COMMENTARY

Direct visualization of ion-channel gating in a native environment

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Pentameric ligand-gated ion channels (pLGICs), also known as Cys-loop receptors, are localized primarily in the postsynaptic membranes, and mediate fast chemical transmission in the central and peripheral nervous systems. Binding of neurotransmitter activates these receptors, causing changes in postsynaptic membrane potential and consequently modulation of neuronal or muscle activity. pLGIC functions are altered by a variety of drugs, making them significant pharmaceutical targets. Indeed, the rise in pLGIC crystal and cryoelectron microscopy structures underscores the magnitude of research efforts that have gone into unraveling the molecular details of channel function. Current structure determination methods of pLGICs capture stationary images and, most often, in nonnative environments. As a result, gaps remain in our understanding of the dynamic properties, intermediate conformational states, and the energetics of gating transitions. In PNAS, Ruan et al. (1) seek to probe some of these missing links using high-speed atomic force microscopy (HS-AFM) by directly visualizing the pLGIC gating process in a membrane environment and under conditions that mimic physiological buffer, temperature, and pressure.

The Cys-loop family in vertebrates includes the cationic acetylcholine receptor (nAChR) and serotonin receptor ($5HT_{3A}R$) and the anionic γ-aminobutyric acid receptor ($GABA_AR$) and glycine receptor (GlyR). Homologs of pLGICs include invertebrate members (2), as well as a growing number of prokaryotic members, including the *Erwinia chrysanthemi* ligand-gated ion channel (ELIC) and *Gloeobacter violaceus* ligand-gated ion channel (GLIC) (3). In general, pLGICs share a conserved architecture (despite low sequence identity) where five identical or homologous subunits are pseudosymmetrically arranged around an ion-conducting pore. Each subunit has an N-terminal extracellular domain (ECD) that binds neurotransmitters, a transmembrane domain (TMD) with four membrane-spanning helices (M1–M4) with M2 helices lining the central pore, and a cytosolic intracellular domain in eukaryotic channels formed by the M3–M4 loop (4).

Impact of Membrane Environment on Channel Gating and Dynamics

Interestingly, high-resolution pLGIC structures solved thus far are in nonnative (detergent) environments, leading to speculations on the possible alteration of channel structures in the absence of membranes. Membrane composition has been shown to be critical for channel gating of the nAChR (5) and possibly other eukaryotic channels. GLIC has served as an archetypal pLGIC because of the overall conservation of its architecture and pharmacological properties (6). GLIC crystal structures in the open and resting conformations were the first high-resolution pLGIC structures available (7–9). Importantly, the global concerted movements resulting in opening of the channel pore in GLIC are comparable to the gating mechanism emerging from recent eukaryotic pLGIC structures. This conservation is remarkable considering that GLIC is activated by protons rather than a neurotransmitter, and the gating kinetics are approximately two orders-of-magnitude slower in comparison.

The crystal structures of GLIC solved at pH 7.0 and pH 4.0 reveal distinct pore profiles at the level of pore-lining M2, clearly revealing changes leading to channel opening (7). However, conformational changes at the ECD and most notably at the peripheral TM helices are subtle. This is surprising because global structural movements are implicated in occurring that couple the conformational changes across the ECD and TMD to facilitate gating. Consistent with this idea, electron paramagnetic resonance spectroscopic measurements of membrane-reconstituted GLIC show that the lipid-sensing M4 undergoes an outward motion away from the fivefold axis in transition from the closed to the open state (10). While GLIC may not be stringent on membrane composition (11) compared with eukaryotic channels, the importance of a membrane environment on channel dynamics cannot be overstated. Crystallographic constraints and cryofreezing in a detergent environment have the potential to mask dynamic properties of channels, making unambiguous mechanistic interpretations a challenging task in the absence of appropriate complementary approaches.

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In light of these observations, Ruan et al. (1) employed HS-AFM to probe the conformational changes of GLIC in a near-native membrane environment and physiological buffer conditions. AFM is a tool for imaging biological samples in aqueous solution upon attachment to a substrate. A short cantilever with a probe oscillates vertically near its resonance frequency, briefly touching the surface of the sample, and the force between the sample and the probe is then measured (12). GLIC was solubilized in detergent and reconstituted at lipid–protein ratios, which produced densely packed vesicles where GLIC molecules were predominantly in an outside-out orientation. The sample was then injected on to an HS-AFM sample support. Titration experiments were performed where the HS-AFM fluid chamber was coupled to a high-precision buffer exchange system starting with pH 3.4 buffer and then pH 7.5 buffer and back to pH 3.4 buffer using multiple syringes. HS-AFM continuously recorded conformational changes from the same ∼70 molecules during the real-time titration. A caveat with contact AFM is the potential for damage as the probe scans the topography of the sample. Ruan et al. (1) are careful about this limitation and explicitly show that the topography of GLIC molecules at the beginning and end of the titration experiments at pH 3.4 was similar, and it is comparable to the open GLIC crystal structure (9).

Ruan et al. (1) report fascinating reversible supramolecular rearrangements and single-molecule conformational changes of GLIC channels during pH titration. It is important to note that HS-AFM images present only a top view of the channels and any implied changes in the TMD can only be alluded to, perhaps, based on the dramatic modifications in the ECD. Nevertheless, it is very interesting that the supramolecular arrangement of reconstituted channels is akin to the acetylcholine receptor in the *Torpedo* postsynaptic membrane captured by cryoelectron tomography (13) in the absence of ligand. The nAChRs are highly clustered at synaptic membranes due to the scaffolding protein rapsyn. However, it would seem that even in an artificial system with channel densities less than what is seen in situ, the overall assembly is still similar, possibly due to the conserved architecture between the channels. GLIC at pH 3.4 showed dense packing with at least six interacting angles of five nearest neighbors arranged in higher-order patterns. Ruan et al. (1) suggest that this pattern of interaction reflects favorable protein–protein contacts denoting positive cooperative gating. A physiological relevance of positive cooperative gating would be in the amplification of postsynaptic signals. Furthermore, the inclination to think that channels gate independently may not be entirely correct, at least for some systems. In fact, intermolecular interactions in gating have been described in a number of ligand and voltage-gated ion-channel types (14–16). In the case of ligand-gated ion channels, an allosteric mechanism has been put forward where the conformational changes associated with channel opening of one channel will influence conformational changes in the neighboring channels perhaps by altering ligand-binding affinity (14). Through HS-AFM, Ruan et al. (1) show for the first time direct imaging of allosteric changes spatio-temporally. Videos are recorded for about an hour and in that time frame we see changes in the ECD and lateral diffusion of channels in titrations from low to high pH buffers.

New Insights From Noncanonical Extracellular Domain Changes?

A question that remains to be answered, however, is whether the reversible conformational changes evident are functionally coupled to gating. At the single-molecule level, titrations with pH 3.4 buffer show channels with a diameter akin to the activated GLIC structure (9). At this low pH, HS-AFM shows the ECDs of individual GLIC subunits exhibiting fivefold symmetry with a visible pore that is similar to other active/desensitized pLGICs. As the titration changes to pH 7.5, the channels have collapsed ECDs that appear to shut the pore. These ECD changes are accompanied by channels having a significantly larger diameter. Titration back to low pH shows a notable asymmetry of the channels, which Ruan et al. (1) suggest may correspond to an intermediate state where some of the subunits have undergone activating conformational changes and others have not. A preactive intermediate conformation for GLIC has been described where the ECD undergoes activating changes while the TMD remains closed to ion permeation (17). The low resolution of HS-AFM does not allow us to decipher the molecular details of the striking ECD conformational changes and the possible coupling to the TMD, which continue to be of fundamental interest. The increase in channel diameter from the activated/desensitized state to the closed state is a curious observation, which Ruan et al. (1) propose is likely to correspond to an outward movement of the extracellular end of the TM helices. This result is opposite to the current view of pLGIC gating, where the TMD helices (mainly M2 but also M4 in some structures) expand away from the pore axis from closed to activated conformations (9, 18). The ECD changes at neutral pH also deviate from the GLIC crystal structure under similar conditions (7) where the ECDs are upright, and display fivefold symmetry with an apparently visible pore. Overall, these results differ from the existing pLGIC structures, where transition from closed to open conformations shows ECDs that are loosely packed with relatively few intersubunit contacts to more compact arrangements with increased subunit–interface contacts, respectively (19).

Ruan et al. present several new insightful features of GLIC gating, some of which differ from mechanisms proposed based on pLGIC cryoelectron microscopy and crystal structures.

In summary, Ruan et al. (1) present several new insightful features of GLIC gating, some of which differ from mechanisms proposed based on pLGIC cryoelectron microscopy and crystal structures. A future direction of this study would be to functionally identify mutations and experimental conditions, such as changing lipid compositions, which uncouple agonist binding to gating as was done for the nAChR (20). Visualization by HS-AFM of such samples would then provide a clearer picture of what these physical changes mean in light of channel gating.

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