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Review

The influence of cholesterol on membrane protein structure, function, and dynamics studied by molecular dynamics simulations☆

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ABSTRACT

The plasma membrane, which encapsulates human cells, is composed of a complex mixture of lipids and embedded proteins. Emerging knowledge points towards the lipids as having a regulating role in protein function. Furthermore, insight from protein crystallography has revealed several different types of lipids intimately bound to membrane proteins and peptides, hereby possibly pointing to a site of action for the observed regulation. Cholesterol is among the lipid membrane constituents most often observed to be co-crystallized with membrane proteins, and the cholesterol levels in cell membranes have been found to play an essential role in health and disease. Remarkably little is known about the mechanism of lipid regulation of membrane protein function in health as well as in disease. Herein, we review molecular dynamics simulation studies aimed at investigating the effect of cholesterol on membrane protein and peptide properties. This article is part of a Special Issue entitled: Lipid–protein interactions.

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Contents

Abbreviations: AA, all-atom; AD, Alzheimer's disease; AFM, atomic force microscopy; BK channels, big potassium channels or large conductance, Ca^{2+}- and voltage-gated K^+ channels; CCM, cholesterol consensus motif; CG, coarse-grained; CRAC, cholesterol recognition/interaction amino acid consensus; CTD, cytosolic C tail domain; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; EM, electron microscopy; ErbB, epidermal growth factor receptor; FF, force field; $GABA_AR$, the γ-aminobutyric acid type A receptor; GluCl, glutamate-gated chloride channel; GPCR, G-protein coupled receptor; Kir, strongly inwardly rectifying K^+ channel; MD, molecular dynamics; MM/PBSA, Molecular Mechanics/Poisson–Boltzmann Surface Area; nAChR, the nicotinic acetylcholine receptor; NMR, nuclear magnetic resonance; PG, phosphatidylglycerol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SERCA, the sarco/endoplasmic reticulum Ca^{2+}-ATPase; TEM, transmission electron microscopy; VDAC, the mitochondrial voltage-dependent anion channel.

☆ This article is part of a Special Issue entitled: Lipid–protein interactions.

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1. Introduction

One of the main challenges in structural biology is to understand the interplay between proteins and molecular constituents of the plasma membrane [1]. Lipid membranes encapsulate cells separating their interior from the extracellular matrix. The lipid bilayer is a complex mixture of phospholipids [2], glycolipids [3], and cholesterol arranged into two asymmetric leaflets [4]. The cholesterol present in cell membranes has been shown to be important for maintaining a healthy body, e.g., in regulating neurotransmission [5], cell signaling [6], and protein sorting [7,8]. An imbalance in the cholesterol level has similarly been implicated in many diseases, such as cancer [9], diabetes mellitus type 2 [10], and Alzheimer's disease (AD) [11,12] among others. However, a molecular understanding of how cholesterol is involved in these essential biochemical processes or how it may result in disease development is still lacking. Computational approaches applied to study how cholesterol influences the function of membrane-embedded proteins have added to the understanding of these processes and directed further experiments. This review provides a summary of these studies, with particular emphasis on molecular dynamics (MD) simulations and the insights they provide. Furthermore, an outlook into the future application of MD simulations to study interactions between cholesterol and various membrane components, focusing on how the function of the peptide and protein may be affected, is also included.

In this review, we aim to provide molecular-level insight into protein–cholesterol interactions and its effects on protein function. The review is divided into two parts. The first part provides an introduction to the cell membrane focusing on cholesterol, which includes a brief overview of regulation of membrane proteins and peptides by the lipid environment. Experimentally, such regulatory effects are extremely challenging to study, due to the dynamic nature of the cell membrane, which accordingly renders computational MD simulations very suitable for studying the effects of cholesterol on membrane proteins and peptides. We will conclude the first part with a short outline of how computational methods can assist in describing the effect of cholesterol on membrane proteins. The second part of the review highlights a number of studies in which MD simulations have shed light on membrane protein–cholesterol interactions. For the second part of the review, a comprehensive literature search was performed, and to the best of our knowledge it includes all studies in which the influence of cholesterol on the function of a membrane embedded protein or peptide has been studied using MD simulations. GPCRs are not included in the review rather the interested reader is referred to another contribution in this special issue [74].

1.1. Modulation of membrane proteins by the lipid environment

The cell membrane (or plasma membrane) separates the interior of a cell from the exterior, hereby protecting the cell. Membrane proteins encompass about 50% of the plasma membrane and are involved in many cellular processes, such as signaling across the membrane, cell–cell communication and the regulation of the access of nutrients and ions to and from the cell [13]. The last decade has provided increasing evidence that membrane lipids play important roles in shaping membrane–protein function [14]. Protein crystallography has further substantiated the importance of the lipid–protein interplay by reporting several structures with intimately bound lipids; cholesterol, cardiolipins, phosphatidylglycerol (PG) lipids, etc. have been found co-crystallized with both α-helical and β-barrel membrane proteins, prompting the question of how these lipids are influencing structure, function, and dynamics of membrane proteins [15]. Specifically, structures of proteins with co-crystallized cholesterol have been published over the past few years, including G-protein coupled receptors (GPCRs) [15], and most recently also transporters [16–18].

Recent research has revealed the existence of organization in the plasma membrane, such as the presence of highly ordered, cholesterol-enriched lipid raft microdomains [19,20]. Evidence also shows that some proteins are prone to segregate into these microdomains [19]. The relevance of such microdomains in the membrane, and their role in modulation of embedded membrane proteins is still much debated [21], though they are believed to be essential for many biological processes [22] including neurotransmission [23,24] and amyloid diseases [25]. The presence of integral membrane proteins also modulates the dynamics of the lipid environment. The lipids in the first ring surrounding the protein, referred to as the annulus, have a constrained motional freedom due to their interactions with the

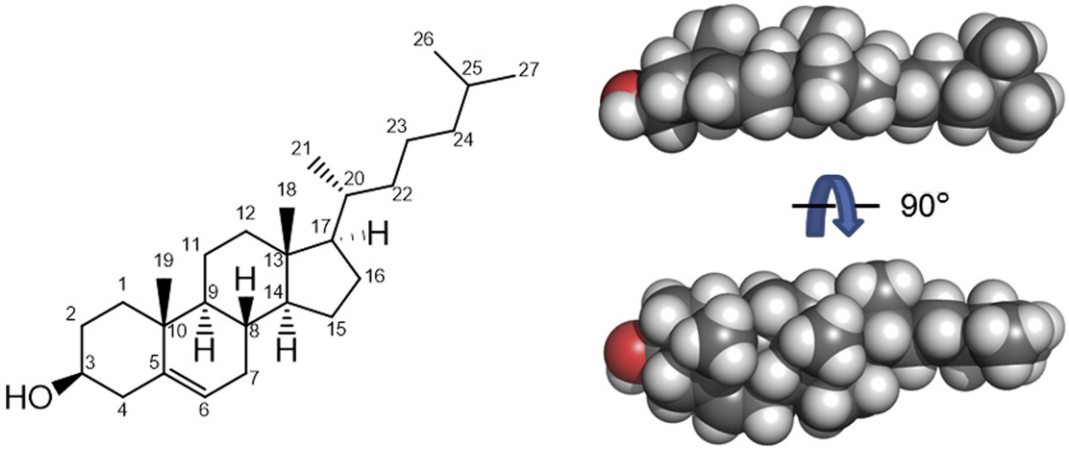

Fig. 1. The molecular structure of cholesterol.

protein, and are known as annular lipids [26]. Additionally, lipids may bind with a higher affinity in so called non-annular sites such as clefts on the protein surface or at the interface between protein subunits. Non-annular lipids are tightly bound and the sites are less accessible to the bulk lipids. Therefore the exchange between non-annular lipids and bulk lipids is significantly slower than what is observed for the exchange between annular and bulk lipids [26]. It has been speculated that non-annular lipids may mediate protein function and/or oligomerization [27].

The specific interaction between lipids in the membrane and membrane proteins has been somewhat neglected in the study of cell membranes and embedded proteins. This field of research has traditionally been divided into two areas: *i*) studies of the biophysics of lipid membrane components, often in simplified model systems [28], and *ii*) studies of membrane protein structure and function neglecting the effects of the surrounding membrane [29]. This dichotomy ignores that essential biological processes can only be understood in the complex interplay between membrane lipids and membrane proteins. This division has been employed partly due to unawareness of the interplay, and partly due to technical limitations. New emerging technologies, both experimental, such as cryo-electron microscopy (EM) [30] and femtosecond crystallography [31], as well as computational, such as coarse-grained (CG) methods [32,33], continuum-MD [34] and advances in computing hardware [35,36], has opened the door for exploring the intriguing question of how lipids regulate properties of membrane proteins at a molecular and atomistic level.

1.2. Cholesterol

Cholesterol is a major constituent of animal plasma membranes [37]. Cholesterol contains a rather rigid planar tetracyclic ring with an angular methyl group on one side and an isooctyl chain attached to C17 as well as a small head group consisting of a β-hydroxyl group at C3 (see Fig. 1). Cholesterol modulates membrane fluidity and membrane thickness, and is non-randomly distributed in the membrane with a preference for ordered microdomains [37]. A large number of membrane proteins, including receptors [27], ion channels [38], transporters [39,40] and peptides [41,42], have been shown to be affected by the presence of cholesterol. Modulation of proteins by cholesterol can either be direct through binding of cholesterol to the protein or indirect through changes in the physiochemical properties of the membrane, such as the fluidity and membrane thickness, or it can be a combination of both [38]. To distinguish between direct and indirect effects, cholesterol can be substituted with other sterols that have similar physiochemical properties but which may not fit into a cholesterol binding pocket. Epi-cholesterol, which differs from cholesterol with respect to the stereochemistry of the hydroxyl group, and ent-cholesterol, which has inverted stereochemistry at all chiral positions, are both widely used for this purpose [38]. Direct binding of cholesterol to proteins can in principle also be measured using radiolabelled cholesterol, although the insolubility of cholesterol in water makes this a very difficult task [43]. Alternatively, a recombinant His-tagged version of the proteins of interest can be used in combination with affinity chromatography on nickel agarose columns, which allows for separation of bound and free cholesterol. Fluorescent analogs of cholesterol have similarly been used to study cholesterol binding, either by using intrinsically fluorescent sterols or cholesterol with a chemically linked fluorophore [38].

Based on proteins which are known to interact with cholesterol, a cholesterol recognition/interaction amino acid consensus (CRAC) pattern with the sequence $(L/V)–X_{1-5}–(Y)–X_{1-5}–(K/R)$ has been suggested to be related to cholesterol recognition [44]. The X_{1-5} segment can consist of 1 to 5 residues with an arbitrary sequence. Similarly, an inverted CRAC motif, known as CARC and defined as $(R/K)–X_{1-5}–(Y/F)–X_{1-5}–(L/V)$, has also been suggested as a cholesterol-recognition pattern [45]. The looseness of the definition of the CRAC and CARC motifs make them difficult to use for reliable prediction of cholesterol binding sites, although the predictive value does increase if only protein segments close to the membrane are considered [46]. A cholesterol consensus motif (CCM) has been proposed for the GPCR family of proteins [47] based on the position of a cholesterol molecule in the crystal structure of the β2-adrenergic receptor. However, as the CCM has also been found in GPCRs speculated to be only indirectly regulated by cholesterol as well as GPCRs which are functionally active in cholesterol-free *Escherichia coli* membranes, the predictive value of the motif may be limited [48].

1.3. Computational approaches for studying cholesterol–membrane protein interactions

In order to study any system with a computational tool the first requirement is a reasonable starting structure. In the case of cholesterol–membrane protein interactions, the 3D-structure of cholesterol is well known, and for a number of membrane proteins the structures have also been characterized. For those systems where the structure of the membrane protein is not available, tools like homology modeling are applied to find a close model that can be used in place of an experimentally determined structure [49]. Along with knowledge of the protein structure, it is crucial to know the position of cholesterol with respect to the protein. However, only very few 3D-structures have been published of a membrane protein co-crystallized with cholesterol, and identification of specific binding sites, if present, is necessary for studying the effects of cholesterol on the protein. This knowledge is achieved either via experimental techniques such as nuclear magnetic resonance spectroscopy (NMR) [50], mass spectrometry based methods [51], or through chemical cross-linking of sterols to residues in the putative cholesterol binding site [52]. In addition to experiments, computational methods, such as molecular docking has been used to identify binding sites [49,53,54]. In a docking calculation a large number of different cholesterol–protein complexes are generated [55]. The complexes are then assessed based on a scoring function, and the output corresponds to the best-scoring complexes. Since most docking algorithms are not optimized for locating shallow pockets on the protein surface and, in particular, do not take the lipid environment surrounding a membrane protein into account, thorough inspection and validation of the proposed cholesterol binding modes should be performed. In this context, MD simulations can be used to evaluate the quality of the proposed cholesterol binding pockets. However, it should be noted that fairly long simulation times on the order of hundreds of ns to μs may be needed to obtain converged results [56], due to the rather slow lateral diffusion of molecules in the lipid bilayer compared with molecules in water. Alternatively, experimental validation of the proposed modes may be performed, e.g., by investigating changes in cholesterol modulation upon introduction of mutations in the proposed pocket(s).

MD simulations capture the time-dependent behavior of biological macromolecules in full atomic detail. To perform the simulations, a description of the potential energy landscape is required, and empirical molecular mechanics force fields (FF), are typically used for this purpose [57]. Using the computer as a laboratory allows full control of all variables in the system (e.g., lipid composition, oligomeric state of the protein, presence of ligands) and makes it comparably easy to introduce changes in the studied system, such as point mutations. However, MD methodology does suffer from three challenges; *i*) a time-scale problem resulting in incomplete sampling of phase-space, *ii*) inherent uncertainties in the empirical FF, and *iii*) approximations in the chemical system with regard to size and complexity. Dramatic improvements in sampling and the underlying physical models [58] have nowadays enabled atomic-level simulations at biologically relevant timescales

[59]. Furthermore, biased MD methods are constantly being developed to improve sampling and the exploration of the energy landscape. They typically fall into two classes; either the time step is increased by eliminating the fastest motions of the molecules by use of CG FFs [60,61], or the energy landscape is manipulated by adding biasing forces to accelerate the events of interest [62]. Multi-scale MD simulations, in which CG simulations and all-atom (AA) simulations are combined, can thus be performed to simulate relevant timescales [63,64]. In terms of investigations of membrane proteins, a promising example of this is to use CG MD simulations for the rather slow process of lipid bilayer self-assembly around the protein followed by AA MD simulations to study the protein–lipid interactions in atomistic detail [65]. Computational studies of cholesterol and membrane proteins necessitate FFs which have been parameterized to describe the potential energy landscape of proteins and lipids, as well as protein–lipid interactions. Among the popular choices for such simulations are the atomistic CHARMM [66,67] and AMBER [68,69] FFs and the CG MARTINI FF [70,71], all of which include compatible parameters for cholesterol [71–73]. Most MD studies on protein–lipid interactions are based on single-component bilayers, although the increase in computational power and available parameters is moving the field towards more realistic membrane models. Parameters for a large number of lipid membrane components have recently been included in the MARTINI FF [33], which opens up the possibility of performing simulations with very large, complex, and in vivo like membranes.

2. MD studies investigating how cholesterol modulates membrane protein function

In the following section we will review a number of studies aimed at investigating the interactions between cholesterol and membrane proteins and peptides by applying computational tools and focusing on the effect cholesterol plays on membrane protein and peptide function. We have included, to the best of our knowledge, all available computational molecular dynamics simulation studies, which have focused on how cholesterol in the membrane affects the function of membrane-spanning proteins, however, excluding studies of the GPCRs which are covered separately in this issue [74]. This provides an overview of both the range of techniques applied in such MD studies as well as the types of problems that may be tackled through a computational approach. There are also a number of computational studies in the literature concerning the influence of proteins on the properties of cholesterol-containing membrane, such as domain formation, as well as MD studies of the influence of cholesterol on proteins that are membrane-anchored, rather than membrane-spanning. However, a review of these studies is beyond the scope of this account.

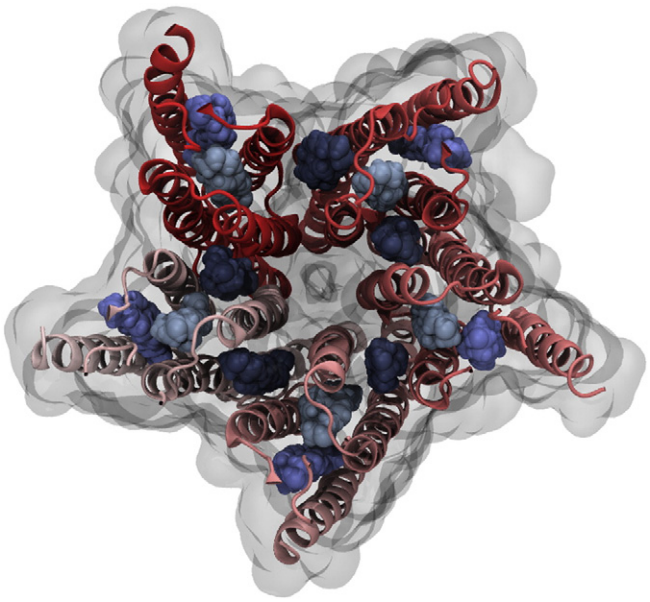

Fig. 2. Cholesterol binding to nAChR. The transmembrane domain of nAChR is shown from the extracellular side. Each subunit is colored in a different shade of red. The cholesterol molecules occupying the three binding sites in each subunit as identified by Brannigan et al. [53] are shown in blue colors. The PDB-file used for creating the figure is Dataset 1 from Brannigan et al. [53].

2.1. Ion channels

Ion channels allow passive diffusion of ions across lipid membranes. A variety of ion channels have been shown to be sensitive to the level of membrane cholesterol. For the majority of channels, cholesterol suppresses channel activity e.g., by decreasing the probability of opening, the conductance, or the number of active channels present in the membrane [38]. However, for some channels, such as the nicotinic acetylcholine receptor (nAChR) and the γ-aminobutyric acid type A receptor ($GABA_AR$), cholesterol is a requirement for the ligand-mediated channel activity [75,76].

2.1.1. Cys-loop receptors

nAChR is a ligand-gated cation-selective pentameric channel in the cys-loop superfamily [77]. It has been shown that the conformational equilibrium of nAChR is modulated by anionic lipids and cholesterol [78]. Incorporation of cholesterol into the bilayer enhances the functional activity of nAChR in reconstituted lipid vesicles, and the nAChR-mediated ion flux increases proportionally to the amount of cholesterol in the membrane up to a certain concentration threshold [75]. Furthermore, photolabeling studies have shown that sterols interact directly with the lipid-exposed transmembrane helices in nAChR [79]. In order to determine potential locations for cholesterol binding sites, Brannigan et al. performed a docking study of cholesterol into nAChR [53] and found three buried non-overlapping sites per subunit, two inside the subunit and one at the interface between subunits (see Fig. 2). All sites correspond to gaps in the electron density of a 4 Å resolution structure of nAChR, gaps which have previously been speculated to be occupied by water. MD simulations of nAChR illustrated that the gaps between the transmembrane helices observed in the crystal structure collapsed if the gaps were initially filled with water whereas simulations with cholesterol bound in the gaps produced results that are more consistent with the experimental structure [53]. Additionally, contacts previously speculated to be important for gating were also observed to be maintained more consistently when cholesterol was included in the simulations. Hence, the results suggest that nAChR can bind up to 15 cholesterol molecules and that cholesterol may aid in shifting the conformational equilibrium towards functionally relevant conformational states. The deeply buried binding sites described in the study could potentially be very difficult to access experimentally due to a slow exchange rate of cholesterol with the bulk environment, which serves as an example of how computational techniques may be used to investigate scenarios which are otherwise inaccessible. There are no CRAC motifs in the transmembrane part of nAChR [45]. However, the suggestion of the CARC motif as an indicator of a cholesterol interaction site was actually made based on studies of nAChR, in which a CARC motif was found in one of the transmembrane helices [45]. Interestingly, the residues in this motif partially overlap with one of the three sites found in the MD study by Brannigan et al., although the potential importance of this motif was not discussed in their paper, since the paper was published several years before the CARC motif was first mentioned [53].

In another MD study with simulations of nAChR in a membrane containing both anionic lipids and cholesterol [80], several annular and non-annular cholesterol binding sites were observed both at the protein lipid interface and between subunits. In this context

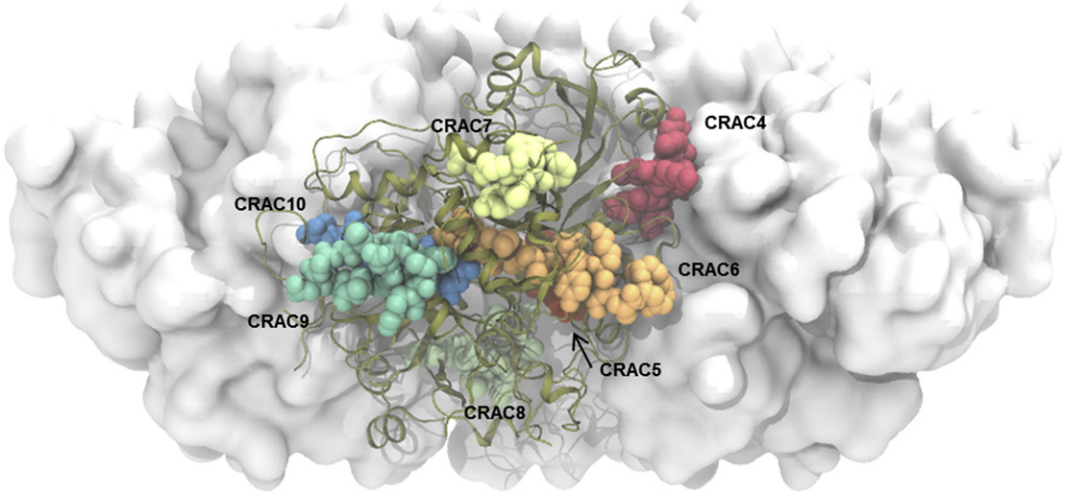

Fig. 3. CRAC motifs in the cytosolic part of the BK channel forming protein Cbv1. A single CTD (PDB code 3NAF [84]) is shown in tan with the residues in CRAC4 to 10 highlighted as differently colored spheres. The three other CTDs in the tetrameric channel are shown as a white surface and the tetramer is viewed from the side such that the transmembrane part of the channel would be positioned above the four CTDs.

any cavity within a subunit or between subunits was defined as non-annular. Cholesterol was not observed to reach the more buried sites inside the subunits, suggested in the above-mentioned study, although it cannot be excluded that this is due to the rather short simulation time of 10 ns. Overall, the two studies both point to the modulating effect of cholesterol on nAChR through direct interactions by binding to either annular or non-annular sites.

The function of another member of the cys-loop superfamily, $GABA_AR$, has also been shown to be modulated by cholesterol [76,81]. Interestingly, both depletion and enrichment of cholesterol decreases the potency of the receptor for the endogenous ligand GABA [76]. It has been speculated that the decreased potency at low cholesterol concentrations may reflect direct interactions between $GABA_AR$ and cholesterol, whereas the decreased potency at high cholesterol concentrations may be due to changes in the physical properties of the membrane, such as fluidity and thickness [76]. Similar to the studies of nAChR, possible cholesterol binding sites on $GABA_AR$ have been investigated through the use of docking calculations and MD simulations [49]. Since no high resolution structures existed for $GABA_AR$ at the time of the study, a homology model based on the crystal structure of a bacterial glutamate-gated chloride channel (GluCl) was used for the simulations. GluCl has been crystallized with a lipophilic agonist, ivermectin, bound in the interface between the transmembrane subunits [82]. Two models of cholesterol-bound $GABA_AR$ were constructed, one in which cholesterol was placed according to the position of ivermectin in the crystal structure of GluCl and one based on docking of cholesterol to $GABA_AR$ [49]. The two models predict the same binding pocket, but differ in the orientation and tilt of cholesterol within the pocket. MD simulations of cholesterol-bound $GABA_AR$ led to two instances of cholesterol unbinding and rebinding in the predicted pocket with a binding mode similar to the docked conformation. Overall, the simulations suggest the existence of a deeply buried pocket in which cholesterol is generally stable although it fluctuates between several orientations. Furthermore, it was observed based on measurements of the pore radius that cholesterol tends to promote pore opening, which may explain the suggested direct effect of cholesterol on $GABA_AR$. Although this study is focused on the direct effect of cholesterols on $GABA_AR$, indirect effects of cholesterol on membrane properties could also be investigated with current developments being made for improved lipid force fields that will enable reliable CG and AA multi-scale simulations.

2.1.2. Voltage gated ion-channels

Large conductance, Ca^{2+}- and voltage-gated K^+ channels, also known as "big potassium" (BK) channels, are found in most human cell membranes and control a variety of biological processes [83]. BK channels have a tetrameric structure and consist of a voltage-sensing transmembrane region and a large C-terminal intracellular part, which is responsible for sensing of Ca^{2+} and other intracellular stimuli [84]. In a study by Singh et al. [85], the structural basis for cholesterol inhibition of BK channels was investigated for the BK channel forming protein Cbv1 by a combination of MD simulations, site-directed mutagenesis, and single channel electrophysiology. The study showed that cholesterol action is mediated by the cytosolic C tail domain (CTD), in which seven CRAC motifs, named CRAC4 to 10, are present (see Fig. 3). Specifically, truncation and mutation experiments showed that cholesterol sensitivity is mainly provided by the membrane-adjacent CRAC4, although CRAC5 to 10 were also shown to play a role in the cholesterol-BK channel interaction. MD simulations were performed to identify the interactions involved in ion channel-cholesterol recognition. Simulations of four versions of CTD with cholesterol placed in the vicinity of the CRAC4 motif were performed; full-length CTD (*WT Cbv1 CTD*), a C-terminally truncated version containing only CRAC4 (*Trcbv1 CTD-CRAC4*), and two different cholesterol insensitive mutants, Y450F and K453A, both with mutations in the CRAC4 motif. For each simulation system, three independent, rather short, simulations of 5 ns each were performed. Comparison of the results from the simulations of each of the four systems showed that in the *WT Cbv1 CTD* and *Trcbv1 CTD-CRAC4* simulations, which correspond to cholesterol-sensitive ion channel constructs, cholesterol was more mobile than in the simulations with the cholesterol-insensitive mutants. Based on this, the authors suggest that an increased entropic penalty for the mutants may contribute to the experimentally observed lack of cholesterol sensitivity for these constructs. Additionally, the lack of hydrogen bonding between the cholesterol hydroxyl and residue 453 in the K453A mutant causes a reorientation of cholesterol, which could also be a contributing factor to the lack of response to cholesterol in this mutant. Thus, it was possible to hint to the origin of cholesterol interaction even from these relatively short simulations. Increasing computational resources enabling the possibility of running simulations for longer time scales provide a promising future for application of computational tools to get a deeper understanding of the role of cholesterol in BK channel function.

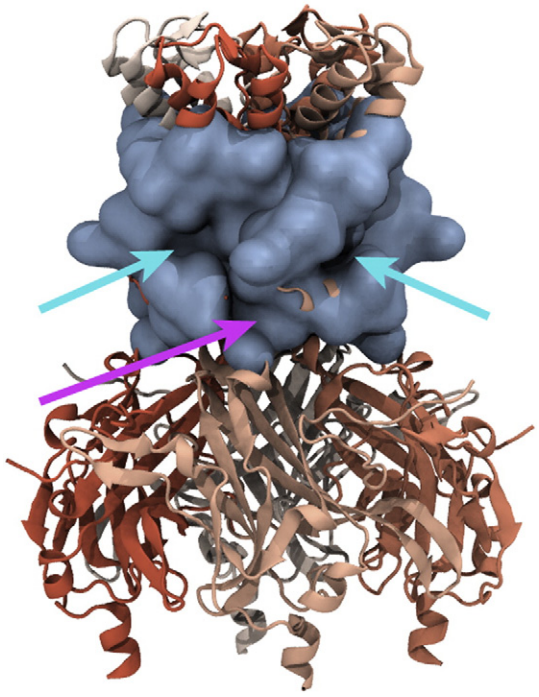

Fig. 4. Cholesterol binding to the Kir2.1 channel. The Kir2.1 channel is shown in cartoon representation with the four subunits in the tetramer in different shades of red. The residues identified by the MD simulations to be within 4 Å of cholesterol are shown in blue surface. The arrows point to the two different binding regions that were identified by Rosenhouse-Dantsker et al. [93]; the transmembrane binding region between the different subunits (region 1, cyan), and the binding region between the transmembrane and the cytosolic domain (region 2, purple). PDB code of protein structure: 2QKS [94].

A recent MD study on cholesterol interactions with the mitochondrial voltage-dependent anion channel (VDAC) displays how the advances in computational power and algorithms increase the accessible simulation times and thus enable more converged results to be obtained [54]. Experiments have shown that VDAC binds cholesterol directly, and it has been suggested that sterols are essential for proper folding and activation of mitochondrial VDACs [86,87]. In the computational study of VDAC, cholesterol was docked into five different sites on the membrane-exposed surface of VDAC, according to positions determined by NMR to be involved in cholesterol binding [50]. Five independent 100 ns simulations were performed of VDAC embedded in a 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)/cholesterol lipid membrane with a cholesterol molecule bound in all five sites simultaneously. To assess the effect of cholesterol on VDAC the results were compared to five independent 100 ns simulations of VDAC with no cholesterol bound in either of the proposed sites. For the cholesterol-bound VDAC system, each of the five sites was essentially occupied for the entire 100 ns in at least three of the simulations. However, unbinding was observed in some instances, suggesting that some of the sites may be low-affinity binding sites. If only a single simulation had been performed for each of the two simulation setups, such unbinding events might not have been observed, which highlights the importance of running multiple independent simulations to obtain improved sampling and consistent results. In one instance, replacement of one of the bound cholesterol molecules with another cholesterol from the membrane occurred, which substantiates the validity of the docking approach. In general, no large differences between the simulations with cholesterol bound and those without were observed. Small changes in the flexibility of the protein were observed when measuring the root-mean-square fluctuation for each residue, corresponding to cholesterol stabilizing VDAC. Additionally, the size of the pore through the channel was also slightly larger in simulations including cholesterol. However, the ion diffusion through the pore and the potential of mean force for movement of ions through the pore were very similar, and thus no changes in channel activity as a consequence of the presence of bound cholesterol were observed. In spite of the reasonably long simulations performed in the study, it is possible that further sampling is needed to observe cholesterol-induced effects, and in this context, methods that accelerate the exploration of the energy landscape, such as accelerated MD [88], could be beneficial.

2.1.3. Kir channels

Strongly inwardly rectifying K^+ (Kir)-channels are found in a wide variety of cells, where they, among other things, are involved in the maintenance of the membrane resting potential [89] as well as regulation of the action potential duration in excitable cells. Kir channels consist of four subunits with each subunit containing two transmembrane regions, a pore-forming loop, and cytosolic amino- and carboxy-terminal domains [89]. It has been shown that the activity of Kir2 channels is decreased in response to cholesterol [90]. Although this effect could be caused by changes in the properties of the lipid membrane, several studies have indicated that cholesterol may also affect Kir channels through direct binding. It has been shown that cholesterol binds to purified KirBac1.1 channels [91] and that cholesterol and *epi*-cholesterol have opposite effects on endothelial Kir currents [89]. Furthermore, *ent*-cholesterol has no effect either on KirBac1.1 or Kir2.1 activity [92], all of which points to a direct effect of cholesterol. Rosenhouse-Dantsker et al. used a combination of molecular docking, MD simulations, and mutagenesis studies to determine putative cholesterol binding sites for the Kir2.1 channel [93]. Based on clustering of the results of the cholesterol docking, six possible binding sites were suggested, and a 50 ns MD simulation was performed for each of the binding modes. During the simulations, five of the initially proposed modes merged into two distinct binding regions while a sixth position led to dissociation of cholesterol from the protein. As the authors also mention, this highlights the importance of combining docking and MD simulations, rather than relying on the docking results alone. One of the cholesterol-binding regions is positioned between transmembrane helices from two adjacent subunits, while the other region is found between the transmembrane domain and the cytosolic domain (see Fig. 4). The two regions do not contain CRAC motifs or CCMs and would thus probably not have been predicted to be important for cholesterol binding based on sequence analysis. From the simulations, binding enthalpies and free energies of binding were calculated using a Molecular Mechanics/Poisson–Boltzmann Surface Area (MM/PBSA) approach and the results suggested a slight preference for region 1 over region 2. However, the binding free energy for region 1 is quite close to 0, corresponding to a weak binding affinity. An analysis of the contact frequency between the residues in the two regions and lipids/cholesterol revealed that while cholesterol has an average of 1.7 contacts/residue the phospholipids have less than 0.4 contacts/residue. This indicates that the two regions correspond to cholesterol-selective non-annular sites in which phospholipids do not bind for extended periods of time. Residues predicted to interact with cholesterol in the two possible binding regions were mutated and basal currents were measured in the presence and absence of cholesterol. In support of the MD results, a number of mutations in each region caused significant changes in cholesterol sensitivity, suggesting that they are important for cholesterol recognition. The study thus serves as a good example of how computational studies can provide hypotheses on possible binding regions, which can then be tested experimentally. The two suggested cholesterol binding regions overlap with structural elements that have been proposed to be involved in channel gating, which may explain how cholesterol binding to these sites can cause a decrease in the channel activity.

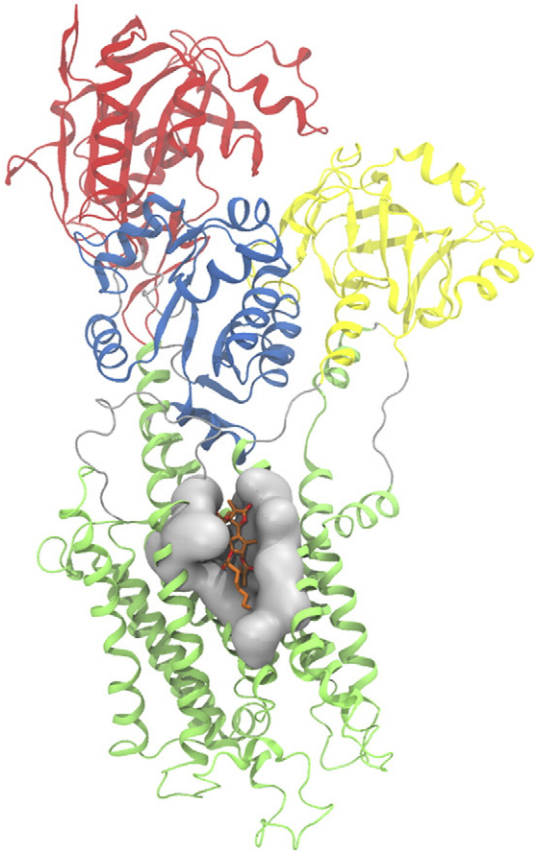

Fig. 5. The structure of SERCA and the thapsigargin binding site (PDB code 2C8K [101]). The transmembrane helices in SERCA are shown in green, while the three cytosolic domains are shown in red, blue and yellow. Thapsigargin is shown in sticks with orange-colored carbon atoms and the residues in the binding pocket are shown as a white surface.

2.2. Aquaporins

Similar to ion channels, aquaporins allow water to passively move from one side of the membrane to the other. Aquaporin 0 is a water transport protein found in high concentrations in the cells in the human ocular lens where it exists as a tetramer [95]. The molar ratio of cholesterol to phospholipid in the ocular lens ranges from 1 to 4, which is in contrast with plasma membranes of typical eukaryotic cells, which have ratios in the range of 0.5 to 1 [96]. MD simulations of an Aquaporin 0 tetramer in a pure 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) bilayer and a mixed DMPC:cholesterol bilayer (1:1 ratio) was performed by O'Connor and Klauda to investigate how Aquaporin 0 has evolved to prefer membranes with high levels of cholesterol [97]. During the 100 ns simulations, Aquaporin 0 remained stable both in the presence and absence of cholesterol according to the root-mean-square-deviation of the atomic positions. In the simulation with a cholesterol-containing membrane, larger hydrogen bond occupancies and longer hydrogen bond lifetimes between Aquaporin 0 and the surrounding lipids were observed than in the simulation with a cholesterol-free membrane, indicating that dynamical properties change with the presence of cholesterol. Furthermore, several aromatic residues on the surface of Aquaporin 0 were seen to form interactions with cholesterol. Both of these observations suggest that the membrane-exposed parts of Aquaporin 0 have evolved to favor cholesterol-rich membranes. Although not mentioned by the authors, the protein contains both a CRAC motif (residues 143–153) and a CARC motif (residues 85–95), however, these residues are not among the ones observed by the authors to interact most strongly with cholesterol. Thus, for Aquaporin 0 these motifs do not appear to play an important role in cholesterol recognition.

2.3. Membrane transport proteins

In contrast to ion channels, transport proteins actively transport their cargo across the membrane. Cholesterol modulation has been observed experimentally for both primary active transporters, such as the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), which uses ATP hydrolysis to drive the transport, and secondary active transporters, such as the human dopamine and serotonin transporters, which use the ion gradient to facilitate the counter-gradient transport.

2.3.1. P-type ATPases

SERCA facilitates the reuptake of Ca^{2+} from the cytosol into the lumen of the sarco- or endoplasmic reticulum. The membrane in the sarco- and endoplasmic reticulum is rather thin and contains a low amount of cholesterol compared to other mammalian membranes, and SERCA is thus expected to function optimally at low cholesterol concentrations [98]. In line with this, cholesterol overload causes inhibition of SERCA activity [99]. However, it is unclear whether this is an indirect effect due to increased membrane thickness or an effect of direct cholesterol binding. In a recent study, a combination of AA and CG MD simulations were applied to address this question [100]. To locate possible cholesterol binding sites, 30 independent CG MD simulations of SERCA embedded in a 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)/cholesterol bilayer were performed. Each simulation was initiated with a self-assembly of the bilayer around the transmembrane part of the protein. Based on the CG MD simulations, two cholesterol–SERCA interaction hotspots were defined; a pocket located near the C-terminus of SERCA and a pocket previously found to bind sarcolipin. Binding of cholesterol in the former pocket was observed to improve the lipid packing around SERCA. However, due to the outline of the pocket, the authors deem it unlikely that occupation of this pocket by cholesterol would affect SERCA activity. Similarly, for the sarcolipin binding pocket, the observed interactions between cholesterol and SERCA would not explain why cholesterol has a different effect on SERCA than other lipids present in the membrane. It is known from X-ray crystallography that SERCA also has a pocket capable of binding the high-affinity inhibitor thapsigargin (see Fig. 5). Since thapsigargin and cholesterol share several structural features, it could seem plausible that cholesterol may bind in the same pocket. However, the cholesterol occupancy of this pocket was very low during the CG MD simulations. To further explore the possibility of cholesterol binding in the thapsigargin pocket, atomistic simulations of SERCA with cholesterol bound in the thapsigargin pocket were performed. The simulation revealed that cholesterol is very flexible in the pocket, and both rotation around the longitudinal axis of the molecule and dislocations in the pocket were observed. It was therefore concluded that cholesterol is not suited to occupy the pocket in a thapsigargin-like manner. Overall, based on the CG and AA MD simulations, the authors suggest that cholesterol affects SERCA exclusively through an indirect effect and that SERCA contains no regulatory cholesterol binding sites [100].

2.4. Peptides

Peptides also play a fundamental role in regulating various processes in relation to cell membrane function and malfunction. Amyloid peptides are speculated to be cytotoxic due to a disruption of the lipid bilayer through oligomer formation and aggregation, resulting in several harmful diseases, such as Parkinson's disease (α-synuclein peptide), diabetes mellitus type 2 (amylin peptide) and AD (Aβ peptide) [25]. Other peptides serve protecting roles,

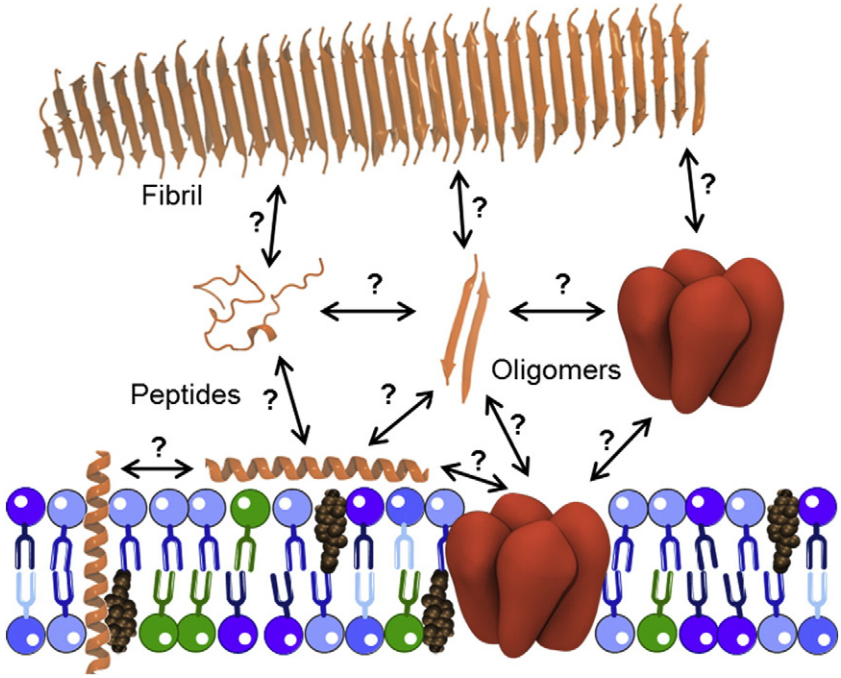

Fig. 6. Different possible stages of amyloid fibril formation. Amyloid fibrils (orange) are formed from oligomers, from monomers adding to ends of fibrils, or from peptides. The role of the membrane in fibril formation and in the toxicity of the oligomers is not well understood yet. A schematic representation of a multicomponent membrane (blue, purple and green) is shown with cholesterol represented in brown spheres.

e.g., in the human innate immune system (e.g., LL37) [102] and in fungi (e.g., antimicrobial peptides) [103], where they punctuate the cell membrane of intervening pathogens. The mechanism by which amyloid and antimicrobial peptides function is largely unknown, however, transmission electron microscopy (TEM) and atomic force microscopy (AFM) have revealed circular assemblies suggested to form membrane pores, which cause the contents of the cell to leak [104,105]. Similar to membrane proteins, the lipid composition of the cell membrane has been found to influence peptide function; especially cholesterol and gangliosides have been found to play important roles in regulation [41,106,107]. MD studies of how cholesterol may influence two selected amyloid peptides are reviewed below.

2.4.1. Aβ peptide

Diseases associated with old age are often suspected to be influenced by cholesterol, and this is also the case for AD [12]. Although it is well established that the deposition of Aβ fibrils in the brain is characteristic of AD, it is not clear if the toxic species are the fibrils themselves or the oligomer intermediates that are formed in the early stages of the fibril formation (see Fig. 6) [108–110]. However, evidence does point to the oligomers as being the toxic species. There is no consensus on how oligomers are formed: in solution, on membrane surfaces, or within the membrane. Thus, MD studies that investigate the role of cholesterol in AD either focus on how cholesterol affects oligomer formation on the surface of lipid membranes or when the peptides are embedded in lipid membranes [111–114].

The influence of cholesterol on AD may be caused either by indirect effects of cholesterol on the membrane properties or other membrane components, or by cholesterol interacting directly with Aβ peptides. A number of different simulation studies have investigated the possibility of a direct interaction between cholesterol and Aβ, focusing either on specific interactions between cholesterol and the assumed cholesterol-binding domain of Aβ, corresponding to residues 22–35 [111,115–117] or on the effect of cholesterol on Aβ membrane pores [112,114]. In terms of indirect effects, an MD study has also looked at how Aβ insertion is affected by differences in the distribution of cholesterol in the inner and outer membrane leaflets [118]. Additionally, the effect of cholesterol on the conformation of the ganglioside GM1, which has been shown to form clusters that bind Aβ [119], has also been explored through MD simulations [120].

There is strong epidemiological evidence supported by several experiments on the role of cholesterol in the toxicity of AD and cholesterol-lowering drugs are considered as potential drug candidates for the prevention of AD [12,121,122]. However, depending on how experiments and simulations are designed and interpreted, there is also evidence for protective effects of cholesterol with regard to AD [115,123,124]. The main cause of the ambiguity is whether membrane association and insertion of Aβ is interpreted as toxic, due to the formation of membrane-spanning pores, or as protective, due to the decrease in free peptides, which may form fibrils. Thus, experimental and computational studies have observed that cholesterol prevents Aβ from leaving the membrane environment and entering into solution and, based on this, suggested a protective role of cholesterol in terms of Aβ fibril formation [115,123,124]. Cholesterol has also been observed to promote membrane-association of Aβ peptides in another MD simulation study, although the conclusion in this case was that this would facilitate Aβ aggregation [116]. In contrast, a CG MD study, investigating the energy associated with Aβ insertion, found that high concentrations of cholesterol in the outer membrane leaflet favors extrusion of the N-terminus of Aβ, which could lead to aggregation [118]. It is possible that this contrasting result partly is caused by the use of a CG representation of the peptide, as this prevents changes in the secondary structure. In support of cholesterol promoting neurotoxicity, MD simulations of an octameric pore, made up of residues 22 to 35 of eight Aβ peptides, showed that cholesterol facilitates tilting of $Aβ_{22–35}$ which is required for channel formation [112]. Based on the results, it was proposed that inhibitors that target cholesterol–Aβ interactions and prevent Aβ insertion could be used in the treatment of AD. This is supported by a recent combined experimental and computational study in which the anticancer drug bexarotene,

which shares structural similarities with cholesterol, was seen to prevent Aβ pore formation [125]. It should be noted that computational studies on Aβ channels needs to be interpreted with caution as there is very little structural information on Aβ channels. Thus, models may not be accurate. In general, MD simulations are limited by the accuracy of the starting structure, and the choice of the starting structure of Aβ may influence the results strongly, particularly in studies focused on how cholesterol influences the secondary structure of Aβ [111,113,116]. So far, NMR structures of Aβ monomers have only been characterized in solution or in organic solvents which mimic a membrane environment, and not in the presence of an actual membrane environment. In the case of Aβ fibrils, the first attempt to characterize the molecular structure of a fibril in the presence of phospholipid vesicles report significant differences compared to the reported fibril structures characterized in solution [126]. In light of this, careful considerations should be made when using Aβ monomer structures obtained in solution for MD studies of Aβ in membrane environments.

There are many uncertainties in the understanding of AD. Hence, identification of the toxic species in AD, the mechanism and environment of oligomer formation, the role of membrane components like cholesterol and gangliosides, and the lack of structural knowledge of the peptides and peptide–lipid complexes, all pose significant challenges in designing experiments and simulation studies. However, advances in experimental techniques and improved computational resources lead the way towards untangling the cooperative effects in these systems and refining our understanding.

2.4.2. Amylin

Amylin is a 37-residue peptide hormone, which is stored along with insulin by the β-cells in the pancreas. In response to elevated blood-glucose levels, amylin and insulin are secreted by the β-cells, both contributing to maintaining glucose homeostasis. In diabetes mellitus type 2 patients, amylin forms extracellular deposits termed islet amyloid, which are mainly composed of β-sheet rich amyloid fibrils formed by amylin, but which also contains different lipids, glucosaminoglycans, and heavy metal ions [127,128]. During the formation of these highly insoluble amyloid fibrils from amylin monomers, a cytotoxic event occurs which has been proposed to be caused by the formation of toxic oligomeric membrane pores made up of amylin monomers [104]. This hypothesis is based on the observation of circular oligomer structures by TEM [105] and AFM [104] combined with membrane leakage experiments showing the membrane permeating ability of these circular structures [104,129]. Very little is known about the actual mechanism by which the cytotoxicity occurs, however it is clear that different components of the plasma membrane can modulate this process. The presence of anionic lipids such as gangliosides has been shown to promote formation of amylin fibrils [106], while the presence of cholesterol in the membrane has been shown to reduce the rate of formation of amylin fibrils [10]. Surface tension experiments have revealed that the first 19 residues in the N-terminal are responsible for the interaction of amylin with the membrane, while residues 20–29 are responsible for initiating aggregation and do not interact with the membrane [130,131]. Xu et al. took advantage of this knowledge to study the aggregation of amylin in mixed anionic and zwitterionic membrane bilayers with and without cholesterol [132]. They employed MARTINI CG MD simulations to study the self-assembly of 20 truncated amylin peptides containing the N-terminal 19 residues in a simulation system with 512 lipid molecules and varying concentrations of cholesterol. Each peptide was constrained to an α-helical structure during the simulations, which is consistent with an NMR structure of amylin bound to a sodium dodecyl sulfate micelle [133]. Without cholesterol in the simulation, peptide aggregates were formed in the membrane in the form of a pore-like structure, however, when cholesterol was present, the peptide aggregates were formed on the surface of the membrane. Furthermore, a difference was also observed in the size of the aggregates; without cholesterol in the system, the aggregates were larger than when cholesterol was present. This is, however, in contrast to the observation by AFM that the size of aggregates on a membrane containing cholesterol was larger than on a membrane without cholesterol [10]. It was suggested by the authors, based on radial distribution functions from simulations of two-component systems, that the effect was caused by the affinity of cholesterol for the lipids over amylin, thereby pushing amylin out of the membrane. As also noted by Xu et al., due to the CG representation employed in this study, the precise internal conformation of the amylin pore cannot be investigated using this approach. Furthermore, it is also possible that the constrained conformation of the peptide has an influence on the results, as experience in our group is that the position of a MARTINI CG representation of the N-terminal 19 residues of amylin in a phospholipid bilayer depends highly on the initial conformation of the peptide (unpublished results).

2.5. Other proteins

In this section, a few studies of other types of membrane proteins are described again focusing on how cholesterol may affect the protein properties, as revealed from MD simulations.

2.5.1. The transmembrane domain of ErbB2

The epidermal growth factor receptor (or ErbB) family is an important class of receptor tyrosine kinases involved in cell proliferation and differentiation, and implicated in several types of cancer [134,135]. The binding of ligands to the extracellular domain of the receptor triggers the dimerization of receptor monomers, which in turn leads to activation. Experimental studies have revealed that the transmembrane domain of ErbB, which consists of a single helix, plays an active role in the dimerization process and associate strongly in the absence of extracellular ligand-binding and cytoplasmic kinase domains [136]. Growth factor receptors have been shown to be localized in cholesterol-rich microdomains in the membrane, and the cholesterol content has an effect on ligand binding as well as kinase activity [137]. The influence of cholesterol on the transmembrane domain of the ErbB2 receptor has been studied using CG MD simulations of both the monomer and dimer in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) bilayers with varying amounts of cholesterol [138]. For a single monomer, the simulations showed that increasing the cholesterol content in the membrane causes a decrease in the tilt angle of the helix with respect to the membrane normal, making it closer to parallel with the membrane normal. This is expected, since the increased cholesterol content causes a thickening of the membrane. The transmembrane helix from ErbB2 contains two GxxxG sequences that have been suggested to be important for dimerization, as the lack of side chains allow the two helices to pack closer. In the study, it was observed that cholesterol generally packs more closely around the helix, than DPPC, and that the position of cholesterol along the membrane normal overlaps with the position of each of the two GxxxG sequences. However, at one particular position, namely Phe671 in the C-terminal of the helix, packing of cholesterol was not observed, leading to an uneven distribution of cholesterol around the helix. Based on Monte Carlo simulations, a free energy profile for the association of two helices was obtained for varying concentrations of cholesterol. The observed mechanism by which the dimer is formed appears to be highly dependent on the cholesterol content in the membrane. Thus, with no cholesterol present, Phe671 from each helix is first seen to associate, followed by a rotation of the helices which removes the Phe residues from the interfaces, and allows a closer packing of the helices. With 20% cholesterol in the membrane, the N-termini of the helices initially associate with the Phe residues pointing away from each other followed by a close packing of the remaining part of the helices. Increasing the cholesterol content further to 30%, causes dimerization to occur via another pathway. Thus, in this simulation the helices are almost aligned with the membrane normal and form a dimer in which the two phenyl rings points towards each other. Overall, the simulations therefore point to cholesterol having a modulating effect both on the association pathway and on the resulting dimer interface.

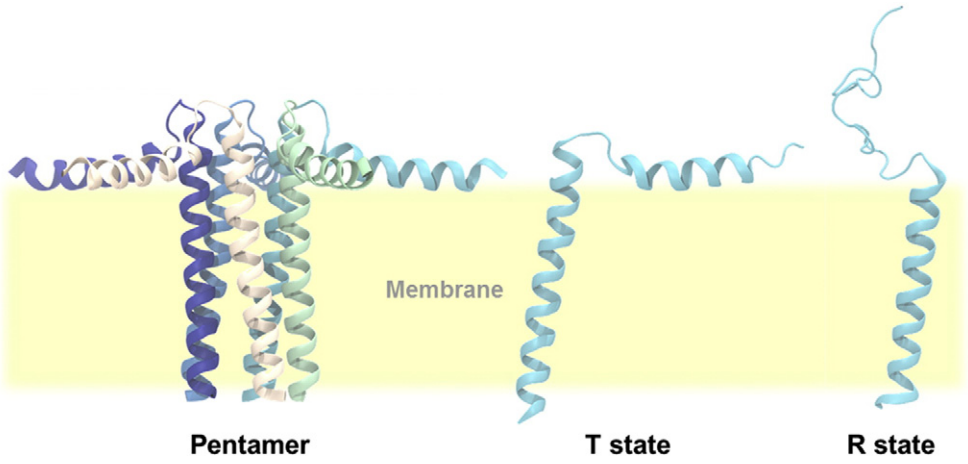

Fig. 7. The three states of phospholamban in the membrane. Representative structures of phospholamban in a homopentamer (PDB code 2KYV [143]), in the inactive T state (PDB code 2KB7 [142]) and in the active R state (PDB code 2LPF [144]) are shown.

Furthermore, the study gives insight into how residues which are not part of the final dimer interface, such as Phe671, may still influence the dimerization process, e.g., by forming the initial contacts. The results presented in this paper are much in line with our studies on SERCA embedded in different lipid bilayers [139], where we observed mutual adaption in the lipid environment and for the protein, by adjusting lipid bilayer thickness and protein tilting, respectively.

2.5.2. Phospholamban

Phospholamban is a 52-residue protein which inhibits the function of SERCA [140]. The inhibitory effect is switched off when phospholamban is phosphorylated. As previously mentioned, the activity of SERCA is influenced by the presence of cholesterol in the membrane. Apart from having a direct effect on SERCA, cholesterol could also influence the activity of SERCA by affecting the conformation and dynamics of phospholamban. The effect of cholesterol on phospholamban was investigated in a study where MD simulations of phospholamban in a POPC/cholesterol bilayer were performed for five different concentrations of cholesterol between 0 and 50% [141]. Phospholamban is stored in the membrane as a homopentamer but it is the monomeric form that is responsible for the inactivation of SERCA [140]. The phospholamban monomer consists of a transmembrane helix and a short cytoplasmic helix connected by a small loop. Phospholamban has been shown to exist in two major conformational states, T and R (see Fig. 7). In the T state, which is inactive, phospholamban has an L-shaped conformation with the cytoplasmic helix interacting with the membrane, while in the active, more disordered R state, phospholamban is in an extended conformation [142]. The simulations showed that in a pure POPC bilayer, phospholamban is flexible and samples conformations in the vicinity of both the T and R states, although conformations similar to the T state dominate. Increasing the cholesterol content leads to decreased flexibility of the protein. Further analysis showed that this is caused by an increased number of interactions between the cytoplasmic helix and the membrane, which keeps the protein locked in an L-shaped conformation. The authors hypothesize that this is likely caused by the ability of cholesterol to act as both a hydrogen bond acceptor and donor, in contrast to POPC, which can only act as an acceptor. Interestingly, the cytoplasmic helix does contain a CRAC motif which could also be part of the explanation for the increase in membrane contacts observed for increased amounts of cholesterol. The effect of cholesterol on the lateral diffusion of phospholamban was also determined for the simulations and it was found that increased cholesterol led to a decrease in the lateral diffusion of phospholamban. This will most likely have an effect on the dynamic interplay between phospholamban monomers and pentamers, which will in turn affect phospholamban activity. Thus, the results suggest that in this case cholesterol affects phospholamban both through direct lipid–protein interactions and through indirect changes of the membrane properties such as the lateral mobility.

3. Discussion and future perspectives

The studies highlighted in this review display the advantages and limitations of using computational techniques, and in particular MD simulations, to investigate the influence of cholesterol on membrane protein function. It is clear that MD simulations can shed light on both direct and indirect effects of cholesterol, as well as help to differentiate between the two types of modulation. Several of the studies reveal that combining docking calculations with MD simulations can be a powerful approach for locating non-annular cholesterol binding sites, which is particularly important since such sites may be difficult to predict based on experimental data. As seen in the study of the Kir2.1 channel, residues important for cholesterol binding can be suggested based on simulations and mutation experiments can then be performed to test their relevance. This approach could also be used to distinguish between relevant and non-relevant sites, if multiple potential binding sites are found in docking calculations and/or the MD simulations. Based on the presented studies, non-annular cholesterol binding sites do appear to be important for several different protein families, as least within the group of ion channels. In terms of the importance of CRAC and CARC motifs in cholesterol recognition, no consensus can be made from the presented examples, as some studies suggest that cholesterol does bind in the vicinity of such motifs, while for others, this is not the case. The difficulty of using such sequence patterns for predicting cholesterol recognition sites, was recently highlighted in an MD study where simulations showed that the amount of disorder in the secondary structure of CRAC-containing peptides affect their ability to bind cholesterol [145].

As detailed in this review, computational studies of the interactions between cholesterol and membrane proteins and peptides hold great promise. During the last couple of years, the applicability of MD simulations has been enhanced due to an increase in computational power, advances in the massively parallel algorithms needed for simulations of large systems, and the development of specialized hardware. This enables MD simulation studies of membrane proteins to reach μs to ms timescales, and allows simulations of large complex membranes. Hence, future MD-based studies may aid in answering questions on how microdomains are formed in the plasma membrane, the segregation of proteins into such domains as well as local and global effects of cholesterol on membrane protein dynamics. Insight into the interactions between cholesterol and membrane proteins is continually gained through the unraveling of high resolution structures of membrane

proteins in complex with cholesterol. These structures may serve as starting points for MD simulations, which then further explores the effects of cholesterol binding at an atomic level. Hence, models for cholesterol (and other lipids as well) can be produced computationally, suggesting direct interaction sites. These models should be challenged through experiments, showing the synergy between theory and experiments for studies of very complex chemical systems. In the future, it can be imagined that such knowledge may find active use in new drug design strategies, such as lipid replacement therapies [146] and for drugs targeting non-annular lipid binding sites [125,147].

Transparency document

The Transparency document associated with this article can be found, in the online version.

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