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Cholesterol and nicotinic acetylcholine receptor: An intimate nanometer-scale spatial relationship spanning the billion year time-scale

Francisco J. Barrantes

Laboratory of Molecular Neurobiology, Institute of Biomedical Research (BIOMED) UCA-CONICET, Faculty of Medical Sciences, Pontifical Catholic University of Argentina, Av. A. Moreau de Justo 1300, 1107 Buenos Aires, Argentina

Tel.: +54 11 4322 3795; E-mail: rtfjb1@gmail.com

Abstract. Once the sterol biosynthetic machinery had progressed over the course of several million years to yield cholesterol, this neutral lipid became an omnipresent and essential component of biomembranes in Eukaryotes. The hopanoids in Prokaryotes and eukaryotic sterols share the ability to provide stability and domain compartmentalization in membranes. Even more important is the intimate association of cholesterol with a wide range of cell-surface membrane proteins, probably responsible for its modulatory effects on neurotransmitter and hormone receptors and ion channels. These effects appear to be exerted via the membrane-embedded segments of essentially all members of the pentameric ligand-gated ion channel and G-protein-coupled receptor superfamilies, which possess consensus linear arrays of amino acid residues recognizing cholesterol with relatively high affinity and specificity, an early evolutionary acquisition already present in ancient bacteria, conserved, and further improved in Eukaryotes. This review focuses on the long-term relationship between cholesterol and these functionally important membrane protein superfamilies, and the ability of cholesterol to induce lateral segregation and ordered domain formation at the nanoscale in cell membranes.

Keywords: Cholesterol, cholesterol-recognition motifs, neurotransmitter receptor, ion channels, phylogenomics, sterol evolution, cholesterol–protein interactions, membrane proteins, neurotransmitter receptors, pentameric ligand-gated ion channels, evolution

Abbreviations

nAChR nicotinic acetylcholine receptor
pLGIC pentameric ligand-gated ion channels
GPCR G-protein coupled receptors

1. Introduction

This review is part of a series celebrating the 200th anniversary of the discovery of cholesterol. The finding of the neutral lipid in gallstones and the coining of the term “cholesterine” (from the Greek *chole* for bile and *sterEOS* for solid) in 1816 is credited by some authors (e.g. [59,135]) to the French chemist

Michel Eugène Chevreul [34], although other authors (e.g. [101]) attribute the discovery, also in gallstones, to the French chemist and physician François Poulletier de la Salle, in 1789. It was only in 1859 that cholesterol was identified as an alcohol by Berthelot [59] and in 1888 F. Reinitzer determined its empirical formula. The 1926 Nobel prize in Chemistry was awarded to Heinrich Wieland for his work on the structure of cholesterol and bile acids – although the tetracyclic ring structure as we know it today was proposed by Wieland only in 1932 [20]. It took the two subsequent decades to clarify the stereochemistry and ring conformations of cholesterol. The 1964 Nobel award in Physiology and Medicine went to Konrad Bloch and Feodor Lynen for their discoveries in the areas of cholesterol biosynthesis and metabolism of fatty acids [135]. In 1985 Michael S. Brown and Joseph L. Goldstein were awarded the Nobel prize for their discoveries concerning the regulation of cholesterol metabolism and the treatment of diseases caused by abnormally elevated cholesterol levels in the blood [26]. The 200th anniversary may prove an interesting opportunity to revisit some of these milestones and to assess how much we have actually learned about this lipid since its “recent” bicentennial discovery – considering that the enzymatic machinery for sterol biosynthesis may have appeared on Earth between 2.7 and 2.4 billion years ago [19], after the emergence of oxygenic photosynthesis with the concomitant oxygenation of the planet [129]. It has been surmised that the early role of sterols in eukaryotes may have been that of protection against oxidative stress when oxygen levels rose [25,56]. High oxygen concentration is required for the generation of sterols [19] and this has been postulated to be at the root of probably one of the most important changes in phylogenetic evolution, i.e. the prokaryote-to-eukaryote transition [32], coincident with the acquisition of new intracellular membrane structures and mechanisms.

A search through Pubmed with the single noun “cholesterol” yields more than 250,000 citations of scientific publications, and today these are clearly outnumbered by references with medical connotations of a dietary, metabolic or physiopathological nature, related to supra-normal levels of cholesterol (e.g. familial or acquired hypercholesterolemia, atherosclerosis and cardiovascular diseases, Alzheimer’s disease, some forms of Niemann–Pick disease) [3,28,45,50,57,76,122,126,130,144], medical conditions associated with sub-normal cholesterol levels (hypocholesterolemia, a relatively common dyslipidemia) or the more rare diseases related to genetic mutations of enzymes involved in the absorption, biosynthesis, transport or metabolism of cholesterol (e.g. chylomicron retention disease, hypobetalipoproteinemia, abetalipoproteinemia, Tangier disease, secondary hypocholesterolemias in hepatic diseases or cancer, malabsorption syndromes, excessive doses of statins, and panic disorder, among several other pathological conditions) [2,60,111,131].

Sterols in general and cholesterol in particular serve key functions in cells, ranging from structural building blocks of cell membranes to dynamic modulators of membrane physicochemical properties, like fluidity, or biological properties, like developmental regulators and precursors of steroid hormones, bile acids, and vitamin D in metazoa. Among the more than 200 lipid species in a cell membrane, cholesterol stands out as a singular lipid in terms of structure, physicochemical properties and unique effects on other lipids and membrane proteins. The mere fact that it accounts for 30–50 mol % of the total lipids of the cell membrane is already quite telling. In the central nervous system, cholesterol is a key component of neuronal and glial membranes, synaptic vesicles and, most importantly, myelin (see recent review in ref. [31]). The principal site of synthesis of cholesterol in brain is the astrocyte [86,101,102]. Although the human brain accounts for about 2% of the body weight, this organ contains almost a quarter of the total pool of cholesterol in the whole organism. Cholesterol controls synapse formation [40,77,86,102,146] and function [23], and alterations of its metabolism have been associated with various neurodevelopmental and neurodegenerative diseases, including psychiatric disorders [15, 31,52].

This review will not attempt to cover the clinical aspects associated with cholesterol physiology or its alterations in disease, but will put emphasis on a narrower subject, the influence of cholesterol on membrane proteins belonging to the superfamily of pentameric ligand-gated ion channels (pLGIC). Towards this end, I will use the receptor for the neurotransmitter acetylcholine, namely the nicotinic acetylcholine receptor (nAChR), as a paradigm to illustrate the pleiotropic functional effects of cholesterol on this “founding member” of the pLGIC superfamily. As I develop the subject of the characteristic CARC cholesterol-recognition motif first recognized in these membrane proteins [8], it will become apparent that its presence extends beyond pLGIC, spreading out to the largest class of transmembrane receptors, the G-protein (heterotrimeric guanine nucleotide-binding protein)-coupled receptors (s), also called 7 transmembrane helical (7TM) receptors, and several other proteins, including soluble ones.

Cholesterol binds to and affects the ligand-recognition, gating, and ion permeation properties of a number of hormone and neurotransmitter receptors, as well as ligand- and voltage-gated ion channels [72,73]. In addition, cholesterol modulates the cell-surface organization, exocytic and endocytic trafficking, and various other physiological properties of these membrane proteins. Cholesterol influence on cell-surface organization of membrane proteins is clearly manifested in the cholesterol-mediated dynamic compartmentalization via the recruitment or exclusion of proteins in/from specialized micro- and nano-domains, on the one hand, and in the degree of oligomerization of receptors, on the other, as discussed in two sections of this review. Protein exocytic and endocytic trafficking is also affected by cholesterol homeostatic equilibrium in the cell, as analyzed in another section. Cell-surface receptor function is also subject to regulation – probably the most important of cholesterol effects – in a pleiotropic manner which is highly intertwined with the two other targets of sterol modulation. Cholesterol effects can be exerted in a specific manner involving direct interactions with the membrane-spanning domains of the receptors [8,10,49,105,106] or in a rather diffuse, less-specific manner in the form of changes in the physical state of the bulk lipid bilayer, e.g. on membrane thickness, fluidity [41,69,78] or curvature [70,153]. These two modes of action of cholesterol are widely distributed, being shared by membrane proteins unrelated in structural and evolutionary terms, and have adapted both to rapid responses like the ion channels and receptors in the pLGIC superfamily [14] and to the slow-acting and often intricate metabotropic responses as is the case with the GPCR superfamily [53]. Millions of years ago, before the appearance of the cholesterol-synthesizing enzymatic machinery in eukaryotic organisms, the hopanoids – prokaryotic sterol surrogate compounds – exerted on bacterial ion channels some of the functional effects displayed by cholesterol in Eukaryotes [16]. A section of this review will briefly analyze the intriguing connection between the two regulatory mechanisms.

2. The site of cholesterol–protein contacts: The example of the nAChR

Membrane-embedded proteins necessarily exhibit close and relatively extensive contacts with the surrounding lipids. At the hydrophobic periphery of the protein a first shell [141] of lipids constitutes the “belt” or “boundary” region, the lipid “annulus” [142], a partially ordered but dynamic interface in active exchange with the membrane bilayer bulk lipid [83]. Cholesterol was reported to be excluded from the annular lipid region [142]. Nuclear magnetic resonance (NMR), electron spin resonance (ESR), and fluorescence spectroscopy studies have provided information on the biophysical properties of annular lipids in the immediate periphery of several membrane proteins [82,121] and recently gas-phase mass spectrometry has been applied to explore the annular lipid in great detail, establishing the number of lipid molecules in this membrane subdomain [85]. The fatty acyl chains of the annular lipids are in most

cases tightly packed against the hydrophobic surface of the TM region of the protein, and experience a transiently less mobile state relative to that of the bulk bilayer lipid in terms of rotational and translational dynamics [83,116,143]. The rate of exchange between annular shell lipid and the bulk lipid is remarkably fast ($1\text{--}5 \times 10^8 \text{ s}^{-1}$ [83]) and the slower rotational motion (50 ns) of this less mobile lipid with respect to the rotational motion along the main molecular axis of the bulk lipid is in the order of $\sim 1 \text{ ns}$ [84]). The height of the annular lipid shell undergoes only small variations among different membrane proteins, whereas the total surface of the annular shell varies with the extent of the transmembrane region of the protein and to a lesser extent with the composition of the annular lipid. Spin-labeled sterols like androstanol and ergosterol preferentially partition in the less mobile lipid region [84]. Subsequent work combining the ESR data with biochemical information on the lipid repertoire of the nAChR-rich native membrane of *Torpedo* electrocytes [115] enabled us to calculate thermodynamic properties of the lipid-nAChR interactions, the relative stoichiometries and the affinities of different lipid species for the nAChR protein [79]. The stoichiometry of cholesterol sites: nAChR was calculated to be ~ 15 molecules. The combined data can be envisaged as a snapshot depicting the “instantaneous” average composition of the shell lipid surrounding the nAChR immediate perimeter. Figure 1 shows the distribution of cholesterol molecules around the waist of the nAChR TM domain.

In contrast to annular lipids, non-annular sites in most membrane proteins are heterogeneous in nature, and are located in grooves or cavities between TM helices and/or at the clefts between individual subunits in multi-subunit proteins [70]. Because of their intimate contact with the TM protein surfaces

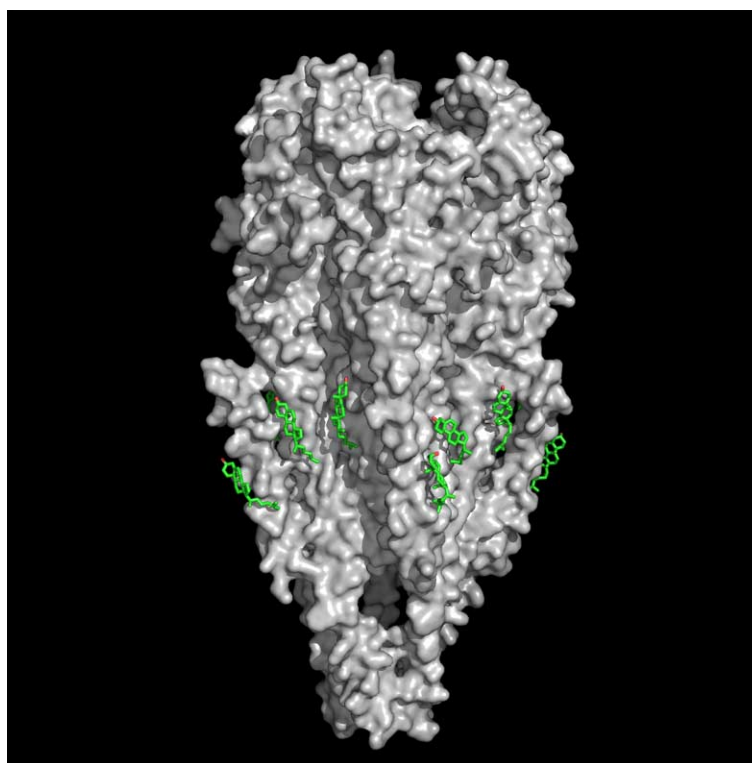


Fig. 1. CPK molecular model of the nicotinic acetylcholine receptor protein (surface rendered in gray) (MW ca. 270,000 Daltons, PDB 2bg9) and cholesterol molecules (stick rendering, green) (PDB entry 1MT5) at cholesterol-recognition sites located on the rough membrane-embedded surface of the nAChR TM region.

and their limited accessibility to the bulk lipids, non-annular lipids often exhibit higher specificities for the protein than annular sites. Their rate of exchange with the bulk lipid is presumably much slower than that of annular lipids, especially for those sites located deeper in the interior of the protein, farther away from the belt region. In contrast to the early ideas of Warren et al. explicitly indicating that cholesterol was excluded from the annular lipid region [142], subsequent work pointed to non-annular lipids as the sites that excluded cholesterol but accommodated phospholipids (Jones and McNamee, 1988). Free fatty acids resulting from controlled phospholipase A2 enzymatic treatment of *Torpedo* native nAChR-rich membranes or exogenously added arachidonic acid were found to localize at both annular and non-annular sites [51]. Attempts to identify the location of cholesterol sites using molecular dynamics simulations suggested the occurrence of cholesterol sites deep inside the nAChR TM region [24]. The crosstalk between the two classes of sites provided a plausible explanation for the distinct resting (R) and desensitized (D) states of the nAChR observed in the equilibrium binding studies: transitions between these conformers were interpreted in terms of structural rearrangements of the nAChR TM region involving either the occlusion or decreased availability of the non-annular sites [51].

3. Identifying cholesterol-recognition motifs in membrane proteins

Since the first report of a membrane protein structure in 1985, Stephen White's laboratory at the University of California at Irvine has compiled 585 new structures solved up to the year 2015 (<http://blanco.biomol.uci.edu/mpstruc>). At the time of writing this review, the number of membrane proteins of known 3D structure listed in this compilation totals ca. 700 entries. Similarly, the MemProtMD data base created by Samson's lab in Oxford University lists all known transmembrane proteins embedded in lipid membranes, and the group recently reported that about 2,000 structures have been solved by the combined efforts of X-ray diffraction, single-particle cryoelectron microscopy and solution nuclear magnetic resonance spectroscopy studies [125]. Thus the number of macromolecules solved at the atomic level effectively represents only a minor fraction of the genome coding for membrane proteins, which accounts for no less than 25% of the genes in a cell. It is therefore not surprising that the direct demonstration of cholesterol recognition sites in membrane protein structures is so elusive.

Which alternative approaches can be used to experimentally establish the direct contact between cholesterol and membrane proteins? A spin-labeled cholesterol analogue has not been synthesized to date; cholesterol-like analogues for electron spin resonance spectroscopy studies such as androstanol- or cholestane-spin labels are available, but they differ from cholesterol in some essential structural details. Similarly, cholesterol probes for fluorescence studies devoid of relatively bulky fluorophores are still not available. The smallest of such extrinsic probes is nitrobenzoxadiazole ("NBD") [94], and this fluorophore adds not only a non-negligible mass to the cholesterol moiety but may also modify its location and orientation in the membrane. An alternative minimally perturbing cholesterol-like probe is dehydroergosterol [113], which inconveniently has low quantum yield, bleaches rapidly, excites in the high-energy UV region of the spectrum in conventional wide-field microscopy [148] and is thus prone to deleterious effects on cell integrity, and requires costly UV-transparent optics for fluorescence microscopy [93]. Dehydroergosterol can also be studied using 2-photon fluorescence microscopy, circumventing one of the above problems, i.e. excitation in the UV region of the spectrum [147,148]. Furthermore, fluorescent cholesterol probes can partition differentially in the plasma membrane as compared to the endogenous lipids [30]. Better extrinsic cholesterol probes, such as BODIPY-cholesterol, or the photoactivatable Ever-Fluor-fluorescein cholesterol have appeared in recent years (reviewed in

[30,149]) but the essential difficulties associated with the bulky fluorophores still remain. In other words, the *ideal* extrinsic cholesterol probe – the *bona fide* qualitative tracer of ref. [30] for biophysical studies having a non-perturbing reporter group is still not available. For all these reasons other methodologies are needed to identify cholesterol recognition motifs in hormone or neurotransmitter receptors and ion channels without perturbing the sterol recognition region of these proteins. Various groups, including ours, have resorted to analyzing sequences in protein data banks and applying *in silico* computational methods to explore and detect the presence of putative cholesterol-binding linear domains in the transmembrane (TM) regions of proteins. This methodology resulted in the definition of consensus motifs with predictive value, which can be further applied for identifying cholesterol-binding linear domains [8,42–44,66]. The first such consensus motif to be identified was defined by the linear amino acid sequence (L/V)-X₁₋₅-(Y)-X₁₋₅-(K/R), and termed “*cholesterol* recognition amino acid consensus” (CRAC) [66]. The linear amino acid array (K/R)-X₁₋₅-(Y/F)-X₁₋₅-(L/V) was found later on in the nAChR and other membrane proteins; it is essentially the mirror version of the CRAC algorithm, and hence referred to as the “CARC” consensus motif [8].

The free energy of interaction between cholesterol molecules and the nAChR amounts to about $-510/-530 \text{ kJ} \cdot \text{mol}^{-1}$, i.e. more than $-100 \text{ kJ} \cdot \text{mol}^{-1}$ per subunit. The particularly favorable fit between the “CARC-like” TM4 segment in the γ subunit of the human nAChR (428RVCFLAMLL435) and cholesterol is remarkable, with an energy of interaction of about $-60 \text{ kJ} \cdot \text{mol}^{-1}$, i.e. $\sim 60\%$ of the total energy of interaction of the entire γ subunit, which displays the highest affinity for cholesterol among all nAChR TM segments and in fact among most transmembrane peptide segments. Comparative analyses of the energy of interaction of cholesterol with pLGIC, GPCRs and various other membrane proteins revealed that the CARC motif generally exhibits higher affinity for cholesterol than the CRAC motif [49]. Essentially two explanations lie at the root of the difference in the predictive value of the CRAC and CARC linear algorithms: the snorkeling effect of Lys/Arg residues, which places the polar residues exposed to the aqueous milieu and the hydrophobic residues buried in the bilayer hydrophobic core [128] on the one hand and the structure of cholesterol itself [48], as reviewed in [46,49] on the other.

Analyses of cholesterol-recognition motifs in membrane [8,49,132] as well as soluble [89–91] proteins have provided strong evidence to sustain the hypothesis that these linear motifs bear functional relevance in cholesterol–protein interactions. Until recently, though, direct experimental demonstration of physical crosstalk between the sterol and the linear peptide sequences was missing for proteins other than GPCRs. We have recently challenged the hypothesis and submitted it to experimental test using two biophysical techniques: the interaction of a prototype CARC domain with cholesterol was studied using lipid monolayer techniques and nuclear magnetic resonance (NMR) spectroscopy. A representative CARC domain for these experimental corroborations was chosen on the basis of previous photolabeling studies of the *Torpedo* nAChR with the cholesterol analogue probe [³H]azicholesterol, which led to the identification of a (predominant) cholesterol-binding domain in the 4th transmembrane domain (TM4) of the human nAChR γ subunit [62]. As mentioned in preceding paragraphs, our *in silico* computational approaches [8] led us to identify a typical CARC motif: 455-**R**V**C**F**L**A**M**L**L**-462 (the characteristic Arg, Phe, and Leu amino acid residues outlined bold and underlined) in the human γ TM4 that incorporated most of the label in the photoaffinity studies. In addition, molecular modeling simulations (Fig. 2) showed that this TM segment displayed the highest energy of interaction (in the order of $-60 \text{ kJ} \cdot \text{mol}^{-1}$) with cholesterol when compared to all other subunits of the nAChR in humans and other species [8]. The homologous γ TM4 segment in the *Torpedo* nAChR possesses a CARC motif similar to the human

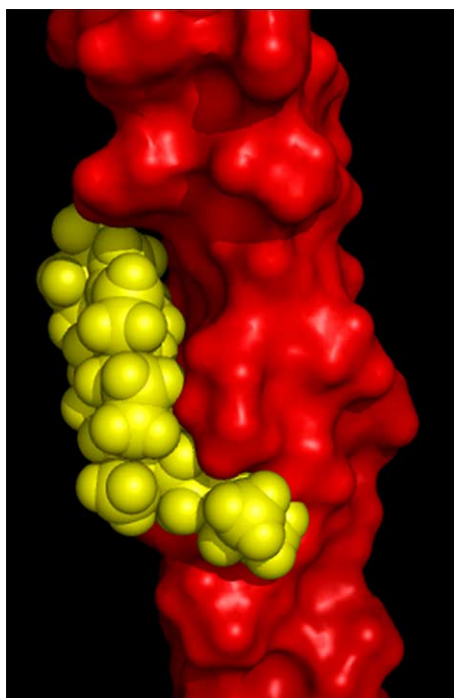


Fig. 2. Molecular model of the human nAChR γ subunit 4th transmembrane domain (surface rendered in red) with bound cholesterol (CPK rendering, in yellow). This TM segment (Swiss Prot entry P02708), located in the outer membrane leaflet, exhibits the highest energy of interaction with cholesterol, i.e. ca. $-60 \text{ kJ} \cdot \text{mol}^{-1}$ [8].

form: 449-KACFWIAL-456. The strongest [^3H]azicholesterol labeling in *Torpedo* TM4 is observed in Asp-448, the second residue after Lys-449, which is the first amino acid of the N-term CARC motif.

Additional molecular dynamics studies on the cholesterol derivative-*Torpedo* γ TM4 segment comprising the CARC domain plus a few upstream and downstream amino acid residues (445–460) indicated that azicholesterol did interact tightly with the residues defined by the CARC motif (Lys-449, Phe-452 and Leu-456), although it seemed unlikely that the strength of the interaction sufficed to account for the reduced mobility observed in the molecular simulations; instead, we proposed that the reduced mobility arises from the cholesterol-induced oligomerization of γ TM4, a hypothesis consistent with the results of fluorescence studies of γ TM4 in POPC vs POPC-cholesterol bilayers [38]. The molecular dynamics studies further indicated that the cholesterol contacts with the CARC motif within γ TM4 involved the β -face, leaving the α -face exposed. This suggested that the cholesterol-mediated oligomerization of the peptide is dictated by cholesterol–cholesterol interactions rather than by protein–protein interactions [48]. We next compared the interaction between cholesterol and either wild-type or mutant TM4 peptides. The WT CARC motif was found to exhibit a higher affinity for cholesterol. The three amino acids defining the CARC domain, and the central Phe-452 residue in particular, were found to interact with cholesterol. Replacement of this aromatic residue with alanine (F-452/A mutant) resulted in a significant loss of affinity. This computational finding was challenged by physicochemical lipid monolayer studies using a CARC-carrying peptide and cholesterol. The fact that the F-452/W mutation had no effect led us to suggest that it is the aromatic nature of Phe-452, and not its specific structure, that is required for optimal binding [47], in agreement with previous studies suggesting that CARC motifs could contain any of the three aromatic residues, i.e. Phe, Trp or Tyr [8,49]. Importantly, the monolayer studies also

demonstrated the lipid-specificity (CARC recognized cholesterol but not phosphatidylcholine) and the concentration dependency of the binding (saturation was reached for peptide concentrations $<10 \mu\text{M}$) [47]. NMR spectroscopy experiments (MAS triple resonance magic-angle spinning deuterium NMR using deuterated Ala471) [47] showed that cholesterol addition to phospholipid bilayers containing a synthetic $^{13}\text{C}/^{15}\text{N}$ -labeled peptide (Asp464-Val492 in the intact *Torpedo* γTM4) caused a reduction in the rotational motion of the peptide within the bilayer, further reinforcing the cholesterol-mediated peptide oligomerization hypothesis. It is tempting to extrapolate the results of the isolated peptide experiments to the possible role of the CARC domains in situ, where they could play a role in cholesterol-mediated oligomerization of the receptor. The high affinity, lipid-specific and saturable nature of the converging lipid monolayer and NMR spectroscopy studies, together with the molecular modeling simulations, constitute a much sturdier argument for the direct physical interaction of cholesterol with a CARC cholesterol-recognition motif [47].

4. The same transmembrane domain can accommodate two cholesterol molecules

Probably one of the most valuable aspects of in silico molecular simulation studies is their predictive value. One prognostic outcome of our molecular modelling exercises was the unexpected finding that two cholesterol molecules could actually be accommodated on the same TM domain [47,49]. This inference follows from the vectorial nature of the CRAC and CARC motifs, mirror images on the linear sequence (“apolar” Leu/Val \rightarrow “basic” Lys/Arg for CRAC and “basic” Lys/Arg \rightarrow “apolar” Leu/Val for CARC, from the N-terminus to the C-terminus sequence). The CARC sequence starts with a basic residue (Arg or Lys) and this feature makes the CARC motif ideally suited for interaction with cholesterol in the outer leaflet of biological membranes (Fig. 3). The N-terminal domain of type I membrane proteins is extracellular, such that the carbon chain enters the membrane bilayer in the N- to C-terminus direction. A search for cholesterol-recognition motifs over a large series of membrane proteins in combination with molecular dynamics simulations of the whole TM regions showed that, indeed, double cholesterol-recognition motifs occur within the same TM segment in various members of the GPCR superfamily [47]. Interestingly, some commonalities were apparent: a) the three key amino acid residues defining the CARC and CRAC motifs were always involved in the interaction; b) the central aromatic residue could be either Phe or Tyr (and even Trp in the case of CARC). This finding is consistent with the nature of the interaction between cholesterol and aromatic rings in amino acid residues, i.e. the CH– π stacking interaction [96]; c) the branched aliphatic residues (Leu/Val) are well suited to accommodate the protruding methyl groups of cholesterol; d) the terminal basic residue of the motif often forms a hydrogen bond with the oxygen atom of the –OH group of cholesterol [49]; e) the consensus CRAC sequence starts with an aliphatic residue (Leu or Val), and therefore its N-terminal is expected to interact with the apolar groups of cholesterol (sterane, methyl and iso-octyl) in the inner leaflet (Fig. 3). Thus, the selection of CARC and CRAC as cholesterol-binding motifs is justified by robust physicochemical rules [47]. The coexistence of a CARC and a CRAC motif within the same TM segment, one in each leaflet of the membrane, has an important consequence: the host transmembrane protein segment can accommodate two opposite (tail-to-tail) cholesterol molecules (Fig. 3). This ensures that the polar amino acid residues of the motif (Lys/Arg) face the intra- and extracellular milieu, whereas the apolar ends of the motifs (Leu/Val) are deeply buried in the most hydrophobic region of the lipid bilayer. The coexistence of CARC and CARC motifs in the same TM domain is found in type I membrane proteins and in domains 1, 3, 5, and 7 of the GPCRs; the CARC motif is always located in the outer leaflet,

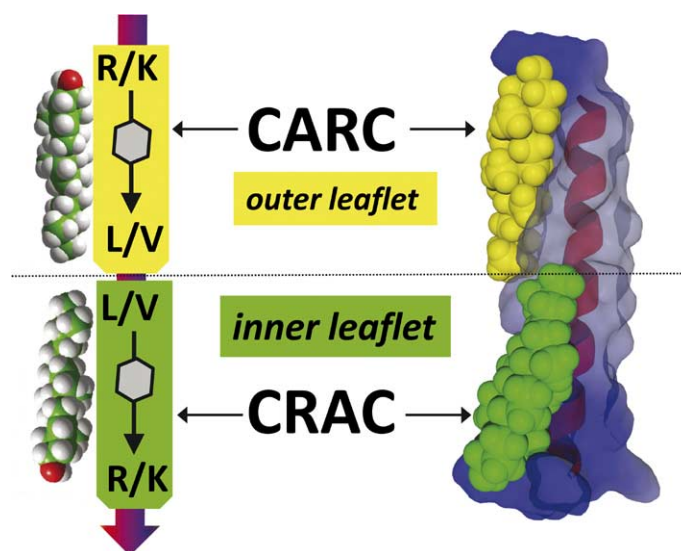


Fig. 3. Coexisting CARC and CRAC motifs within the same TM domain. The amino acid residues in the CARC and CRAC motifs exhibit a defined hydrophilic/hydrophobic vectoriality. Furthermore, the predominant occurrence of the CARC motif is in the outer membrane leaflet, and the CRAC array is preferentially located in the inner leaflet. Their vectorial orientation is schematically depicted in the left panel. The cholesterol molecule binding the CARC domain is shown with its $-OH$ group pointing towards the aqueous compartment of the extracellular space. Correspondingly, the hydrophobic/hydrophilic vectoriality of the CRAC motif on the inner leaflet matches the orientation of cholesterol, with its $-OH$ group pointing towards the cytoplasmic compartments. On the right, the example of the CARC/CRAC doublet in the 1st TM domain of the human VIP receptor. The 3D structure of cholesterol was retrieved from PDB entry 1MT5. From ref. [46].

whereas the CRAC sequence is found in the inner leaflet. Membrane receptors such as the somatostatin, GABA, serotonin, adenosine, VIP and cannabinoid receptors, as well as the voltage-dependent TRPV1 channel exhibit the mirror cholesterol-recognition motifs. Overall, the mean energy of interaction was in the order of $-58 \text{ kJ} \cdot \text{mol}^{-1}$ for CARC and $-48 \text{ kJ} \cdot \text{mol}^{-1}$ for CRAC, indicating that the CARC domain generally exhibits more affinity for cholesterol than a CRAC domain [8,47,49]. Exceptions to this rule can be found e.g. for neuropeptide FF and corticotrophin-releasing factor receptors.

In weighing the possible biological implications of the coexisting CARC-CRAC motifs within the same membrane-embedded peptide we have noticed the special case of the yeast heterologous protein expression system. Functional expression of some human receptors is not always sustained by yeasts [97]. Yeast cells have an essential requirement for ergosterol for their growth, and cholesterol is not an effective substitute. However, *Saccharomyces* yeast cells transfected with the human μ -opioid receptor increased ligand binding when ergosterol was replaced by cholesterol [68]. In order to account for this observation, we have hypothesized [47] that human CARC/CRAC domains of human origin might exhibit species-specificity for cholesterol as manifested in the yeast expression system due to subtle differences between the two sterol molecules, cholesterol being more flexible due to the presence of several extra-double bonds in ergosterol [7,37].

5. Cholesterol effects on the oligomeric state of G-protein-coupled receptors

GPCRs constitute the largest superfamily of transmembrane proteins, with more than 900 members encoded by the human genome [55]. Their biomedical and economic importance is outlined by the fact

that 40% of the marketed drugs produced by the pharmaceutical industry act on GPCRs [35,65,67]. This group of macromolecules is involved in a great variety of physiological processes, including the decoding of signals encoded in neurotransmitters, hormones, chemokines, proteinases, odorant and taste molecules... and photons. They bear in common the ability to transduce external cell surface-targeted stimuli into relatively slow, downstream chemical metabolic cascades inside the cell. The common features also encompass molecular structure: GPCRs are built of seven transmembrane segments (TM1–TM7) and hence are also referred to as 7TM receptors. Upon signal recognition (I avoid the terminology “ligand-recognition” here, because GPCRs recognize not only chemical ligands like hormone peptides or even proteins, but also ions, and even photons as is the case with the GPCR rhodopsin), GPCRs transduce the message to a G-protein at its C-terminal, intracellular domain. The heterotrimeric G-proteins also come in a variety of combinations, transducing inhibitory and excitatory downstream signals. In the context of this review, cholesterol is gaining increasing relevance in GPCR function as a possible modulator of GPCR oligomerization. A matter of debate for more than a decade, it is now more widely accepted that various types of GPCRs undergo dimerization or higher-order oligomerization prior to activation [5], and although monomers in family type A GPCRs are functional, heteromerization appears to be fundamental in receptor trafficking and pharmacology [55]. Current views strongly point to the membrane microenvironment as the key modulator of GPCR oligomerization [54] and more specifically to cholesterol as the dimerization-inducing membrane component [54,104]. The availability of the crystal structure of the $\beta 2$ adrenergic receptor provided not only the first experimental observation of direct interactions between a GPCR and cholesterol [33,63,114] but also led to elaborating about the importance of the sterol, necessary for the crystallization of the protein, in establishing the dimeric form of this receptor in the crystal lattice [33]. These seminal crystallographic structures of the $\beta 2$ adrenergic receptor expedited subsequent X-ray studies on binding sites in various other GPCRs like the $\beta 1$ adrenergic receptor in complex with a G-protein [140] or the human A2A adenosine receptor bound to an agonist [64], and led to the identification of cholesterol sites in several other members of the GPCRs (reviewed e.g. in [133]). Up to the end of 2015, the X-ray structures of more than 60 ligands and 20 receptors had been reported, corresponding to GPCRs class A, B, C and F [35]. Cholesterol-GPCR interactions have been reported to increase the compactness of β -adrenergic and 5-HT1A receptor structures and to enhance the conformational stability towards active or inactive receptor states [58,107]. An additional actor in GPCR dimerization is the fatty acid, palmitic acid [6]. The covalent palmitoylation of the β -adrenergic receptor at two sites appears to recruit cholesterol molecules involved in the stabilization of the receptor dimers (reviewed in [54]) and in the localization of the 5-HT1A serotonin receptor in lipid microdomains [112]. Cholesterol was reported to modulate the membrane partitioning in the plasma membrane [109] and the ligand binding properties [108,120] of this GPCR.

6. Properties of cholesterol in liquid-ordered (Lo) lipid domains

As analyzed in a preceding section, cholesterol can occur in the inner or outer leaflet of the membrane bilayer, or in both leaflets. In general, the outer leaflet possesses a more rigid apolar surface than glycerophospholipids, and this facilitates the preferential interaction with cholesterol. The cholesterol-sphingomyelin domains are also enriched in glycerophospholipids with saturated fatty acyl chains (relative to the average saturation in the rest of the bilayer). The lipid raft hypothesis proposes that these ternary complexes formed by specific self-associated lipid species constitute microdomains or platforms that can intervene in protein partition, signaling and other functional events in cell physiol-

ogy [4,75,123,124]. Favorable and unfavorable lipid-lipid interactions result in transient lateral heterogeneities that put together or segregate their constituent molecules, respectively. Above/below certain critical lipid and water concentrations and/or temperatures these lateral heterogeneities generate transiently separated lipid phases, the two most prominent of which are the liquid-disordered (Ld) and liquid-ordered (Lo) phases [81,152]. Other factors are also influential in the constitution and transitions between these two (and additional) phases, such as variations in the ionic strength, pH and pressure. Furthermore, the action of lipases can retailor the composition and topography of lipids in the membrane, and hence redesign the Ld and Lo. For instance endogenous flippases (e.g. the scramblases) acting in situ can alter the distribution of lipids across the two leaflets of the membrane whereas other enzymes can modify the fatty acyl chain composition and hence modify the degree of saturation of the membrane phospholipids, a key factor affecting the phase state of the membrane. The temperature and compositional range over which these lateral separations into liquid phases occur is rather large [136–139]. Lipid domains enriched in cholesterol–sphingomyelin-saturated glycerophospholipids are generally considered the “consensus” Lo phase in “test tube” model systems. These ternary systems constitute a more condensed, rigid and thicker bilayer which has been associated with the concept of “lipid raft” [103], although not all rafts appear to contain sphingomyelins. The tighter packing of the phospholipid acyl chains in the presence of the sterol has been termed “condensing effect”. Outside these Lo domains cholesterol associates with other glycerophospholipids (mainly phosphatidylcholines) in a rather loose manner, and at relatively lower concentrations. There is abundant experimental evidence to support the notion that Lo domains exist in binary phospholipid (DPPC)-cholesterol mixtures (see recent review in ref. [119]).

Lipid domains apparently cover a wide range of sizes, from assemblies with <5 nm radius (“utrananodomains” [99]), comprising a couple of hundred lipid molecules per bilayer, to micron-sized platforms with thousands of molecules readily observable by conventional wide-field light microscopy [61,87,110,134]. Functionally, lipid domains play important roles in the cell by way of the lateral separation of chemical species in the plane of the membrane. Membrane-embedded proteins with preferential affinities for Lo or Ld domains could influence both the lifetime and size of the domains in which they are located by selecting their local lipid environment.

7. Possible raisons d’être for cholesterol induction of ordered lipid domains

The ability of cholesterol to promote the formation of liquid-ordered domains in a biological membrane stems to a large extent from the long evolutionary process towards optimizing the sterol surface chemistry to two complementary but somewhat contrasting demands: good match with other lipids on the one hand, and with membrane proteins, on the other. The bifacial geometry of cholesterol appears to be the outcome of this struggle [48]. The dissymmetrical nature of the molecule is manifested in the contrast between the rough β face, with methyl substituents in C10 and C13 and an iso-octyl chain in C17, with the featureless, smooth α face. The planar α face is optimally suited to interact with sphingomyelin or the phospholipid fatty acyl chains, a requirement probably driven towards the induction of order in the acyl chain conformations, to ensure tight packing of the sterol with phospholipid saturated acyl chains, a collective property that promotes the formation of Lo domains [150,151]. This capacity varies among sterols; using spin-label fluorescence quenching and DPH polarization techniques we found that most sterols have a tendency to disrupt lipid ordered domains, whereas it is precisely cholesterol and to a lesser extent 25-hydroxycholesterol that exhibit the highest propensity to form and

stabilize ordered lipid domains [145]. This obviously implied the tuning of the enzymatic machinery to progress from lanosterol to cholesterol. From *in vivo* experiments with eukaryotic sterol auxotrophs and microviscosity measurements of sterol-containing artificial membranes with the cholesterol precursor lanosterol (4,4',14 trimethyl cholastadienol) Bloch has argued that lanosterol is “incompetent for membrane function” [19]. In this sense, lanosterol would not only be the precursor of cholesterol in the sterol biosynthetic pathway but also its predecessor in the molecular evolution to cholesterol; that is, the temporal sequence of cholesterol biosynthesis can be assumed to recapitulate the evolutionary sequence. And what is the purpose of the appearance of cholesterol in the phylogenetic scale? Using a combination of deuterium NMR spectroscopy on multilamellar lipid-sterol systems in combination with Monte Carlo simulations of microscopic models of lipid-sterol interactions, Mouritsen and coworkers [88] concluded that the evolution in the molecular chemistry from lanosterol to cholesterol is manifested in the increase in the ability of the sterols to promote and stabilize lipid order, in the form of the *L_o* phase in model phospholipid-sterol membranes. However, experimental work using the simplest of bacterial sterol surrogates, the hopanoid diploptene, demonstrated that this property was also present in bacteria early in evolution [117,118]. The second requirement, *i.e.* the optimal adjustment of a sterol surface to the complementary surface of a membrane protein [48] is also likely the consequence of evolutionary pressures exerted on both partner molecules in the course of phylogeny [16]. As can be seen in Fig. 4, the

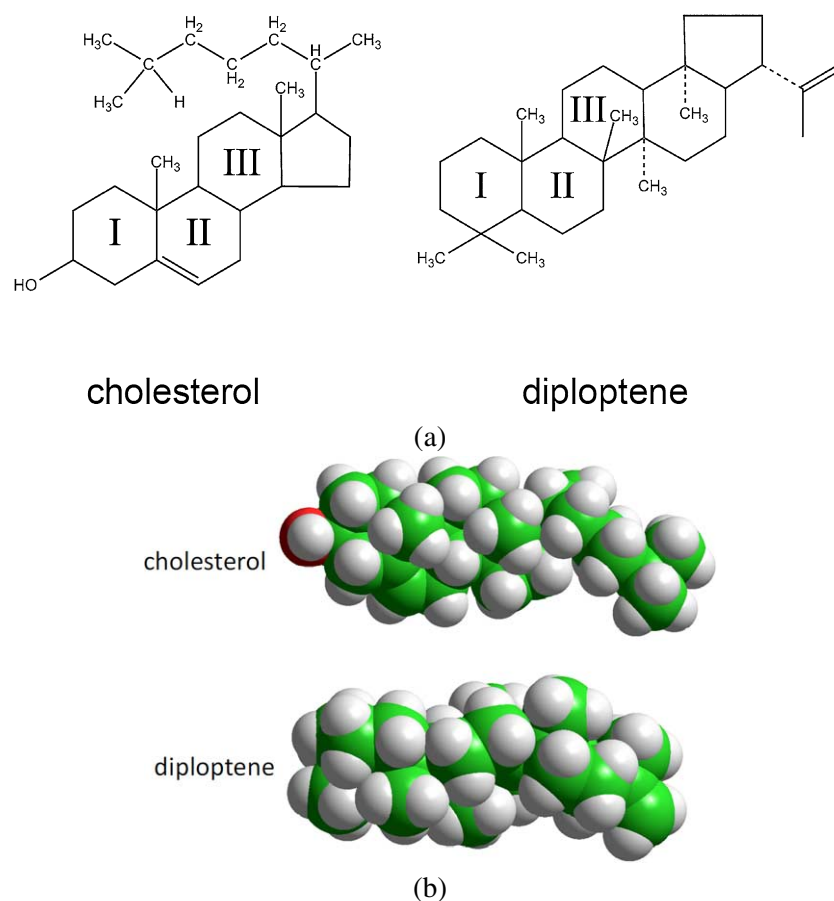


Fig. 4. Chemical formulae (a) of cholesterol and the hopanoid diploptene and (b) CPK representation of the same molecules.

main difference between hopanoids and sterols is the loss of a ring from the pentacyclic triterpenoids, the hopanoids, to the tetracyclic triterpenoids, the sterols. This long-term tuning was associated with the stabilization of amino acid motifs in contact with the rough surface of the sterol molecule [48], which is manifested in the strong conservation of amino acid residues in tight contact with membrane lipids exhibited by membrane proteins [1].

8. Occurrence of receptors in postsynaptic ordered lipid domains

The chemical analysis of the postsynaptic apparatus in the peripheral nervous system shows that cholesterol is a very abundant component (see review in [9]) of this specialized membrane. This sterol is an essential partner of the nAChR, affecting its distribution and several of its functional properties in the peripheral synapse, the neuromuscular junction [12,13]. The lateral heterogeneity of lipids in the postsynaptic membranes of the *Torpedo* electrocyte was an early biophysical finding: protein-associated lipids were shown to be immobilized with respect to bulk membrane lipid [83], and subsequent work has shown that cholesterol-like molecules form part of this protein-immobilized pool [11]. The functional implications of this finding became apparent when it was demonstrated that cholesterol is an essential component for maintaining nAChR agonist-dependent state transitions in the postsynaptic membrane [36]. It has been proposed that there are two cholesterol populations in nAChR-rich membranes from *Torpedo*: an easily extractable fraction that influences the bulk fluidity of the membrane and a tightly bound receptor-associated fraction [71].

Signaling via the agrin/MuSK complex and interaction between the receptor and rapsyn appears to involve Lo-type lipid platforms [154]. The formation of micron-sized nAChR clusters induced by agrin in muscle cells has been found to be influenced by cholesterol [29]. Laurdan two-photon fluorescence microscopy was used to image nAChR clusters in association with Lo membrane domains [127]. Another study [146] proposed that these cholesterol-rich lipid microdomains and Src-family kinases both contribute to stabilizing nAChRs and the postsynaptic apparatus. A pool of nAChRs has indeed been found in Lo domains in mammalian cells [27,29,80,127,146,154]. In contrast, when reconstituted in a sphingomyelin–cholesterol–POPC (1:1:1) model system, purified nAChR protein from *Torpedo* was found not to exhibit any preference for Lo domains in vitro [17]. However, inclusion of some sphingomyelin molecular species (brain sphingomyelins, 16:0, 18:0, or 24:1 sphingomyelins) that generate bilayer asymmetry by enriching the sphingolipid content of the outer leaflet of the lipid bilayer appear to favor the partitioning of the nAChR in Lo domains [100]. This can be correlated with the observation that Lo domains in the outer leaflet of a bilayer can induce liquid order in the inner leaflet by a coupling mechanism involving in-register Lo domains in the two halves of the bilayer [74].

9. Evolutionary aspects of hopanoid/sterol-protein interactions in membranes

The appearance in phylogeny of the sterol biosynthetic machinery – that is the enzymes involved in the synthetic pathways of phytosterols in land plants, ergosterol in yeasts, and cholesterol in many other organisms from fungi to vertebrates – is considered a major hallmark in the development of eukaryotic cells [18,19,21]. Although cholesterol is absent from Prokaryotes [19,22,92] a selected group of bacteria such as the myxobacterium *Plesiocystis pacifica* possess surrogate enzymes of the sterol pathway, a singularity postulated to have been acquired via horizontal gene transfer from eukaryotes [39]. Biosynthesis

of cholesterol is an extremely oxygen-intensive process necessitating sufficient terrestrial oxygen to proceed; eleven molecules of oxygen are required for the synthesis of one molecule of cholesterol [129]. Several lines of evidence suggest that cholesterol evolved at least in part as an adaptation to oxygenic conditions on planet Earth following the advent of oxygenic photosynthesis [25,56].

Isopentenyl diphosphate is the universal precursor of the triterpenoids, a class of organic compounds common to Prokaryotes and Eukaryotes. Isopentenyl diphosphate is synthesized by the 2-C-methyl-D-erythritol 4-phosphate pathway in bacteria, and through the mevalonate pathway in Archea and some Eukaryotes [98]. In bacterial cells, isopentenyl diphosphate gives rise to squalene, which is followed via the anaerobic squalene hopane cyclase activity to its cyclization products, the hopanoids (pentacyclic triterpenoids). In eukaryotic cells, isopentenyl pyrophosphate is synthesized starting with the formation of 3-hydroxy-3-methylglutaryl Co A from acetyl-CoA. Initially two molecules of acetyl-CoA condense to form acetoacetyl-CoA, a reaction catalyzed by a thiolase, and acetoacetyl-CoA further condenses with another acetyl-CoA molecule to yield HMG-CoA that is reduced to mevalonate by NADPH catalyzed by HMG-CoA reductase. This is the so-called committed, irreversible and control site in cholesterol biosynthesis. Mevalonate is further converted to 3-isopentenyl pyrophosphate. Six molecules of isopentenyl pyrophosphate, the activated isoprene form, are then condensed to form squalene. Acquisition of oxygen by squalene produces squalene epoxide, subsequently cyclized to lanosterol or to cycloartenol, tetracyclic sterols [95]. Demethylation of the methyl groups of lanosterol yield desmosterol, and the reduction of its side chain double bond finally produces cholesterol. In total, 19 steps are involved to produce cholesterol from its precursor lanosterol, out of the total 37 steps that mediate the elaborate synthesis of cholesterol in animal cells.

The appearance, improvement and maintenance of cholesterol-recognition linear amino acid motifs cover a wide evolutionary span, from *Homo sapiens* back to the bacterial pentameric channels, structural homologs of the nAChR, e.g. the cyanobacterium *Gloebacter violaceus* and its orthologue from *Erwinia chrysanthemi*. The former bacterium expresses hopanoids, which are structurally and functionally similar to sterols. The remarkable preservation of structural features in pLGIC proteins – including lipid recognizing consensus motifs through millions of years has led us to suggest that these domains fulfill important physiological roles [14,16]. In support of this hypothesis is the extensive experimental work showing that mutations in amino acid residues in the TM regions of the nAChR alter channel gating (see review in [11]). Some of these functionally relevant mutations are very close to or within CARC/CARC-like domains. It has been proposed that the hopanoids may have originally participated in the ordering of the membrane bilayer in bacteria [117,118]. We surmise that the counterpart, interacting lipid-recognition moiety in the transmembrane protein segments (the CARC or CRAC recognition domains), may have co-evolved with the lipid biosynthetic apparatus along phylogeny thus facilitating the capacity of some membrane proteins to be recruited into Lo domains in prokaryotic channel-forming and other membrane-embedded proteins; however, upon appearance of oxygenic photosynthesis and the cholesterol synthesizing machinery in the course of phylogeny, this lipid probably acquired protagonism in Eukaryotes and added further functions, such as aiding the process of transducing regulatory signals from the plasma membrane to the protein moiety. Cholesterol-recognition sequences concomitantly became integrated into the genes coding for many hormone and neurotransmitter receptors as well as channel proteins. This is, in essence, the idea behind the premise that hopanoids in Prokaryotes and cholesterol in Eukaryotes share some common roles; cholesterol added complexity to the protein-lipid interplay.

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