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Allosteric regulation of pentameric ligand-gated ion channels

An emerging mechanistic perspective

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Pentameric ligand-gated ion channels (pLGICs) play a central role in intercellular communications in the nervous system by converting the binding of a chemical messenger—a neurotransmitter—into an ion flux through the postsynaptic membrane. They are oligomeric assemblies that provide prototypical examples of allosterically regulated integral membrane proteins. Here, we present an overview of the most recent advances on the signal transduction mechanism based on the X-ray structures of both prokaryotic and invertebrate eukaryotic pLGICs and atomistic Molecular Dynamics simulations. The present results suggest that ion gating involves a large structural reorganization of the molecule mediated by two distinct quaternary transitions, a global twisting and the blooming of the extracellular domain, which can be modulated by ligand binding at the topographically distinct orthosteric and allosteric sites. The emerging model of gating is consistent with a wealth of functional studies and will boost the development of novel pharmacological strategies.

Introduction

Ligand-gated ion channels (LGICs) mediate intercellular communication by converting a chemical signal, the neurotransmitter released from the nerve ending, into a transmembrane ion flux in the postsynaptic cell: neuron, muscle fiber, or gland cell. They are oligomeric membrane proteins allosterically regulated by the binding of a neurotransmitter—the agonist—to an orthosteric site that is topographically distinct from the transmembrane ion channel.[1,2] At rest, the ion channel is closed, and binding of the agonist to the extracellular domain triggers a rapid conformational change that results in the opening of the transmembrane pore, a process referred to as «gating»[3]. This process, which takes place in the microsecond-millisecond time scale, represents one of the most rapid conformational changes ever observed in oligomeric proteins. Channel opening allows cations (or anions) to diffuse through the membrane at rates approaching tens of millions of ions per second. In addition to the well established role in neurotransmission, some LGICs were found expressed in non-excitable cells, such as lung cells[4] or fat cells[5] suggestive of a wider function for these receptors.[6] LGICs thus present attractive targets for which more than 150 years of research have been dedicated since the pioneering work of Claude Bernard on curare's action.[7]

There are three major, genetically unrelated vertebrate superfamilies of LGICs, each folded in unique protein architectures. Besides the pentameric LGICs (pLGICs) are the tetrameric ionotropic glutamate receptors (iGluR), which carry cation (Na^+, K^+, Ca^{2+})-selective channels activated by glutamate, and the trimeric P2X receptors (P2XR), whose cationic channels are gated by ATP. The pentameric superfamily comprises, in vertebrates, the excitatory, cation-selective, nicotinic acetylcholine receptor (nAChR),[8] 5-hydroxytryptamine receptor ($5\text{-}HT_3$ R) and the zinc-activated channels (ZAC);[9] the inhibitory, anion-selective, $GABA_A$ Receptor[10] and the strychnine-sensitive glycine receptor;[11] and, in invertebrates, the glutamate-gated chloride channel (GluCl)[12] (see also refs. 13 and 14). These pLGICs are formed by the assembly of five identical or homologous subunits and were in the past referred to as «Cys-loop receptors», due to the presence in the extracellular domain of a loop of approximately 13 residues flanked by two canonical cysteines linked through an intrasubunit disulfide bridge. All subunits of the superfamily are homologous, and thus have evolved from a common ancestral gene.[15,16] As a consequence, the biochemical and subsequent site-directed mutagenesis experiments gathered on the nAChR made this receptor a privileged model of the superfamily for more than two decades. During this time, it was established that: (1) the N-terminal domain of ~200 amino acids is extracellular and contains the orthosteric-binding site, which lies at the interface of two adjacent subunits (ref. 17); (2) there are several allosteric-binding sites including the benzodiazepine and the general anesthetic-binding sites for $GABA_A$ receptors[18]; (3) there are four transmembrane segments that follow the N-terminal domain, and consequently the C-terminus is located extracellularly; (4) the second segment, M2, lines the ion pore in such a way that the channel is formed from the association of five M2 segments[19-24];

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and (5) the second intracellular loop (also called M3-M4) is of variable size and amino acid sequence.[2]

At the turn of the century, both prokaryotic and eukaryotic members were identified in the family of K^+ and Na^+ voltage-dependent channels[25] pointing to the occurrence of ion channels far before the development of the nervous systems in eukaryotes. This observation motivated the quest for prokaryotic homologs of pentameric LGICs (pLGICs). Sequence searches using the signature loop of the α7 nAChR as a starting point identified gene sequences coding for putative LGICs (up to 15 in bacteria and one in archae)[26] and two of them were subsequently shown to behave as ligand-gated ion channels.[27,28] Yet, the structure of the prokaryotic pLGICs is simpler than their eukaryotic counterpart: they have an extracellular domain folded as a β-sandwich, like AChBP (and the eukaryotic pLGICs) but they lack the N-terminal helix and the two cysteines that border the signature loop, followed by four transmembrane helices connected by short loops without cytoplasmic domain.

Because the sequence identity between eukaryotic and prokaryotic pLGICs is low (~20%) their belonging to the family was tested experimentally. The gene from *Gloeobacter violaceus* (GLIC) was cloned and the protein expressed showing a pentameric assembly.[27] It was found to be a cationic ion channel activated by low pH.[27] The results obtained with the prokaryotic homologs, in particular their structural determination at high resolution, which will be discussed in the next section, are of considerable importance for a molecular understanding of the allosteric transitions of these channels and LGICs more generally.[1,29]

Since the 60s the signal transduction mechanism carried by the nAChR, which globally links the topographically distinct sites, has been proposed to be a global isomerization of the protein linking the extracellular and the transmembrane domains, which was referred to as an "allosteric transition".[30-33] Several models have been proposed for the process of activation and deactivation. Among them, the Monod-Wyman-Changeux[34] (MWC) model postulates that allosteric LGICs spontaneously undergo reversible transitions between a few—at least two—**discrete** and **global** conformational states even in the **absence of agonist**[2] and that a conformational selection—or shift of conformers population—takes place in the presence of agonist.[2,35] This model accounts for the signal transduction mechanism mediated by the nAChR between the "active" open-channel form, which preferentially binds agonists, and the "resting" closed-channel form, which preferentially binds the competitive antagonists, and for the cooperativity of agonist binding, which arises from the assembly of the repeated subunits into a symmetric oligomer. Most importantly, it predicts that agonists and antagonists binding would select and stabilize **structurally different** conformations. Also, it accounts for the spontaneous opening of the channel in the absence of ACh[36] as well as the unexpected "gain of function" associated with some of its pathological mutations (see ref. 37). However, to account for desensitization, additional slowly accessible, high affinity, closed-channel states (intermediate and slow) need to be introduced for both eukaryotic[3,38-41] and prokaryotic receptors.[42]

Overall, pLGICs (along with hemoglobin and other regulatory proteins[43]) present a prototypical example of allosterically regulated proteins where the conformational equilibrium between a resting, an active and one or more desensitized states is modulated by the binding of ligands at topographically distinct sites. The increasing availability of high-resolution structures of pLGICs both from prokaryotic and eukaryotic organisms thus delineates an ideal framework to elucidate the allosteric transitions at atomic resolution.

In this review, we give an overview of the recent advances on the structure of pLGICs and their conformational transitions using presently available structures and recent simulation analyses as starting point. The link between the structural isomerization(s) and ligand binding is also presented.

Structural Background

Structural data are of primordial importance for the molecular dynamics studies discussed below. The present knowledge of pLGIC structures and relevant limitations has been recently reviewed.[1] Its highlights are summarized as follows.

Structures of pLGICs

Early electron microscopy data of the nAChR from the *Torpedo* electric organ revealed a cylinder of approximately 8 nm in diameter and 16 nm in length which, when viewed from the synaptic cleft, looked like a rosette of five subunits arranged around a symmetrical 5-fold axis perpendicular to the membrane plane.[44,45] Further structural analysis of purified and/or receptor–rich membranes from fish electric organ[46-49] revealed a heteropentameric organization and a non-symmetrical distribution of the α toxin sites. The discovery that nAChR-rich membranes of the electric organ of *Torpedo* form tubular 2D crystals[50,51] enabled for a significant increase in the resolution of the cryo-EM data up to 4 Å (ref. 52), yet under preparation conditions that are known to abolish or uncouple receptor function.[53,54] By taking advantage on the high-resolution structure of the homopentameric, water soluble, Acetylcholine Binding Protein (AChBP) from *Lymnaea stagnalis*,[55,56] which presents significant sequence homology with the extracellular (EC) domain of the nAChR (roughly 30%) and remarkable conservation of the binding site residues (reviewed in ref. 57), Unwin and coworkers developed atomic models, first of the transmembrane (TM) domain alone,[58] and then of the full-length nAChR.[52,59, See note a.]

The situation changed dramatically with the discovery in bacteria[26] of DNA sequences homologous of the eukaryotic nAChR. The cloning and expression[27] of two prokaryotic pLGICs combined with improved techniques for growing regular 3D crystals of integral membrane proteins led to the resolution of the first X-ray structure of a pLGICs from *Erwinia chrysanthemi* (ELIC) in a closed state (at 3.3 Å resolution)[60,61] and from *Gloeobacter violaceus* (GLIC) in an open channel conformation (at 2.9 Å resolution).[62,63] Last, the first structure of an eukaryotic member of the family, the anionic glutamate receptor from *Caenorhabditis elegans* (GluCl), was recently solved in complex with the positive allosteric modulator ivermectin at atomic resolution[12] revealing a remarkable similarity with the 3D structure of GLIC.

All the available sequence data of prokaryotic and eukaryotic pLGICs show the same organization of the constitutive subunits into an EC domain and a TM domain (**Figure 1**). The EC subunits are folded into a highly conserved immunoglobulin-like β sandwich stabilized by inner hydrophobic residues with connecting loops and the N-terminal α helix that are variable in length and structure. Consistent with the early EM structures of *Torpedo* nAChR,[52] the four transmembrane segments fold into α helices and are organized as a well-conserved bundle. The second segment, M2, lines the channel walls[19,20,22-24] and is surrounded by a ring of α helices made of M1 and M3. The fourth transmembrane helix, M4, lies on the side and interacts extensively with the lipid bilayer, as shown by the crystal structures of GLIC.[62,64]

The Orthosteric Binding Site

The neurotransmitter or "orthosteric" binding site lies in the EC domain at the interface between subunits in the whole pLGIC family (**Figure 1**). Three regions from the "principal" or (+) subunit, named loops A, B, and C, and four from the "complementary" or (–) subunit, named loops D, E, F, and G, contribute to the binding pocket.[17] Corresponding X-ray structures have been reported in AChBP, GLIC, ELIC, and GluCl receptors. In AChBP, loops A (Tyr), B (Trp), C (two Tyr), and D (Trp) form an aromatic "box" chelating the quaternary ammonium group of ACh, among which the tryptophane from loop B forms a direct cation π interaction with it.[65] In the eukaryotic GluCl, the endogenous agonist L-glutamate binds through the ammonium moiety to aromatic residues from loops A (Phe), B (Tyr), and C (Tyr), whereas the lateral carboxylate moieties interacts mainly with Arg and Lys residues from loops D and F of the complementary subunit.[12] Cocrystallization of ELIC in complex with the mild agonist bromopropylamine at 4 Å resolution[66] or the competitive antagonist acetylcholine at 2.9 Å resolution[61] showed that both ligands bind to the orthosteric site. Interestingly, the structure of ELIC with ACh shows that ligand binding to an aromatic cage at the subunit interface causes a significant contraction of loop C along with a slight increase in the pore diameter, which is thought insufficient to open the pore. Cinnamic acid derivatives antagonize the GLIC proton-elicited response and structure-activity analysis has a revealed key contribution of the carboxylate moiety to GLIC inhibition. Molecular docking coupled to site-directed mutagenesis has suggested that the binding pocket is located at the EC subunits interfaces yet slightly below the classical orthosteric site.[67] Overall, the structure of the orthosteric neurotransmitter site appears to be remarkably conserved from bacteria to brain.

The Ion Permeation Pathway

An abundant series of X-ray structures data[60,62,63] (reviewed in ref. 1) demonstrates a remarkable conservation of permeation and selectivity structure/function relationships in the transmembrane domain from prokaryotic to eukaryotic pLGICs.[14,68] Crystallographic data with GLIC at 2.4 Å resolution reveal, within the ion channel, ordered water molecules at the level of two rings of hydroxylated residues (named Ser6' and Thr2') that contribute to the ion selectivity filter.[69]

The Allosteric Binding Site(s)

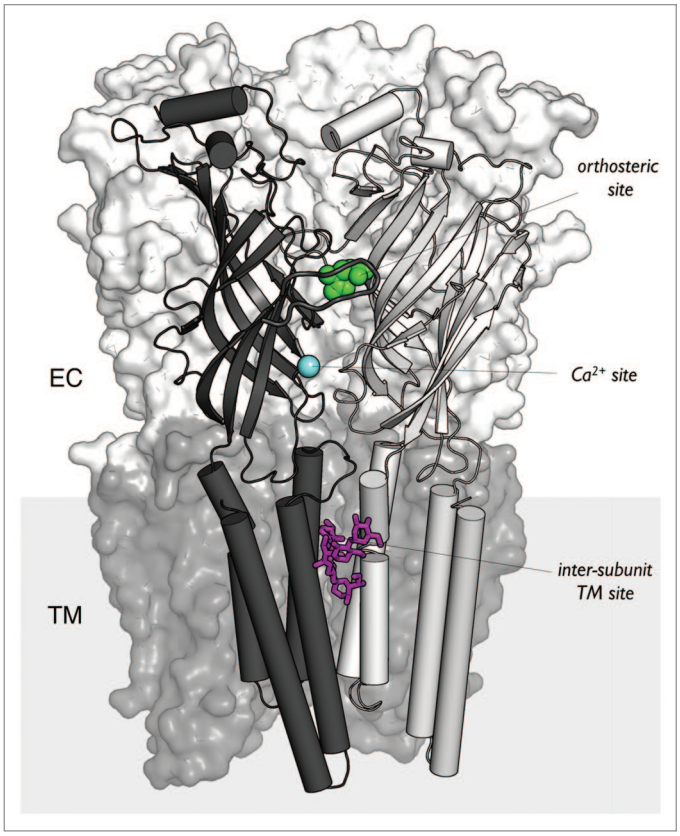

Figure 1. Structure of pLGICs. The side view of the ion channel along the membrane is shown as visualized by the crystal structure of GluCl.[12] The two front subunits of the homopentamer, which correspond to the principal (dark gray) and the complementary (white) subunits, are shown in cartoon representations. The remaining three subunits are shown as solvent-accessible surfaces, which are color-coded according to the EC (white) and TM (light gray) domains. Ligand binding at the subunits interfaces is highlighted in colors. The endogenous agonist L-glutamate, which binds to the orthosteric site, is shown as green spheres. The positive allosteric modulator ivermectin, which binds to the allosteric intersubunit site in the TM domain, is shown as magenta sticks. A cyan sphere shows the location of the allosteric Ca^{2+} binding site for the modulation of pLGICs by divalent cations. The coordinates of the Ca^{2+} ion were taken from the structure of ELIC in complex with the allosteric modulator Ba^{2+} (ref. 105) after optimal superimposition of the TM domain.

Several **allosteric sites** topographically distinct from the orthosteric neurotransmitter-binding site and ion channel binding sites have been identified in pLGICs, and are exploited to regulate the ion channel activity through the binding of a variety of small molecules.

Ca^{2+} ions were the first positive allosteric modulator identified with α7 and α4β2 neuronal nAChRs.[70,71] Site-directed mutagenesis of the Ca^{2+} binding sites in α7-nAChRs identified residues in close proximity to one another but on the opposite sides of the subunit interface in the EC domain, below the orthosteric site near the TM domain.[72,73] Homologs of the Ca^{2+} sites have been more recently recognized in the structure of ELIC where divalent cations including Ba^{2+} behave as negative modulators[66] and in GLIC where it forms a well-delimited pocket for still unidentified ligands[74]; see **Figure 1**.

Another important site for the allosteric modulation of pLGICs was identified in the transmembrane domain. The anti-helmintic ivermectin was found to strongly enhance the ACh-evoked response of α7-nAChR at micromolar concentration (with increased apparent affinity, cooperativity and maximal response) and the effect to be altered by mutations in the transmembrane domain.[75] The recent structural determination of GluCl in complex with ivermectin, which potently activates the ion-channel response, has shown that the binding site is located on the periphery of the transmembrane domain **between** the channel subunits wedged by the helix M3 of the (+) subunit and the helix M1 of the (–) subunit; see **Figure 1**. Also, the ethanol binding sites identified in the crystal structure of an ethanol-sensitized GLIC variant are closely related to the binding site of ivermectin in GluCl.[76] Finally, this transmembrane cavity was shown by homology modeling to be conserved in human ethanol-sensitive glycine and $GABA_A$ receptors and to involve residues previously recognized as influencing alcohol and anesthetic action on these proteins.[77]

General anesthetics such as propofol and desflurane, which behave as negative modulators of GLIC,[78] were shown to possess a common binding site located **within** the upper part of the transmembrane subunits in a cavity delimited by the helices M1, M2, and M3.[64] The structure of GLIC shows that this intrasubunit binding site is accessible from the lipid bilayer. Interestingly, because its entrance is obstructed by a lipid alkyl chain in the structure of GLIC at pH = 4, which would clash with propofol binding, it was argued that lipids could be endogenous ligands for this transmembrane allosteric site.[64]

Homologous inter- and intra-subunit binding sites in the transmembrane domain are present on glycine, $GABA_A$ or ACh receptors, and are of considerable pharmacological importance as they bind to a large variety of anticonvulsants, anesthetics, and diuretics (reviewed in refs. 79–81*).*

Last, in heteropentameric pLGICs such as the neuronal α4β2-nAChR, not all five homologous sites bind ACh. The **non-agonist-binding interface** may accommodate modulatory ligands different from the neurotransmitter. Using AChBP as a structural model, ligands as galanthamine, strychnine, cocaine, and morphine were found to be allosteric effectors at micromolar concentrations.[82-84] Based on data collected on the nAChR, the binding of allosteric modulators at interfaces that do not normally bind the neurotransmitter in the EC domain was initially suggested to be homologous to the benzodiazepines binding site in $GABA_A$ receptors.[85] Although the direct structural evidence is still missing, considerable biochemical, pharmacological and modeling evidence has since then demonstrated that benzodiazepines allosterically potentiate $GABA_A$ receptors by binding to intersubunit sites in the extracellular domain that are homologous to the GABA sites but do not bind GABA.[86,87]

Other allosteric modulatory sites are present in the cytoplasmic domain and may play important roles in the clustering, stabilization, and modulation of receptor functions (reviewed in ref. 18).

Functional Interpretation of Structures

Two methods have been used in the past decades to elucidate the **three-dimensional structure** of pLGICs: electron microscopy (EM) and X-ray crystallography. At a glance the data obtained by these techniques look consistent. However, the intrinsically low resolution of the EM data as well as crystallographic artifacts possibly arising from the use of detergents, non-natural ligands, and mutations imposed by the crystallization conditions, make the functional interpretation of the structural results challenging. Until recently, the only well characterized state of pLGICs was the open state described by the structure of GLIC pH4.[62,63] In particular, the striking similarity with the open-channel form of the eukaryotic GluCl, which was solved in complex with the allosteric agonist ivermectin, strongly supports the interpretation of GLIC pH4 as representative of the active state. Finally, the recent structural determination of GLIC at 2.4 Å resolution[76] helped solving the remaining ambiguities. For instance, it was argued that the conserved Proline at the tip of the "Cys-loop" must adopt a cis configuration, which was found to better account for the crystallographic data not only for GLIC, but also for the structures of ELIC and GluCl.[76]

The structure of ELIC, although well resolved and with a closed channel,[60] is not universally accepted as a model of the resting state.[88] In this respect, the most recent structure of GLIC, which was solved at pH=7,[74] presents a closed conformation of the ion pore that is different from that observed in ELIC and shows a profound rearrangement of the extracellular domain. In fact, whereas in ELIC the conformation of the EC domain is virtually unaffected by co-crystallization with agonists,[89,90] in GLIC pH7 the extracellular subunits tilt radially in the outward direction promoting the **blooming** of the EC domain.[74] Finally, the conformation of the C loop in ELIC, which is supposed to contribute to neurotransmitter binding, is strikingly more similar to the conformation observed in GLIC pH4 than that in GLIC pH7, thus suggesting a possible assignment to a desensitized conformation for ELIC. One possible reason for the resting state to elude its structural determination has been the larger flexibility of the EC domain as compared with the more rigid structure of the active state.[74]

In addition to problems concerning the functional interpretation of structures, prokaryotic pLGICs present functional kinetics that are markedly different from those of their heteropentameric eukaryotic homologs. In fact, under conditions of ultra-fast application of agonist at saturating concentrations, both GLIC and ELIC current activations are two to three orders of magnitude slower than that in the $GABA_A$ receptor. Moreover, the prokaryotic channels show a much slower current desensitization, which occurs on the timescale of seconds.[42] Yet, patch clamp studies show rise times in the microsecond timescale as in the case of eukaryotic receptors.[27] It follows that prokaryotic receptors, which are easier to crystallize, may be used as structural models of pLGICs, yet with peculiarities of their own.

On the other hand, the lack of resolution in the structural determination of heteropentameric pLGICs by cryo EM has led

to at least one serious problem: a residue misassignment in the transmembrane helices M2 and M3 of the first atomic model of the TM domain.[58] The residues are shifted by one helical turn from their correct location, which affects the identity of residues in the functionally critical M2-M3 loop at the EC/TM domains interface; see **Figure 2**. The error was identified when prokaryotic structures were first resolved[62,63] and it was later confirmed by comparison with the eukaryotic GluCl.[12] The ultimate demonstration of the misassignement was recently provided by direct M2-M3 cross-linking experiments.[91] As we shall see, this error has affected the interpretation of functional studies based on site-directed mutagenesis and electrophysiology recordings and has led to the development of incorrect models of gating. More generally, the modest resolution of the EM data unfortunately does not allow for a functional interpretation of the reconstructed models. Indeed, the most recent models of the *Torpedo* nAChR[92], which were obtained both in the presence (assumed open) and the absence (assumed closed) of acetylcholine,[92] are surprisingly similar (Cα-RMSD of 0.6 Å) particularly with respect to the structural variance observed in GLIC pH4 vs. GLIC pH7.[74]

In conclusion, X-ray studies of 3D crystals of both prokaryotic and invertebrate eukaryotic pLGICs, which offer the best structural resolution, in conjunction with atomistic simulations should be used as models for a structural interpretation of gating.

The Molecular Mechanism of Gating

Comparison of the crystal structures of the prokaryotic homologs GLIC pH4 (open) and ELIC or GLIC pH7 (closed) unambiguously shows the occurrence of a large twist on receptor activation.[62] This conformational change, which is usually referred to as a concerted opposite-direction rotation of the EC and the TM domains around the pore axis, was first identified by a coarse-grained normal mode analysis (NMA) of a homology model of the α7 nAChR.[93] As pointed out by Taly et al. (2005) the twisting motion has a large quaternary component and couples the global movement of the ion channel to a significant reshaping of the subunits interfaces, which was thought to open and close the orthosteric binding site(s). These observations were further corroborated by atomistic NMA of another model of α7[94] as well as the crystal structure of ELIC.[95] In all computational studies the **quaternary twisting** was found to be described by one or a few low-frequency (i.e., low energy) modes. Moreover, in another computational study on α7 nAChR it was reported that most pathological mutations associated with congenital myasthenia and autosomal dominant nocturnal frontal lobe epilepsy were found to stiffen the twisting mode.[96] Taken together these results support the conclusion that quaternary twisting is a functional motion that is built in the topology of pLGICs.[35]

The coupling between the quaternary twist and the opening of the ion channel, which was referred to as the **twist-to-open** model,[97] has been challenged by the structural determinations of the bacterial pLGICs.[60,62,63] In fact, these structures show the occurrence of important tertiary changes on activation in particular in the TM domain that could not be accounted for by a pure twisting model. Also, the structure of the "locally closed" state of GLIC,[98] which captures a closed pore conformation in a channel preserving most features of the open form, has recently suggested that the quaternary twist and the **tilting** of the pore-lining helices could be non-correlated events.

Recent computational analyses based on all-atom MD simulations of the crystal structures of GLIC[99] and GluCl[29] have shed new light on the coupling mechanism. Based on the spontaneous relaxation of the open-channel structure elicited by agonist unbinding, i.e., an increase of pH for GLIC or the removal of ivermectin from GluCl, these analyses have developed independent models of gating with atomic resolution, which are quite related. Although the precise sequence of events is somewhat different, these models rely on the existence of an indirect coupling mechanism, which involves a concerted quaternary twisting of the channel to initiate the closing transition that is followed by the radial reorientation of the M2 helices to shut the ion pore.[29,99] Interestingly, the mechanistic scenario emerging from these simulations suggests that the twisting transition contributes to activation by preventing the spontaneous re-orientation of the pore-lining helices in the active state, thus "locking" the ion channel in the open pore form. In addition, the model of Calimet et al[29] introduces a new element in the gating isomerization proposing that a large reorientation or **outward tilting** of the β-sandwiches in the EC domain is crucial for coupling the orthosteric binding site to the transmembrane ion pore. Indeed, this movement was shown in simulation to facilitate the inward displacement of the M2-M3 loop at the EC/TM domains interface, on closing the ion pore. Most importantly, because the outward tilting of the β-sandwiches was found to correlate with orthosteric agonist unbinding, the model of Calimet et al.[29] provides the first complete description of the gating reaction, with notion of causality between ligand binding/unbinding and the isomerization of the ion channel.[29]

This model of gating makes it clear that the allosteric coupling in pLGICs is mediated by the reorganization of the loops at the EC/TM domains interface, whose position is controlled by structural rearrangements of the ion channel elicited by agonist binding\unbinding at the orthosteric or the allosteric site(s). In this framework, the position of the β1-β2 loop in the active state of pLGICs, which "senses" the agonist at the orthosteric site, acts as a brake on the M2-M3 loop to keep the ion pore open. Conversely, neurotransmitter unbinding removes the steric barrier by displacing the β1-β2 loop at the EC/TM domains interface and facilitates the inward displacement of the M2-M3 loop that mediates the closing of the pore.[29] Taken together, these observations suggest that controlling the position of the interfacial loops by structural changes that are coupled to chemical events may provide the basis for establishing the **allosteric communication** between functional sites in pLGICs.

The occurrence of a large reorientation of the extracellular β-sandwiches on ion-channel's deactivation, first observed in simulation,[29] has been recently demonstrated by the X-ray structure of GLIC pH7.[74] Indeed, the same radial opening of the β-sandwiches[9] is present in the resting state structure of GLIC and was referred to as the blooming of the EC domain.[74] Also, Sauguet et al. described the blooming motion as a distinct quaternary component of the gating isomerization, which precedes

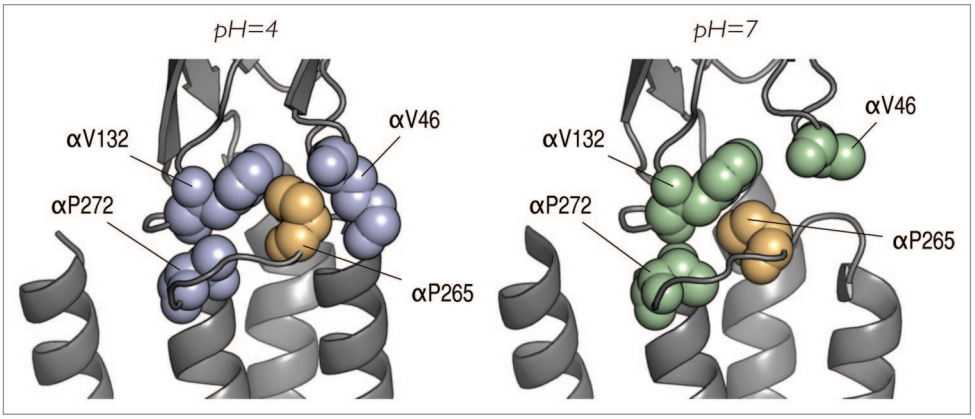

Figure 2. Energetic coupling of residues at the EC/TM domains interface. The structure of the active vs. the resting state of pLGICs are compared as visualized by the structures of GLIC at pH4[69] and pH7[74], respectively. Residues corresponding to αV46 (K33), αV132 (F116), αP272 (T253), and αP265 (P247) in *Torpedo* nAChR are shown as van der Waals spheres; corresponding residues in GLIC are given in parenthesis. The high-resolution structures of GLIC demonstrate that residues V46, V132, and P272 (blue in A, and green in R) do not form a pin-in-socket assembly at the EC/TM domains interface, as suggested by the EM reconstruction of the *Torpedo* nAChR, but cluster in a rather loose arrangement. Strikingly, these structures demonstrate that the absolutely conserved Proline on the M2-M3 loop, P265 (light orange) rather than P272, forms a pin-in-socket assembly with V46 and V132 in the active state (on the left) and disassemble in the resting state (on the right).

ion-channel twisting on activation. Strikingly, this model of gating closely corresponds to the reverse of the transition path for closing inferred by Calimet et al from the simulation of GluCl.[29]

Taken together, the most recent structural and simulation data consistently point to a mechanism that involves a large structural reorganization of the ion-channel mediated by **two distinct quaternary transitions**, i.e., a global twisting and the blooming of the EC domain; see **Figure 3**. As both transitions result in a significant restructuring of the subunits interfaces at both the EC and the TM domains, which host the orthosteric site[68] and both the Ca^{2+}-binding[74] and the transmembrane inter-subunit[12] allosteric sites, this model explains how ion-pore opening/closing in pLGICs could be effectively regulated by small-molecule binding at these interfaces.

Interpretation of Gating in the Previous Context

In the following we compare the new model of gating with previous experimental efforts to probe the sequence of structural events leading to activation/deactivation in pLGICs. The comparison with past electrophysiological analyses, which capture the functional behavior of pLGICs in the physiologically relevant context, is an important step for the validation of the emerging mechanistic perspective.

One previous model of gating based on electrophysiological recordings and double mutant cycle thermodynamic analyses of the human muscle nAChR was proposed by Lee et al.[100] In this analysis, site-directed mutagenesis was systematically performed at three residues of the α-subunit, i.e., V46 on the β1-β2 loop, V132 on the Cys loop, and P272 on the M2-M3 loop, which were thought to be located at the EC/TM domains interface based on the first cryo-EM reconstruction of the *Torpedo* nAChR.[52] In short, Lee et al. (2008) found that: (1) mutagenesis at αP272, αV46, and αV132 result in quantitative changes at both the opening rate and the equilibrium constant of gating, i.e., the difference in free energy between the active and the resting states of the ion channel; (2) the removal of the bulky side chains of αP272, αV46, and αV132 by residue substitution with a series of less hydrant aliphatic side chains result in significant reductions of the dwell time in the open conformation (i.e., by one order of magnitude upon mutation to Glycine); (3) these three residues are strongly (energetically) coupled and contribute to ion-channel activation in a context-dependent manner, e.g., when V132 is mutated into Alanine the coupling between αP272 and αV46 essentially disappears; (4) a triple substitution to Alanine residues (P272A-V46A-V132A) suppresses channel gating even in the presence of agonist.

Based on the low-resolution structure of the *Torpedo* nAChR,[52] which was thought to represent the resting state and shows that these residues form a pin-in-socket assembly at the EC/TM domain interface, Lee et al. concluded that αP272, αV46, and αV132 are engaged in the closed-channel form, move together while approaching the transition state, and possibly disengage to reach the full open-channel form.[100] Thus, it was speculated that the EC domain acts as a brake to maintain the pore in the closed state and mediates channel opening through the disengagement from the TM domain.

The interpretation of Lee et al. (2008) may be challenged for the following reasons: (1) it is based on a low-resolution structure whose functional significance is unclear (see above); (2) it does not explain the surprising gain-of-function resulting from Alanine substitution at αP272, which shifts the equilibrium to the active state of AChR even in the absence of agonist[101]; (3) it does not explain why Alanine substitution at αV132 suppresses the strong coupling between αV46 and αP272; and (4) it is inconsistent with the functional behavior of the triple mutant P272A-V46A-V132A, which is expected to favor and not suppress gating. Interestingly, the same data can be reinterpreted using the high-resolution structures of GLIC pH4[62] and GLIC pH7[74] as representative of the active and the resting state of pLGICs, respectively.

First, if one considers the residue misassignment at helices M2 and M3 in the structure of the *Torpedo* nAChR (see above), αP272 does not correspond to the totally conserved Proline on the M2-M3 loop (P247 in GLIC) but to T253, which sits on top of the M3 helix in close proximity to the Cys loop. As such, the interfacial residues of GLIC corresponding to αV46, αV132 and αP272 do not form a pin-in-socket assembly but cluster in a rather loose arrangement with F116 (αV132) in between the other two; (see **Figure 2**). This local change in topology already explains why the coupling between αV46 and αP272 depends upon residue substitution at αV132 and why nAChR gating, which is profoundly decreased by the triple mutant P272A-V46A-V132A, is totally suppressed by the apparently more conservative double mutant V46A-V132A; see Table 3 of ref. 100. Also, it suggests that the surprising gain-of-function observed upon Alanine substitution at αP272 could be related to the helicity of the M3 helix more than tertiary contacts at the EC/TM interface. Last, if one considers the homologous mutation P272S, which corresponds to a moderate loss of function resulting most probably from a reduction of the side chain volume, the double-mutant data of Lee et al. (2008) (i.e., V123A-P272S, V46A-V123A, and V46A-P272S) demonstrate the existence of energetic coupling between αV132 with αV46 and αP272 but not between αV46 and αP272, which is consistent with the structure of GLIC pH4; see blue residues in **Figure 2**.

Second, the comparison of GLIC pH4 (A) with GLIC pH7 (R) clearly shows that the interfacial residues corresponding to αV46 (on the β1-β2 loop), αV132 (on the Cys loop), and αP272 (on the M2-M3 loop) do form a pin-in-socket assembly that functionally links the EC to the TM domain, but they do so in the open state and disengage in the closed state which thus explains the drop in the gating equilibrium constant upon triple Alanine mutagenesis at these residues.

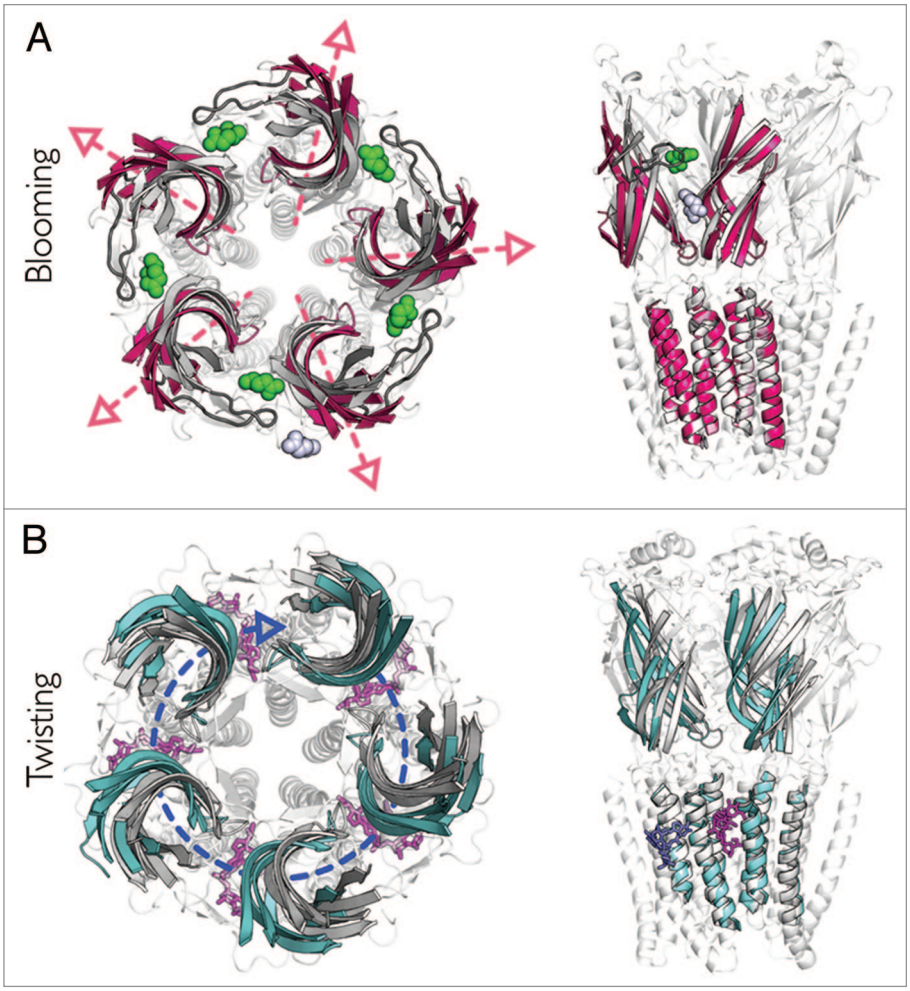

Figure 3. The blooming and twisting components of the isomerization underlying gating in pLGICs. (**A**) The blooming transition is shown. The conformation of the A state as captured by the X-ray structure of GLIC pH4[69] is shown in a cartoons representation in light gray with the C-loop closed on top of the orthosteric site in gray. For illustration, a hypothetical agonist bound to the EC domain is shown as green spheres; its coordinates correspond to those of L-glutamate in the active state of GluCl after optimal superposition of the TM domain. The position of the extracellular β-sandwiches in the resting state of pLGICs is shown in pink; coordinates were extracted from the crystal structure of GLIC pH7[74] and are shown upon optimal superposition of the TM domain. The pink dashed arrows illustrate the direction of the blooming motion from the active to the resting state. The blooming transition results in a significant reshaping of the EC subunits interfaces, which open the orthosteric site and presumably reduce the affinity for the agonist (light blue spheres). (**B**) The twisting transition is shown. The conformation of the active state of pLGICs as captured by the X-ray structure of GluCl in complex with the allosteric agonist ivermectin[12] is shown as light gray cartoons. Ivermectin bound at the subunits interfaces in the TM domain is shown as magenta sticks. The orientation of the extracellular β-sandwiches captured at the end of the twisting transition by the simulation of GluCl with ivermectin removed[29] is shown in cyan; the coordinates of the channel taken after 100ns relaxation without ivermectin are shown upon optimal superposition of the TM domain. The blue arrow illustrates the direction of the twisting transition from the active (untwisted) to the resting (twisted state). The quaternary twisting results into a small but significant reshaping of the TM subunits interfaces, which impairs ivermectin binding (violet sticks) to the untwisted or R-like conformation of the channel.

Quite interestingly, the physiological data of Lee et al. (2008) reinterpreted in light of the high-resolution structures of GLIC (see **Figure 2**) appear to be fully consistent with the emerging model of gating[29] where the tip of the β1-β2 loop acts as a brake on the M2-M3 loop through interaction with the conserved Proline (αP265 in nAChR), whose position is controlled by agonist binding at the orthosteric site. Importantly, the present interpretation predicts the existence of strong coupling of αP265 with αV132 and αV46 in the muscle nAChR, which should be urgently tested experimentally.

Another model of gating in pLGICs has been proposed by Auerbach and coworkers based on a φ-value analysis of the murine nAChR.[102] Based on an extensive set of mutants and corresponding electrophysiology recordings, these authors have determined φ-values for a large number of residues and shown that amino acids with similar values of φ tend to cluster when mapped on the structure of the nAChR.[102] Also, the structural map of the φ-values reveals a spatial gradient going from the EC orthosteric site to the TM gate region. As the φ-values can be used to measure the fractional time at which the mutated residues change their local environment on going from closed-like to open-like,[103] Auerbach and coworkers proposed that ion-channel activation proceeds through a conformational "wave" that starts from the ligand-binding site (loops A, B, and C), propagates to the EC/TM interface (β1-β2 loop and Cys loop) and moves down to the transmembrane helices (first M2, then M4 and M3) to open the ion pore.[102] Remarkably, this model of activation involves the same sequence of events described for the tertiary changes associated with the blooming transition, which is supposed to be the first step of the gating reaction.[74] In fact, the tighter association of the loops B and C at the orthosteric pocket as a consequence of agonist binding, the relative rotation of the inner and outer β-sheets of the EC domain, which causes a redistribution of the hydrophobic contacts in the core of the β-sandwiches followed by changes in the network of interactions between the β1-β2 loop, loop F, the pre-M1, and the Cys loop, the repositioning of the Cys loop and the M2-M3 loop at the EC/TM domains interfaces, and the tilting of the M2 helices to open the pore, have been described by Sauguet et al.[74] as associated with the unblooming of the EC domain in this precise order, and thus provide the structural basis for Auerbach's conformational "wave".

Modulation of Gating by Small-Molecule Binding

The recent simulation analysis of the active state of GluCl with and without ivermectin has shown that quaternary twisting can be regulated by agonist binding to the inter-subunit allosteric site in the TM domain.[29] According to the MWC model, this global motion would be the (only) quaternary transition mediating ion-channel activation/deactivation and one would predict that the twisting barrier, which is thought to be rate determining for closing,[29] should be modulated by agonist binding at the orthosteric site. Surprisingly, recent single-channel recordings of the murine AChR activated by a series of orthosteric agonists with increasing potency unambiguously show that orthosteric agonist binding has no effect on the rate for closing[104] although the series of agonists used (listed in ref. 104) modulate the di-liganded gating equilibrium constant over four orders of magnitude. The model of gating presented above provides a plausible explanation for these apparently contradictory observations even if, at this stage, it remains to be tested. In fact, the introduction of a second quaternary transition corresponding to the blooming of the EC domain, which is supposed to initiate the ion-channel activation would lead to the development of a two-step gating mechanism in which the rate-determining event would differ in the forward and the backward direction. As such, the isomerization of ion-channel on activation or deactivation might be controlled by ligands binding at topographically distinct sites. In this view, agonist binding at the orthosteric site (EC domain) is expected to primarily regulate the blooming transition, which would be rate-determining on activation, whereas the binding of positive allosteric modulators at the inter-subunit allosteric site (TM domain) would primarily control ion-channel twisting, which is rate-determining for closing. Repeating the analysis of Jadey et al[104] for a series of allosteric agonists with increasing potency, which are expected to modulate the closing rate with little or no effect on the opening rate, would provide an experimental test for the model.

The putative conformation of the resting state of pLGICs recently captured by the structure of GLIC pH7 shows that during activation a large structural change occurs between adjacent subunits in the EC domain near the interface with the TM domain. Interestingly, this region involves residues, that were shown to be implicated in binding of regulatory Ca^{2+} ions in neuronal nAChRs[72] and the prokaryotic channel ELIC.[105] The structural comparison of GLIC pH4 (A) with GLIC pH7 (R) demonstrates that the change at Ca^{2+} binding site results from a tertiary rearrangement of the extracellular β-sandwiches in response to orthosteric agonist binding, which increases the distance between residues located on opposite sides of the subunits interface.[74] Thus, the crystal structures of GLIC provide a structural understanding for the modulation of pLGICs by divalent cations and offer unprecedented opportunities for the rational design of novel allosteric modulators. Predicting whether divalent cations binding would act more on the twisting or the blooming transition is not possible at this stage and requires further simulation analysis.

Engineering chemical events solely affecting the interconversion rate (or the free-energy barrier) of each or both quaternary transitions of pLGICs would thus provide rational strategies for the design of novel small-molecule modulators of ion-channel conductance. In light of this, the positive allosteric modulatory effect of ivermectin in GluCl[12] or the endogenous cholesterol (as well as other lipids) in the nAChR[106] would arise from the ability of these ligands to stabilize the untwisted conformation of pLGICs.

Conclusion

Although the precise sequence of tertiary changes involved in the gating reaction is still debated, the mechanistic scenario put forward by the recent structural and simulation results of homopentameric prokaryotic and eukaryotic pLGICs is consistent with a wealth of experimental data collected on the nAChR eukaryotic homologs.[101] The emerging model of gating, which introduces the notion of causality between agonist binding/unbinding and the functional isomerization of the channel, in combination with a more detailed description of the gating reaction and the availability of high-resolution structures of corresponding pLGICs in humans is expected to pave the way to the development of novel strategies of rational drug design.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed for all the authors except for JPC which is consultant to Institut de Recherche Servier, Croissy-sur-Seine France for the design of anti-Alzheimer drugs.

Notes

[A]The complete model of Unwin was deposited in the PDB (access number 2BG9) in 2005 without accompanying experimental data. Lack of access to the raw data made it impossible for a third party to refine these models incorporating, for instance, knowledge from the X-ray structures of the prokaryotic homologs.

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