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SYMPOSIUM REVIEW

Atomic structure and dynamics of pentameric ligand-gated ion channels: new insight from bacterial homologues

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Pentameric ligand-gated ion channels (pLGICs) are widely expressed in the animal kingdom and are key players of neurotransmission by acetylcholine (ACh), γ-amminobutyric acid (GABA), glycine and serotonin. It is now established that this family has a prokaryotic origin, since more than 20 homologues have been discovered in bacteria. In particular, the GLIC homologue displays a ligand-gated ion channel function and is activated by protons. The prokaryotic origin of these membrane proteins facilitated the X-ray structural resolution of the first members of this family. ELIC was solved at 3.3 Å in a closed-pore conformation, and GLIC at up to 2.9 Å in an apparently open-pore conformation. These data reveal many structural features, notably the architecture of the pore, including its gate and its selectivity filter, and the interactions between the protein and lipids. In addition, comparison of the structures of GLIC and ELIC hints at a mechanism of channel opening, which consists of both a quaternary twist and a tertiary deformation. This mechanism couples opening–closing motions of the channel with a global reorganization of the protein, including the subunit interface that holds the neurotransmitter binding sites in eukaryotic pLGICs.

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Introduction

Pentameric ligand-gated ion channels (pLGICs) are widely expressed in the animal kingdom where they mediate primarily cellular communication. In mammals, they are key players of neurotransmission by acetylcholine (ACh), γ-amminobutyric acid (GABA), glycine and serotonine in the central and peripheral nervous system (Corringer *et al.* 2000; Sine & Engel, 2006; Kalamida *et al.* 2007; Taly *et al.* 2009).

pLGICs are transmembrane proteins, composed of five symmetrically arranged subunits, which carry several neurotransmitter binding sites located within their extracellular domain (ECD), and a single ion channel within their transmembrane domain (TMD). Upon binding, neurotransmitters typically trigger a rapid (less than 1 ms) opening of the channel (also called activation or gating process), while prolonged agonist residence promotes a slower process of desensitization. Since the

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Pierre-Jean Corringer trained as a chemist and did his PhD (Paris) and post-doctoral fellowship (Brighton) in organic synthesis. He then joined the Pasteur Institute as a CNRS researcher to work on the functional architecture and biosynthesis of nicotinic acetylcholine receptors. His work contributed to the discovery of bacterial homologues of these neurotransmitter receptors. In 2008 he created his own research group on Channel Receptors in the Pasteur Institute, which has already produced, in collaboration with Marc Delarue, one of the first atomic resolution structures of the bacterial homologues of the nicotinic receptors.

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agonist binding sites are more than 60 Å away from the ion channel, the agonist-elicited activation and desensitization processes involve global allosteric motions of the protein. These functional properties imply the occurrence of at least three allosteric conformations of the protein: a basal activatable B state (also called resting), an active A state with an open channel, and a desensitized D state with a closed channel but displaying a high affinity for agonists (Changeux & Edelstein, 2005).

Our knowledge of the structural organization of pLGICs from animals comes mainly from nicotinic acetylcholine receptor subtypes (nAChRs). Each subunit contains an N-terminal extracellular domain, followed by four transmembrane segments (named M1 to M4) and a cytoplasmic domain between M3 and M4 of high sequence and length variability (Fig. 1). Electron microscopy analysis of the nAChR from *Torpedo* yielded a 4 Å resolution structure of the protein, notably of the TMD, which contains α-helical transmembrane segments (Miyazawa *et al.* 2003). This structure confirmed the positioning of the M2 segment along the pore, close to the axis of symmetry, as previously established by affinity labelling experiments (Giraudat *et al.* 1986, review in Corringer *et al.* 2000). The discovery of the acetylcholine binding protein (AChBP), a naturally truncated ECD found in invertebrates (Smit *et al.* 2001), led to its X-ray atomic structure (Brejc *et al.* 2001). Each AChBP subunit presents a β-sandwich fold reminiscent of immunoglobulins, and is very close to that of the ECD of the α1 nAChR subunit (Dellisanti *et al.* 2007). The ACh binding sites are located at the interface between the subunits (Celie *et al.* 2004).

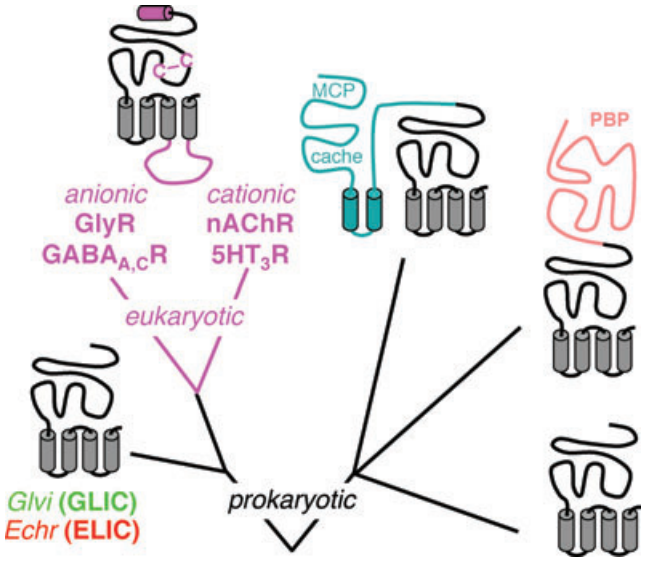


Figure 1. Schematic phylogenetic tree of the pLGIC family
The different subfamilies of genes are shown as cartoons representing their transmembrane topology (transmembrane helices as vertical cylinders). The minimal structure required for ligand-gated ion channel function is shown in grey, and additional modules are shown in colour.

Despite these advances, an X-ray structure of an integral mammalian pLGIC is still lacking. In the last few years, the field has moved to the atomic level thanks to the discovery of pLGIC homologues in prokaryotes.

A large and diverse family of pentameric ligand-gated ion channels in prokaryotes

In 2005, Tasneem *et al.*, using sensitive sequence-profile searches, identified 15 putative homologues of pLGICs in several bacteria and in a single archaeal genus (Fig. 1). The confirmation that their genes code for homologues of eukaryotic pLGICs was established with the genes from *Gloeobacter violaceus* (named *GLIC*) and *Erwinia chrysanthemi* (named *ELIC*). The former codes for a pentameric membrane protein (GLIC) which functions as a ligand (proton)-gated ion channel (Bocquet *et al.* 2007). This channel shows slow activation by protons and displays an ionic selectivity typical of nAChRs with similar permeabilities for sodium and potassium but no permeability for chloride ions. Next, the structure of ELIC was determined by X-ray crystallography at 3.3 Å resolution (Hilf & Dutzler, 2008), clearly showing a 3D organization homologous to nAChRs.

A basic local alignment at present reveals 12 additional genes belonging to the pLGIC family (from *Acidovorax delafieldii*, *Beggiatoa*, *Candidatus pelagibacter*, *Cyanothece*, *Dickeya dadantii*, *Francisella tularensis*, *Lyngbya*, *Marinomonas*, *Plesiocystis pacifica*, *Prochlorococcus marinus* and *Roseobacter*). Thus prokaryotic homologues constitute a large and growing family of genes, whose high divergence in sequence suggests a wide repertoire of pharmacologies and ion channel selectivities. The shorter bacterial members encompass an ECD corresponding to the β-sandwich of the AChBP without its N-terminal α-helix, followed by the four transmembrane segments connected by short linkers, and no cytoplasmic domain. GLIC fits this description and probably represents the minimal structure required for a ligand-gated ion channel to be functional. This minimal structure has been enriched during evolution: some bacterial homologues correspond to the minimal structure fused at its N-terminus to periplasmic binding protein modules, or to two predicted transmembrane segments, a 'cache' and a methyl-accepting chemotaxis protein-N (MCP) domain. The impact of these extracellular modules on the ligand-gated ion channel function remains to be explored. An interesting idea would be that they modulate the intrinsic and pharmacological activity of the channel in a manner similar to what is observed for the N-terminal domain of NMDA receptors (Gielen *et al.* 2009). Eukaryotic subunits consist of the minimal structure fused at the N-terminus with an α-helix, fused between M3 and M4 with a large and variable cytoplasmic loop. They carry a conserved disulfide bridge within their

N-terminus which gives them their cys-loop receptor name. Still, the cytoplasmic domain is not required for signal transduction, since its deletion within the $GABA_A$ and $5HT_3$ receptors generates functional ligand-gated ion channels (Jansen *et al.* 2008).

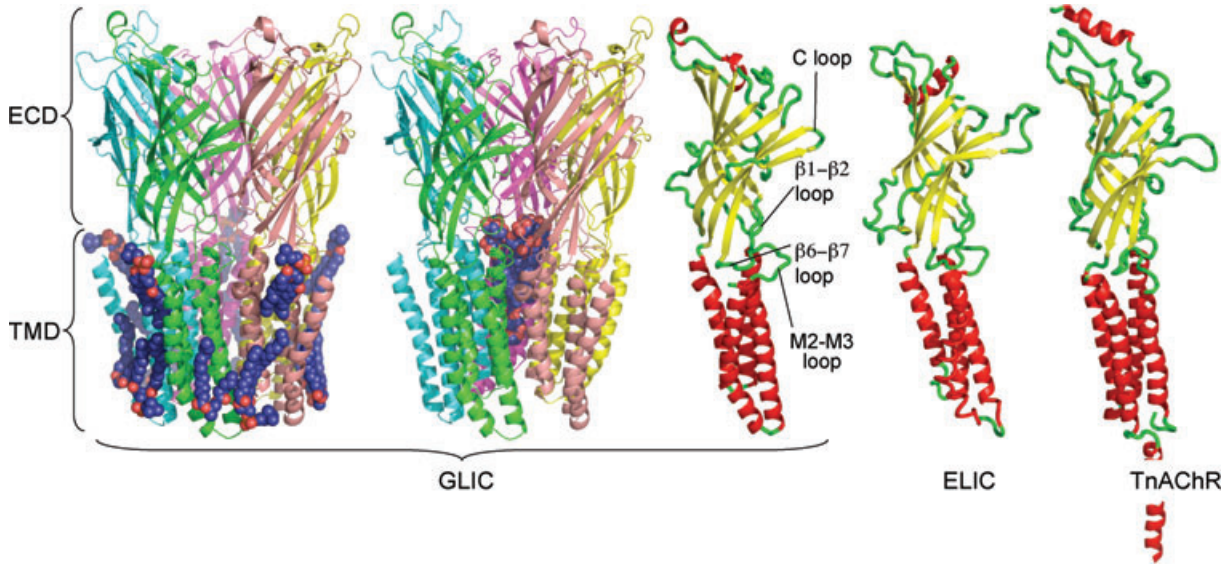
Finally, the physiological significance of this large family of prokaryotic channel receptors remains unknown, and their presence in the inner membrane of gram-negative bacteria suggests their contribution to adaptation to the external environment, possibly through chemotaxis and adaptation to external pH in the case of GLIC.

X-ray structure of bacterial channel receptors

The prokaryotic origin of GLIC allowed for the use of the highly efficient *E. coli* expression system. The expression of GLIC was found toxic for the bacteria (for still unknown reasons), but an N-terminal fusion with the maltose binding protein (MBP, including its signal peptide), designed on the basis of bacterial members naturally fused to periplasmic proteins, enabled overexpression in amounts compatible with X-ray crystallography (Bocquet *et al.* 2007). N-terminal fusion with MBP is indeed known to increase the expression of membrane proteins (Korepanova *et al.* 2007), probably through a better recognition of the secretory pathway and/or a reduction of aggregation during biosynthesis.

The first member to be crystallized was ELIC at 3.3 Å resolution (Hilf & Dutzler, 2008), followed by GLIC at 2.9 Å (Bocquet *et al.* 2009) and 3.1 Å (Hilf & Dutzler, 2009) resolution. The overall architecture is clearly homologous to nAChRs, with an ECD folded as a β-sandwich that superimposes well with AChBP and an all α-helical TMD (Fig. 2). M2 lines the ion channel, which is composed of rings of equivalent residues from each subunit: from the top to the bottom, one ring of polar/charged residues (at position 20′), three rings of hydrophobic residues (9′, 13′ and 16′), two rings of polar residues (2′ and 6′) and one ring of negatively charged residues (called intermediate, −1′ or −2′ for GLIC) that were initially identified by affinity labelling using channel blockers (Giraudat *et al.* 1986, reviewed in Corringer *et al.* 2000) (Fig. 3). The M1 and M3 segments surround M2, and M4 is peripheral. Regarding the structural model of *Torpedo* nAChR derived from EM data and from the AChBP crystal structure (Unwin, 2005), two local but important differences can be noted. First, the loop linking the M2 and M3 segments at the interface between the ECD and the TMD protrudes within the subunits in the nAChR model, while it is clearly at the subunit interface in the bacterial receptor structures. Second, the axis of the M2 helices can be nicely aligned between the nAChR and GLIC structures, but the structural alignment shows a one helix turn shift, with the intermediate ring of charged residues being at the base of the M2 helix in the *Torpedo* nAChR model, and one turn of helix higher in bacterial receptors. This difference has important implications for the ion permeation process (see below).

The structure of GLIC at 2.9 Å resolution shows electron density outside the protein that allows us to partially build



Figures 2. X-ray structure of GLIC and comparison with ELIC and nAChR
Left, cartoon representation of the X-ray structure of the GLIC protein at 2.9 Å resolution. Each subunit is coloured differently, and lipids partially seen in the electron density map are represented as spheres. The panel on the right shows the same structure with the bundle of detergent (dodecyl-β-D-maltopyranoside) bound within the ion channel. Right: cartoon representation of a single subunit of GLIC, ELIC and nAChR (TnAChR, from EM data of the *Torpedo* receptor). Subunits are coloured according to the secondary structure, illustrating the common core of β-sandwich (yellow) and α-helices (red) that is conserved throughout the family, while connecting loops (green) are on the whole highly divergent in structure.

three lipid molecules per subunit, and six molecules of detergent that are bound within the ion channel and shield the three rings of hydrophobic residues from the solvent (Fig. 2). In all cases, the alkyl chains are well resolved but the polar heads are not seen or weakly seen, indicating structural flexibility. It is noteworthy that no lipids were added during the purification of the protein, indicating that they remain bound within crevices formed between the M1–M4 and M3–M4 segments after the extraction of the protein from the membrane. The lipids thus seem to hold M4 in its position and may be required for the proton-gated ion channel function, a feature reminiscent of the nAChR, a function which requires the presence of cholesterol and anionic lipids (Nievas *et al.* 2008; Dacosta & Baenziger, 2009). In contrast, the bound detergent within the channel could be removed at least partially without altering the structure.

Despite the relatively low sequence identity between GLIC and ELIC (18%), which probably reflects the phylogenetic distance between cyanobacteria and proteobacteria, the striking conservation of secondary/tertiary motifs permits us to delineate a 'core' structure conserved in the superfamily. It includes the interior of the β-sandwich also found in AChBP, and the four transmembrane α-helices also observed by electron microscopy. This core structure is decorated by loops that are highly variable in structure and/or in length, with the exception of two loops at the ECD–TMD interface: (1) the $\beta6-\beta7$ loop, a canonical DxRxxPFDxQ motif, flanked in eukaryotes by two bridged cysteines, and (2) the $\beta1-\beta2$ loop, variable in sequence but highly conserved in length.

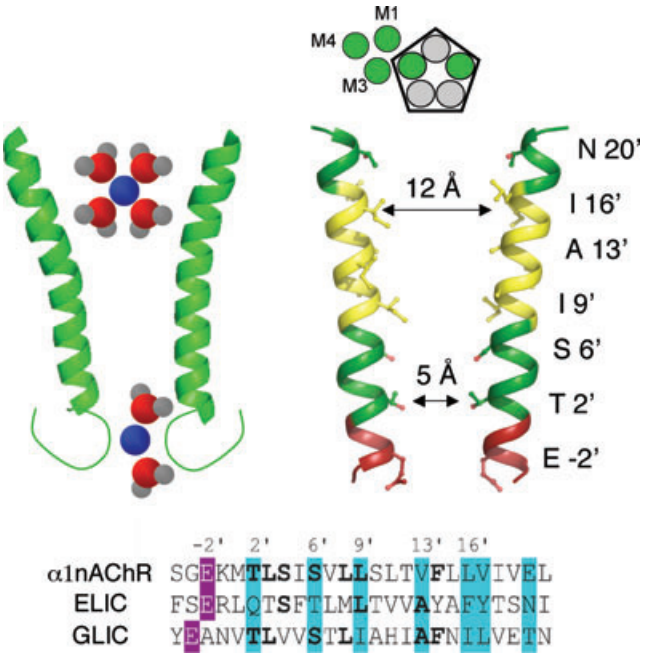


Figure 3. The ion channel of GLIC
Right: cartoon representation of the ion channel of GLIC. Only two M2 segments are represented for clarity, with rings of charged residues in red, of polar residues in green and of hydrophobic residues in yellow. Left: hypothesized ion selection mechanism proposed on the basis of mutational analysis; adapted from Corringer *et al.* 1999.

X-ray structures provide insights into the pLGIC allosteric transition

The above presentation provides a static picture of the pLGIC fold, but a key property of these molecules is their ability to isomerize between the B, A and D allosteric states. The available structural information has therefore to be evaluated in this context.

The numerous structures of AChBP–ligand complexes overall showed that agonists tend to stabilize a compact conformation of the binding pocket, but antagonists do not, yet exceptions to this rule have recently been reported (Hansen *et al.* 2005; Hibbs *et al.* 2009). However, ligand binding is always associated with no change in the conformation of the core β-sandwich suggesting that AChBPs adopt a 'frozen' structure, which is not allosterically modified by ligands, even though its fusion to the TMD of the $5HT_3$ receptor was reported to form a functional ACh-gated ion channel (Bouzat *et al.* 2004).

A gating model has been proposed from the EM data of the *Torpedo* nAChR (Unwin, 2005). The X-ray structure of AChBP was used to construct a detailed model of the ECD/TMD portion of *Torpedo* nAChR, where the conformation of the two $\alpha1$ subunits is 'distorted' compared to that of both non-α subunits and AChBP–agonist complexes. It suggests that in the course of agonist binding the α subunits transit from a distorted (inner β-sheet rotated 10 deg around an axis perpendicular to the membrane, open loop C) to an AChBP-like conformation. This is proposed to release the M2 segment from specific interactions with the $\beta1-\beta2$ loop, allowing channel opening.

Recently, the available X-ray structures of bacterial receptors have allowed us to propose a global model of the activation transition at atomic resolution (Bocquet *et al.* 2009). Integral channels investigated in the absence of agonists, the *Torpedo* nAChR and ELIC, are thought to correspond to a basal state. In contrast, the GLIC structure has been solved in acidic conditions compatible with stabilization of an active state, since GLIC shows persistent current upon prolonged activation by protons (Bocquet *et al.* 2007). Comparison of the core structure of GLIC and ELIC shows a wide opening of the pore of GLIC, associated with a reorganization of the whole structure (Fig. 4). The structural superimposition (Fig. 5) suggests that upon activation: (1) pentamers undergo a quaternary twist, resulting from a tilt of each subunit that causes anticlockwise (*vs.* clockwise) motion in the upper (*vs.* lower) part of the pentamer, in a manner similar to what has been predicted from normal mode analysis on

nAChRs (Taly *et al.* 2005), and (2) each subunit undergoes a tertiary deformation, the β-sandwich rotating in a rigid body manner around an axis roughly parallel to the inner β-sheet. This is concomitant with a downward motion of the $\beta1-\beta2$ loop, and with a concerted tilt of the M2 and M3 segments away from the central axis. This twist/deformation-to-open scheme couples the opening/closing motion of the gate of the channel with a global reorganization of the protein, in particular with the subunit interface, which holds the neurotransmitter binding pocket in eukaryotic pLGICs.

However, the twist/deformation-to-open mechanism raises two paradoxes related to structural data on eukaryotic pLGICs, especially in regard to the gating model suggested by the EM data. First, the β-sandwich of ELIC precisely superimposes with that of AChBPs, indicating that AChBP would adopt a basal conformation, while its structure is attributed to an active conformation in the EM model. Second, the conformation of the TMD of the *Torpedo* nAChR EM structure is much closer to that of GLIC than to ELIC, suggesting that it would correspond to an active conformation, despite the absence of ACh. Further work will be required to address these issues, which might be due to species differences or to the resolution limit of electron microscopy.

X-ray structures provide insights into the functional organization of ion channels

The X-ray structures of bacterial pLGICs offer for the first time the atomic structure of the TMD in both open and closed conformations, providing new insights into the mechanisms of both gating and ion permeation/selection.

The gate of the pore. Substituted cysteine and histidine accessibility monitored with respectively impermeant reagents (nAChR) and zinc ($5HT_3R$) point to a cytoplasmic location of the gate of the pore in the basal state (Wilson & Karlin, 1998; Paas *et al.* 2005), while similar studies performed on $GABA_A$ and $5HT_3$ receptors point to a location in the midsection of the channel (Panicker *et al.* 2002; Bali & Akabas, 2007). These discrepancies probably arise from intrinsic problems of the mutational approach that may alter the allosteric properties and/or the local conformation of the channel. However, the available agonist-free structures of the *Torpedo* nAChR, and even more of ELIC, reveal a constriction at respectively L9′ (3 Å radius) and L9′–A13′–F16′ (1–2 Å radius) (Fig. 4). These data suggest that the gate is constituted of one or several narrow rings of hydrophobic residues, termed the 'hydrophobic girdle', as supported by extensive computer modelling that indicates that such hydrophobic 'nanopores' are weakly hydrated and prevent ion permeation (Beckstein & Sansom, 2006; Cheng *et al.* 2006; Corry, 2006; Ivanov *et al.* 2007). In line with these experiments, mutation of these hydrophobic rings into hydrophilic ones produced a strong 'gain of function' phenotype (Revah *et al.* 1991), characterized by a large destabilization of the closed state as compared to the active state, further supporting the occurrence of such stabilizing hydrophobic clusters in the closed-pore states.

The hydrophilic pathway and the selectivity filter of the pore. To reach the ion channel, extracellular ions must cross the wide vestibule at the centre of the ECD, and intracellular ions must cross the intracellular domain, possibly at the level of lateral openings seen in electron microscopy (Unwin, 2005). Both compartments contribute to the conductance of the channel, probably by concentrating the permeant ions at both sides of the pore (Kelley *et al.* 2003; Hansen *et al.* 2008).

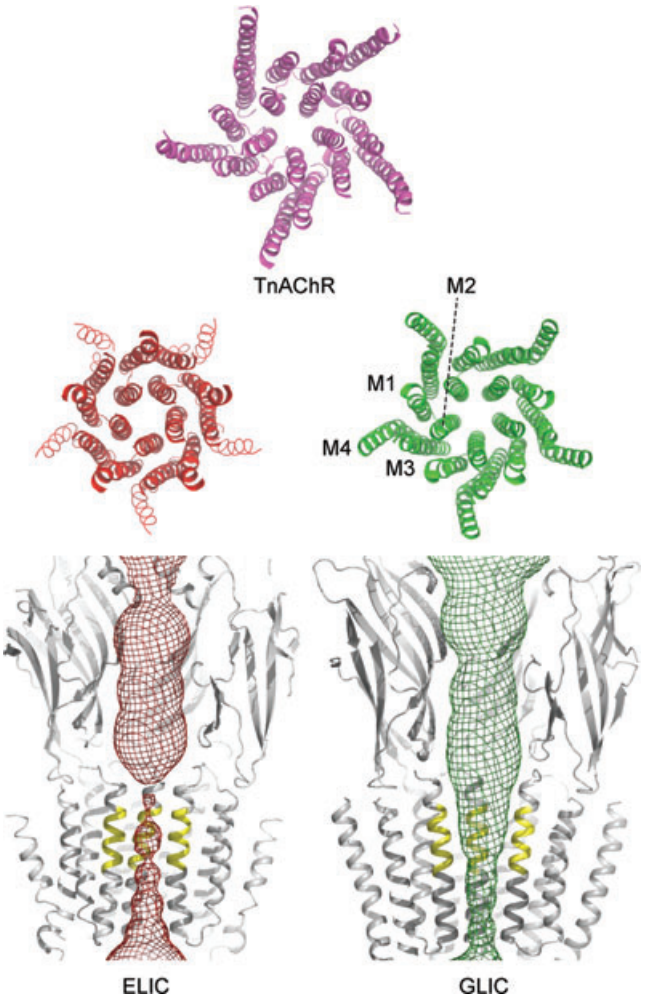


Figure 4. The permeation pathway of GLIC and ELIC
Mesh representation of the aqueous pathway in the ELIC and GLIC structures, illustrating that ELIC is in a closed conformation, and GLIC is apparently open. The top view shows the transmembrane helices alone, illustrating that the conformation of the pore and of the M2 helices are similar between GLIC and TnAChR.

It is well established that the transition from the closed to the open channel conformation does not involve strong rotation of the M2 helix, since its same side has been identified as contributing to the open and closed pore using SCAM (substituted cysteine accessibility method) (Wilson & Karlin, 2001) or titrable amino acids as probes (Cymes *et al.* 2005). These studies located the constriction of the open conformation at the intracellular end, between residues −2′ and 2′, since this region is not accessible to reagents applied both intracellularly and extracellularly, and since single channel conductance of mutants is critically decreased when positively charged residues are incorporated at this level.

Still, the key property of the ion channels in this superfamily is that they select ions mainly according to their charge, but weakly discriminate between monovalent ions of the same charge. Chimeric analysis between the cationic α7 nAChR and the anionic α1 glycine receptor (Galzi *et al.* 1992; Corringer *et al.* 1999; Gunthorpe & Lummis, 2001) established that the region −2′ to 2′ is critical for selectivity and permeation: first, anionic channels contain an additional proline residue at position −1′, whose insertion in an α7 mutant switches the selectivity from cationic to anionic, and whose deletion in anionic channels increases the cationic conductance. Second, cationic channels carry a ring of negatively charged residues at position −1′, whose mutation to neutral amino acids critically decreases the cationic conductance and is required to yield an anionic channel. Introduction of a homologous charged ring in anionic receptors was found to increase their cationic conductance (Keramidas *et al.* 2004; Jensen *et al.* 2005). The region −2′ to 2′ controls charge discrimination and contributes to the narrowest portion of the channel, and thus constitutes the charge selectivity filter of the ion channel.

Interestingly, the minimum pore diameter estimated from permeation analysis of ions of increasing size indicates that anionic channels are narrower than cationic channels, of 0.7–0.8 nm diameter for $5HT_3$ and nAChRs, and 0.5–0.6 nm for $GABA_A$ and glycine receptors (reviewed in Keramidas *et al.* 2004). The minimum pore diameter of these channels requires at least a partial dehydration of the ions to pass through. Anions such as chloride are more easily dehydrated than cations, which interact more tightly with water molecules. Therefore, the easily dehydrated chloride ions may interact more closely with the selectivity filter, where they lose laterally bound water molecules, whereas the wider cationic selectivity filters might permit cation permeation in a more hydrated state (Keramidas *et al.* 2004).

Overall, these data are consistent with the structure of the GLIC channel that was solved in the presence of its agonist. Its channel is funnel shaped, widely open at the outer side (6 Å radius) and progressively narrowing to the channel constriction made up by the 2′ ring (Fig. 3). In the structure, T2′ is flanked at both sides by the polar S6′ and E-2′ rings. This organization is consistent with a dehydration to select mechanism, since the narrow radius

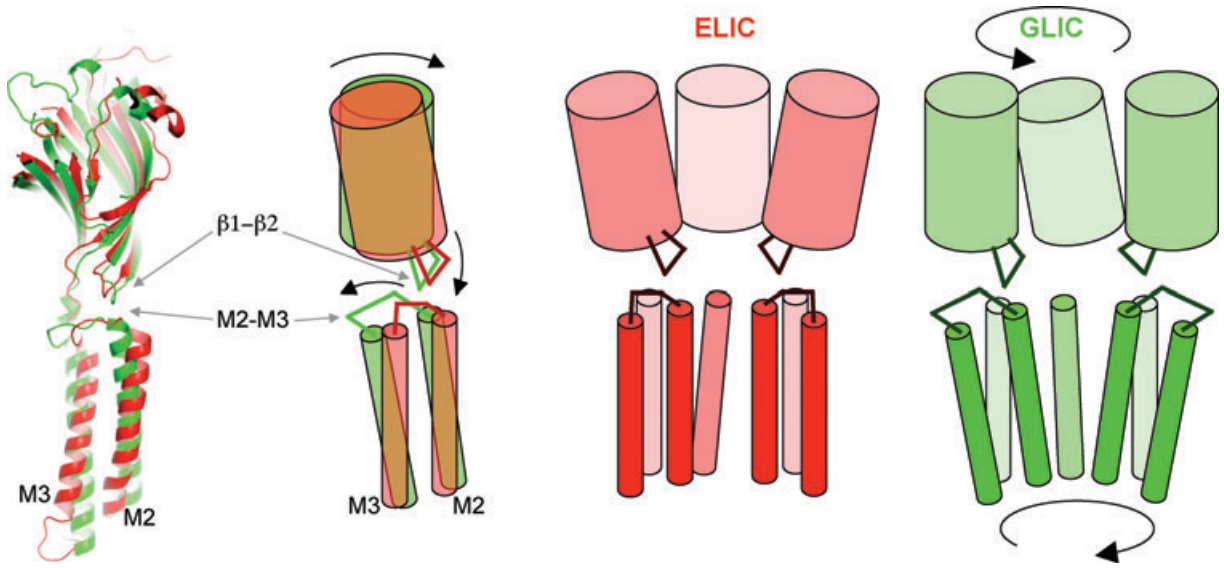


Figure 5. A twist/deformation mechanism for channel opening
Left, superimposition of the GLIC (green) and ELIC (red) structures. Only one subunit is shown, and the tertiary deformation is schematically represented on the right. From ELIC to GLIC, the extracellular β-sandwich undergoes an 8 deg rotation, which, apparently through the interaction between the β1–β2 and M2–M3 loop, is concomitant with a rigid-bogy tilt of the M2 and M3 helices, thereby opening the pore. Right, scheme showing the transition of three subunits together, illustrating the twist motion, with opposite rotations in the upper and lower parts of the protein.

at 2′ (less than 3 Å) is not compatible with permeation of fully hydrated cations. The structure shows that the polar hydroxyl groups can transiently interact with cations, and thus substitute for water molecules, thereby decreasing the energetic barrier required for ion translocation. This idea is consistent with X-ray data, which locate electron density of bound ions (Rb^+, Cs^+ Zn^{2+}) at this constriction (Hilf & Dutzler, 2009), further supporting its potential role for conductance and ion selectivity.

Conclusion

This short review shows the advances in our understanding of the signal transduction mechanisms mediated by pLGICs, enabled by the recent resolution of the X-ray structure of bacterial homologues in two different conformations. The clear homology between prokaryotic and eukaryotic structures establishes that bacterial homologues are excellent prototypes for investigating the fundamental mechanism of channel gating, and its regulation by orthosteric (neurotransmitters) and allosteric ligands.

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