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EPR Studies of Gating Mechanisms in Ion Channels

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Abstract

Ion channels open and close in response to diverse stimuli, and the molecular events underlying these processes are extensively modulated by ligands of both endogenous and exogenous origin. In the past decade, high-resolution structures of several channel types have been solved, providing unprecedented details of the molecular architecture of these membrane proteins. Intrinsic conformational flexibility of ion channels critically governs their functions. However, the dynamics underlying gating mechanisms and modulations are obscured in the information from crystal structures. While nuclear magnetic resonance spectroscopic methods allow direct measurements of protein dynamics, they are limited by the large size of these membrane protein assemblies in detergent micelles or lipid membranes. Electron paramagnetic resonance (EPR) spectroscopy has emerged as a key biophysical tool to characterize structural dynamics of ion channels and to determine stimulus-driven conformational transition between functional states in a physiological environment. This review will provide an overview of the recent advances in the field of voltage- and ligand-gated channels and highlight some of the challenges and controversies surrounding the structural information available. It will discuss general methods used in site-directed spin labeling and EPR spectroscopy and illustrate how findings from these studies have narrowed the gap between high-resolution structures and gating mechanisms in membranes, and have thereby helped reconcile seemingly disparate models of ion channel function.

A complete mechanistic description of ion channel function requires both the structure of the channel in multiple conformational states and the information of protein dynamics during transitions between these states. The functioning of ion channels is governed by their ability to undergo stimulus-driven movements that enable opening of the gate, and in most cases, these processes are accompanied by global protein motions. From a crystallographic point of view, “moving parts” and intrinsic flexibility of ion channels pose serious hurdles in crystallization and structure determination. Further, stabilizing channels in multiple conformational states under crystallographic conditions is yet another barrier. As a result, the structural basis for gating mechanisms in many channel types has been inferred from comparisons across homologues trapped in crystallographically distinct states. Although this strategy has proved informative, it is clearly plagued by two major issues: first, uncertainties in assigning functional states to crystallographically captured conformations, and second, issues with distinguishing intrinsic structural variations among homologues from differences between the conformational states. In some cases, truncation, limited proteolysis, and

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antibodies have been used to stabilize proteins with considerable success (Hassaine et al., 2014; Hibbs & Gouaux, 2011; Jiang et al., 2003; Miller & Aricescu, 2014; Zhou, Morais-Cabral, Kaufman, & MacKinnon, 2001), although these strategies suffer from limiting conformational transitions. Quite often, detergent solubilization, lattice forces, and crystal contacts have led to stabilization of states that are not part of the functional scheme or constitute only a minor population of the channels in the membrane (Cross, Sharma, Yi, & Zhou, 2011; Jiang, Lee, et al., 2003; Kim, Xu, Murray, & Cafiso, 2008). These limitations have spurred much of the discrepancies between various gating mechanisms and models. This necessitates strategies that allow structural and functional measurements under similar conditions. In this aspect, site-directed spin labeling (SDSL) and electron paramagnetic resonance (EPR) spectroscopy offer a unique perspective to multistep gating schemes (Columbus & Hubbell, 2002; Fanucci & Cafiso, 2006; Hubbell, Cafiso, & Altenbach, 2000; McHaourab, Lietzow, Hideg, & Hubbell, 1996; Sahu, McCarrick, & Lorigan, 2013). In comparison, comparison to X-ray and nuclear magnetic resonance, information from EPR is of relatively low resolution, however there are several advantages of this technique that surpass its limitations and make it highly complementary to other structural methods. Most notably, EPR is not constrained by the molecular size or the optical property of the system, and thereby allows measurements of the full-length constructs in a native lipid environment. In addition, this technique is extremely sensitive and the minimum sample requirements are in the pico- to micromolar range.

Structural understanding of ion channels was greatly facilitated by the discovery and cloning of prokaryotic homologues (Bocquet et al., 2007; Ruta, Jiang, Lee, Chen, & MacKinnon, 2003; Schrempf et al., 1995; Tasneem, Iyer, Jakobsson, & Aravind, 2005). These channels reveal remarkable conservation of the overall scaffold and basic channel functions, representing a minimal functional core of the channel. These model systems have therefore offered new avenues for determination of high-resolution structures and uncovering protein dynamics of the previously elusive channel class (Bocquet et al., 2009; Doyle et al., 1998; Hilf & Dutzler, 2008, 2009; Jiang, Lee, et al., 2003; Zhou et al., 2001). Advantages of using these channels include functionality in isolation from their native membranes, a smaller number of native cysteines, and strong resilience to mutagenesis (Gross, Columbus, Hideg, Altenbach, & Hubbell, 1999; Jiang, Lee, et al., 2003; Perozo, Cortes, & Cuello, 1998; Ruta et al., 2003; Velisetty & Chakrapani, 2012; Velisetty, Chalamalasetti, & Chakrapani, 2012, 2014). Thus, a complete understanding of structure, function, and dynamics has been made feasible.

1. SDSL AND EPR SPECTROSCOPY TO STUDY GATING MECHANISMS IN ION CHANNELS

The use of EPR spectroscopy in combination with SDSL was pioneered by Hubbell and colleagues and has been adopted for studying the molecular architecture and protein motions in a number of proteins (Altenbach, Marti, Khorana, & Hubbell, 1990; Bordignon, 2012; Columbus & Hubbell, 2002; Drescher, 2012; Fanucci & Cafiso, 2006; Hubbell et al., 2000; Hubbell, Lopez, Altenbach, & Yang, 2013; Hubbell, McHaourab, Altenbach, & Lietzow, 1996; Klare & Steinhoff, 2009; McHaourab et al., 1996; McHaourab, Steed, & Kazmier,

2011; Perozo, Cuello, Cortes, Liu, & Sompornpisut, 2002; Sahu et al., 2013). These techniques are best suited for studying the conformational dynamics of membrane proteins, especially in a native membrane environment. This method involves engineering cysteine substitutions at target positions by site-direct mutagenesis. Since SDSL requires a unique cysteine for site-specific introduction of the spin label, native cysteines within the protein need to be substituted with another amino acid to create a cysteine-less template. Work in numerous systems, including membrane proteins, has established that cysteine mutagenesis is generally well-tolerated in most parts of the protein without loss of function. In addition, the relatively small size of the spin probe causes minimal perturbation, and in many cases, the functionality of labeled proteins has been confirmed. Individual cysteine mutants are expressed, purified, and labeled with a nitroxide spin probe. To maximize reactivity, the labeling reaction of the protein is carried out in the detergent-solubilized form. After separation of the excess free spin label by size-exclusion chromatography, the protein is reconstituted into liposomes or bilayer-mimicking systems of defined lipid composition. The most commonly used spin label is a thiol-specific methanethiosulfonate derivative (1-oxy-2,2,5,5-tetramethyl-3-pyrroline-3-methyl) that attaches to the protein through a disulfide bond bridge. Due to its high specificity, relatively small size (slightly larger than tryptophan), and flexibility of the linker region, this spin label has been shown to have excellent reactivity even with buried cysteine. Continuous wave (CW) is the most commonly used EPR technique where the sample is subjected to varying magnetic fields in the presence of a fixed microwave frequency. The unpaired electron from the nitroxide group absorbs microwave radiation as it undergoes reorientation to the higher energy level. Spin-flip transitions occur when the energy separation between the two electron spin states matches the energy of the applied microwave radiation. EPR spectrum is usually represented as a first derivative of the absorption as a function of the magnetic field strength. EPR analyses reveal three different pieces of structural information (Altenbach, Froncisz, Hemker, McHaourab, & Hubbell, 2005; Altenbach, Greenhalgh, Khorana, & Hubbell, 1994; Altenbach et al., 1990; Farahbakhsh, Altenbach, & Hubbell, 1992; Hubbell et al., 2000, 1996): (1) *Line-shape analysis* of motional freedom or the dynamics of the spin probe. At room temperature, the spin probe can adopt multiple rotameric conformations, and the spectral line shape is sensitive to the motion of spin label side chain relative to the peptide backbone, backbone fluctuations, and global rotational motion of the protein. The mobility of the probe can be reasonably quantified as an empirical parameter (ΔH_0^{-1}), which is measured as an inverse of the central linewidth of the first-derivative absorption spectra (McHaourab et al., 1996). For a nitroxide spin probe in a motionally restricted environment, the linewidth is larger (and hence smaller ΔH_0^{-1}) compared to that of a spin probe in a location with high freedom of movement. This is a useful parameter to detect protein conformational changes or protein-protein interactions. (2) *Solvent accessibility* of the spin probe evaluated by collisional relaxation methods. Paramagnetic agents such as lipid-soluble O_2 (lipid-soluble) or water-soluble metal complex NiEDDA have different diffusional profiles across the membrane. Collision with these paramagnetic relaxing agents enhances the spin-lattice relaxation rate (T_1) of the nitroxide by Heisenberg spin exchange (Altenbach et al., 2005). The accessibility parameter (Π) is estimated from power saturation experiments in which the vertical peak-to-peak amplitude of the central line of the first-derivative EPR

spectrum is measured as a function of increasing incident microwave power (Farahbakhsh et al., 1992). A spin label at a specific position on the protein can be classified as one of the following: buried within the protein core inaccessible to either water or lipid, on the surface exposed to aqueous solution, or on the surface exposed to lipids. The accessibility parameters obtained for a series of consecutive sites can then be used to determine the secondary structure of a region, probe areas of protein–protein contact, measure the tilt of a protein within the membrane, estimate the immersion depth of interfacial loops, and identify water vestibules and lipid-bound pockets within the transmembrane helices (Altenbach et al., 1994). (3) *Interspin distances* between pairs of nitroxide. In systems with two or more spin labels, electron–electron dipolar interactions depend on the interspin distance and the angle between the paramagnetic centers relative to the external magnetic field. Measurements of these distances involve either CW or pulsed (double-electron electron resonance or DEER) EPR techniques. In CW, through-space dipolar interactions lead to spectral broadening and are a function of proximity between the labels. It is also influenced by relative orientation of the spin labels, and the frequency and amplitude of the motion averaging the orientation of the spin vector relative to the magnetic field. Line broadening is accompanied by a decrease in signal amplitude, and for distances up to ~15–20 Å, a decrease in peak height for spectra normalized to the same spin concentration can often be used as a qualitative indication of spin–spin interaction. Extent of broadening in comparison to the sum of singly labeled mutant spectra can also be used to semi-quantitatively estimate distances in 8–20 Å range (Rabenstein & Shin, 1995). While the information from the methods described above is intrinsically local in nature, mobility and accessibility data from a series of spin-labeled positions in combination with distance constraints allow modeling of the protein structure with a backbone level spatial resolution. In addition, more advanced pulsed EPR methods (DEER) have been developed to determine global changes in distances (Borbat, McHaourab, & Freed, 2002; Chiang, Borbat, & Freed, 2005; Jeschke, 2012; Jeschke & Polyhach, 2007; Jeschke, Wegener, Nietschke, Jung, & Steinhoff, 2004). In DEER, the oscillations in the spin-echo amplitude are used to determine distances and distance distribution in the 20–70 Å range. Taken together, sequence-specific secondary structure, tertiary contacts, and distance constraints can be used to measure conformational changes under various conditions. The following examples of voltage- and ligand-gated channels will highlight how EPR studies have helped evaluate crystal structures, determine the structure of missing regions or distorted elements in the crystal structures, and finally, validate structural models for gating.

2. VOLTAGE-GATED ION CHANNELS

Voltage-gated channels play a critical role in cellular excitability and thereby form the basis for initiation and propagation of nerve impulses (Hille, 2001). In addition, they are involved in muscle contraction, neuronal activity, hormone secretion, osmotic balance, and immune response. Mutations in voltage-gated channels have been associated with cardiac arrhythmias, episodic ataxia, and epilepsy. These channels are primarily composed of two interacting yet modular units: a voltage sensor domain (VSD) formed from the first four transmembrane segments (S1–S4), and a pore domain (PD) formed from the remaining two transmembrane segments (S5 and S6). During voltage-dependent activation, gating charges

on the S4 segment translocate from the “down” to the “up” conformation. Movement of S4 in turn is coupled with opening of the pore through the S4–S5 linker. The structure of the voltage sensor and the mechanisms underlying S4 movement leading to the opening channel gate have been the subject of intense debate for the past decade.

Much of this controversy originated with the first crystal structure of a voltage-gated channel from the archaea *Aeropyrum pernix*, KvAP (Jiang, Lee, et al., 2003). The crystal structure, obtained in detergent and in the presence of Fab fragments, was found to be largely distorted and in a non-native conformation at the level of the VSD. With respect to the PD, S1 and S2 were oriented parallel to the membrane, and S3 and S4 were positioned close to the membrane inner-leaflet and perpendicular to the pore axis. However, transmembrane orientation of S1 relative to the rest of the VSD helices was partly reconciled based on the structure of the isolated VSD of KvAP (also solved in the presence of antibody) (Jiang, Lee, et al., 2003). Based on this structure, it was proposed that the S3b–S4 forms a “paddle” motif that is exposed to the membrane and moves as a hydrophobic cation upon membrane depolarization (Jiang, Ruta, Chen, Lee, & MacKinnon, 2003). This structure essentially challenged two of the long-held ideas in the field. The first was that the S1, S2, and S3 segments shield S4 and its charges from the lipid bilayer. The second was that the S4 segment undergoes translation or rotation within a canaliculi or gating pore (Goldstein, 1996; Yang, George, & Horn, 1996).

To address this controversy, Perozo’s group sought to determine the architecture of the KvAP voltage sensor in a native environment by carrying out an extensive EPR analysis of spin probes at ~140 positions covering the entire VSD (Cuello, Cortes, & Perozo, 2004). EPR measurements were carried out in the absence of a membrane potential (0 mV), which in principle would favor the activated or “up” conformation of the VSD. Clear boundaries of probe dynamics and NiEDDA accessibility of the S1–S2/S3–S4 loops interspaced between the TM helices allowed the authors to conclude that the VSD domain indeed adopts a transmembrane orientation. While the overall architecture of the VSD predicted by EPR was incompatible with the KvAP full-length structure, it was in agreement with the KvAP-isolated VSD structure. A major finding of this work was that the spin labels on one face of the S4 helix showed high O₂ accessibility revealing exposure to the membrane and thereby provided the first direct evidence for a peripheral location of S4 in the lipid bilayer. Interestingly, the spin probes at positions corresponding to the S4 gating charges showed low mobility and limited accessibility to O₂ or NiEDDA suggesting that they lie on the protected face, away from the membrane lipid, and presumably interact with the rest of the TM helices. In addition, the pattern of mobility and high NiEDDA accessibility of several positions within the S3 segments were indicative of water crevices penetrating the VSD. These crevices have been implicated in reducing the energetic cost of placing the gating charges in a hydrophobic environment while at the same time focusing the membrane field which will allow an efficient transduction of the electric field into S4 movements. Another surprising result from this study was that the spin probes at positions along S1 were motionally restricted and solvent inaccessible. This was in contrast to findings from perturbation analysis in eukaryotic channels, which position S1 in the channel periphery with minimal interaction with the rest of the protein.

To unequivocally define the interacting footprint of PD on VSD, EPR analysis was then carried out at the same positions in the isolated VSD (Chakrapani, Cuello, Cortes, & Perozo, 2008). The expectation was that the region of VSD in contact with PD in the full-length channel would show an increase in probe mobility and O₂ accessibility in the isolated VSD, while the membrane-exposed region would be minimally perturbed. Indeed, spectra from S3 and S4 were essentially identical and there were no changes in the overall O₂ accessibility trends (Fig. 1). On the other hand, profound increases in spin label dynamics and lipid exposure were noted in the S1 and S2 segments, and more so for residues facing away from the VSD helical bundle. These results clearly showed that in the activated conformation of the KvAP sensor, S3 and S4 segments are at the periphery of the channel and make minimal contact with the PD. In contrast, the S1 and S2 segments are in close proximity to the PD. This finding was in agreement with DEER measurements in full-length KvAP, which showed shorter intersubunit distances for probes placed on S1 in comparison to those on S4 (Vamvouka, Cieslak, Van Eps, Hubbell, & Gross, 2008). These data were compared with the VSD orientation in the open-state structure of Kv1.2–Kv2.1 chimera (Long, Tao, Campbell, & MacKinnon, 2007). While there was a remarkable agreement with the arrangement of individual side chains, a discrepancy in the positioning of the VSD with respect to the PD was noted. The structure shows that VSD in the chimera interacts with the PD through S1 and S4, while the EPR data in KvAP indicated that the interaction with the PD was via S1 and S2, with S3 and S4 clearly placed in the periphery (Fig. 2). This arrangement of the two domains requires a rotation of ~70–100° (from the intracellular side) about an axis perpendicular to the membrane plane relative to the PD such that S1 and S2 are more closely placed to the PD.

Interestingly, water crevices were also identified in the isolated VSD and they were not considerably different from those in the full-length channel. It was thereby proposed that these cavities could be a common feature underlying the conductance of protons and other cations through the VSD, and that the VSD scaffold fully supports this pathway in the absence of auxiliary proteins and even in the monomeric state. Consistent with idea, EPR studies in a voltage-gated sodium channel (NaChBac) revealed conservation of fundamental features of the VSD in K_v and Na_v channel families (Chakrapani, Sompornpisut, Intharathep, Roux, & Perozo, 2010), although there were a few notable differences particularly at the packing of the helices and location of the S4 charges. While the periodicity of S1 and S2 accessibilities suggested that they are straight helices, a break in periodicity for S3 and S4 was reflective of a bending or kinking of the α -helix. In addition, patterns of ΠO_2 in NaChBac revealed that the S4 segment is not a simple α -helix; positions at the upper half of S4 show low values every third or fourth position, while at the bottom end it is every third position. The derived power spectrum of the N-terminal half of S4 shows a distinct peak centered at 99.8° reflecting its α -helical nature while the C-terminal end reveals a peak at 118.1°, which is characteristic of a 3_{10} helix. The occurrence of a 3_{10} helical hydrogen-bonding pattern in S4 was observed in the Kv1.2–2.1 chimera (Long et al., 2007), MlotiK (Clayton, Altieri, Heginbotham, Unger, & Morais-Cabral, 2008), and later in the Na_v structures (Payandeh, Scheuer, Zheng, & Catterall, 2011; Zhang et al., 2012). A varying length of 3_{10} stretched has been observed in these structures. In Na_vAb, the entire S4 is in a 3_{10} -helix conformation (Payandeh et al., 2011), while in Na_vRh structure the S4

segment is further outward and the 3_{10} -helix is partially relaxed to an α helix (Zhang et al., 2012). There is no evidence of 3_{10} in the KvAP sensor crystal structure (Jiang, Lee, et al., 2003) or in the EPR accessibility parameters of the KvAP sensor in the membrane (Chakrapani et al., 2008; Cuello et al., 2004). A 3_{10} helical conformation of S4 positions the series of charged arginine in a linear fashion pointing toward a water-filled crevice at the center of the VSD core, which would be energetically favorable (Long et al., 2007). An α -helix-to- 3_{10} transition might allow S4 to stretch and spring back, changing the position of the gating charges within the electric field, as previously suggested (Long et al., 2007). Based on the extent of immobilization and NiEDDA accessibility, it was proposed that in the “up” conformation R4 and R5 occupy the narrowest constriction, separating the inner and outer vestibules in KvAP, while this position is occupied by R3 in NaChBac. These findings were in remarkable agreement with the NavAb structure (Payandeh et al., 2011) and the gating pore current measurements in NaChBac (El-Din, Scheuer, & Catterall, 2014). Mutation of R3 to a smaller hydrophilic residue in skeletal muscle Na_v creates gating pore currents in the “up” state of the voltage sensor and underlies potassium-sensitive normokalemic periodic paralysis (Sokolov, Scheuer, & Catterall, 2007). These findings collectively reinforce the modular nature of the VSD and PD in the voltage-gated channels.

Understanding the architecture of the voltage sensor completes just one piece of the giant puzzle, and another major part is the determination of S4 movement during voltage-dependent activation. Based on multitudes of experimental findings and predictions from molecular dynamic simulations (reviewed in (Bezanilla, 2008; Catterall, 2010; Swartz, 2008; Tombola, Pathak, & Isacoff, 2006), at least three different mechanistic models have been proposed: first, the voltage sensor paddle model, where the S3b–S4 paddle moves 15–20 Å across the membrane as a hydrophobic cation (Jiang, Ruta, Chen, Lee, & MacKinnon, 2003); second, the transporter-like model (Cha, Snyder, Selvin, & Bezanilla, 1999; Chanda, Asamoah, Blunck, Roux, & Bezanilla, 2005; Starace & Bezanilla, 2004), where a highly focused field across the membrane necessitates only small-scale (2–4 Å) movement of charges; and third, the helical-translocation/helical-screw model, where the S4 segment undergoes a vertical displacement/rotation to varying degrees (Catterall, 1986; Glauner, Mannuzzu, Gandhi, & Isacoff, 1999; Guy & Seetharamulu, 1986; Yang et al., 1996).

Understanding S4 movements and the associated gating charge translocation requires the VSD in its two end states: the “up” and “down” conformations. While crystal structures and EPR studies at 0 mV have provided a detailed understanding of the “up” conformation of the VSD from diverse sources (Clayton et al., 2008; Jiang, Lee, et al., 2003; Li, Wanderling, Paduch, et al., 2014; Long, Campbell, & MacKinnon, 2005; Long et al., 2007; Payandeh et al., 2011; Zhang et al., 2012), the challenge thus far has been to identify conditions that would stabilize the “down” state in the absence of a membrane potential. There is growing evidence that lipids influence the gating transitions in voltage-gated channels.

Electrophysiological studies have shown that protein–lipid interaction plays a key role in shifting the conformational equilibrium between multiple functional states (Ramu, Xu, & Lu, 2006; Schmidt, Jiang, & MacKinnon, 2006; Xu, Ramu, & Lu, 2008; Zheng, Liu, Anderson, & Jiang, 2011). In Kv2.1, hydrolysis of sphingomyelin to ceramide by sphingomyelinase (SMase) D treatment led to a left shift in voltage-dependent activation and thereby an increase in open probability at resting membrane potentials (Ramu et al.,

2006). Similarly, reconstitution of KvAP in nonphospholipid bilayers (DOTAP) led to a significant right shift in the activation curves, suggesting that the gating charge interaction with surrounding phospholipids is essential for the transition to the “up” conformation (Schmidt et al., 2006). Antibodies specific to the “down” state bind to KvAP in DOTAP membranes, further confirming that the conformation of the VSD in DOTAP is similar to the physiological hyperpolarization-driven resting state (Zheng et al., 2011). Perozo’s group used this strategy to investigate the dynamics of the “down” state of isolated VSD of KvAP using EPR (Li, Wanderling, Sompornpisut, & Perozo, 2014). Probe mobility, solvent accessibility, and interhelical distance determinations from spin probes attached to the VSD in both phospho (POPC/POPG) and nonphospholipids (DOTAP) were used to elucidate the conformational changes associated with voltage-dependent activation of the VSD. In comparison to PC membranes, a dramatic increase in NiEDDA accessibility was observed for residues lining the intracellular crevasse. As a consequence, the water-excluded region was limited to 5 Å roughly at the center of the bilayer, in contrast to 15 Å for the VSD in PC membranes. Furthermore, the extracellular end of S4 showed a decrease in dynamics and NiEDDA accessibility, while the intracellular end of S4 showed an opposite effect consistent with an inward motion of S4 during transition to the “down” state. The extent of S4 movements with respect to relatively rigid ends of S1 and S2 was quantified by DEER measurement using bifunctional spin labels (reduce intrinsic flexibility) at positions in S4 and S1/S2. Overall, a 3 Å downward tilt away from S1/S2 and a 2 Å downward movement for S4 was proposed during the “down” state transition. A new “tilt-shift” model was proposed, suggesting a modest ~3 Å S4 displacement. An extensive analysis of NiEDDA accessibility in the “up” and “down” states suggested that the S4 reorientation to the “down” state led to a large increase in intracellular water penetration within the VSD, thus putatively refocusing the electric field. These studies show how lipid–channel interactions can be used as a key tool in stabilizing specific channel conformation for structure determination by EPR.

3. LIGAND-GATED ION CHANNELS

Signal transduction at the postsynaptic membranes is mediated by neurotransmitter-gated channels. Pentameric ligand-gated channels (LGIC), also called Cys-loop receptors, mediate fast synaptic transmissions in the central and peripheral nervous systems and include both excitatory and inhibitory neurotransmitter receptors. The vertebrate members of this family consist of nicotinic acetylcholine receptor (nAChR), the γ -aminobutyric acid (GABA_{A/C}R) receptors, glycine receptors (GlyR), and serotonin receptors (5-HT₃R). Dysfunctions in LGIC underlie a number of neurological disorders, including Alzheimer’s disease, Parkinson’s disease, schizophrenia, epilepsies, myasthenia gravis, and congenital myasthenic syndromes, and are implicated in nicotine addiction and lung cancer. LGIC are targets for several classes of clinically used drugs, including general anesthetics, alcohols, benzodiazepines, neurosteroids, and barbiturates. In addition to these channels in humans, several orthologs within the LGIC are widely expressed in invertebrates and were recently discovered to be present in bacteria and archaea (Tasneem et al., 2005). While the invertebrate channels are activated by GABA, 5-HT₃, glutamate, histamine, or protons, the bacterial channels are shown to respond to primary amines (including GABA) or protons

(Bocquet et al., 2007; Lynagh & Pless, 2014; Miller & Smart, 2010; Zimmermann & Dutzler, 2011). Members within the family are known to have evolved from a single ancestral gene and share a common overall architecture with significant homology. These channels have served as important structural surrogates for understanding mechanisms in humans.

LGIC are comprised of five subunits (identical or homologous subunits) arranged around a central channel axis in a pseudo-symmetric manner. Each subunit in turn is composed of an extracellular domain (ECD) that hosts the neurotransmitter binding site, a transmembrane domain (TMD) containing four transmembrane helices (M1–M4) that provides the channel gating machinery, and an intracellular domain consisting of the M3–M4 linker and governs assembly, localization, and membrane trafficking through interactions with scaffolding proteins. Ligand binding within the ECD initiates conformational changes that traverse 50–60 Å across the channel and result in channel opening at the TMD. The allosteric transition is believed to involve large-scale global change in quaternary structure (Bocquet et al., 2009; Hilf & Dutzler, 2009; Taly et al., 2005; Unwin & Fujiyoshi, 2012). The molecular details of protein motions are not completely clear. Most channels within the family undergo desensitization subsequent to opening, which renders them refractory to the ligand. These gating mechanisms are modulated by a range of allosteric ligands of both endogenous and exogenous origin, and some of which have important therapeutic potential. Overall, there is tremendous interest in terms of understanding the molecular details of channel activation and desensitization, and, in turn, the basis of allosteric modulation in these channels.

Structural insights for human Cys-loop receptors have come from members of both prokaryotic and eukaryotic origin (Corringer et al., 2010; Labriola et al., 2013; Sauguet et al., 2014). Our first comprehensive view of the full-length channel in a membrane comes from a 4-Å resolution structural model of a heteromeric *Torpedo* nAChR generated using cryo-electron microscopy data (Unwin, 2005; Unwin & Fujiyoshi, 2012). First atomic-resolution snapshots of the agonist/antagonist-bound ECD came from structures of the acetylcholine-binding protein (AChBP), a naturally truncated homologue of the ECD of nAChR (Brejc et al., 2001; Celie et al., 2004; Hansen et al., 2005). The past decade has seen several landmarks in the LGIC field with structure determination of prokaryotic homologues GLIC and ELIC (Bocquet et al., 2009; Hilf & Dutzler, 2008, 2009), an invertebrate glutamate-gated chloride channel GluCl (Hibbs & Gouaux, 2011), and more recently, the mammalian GABA_A β 3 receptor (Miller & Aricescu, 2014) and the mouse serotonin 5-HT₃ receptor (Hassaine et al., 2014). In spite of limited overall sequence identity, these solved structures superimpose well, revealing a remarkable conservation of the overall fold and architecture. In all these structures, the ECD has an archetypal arrangement of antiparallel β -sheet with the subunits arranged around a central ion pathway perpendicular to the membrane and lined by the M2 segments from individual subunits. However, interesting differences can be noted in the extent of rotation of the β -sandwich core of the ECD, the positioning of the helical segments within the TMD, and the relative orientation of the ECD with respect to the TMD ($\sim 20^\circ$). LGIC homologues have been crystallized under different conditions that, in principle, stabilize the channel in the closed, open, or desensitized conformation, and structural differences among them have been used to infer gating

mechanisms. In general, it is believed that ELIC, GLIC_c (at pH 7.0), and GluCl (Apo) structures may represent closed/resting states, while GLIC_o (at pH 4.0) (Fig. 3) and GluCl (ivermectin bound) are in the putative open conformation (Althoff, Hibbs, Banerjee, & Gouaux, 2014; Bocquet et al., 2009; Hibbs & Gouaux, 2011; Hilf & Dutzler, 2008, 2009; Sauguet et al., 2014). The 5-HT₃ structure in the presence of nanobodies (Hassaine et al., 2014), which are potent inhibitors of channel activation, is proposed to be in an intermediate conformation, while the GABA_A $\beta 3$ bound to benzamidine, a novel activator of the channel, might be in a desensitized conformation (Miller & Aricescu, 2014). An overlay of these structures suggests that gating may involve rigid body motions of ECD and TMD relative to each other.

Overall, these structures have provided us atomic details of structural organization in LGIC, and there is reasonable consensus about the general architecture of the family. However, drawing mechanistic information from these structures is a goal far from achieved. Besides, LGIC are well known to be extensively modulated by membrane lipid composition; particularly in nAChR, transitions between closed-to-open-to-desensitized conformations are abrogated in the absence of negative lipids and cholesterol (Fong & McNamee, 1986). Molecular dynamic simulations predict that cholesterol binding at the lipid–protein interfacial sites of nAChR stabilizes ECD–TMD contacts and thereby regulates channel activation (Brannigan, Henin, Law, Eckenhoff, & Klein, 2008). Interestingly, GLIC structure reveals bound lipid molecules within the TMD cavity that are displaced in the presence of a general anesthetic (propofol), suggesting the modulatory role of tightly associated lipids (Nury et al., 2011). Similarly, POPC molecules were found to insert into the intersubunit crevice of GluCl, and quite remarkably this structure is stabilized in an expanded open-like conformation (Althoff et al., 2014). These observations clearly underscore the importance of membranes in channel dynamics.

The ability to use defined lipid composition in membrane reconstitution allows one to study the role of specific membrane constituent such as cholesterol, PIP₂, or negative phospholipids at the functional level by patch-clamp measurement in proteoliposomes and monitor conformational changes by EPR spectroscopy. GLIC is chosen as an example to illustrate how this approach can be used to study gating mechanisms in this channel class, and particularly to probe the organization and packing of the TM helices in different conformational states, determine the extent of M2 movements during gating, and define the nature of conformational changes at the ECD–TMD interfaces.

GLIC is a pH-activated channel from *Gloeobacter violaceus* which was initially crystallized under acidic conditions (Bocquet et al., 2009; Hilf & Dutzler, 2009). The structure was proposed to be in an open conformation based on functional measurements in heterologous expression systems where channels were shown to display nondecaying macroscopic currents in response to pH changes (Bocquet et al., 2009, 2007; Hilf & Dutzler, 2009), although later studies showed evidence for GLIC desensitization (Gonzalez-Gutierrez & Grosman, 2010; Parikh, Bali, & Akabas, 2011). From the structure point of view, the pore-lining M2 region of GLIC was clearly in a more open conformation than the unliganded ELIC structure. However, this region was essentially identical to unliganded AChR model from *Torpedo* leading to questions about the mechanism of channel opening and the extent

of M2 movement underlying activation. To directly study structural and functional correlates of GLIC gating in a membrane environment, we measured the functional properties of purified and reconstituted channels in proteoliposomes through patch-clamp recordings and investigated underlying conformational transitions by EPR spectroscopy (Velisetty & Chakrapani, 2012; Velisetty et al., 2012, 2014). Interestingly, GLIC currents showed robust desensitization within 1–3 min of exposure to protons (Fig. 4). In addition, GLIC desensitization had several hallmark features of the mechanism in eukaryotic channels, allowing us to investigate this far less understood mechanism in LGIC (Velisetty & Chakrapani, 2012).

We carried out extensive cysteine mutagenesis and SDSL at over 100 positions covering key regions of the protein, namely the M2 helix and the intra- and intersubunit interfaces (Velisetty & Chakrapani, 2012; Velisetty et al., 2012, 2014). Functionality of cysteine mutants at several key positions in the presence of spin label was confirmed by patch-clamp measurements of macroscopic currents. Since EPR measurements were carried out under steady-state conditions, GLIC mutants are expected to reside predominantly in the closed (at neutral pH) and desensitized conformations (at acidic pH). In the closed conformation, pore-facing positions in M2 revealed line-shape characteristic of motionally restricted spin labels, suggesting an overall sterically packed environment of M2. Furthermore, a number of pore-facing residues revealed broadening by short-range dipolar coupling arising from intersubunit proximity. Under acidic conditions, residues beyond the 9' position showed a dramatic decrease in broadening and concomitant increase in amplitude, demonstrating that residues in this region move farther away from each other (Fig. 5). To quantify through-space intersubunit spin–spin dipolar coupling, we calculated the Ω parameter (Perozo, Cortes, & Cuello, 1999) as the ratio of spin-normalized amplitudes of the central resonance line in the desensitized and closed conformations. The Ω values lower than 1 indicate that the spin-labeled residues move closer to the symmetry axis, while values larger than 1 reflect motions away from the axis. The largest change in Ω was observed at the extracellular end of M2 in the region lined by hydrophobic residues (10'–20') and suggests that this region tilts away radially during channel opening. In clear contrast, residues at the intracellular end (–4'–1'), comprising the selectivity filter region showed no major change in Ω values, suggesting that this region does not undergo significant movement. Interestingly, the region immediately above (residues 4'–9', consisting mostly of polar residues) shows a decrease in Ω , presumably resulting from these residues moving closer to the pore axis. The polar region may therefore undergo a tighter packing during desensitization and thereby contribute to the desensitization gate of the channel. Consistent with this idea, bulky pore blockers such as lidocaine slow desensitization in GLIC presumably by a “foot-in-the-door” effect (Velisetty & Chakrapani, 2012), and small hydrophilic substitution at the 9' position also decreases the rate and extent of desensitization (Gonzalez-Gutierrez & Grosman, 2010). Interestingly, in the GABA structure, M2 tapers downward toward –2', where the narrowest point is 3.15 Å (Miller & Aricescu, 2014). The compression of the intracellular portion has been proposed to be a region of pore occlusion in the desensitized state, although this mechanism might be unique to GABA_A β 3 receptors. In contrast to ELIC, the helical packing in GLIC in the closed conformation creates a deep water-accessible vestibule behind M2 that extends roughly to the middle of the membrane (closer to the 2' position)

(Fig. 6). These vestibules have been implicated in anesthetic and alcohol modulation in this class of channels (Howard et al., 2011; Mascia, Trudell, & Harris, 2000; Nury et al., 2011; Weng, Yang, Corringier, & Sonner, 2010; Williams & Akabas, 1999). The EPR data of GLIC at acidic pH show general similarities with the overall arrangement of M2, as seen in the crystal structures, especially in the wide-open extracellular and tightly packed intracellular end (Bocquet et al., 2009; Hilf & Dutzler, 2009). A similar pore profile is also seen in GluCl_o, GABA_A β 3, and 5-HT₃. Overall, these results indicate that the activation gate is at the extracellular end of M2 and channel activation involves an outward motion of this region. This model for activation was in remarkable agreement with the subsequently solved GLIC structure at neutral pH that showed a narrow extracellular vestibule (Sauguet et al., 2014).

Ligand-binding events at the ECD are transduced to the M2 gate through the ECD–ECD and ECD–TMD interfacial regions (Fig. 3). The ECD–TMD interface is framed by tightly coupled interactions between the β 6– β 7 (Cys-loop), the M2–M3 linker, and the β 1– β 2 loop. In the closed conformation, residues within these loops were found to be sterically constrained and solvent occluded (Velisetty et al., 2014). In the desensitized conformation, these interactions were replaced by extensive accessibility to O₂ and NiEDDA. A decrease in dipolar broadening for labels at the N-terminal end of M2–M3 was indicative of large outward movement of the M2–M3 linker away from the pore axis. An outward motion of the M2–M3 segment (5 Å away from the channel pore) is evident in GLIC structures (Sauguet et al., 2014). Consistent with this idea, the tip of the M2–M3 segment was found to be disordered in 5HT₃ structures, indicating a high degree of flexibility of this region (Hassaine et al., 2014). In addition to these movements, there was an overall increase in probe dynamics and O₂ accessibility for residues lining the intrasubunit cavity (four-helix TM bundle), which would indicate an expansion of the intrasubunit cavity upon activation. The strategic position and increased membrane interaction of the β 6– β 7 linker suggested a downward movement into this cavity interacting with tightly associated membrane lipids. These protein motions may allow general anesthetics to access the intrasubunit upon channel activation and thereby play a role in the state-dependent binding of allosteric modulators.

The widely probed regions of the ECD–ECD interface are the β 9– β 10 (loop C) and β 8– β 9 (loop F). Studies in AChBP and LGIC have shown that agonists affect the mobility of loop C at the binding site and elicit closure of the binding cavity via capping this loop. This movement has been proposed to be the initial conformational change during channel activation (Celie et al., 2004; Hansen et al., 2005; Mukhtasimova, Lee, Wang, & Sine, 2009). Even though GLIC does not have a canonical ligand-binding site, EPR line-shape changes and accessibility profiles are suggestive of a pH-dependent immobilization of loop C (Velisetty & Chakrapani, 2012). Such enhanced interaction across the ECD subunit interface was also noted in the β 8– β 9 loop on the complimentary face. Extensive movement of β 8– β 9 has been detected in AChBP and members of the LGIC (Hansen et al., 2002; Hansen, Sulzenbacher, Huxford, Marchot, Taylor, & Bourne, 2005; Hanson & Czajkowski, 2008; Khatri, Sedelnikova, & Weiss, 2009; Lyford, Sproul, Eddins, McLaughlin, & Rosenberg, 2003; Pless & Lynch, 2009; Thompson, Padgett, & Lummis, 2006). Intersubunit distance measurements by DEER for a key position in β 8– β 9 revealed a large inward motion (~9 Å) during activation (Dellisanti et al., 2013). Interestingly, the study noted the absence

of $\beta 8$ – $\beta 9$ movements when the channel was in detergents. Overall, these studies provide a detailed view of the dynamic changes with the TM helices and the subunit interfaces during gating, and how they relate to the conformations in the currently available structural models.

4. FUTURE DIRECTIONS

In combination with other structural techniques, SDSL–EPR spectroscopy has enhanced our understanding of conformational dynamics in large and complex systems. Several recent technological innovations have been geared toward improving sensitivity and extending the upper limit of distance determination (~ 80 – 100 Å) (Borbat, Georgieva, & Freed, 2013; Ghimire, McCarrick, Budil, & Lorigan, 2009; Hubbell et al., 2013; Lovett, Lovett, & Harmer, 2012; Ward et al., 2010). DEER measurements with a Q-band (35 GHz) spectrometer have substantially improved sensitivity with smaller sample requirements and shorter data collection times (Ghimire et al., 2009). Improved pulse protocols have helped achieve longer dipolar evolution times and hence an increase in distance resolution and measurable distances (Borbat et al., 2013; Ward et al., 2010). Multifrequency EPR approaches have the capabilities to resolve fast and slow dynamics corresponding to internal and overall motions, respectively (Nesmelov & Thomas, 2010; Zhang et al., 2010). Recent use of Nanodiscs (nanoscale bilayers surrounded by a belt of amphipathic protein derived by apolipoprotein A1; Bayburt & Sligar, 2010) has shown remarkable improvement in DEER sensitivity by decreasing background contribution that arises from intermolecular dipolar interactions. The next rapidly emerging areas are to measure dynamics over the entire range of protein motions (picosecond to millisecond timescales) and to further extend the determination to physiological temperatures (Hubbell et al., 2013). Furthermore, for systems where mutating native cysteines is not feasible (as is the case for LGIC), alternative nitroxide side chains based on site-specific incorporation of unnatural amino acids have been introduced (Fleissner et al., 2009). The recent structures of mammalian channels have ushered in a new era of structural biology, and the stage is set for an in-depth understanding of structural dynamics of these complex channel systems.

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Figure 1.

A comparison of spectral line-shape and residue-specific environmental parameters in the KvAP full-length (FL) channel, and the isolated VSD. (A) An overlay of representative X-band CW EPR spectra of spin-labeled mutants from S1 and S4 in the full-length channel (black) and the isolated VSD (red (light gray in the print version)). The green (gray in the print version) arrow head shows the location of immobile component of the spectra. Distinct differences in line shapes can be noted for positions in S1 that reflect increased mobility for side chains in the isolated VSD. The spectra for S4 are identical in the two constructs. (B) Profile of changes in the normalized O_2 accessibility. The arrow head marks the break in the α -helical periodicity of the accessibility. Positions of the gating charges in S4 are highlighted by ovals. While there are minimal changes in the membrane exposure in S4, dramatic differences in S1 clearly proves that interaction of the VSD with the pore occurs via S1 rather than S4. *This figure is adapted from the originally published data in Chakrapani et al. (2008).*

Figure 2.

Mapping the molecular interacting surface of the VSD with the PD. Fractional differences in ΠO_2 mapped on the sensor structure and color coded with a gradient from red–white–blue. Red denotes increase and blue represents decrease in the environmental parameter. The surface of highest impact (S1 and S2) is marked by dotted black line. (B) Evaluation of the open-state structures for the K_v channels. The delta values mapped on to the coordinates of Kv1.2–Kv2.1 chimera structure (2R9R.pdb). A view from the intracellular part of the channel is shown. The EPR data from KvAP in the open-inactivated state predict a rotation of the VSD (~ 70 – 100°) to place S1 and S2 in the proximity of the pore as shown in the cartoon. *This figure is adapted from the originally published data in Chakrapani et al. (2008).*

Figure 3. GLIC structures at neutral and acidic pH. An alignment of two adjacent subunits from GLIC structures representing putative closed and open states. The regions contributing toward the ECD-ECD and ECD-TMD interfaces are labeled.

Figure 4. Functional reconstitution of GLIC in asolectin membranes. Macroscopic current elicited in response to pH jumps from excised inside-out patch under symmetrical 150 mM Na^+ at -50 mV holding membrane potential. GLIC shows rapid activation (10–30 ms) followed by desensitization (1–3 s) that results in 10–15% steady-state currents. *This figure is adapted from the originally published data in Velisetty and Chakrapani (2012).*

Figure 5.

Changes in the spin label proximity during gating. (A) pH-dependent conformational changes at the putative activation gate L241 (highlighted in the GLIC crystal structure. Only diagonal subunits are shown). Spin-normalized EPR spectra show changes in amplitude and extent of broadening (indicated by arrows) revealing pH-dependent changes in proximity at this position in M2. (B) Profile of changes in the amplitude of the EPR signal (Ω) reveals differences in the spin–spin dipolar coupling in the closed and desensitized conformation. The $\log \Omega$ parameter mapped on to the GLIC crystal structure. Residues displayed in red (gray in the print version) and blue (dark gray in the print version) show positive and negative $\log \Omega$ values, respectively. Residues colored in white show values closer to 0. The boxed areas highlight regions moving away from the pore axis ($\log \Omega > 0$), getting closer toward the pore axis ($\log \Omega < 0$), and regions of no significant movement ($\log \Omega \sim 0$). *This figure is adapted from the originally published data in Velisetty et al. (2012).*

Figure 6.

Aqueous vestibules in the GLIC closed state. (A) Periodicity of IINiEDDA values for the M2 residues in the closed state. (B) Helical wheel representation of IINiEDDA values superimposed in a polar coordinate. The resultant vector points toward the more solvent accessible face of the helix; in this case, the surface of M2 away from the pore lumen. The shaded area within the dashed lines highlights the projection for complete set of accessibility data relative to the maximal accessibility vector. (C) IINiEDDA values mapped on the GLIC closed structure. *This figure is adapted from the originally published data in Velisetty et al. (2012).*