Gating of Pentameric Ligand-Gated Ion Channels: Structural Insights and Ambiguities

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Pentameric ligand-gated ion channels (pLGICs) mediate fast synaptic communication by converting chemical signals into an electrical response. Recently solved agonist-bound and agonist-free structures greatly extend our understanding of these complex molecular machines. A key challenge to a full description of function, however, is the ability to unequivocally relate determined structures to the canonical resting, open, and desensitized states. Here, we review current understanding of pLGIC structure, with a focus on the conformational changes underlying channel gating. We compare available structural information and review the evidence supporting the assignment of each structure to a particular conformational state. We discuss multiple factors that may complicate the interpretation of crystal structures, highlighting the potential influence of lipids and detergents. We contend that further advances in the structural biology of pLGICs will require deeper insight into the nature of pLGIC-lipid interactions.

Cys-loop receptors, including nicotinic acetylcholine (nAChR), serotonin (5-HT3), glycine, and GABA<sub>A</sub>/GABA<sub>B</sub> receptors, mediate fast chemical to electrical transduction at synapses throughout the nervous system. Insight into Cys-loop receptor structure has expanded rapidly in recent years with structures of water-soluble homologs of the acetylcholine receptor agonist-binding domain (ABD) (Brejc et al., 2001; Celie et al., 2005; Dellisanti et al., 2007; Hansen et al., 2005; Li et al., 2011), the Torpedo nAChR (Unwin, 2005), the prokaryotic pentameric ligand-gated ion channels (pLGICs), Erwinia ligand-gated ion channel (ELIC) (Hilf and Dutzler, 2008) and Gloeobacter ligand-gated ion channel (GLIC) (Bocquet et al., 2009; Hilf and Dutzler, 2009), and the Caenorhabditis elegans glutamate-activated chloride channel (GluCl) (Hibbs and Gouaux, 2011). These studies firmly establish the structural architecture of the pLGIC superfamily, in which all members adopt a similar quaternary structure formed from five subunits (Figure 1).

The basic mechanism that emerges from accumulated structural, biochemical, and physiological studies is that agonist binding triggers a structural transition from a channel-closed to a channel-open conformation, followed by a relatively slow transition to an agonist-unresponsive desensitized state (Figure 6A). Despite increasing structural data obtained in both the presence and absence of bound agonist, however, our understanding of channel activation remains clouded by our inability to unequivocally relate pLGIC structures to these canonical conformations. With this in mind, we introduce the pLGIC superfamily, review current pLGIC structures, and focus on the insight these structures provide into the conformational changes underlying agonist-induced channel gating. We then review evidence supporting the interpretation of each structure in terms of a specific conformation and discuss plausible causes for discrepancies. We also explore the possible consequences of detergent-solubilization on pLGIC structure. We conclude that a better understanding of the effects of lipids/detergents on pLGIC structure will be necessary to advance the structural biology of these important receptors.

From the nAChR to Cys-Loop Receptors and the pLGIC Superfamily

The identification of an abundant source of a “nicotinic receptive substance” in the electroplax tissues of both electric eels, Electrophorus, and electric fish, Torpedo (Changeux et al., 1970; Miledi et al., 1971), originally led to the extensive biochemical characterization of the nAChR. The nAChR is an acetylcholine-gated, cation-selective ion channel. It is a heteropentamer, with a subunit stoichiometry of 2αβδ (Weill et al., 1974). Each subunit contains a large N-terminal ABD, a transmembrane domain (TMD) consisting of four membrane-spanning α helices (M1–M4), and a cytoplasmic domain, consisting of an amphipathic helix, MA, located between M3 and M4 (Finner-Moore and Stroud, 1984). The M2 α helix from each subunit lines the ion channel pore (Akabas et al., 1994; Akabas et al., 1992; Imoto et al., 1986), whereas M4 faces the lipid bilayer (Blanton and Cohen, 1992, 1994). Key residues in the agonist binding site, including conserved aromatic residues and a vicinal disulfide, were located in loops “A” to “F” at the interfaces between the α/γ and α/δ subunits (Figure 2A).

Further work led to the discovery that the Torpedo nAChR is part of a family of nicotinic receptors that includes both muscle and neuronal nAChRs (Changeux, 2012; Taly and Changeux, 2008). In humans, this nicotinic family is part of an even broader superfamily comprised of both excitatory (acetylcholine and 5-HT<sub>3</sub>) and inhibitory (glycine and GABA<sub>A</sub>/GABA<sub>B</sub>) channels, in which all members exhibit a similar pentameric architecture (Schofield et al., 1987; Sine and Engel, 2006). Collectively, this superfamily is referred to as the “Cys-loop receptors,” because each subunit contains a conserved 13 residue loop (the β6–β7, see below) in the ABD that is bracketed by two cysteine residues. The diversity of Cys-loop receptor subunits leads to a wealth of...
The Acetylcholine Binding Proteins

Despite considerable effort (Hertling-Jaweed et al., 1988; Paas et al., 2003; Padilla-Morales et al., 2011), the Torpedo nAChR has proven refractory to crystallization, limiting its use for X-ray crystallographic studies of pLGIC structure. Atomic resolution insight into the nAChR ABD began to emerge with the discovery of the so-called acetylcholine binding proteins (AChBP), water-soluble homologs of the nAChR ABD (Smit et al., 2001). To date, structures have been solved for four AChBP orthologs in both the presence and absence of agonists and competitive antagonists (Billen et al., 2012; Brejc et al., 2001; Celle et al., 2005; Hansen et al., 2005). The AChBP structures are all homopentamers. Each subunit exhibits a conserved tertiary fold consisting of a short N-terminal α helix followed by a ten-strand β sandwich core. The β sandwich is formed from an inner β sheet of six strands (β1, β2, β3, β5, β6, and β8) that is located toward the central axis of the pentamer and an outer sheet of four strands (β4, β7, β9, and β10) that is located distally from the central axis (Figure 2). This same tertiary fold is conserved in structures of the water-soluble ABDs of the mouse muscle α1 subunit (Dellisanti et al., 2007), the prokaryotic pLGIC, GLIC (Nury et al., 2010), and an α7 nAChR ABD/AChBP chimera (Li et al., 2011). The same tertiary fold is also observed in the full-length structures of the prokaryotic pLGICs, GLIC and ELIC, and the C. elegans, GluCl.

The agonist-bound structures of the AChBP confirm that the agonist-binding sites are formed by loops from both the principle and complementary subunits (Figure 2A) (Brejc et al., 2001; Sine, 2002). Conserved aromatic residues, at positions analogous to those in loops A, B, and C on the principle face of the nAChR α subunit, form an “aromatic box” that surrounds the charged amine of bound agonists. AChBP-specific contributions to agonist binding are located on the complementary face of the agonist site and account for variations in agonist affinity between the different AChBP orthologs and between AChBP and different nAChRs (Hansen et al., 2004; Rucktooa et al., 2009; Smit et al., 2001). AChBP has proven to be an excellent structural surrogate for probing agonist recognition (Cromer et al., 2002; Reeves and Lummins, 2002; Rucktooa et al., 2009), especially for the homopentameric α7 nAChR with which it shares a similar pharmacological profile (Brejc et al., 2001; Smit et al., 2001).

Agonist binding to AChBP elicits conformational change, suggesting that AChBP could serve as a surrogate for studying the structural rearrangements in the binding site that initiate pLGIC gating (Bourne et al., 2005; Hansen et al., 2002; Hansen et al., 2005). Support for this was obtained with the creation of a functional chimera between AChBP and the TMD of the 5-HT3 receptor (Bouzat et al., 2004), although function was only obtained after replacement of the three TMD-facing loops, β1–β2, β6–β7, and β8–β9, of AChBP with the corresponding loops of the 5-HT3 receptor. Nevertheless, this chimera suggests that the conformational changes induced in AChBP upon agonist binding resemble those leading to gating in full-length pLGICs.

The structural changes elicited by agonist binding to the AChBP orthologs, as well as to the α7/AChBP chimera, suggest common themes. First, residues in both the agonist-binding pocket and the two β sheets exhibit conformational flexibility but lock into a defined conformation in the agonist-bound state. This is most notable with the agonist-site C-loop, which in the absence of agonist extends tangentially from the protein exposing the binding site to solvent (Figure 3A). The precise position of the C-loop, however, varies between structures and within subunits of individual structures (Li et al., 2011). In
fact, the C-loop is most open when AChBP is complexed with neurotoxin antagonists, which are thought to stabilize the resting state (Baenziger et al., 1992; Herz et al., 1987; Moore and McCarthy, 1995). Upon agonist binding, the C-loop moves toward the channel pore, thus capping the bound agonist, as suggested by both molecular dynamics (MD) simulations and fluorescence quenching studies (Gao et al., 2005).

Agonist binding draws together aromatic residues to stabilize the quaternary ammonium of acetylcholine and recruits additional residues to mediate signal transduction (Li et al., 2011). In particular, aromatic residues in loops A (β7 Tyr91) and C (β7 Tyr184) move toward the anchoring loop B Trp residue (β7 Trp145) in the aromatic box. The F-loop in the complementary subunit moves toward the bound agonist. These local changes propagate to the rest of the protein primarily by subtle rotations of the outer β sheet, leading to a repacking of the β sandwich core. The repacking of the β sandwich is highlighted by a rotamer switch of a conserved aromatic (Phe or Tyr) residue in β10 (β7 Phe196). These rotations cause the outer β sheet to bend toward the central pore axis, while the position of the inner β sheet remains essentially unchanged.

Although both the detected movements of the C-loop and the altered packing of the β sandwich core are conserved features in the gating of the Torpedo nAChR, a complete description of the conformational pathway leading from the agonist site to the TMD has, unfortunately, not been obtained. This is partly due to the fact that structures of AChBP cannot be unequivocally attributed to the resting and open conformations observed with full-length pLGICs. For example, some of the Apo AChBP structures exhibit solute electron density in the aromatic box, leading to the

Figure 2. The Agonist Binding Domain

(A) Residues contributing to the agonist binding site of the Torpedo nAChR were identified by biochemical studies on noncontiguous loops A, B, and C from the principle (+) (Dennis et al., 1988; Fu and Sine, 1994; Gatz et al., 1992; Kao and Karlin, 1986; Middleton and Cohen, 1991; Silman and Karlin, 1969), and loops D, E, F from the complimentary (-) (Corringer et al., 1995; Czajkowski et al., 1993; Fu and Sine, 1994; Martin et al., 1996; O’Leary et al., 1994; Prince and Sine, 1996; Sine et al., 1995), subunits (top panel). Cartoon modified from Corringer et al. (2000). A close-up view of the agonist binding site of Lymnaea stagnalis AChBP with bound agonist; carbamylcholine (PDB ID code 1UV6) is shown in the lower panel, looking at the binding site from the side (same view as in B). The principle and complimentary faces are highlighted in yellow and blue, respectively. Interacting residues are highlighted as sticks. The polypeptide backbone is shown as a transparent cartoon. Carbamylcholine is highlighted as multicolored spheres. Aromatic residues interact with the quaternary amine of acetylcholine/ carbamylcholine via cation–π electron interactions (Dougherty, 1996; Fu and Sine, 1994).

(B) Structure and tertiary fold of the “Holo” form of L. stagnalis AChBP (PDB ID code 1I9B) viewed from both the side and the top (top and bottom panels, respectively). Subunits are colored to highlight the pentameric quaternary structure. A HEPES molecule bound to one site is highlighted as multicolored spheres in the side view.

(C) The tertiary fold of a single AChBP subunit shown in an orientation similar to that of the yellow subunit in the top panel of (B). The β strands comprising the inner and outer β sheets are highlighted in blue and red, respectively. The eponymous Cys-loop is green, whereas the C-loop is yellow. Both the vicinal disulfide bond in the C-loop and the disulfide bond closing off the Cys-loop are shown as orange sticks. A topology map of the AChBP protomer is shown in the bottom panel with the same color scheme, highlighting loops A–F.
suggestion that they resemble more closely a desensitized state (Brejc et al., 2001; Celie et al., 2005; Grutter and Changeux, 2001). Even Apo AChBP structures that lack solute electron density in the agonist site undergo a further opening of the C-loop upon binding toxins, which are thought to stabilize the resting nAChR (Figure 3B). In addition, the formation of a resting state-like AChBP/5-HT₃ chimera was only achieved when AChBP was appropriately complexed with a complementary TMD (Bouzat et al., 2004). As the presence of a TMD appears to influence the conformations of the ABD, soluble AChBPs cannot offer definitive insight into the structural changes leading to pLGIC gating.

The Torpedo nAChR

Nigel Unwin and colleagues have used cryo-electron microscopy (cryo-EM) since 1984 to image membrane-imbedded nAChR-enriched tubes formed from native Torpedo electroplaques. Extensive studies culminated in a 4.0 Å resolution structure of the closed/resting nAChR pore in 2003 (Myazawa et al., 2003), which was subsequently refined by incorporating the AChBP crystal structure in 2005 (Unwin, 2005). The 4.0 Å model provided the first direct insight into the structure of the TMD, confirming the existence of four membrane-spanning α helices in each subunit, as initially predicted by hydropathy plots (Schofield et al., 1987). The α-helical nature of the TMD was subsequently reinforced by nuclear magnetic resonance (NMR) structures of the TMD of the neuronal α4β2 nAChR (Bondarenko et al., 2012, 2013) and by crystal structures of full-length prokaryotic pLGICs (see below). Unwin’s refined model highlights the structurally distinct ABD, TMD, and cytoplasmic domains, although much of the latter structure is still unresolved.

Unlike the AChBP, the nAChR is a heteropentamer formed from four different but homologous subunits. The conformations of the β₁, γ₁, and δ-subunit ABDs are similar to the agonist-bound AChBP protomer, whereas the ABDs of the two α subunits adopt a distinct “strained” conformation. In the α subunits, the inner β sheets are rotated counterclockwise relative to the other subunits (Figure 3B) (Unwin, 2005; Unwin et al., 2002). In addition, the C-loops in the α subunits are extended, similar to that seen in unliganded AChBP structures (Celie et al., 2004; Hansen et al., 2005; Unwin et al., 2002). These differences led to the suggestion that agonist binding leads to closure of the C-loop and relaxation of the inner β sheets to a more non-α/AChBP-like conformation. Because the inner sheet of the ABD is directly above the channel lining M2 α helix, it was originally suggested that a change in structure of this β sheet couples directly to a change in structure of the pore. Coupling was proposed to be mediated primarily by interactions between αVal46 of the β1–β2 loop and αPro272 of the M2–M3 linker. More recent structures, however, suggest that asymmetric motions of the different subunits ultimately lead to relatively large changes in conformation of the β subunit, which plays the key role in transmitting changes from the agonist-binding site to the channel gate (Unwin and Fujiyoshi, 2012).

Several loops at the interface between the ABD and the TMD are key to the allosteric transition that links the ABD to the channel gate (Andersen et al., 2011; Bouzat et al., 2004, 2008; Chakrapani et al., 2004; Grutter et al., 2005; Jha et al., 2007; Kash et al., 2003; Lee et al., 2009; Lee and Sine, 2005; Reeves et al., 2005; Shen et al., 2003; Xiu et al., 2005), as reviewed elsewhere (Bouzat, 2012). The ABD is linked covalently to the TMD by a loop between β10 and M1. The β1–β2 loop and β6–β7 (Cyss-loop), and the β8–β9 loops extend down toward the TMD, with the former two directly contacting a short linker between the M2 and M3 transmembrane α helices, known as the M2–M3 linker. The M2–M3 linker is a well-established gating control element (Campos-Caro et al., 1996; Grosman et al., 2000; Jha et al., 2007; Lee and Sine, 2005; Lynch et al., 1997; Rovira et al., 1998, 1999). Mutations within this linker affect nAChR apparent gating kinetics without altering the nAChR’s affinity for agonist (Grosman et al., 2000). Unnatural amino acid substitutions suggest that channel opening in the homologous 5-HT₃ receptor is directly related to the cis-trans conformational preference of an
M2–M3 linker proline residue (αP272) (Lummis et al., 2005). Unfortunately, the specific changes in conformation at the interface between the ABD and TMD that lead to movement of the pore-lining TMD α helices remain poorly defined at the current resolution of the open structure (~6 Å).

The TMD pore is lined with the M2 α helices from each subunit. These pore lining α helices contain three rings of charged/polar residues that confer ion selectivity (αE241, αE262, αS266, and the corresponding residues from other subunits) (Figure 4A). In the nAChR’s closed conformation, the α helices bend toward the center of the pore leading to a narrow region formed by three rings of hydrophobic residues (αL251, αV255, αV259, and the corresponding residues from other subunits). At the level of αL251 and αV255, the pore constriction is both too narrow (~8.0 Å diameter) and too hydrophobic to allow the passage of a hydrated sodium or potassium ion (~8.0 Å). By making it energetically unfavorable for a permeating ion to shed its solvating waters, these residues are thought to constitute a “hydrophobic gate” (Beckstein and Sansom, 2006; Ivanov et al., 2007; Miyazawa et al., 2003). This constricted structure is stabilized by hydrophobic interactions between residues on adjacent M2 α helices, and structural rearrangements underlying gating are thought to break these interactions leading to a widening of the pore. Also, the narrowest constriction of the pore in the open state is closer to the cytoplasmic leaflet of the bilayer (Unwin and Fujiyoshi, 2012), consistent with a recent analysis of ion permeation through the prokaryotic homolog, GLIC (Sauguet et al., 2013b).

Cryo-EM images recorded from nAChRs trapped in the open state at 9.0 Å resolution originally suggested that pore opening results from a clockwise rotation of the five pore lining M2 helices, causing them to collapse against the outer ring of TMD helices (Law et al., 2005; Unwin, 1995), although functional studies suggest rigid body tilting (Cymes and Grosman, 2008; Cymes et al., 2005; Paas et al., 2005). Both models are consistent with the observation that the relative stability of the open state is dependent upon the volume and stereochemistry of residues within M3 (Wang et al., 1999). More recent and higher resolution images suggest that although each of the pore lining M2 α helices move most in the extracellular leaflet, individual M2s exhibit distinct and complex movements (Unwin and Fujiyoshi, 2012). Some helices tilt outward, whereas others straighten from a “bowed” conformation. Although these rearrangements only increase the limiting diameter of the pore by about 1 Å, they also diminish pore hydrophobicity by exposing previously buried polar residues. These subtle, yet complex, movements are consistent with those suggested by both MD simulations and functional measurements examining gating (Beckstein and Sansom, 2004; Cymes and Grosman, 2008; Cymes et al., 2005; Song and Corry, 2009; Wang et al., 2008, 2009).

Note that as the resolution of available structures has increased, so has the depth of understanding. In fact, structural models of gating have evolved, with some original features maintained and others discarded. The evolution in our understanding of nAChR gating with increasing resolution of the solved structures serves as a reminder that further improvements in resolution will likely lead to further insight.

pLGIC Crystal Structures and Comparison with the Torpedo nAChR

A major breakthrough in the structural characterization of pLGICs arrived with the identification of several prokaryotic pLGICs (Bocquet et al., 2007; Tasneem et al., 2005). Two of these, GLIC and ELIC, are cation-selective channels gated by protons and primary amines (including GABA), respectively (Bocquet et al., 2007; Zimmermann and Dutzler, 2011). Both homopentamers are expressed in Escherichia coli at levels sufficient for biochemical and structural studies. Both also exhibit drug sensitivities similar to other pLGICs (Thompson et al., 2012; Weng et al., 2010). GLIC and ELIC thus serve as excellent representatives for further comparison with the nAChR structure.

Figure 4. The Transmembrane Pore

(A) Right: side view surface diagram of the Torpedo nAChR pore (PDB ID code 2BG9) with the γ subunit removed to provide an unobstructed view of the ion channel lumen. The M2 helices lining the pore are colored blue, whereas the rings of negative/polar residues that play a role in ion selectivity are red. The narrowest region of the pore corresponding to the hydrophobic gate is shown in yellow. Left: a ribbon representation of the αM2 transmembrane α helix showing the relative position of the highlighted residues lining the pore in the accompanying surface diagram.

(B) Top row: view looking toward the membrane surface of the transmembrane pore formed by the M2 α helices of each subunit in the Torpedo nAChR (left; PDB ID code 2BG9), ELIC (center; PDB ID code 2VLO), and GLIC (right; PDB ID code 3EAM). The residues highlighted as yellow spheres in the nAChR and ELIC form the narrowest constriction of each pore. In GLIC, the highlighted yellow residues are at positions analogous to those in ELIC. Bottom row: view of both the pore and the interface between the agonist binding and transmembrane domains from within the plane of the membrane showing potential movements of the pore-lining α helices upon channel gating and/or uncoupling (see text). For clarity, only two subunits are shown for each pLGIC (for the nAChR two α subunits). For each subunit, the transmembrane α helices M2 and M3 (blue), the M2–M3 linker (red), and both the β1–β2 (light blue) and β6–β7 (dark green) loops are shown. A conserved proline residue is highlighted as red spheres in analogous positions of the M2–M3 linker in the nAChR (αP286), ELIC (P253), and GLIC (P247).
models for probing both the mechanisms of channel activation and pLGIC-drug interactions.

The crystal structure of ELIC solved in 2008 (Hilf and Dutzler, 2008) was quickly followed by crystal structures of GLIC (Bocquet et al., 2009; Hilf and Dutzler, 2009) and the *C. elegans*, GluCl (Hibbs and Gouaux, 2011). These structures revealed conserved tertiary and quaternary folds, as well as conserved motifs, such as the rings of charged and hydrophobic residues that line the ion channel pore conferring ion selectivity and forming the channel gate (Figure 4A). The prokaryotic pLGICs, however, lack both the cysteine residues that bracket the Cys-loop and the cytoplasmic domain located between M3 and M4. The pore lining M2 α helices in the GLIC, ELIC, and GluCl structures also display a whole helix turn shift in register. Structures of ELIC, GLIC, and GluCl have been solved in the presence of a variety of ligands (Corringer et al., 2012; Gonzalez-Gutierrez et al., 2012; Hilf et al., 2010; Nury et al., 2011; Pan et al., 2012a, 2012b; Sauguet et al., 2013a; Zimmermann and Dutzler, 2011; Zimmermann et al., 2012).

**The TMD Pore**

The first crystal structure of the pLGIC, ELIC, was solved in the absence of agonist and attributed to the closed conformation (Hilf and Dutzler, 2008). In agreement with this interpretation, the TMD pore is occluded with hydrophobic side chains sterically blocking the pore (Figure 4B). Crystal structures of GLIC and GluCl were subsequently solved in the presence of activating ligands and thus proposed to be stabilized in an open state (Bocquet et al., 2009; Hibbs and Gouaux, 2011; Hilf and Dutzler, 2009). Consistent with this hypothesis, the C termini of the pore-lining α helices in the GLIC and GluCl structures tilt away from the central channel axis (relative to the ELIC structure), leading to an increase in pore diameter suggestive of an open and conductive conformation (Figure 4B). In further support of an open conformation, disulfide-linked cystine mutants of GLIC designed to lock the channel in a closed ELIC-like conformation reduce the pore diameter, demonstrating that closed conformations of GLIC with narrower pore diameters exist. This latter closed structure may represent an intermediate in the pathway, leading to channel gating (Prevost et al., 2012). GluCl was co-crystallized with the open channel blocker, picrotoxin. Electron density for the blocker was observed in the channel pore, suggesting that the GluCl channel is open (Hibbs and Gouaux, 2011). It should be mentioned that picrotoxin can be trapped in the closed pore of the homologous GABA _A_ receptor (Bali and Akabas, 2007; Xu et al., 1995). Finally, a structure solved of GLIC at 2.4 Å reveals the detailed hydration of a sodium ion within the pore of the open state. Computer simulations describe the changes in hydration that occur as a sodium ion transitions through the channel pore of this structure (Sauguet et al., 2013b). Collectively, these data are consistent with the assignment of the ELIC and the GLIC/GluCl structures to closed and open pore conformations, respectively.

The interpretations of the ELIC and the GLIC/GluCl structures in terms of closed and open conformations, however, are not easily reconcilable with the closed and open structures of the *Torpedo* nACHR (Figure 4B). The nACHR structure solved in the absence of agonist is presumably in the closed conformation (Unwin, 2005). In contrast to ELIC, the closed nACHR structure exhibits a channel pore that narrows to only 6 Å in diameter. The narrow constriction forms a hydrophobic, rather than a steric, barrier to ion flow (Beckstein and Sansom, 2006). In addition, the orientations of the pore lining α helices in the nACHR structure are similar to the orientations of the pore-lining α helices in the structures of both GLIC and GluCl. In fact, the pore diameter of the “closed” nACHR resembles more closely that of the open GLIC (and GluCl) than that of the closed ELIC (Figure 4B). Furthermore, cryo-EM images of the nACHR in both the closed and open conformations suggest that the changes in orientation of the TMD α helices upon gating are subtle and lead to only minor changes in pore diameter—not the relatively large changes observed between the GLIC and ELIC structures (Bocquet et al., 2009; Hilf and Dutzler, 2008, 2009).

One possible interpretation is that the apparent conformational ambiguities between the nACHR and ELIC/GLIC/GluCl structures reflect intrinsic structural differences between the pores and/or differences in the gating mechanisms of the different orthologs. In an attempt to eliminate this ambiguity, crystal structures of ELIC have been solved in both the presence and absence of an activating ligand (Gonzalez-Gutierrez et al., 2012; Zimmermann and Dutzler, 2011). Although additional electron density was observed in the agonist binding pockets of ELIC, suggesting agonist binding, no structural rearrangements were detected in the TMD pore. In fact, no movement of the pore-lining M2 α helices was detected with mutants that prolong channel opening and exhibit no propensity to desensitize (Gonzalez-Gutierrez et al., 2012). It has been suggested that the ELIC crystal structures reflect a state distinct from the expected closed, open, or desensitized states (Gonzalez-Gutierrez and Grosman, 2010).

**The Coupling Interface between the ABD and TMD**

Intimate interactions between structures at the interface between the ABD and TMD mediate coupling of agonist binding to channel gating. Given the ambiguity in the pore diameters of ELIC/GLIC/GluCl versus the nACHR, it is intriguing to compare the conformations of the loops at this interface. Subtle, but significant, differences are observed between the closed ELIC and open GLIC/GluCl structures (Bocquet et al., 2009; Hibbs and Gouaux, 2011; Hilf and Dutzler, 2009). Relative to ELIC, there is a downward motion of the β1–β2 loop in GLIC/GluCl and a displacement of both the β6–β7 loop and the M2–M3 linker away from the central pore axis so that the β1–β2 loop is now located on the proximal side (relative to the channel pore) of a conserved proline (*Torpedo* αP272) in the M2–M3 linker (Figure 4C). These movements are accompanied by an outward tilt of the C termini of the five M2 α helices, leading to an expansion of the pore diameter. The largest changes in the diameter of the pore occur in the extramembranous leaflet of the bilayer, consistent with the most recent structural data for the open state of the nACHR (Unwin and Fujioyoshi, 2012).

Surprisingly, the structural differences at this interface between the closed and open conformations of ELIC and GLIC are subtle in comparison to those between ELIC/GLIC and the nACHR (Figure 5A). In the nACHR, the extended side chains of the β1–β2 and β6–β7 loops engage the M2–M3 linker in a fashion reminiscent of vice grips attached to a metal pipe. Specifically, the side chains αE45 and αV46 in the β1–β2 loop form an inverted “V” that interacts with both faces of αP272 in the M2–M3 linker. The β6–β7 loop forms an inverted “Y” with side chains αV132
and αF135 extending to surround the polypeptide backbone of the M2–M3 linker. In contrast, substantially less contact between the loops of the ABD and the M2–M3 linker of the TMD is observed in the ELIC, GLIC, and GluCl structures (Figures 5B and 5C). In the latter, the β1–β2 and β6–β7 loops no longer surround the M2–M3 linker, which itself tilts down toward the membrane surface so that it approaches the β8–β9 loop on the complementary face of the adjacent subunit. The side chains of the β1–β2 loop extend away from the M2–M3 linker flattening the inverted “V” so that it interacts with only one surface of the conserved proline residue. In addition, the β6–β7 loop forms an extended rod-like structure that interacts with a single side of the M2–M3 linker. In the nAChR the β1–β2 loop forms numerous atom-atom contacts with the M2–M3 linker proline that are ≤3.0 Å apart, whereas in ELIC, GLIC, and GluCl, the analogous contacts are typically ≥4.0 Å apart.

These comparisons show that the conformations of the loops at the interface between the agonist-binding and TMDs in the nAChR bear little resemblance to the conformations of the same loops in ELIC, GLIC, and GluCl, raising further questions regarding the conformational interpretation of the various structures. Significantly, whereas the ELIC, GLIC, and GluCl structures suggest that gating results from increased interactions between the ABD and TMD upon agonist binding, the nAChR structure suggests that tight interactions between the two domains already exist in the agonist-free state.

Factors Contributing to the Conformational Ambiguity

Based on both pore diameter and the geometry of the M2 α helices, the nAChR in the absence of agonist appears to be in a similar open structure to that of GLIC and GluCl, whereas the ELIC structure appears to be closed. The assignment of the nAChR to an open conformation, however, is not easily reconcilable with the absence of an activating ligand. The relatively large differences in diameter of the channel pores between the closed ELIC and open GLIC structures are also not consistent with the subtle changes in nAChR pore diameter that are observed by cryo-EM upon activation and that are further supported by functional studies and MD simulations (Beckstein and Sansom, 2004; Cymes and Grosman, 2008; Cymes et al., 2005; Song and Corry, 2009; Unwin and Fujiyoshi, 2012; Wang et al., 2008, 2009). On the other hand, based on the structures of the coupling interface between the ABD and TMD, the nAChR structure suggests that tight interactions between the two domains already exist in the agonist-free state.
One possible explanation might be the differences in resolution of the various structures. The closed and open nAChR structures were solved at 4.0 and ~6 Å resolution, respectively, whereas the structures of GLIC, ELIC, and GluCl were all determined at higher resolution (2.4–3.3 Å). Although the lower precision of the nAChR structures undoubtedly leads to some ambiguity, it seems unlikely that the lower precision by itself accounts for the relatively large differences in pore diameter between the closed nAChR and ELIC structures, as well as the substantial differences observed between the cryo-EM and crystal structures at the interface between the ABD and TMD. In addition, the lower precision of the nAChR structures does not explain the lack of structural change in the pore upon agonist-binding to ELIC (Gonzalez-Gutierrez et al., 2012; Zimmermann and Dutzler, 2011).

Another possibility is that the structural discrepancies between the pLGICs reflect intrinsic structural differences and/or distinct activation mechanisms. The open GluCl structures were solved in the presence of both agonist and the allosteric ligand, ivermectin, which contributes to channel opening by wedging between the M1 and M3 α helices of the TMD (Hibbs and Gouaux, 2011). The activating ligand of GLIC is a proton, which does not bind to the typical aromatic box found in the binding sites of other pLGICs. A protonation site in the TMD of GLIC has also been implicated in gating (Wang et al., 2012), although a chimera formed between the ABD of GLIC and the TMD of the glycine receptor still responds to protons (Duret et al., 2011). The activation mechanisms revealed by these structures may thus differ from the classical activation of the nAChR resulting from agonist binding to its canonical site. The similarity of the open structures of GLIC and GluCl, despite proton and ivermectin binding to distinct sites, however, suggests a common activation mechanism for both GLIC and GluCl. Furthermore, the possibility of distinct activation mechanisms, and thus potentially distinct closed states, does not explain the absence of conformational change in the pore structure of agonist-bound ELIC.

In this light, it may be significant that the Torpedo nAChR is a heteropentamer, whereas ELIC, GLIC, and GluCl are all homopentamers. The proposed gating mechanism of the Torpedo nAChR suggests differential roles for the agonist-binding α versus the non-α subunits. In addition, the Torpedo nAChR requires only two bound agonist molecules for maximal stabilization of the open state, whereas an α7/5-HT3 homopentameric chimera requires occupation of three agonist sites for maximal opening (Rayes et al., 2009). There may be subtle differences in the activation mechanisms of hetero- versus homopentameric channels. The fact that some subunits form both homo- and heteropentamers (Lummis, 2012), however, argues against distinct activation mechanisms in homomeric ELIC/GLIC/GluCl versus the heteromeric nAChR.

The transient nature of the open state may also complicate interpretation of the pLGIC structures. The prolonged stabilities of the open conformations of crystallized GLIC, GluCI, and ELIC in the presence of activating ligands have not been unequivocally demonstrated. ELIC undergoes desensitization after exposure to activating ligands, such as cystamine, although nondesensitizing mutants have been identified (Gonzalez-Gutierrez et al., 2012). Although it was originally suggested that GLIC did not desensitize in response to proton binding (Bocquet et al., 2007), this interpretation has been challenged (Gonzalez-Gutierrez and Grosmann, 2010; Parikh et al., 2011; Velisetty et al., 2012). Membrane-reconstituted GLIC rapidly desensitizes, albeit at activating pH values that are below those used in crystallographic studies (Velisetty and Chakrapani, 2012). These recent findings raise the possibility that the agonist-bound structures of both GLIC and ELIC are influenced by desensitization (Parikh et al., 2011; Velisetty and Chakrapani, 2012; Velisetty et al., 2012). The possibility that GLIC and ELIC adopt a desensitized conformation, however, does not explain the substantial structural differences between GLIC and ELIC, particularly in the diameters of their TMD pores.

**Lipid and Detergent Sensitivity of pLGICs**

Another potential complication arises from the fact that the nAChR structure was solved by cryo-EM in its native Torpedo membrane, whereas the structures of ELIC, GLIC, and GluCl were each solved by X-ray diffraction in the detergent-solubilized state. Crystal packing forces may influence the conformations adopted by the crystallized pLGICs (Kollman et al., 2009). Detergent molecules interact closely with the pore-lining M2 α helices in structures of GLIC (Bocquet et al., 2009; Sauguet et al., 2013b). Given that detergent-solubilization can influence the conformation of any membrane protein extracted from its lipid environment, it is prudent to question whether the crystallized pLGIC structures are influenced by detergent solubilization and whether this might be the source of conformational ambiguity. In this context, it should be noted that there are currently no published data directly characterizing the functional properties of ELIC, GLIC, or GluCl in the detergent-solubilized state. In fact, it remains to be determined whether agonist-induced conformational transitions even occur after detergent solubilization.

Although the conformational sensitivity of ELIC, GLIC, and GluCl to lipids and detergent has not been extensively studied (Labriola et al., 2013; Velisetty and Chakrapani, 2012), lipids are observed bound to the surface of crystallized GLIC (Figure 5C) and may thus play a role in GLIC function (Bocquet et al., 2009). Recent studies highlight the sensitivity of the GLIC TMD to allosteric modulators (Brömstrup et al., 2013; this issue of Structure). On the other hand, complex relations have been documented between lipids and the conformational landscape of the Torpedo nAChR (Asmar-Rovira et al., 2008; Baenziger and daCosta, 2013; Barrantes, 2002; Criado et al., 1984; daCosta et al., 2009; Fong and McNamee, 1986; Hamouda et al., 2006; Rankin et al., 1997). Some reconstituted membranes stabilize the nAChR in an activatable conformation, whereas others favor either a desensitized or a distinct conformation that binds agonist and other ligands with low resting-state like affinities but does not undergo agonist-induced conformational change (daCosta and Baenziger, 2009). In the latter, binding of agonist is uncoupled from both channel gating and desensitization, as is apparently the case with the crystallized ELIC (Gonzalez-Gutierrez et al., 2012). A similar uncoupled conformation has been observed upon detergent solubilization of the nAChR (Heidmann et al., 1980), raising the concern that detergent solubilization could have similar effects on other pLGICs.

One defining property of the uncoupled nAChR is that regions of the polypeptide backbone that are buried from solvent
in the resting and desensitized conformations become exposed to solvent in the uncoupled state (daCosta and Baenziger, 2009; Méthot et al., 1995). Given the importance of the coupling interface in nAChR function, it has been suggested that lipid-dependent uncoupling results from weakened interactions, and increased physical separation, between the ABD and TMD (daCosta, 2006; daCosta and Baenziger, 2009), similar to the mechanism of coupling/uncoupling associated with the PIP2-activated potassium channel (Hansen et al., 2011). Lipids and detergents could influence interactions between the ABD and TMD via the C terminus of the lipid-exposed M4 α helix, which in the nAChR structure interacts directly with the β6–β7 loop (Figure 4B). Although the functional significance of this putative interaction has yet to be demonstrated, the importance of the C terminus of M4 in nAChR function is well documented (Hosie et al., 2006; Jin and Steinbach, 2011; Paradiso et al., 2001; Pons et al., 2004; Tobinatsu et al., 1987).

As noted, the coupling interface of the Torpedo nAChR exhibits tight interactions between the ABD and TMD, whereas in ELIC, GLIC, and GluCl, these contacts are weaker. Given that the structures of ELIC, GLIC, and GluCl were solved in the detergent-solubilized state, it is conceivable that contact between the ABD and TMD is influenced by detergent-solubilization. One possible interpretation is that the weakened interactions observed between the two domains could be similar to those thought to underlie the increased solvent accessibility detected with the uncoupled Torpedo nAChR in unfavorable lipid environments. In this vein, it is interesting that the M4 α helix in the ELIC structures is partially unwound and tilted away from the remaining TMD with several C-terminal M4 residues not modeled (Figure 5B). Contrasted with the nAChR structure (and that of GLIC and GluCl), the ELIC structure does not exhibit contacts between the C terminus of M4 and the β6–β7 loop, precisely the type of structural rearrangements thought to underlie lipid-dependent uncoupling (Figure 6B).

A lipid-dependent uncoupled conformation of ELIC would explain both the lack of agonist-induced structural change in the crystal structures of ELIC (Gonzalez-Gutierrez et al., 2012) and would largely reconcile the conformational ambiguities between available pLGIC structures. Rather than representing the canonical closed state, the conformation of the pore in the ELIC structure would instead reflect the distinct uncoupled state. If this interpretation is correct, then the GLIC/GluCl and the 4.0 Å nAChR structures may still reflect the open state and closed states, respectively. In this scenario, these structures would suggest that only subtle changes in pore diameter occur upon channel gating, consistent with the cryo-EM studies of nAChR gating, broader functional studies, and MD simulations (Beckstein and Sansom, 2004; Cymes and Grosman, 2008; Cymes et al., 2005; Song and Corry, 2009; Unwin and Fujiiyoshi, 2012; Wang et al., 2008, 2009). Strictly speaking, a definitive interpretation of each crystal structure in terms of a specific conformation still requires experimental characterization of the conformation adopted in the crystalline state.

**Summary and Future Directions**

Our current understanding of pLGIC structure stems from several major breakthroughs over the past 50 years, including the identification of the Torpedo nAChR as an abundant model for biochemical studies, the discovery and structural characterization of molluscan AChBPs, continued technical improvements in the EM imaging of Torpedo nAChRs, the discovery and structural characterization of prokaryotic pLGICs, and the first X-ray structure of a eukaryotic pLGIC, GluCl. Each of these advancements generated new lines of investigation that collectively led to detailed insight into both structure and function, but each line of investigation has its challenges and limitations. The Torpedo nAChR is abundant and amenable to biochemical studies but has proven refractory to crystallization. The AChBP and other water-soluble ABDs are amenable to crystallization without the complicating issues of detergent-solubilization, but...
they cannot be used to study gating as they lack a TMD pore. Cryo-EM studies of the Torpedo nAChR have yielded critical insight into the structures of both the closed and open states and are unaffected by potential effects of detergent solubilization, but the interpretation of these structures is limited by current resolution. Prokaryotic pLGICs are relatively easy to express and crystallize, but little is known about their functional properties in the detergent-solubilized state.

Thus, despite exciting progress, a number of open questions remain surrounding the interpretation of different conformations captured for different channels by different techniques. The conformational ambiguities between the Torpedo nAChR structure and those of ELIC, GLIC, and GluCl could reflect many factors, including the effects of crystal packing forces, differences in the resolution of the determined structures, intrinsic differences in the activation mechanisms of each pLGIC, and the possible transient nature of the open conformation in the presence of activating ligands. We contend that detergent-solubilization has the potential to exert significant influence on the conformations of the crystallized pLGICs and that some of the crystallized structures exhibit features consistent with the uncoupled nAChR conformation observed in unfavorable membranes. Further work is required to develop probes that can be used to examine the functional properties of pLGICs in the detergent solubilized state and thus to unequivocally assess conformations adopted during crystallization. An understanding of how lipids and detergents influence pLGIC structure may also assist in defining conditions for crystallization in unambiguous conformations. Ultimately, definitive conformational insight is essential for a structural understanding of pLGIC function. Conformational insight is also essential for understanding the nature of drug action at these important therapeutic targets.

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