From hopanoids to cholesterol: Molecular clocks of pentameric ligand-gated ion channels

Francisco J. Barrantes, Jacques Fantini

PII: S0163-7827(15)30030-8
DOI: doi: 10.1016/j.plipres.2016.03.003
Reference: JPLR 909

To appear in:

Received date: 29 November 2015
Revised date: 22 March 2016
Accepted date: 24 March 2016

Please cite this article as: Barrantes Francisco J., Fantini Jacques, From hopanoids to cholesterol: Molecular clocks of pentameric ligand-gated ion channels, (2016), doi: 10.1016/j.plipres.2016.03.003

From hopanoids to cholesterol: molecular clocks of pentameric ligand-gated ion channels

Francisco J. Barrantes[1]* and Jacques Fantini[2]

[1]Laboratory of Molecular Neurobiology, Institute for Biomedical Research (BIOMED), Faculty of Medical Sciences, UCA-CONICET, Av. Alicia Moreau de Justo 1600, C1107AFF Buenos Aires, Argentina, and [2]EA-4674, Interactions Moléculaires et Systèmes Membranaires, Aix-Marseille Université, Marseille, France.

*To whom correspondence should be addressed at rtfjb1@gmail.com

Running head: Lipid evolution and channel tuning

The abbreviations used are: nAChR, nicotinic acetylcholine receptor; ELIC, pentameric ligand-gated ion channel protein found in *Erwinia chrysanthemi;* GluCl, anion-selective glutamate-gated chloride channel from the planarian worm *Caenorhabditis elegans;* GLIC, pentameric ligand-gated ion channel from the cyanobacterium *Gloeobacter violaceus;* pLGIC, pentameric ligand-gated ion channels.

ABSTRACT

Pentameric ligand-gated ion channels (pLGIC) and their lipid microenvironments appear to have acquired mutually adaptive traits along evolution: 1) the three-ring architecture of their transmembrane (TM) region; 2) the ability of the outermost TM ring to convey lipid signals to the middle ring, which passes them on to the central pore ring, and 3) consensus motifs for sterol recognition in all pLGIC. Hopanoids are triterpenoid fossil lipids that constitute invaluable biomarkers for tracing evolution at the molecular scale. The cyanobacterium *Gloeobacter violaceus* is the oldest known living organism in which the X-ray structure of its pLGIC, GLIC, reveals the presence of the above attributes and, as discussed in this review, the ability to bind hopanoids. ELIC, the pLGIC from the bacillum *Erwinia chrysanthemi* is the only other known case. Both prokaryotes lack cholesterol but their pLGICs exhibit the same sterol motifs as mammalian pLGIC. This remarkable conservation suggests that the association of sterols and hopanoid surrogate molecules arose from the early need in prokaryotes to stabilize pLGIC TM regions by means of relatively rigid lipid molecules. The conservation of these phenotypic traits along such a long phylogenetic span leads us to suggest the possible co-evolution of these sterols with pLGIC.

Keywords: evolution; pentameric ligand-gated ion channels; steroids; cholesterol; hopanoids; ion channel evolution; *Gloeobacter*; *Erwinia*.

1. Prokaryotic and eukaryotic ion channels and their membrane habitats

The appearance of a thin sub-nanometric lipid barrier in autonomous self-replicating units of prokaryotic stem to isolate their internal contents from the environment was an attainment common to Archaea and Bacteria. This trait was adopted by Eukaryotes billions of years later. Proteins localized in these boundary interfaces called membranes had to optimize their relationship not only with the external and internal milieu but also with their constituent surrounding lipids. It is likely that this process involved multiple choices of partner molecules along evolution, making it all the more remarkable that certain partnerships appear to have been implemented early on and conserved over billions of years ever since. One superfamily of membrane proteins exemplifies this phenomenon: the pentameric ligand-gated ion channels (pLGIC)[1-3] .

pLGICs descend from an ancestral "proto-channel" which occurred before the Prokaryote-Eukaryote dichotomy [4].The identification of several structural homologues of the eukaryotic pLGIC in unicellular prokaryotic organisms [4] constitutes a pioneer work establishing the structural conservation of key features in this superfamily of ion channel proteins along phylogeny. Seven years were to elapse before Hilf and Duzler [5] produced the first crystallographic structure of a full-length prokaryotic pLGIC: ELIC, a protein found in *Erwinia chrysanthemi* (Figure 1). The causal agent of an outbreak of bacterial heart rot of pineapple in Hawaii in 2003, *Erwinia chrysanthemi* (now reclassified as *Dickeya sp.*) is a Gram-negative phytopathogenic bacillus in the family Enterobacteriaceae, closely related to *Escherichia coli*, causing soft rot diseases in many crops, and is also an aphid pathogen. The

structure of ELIC presumably corresponds to a non-conducting, closed conformation of the ion channel [6-8]. The structure of GLIC, a prokaryotic pLGIC orthologue present in the cyanobacterium *Gloeobacter violaceus* [7, 9-11], was elucidated next and its crystal structure is consistent with an open channel conformation [7, 10-12] (Figure 1).

In physiological terms, both GLIC and ELIC are cation-selective ion channel proteins, as are the nicotinic acetylcholine receptor (nAChR) and the serotonin 5-HT3 receptor, two representative members of the eukaryotic pLGIC. GLIC is a peculiar channel which upon activation by low pH -generated by an increase in extracellular H^+ concentration [9]- exhibits a low single-channel conductance (~8 pS) resulting in an unknown number of protonated amino acid side chains in the protein –at least one in its TM region [13]. Unlike several eukaryotic pLGIC and ELIC, GLIC does not undergo desensitization. Its gating kinetics, in parallel with the crystal structures of the liganded-closed and liganded-open states, have recently been characterized using a combination of electrophysiological and X-ray crystallographic techniques[14].

ELIC conforms more closely to the characteristics of eukaryotic channels, being activated upon ligand binding to canonical sites in the extracellular moiety: it can be stimulated by primary amines such as propylamine and cysteamine and, more interestingly, γ-aminobutyric acid, GABA [8, 15], the endogenous neurotransmitter acting on $GABA_A$ and $GABA_C$ receptors, also members of the pLGIC superfamily and main mediators of inhibitory signals in mammalian brain. ELIC has a high single-channel conductance (84-96 pS) and its channel behavior resembles in some respects that of the eukaryotic nAChR [8, 15], although curiously

acetylcholine acts as a competitive antagonist [16]. ELIC shares most mechanistic channel properties with the $GABA_A$ receptor, albeit with slower kinetics [15, 17-20]. Marabelli et al. [21] have produced a detailed analysis of ELIC's channel kinetics, concluding that this bacterial pLGIC behaves very much like an ancestral ortholog of the eukaryotic $GABA_A$ receptor, to the point that benzodiazepines, prototypic man-tailored ligands of this receptor, also modulate GABA-activated currents in ELIC[8].

The first crystal structure of an anion-selective glutamate-gated chloride channel, GluCl, from a metazoan (the planarian worm *Caenorhabditis elegans*) was solved by X-ray diffraction at a resolution of 3.3 Å [22]. The dimensions of this eukaryotic channel appear to correspond to an open conformer, similar to that of the prokaryotic homologue GLIC [7, 10]. In addition to the GluCl, the collection of high resolution X-ray crystallographic data on pLGIC and homologous proteins like the acetylcholine binding protein, AChBP (reviewed in [23]), has recently grown with the incorporation of the human $GABA_A$ receptor β3 subunit homopentamer [24], the mouse 5-HT_3 receptor A subunit homopentamer [25], a chimaeric α1 glycine receptor-GLIC protein coined "Lily"and the glycine receptor α1 subunit in complex with strychnine, glycine or ivermectin (solved by cryoelectron microscopy at resolutions of 3.8-3.9 Å)[26]. The pharmacological implications of the crystallographic data have recently been reviewed [27].

2. Evolutionarily conserved three-ring design in pLGIC TM architecture

Various topographical regions have been distinguished in the eukaryotic pLGIC macromolecules: an extracellular domain exposed to the synaptic gap, a TM region composed of hydrophobic segments having 20–30 amino acids each, and the cytoplasmic domain made up of loops linking the TM segments [28]. All members of the pLGIC share a conserved overall architecture, with five identical or homologous subunits symmetrically arranged around a central ion channel (Figure 1). But as recently reviewed [3], there is more that has been conserved: in addition to the oligomeric organization of the pentamer, observation of their structures reveals a concentrically layered topology of the TM region, as early gained from the cryoelectron microscopy experiments of Unwin and coworkers on the nAChR [29-31], currently reaching a resolution of ~4 Å [32]. The early data already revealed hitherto unknown features of the membrane-embedded domains. One such feature is the arrangement of the TM region in three coaxial rings [3, 33]: (i) the narrow inner ring exclusively made up of five TM2 segments, constituting the walls of the nAChR ion pore. This inner ring has no contact with the membrane bilayer lipid since its outer rim is shielded by the middle ring; (ii) the middle ring, formed by 10 helices from the TM1 and TM3 segments. This middle ring is separated from the inner five-member ring of TM2s, and its outer face is exposed to lipids and also to (iii) the outermost ring, which consists of five TM4 segments. Although the proton-gated ion channel GLIC shares only 20% amino acid identity with the homomeric α7 neuronal nAChR [9], both share the same three-ring motif present in the TM region of all pLGIC known to date (Figure 2). The latter feature is clearly observed in the X-ray structure of GLIC [10, 34] as well as in that of its orthologue, the ELIC protein from *Erwinia chrysanthemi*, also resolved by X-ray diffraction at a

resolution of 3.3 Å, which exhibits only 16% sequence identity with the nAChR [34]. Yet, the crystal structure of ELIC, the first pLGIC high-resolution structure solved by X-ray diffraction techniques, clearly shows the three concentric ring motif in the TM region [35]. Not surprisingly, the 3-ring architecture is conserved in all eukaryotic nAChRs, $GABA_A$, $GABA_C$, GluCl and 5-HT_3 receptors [33]. When we noted an identical design in the bacterial homologues GLIC and ELIC, the finding came as a total surprise [36, 37].

3. Shared phospholipid and lipophilic ligand (e.g. general anesthetic) sites in pLGIC TM rings

General anesthetics are known to be positive allosteric modulators of some members of the eukaryotic pLGIC, the $GABA_A$ or glycine receptors [38], whereas they exert the opposite effect, i.e. negative allosteric modulation, on the nAChR [39] and on the prokaryotic pLGIC, GLIC [40]. In general, small volatile general anesthetics like halothane exhibit multiple sites in pLGIC. Halothane binds to both intra- and inter-subunit sites in the muscle-type and in the neuronal-type α4β2 nAChR [41]. Fluorescence quenching by halothane of intrinsic Trp fluorescence helped to define the binding site of this anesthetic on the bacterial homologue GLIC [42]. Cannabinoids, which exert analgesic effects via glycine receptors, appear to establish direct contacts at a cavity in the TM region of the protein, involving the lipid-exposed residue Ser296 in the TM3 middle ring and surrounding lipids, as suggested by solution NMR experiments [43]. Similarly, when studied by X-ray diffraction techniques, the general anesthetic molecules propofol and desfluorane were found to bind to GLIC at sites located at the TM-lipid interface, close to the surface of the extracellular membrane hemilayer. The sites are also cavities,

accessible from the lipid bilayer, and preexisting in the anesthetic-free, apo-form of the bacterial protein [44]. In the crystal structure, propofol lies closer to the entrance of the general-anesthetic cavity and competes for occupancy with the phospholipid molecule seen in the apo- and desflurane structures lying in the crevice between TM1 and TM4: the lipid "guarding" the entrace of the cavity is displaced in the propofol-bound structure.

Well-defined densities are observed in the grooves between the TM4 outer ring and the TM1-TM3 middle ring in the crystal structure of GLIC, surrounding the protein like a belt, with the five TM4 segments wedged in between and interrupting the continuity of the boundary[10]. The densities lie close to residues that are photoaffinity labelled by hydrophobic probes in *Torpedo* nAChR [45-47]. The densities in GLIC correspond to endogenous glycerophospholipids present in the native membrane where the protein is inserted, and not to exogenous lipids added in the purification/crystallization steps [10]. The finding of tightly-bound lipids in a pLGIC, early disclosed by electron-spin resonance (ESR) experiments with *Torpedo* nAChR[48] constitutes the first direct experimental observation of the protein-vicinal lipid or lipid belt region in a channel protein. Nury and coworkers suggested that the alteration of GLIC-phospholipid interactions caused by binding of the general anesthetic molecules might contribute to functional inhibition by competing with endogenous allosteric modulators [44]: phospholipids in the case of GLIC, or phospholipids, cholesterol, neurosteroids or fatty acids in the case of eukaryotic pLGIC. In fact, an intra-subunit cavity similar to that found in GLIC appears to be present in the *Torpedo* nAChR TM domain [49].

In the prokaryotic channel ELIC, general anesthetics bind more promiscuously to multiple sites. One site is located at the ion channel proper and is presumably responsible for the non-competitive type of blockage that general anesthetics exert on ELIC and other ion channels [50]. The second site is present at the TM region in a crevice formed at the interface between two subunits. The walls of the crevice are formed by the middle (TM1) and outer (TM4) rings of one subunit and the middle ring (TM3) of another subunit. General anesthetics may thus compete with endogenous lipids not only in the case of GLIC but also in ELIC. In the latter case, given the functional similarities with the $GABA_A$ receptors, competition may be established with cholesterol or with neurosteroids [51-53]. Using molecular modeling simulations, changes in the lipid cavities caused by motions of TM2 and TM3 were also reported [44], suggesting that the shape and volume of the cavity are coupled to channel gating. The observation of changes in the shape of the lipid cavities upon binding of the general anesthetic propofol was also recently reported by Gosh et al. [54] for GLIC.

The nematode *C. elegans* does not biosynthesize but does require cholesterol [55]. The worm harbors an anion-selective glutamate-activated chloride channel (GluCl) that is an important member of the rapid pLGIC superfamily present in neurons and a target of pharmacologically diverse compounds such as barbiturates, benzodiazepines, volatile anesthetics, alcohols and strychnine. The structure of the channel was first resolved in a co-crystal with the antiparasitic drug ivermectin[56]. A subsequent crystallographic study revealed the presence of the phospholipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) at a cavity within the membrane-embedded region of the protein. The lipid site, found at the

interface between two different subunits, was flanked by the TM1 helix from one subunit and the TM3 helix from an adjacent subunit, and delimited by one amino acid at the outer surface of TM2 at its deepest level [57] (Figure 3). Molecular dynamics simulations also identified the phospholipid pocket as an inter-subunit crevice transiently occupied by, on average, four lipid molecules per pentamer [58]. Interestingly, the phospholipid site at the protein–lipid interface extensively overlaps with the binding site of ivermectin, and has been proposed to correspond to the site used by several other lipophilic molecules in pLGIC, such as steroids in the $GABA_A$ receptor [52, 59] or cholesterol in the nAChR [60, 61]. In fact the recent work of Hénin and coworkers on cholesterol sites in another pLGIC, $GABA_A$, places the cyclohexanol ring of cholesterol in the position of the six-membered ring of the benzofuran moiety of ivermectin [59]. Further proof of the commonality of these sites across the pLGIC superfamily is the observation that ivermectin behaves as a positive allosteric modulator of the α7-type neuronal nAChR [62]. This type of compound binds to nAChR TM regions.

4. Lipid sensing, the role of the outermost TM4 ring, is another evolutionarily conserved trait

With the aim of learning about the gating kinetics of nAChR, molecular dynamics simulations were conducted on the entire TM region of the receptor in a hydrated lipid bilayer [63]. An unexpected outcome of the work was the finding that the most external TM4 ring undergoes substantial motions in the time window explored, extensively alternating between contact with the lipids surrounding its outside boundaries and with the middle ring (TM1-TM3) helix bundle inside its perimeter. The data led us to explicitly suggest: "*the outer ring of TM4*

acts as the vehicle to transfer the influences of the lipid surroundings to the conformational changes of the whole channel" [63]. We further expanded that this ability of the TM4 ring provided a foundation for the effect of mutations in αTM4, βTM4 and γTM4 on channel function, and for the influence of the lipid environment on the stability of nAChR functional states, as well as on how several pharmacologically relevant ligands that partition in the lipid bilayer affect nAChR channel function [63, 64]. Electrophysiological studies of Auerbach and his group [65] pointed to the extensive motions of TM4 in the course of nAChR gating.

If lipids in contact with the outermost perimeter of the nAChR exert influence on channel gating, governed by the relatively distant ion permeation pathway lined by the inner ring of TM2s residues, mutations of amino acids in the TM4 lipid-sensing region should affect the TM4-TM2 functional coupling. Furthermore, in order to sustain the hypothesis that the TM4 is the locus of a phylogenetically maintained lipid-sensing function, this should be conserved along evolution. Both premises appear to be the case. Firstly, a wealth of electrophysiological studies show that nAChRs mutated in the outer TM4 ring exhibit changes in channel kinetics, the rate of the transitions between channel states being most affected[66-74]. These mutations in the TM4 region of the eukaryotic nAChR are similarly observed in prokaryotic pLGIC. Recent alanine-scanning mutagenesis of residues on either the protein-facing or the lipid-facing surfaces of TM4 in GLIC and ELIC showed altered EC50 values for channel activation [75]. TM4 mutations in GLIC led to loss of function, whereas in ELIC they typically led to gain-of-function phenotypes. The latter was interpreted by the cited authors to suggest that, unlike GLIC, TM4 and/or the interactions between TM4 and TM1-TM3 in ELIC are

not optimized for channel gating. Furthermore, deletion of aromatic residues at the interface region between TM4 and the TM1-TM3 in GLIC reduces the interaction between the two rings, affecting the normal assembly of the pentamer [76]. More recent work provides additional evidence that mutations in the TM4 region of ELIC and GLIC, which alter the interactions of the TM4 lipid sensing domain with the TM1-TM3 helix bundle, reduce the crosstalk of TM4 with the lipid microenvironment [77]. Conversely, engineering new aromatic interactions at the TM4-TM1/TM3 interface promotes effective interactions between the outer and middle rings [77].

The concept of TM4 acting as a lipid sensor has been extended to assign a functional role to a region of the nAChR beyond the lipid bilayer, and hence termed “post-M4” [78]. In the extended hypothesis, residue Gln435, located in the post-M4 extracellular region, interacts with Phe137, located in the extracellular Cys-loop[78], and thus functionally couples agonist binding with channel gating. Moreover, the post-M4 region outside the lipid bilayer would also be subject to lipid modulation, and ineffective coupling would lead to a non-functional, “uncoupled” state of the channel [78, 79]. Early mutagenesis studies which truncated part of TM4, thus effectively shortening its length, were invoked to claim lack of influence of TM4 on nAChR channel function [80], a statement which is currently overturned by the wealth of information on the involvement of TM4 in channel activity. It has also been argued that the post-M4 C-term domain in bacterial homologues of pLGIC is too short to reach the β6-β7 loop in the extracellular domain, and that the shorter TM4 length would preclude its involvement in the extracellular-TM coupling [78, 79]. These authors further speculate that the emergence of lipid sensitivity coincided with the increasing demands of an evolving nervous system. Here we

argue that lipid sensitivity had already emerged in unicellular prokaryotic species much earlier than the appearance of the nervous system. Cumulatively, the data provide strong support to the hypothesis that the alternating crosstalk of TM4 with its surrounding lipids outside, or the TM1-TM3 bundle inside, coupled the lipid sensing region with the ion permeation domain of pLGIC, giving rise to the lipid sensing hypothesis [63] -which appears to be conserved in prokaryotic and eukaryotic pLGIC.

5. Cholesterol motifs are conserved among prokaryotic and eukaryotic pLGIC

Photoaffinity labeling techniques have been the experimental choice in the search for cholesterol binding sites on the nAChR protein. Early experiments aimed at the labeling of individual subunits [81, 82]. A decade later photoactivatable cholesterol analogues were used to dissect subunit labeling into individual TM segment labeling [83, 84]. The most recent work along these lines [85] confirmed the occurrence of sterol-recognition sites in the TM4, TM3, and TM1 segments of each subunit, fully overlapping the lipid-exposed rings of the nAChR. The TM4 segment showed the most extensive labelling with the cholesterol analogues. Incorporation of azido-cholesterol in αTM4 occurred in amino acid residues that lie in a rather shallow region of the membrane. Using fluorescence quenching techniques, sterol sites were also identified in all nAChRs TM segments, and their depth in the membrane bilayer was also found to be close to the polar head region [86].

Early ESR experiments revealed that steroid spin label androstanol displayed affinity for the immediate perimeter of the nAChR protein [48]. The interaction was also characterized in

terms of selectivity and stoichiometry [87-91]. A total number of about 15 cholesterol molecules were calculated to be bound to the outer perimeter of the nAChR [92]. In silico studies of the nAChR in the presence or absence of cholesterol revealed multiple cholesterol binding sites [61], some of which correspond to non-annular cavities located between the TM α-helices, deeply buried in the protein, and others to those located at the nAChR-lipid interface identified by photoaffinity labelling, fluorescence and ESR techniques (reviewed in [60]). Cholesterol-recognition motifs have been identified in the TM region of all nAChR proteins using an algorithm which we coined "CARC": (K/R)-X1−5-(Y/F)-X1−5-(L/V) from the N-term ending to the C-term ending [93]. The CARC consensus domain (Figure 4) was found not only in all nAChRs but also in all eukaryotic and prokaryotic members of the pLGIC, as well as in other TM proteins such as the GPCR superfamily [93, 94].

The CARC motif was originally identified in the TM domains of the nAChR protein [93], basically as a reverse version of the previously characterized CRAC algorithm (L/V)-X1-5-(F)-X1-5)-(K/R) [95]. It has since been found in a broad range of cholesterol-binding proteins, including some membrane receptors [94, 96]. Although covalent cross-linking with a radiolabeled cholesterol derivative has experimentally demonstrated the existence of cholesterol-binding sites in the TM domains of *Torpedo* nAChR [97], the membrane-spanning regions of nAChRs from various species do not display CRAC domains. Instead, we found a series of inverse CRAC motifs –CARC motifs- in the terminal part of the TM domains that appeared to be appropriately positioned to interact with cholesterol. In fact there are some CRAC motifs in the nAChR, but not in TM domains [93]. This situation perfectly illustrates a common caveat of the CRAC

definition: the motif can be found in proteins that do not interact with cholesterol or in specific protein regions for which an interaction with cholesterol is unlikely [98-100]. In contrast, single mutations in the CRAC domain of various cholesterol-binding proteins have been found to significantly affect or even abolish cholesterol binding or cholesterol-dependent functions [95, 99, 100]. For instance, the large conductance Ca^{2+}- and voltage-gated K^{+}(BK) channel displays several CRAC domains whose integrity is mandatory for proper cholesterol modulation of channel functions [101]. Convergent results were obtained through site-directed mutations of functional CRAC domains in several proteins, including ATP-gated cation channels [102], GPCRs [103], the glucose transporter GLUT-1 [104] or the major peripheral myelin protein [105]. In most cases, molecular modeling studies comparing wild-type and mutant proteins support the view that cholesterol could bind to these CRAC domains and that the mutations which affect cholesterol-dependent properties impair cholesterol binding [106].

A similar rule applies for the CARC domain. For instance the TRVP1 ion channel displays a linear motif [579-**R**FMFVY**L**-585] which has been erroneously considered as "CRAC" by Picazo-Juárez et al. [107] but is actually a typical CARC domain. This case is particularly interesting because there are two human TRVP1 variants which differ by one amino acid in this CARC domain. The one with a native CARC [579-**R**FMFVY**L**-585] is inhibited by cholesterol. The second one, which has an Ile residue instead of Leu at position 585 [579-RFMFVYI-585] is not cholesterol-sensitive. Moreover, mutations of either R-579 or F-582 in the CARC domain of rat TRVP1 abrogated cholesterol-dependent properties of the channel. On the basis of electrophysiological and molecular modeling data, the authors concluded that all three amino

acid residues that define the CARC motif (R-579, F-582 and L-585) are part of a functional cholesterol-binding domain [107]. In line with these findings, recent experimental data have shown that a synthetic peptide derived from the 4th TM domain of the nAChR γ subunit, which possesses a characteristic CARC domain, binds cholesterol with high affinity [108]. Replacing the central aromatic amino acid of this CARC domain with an alanine residue (F-452/A mutant) strongly affected the insertion of this peptide in cholesterol monolayers. Furthermore, nuclear magnetic resonance studies with reconstituted phosphatidylcholine bilayers with or without cholesterol confirmed the capacity of the CARC motif to functionally interact with cholesterol. Finally, the CARC-cholesterol interaction proved to be both lipid-specific and saturable [108].

Taken together, these data support the notion that the presence of a CARC (or a CRAC) motif within a TM domain is consistent with a specific interaction with cholesterol. Whether cholesterol binding actually occurs may depend on the availability of cholesterol around the TM domain. Both CARC and CRAC are vectorial motifs displaying a basic amino acid (Lys or Arg) at one terminus and a branched aliphatic one (Leu or Val) at the opposite end. This specific topology implies that the basic residue is located near the polar/apolar interface of the TM domain, in either the inner or the outer leaflet of the plasma membrane [94, 108]. Because of their apolar nature, the remaining residues of CARC and CRAC (i.e., Y/F and L/V) are frequently found in TM domains. In this respect, the predictive value of CARC and CRAC algorithms appears to be most accurate when those motifs are found in TM domains [106]. The situation may be different for globular proteins because in these cases a significant part of the motif could be buried in the apolar core of the protein and thus not accessible to any ligand. This

caveat may explain the poor reliability of the CRAC algorithm for the detection of cholesterol-binding sites outside TM domains [94].

The strategies developed for identifying cholesterol-binding sites in ion channels has already led to remarkable findings, as is the case with the $GABA_A$ receptor. This receptor is one of the main targets of endogenous neurosteroids [52, 53]. Cholesterol sites have been recently postulated at the TM surface of the human $GABA_AR$ using the X-ray coordinates of the glutamate-gated GluCl from *C. elegans* as a template [59] and a combination of several molecular modelling approaches, one of them based on the analogy with the crystallographic structure of the ivermectin-GluCl complex [22].

The presence of cholesterol-binding domains in organisms that do not synthesize cholesterol is, at first sight, quite puzzling. Indeed, this finding raises a series of unresolved questions: are these domains functional for binding lipids? And if so, which lipids? Could modern sterols bind to these ancient domains? Resolving these issues will fill a gap in our comprehension of lipid-protein co-evolution over several billion years.

6. Evolutionary sterol chemotaxonomy: from sterol surrogates in ancient bacteria to cholesterol in eukaryotes

All eukaryotes exhibit a requirement for sterols, a diverse class of triterpenoid lipids [109, 110]. Sterols serve important functions in cells, ranging from structural building blocks of cell membranes to developmental regulators and precursors of steroid hormones, bile acids, and vitamin D in metazoa. Cholesterol, the key sterol in eukaryotic membranes, is absent from prokaryotes [111]. However, the hopanoids, a class of pentacyclic triterpenoid lipids with a five-

membered E-ring derived from squalene (Figure 5), appear to play similar roles in bacterial membranes [112]. As such, they are considered the prokaryotic predecessor of eukaryotic sterols [112-114], and therefore referred to as biohopanoids.

The widespread use of nucleic acid sequencing has enabled the bacterial species that synthesize hopanoids to be clearly identified. Approximately 10% of all sequenced bacterial genomes contain genes coding for hopanoid biosynthetic enzymes, the majority being found in cyanobacteria, acetobacters, ethylotrophs and purple nonsulfur bacteria. Most importantly, the gene coding for squalene-hopene cyclase is present in the genome of *Gloeobacter violaceus* [115]. This enzyme catalyzes the conversion of squalene into the pentacyclic triterpenes that form the hopane backbone [116]. In contrast, enterobacteria do not appear to express enzymes involved in hopanoid synthesis [117].

Bloch [118] clearly summarized sterol synthesis in relation to evolution: "*sterol synthesis occurs in two stages; one, the anaerobic phase terminates with squalene, the acyclic sterol precursor; during the second, aerobic phase, squalene is oxygenated, cyclized, and the tetracyclic product lanosterol modified oxidatively to yield eventually cholesterol or related sterols*" and further on: *"…It is therefore tempting to argue that the sterol pathway, although it began in some prokaryotic cells, was fully developed only by eukaryotes*". Hopanoids are products of the mevalonate pathway, but do not require O_2 for their synthesis [119]. As a result, they lack the 3β-hydroxyl group of sterols [120]. Nes [121] was among the first to think about the key appearance of sterol biosynthetic enzymatic pathways along phylogeny, pointing to the Δ^{24}-reduction-alkylation pathway bifurcation as a key landmark in the evolutionary

differentiation of the animal from the plant kingdom. Rohmer et al. [122] have discussed the plausible phylogenetic evolution from hopanoids (pentacyclic triterpenoids) to sterols (tetracyclic triterpenoids); both are cyclization products of squalene, albeit hopanoids are cyclized through a simpler mechanism, via the squalene-hopene cyclases. These cyclases, which produce hopanoids from squalene and sterols and pentacyclic triterpenoids from oxidosqualene, are closely related and clearly reflect evolutionary changes in the sterol biosynthetic pathway over time [123, 124]. The cyclization reaction product is a ternary cation [125] that is subsequently deprotonated to obtain the simplest hopanoid, i.e. diploptene [126]. Lipid analysis of a broad range of bacteria has revealed that the most common hopanoids are diploptene, diplopterol and C_{35} bacteriohopanetetrol [113] (Figure 5). Diplopterol is obtained by hydration of the cyclization product, whereas C_{35} needs a polyfunctionalized side chain whose backbone is derived from a ribosugar [127, 128].

7. Hopanoids, proton gradients and pLGIC function

An intriguing function that has been attributed to bacterial hopanoids is a potential role in the adjustment of cell membrane permeability in adaptation to extreme environmental conditions. Since cell membranes maintain proton electrochemical gradients as energy transducers, proton leakage constitutes a waste of energy. It has been speculated that hopanoids fulfill a unique role in acidophile membrane structure, namely the prevention of inward leakage of protons[129]. Alkaliphile bacteria synthesize the polyisoprene squalene, a critical precursor of cholesterol in higher organisms [130]. Neutrophile bacteria do not usually

synthesize isoprenes, although tetracyclic isoprenoids, referred to as “sporulenes”, have been identified in *Bacillus subtilis* spores [131]. Squalene synthesis increases with increasing pH of the culture medium. At high pH, isoprenes constitute over 40 mol% of the lipids, of which 10-11% is squalene. Hauss et al. [132] speculate that squalene acts as a proton permeability barrier to impede proton leakage through prokaryotic membranes. *B. cenocepacia* is a bacterium of importance in human pathology, causing opportunistic infections in cystic fibrosis patients and other immune-compromised individuals. Deletion of the gene coding for the squalene-hopene cyclase in *B. cenocepacia* halts hopanoids biosynthesis, resulting in altered motility and, in particular, low pH tolerance, and membrane damage at low pH conditions [133]. Similarly, mutation of squalene-hopene cyclase in *Rhodopseudomonas palustris*, resulting in the inability to produce hopanoids, has been associated with sensitivity to low pH [134]. Hopanoid biosynthesis is apparently not needed at neutral pH [133]. Bacteria employ transporters and efflux pumps to move protons in and out of their cytoplasm, and this ability appears to be correlated with their hopanoid content. Acidophile bacteria have the largest concentrations of hopanoids, almost exclusively in membranes holding large proton gradients [129, 132, 135]. It is therefore conceivable that in *G. violaceus*, the cyanobacterium harboring GLIC, the pLGIC gating cation permeation upon lowering the extracellular pH, is subjected to similar hopanoid modulatory mechanisms for stabilizing and regulating membrane fluidity and permeability under conditions of extreme proton gradients. In the last part of this review we will attempt to address how these evolutionary distant lipids interact with structurally conserved ion channels.

8. Hopanoids vs. cholesterol: what chemical structures reveal

The chemical formulae of cholesterol and diploptene are shown in Figure 6. Both lipids are polycyclic compounds that share a common typical triad of 6-carbon rings (numbered I, II and III or A, B and C). Since diploptene lacks the -OH group of cholesterol, it cannot be oriented in a membrane like an amphipathic molecule. Instead, the common ring triad can be used as a topological reference for superimposing diploptene onto the cholesterol structure (Figure 6). This geometry exercise reveals some interesting features, in particular the fact that both lipids occupy approximately the same volume; in fact they can be included in a 6 Å diameter cylinder, with a length of 17 Å for cholesterol and 15 Å for diploptene. These molecular dimensions, which were deduced from energy-minimized structures [94, 136], are consistent with those initially published by Rohmer et al. [122], although the refined models obtained by molecular dynamics simulations are slightly more condensed than the extended theoretical formulas used by these authors.

At first glance, cholesterol could be considered as a hopanoid-derived compound displaying two specific chemical groups located at each end of the molecule, i.e. a polar -OH group and a branched aliphatic chain CH-$(CH_3)_2$. However, there is an important feature that should not be overlooked when comparing the 3D structure of cholesterol and diploptene (Figure 7). In the case of cholesterol, all the aliphatic groups (methyl and iso-octyl) linked to the ring structure are located on the same face, referred to as the β-face according to the nomenclature of cyclic compounds[137]. This unique topology creates a “rough” surface [136]. In contrast, the α-face of cholesterol is “smooth” because it consists exclusively of CH bonds.

This dissymmetry is consistent with the formation of a ternary complex in which the beta face of cholesterol interacts with a TM domain belonging to a channel and the alpha face with a sphingolipid belonging to a raft domain [136]. In the case of diploptene, both faces are rough because its methyl groups protrude equally from each side of the ring backbone (Figure 7). Thus, although diploptene might well be accommodated on the TM domains of eukaryotic ion channels and exert a functional condensing effect on these membrane-spanning domains, it would not be particularly well suited for an interaction with "more modern" lipids such as raft-forming sphingolipids [106]. In this respect, one could consider that an important consequence of the diploptene/cholesterol substitution in eukaryotic membranes is the possibility to form functional ternary complexes involving a TM domain, a sphingolipid and a polycyclic lipid.

Both diplopterol and C_{35} bacteriohopanetetrol are built on the same hopane structure as diploptene (Figure 5). Therefore, all the structural properties discussed above for diploptene also apply for these hopanoids. However, in marked contrast with the neutral diploptene molecule, diplopterol and C_{35} bacteriohopanetetrol display -OH groups (one and four groups, respectively). One might assume that the presence of such -OH groups linked to the hopane backbone would render these bacterial lipids more "cholesterol-like" than diploptene. This would make sense if the -OH groups were linked to the first ring of the hopane backbone, in a "cholesterol-like" topology. However, examination of the chemical structures in Figure 5 shows that this is clearly not the case. In both diplopterol and C_{35} hopanoids, the -OH groups are linked to a chemical group attached to the 5^{th} ring of hopane. If we consider that hydroxylated hopanoids have a "cholesterol-like" orientation in a biological membrane, which is highly

probable from a physicochemical point of view, we obtain a striking “anti-cholesterol” effect in terms of molecular flexibility. Indeed, in cholesterol the -OH group is linked to the more rigid part of the molecule, i.e. the first ring of sterane. The mobility of this -OH group is particularly restricted because it is often fixed by a hydrogen bond formed with a vicinal lipid or protein. This “freezing” is compensated by the conformational flexibility of the iso-octyl chain that can optimally adapt its shape to the apolar environment provided by lipid acyl chains of TM domains of proteins. If we apply this reasoning to diplopterol and C_{35} hopanoids, we can see that in this case the “freezing” may occur at both ends of the lipid, i.e. the -OH group(s) potentially involved in a hydrogen bond network, and in the hopane backbone deeply embedded in the apolar phase of the membrane. In conclusion, although all three hopanoids may induce a common “cholesterol-like” ordering effect on bacterial membranes [112], there are crucial traits of hopanoid molecules that may reflect i) specific capabilities for molecular interactions with vicinal membrane components, and ii) some obvious evolutionary limitation due to the restricted flexibility of the apolar parts of these lipids. In this respect, it is interesting to find that the evolutionary jump from hopanoids to sterols follows robust physicochemical and biochemical rules.

9. Co-evolution of lipid sites and pLGIC

Gloeobacter violaceus constitutes a unique bacterium: it is the only known oxygenic organism that lacks intracellular thylakoid membranes. *G. violaceus* evolved before the appearance of thylakoids and instead harbors the photosynthetic complexes in its single plasma membrane[138]. Besides this singularity, what makes *G. violaceous* particularly interesting in

the context of our current hypothesis are three sets of interrelated data: 1) *G. violaceus* is a hopanoid-producing cyanobacterium [115]; 2) it exhibits lateral membrane heterogeneity [138], one of the common structural features involving sterols or hopanoids for modulating membrane order [139] and 3) its plasma membrane generates a proton gradient and harbors GLIC, one of only two prokaryotic pLGICs crystallized and analyzed by X-ray diffraction to date [9-11]. Cyanobacteria engage in oxygenic photosynthesis; accordingly, the occurrence of hopanoids in 2.7 billion-year-old sediments was taken as evidence that photosynthetically derived O_2 first appeared on Earth at least that long ago. The appearance of cyanobacteria preceded the so-called Great Oxygenation event (GOE) by several hundred million years [123, 140], and *G. violaceus* is a unique early-branching member from the cyanobacterium clad, probably as old as the original progenitor bacterium. This is not too distant from the emergence of the ancestral proto-channel -the "mother of all channels"- on planet Earth [141].

It has been surmised that the emergence of sterol-like ordering was likely a critical step in the evolution of biological membranes, providing the means to modulate their physicochemical status –fluidity in particular- and to organize them into functionally distinct lateral domains. Kannenberg et al. [142] early pointed out that hopanoids "condensed" bacterial membranes. Sáenz et al. [112, 139] have recently provided evidence showing that from a physicochemical point of view hopanoids behave in bacteria as sterols do in eukaryotes, with the ability to order saturated lipids and to form a laterally segregated liquid-ordered phase (Lo) in model membranes. These properties led these authors to propose that all membrane polycyclic isoprenoids share a common, conserved feature along the evolutionary scale. The

presence of C_{27}-C_{30} steranes in 2,700 million-year-old Australian rocks was initially suggested to mark the emergence of eukaryotic cells on Earth [143], but this dating has been recently challenged due to probable surface contamination of Archean rocks by hydrocarbon biomarkers [144]. Others trace this crucial transition in macroevolution to 2,100-1,800 million years ago [145]. In fact, the oldest fossil evidence is ca. 2,150 million years old for cyanobacteria and 1,780–1,680 million years old for eukaryotes [140]. The rare steroid 24-isopropylcholestane constitutes a more recent molecular clock found in pre-Ediacaran to Early Cambrian sedimentary rocks, also present in the Animal Kingdom in unicellular sponges [146].

G. violaceous and *E. chrysantemii* do not possess cholesterol. However, GLIC and ELIC, the two pLGIC proteins of prokaryotic origin so far identified in these two bacteria, possess cholesterol-recognition consensus motifs in their membrane-embedded region, sharing this evolutionarily conserved structural feature with all the other members of the eukaryotic pLGIC [93], reviewed in ref. [94]. The presence of such domains in bacteria is puzzling. Do they mediate the interaction of non-annular lipids with prokaryotic ion channels? If this hypothesis proved to be correct, then we are facing a fascinating case of lipid-protein co-evolution that has arisen very early in the evolution of living organisms. It is assumed that the three domains of Bacteria, Archaea and Eukaryota derive from a primeval cell, probably a hyper-thermophilic "last universal common ancestor" [123]. Others have hypothesized that this ancestor is a non-thermophilic proto-eukaryote from which Archaea and Bacteria emerged by reductive evolution [147]. The evolution of lipid biosynthetic pathways appears to have followed similar steps. Recent studies suggest that the capacity to synthesize hopanoid was likely horizontally

transferred from the Alphaproteobacteria into the Cyanobacteria after the Cyanobacteria's major divergences [148]. Ricci et al. [148] further suggest that the ancestral function of hopanoids was not related to oxygenic photosynthesis but instead to a trait already present in the Alphaproteobacteria, thought to be ancestrally aerobic, leading these authors to infer that hopanoids arose after the oxygenation of the atmosphere. Tasneem et al. [4] speculated that the phyletic patterns and phylogenetic relationships suggest the possibility that the metazoan pLGIC emerged through an early lateral transfer from a prokaryotic source, before the divergence of extant metazoan lineages. It is thus likely that lateral transfer of genes also involved concomitant changes in TM proteins involved in signal transduction –receptors and channels in particular-, as some evidence on the evolution of TM proteins' extracellular domains driven by changes in molecular oxygen suggest [149]. These evolutionary changes in receptors and channels may have also been contemporary with the acquisition of motifs for sterol-like molecules on their bilayer lipid-exposed surfaces, to ensure structural stability in the membrane environment, a trait which apparently entailed a clear gain-of-function, the proof of which lies in the striking conservation in pLGIC along evolution. Nevertheless, one important issue remains to be resolved to demonstrate that this trait is not coincidental: can hopanoids bind to bacterial channels?

10. Hopanoid binding to bacterial channels: what in silico studies reveal

Hopanoids have long been considered as sterol surrogates in living organisms that do not express cholesterol. In this respect, the presence of cholesterol-binding motifs in bacterial

channels raises the possibility that hopanoids are able to physically interact with these channels, a hypothesis which remains to be confirmed by appropriate physicochemical approaches. Meanwhile, in silico approaches could help identify membrane-spanning domains that sustain hopanoid binding. For instance, a potential region to study is the 234-245 segment of the 1st TM domain of GLIC (Uniprot entry Q7NDN8), which fulfills the criteria of the CARC algorithm: 234-RQYFSYIPNIIL-245 (alternatively numbered 192-203 if we take into account the cleavage of the signal peptide). Interestingly, this region in the GLIC protein is involved in the binding of the general anesthetic propofol [44]. It has been suggested that the mechanism of anesthetic action could locally affect the organization of lipid domains through specific effects on protein-lipid coupled systems[150]. Moreover, in silico approaches revealed that propofol has a cholesterol-like ordering effect on phosphatidylcholine in the fluid phase [151]. Thus, the co-localization of the propofol and the potential hopanoid binding sites within a conserved CARC motif is in full agreement with the notion that hopanoids could stabilize bilayer-exposed regions of pLGIC through a general membrane-ordering effect.

Docking of diploptene onto this CARC domain further supports the notion that hopanoid may bind to GLIC. The binding site identified in this study involves Arg-192, Tyr-197, Ile-201 and Ile-202, with a predicted energy of interaction of -42.7 kJ/mol (Figure 8A). Interestingly, the first cycle of diploptene interacts with the apolar part of the arginine side chain, i.e. the methylene groups. As expected, there is no possibility of interaction between hopanoid and the charged guanidinium group. This point is perfectly illustrated by the fact that the position of the lateral side chain of Arg-192 remained virtually unchanged during several nanoseconds of simulations.

Fitting this CARC-diploptene complex with the structure of the whole GLIC channel shed some light on the hopanoid-channel interaction. Indeed, the hopanoid appeared to occupy an internal cavity formed by the TM helices of each channel subunit (Figure 8A). This type of interaction, which involves several TM domains, is consistent with the presence of spiking methyl groups on each side of the hopanoid, as discussed above (Figure 7).

Since the CARC motif of this prokaryotic channel existed long before cholesterol ever appeared on earth, it was especially interesting to assess whether cholesterol could bind to this motif as diploptene does. To explore this possibility, we replaced the diploptene molecule bound to the GLIC structure with cholesterol and performed a series of molecular dynamics simulations of the GLIC/cholesterol system (Figure 8B). Overall, we found that cholesterol could perfectly interact with the CARC motif of GLIC. Most importantly, the -OH group of the sterol comes closer to the guanidinium group so that the energy of interaction rose to -53.9 kJ/mol. The snapshots of the complex during the simulation process clearly indicate that the side chain of Arg-192 is progressively re-oriented so that it eventually faces the -OH group of cholesterol. Moreover, the iso-octyl chain of cholesterol also improves the interaction with Ile-202, which further contributes to raise the energy of interaction of the cholesterol/GLIC complex. Overall, these considerations raise the intriguing possibility that the CARC motif has been in fact primarily "designed" for hopanoids and that evolution has subsequently used this domain for sterol binding. In other words, what worked with hopanoids worked even better for cholesterol and this might explain why such sequences are conserved.

Finally, we would like to comment further on the respective topologies of CARC/cholesterol and CARC/hopanoid complexes. Due to its amphipathic structure, cholesterol is bound to the domain at the polar-apolar interface of the membrane. We have previously shown that the -OH/ε-NH_3^+ interaction between cholesterol and the first basic residue of CARC is at the basis of this phenomenon [93]. Diploptene lacks such an -OH group, but may still bind to the aliphatic chain of those residues which, due to the snorkeling effect of Lys and Arg residues, are generally buried in the apolar phase of the membrane, whereas the charged ε-NH_3^+group "breathes" at the membrane surface [152, 153]. However, the absence of a polar head group in the hopanoid is clearly a severe drawback, because diploptene could otherwise penetrate more deeply into the apolar phase of the membrane, where it might still interact with the aromatic and aliphatic signature amino acids of the CARC motif. One could challenge this hypothesis by arguing that two other important hopanoids, diplopterol and C35 bacteriohopanetetrol, have -OH groups. However, as discussed above, these -OH groups are linked to chemical groups on the 5^{th} ring of hopane, so that they can be considered as functional surrogates for the -OH group of cholesterol. Overall, these elements indicate that although the CARC domain appeared first during the evolution of living organisms, and was initially used for hopanoid binding, it was only after the appearance of cholesterol that it revealed all its striking functional properties. In other words, the CARC domain is evolutionary conserved but although it can bind both hopanoids and cholesterol, it fulfills distinct functions in Prokaryotes and Eukaryotes.

11. Conclusion and perspectives

Hopanoids found in Archean rocks have been used for decades as unique calibrators that serve as molecular clocks to count evolutionary times [126]. The biological origin of these molecules is no longer a matter of debate, even though a considerable time reset has been required to provide a more accurate estimation of the probable date of appearance of Eukaryotes on Earth [140]. In parallel, hopanoids were studied for what they are, i.e. a category of membrane lipids selectively expressed in Prokaryotes. A substantial analogy with the structure of cholesterol was soon recognized [122] and subsequently substantiated through appropriate physicochemical approaches [112, 139]. In this review we have suggested and discussed a third dimension of the striking biological impact and significance of hopanoids. This new level takes into account the chemical structure of these lipids, a basic property that controls the manner in which they interact with vicinal membrane components. Until now, these molecular interactions had received scant attention and one of the goals of this review was therefore to stimulate further structural research. The evolution of pLGIC from bacteria to Eukaryotes is analyzed from the perspective of cholesterol-binding domains that might have initially been designed by Nature to interact with hopanoids, and subsequently evolved to satisfy the structural requirements of sterols, in a striking protein-lipid co-evolution process that took place over several hundred million years. We hypothesize that this molecular evolution pathway is punctuated by two critical milestones, hopanoids and cholesterol, which we suggest to be molecular clocks in the phylogenetic evolution of pentameric ligand-gated ion channels.

Acknowledgements. Experimental work from our laboratory quoted in this article was supported by grant PIP No. N° 112-201101-01023 from the National Scientific and Technical Research Council of Argentina (CONICET). Thanks are due to Dr. C.J. Baier, from the Institute of Biochemistry in Bahía Blanca for providing Figure 4, and to Dr. Gordon Love, Dept. of Earth Science, UCR, for clarifying our query on the age of sterol precursors in geological records.

Figure captions

Figure 1: Common structural organization of eukaryotic and prokaryotic pentameric ligand-gated ion channels.

Two views (top and lateral) of representative examples of the two types of channel are shown. Alpha-helices are in red, beta strands in blue and coil regions in grey. The structure of *Torpedo* nicotinic acetylcholine receptor (nAChR) has been retrieved from PDB # 2BG9 [154]. GLIC, the pentameric ligand-gated ion channel homologue from the bacteria *Gloeobacter violaceus* has been retrieved from PDB # 3EAM [10]. ELIC, the pLGIC from *Erwinia chrysanthemi* has been retrieved from PDB # 2YOE [8].

Figure 2: Common three-ring organization of the TM region in eukaryotic and prokaryotic pentameric ligand-gated ion channels.

The conservation of the three-ring structure from Prokaryotes to Eukaryotes is schematically depicted. The inner ring is formed by TM2 domains (green) and the outer one by TM4 domains (red). The middle ring is formed by TM1 and TM3 domains (blue). The structure of *Torpedo* nicotinic acetylcholine receptor (an eukaryotic nAChR) has been retrieved from PDB # 2BG9 [154]. The bacterial *Gloeobacter violaceus* pLGIC homologue (GLIC) has been retrieved from PDB # 3EAM [10].

Figure 3: A phospholipid pocket within *C. elegans* glutamate-activated chloride channel (GluCl).

A ribbon structure of the whole channel is shown on the left with a phosphatidylcholine molecule (POPC) in yellow spheres. Alpha-helices are in red, beta strands in blue and coil regions in grey. POPC lies in a crevice formed by two adjacent subunits (D and E). In particular, POPC interacts with TM1 of subunit E and TM3 of subunit D. In the TM domains view one can see that POPC also contacts the outer surface of TM2 in the inner ring. The 3D structure of GluCl has been retrieved from PDB # 4TNW [57].

Figure 4: CARC and CRAC, the vectorial cholesterol consensus motifs.

A) Schematic diagram showing the topology of the extracellular loop, the four TM regions and the TM3-TM4 intracellular loop of the pentameric adult muscle type nAChR, a paradigmatic member of the pLGIC[1, 3]. The TM domain TM1 is displayed in the lower panel (B) to show the occurrence of the two vectorial sequences in the five subunits. The CRAC motif is shown in bold underlined characters and the CARC consensus motif is outlined in yellow with the central Y residue in bold[93].

Figure 5: Chemical structure of hopanoids.

A. Diploptene. **B.** Diplopterol. **C.** C_{35} tetrahydroxybacteriohopane (also known as bacteriohopanetetrol). All three hopanoids are shown in the same orientation, with the first ring of the hopane backbone upside. The rings are numbered from I to V (Roman numerals). **D.** Cholesterol. The first ring of the sterane backbone is upside to facilitate the comparison with hopanoids. **E.** Comparison of the structure of cholesterol and hydroxylated hopanoids. In this case, the molecules are oriented with the hydroxyl groups pointing up.

Figure 6: Chemical structures and volume occupancy of cholesterol and diploptene.

Despite significant differences in chemical structure (upper panel), cholesterol and diploptene occupy roughly the same cylindrical volume (lower panel). This is particularly obvious when diploptene (in blue) and cholesterol (colored atoms) are superimposed in the same cylinder.

Figure 7: Topological differences between cholesterol and diploptene.

Cholesterol (A) and the hopanoid diploptene (B) have a multi-ring structure (4 rings in cholesterol and 5 in diploptene, noted A-D and A-E, respectively). However, a most notable difference is that in the case of cholesterol all the aliphatic groups are on the same face, whereas they are homogenously distributed over the two surfaces in diploptene. As a consequence, cholesterol has a dissymmetric topology whereas the hopanoid is almost symmetrical.

Figure 8: Docking of diploptene and cholesterol on a pLGIC.

Molecular modelling simulations were conducted with the aim of determining whether the CARC domain of the bacterial *Gloeobacter violaceus* pLGIC homologue, GLIC, could functionally interact with diploptene (A) and cholesterol (B). In the case of the hopanoid, a high affinity complex was obtained after 1 ns of simulations, and this complex remained remarkably stable after 5 ns (A). The methylene groups of the Arg-192 established a series of van der Waals interactions with diploptene and the aromatic group of Tyr-197 formed a network of CH-π bonds with the hopanoid. The two residues together accounted for more than 50% of the whole energy of interaction of the GLIC-hopanoid complex. The location of diploptene in the structure of the whole pentameric channel is shown in the views of the complex on the right side (hopanoid in yellow). The channel could also accommodate cholesterol (B) but in this case the -OH group of the sterol induced a progressive rearrangement of the side chain of Arg-192 so that at the end of the process (i.e. after 5 ns of simulations) a hydrogen bond (H bond) could be formed. The structure of the GLIC channel has been retrieved from PDB entry 3EHZ [7].

References

[1] Cecchini M, Changeux JP. The nicotinic acetylcholine receptor and its prokaryotic homologues: Structure, conformational transitions & allosteric modulation. Neuropharmacology. 2015;96:137-49.
[2] Taly A, Henin J, Changeux JP, Cecchini M. Allosteric regulation of pentameric ligand-gated ion channels: an emerging mechanistic perspective. Channels (Austin, Tex). 2014;8:350-60.
[3] Barrantes FJ. Phylogenetic conservation of protein–lipid motifs in pentameric ligand-gated ion channels. Biochimica et Biophysica Acta (BBA) - Biomembranes. 2015;1848:1796-805.
[4] Tasneem A, Iyer LM, Jakobsson E, Aravind L. Identification of the prokaryotic ligand-gated ion channels and their implications for the mechanisms and origins of animal Cys-loop ion channels. Genome Biol 2005;6:R4.
[5] Hilf RJ, Dutzler R. X-ray structure of a prokaryotic pentameric ligand-gated ion channel. Nature. 2008;452:375-9.

[6] Hilf RJ, Dutzler R. A prokaryotic perspective on pentameric ligand-gated ion channel structure. Current opinion in structural biology. 2009;19:418-24.
[7] Hilf RJ, Dutzler R. Structure of a potentially open state of a proton-activated pentameric ligand-gated ion channel. Nature. 2009;457:115-8.
[8] Spurny R, Ramerstorfer J, Price K, Brams M, Ernst M, Nury H, et al. Pentameric ligand-gated ion channel ELIC is activated by GABA and modulated by benzodiazepines. Proceedings of the National Academy of Sciences of the United States of America. 2012;109:E3028-34.
[9] Bocquet N, Prado dC, Cartaud J, Neyton J, Le Poupon C, Taly A, et al. A prokaryotic proton-gated ion channel from the nicotinic acetylcholine receptor family. Nature. 2007;445:116-9.
[10] Bocquet N, Nury H, Baaden M, Le Poupon C, Changeux JP, Delarue M, et al. X-ray structure of a pentameric ligand-gated ion channel in an apparently open conformation. Nature. 2009;457:111-4.
[11] Hilf RJ, Bertozzi C, Zimmermann I, Reiter A, Trauner D, Dutzler R. Structural basis of open channel block in a prokaryotic pentameric ligand-gated ion channel. Nature structural & molecular biology. 2010;17:1330-6.
[12] Corringer PJ, Baaden M, Bocquet N, Delarue M, Dufresne V, Nury H, et al. Atomic structure and dynamics of pentameric ligand-gated ion channels: new insight from bacterial homologues. J Physiol. 2010;588:565-72.
[13] Wang HL, Cheng X, Sine SM. Intramembrane proton binding site linked to activation of bacterial pentameric ion channel. The Journal of biological chemistry. 2012;287:6482-9.
[14] Gonzalez-Gutierrez G, Cuello LG, Nair SK, Grosman C. Gating of the proton-gated ion channel from Gloeobacter violaceus at pH 4 as revealed by X-ray crystallography. Proceedings of the National Academy of Sciences of the United States of America. 2013;110:18716-21.
[15] Zimmermann I, Dutzler R. Ligand activation of the prokaryotic pentameric ligand-gated ion channel ELIC. PLoS biology. 2011;9:e1001101.
[16] Pan J, Chen Q, Willenbring D, Yoshida K, Tillman T, Kashlan OB, et al. Structure of the pentameric ligand-gated ion channel ELIC cocrystallized with its competitive antagonist acetylcholine. Nat Commun. 2012;3:1-8.
[17] Laha KT, Ghosh B, Czajkowski C. Macroscopic kinetics of pentameric ligand gated ion channels: comparisons between two prokaryotic channels and one eukaryotic channel. PloS one. 2013;8:e80322.
[18] Gonzalez-Gutierrez G, Lukk T, Agarwal V, Papke D, Nair SK, Grosman C. Mutations that stabilize the open state of the Erwinia chrisanthemi ligand-gated ion channel fail to change the conformation of the pore domain in crystals.
. Proc Natl Acad Sci U S A 2012;109:6331-6.
[19] Zimmermann I, Marabelli A, Bertozzi C, Sivilotti LG, Dutzler R. Inhibition of the prokaryotic pentameric ligand-gated ion channel ELIC by divalent cations. PLoS biology. 2012;10:e1001429.
[20] Gonzalez-Gutierrez G, Lukk T, Agarwal V, Papke D, Nair SK, Grosman C. Mutations that stabilize the open state of the Erwinia chrisanthemi ligand-gated ion channel fail to change the conformation of the pore domain in crystals. Proceedings of the National Academy of Sciences. 2012;109:6331-6.
[21] Marabelli A, Lape R, Sivilotti L. Mechanism of activation of the prokaryotic channel ELIC by propylamine: A single-channel study. The Journal of general physiology. 2015;145:23-45.
[22] Hibbs DE, Gouaux E. Principles of activation and permeation in an anion-selective Cys-loop receptor. Nature. 2011;474:54-60.
[23] Sixma TK, Smit AB. Acetylcholine binding protein (AChBP): a secreted glial protein that provides a high-resolution model for the extracellular domain of pentameric ligand-gated ion channels. Annual review of biophysics and biomolecular structure. 2003;32:311-34.
[24] Miller PS, Aricescu AR. Crystal structure of a human GABAA receptor. Nature. 2014;512:270-5.

[25] Hassaine G, Deluz C, Grasso L, Wyss R, Tol MB, Hovius R, et al. X-ray structure of the mouse serotonin 5-HT3 receptor. Nature. 2014;512:276-81.
[26] Du J, Lu W, Wu S, Cheng Y, Gouaux E. Glycine receptor mechanism elucidated by electron cryo-microscopy. Nature. 2015;526:224-9.
[27] Sauguet L, Shahsavar A, Delarue M. Crystallographic studies of pharmacological sites in pentameric ligand-gated ion channels. Biochim Biophys Acta. 2015;1850:511-23.
[28] Karlin A. Emerging structure of the nicotinic acetylcholine receptors. NatRevNeurosci. 2002;3:102-14.
[29] Miyazawa A, Fujiyoshi Y, Stowell M, Unwin N. Nicotinic acetylcholine receptor at 4.6 Å resolution: transverse tunnels in the channel wall. JMolBiol. 1999;288:765-86.
[30] Unwin N, Miyazawa A, Li J, Fujiyoshi Y. Activation of the nicotinic acetylcholine receptor involves a switch in conformation of the alpha subunits. JMolBiol. 2002;319:1165-76.
[31] Miyazawa A, Fujiyoshi Y, Unwin N. Structure and gating mechanism of the acetylcholine receptor pore. Nature. 2003;423:949-55.
[32] Unwin N. Nicotinic acetylcholine receptor and the structural basis of neuromuscular transmission: insights from *Torpedo* postsynaptic membranes. Q Rev Biophys. 2013;46:283-322.
[33] Barrantes FJ. Modulation of nicotinic acetylcholine receptor function through the outer and middle rings of transmembrane domains. Curr Opin Drug Discov Devel. 2003;6:620-32.
[34] Hilf RJ, Dutzler R. X-ray structure of a prokaryotic pentameric ligand-gated ion channel. Nature. 2008;452:375-9.
[35] Barrantes FJ, Bermudez V, Borroni MV, Antollini SS, Pediconi MF, Baier JC, et al. Boundary lipids in the nicotinic acetylcholine receptor microenvironment. Journal of molecular neuroscience : MN. 2010;40:87-90.
[36] Barrantes FJ. Modulation of nicotinic acetylcholine receptor function through the outer and middle rings of transmembrane domains. CurrOpinDrug DiscovDevel. 2003;6:620-32.
[37] Barrantes FJ, Bermudez V, Borroni MV, Antollini SS, Pediconi MF, Baier JC, et al. Boundary lipids in the nicotinic acetylcholine receptor microenvironment. J MolNeurosci. 2010;40:87-90.
[38] Olsen RW, Li GD, Wallner M, Trudell JR, Bertaccini EJ, Lindahl E, et al. Structural models of ligand-gated ion channels: sites of action for anesthetics and ethanol. Alcohol Clin Exp Res 2014;38:595-603.
[39] Violet JM, Downie DL, Nakisa RC, Lieb WR, Franks NP. Differential sensitivities of mammalian neuronal and muscle nicotinic acetylcholine receptors to general anesthetics. Anesthesiology. 1997;86:866-74.
[40] Weng Y, Yang L, Corringer PJ, Sonner JM. Anesthetic sensitivity of the Gloeobacter violaceus proton-gated ion channel. Anesth Analg. 2010;110:59-63.
[41] Chiara DC, Dangott LJ, Eckenhoff RG, Cohen JB. Identification of nicotinic acetylcholine receptor amino acids photolabeled by the volatile anesthetic halothane. Biochemistry. 2003;42:13457-67.
[42] Chen Q, Cheng MH, Xu Y, Tang P. Anesthetic Binding in a Pentameric Ligand-Gated Ion Channel: GLIC. Biophysical Journal. 2010;99:1801-9.
[43] Xiong W, Cheng K, Cui T, Godlewski G, Rice KC, Xu Y, et al. Cannabinoid potentiation of glycine receptors contributes to cannabis-induced analgesia. Nature chemical biology. 2011;7:296-303.
[44] Nury H, Van Renterghem C, Weng Y, Tran A, Baaden M, Dufresne V, et al. X-ray structures of general anaesthetics bound to a pentameric ligand-gated ion channel. Nature. 2011;469:428-31

[45] Blanton MP, Cohen JB. Mapping the lipid-exposed regions in the *Torpedo californica* nicotinic acetylcholine receptor. Biochemistry. 1992;31:3738-50.

[46] Blanton MP, Cohen JB. Identifying the lipid-protein interface of the *Torpedo* nicotinic acetylcholine receptor: secondary structure implications. Biochemistry. 1994;33:2859-72.
[47] Blanton MP, McCardy EA, Huggins A, Parikh D. Probing the structure of the nicotinic acetylcholine receptor with the hydrophobic photoreactive probes [^{125}I]TID-BE and [^{125}I]TIDPC/16. Biochemistry. 1998;37:14545-55.
[48] Marsh D, Barrantes FJ. Immobilized lipid in acetylcholine receptor-rich membranes from Torpedo marmorata. ProcNatlAcadSciUSA. 1978;75:4329-33.
[49] Jayakar SS, Dailey WP, Eckenhoff RG, Cohen JB. Identification of propofol binding sites in a nicotinic acetylcholine receptor with a photoreactive propofol analog. The Journal of biological chemistry. 2013;288:6178-89.
[50] Spurny R, Billen B, Howard RJ, Brams M, Debaveye S, Price KL, et al. Multisite binding of a general anesthetic to the prokaryotic pentameric Erwinia chrysanthemi ligand-gated ion channel (ELIC). The Journal of biological chemistry. 2013;288:8355-64.
[51] Hosie AM, Wilkins ME, da Silva HM, Smart TG. Endogenous neurosteroids regulate GABAA receptors through two discrete transmembrane sites. . Nature. 2006;444:486-9.
[52] Hosie AM, Wilkins ME, Smart TG. Neurosteroid binding sites on GABA(A) receptors. Pharmacol Ther 2007;116:7-19.
[53] Seljeset S, Laverty D, Smart TG. Inhibitory Neurosteroids and the GABAA Receptor. Advances in pharmacology (San Diego, Calif). 2015;72:165-87.
[54] Ghosh B, Satyshur KA, Czajkowski C. Propofol binding to the resting state of the gloeobacter violaceus ligand-gated ion channel (GLIC) induces structural changes in the inter- and intrasubunit transmembrane domain (TMD) cavities. The Journal of biological chemistry. 2013;288:17420-31
[55] Kurzchalia TV, Ward S. Why do worms need cholesterol? Nature cell biology. 2003;5:684-8.
[56] Hibbs RE, Gouaux E. Principles of activation and permeation in an anion-selective Cys-loop receptor. Nature. 2011;474:54-60.
[57] Althoff T, Hibbs RE, Banerjee S, Gouaux E. X-ray structures of GluCl in apo states reveal a gating mechanism of Cys-loop receptors. Nature. 2014;512:333-7.
[58] Yoluk O, Bromstrup T, Bertaccini EJ, Trudell JR, Lindahl E. Stabilization of the GluCl ligand-gated ion channel in the presence and absence of ivermectin. Biophys J. 2013;105:640-7.
[59] Hénin J, Salari R, Murlidaran S, Brannigan G. A predicted binding site for cholesterol on the GABAA receptor. Biophys J. 2014;106:1938-49.
[60] Barrantes FJ. Structural basis for lipid modulation of nicotinic acetylcholine receptor function. Brain Res Brain Res Rev. 2004;47:71-95.
[61] Brannigan G, Henin J, Law R, Eckenhoff R, Klein ML. Embedded cholesterol in the nicotinic acetylcholine receptor. ProcNatlAcadSciUSA. 2008.
[62] Krause RM, Buisson B, Bertrand S, Corringer PJ, Galzi JL, Changeux JP, et al. Ivermectin: a positive allosteric effector of the alpha7 neuronal nicotinic acetylcholine receptor. Molecular pharmacology. 1998;53:283-94.
[63] Xu Y, Barrantes FJ, Luo X, Chen K, Shen J, Jiang H. Conformational dynamics of the nicotinic acetylcholine receptor channel: a 35-ns molecular dynamics simulation study. Journal of the American Chemical Society. 2005;127:1291-9.
[64] Antollini SS, Xu Y, Jiang H, Barrantes FJ. Fluorescence and molecular dynamics studies of the acetylcholine receptor gammaM4 transmembrane peptide in reconstituted systems. MolMembrBiol. 2005;22:471-83.
[65] Mitra A, Bayley TD, Auerbach A. Structural dynamics of the M4 transmembrane segment during acetylcholine receptor gating. Structure. 2004;12:1909-18.

[66] Lee YH, Li L, Lasalde-Dominicci JA, Rojas L, McNamee MG, Ortiz-Miranda SI, et al. Mutations in the M4 domain of *Torpedo californica* acetylcholine receptor dramatically alter ion channel function. Biophys J. 1994;66:646-53.
[67] Lasalde-Dominicci JA, Tamamizu S, Butler DH, Vibat RT, Hung B, McNamee MG. Tryptophan substitutions at the lipid-exposed transmembrane segment M4 of *Torpedo californica* acetylcholine receptor govern channel gating. Biochem. 1996;35:14139-48.
[68] Cruz-Martin A, Mercado JL, Rojas LV, McNamee MG, Lasalde-Dominicci JA. Tryptophan substitutions at lipid-exposed positions of the gamma M3 transmembrane domain increase the macroscopic ionic current response of the Torpedo californica nicotinic acetylcholine receptor. JMembrBiol. 2001;183:61-70.
[69] Ortiz-Acevedo A, Melendez M, Asseo AM, Biaggi N, Rojas LV, Lasalde-Dominicci JA. Tryptophan scanning mutagenesis of the gammaM4 transmembrane domain of the acetylcholine receptor from Torpedo californica. J BiolChem. 2004;279:42250-7.
[70] Ortiz-Miranda SI, Lasalde-Dominicci JA, Pappone PA, McNamee MG. Mutations in the M4 domain of the *Torpedo californica* nicotinic acetylcholine receptor alter channel opening and closing. JMembrBiol. 1997;158:17-30.
[71] Tamamizu S, Guzman GR, Santiago J, Rojas LV, McNamee MG, Lasalde-Dominicci JA. Functional Effects of Periodic Tryptophan Substitutions in the alpha M4 Transmembrane Domain of the Torpedo californica Nicotinic Acetylcholine Receptor. Biochemistry. 2000;39:4666-73.
[72] Santiago J, Guzman GR, Rojas LV, Marti R, Asmar-Rovira GA, Santana LF, et al. Probing the effects of membrane cholesterol in the Torpedo californica acetylcholine receptor and the novel lipid-exposed mutation alpha C418W in Xenopus oocytes. J BiolChem. 2001;276:46523-32.
[73] Bouzat C, Roccamo AM, Garbus I, Barrantes FJ. Mutations at lipid-exposed residues of the acetylcholine receptor affect its gating kinetics. Molecular Pharmacology. 1998;54:146-53.
[74] Bouzat C, Barrantes FJ, Sine S. Nicotinic Receptor Fourth Transmembrane Domain. Hydrogen bonding by conserved threonine contributes to channel gating kinetics. JGenPhysiol. 2000;115:663-72.
[75] Henault CM, Sun J, Therien JP, daCosta CJ, Carswell CL, Labriola JM, et al. The role of the M4 lipid-sensor in the folding, trafficking, and allosteric modulation of nicotinic acetylcholine receptors. Neuropharmacology. 2014.
[76] Haeger S, Kuzmin D, Detro-Dassen S, Lang N, Kilb M, Tsetlin V, et al. An intramembrane aromatic network determines pentameric assembly of Cys-loop receptors. NatStructMolBiol. 2010;17:90-8.
[77] Carswell CL, Sun J, Baenziger JE. Intramembrane Aromatic Interactions Influence the Lipid Sensitivities of Pentameric Ligand-gated Ion Channels. The Journal of biological chemistry. 2015;290:2496-507.
[78] daCosta CJ, Baenziger JE. A lipid-dependent uncoupled conformation of the acetylcholine receptor. J BiolChem. 2009;284:17819-25.
[79] Baenziger JE, Corringer P-J. 3D structure and allosteric modulation of the transmembrane domain of pentameric ligand-gated ion channels. Neuropharmacology. 2011;60:116-25.
[80] Tobimatsu T, Fujita Y, Fukuda K, Tanaka K-, Mori Y, Konno T, et al. Effects of substitution of putative transmembrane segments on nicotinic receptor function. FEBS Lett. 1987;222:56-62.
[81] Middlemas DS, Raftery MA. Identification of subunits of acetylcholine receptor that interact with a cholesterol photoaffinity probe. Biochemistry. 1987;26:1219-23.
[82] Fernandez AM, Fernandez-Ballester G, Ferragut JA, Gonzalez-Ros JM. Labeling of the nicotinic acetylcholine receptor by a photoactivatable steroid probe: effects of cholesterol and cholinergic ligands. BiochimBiophysActa. 1993;1149:135-44.

[83] Corbin J, Wang HH, Blanton MP. Identifying the cholesterol binding domain in the nicotinic acetylcholine receptor with [125I]azido-cholesterol. BiochimBiophysActa. 1998;1414:65-74.
[84] Blanton MP, Xie Y, Dangott LJ, Cohen JB. The steroid promegestone is a noncompetitive antagonist of the Torpedo nicotinic acetylcholine receptor that interacts with the lipid-protein interface. MolPharmacol. 1999;55:269-78.
[85] Hamouda AK, Chiara DC, Sauls D, Cohen JB, Blanton MP. Cholesterol interacts with transmembrane alpha-helices M1, M3, and M4 of the Torpedo nicotinic acetylcholine receptor: photolabeling studies using [3H]Azicholesterol. Biochemistry. 2006;45:976-86.
[86] Barrantes FJ, Antollini SS, Blanton MP, Prieto M. Topography of nicotinic acetylcholine receptor membrane-embedded domains. JBiolChem. 2000;275:37333-9.
[87] Abadji VC, Reines DE, Dalton LA, Miller KM. Lipid-protein interactions and protein dynamics in vesicles containing the nicotinic acetylcholine receptor: a study with ethanol. BiochimBiophysActa. 1994;1194:25-34.
[88] Abadji VC, Raines DE, Watts A, Miller KW. The effect of general anesthetics on the dynamics of phosphatidylcholine-acetylcholine receptor interactions in reconstituted vesicles. BiochimBiophysActa. 1993;1147:143-53.
[89] Arias HR, Sankaram MB, Marsh D, Barrantes FJ. Effect of local anaesthetics on steroid-nicotinic acetylcholine receptor interactions in native membranes of Torpedo marmorata electric organ. BiochimBiophys Acta. 1990;1027:287-94.
[90] Horvath LI, Arias HR, Hankovszky HO, Hideg K, Barrantes FJ, Marsh D. Association of spin-labeled local anesthetics at the hydrophobic surface of acetylcholine receptor in native membranes from Torpedo marmorata. Biochemistry. 1990;29:8707-13.
[91] Marsh D, Watts A, Barrantes FJ. Phospholipid chain immobilization and steroid rotational immobilization in acetylcholine receptor-rich membranes from Torpedo marmorata. BiochimBiophys Acta. 1981;645:97-101.
[92] Mantipragada SB, Horvath LI, Arias HR, Schwarzmann G, Sandhoff K, Barrantes FJ, et al. Lipid-protein interactions and effect of local anesthetics in acetylcholine receptor-rich membranes from Torpedo marmorata electric organ. Biochemistry. 2003;42:9167-75.
[93] Baier CJ, Fantini J, Barrantes FJ. Disclosure of cholesterol recognition motifs in transmembrane domains of the human nicotinic acetylcholine receptor. Scientific reports. 2011;1:69.
[94] Fantini J, Barrantes FJ. How cholesterol interacts with membrane proteins: an exploration of cholesterol-binding sites including CRAC, CARC, and tilted domains. Front Physiol. 2013;4:31.
[95] Jamin N, Neumann JM, Ostuni MA, Vu TK, Yao ZX, Murail S, et al. Characterization of the cholesterol recognition amino acid consensus sequence of the peripheral-type benzodiazepine receptor. Molecular endocrinology (Baltimore, Md). 2005;19:588-94.
[96] Jafurulla M, Tiwari S, Chattopadhyay A. Identification of cholesterol recognition amino acid consensus (CRAC) motif in G-protein coupled receptors. Biochemical and biophysical research communications. 2011;404:569-73.
[97] Corbin J, Wang HH, Blanton MP. Identifying the cholesterol binding domain in the nicotinic acetylcholine receptor with [125I]azido-cholesterol. Biochimica et biophysica acta. 1998;1414:65-74.
[98] Palmer M. Cholesterol and the activity of bacterial toxins. FEMS microbiology letters. 2004;238:281-9.
[99] Epand RF, Thomas A, Brasseur R, Vishwanathan SA, Hunter E, Epand RM. Juxtamembrane protein segments that contribute to recruitment of cholesterol into domains. Biochemistry. 2006;45:6105-14.
[100] Epand RM. Cholesterol and the interaction of proteins with membrane domains. Progress in lipid research. 2006;45:279-94.

[101] Singh AK, McMillan J, Bukiya AN, Burton B, Parrill AL, Dopico AM. Multiple cholesterol recognition/interaction amino acid consensus (CRAC) motifs in cytosolic C tail of Slo1 subunit determine cholesterol sensitivity of Ca2+- and voltage-gated K+ (BK) channels. J Biol Chem. 2012;287:20509-21.
[102] Robinson LE, Shridar M, Smith P, Murrell-Lagnado RD. Plasma membrane cholesterol as a regulator of human and rodent P2X7 receptor activation and sensitization. J Biol Chem. 2014;289:31983-94.
[103] Potter RM, Harikumar KG, Wu SV, Miller LJ. Differential sensitivity of types 1 and 2 cholecystokinin receptors to membrane cholesterol. J Lipid Res. 2012;53:137-48.
[104] Nowis D, Malenda A, Furs K, Oleszczak B, Sadowski R, Chlebowska J, et al. Statins impair glucose uptake in human cells. BMJ open diabetes research & care. 2014;2:e000017.
[105] Saher G, Quintes S, Mobius W, Wehr MC, Kramer-Albers EM, Brugger B, et al. Cholesterol regulates the endoplasmic reticulum exit of the major membrane protein P0 required for peripheral myelin compaction. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2009;29:6094-104.
[106] Fantini J, Yahi N. Brain lipids in synaptic function and neurological disease. Clues to innovative therapeutic strategies for brain disorders. San Francisco: Elsevier Academic Press; 2015.
[107] Picazo-Juarez G, Romero-Suarez S, Nieto-Posadas A, Llorente I, Jara-Oseguera A, Briggs M, et al. Identification of a binding motif in the S5 helix that confers cholesterol sensitivity to the TRPV1 ion channel. J Biol Chem. 2011;286:24966-76.
[108] Fantini J, Di Scala C, Evans LS, Williamson PT, Barrantes FJ. A mirror code for protein-cholesterol interactions in the two leaflets of biological membranes. Scientific reports. 2016;6:21907.
[109] Desmond E, Gribaldo S. Phylogenomics of sterol synthesis: insights into the origin, evolution, and diversity of a key eukaryotic feature. Genome biology and evolution. 2009;1:364-81.
[110] Pichler H, Riezman H. Where sterols are required for endocytosis. Biochim Biophys Acta. 2004;1666:51-61.
[111] Mouritsen OG, Zuckermann MJ. What's so special about cholesterol? Lipids. 2004;39:1101-13.
[112] Saenz JP, Grosser D, Bradley AS, Lagny TJ, Lavrynenko O, Broda M, et al. Hopanoids as functional analogues of cholesterol in bacterial membranes. Proc Natl Acad Sci U S A. 2015;112:11971-6.
[113] Ourisson G, Rohmer M, Poralla K. Prokaryotic hopanoids and other polyterpenoid sterol surrogates. Annual review of microbiology. 1987;41:301-33.
[114] Ourisson G, Rohmer M, Poralla K. Microbial lipids betrayed by their fossils. Microbiological sciences. 1987;4:52-7.
[115] Nakamura Y, Kaneko T, Sato S, Mimuro M, Miyashita H, Tsuchiya T, et al. Complete genome structure of Gloeobacter violaceus PCC 7421, a cyanobacterium that lacks thylakoids. DNA research : an international journal for rapid publication of reports on genes and genomes. 2003;10:137-45.
[116] Wendt KU, Poralla K, Schulz GE. Structure and function of a squalene cyclase. Science. 1997;277:1811-5.
[117] Kuchta T, Kubinec R, Farkas P. Analysis of hopanoids in bacteria involved in food technology and food contamination. FEMS microbiology letters. 1998;159:221-5.
[118] Bloch KE. Speculations on the evolution of sterol structure and function. CRC critical reviews in biochemistry. 1979;7:1-5.
[119] Hartner T, Straub KL, Kannenberg E. Occurrence of hopanoid lipids in anaerobic Geobacter species. FEMS microbiology letters. 2005;243:59-64.
[120] Brown AJ, Galea AM. Cholesterol as an evolutionary response to living with oxygen. Evolution; international journal of organic evolution. 2010;64:2179-83.
[121] Nes WR. Role of sterols in membranes. Lipids. 1974;9:596-612.

[122] Rohmer M, Bouvier P, Ourisson G. Molecular evolution of biomembranes: structural equivalents and phylogenetic precursors of sterols. Proceedings of the National Academy of Sciences of the United States of America. 1979;76:847-51.
[123] Volkman JK. Sterols and other triterpenoids: source specificity and evolution of biosynthetic pathways. organic Geochemistry. 2005;36:139-59.
[124] Pearson A, Flood Page SR, Jorgenson TL, Fischer WW, Higgins MB. Novel hopanoid cyclases from the environment. Environmental microbiology. 2007;9:2175-88.
[125] Rajamani R, Gao J. Balancing kinetic and thermodynamic control: the mechanism of carbocation cyclization by squalene cyclase. J Am Chem Soc. 2003;125:12768-81.
[126] Pearson A. Lipidomics for Geochemistry. . In: Holland H, Turekian KK, editors. Treatise on Geochemistry, Second Edition. Oxford: Elsevier; 2014. p. 291-336.
[127] Flesch G, Rohmer M. Biosynthesis of a carbocyclic pentose analogue linked to bacteriohopanetetrol from the bacterium Methylobacterium organophilum. Journal of the Chemical Society, Chemical Communications. 1988:868-70.
[128] Neunlist S, Holst O, Rohmer M. Prokaryotic triterpenoids. The hopanoids of the purple non-sulphur bacterium Rhodomicrobium vannielii: an aminotriol and its aminoacyl derivatives, N-tryptophanyl and N-ornithinyl aminotriol. European journal of biochemistry / FEBS. 1985;147:561-8.
[129] Haines TH. Do sterols reduce proton and sodium leaks through lipid bilayers? Progress in lipid research. 2001;40:299-324.
[130] Clejan S, Krulwich TA, Mondrus KR, Seto-Young D. Membrane lipid composition of obligately and facultatively alkalophilic strains of Bacillus spp. Journal of bacteriology. 1986;168:334-40.
[131] Bosak T, Losick RM, Pearson A. A polycyclic terpenoid that alleviates oxidative stress. Proc Natl Acad Sci U S A. 2008;105:6725-9.
[132] Hauss T, Dante S, Dencher NA, Haines TH. Squalane is in the midplane of the lipid bilayer: implications for its function as a proton permeability barrier. Biochim Biophys Acta. 2002;1556:149-54.
[133] Schmerk CL, Bernards MA, Valvano MA. Hopanoid production is required for low-pH tolerance, antimicrobial resistance, and motility in Burkholderia cenocepacia. Journal of bacteriology. 2011;193:6712-23.
[134] Welander PV, Hunter RC, Zhang L, Sessions AL, Summons RE, Newman DK. Hopanoids play a role in membrane integrity and pH homeostasis in Rhodopseudomonas palustris TIE-1. Journal of bacteriology. 2009;191:6145-56.
[135] Kleemann G, Kellner R, Poralla K. Purification and properties of the squalene-hopene cyclase from Rhodopseudomonas palustris, a purple non-sulfur bacterium producing hopanoids and tetrahymanol. Biochim Biophys Acta. 1994;1210:317-20.
[136] Fantini J, Barrantes FJ. Sphingolipid/cholesterol regulation of neurotransmitter receptor conformation and function. BiochimBiophysActa. 2009.
[137] Rose IA, Hanson KR, Wilkinson KD, Wimmer MJ. A suggestion for naming faces of ring compounds. Proc Natl Acad Sci U S A. 1980;77:2439-41.
[138] Rexroth S, Mullineaux CW, Ellinger D, Sendtko E, Rogner M, Koenig F. The plasma membrane of the cyanobacterium Gloeobacter violaceus contains segregated bioenergetic domains. The Plant cell. 2011;23:2379-90.
[139] Saenz JP, Sezgin E, Schwille P, Simons K. Functional convergence of hopanoids and sterols in membrane ordering. Proc Natl Acad Sci U S A. 2012;109:14236-40.
[140] Rasmussen B, Fletcher IR, Brocks JJ, Kilburn MR. Reassessing the first appearance of eukaryotes and cyanobacteria. Nature. 2008;455:1101-4.

[141] Ortells MO, Lunt GG. Evolutionary history of the ligand-gated ion-channel superfamily of receptors. TINS. 1995;18:121-7.
[142] Kannenberg E, Poralla K, Blume A. A hopanoid from the thermo-acidophilic Bacillus acidocaldarius condenses membranes. Naturwissenschaften. 1980;67:458-9.
[143] Brocks JJ, Logan GA, Buick R, Summons RE. Archean molecular fossils and the early rise of eukaryotes. Science (New York, NY). 1999;285:1033-6.
[144] French KL, Hallmann C, Hope JM, Schoon PL, Zumberge JA, Hoshino Y, et al. Reappraisal of hydrocarbon biomarkers in Archean rocks. Proc Natl Acad Sci U S A. 2015;112:5915-20.
[145] Han TM, Runnegar B. Megascopic eukaryotic algae from the 2.1-billion-year-old negaunee iron-formation, Michigan. Science (New York, NY). 1992;257:232-5.
[146] Kodner RB, Summons RE, Pearson A, King N, Knoll AH. Sterols in a unicellular relative of the metazoans. Proceedings of the National Academy of Sciences of the United States of America. 2008;105:9897-902.
[147] Glansdorff N. About the last common ancestor, the universal life-tree and lateral gene transfer: a reappraisal. Molecular microbiology. 2000;38:177-85.
[148] Ricci JN, Michel AJ, Newman DK. Phylogenetic analysis of HpnP reveals the origin of 2-methylhopanoid production in Alphaproteobacteria. Geobiology. 2015.
[149] Acquisti C, Kleffe J, Collins S. Oxygen content of transmembrane proteins over macroevolutionary time scales. Nature. 2007;445:47-52.
[150] Balasubramanian SV, Campbell RB, Straubinger RM. Propofol, a general anesthetic, promotes the formation of fluid phase domains in model membranes. Chemistry and physics of lipids. 2002;114:35-44.
[151] Hansen AH, Sorensen KT, Mathieu R, Serer A, Duelund L, Khandelia H, et al. Propofol modulates the lipid phase transition and localizes near the headgroup of membranes. Chemistry and physics of lipids. 2013;175-176:84-91.
[152] Strandberg E, Morein S, Rijkers DT, Liskamp RM, Van Der Wel PC, Killian JA. Lipid Dependence of Membrane Anchoring Properties and Snorkeling Behavior of Aromatic and Charged Residues in Transmembrane Peptides. Biochemistry. 2002;41:7190-8.
[153] Strandberg E, Killian JA. Snorkeling of lysine side chains in transmembrane helices: how easy can it get? FEBS Lett. 2003;544:69-73.
[154] Unwin N. Refined structure of the nicotinic acetylcholine receptor at 4A resolution. Journal of molecular biology. 2005;346:967-89.