOML3 resulted in a softer membrane and attenuated mechanosensitivity of the channel, as shown by reduced currents due to decreased open-state probabilities. In vivo, tactile allodynia was attenuated by cholesterol depletion, suggesting that STOML3 might act on Piezo through recruiting cholesterol to the channel [345]. Furthermore, MβCD caused a rightward shift in pressure-response curves of Piezo1 channels, associated with slowed channel activation in response to mechanical stimuli. TIRF-STORM super-resolution imaging revealed the presence nano-scale Piezo1 clusters whose density was decreased upon MβCD treatment. In addition, Spatio-Temporal Image Cross Correlation Spectroscopy (STICCS) showed cholesterol-dependent colocalization between Piezo1 channels and CTX-B labeled lipid rafts [346]. Although only a limited number of studies addressed the importance of cholesterol in the gating of Piezo channels, the well-described relationship between these channels and membrane tension/curvature, and between membrane tension/curvature and cholesterol levels point to major relevance of cholesterol, which needs to be confirmed in the future.

5.4. Pentameric ligand gated ion channels

Pentameric ligand gated ion channels (pLGIC) are integral membrane proteins mainly found in the central and peripheral nervous system, involved in signaling processes of neurotransmitters leading to rapid ion fluxes and in the pathogenesis of chronic pain, addiction, neurodegenerative and autoimmune disorders. The "founding father" of the family, the nicotinic acetylcholine receptor (nAChR) is localized in neuronal postsynaptic membranes (neuronal subtypes) and neuromuscular junctions (muscular subtype). Each of the five receptor subunits ($\alpha_2\beta(\gamma/\varepsilon)\delta$ in muscular subtype or most commonly $\alpha_4\beta_2$ 2:3 or 3:2 heteropentamer or α_7 homopentamer in neuronal subtypes) has a large N-terminal extracellular domain, four transmembrane segments (M1-M4), a small cytoplasmic domain between M3 and M4, and a short C-terminal extracellular domain. M1-M4 segments are organized in a three-ring system with the inner ring formed by five M2 segments lining the pore, the middle ring formed by ten M1 and M3 segments partially exposed to lipids and the outermost ring consisting of five M4 segments that act as "lipid sensors". Ligand binding results in complex allosteric structural rearrangement of TM helices leading to opening of the channel selective for cations [347,348].

Cholesterol has long been suggested as an important regulator of nAChR function, since its presence was required for transport activity when reconstituted into vesicles [349]. Direct cholesterol binding to M4, M1 and M3 segments of all channel subunits was initially proposed based on photoactivatable sterol probe incorporation [220] and MD simulations demonstrating three binding sites per subunit [134]. Consistently, CARC and CARC-like motifs were described in membrane embedded regions, rendering a total of 15 cholesterols bound per nAChR [124]. The presence of directly bound cholesterol molecules was

recently confirmed by sausage-shaped voids proposed to correspond to cholesterols in cryo-EM structures of both muscular [10] and neuronal nAChR [58], and MD simulations [57]. Cholesterol binding was suggested to stabilize the conformation and confer local rigidity to the membrane contributing to productive coupling between ligand binding and channel forming regions [10]. Besides cholesterol, its analogues ent-cholesterol, epicholesterol, cholestanol and coprostanol were also able to support carbamylcholine induced channel activity when reconstituted into vesicles. A direct binding site with lax structural requirements and conveying the same effect by different sterols was proposed [135], in line with the presence of such binding sites suggested recently for other channels as well [49]. On the other hand, a recent report emphasized indirect cholesterol action. When examining nAChR reconstituted into vesicles, increasing membrane thickness facilitated agonist induced conformational transitions into the coupled state by reducing the large activation energy barrier through promoting interactions between M4 and adjacent M1 and M3 helices. This suggested that cholesterol, besides shifting conformational equilibrium, may facilitate nAChR activation via a kinetic mechanism due to increased hydrophobic thickness of the membrane [33]. nAChR was also found to be localized in GM1 enriched lipid rafts in a cholesterol dependent manner [81,350] and functionally relevant receptor aggregation was recently demonstrated with super resolution imaging techniques, which was significantly modified upon cholesterol extraction [351,352]. Furthermore, alterations in raft-dependent clustering in response to cholesterol depletion resulted in enhanced internalization kinetics through accelerated endocytosis, which may lead to decreased whole-cell currents [353].

Given the evolutionarily conserved design of pGLIC superfamily, members including $GABA_A$ receptors, $5\text{-}HT_3R$ serotonin receptors, glutamate-gated Cl^- channels and bacterial ELIC and GLIC [354] and their similar gating mechanisms [355], cholesterol may also play modulatory roles in the functions of other pGLICs. Consistently, recent MD simulations [59] and the cryo-EM structure of $GABA_A$ receptor proposed the presence of directly bound cholesterols [60] that, unlike epicholesterol, may exert significant effects on ligand sensitivity of the receptor [218].

6. Effects of cholesterol on ATP-driven transporters

6.1. Na^+/K^+-ATPase

The first discovered ion pump, the Na^+/K^+-ATPase uses the energy derived from ATP hydrolysis to establish Na^+ and K^+ gradients across the cell membrane, which is essential for cellular homeostasis, neural and muscular excitability and proper kidney function. During a transport cycle, ATP accelerates the release of two occluded K^+ and binding of three Na^+ on the cytoplasmic side, which is associated with a conformational change from E2 form (with low Na^+ and high K^+ affinity) to E1 (with high Na^+ and low K^+ affinity), and phosphorylation of the enzyme with Na^+ occlusion. This is followed by a conformational change from E1-P to E2-P and the release of Na^+. At the end of the transport cycle, two K^+ are bound from the extracellular side and occluded as a result of dephosphorylation. The pump is composed of an α subunit that contains 10 TM segments (αM1–10) responsible for ion transport; a small accessory β subunit with a TM segment (βM) responsible for structural and functional maturation of the α subunit; and in certain tissues a γ subunit regulating the pump activity. Cholesterol has an important role in the function of Na^+/K^+-ATPase, as reviewed recently [356,357].

In general, the activity of the Na^+/K^+-ATPase was shown to positively correlate with cholesterol levels in bilayers [178,358]. Consistently, MβCD treatment resulted in decreased Na^+/K^+-ATPase activity in a near native membrane environment, which was restored by cholesterol-MβCD [61]. Cholesterol was shown to modify rate constants of several steps of the working cycle of Na^+/K^+-ATPase by shifting the E2 – E1 transition towards E1 and accelerating phosphorylation of the pump, the E1-P to E2-P conversion and K^+ deocclusion [61,358]. Recent reports suggested direct action of cholesterol based on the presence of three directly bound cholesterol molecules in crystal structures of the Na^+/K^+-ATPase [62,63]. Cholesterol bound to a deep binding pocket formed by αM8–10 and γM was suggested to contribute to the stabilization of the protein, while cholesterol bound to a pocket lined by αM3, αM5, αM7 and βM might influence the inhibitory action of sphingomyelin [359]. Recent MD simulations revealed possible state dependent binding sites in addition to those described in crystal structures, which may differ markedly among protein conformations [61].

Besides direct actions, cholesterol was suggested to affect the Na^+/K^+-ATPase through alterations in bulk membrane biophysical parameters as well. In reconstituted vesicles, an optimal length of phospholipid acyl chains was required to support pump activity, which depended on the presence of cholesterol, suggesting the importance of bilayer thickness for hydrophobic matching [178]. Consistently, recent MD simulations revealed significant changes in the hydrophobic thickness of Na^+/K^+-ATPase during its conformational changes, mainly in the E2 – E1 transition involving regions linked to cholesterol binding (αM9, αM10, βM and γM) [360]. Furthermore, pump activity was shown to positively correlate with the magnitude of the membrane dipole potential and dipole modifiers were suggested to affect conformational kinetics of the protein [34,211]. A significant fraction of Na^+/K^+-ATPases is localized in caveolae directly bound to caveolin proteins through its caveolin binding motif on αM1 and αM10. Instead of canonical pump function, caveolar Na^+/K^+-ATPase might be involved in non-canonical signal transduction processes as a component of a signaling platform activating Src and ERK proteins in response to ouabain binding. This signaling is significantly inhibited by MβCD, which is reversed by addition of cholesterol-MβCD, suggesting cholesterol-dependence of the pathway [82].

6.2. ABC transporters

The human ATP-binding cassette (ABC) transporter superfamily is divided into seven subfamilies (A to G). Functional ATP transporters are composed of 12 transmembrane (TM) segments and two nucleotide binding domains, which are formed by a continuous polypeptide chain (as in ABCB1) or dimerization of two symmetrical “half-transporter” monomers (as in ABCG2). Many members of the superfamily are involved in cholesterol transport or regulated by cholesterol, as reviewed recently [361]. Here, we limit our discussion to the latter group.

Cholesterol substantially modifies the function of the ABCB1/MDR1/P-glycoprotein, a transporter with wide substrate specificity involved in detoxification and protection of various tissues from xenobiotics, regulating digestive absorption, cerebral disposition, biliary and urinary elimination and multidrug resistance of tumors. During transport ABCB1 switches between an inward-facing state for substrate binding (with the cavity open towards the cytoplasm and two portals allowing access of molecules directly from the bilayer) and an outward-facing conformation for substrate release [362]. Cholesterol was shown to positively modulate the ATPase and transport activities of ABCB1 through changing the conformation of the substrate binding site [11,363] or effects on lipid partitioning of substrates [364]. Direct cholesterol binding of ABCB1 proposed by pulldown experiments [11] was recently confirmed by high resolution cryo-EM structures of the transporter, which suggested cholesterol molecules bound to surface grooves on the EC sides of TM regions and, in the inner leaflet, to a membrane exposed pocket near TM3, TM4 and TM6 and at a pseudo-symmetrical site near TM9, TM10 and TM12 [64]. Besides direct actions, cholesterol was suggested to potentiate ABCB1 activity through alterations in the dipole potential as well [35]. Consistent with a caveolin binding motif in its sequence, ABCB1 was shown to directly interact with caveolin and localize in caveolae, which exerts inhibitory effects on the protein, since abruption of caveolin interaction and

subsequently caveolar localization resulted in increased transport activity [365,366].

ABCG2/BCRP (breast cancer resistance protein) is another ATP transporter involved in the efflux of various compounds including chemotherapeutic agents from the cell. Similarly to ABCB1, transporting activity of ABCG2 was shown to positively correlate with cholesterol levels in cell membranes treated with MβCD or cholesterol-MβCD complexes [367]. Cholesterol dependence of pump function was related to the presence of an LxxxL steroid binding element [368] and one of the five potential identified CRAC motifs [12]. MD simulations confirmed the presence of cholesterol molecules bound to LxxxL and CRAC motifs, which were suggested to contribute to conformational stabilization [65,369]. Recent cryo-EM structures also proposed five ordered cholesterols directly bound to an ABCG2 monomer in the dimeric structure, however, in contact with residues outside the previously suggested LxxxL or CRAC motifs [66,67].

7. Effects of cholesterol on cell surface receptors

7.1. G protein coupled receptors

G protein coupled receptors (GPCRs) constitute the largest protein superfamily with more than 800 members involved in all cellular functions and represent the molecular target of 30–40% of currently used drugs [370]. GPCRs are characterized by extracellular regions involved in ligand binding, intracellular regions for coupling with heterotrimeric G proteins and seven transmembrane helices (TM1–7) embedded in the membrane. An intrinsic property of GPCRs is their great conformational flexibility with multiple active and inactive conformations [371,372].

Cholesterol was shown to substantially regulate various steps of GPCR signaling. The dependence of ligand binding on cholesterol levels was found in various GPCRs. In general, a positive correlation was seen between membrane cholesterol levels and affinities. As examples, cholesterol depletion of the cell membrane resulted in decreased ligand binding affinity of oxytocin receptors [36], cholecystokinin receptors (CCK1R) [36], CXCR4 chemokine receptors [86], metabotropic glutamate receptors (mGluR) [83] and serotonin receptors (5HTR) [373]. However, several studies demonstrated elevated binding efficiency in response to MβCD treatments in cannabinoid receptors (CB1R) [374] and adenosine receptors (A2AR) [375], although the latter finding was questioned recently [376]. Consistent with general changes in ligand binding, lowering cholesterol levels reduced agonist induced activation of signaling pathways of mGluR [84], A2AR [376] and 5HTR [373]. On the contrary, MβCD treatment resulted in spatially diffused β_2-adrenerg receptor (β2AR) localization and a consequent agonist induced signaling [85,89]. In general, cholesterol replenishment and/or loading exerted opposite effects.

Recent crystal structures revealed cholesterols directly bound to rhodopsin [21], β2AR [13,68], A2AR [16], P2Y12 receptors [24], mGluR [22], CB1R [69], opioid receptors [14] and 5HTR [17], suggesting that cholesterol may increase the conformational stability of these proteins. In keeping with the common presence of CRAC and CCM cholesterol binding motifs in GPCRs, many of the cholesterols identified in crystal structures occupied these receptor regions [131]. However, recent studies questioned the exclusive importance of these motifs, since cholesterols directly bound to regions outside CRAC or CCM sequences were also demonstrated in the crystal structures and by MD simulations [136–142].

Although the functional units of GPCRs were initially thought to be monomers, dimerization was recently recognized as a functionally relevant common feature in the family [377]. Cholesterol was shown to be directly bound and mediate stabilizing interactions at the dimer interface in X-ray crystallography or cryo-EM structures of A2AR [16], P2Y12 receptors [24] and mGluR [15,22]. Furthermore, directly bound cholesterols can specifically modulate dimeric interfaces contributing to conformational selectivity of possible dimers, which may result in functional consequences due to the different activities of these configurations, as shown in β2AR [140], mGluR [15,23], CXCR4 [18], opioid receptors [20] and 5HTR [19].

Besides direct action, alterations in membrane biophysical parameters could mediate cholesterol effects on GPCR function. When examining the effects of various sterols in cholesterol depleted cells, functional restoration correlated with the ability of derivatives to induce alterations in membrane fluidity in the cases of CCK1R [36]. Indirect cholesterol effects were also proposed by studies showing that formation of the active metarhodopsin II form and binding to its signaling partner transducin correlated with good hydrophobic matching resulting from optimal membrane thickness [42] and the tendency of phospholipid components to favor negative spontaneous curvature of the bilayer [38,43]. MD simulations suggested that cholesterol induced increased membrane thickness can influence membrane embedding and conformation of helix-8 of mGluR [39]. Membrane thickness was also shown to regulate the association tendency of β2AR [41] and modulate dimeric interfaces of 5HTR [19]. The signaling activity of 5HTR also depended on bilayer order and curvature elastic stress [40]. Furthermore, after solubilization of 5HTR, decreased ligand binding was restored by cholesterol and its enantiomer ent-cholesterol, but not its diastereomer epicholesterol [44]. These effects correlated with sterol induced alterations in the magnitude of dipole potential, proposing its role in the modulation of 5HTR [37].

Palmitoylation was shown to be a general property of many GPCRs [378] and, consistent with the palmitoyl group being a raft targeting signal [119], lipid raft localization was demonstrated in β2AR [85,89], CXCR4 [86], mGluR [83,84], CB1R [87] and opioid receptors [88]. GPCR distribution between raft and non-raft regions of the cell membrane was suggested to be of functional significance in the ligand binding of CXCR [86], mGluR [83] or opioid receptors [379]. Raft localization also correlated with the activation of signaling pathways induced by mGluR [84], CB1R [87] or opioid receptors [380]. On the other hand, caveolar localization of β2AR was shown necessary for the spatial regulation of agonist induced signals, as these microdomains sequester the receptor from its signaling partners, limiting its activity in cardiomyocytes [85,89].

7.2. Proteins of the Hedgehog signaling pathway

The extensive and substantial role of cholesterol has been demonstrated in various steps of the Hedgehog signaling pathway that is functionally relevant in embryonic development and the function of adult stem cells with pathogenic roles in developmental malformations or medulloblastoma [381]. In the absence of Hedgehog ligands, Patched, a cell surface receptor, inhibits Smoothened, a class F GPCR acting as the main component of the pathway. Hedgehog ligands are secreted into the extracellular space and bind to their receptor Patched on the surface of the target cell. Hedgehog binding inhibits the function of Patched, leading to the activation of Smoothened, as reviewed recently [381]. The Sonic hedgehog morphogen was the first protein shown to be covalently modified by cholesterol [382], which is essential in normal range of its action by limiting spreading of the ligand in the EC space through modifying its interaction with Patched [383]. Patched is a sterol-sensing-domain-containing cell surface receptor with 12 transmembrane helices (TM1–12) and 2 extracellular domains (ECD1–2), and the protein was suggested to mediate cholesterol transport [143]. Consistently, cholesterols directly bound to a sterol binding pocket formed mainly by ECD1 or the sterol sensing domain created by TM2–6 was visualized in cryo-EM structures [25,26]. Furthermore, a central hydrophobic conduit was described that could mediate cholesterol transport between the EC and TM regions [384]. The cholesterol moiety of Hedgehog ligands can bind to sterol binding sites of Patched blocking its sterol channel [26,385], resulting in decreased cholesterol transport activity and increased cholesterol accessibility in the membrane [26,384,385], which leads to the activation of Smoothened. Consistently, recent crystallographic structures

revealed cholesterols directly bound to an extracellular cysteine rich domain [27,29] and a transmembrane binding site [28] of Smoothened. These studies described cholesterol induced activating conformational changes similar to those observed during the activation of class A GPCRs and, as a unique feature, opening of a hydrophobic tunnel for steroid transport [27–29,386]. The endogenous modulator role of cholesterol was demonstrated by studies showing decreased Hedgehog induced signaling in response to mutations in sterol binding sites [27,28] or cholesterol depletion with MβCD [386]. Consistently, developmental abnormalities described in Smith-Lemli-Opitz syndrome can occur due to abnormal Hedgehog signaling resulting from decreased cholesterol levels in the disorder [387].

7.3. ErbB receptors as prototypical receptor tyrosine kinases

ErbB proteins are the best-characterized historically prototypical members of receptor tyrosine kinases and represent targets of novel anticancer drugs due to their pathogenic roles in a great variety of cancers including breast, glial, lung, gastric, endometrium, head and neck, and colorectal tumors [388]. ErbB receptors are characterized by an extracellular domain (ECD) with two regions involved in ligand binding (ECD1 and ECD3) and two cysteine-rich portions mediating dimerization (ECD2 and ECD4), a single-pass transmembrane domain (TMD) with two GxxxG dimerization motifs and an intracellular domain composed of a short juxtamembrane region (JMD) with an N-terminal (JM-A) and a C-terminal segment (JM-B), a tyrosine kinase domain (TKD) and a C-terminal tail serving as sites for phosphorylation and docking for signaling partners. These domains undergo large conformational changes during activation, as reviewed recently [389].

According to the classical dogma, receptors are in monomer forms in the membrane in inactive state and ligand binding leads to their dimerization and activation [390], however, inactive dimers and higher-order oligomers were recently described [48,391]. In general, the monomeric state of ErbBs is characterized by a tethered ECD configuration due to interactions between ECD2 and ECD4, resulting in an occluded canonical dimerization interface preventing the intrinsic dimerization tendency of TMD and ICD, thus stabilizing the inactive conformation [392]. Alternatively, inactive dimers and oligomers may form through contacts other than that of the canonical interface with keeping the closed configuration [48,391]. Since their N-termini are consequently held apart, TMDs are monomers or dimers with an interface provided by a GxxxG motif in the C-terminal part of the helix [393,394]. As a result, the JM-B segment is embedded into the membrane due to interactions between the positively charged amino acids of JM-B and negatively charged lipids of inner leaflet [395,396], leading to an intrinsically autoinhibited conformation of the kinase domain similar to inactive CDKs [397] (Fig. 5).

During ErbB activation, ligand binding by ECD1 and ECD3 (or an altered primary structure in ErbB2) results in conformational opening with the protrusion of a dimerization arm of ECD2 mediating canonical back-to-back dimerization of receptor monomers [398]. This leads to the formation of a TMD dimer where the binding surface is provided by the N-terminal GxxxG dimerization motifs [393,394], enabling release of the JMD from the membrane and antiparallel dimerization of JM-A segments [399]. As a result, extensive interactions between JM-B and TKD promote consequent formation of an asymmetric kinase dimer [395,396], in which the C-terminal lobe of an activator kinase binds to the N-terminal lobe of the acceptor kinase, thereby stimulates it allosterically initiating signaling cascades [397]. Recent observations added additional levels of complexity to the activation process by demonstrating that different ligands might induce ECD dimers with different

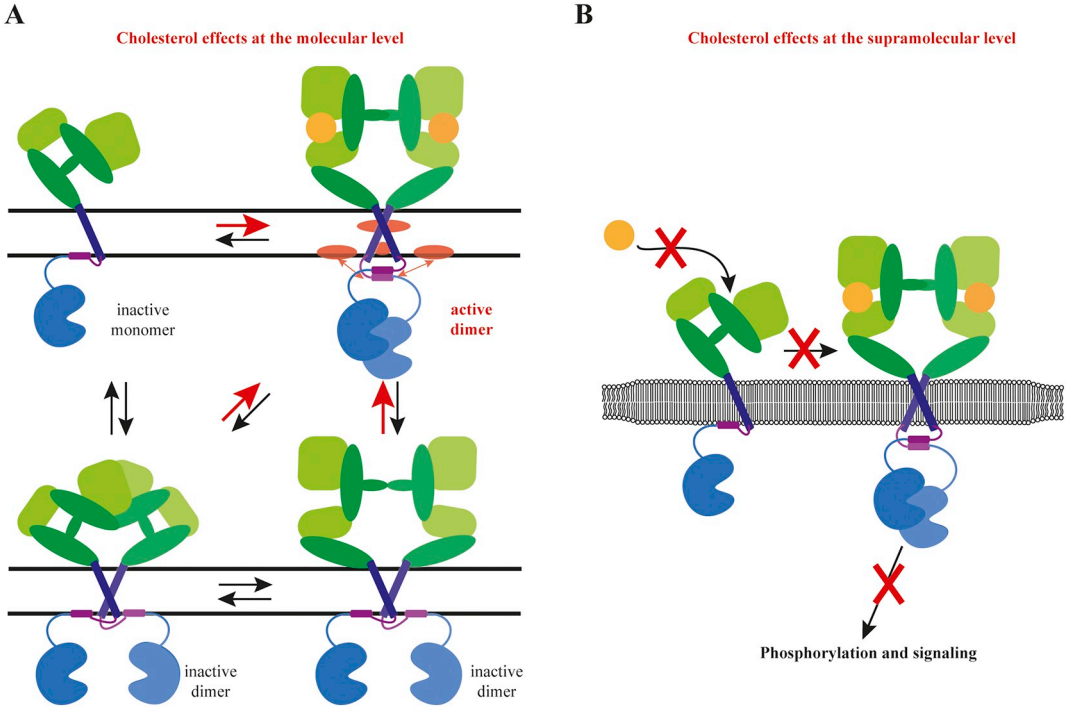

Fig. 5. Dual action of cholesterol on ErbB receptor tyrosine kinases.
(**A**) At the molecular level, cholesterol may contribute to the activation of ErbB proteins by promoting formation of the active dimer (shown at the top right corner of the panel) through stabilization of TM dimer formation (*dark purple*) mediated by the N-terminal GxxxG motif by direct binding to residues neighboring this motif (upper *red* ellipse) and building a "frozen" pad of lipids (lower middle *red* ellipse) at the C-terminal TM region holding them apart, thereby stabilizing N-terminal dimerization. Additionally, cholesterol induced increases in the magnitude of the dipole potential (shown as lower lateral *red* ellipses) increases the repulsion of JM segments (*light purple*) from the membrane, thus aiding formation of JM dimers and subsequently asymmetric activating dimerization of IC kinase domains (*blue*). (**B**) At the supramolecular level, cholesterol-dependent lipid raft microdomains of the cell membrane exert an inhibitory role on ErbB function by decreasing ligand (*orange*) binding to EC ligand binding domains (*light green*), decreasing EC dimerization through dimerization domains (*dark green*), and subsequently attenuating ligand induced signaling cascades.

configurations and stability [400], various TMD [394,401] and kinase dimers having different conformations might exist [402,403], and higher-order oligomeric receptor complexes can act as signal initiators besides dimers [404,405], all of which can lead to substantially altered signaling dynamics and atypical cellular responses (Fig. 5).

The function of ErbB proteins is substantially modulated by its lipid environment in the cell membrane. Effects of cholesterol mediated by direct binding are barely documented (summarized in Fig. 5 panel **A**), possibly due to the inability of a single transmembrane ErbB helix to sterically accommodate cholesterol molecules. Instead, effects of cholesterol on ErbBs are generally attributed to alterations in membrane biophysical parameters or consequences of membrane microdomain formation in keeping with the preferential localization of these proteins in lipid rafts in a cholesterol dependent manner [45,47,90,91]. In general, cholesterol (and raft localization) is thought to exert inhibitory effects on ErbBs (as summarized in Fig. 5 panel **B**), since its depletion with MβCD resulted in increased ligand binding affinity [90,91], increased ligand induced dimerization and clustering [46,91,406], and autophosphorylation of the receptors [90,150,406], accompanied by increased activity of downstream elements of the signaling pathway [47,90]. These effects were generally reversed upon addition of MβCD-complexed cholesterol [90,91,150,406] and, according to a study, ent-cholesterol [150], corroborating indirect effects of sterols. Consistent with the proposed mutual connection between membrane biophysical parameters and ErbB receptors, overexpression of these proteins might lead to deformation of the cell membrane [407] and alterations in local membrane thickness or curvature might affect receptor clustering [48].

On the other hand, recent MD simulations revealed cholesterol molecules found predominantly coincident with GxxxG motifs modulating TMD dimerization interfaces with a tendency to favor activating N-terminal association, an effect that was attributed to changes in membrane thickness [31]. NMR spectroscopy and MD simulations demonstrated substantial changes in lipid ordering associated with conformational changes in ErbB TMDs, with a “frozen” pad of lipids at the C-terminal part of helices stabilizing N-terminal dimerization and favoring the release of the JM-A segment from the membrane, thereby promoting kinase activation [30]. Furthermore, an increase in the magnitude of dipole potential was shown to facilitate ErbB homo- and heterodimerization associated with increased receptor phosphorylation (despite decreased ligand binding), possibly through favoring TMD dimerization via the N-terminal GxxxG (by decreasing repulsion between helical dipoles) or increased repulsion of the positively charged JM-A segment resulting from more positive intramembrane potential [45] (Fig. 5 panel **A**).

These seemingly contradictory results regarding sterol effects on ErbB proteins might be resolved by a dual role of cholesterol. It might favor receptor activation on a single molecular basis through direct or dipole potential mediated effects [30,31,45], while inhibiting activation on the supramolecular level through the special membrane microenvironment provided by lipid rafts [46,47,90,91,150,406]. Consistent with this hypothesis, ErbB receptors were proposed to exit raft microdomains during activation [45].

8. Summary and conclusion

As detailed in the pages above and summarized in Table 1, cholesterol exerts a multitude of diverse and often opposing effects on the function of proteins embedded in the plasma membrane. Beyond its structural and membrane organizing role, the effects of cholesterol on the physical properties of the membrane and the consequential modulation of membrane protein function were recognized early. Accordingly, most such observed alterations were initially attributed to changes in membrane fluidity, lateral stress, curvature and thickness.

With the discovery of protein-organizing dynamic lipid membrane regions, the lipid rafts, which were found to be richer in cholesterol and sphingolipids than the bulk of the bilayer, attention shifted to the raft or non-raft localization of membrane proteins. As cholesterol was identified as an essential component of raft assembly, depleting the membrane of cholesterol by MβCD and thereby disrupting raft structure became a common way of investigating the effect of protein recruitment into or out of rafts on their functions. However, the mere fact of a protein being localized to rafts does not say much about the mechanism of modulation. The originally proposed indirect physical effects must still be considered due to the special composition and increased thickness of rafts. In addition, rafts act as organizing platforms for protein complexes for efficient interaction in signaling pathways. Thus, disruption of rafts may also separate interacting partners, thereby modifying protein function. Lipid rafts have also been implicated in protein trafficking or determining the residency time of proteins in the membrane, in that way regulating their expression level and overall activity.

Along with described indirect effects, for certain proteins modulation via direct cholesterol binding was suspected, but the distinction of the two pathways required new techniques. Using stereoisomers of cholesterol, several observed effects were found to be cholesterol-specific and as such, could not be explained by indirect membrane effects. The generally accepted view that identical effects by cholesterol and its isomers on protein function imply a non-specific membrane action has recently been challenged by showing that all sterols may bind to the same site of lax constraints and induce similar effects. Moreover, it is also possible that despite binding to the same site isomers evoke different responses. Thus, results obtained with cholesterol isomers or analogues require careful interpretation.

With the advancement of high-resolution structural techniques and MD simulations, the exploration of specific modulatory cholesterol binding sites received a boost. Various cholesterol binding motifs have been identified, which revived interest in the topic. This proved useful as they improved our structural understanding of cholesterol effects and intensified the search for specific ligand-like regulatory mechanisms. As a result, numerous such motifs (CRAC, CARC and CCM) have been found in the sequence of the majority of the investigated proteins. However, the motifs have very lax requirements as to the identity of the residues involved and no 3-dimensional arrangement constraints on the sequences. This explains the high number of copies found and the fact that many of them could not be proven functionally relevant. Recent high resolution structures and improved MD simulations questioned their active role by showing that cholesterol does not bind to these motifs, mutations in them do not affect cholesterol sensitivity, and that cholesterol could bind to other regions, sites or domains that were not part of the motifs. Thus, these recent results indicate that the role of CRAC and similar motifs may be overrated, and experimental verification of their real functional relevance is required.

Since cholesterol is a membrane component, most studies focus on its interaction with the transmembrane domains of proteins, which are in actual contact with the surrounding lipids. However, a number of reports have shown the role of juxtamembrane or intracellular domains in mediating cholesterol effects on protein function. CRAC motifs have been found outside of the TMD, and in some proteins the deletion of the IC domains abolished cholesterol action. It has been suggested for several ion channels and ErbB proteins that the JMD and ICD domains may have dynamic interactions with the inner leaflet of the membrane by folding back and that this is accomplished by cholesterol. This mechanism and its presence in other membrane proteins need further investigations.

When analyzing cholesterol effects on transmembrane proteins with highly variable transmembrane domains, in most protein families some general trends emerge from the vast amount of accumulated data summarized in Table 1. For example, while in most GPCRs cholesterol enhances function, in many voltage-gated K^+ channels a suppression of current amplitude, a right-shift in the voltage-dependence of activation and a slowing of activation kinetics have been reported following cholesterol enrichment, which is likely due to indirect membrane parameter effects. However, concurrently more specific regulatory

Table 1
Summary of cholesterol effects on transmembrane proteins.

Protein family	Structure	Cholesterol effect	Indirect mechanism suggested (ref)	Direct mechanism suggested (ref)	Role of rafts (raft partitioning/raft mediated effects) (ref)
K_V channels	Tetrameric, 6TM	Positive modulation: K_V4.2, K_V7.2/K_V7.3?	[251,252]	[252]	[9,72–76,241–246,248,251]
		Negative modulation: K_V1.3, K_V1.5, K_V10.1, K_V2.1, K_V7.2/K_V7.3?	[9,247,252]	[252,253]	
Kir channels	Tetrameric, 2TM	Positive modulation: Kir3.2, Kir3.4, Kir7.1, Kir3.1/Kir3.4, Kir3.1/Kir3.3/Kir3.4	–	[267,272–275,277]	[255,264–266]
		Negative modulation: Kir1.1, Kir2.1, Kir2.2, Kir4.1, Kir6.2		[2,7,50,128,133,215,216,260,267,269,270]	
BK channel	Tetrameric, 7TM	Negative modulation	[295,303,305]	[54,125,126,306]	[298–300]
Na_V channels	4-Domain monomeric 4 × 6 TM	Positive modulation: Na_V1.4, Na_V1.8	[79,177]	[78]	[78,79]
		Negative modulation: Na_V1.4, Na_V1.5, Na_V1.9			
Ca_V channels	4-Domain monomeric 4 × 6 TM	Positive modulation: Ca_V2, Ca_v1	[77,312,316]	[318]	[77]
		Negative modulation: Ca_v1			
TRP channels	Tetrameric, 6TM	Positive modulation: TRPV3, TRPV1, TRPC1	[321,324,325]	[56]	[80,323]
		Negative modulation: TRPM8, TRPV1			
Piezo channels	Trimeric, 38 TM	Positive modulation	[345,346]	–	[346]
pLGIC	Pentameric, 4 TM	Positive modulation: nAChR, $GABA_AR$	[33]	[10,57–60,124,134,135,218,220]	[81,170,350,351]
Na^+/K^+ ATPase	Dimeric (trimeric), α:10, β:1 (+ γ:1) TM	Positive modulation	[34,178,211,358,360]	[61–63,359]	[82]
ABC transporters	Mono/dimeric, total 12 TM	Positive modulation: ABCB1, ABCG2	[35,364]	[11,12,64–67,368,369]	[365,366]
GPCR	Mono/dimeric, 7 TM	Positive modulation: oxytocin R, CCK1R, CXCR4, mGluR, A2AR?, rhodopsin, 5HTR, opioid R	[36–40,42–44]	[14,15,18–23,373,376]	[83,84,86–88,379,380]
		Negative modulation: CB1R, A2AR?, β2AR	[41]	[13,16,68,375]	[85,89,374]
ErbB R	Mono/dimeric, 1 TM	Positive modulation	[45]	[30,31]	–
		Negative modulation	[48,407]	–	[45–47,90,91,150,406]

pathways via binding sites, protein partners in rafts, modifications in signaling cascades may also be activated, and the relative weight of these parallel processes will determine the final outcome of protein function modulation. Thus, in certain channels or in different expression systems the generally observed phenomena may be overridden by more robust, channel- and cell-specific effects. The alteration of membrane physical parameters is a factor for all proteins, but multi-TM proteins, such as ion channels are more likely to have specific cholesterol binding sites formed for example among neighboring TMDs, than single TM receptors, such as the ErbB proteins, and consequently are more susceptible to direct modulation. Altogether, these factors can lead to contradictory cholesterol effects on proteins, which are indeed often observed, as also indicated in Table 1.

These controversies could particularly arise from the fact that most studies used a limited number of different techniques and focused on a single aspect (direct, indirect or raft-mediated) of cholesterol effect without considering other simultaneous actions at a different level. For example, when searching for specific ligand-like binding sites on protein surfaces, the non-specific effects of the surrounding lipids and the possible protein-protein interactions within rafts are mostly disregarded. Although cryo-EM and X-ray crystallographic images provide high-resolution structures to identify cholesterol binding sites, they only represent a snapshot of a possible conformation in a non-physiological environment in the absence of cellular functions, membrane potential and interacting molecules. Likewise, MD simulations may give powerful predictions about specific cholesterol binding sites, but the model systems are highly artificial and oversimplified, neglecting interacting proteins or changes in the biophysical parameters of the membrane. Methods previously used to examine indirect cholesterol effects mostly applied non-physiological oversimplified model membranes or, when cells were examined, the spectrofluorometric techniques provided no visualization and no information about individual cells or whether the signal originated from the cell membrane or intracellular compartments. Additionally, most studies lack the use of stereoisomers or cholesterol analogues to differentiate between direct and indirect effects, and as a result the conclusions about the mechanism of action are often speculative without sufficient experimental support. Studies about the raft-mediated cholesterol effects also have serious intrinsic limitations due to the application of detergent-based techniques with totally non-physiological conditions and arbitrary definitions of membrane microdomains depending on the type of the applied substance for solubilization, or, alternatively, the use of conventional microscopy with limited spatial resolving power not reaching the size of lipid rafts. Furthermore, raft-mediated alterations are often described as simply the inverse of MβCD effects, which is oversimplification again, since this idea does not consider plausible direct MβCD or direct and indirect cholesterol (but not raft-mediated) effects on proteins.

Controversies arising from these one-aspect views could be resolved by considering a “holistic” multi-level approach to describe cholesterol-induced alterations in protein function. Examinations of cholesterol action should include molecular interactions between cholesterol and proteins (level of direct modulation), effects on membrane biophysical parameters (indirect level) and raft-mediated alterations with possible changes in the interaction efficiencies with other membrane proteins or interacting cytosolic molecules (raft level), as summarized in Fig. 1. In summary, all possible modulatory pathways by cholesterol should be thoroughly investigated by simultaneously using multiple experimental techniques, because this is the only way to describe precisely the continuum between concurrently existing direct, indirect and raft-mediated effects of cholesterol on all membrane proteins. The constant methodological advancements give us more tools than ever before to accomplish this: the availability of high resolution structures by X-ray crystallography, and especially cryo-EM grows day by day increasing the number of sequences involved in direct cholesterol binding; newly developed fluorophores yield information about the biophysical parameters of membranes in living systems (and can possibly be used in superresolution applications); raft structure and protein recruitment can be studied with superresolution imaging techniques, such as STED, STORM or PALM, and can be combined with microscopic FRET imaging to study direct protein interactions; the ever-increasing computing power enables the running of more complex MD simulations for longer periods; special electrophysiological techniques, such as TEVCF make it possible to distinguish between effects on different functional domains of an ion channel; and of course the use of cholesterol stereoisomers and other analogues allows the separation of direct and indirect effects. Finally, the relevance of findings obtained with the previous methods should be necessarily tested by examining the consequences of cholesterol-induced alterations on cellular functions, such as proliferation, differentiation, survival, activation, migration, etc. (which can be considered as an additional synthesizing level of cholesterol action on membrane proteins in our suggested multi-level approach).

Altogether, the combined use of these techniques should aid us in understanding the effect of cholesterol on the function of embedded membrane proteins, as it is one of the most abundant and influential lipids in the plasma membrane. In numerous diseases, such as hypercholesterolemia, neurodegenerative disorders, immunological disorders, tumors, and in aging, the malfunctioning of membrane proteins is involved, which in many cases may be partly due to altered membrane composition, especially cholesterol content. Therefore, future studies should aim to elucidate the simultaneous direct and indirect mechanisms of cholesterol regulation using the latest arsenal of methodologies.

Abbreviations

5-HT_3R	serotonin receptor
7DHC	7-dehydrocholesterol
A2AR	adenosine receptor
ABC	ATP-binding cassette
AFM	atomic force microscopy
AP	action potential
BK	voltage- and calcium activated large conductance potassium channel
CARC	reverse or mirror version of the CRAC, cholesterol recognition motif
Ca_V	voltage gated calcium channel
CBR	cannabinoid receptor
CCKR	cholecystokinin receptor
CCM	Cholesterol Consensus Motif
CRAC	Cholesterol Recognition Amino Acid Consensus
CTD	cytosolic tail domain
DP	dipole potential
DPH	diphenylhexatriene
DRG	dorsal root ganglion
EC	extracellular
ECD	extracellular domain
ER	endoplasmic reticulum
ESR	electron spin resonance
FLIM	fluorescence lifetime imaging
F-V curve	fluorescence-voltage curve
GABA	gamma-aminobutyric acid
GIRK	G-protein gated inwardly rectifying potassium channel
GPCR	G-protein coupled receptor
GPI	glycosylphosphatidylinositol
G-V curve	conductance-voltage curve
GxxxG	a dimerization motif
HEK	human embryonic kidney
HMGCoA	3-hydroxy-3-methylglutaryl-CoA
IC	intracellular
ICTD	intracellular C-terminal domain
JM	juxtamembrane

JMD	juxtamembrane domain
KCHIP	potassium channel interacting protein
KCNE	potassium voltage-gated channel subfamily E
Kir	inward rectifier potassium channel
K_V	voltage gated potassium channel
LRRC	leucine-rich repeat-containing domain
LxxxL	a steroid binding element
MβCD	methyl-beta-cylclodextrin
MD simulations	molecular dynamics simulations
mGluR	metabotropic glutamate receptor
MscL	large-conductance mechanosensitive channel
MscS	small-conductance mechanosensitive channel
MTS	methane thiosulfonate
nAChR	nicotinic acetylcholine receptor
Na_V	voltage gated sodium channel
NMR	nuclear magnetic resonance
NPA	non-physiological amphiphiles
PD	pore domain
PIP_2	phosphatidylinositol-4,5-bisphosphate
pLGIC	pentameric ligand gated ion channels
Po	open channel probability
PUFA	polyunsaturated fatty acid
RCK	Regulatory of Conductance for K^+ domain
SOCE	store-operated Ca^{2+} entry
STOML3	stomatin-like protein-3
STORM	stochastic optical reconstruction microscopy
TEVCF	two-electrode voltage-clamp fluorometry
TIRF	total internal reflection fluorescence
TKD	tyrosine kinase domain
TM	transmembrane
TMD	transmembrane domain
TRAAK	TWIK-related arachidonic acid-stimulated K+ channel/potassium channel subfamily K member 4
TREK	TWIK-related K+ channel/potassium channel subfamily K member 2
TRP	transient receptor potential channel
TRPA	transient receptor potential ankyrin channel
TRPC	transient receptor potential canonical channel
TRPM	transient receptor potential melastatin-related channel
TRPML	transient receptor potential mucolipin channel
TRPP	transient receptor potential polycystin channel
TRPV	transient receptor potential vanilloid channel
TX100	Triton X-100
VSD	voltage-sensor domain

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the Hungarian Academy of Sciences projects KTIA_NAP_13-2-2015-0009 and KTIA_NAP_13-2-2017-0013 (Z.V.); OTKA K132906 (Z.V.), the ÚNKP-18-3-IV-DE-54 New National Excellence Program of the Ministry of Human Capacities (F.Z.); ÚNKP-19-3-III-DE-92 New National Excellence Program of the Ministry for Innovation and Technology (F.Z.), the National Research Development and Innovation Office (GINOP-2.3.2-15-2016-00020) (T.K.), EFOP-3.6.2-16-2017-00006 (G.P.), GINOP-2.3.2-15-2016-00015 (G.P.) and OTKA K119417 (G.P., F.Z.) and K120302 (T.K.).

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