Desensitization Mechanism in Prokaryotic Ligand-gated Ion Channel

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Background:
GLIC, a prokaryotic homologue of pentameric ligand-gated ion channel (LGIC), is activated by protons, and crystal structures suggest a putative open conformation. 

Results:
GLIC function characterized in proteoliposomes reveals rapid activation and slow desensitization. Desensitization is modulated by voltage, blockers, and membrane cholesterol.

Conclusion:
GLIC desensitization shows many hallmark features of the mechanism in LGIC.

Significance:
Understanding GLIC desensitization is an important step toward structure-function characterization of LGIC.

Crystal structures of *Gloeobacter violaceus* ligand-gated ion channel (GLIC), a proton-gated prokaryotic homologue of pentameric ligand-gated ion channel (LGIC) from *G. violaceus*, have provided high-resolution models of the channel architecture and its role in selective ion conduction and drug binding. However, it is still unclear which functional states of the LGIC gating scheme these crystal structures represent. Much of this uncertainty arises from a lack of thorough understanding of the functional properties of these prokaryotic channels. To elucidate the molecular events that constitute gating, we have carried out an extensive characterization of GLIC function and dynamics in reconstituted proteoliposomes by patch clamp measurements and EPR spectroscopy. We find that GLIC channels show rapid activation upon jumps to acidic pH followed by a time-dependent loss of conductance because of desensitization. GLIC desensitization is strongly coupled to activation and is modulated by voltage, permeant ions, pore-blocking drugs, and membrane cholesterol. Many of these properties are parallel to functions observed in members of eukaryotic LGIC. Conformational changes in loop C, measured by site-directed spin labeling and EPR spectroscopy, reveal immobilization during desensitization analogous to changes in LGIC and acetylcholine binding protein. Together, our studies suggest conservation of mechanistic aspects of desensitization among LGICs of prokaryotic and eukaryotic origin.

Pentameric ligand-gated ion channels (LGIC)\(^2\), also called Cys-loop receptors, mediate fast neurotransmission in the central and peripheral nervous systems and thereby regulate excitability, neurotransmitter release, and muscle contraction (1–8). The vertebrate members of this family include receptors activated by acetylcholine (ACh), serotonin (5-HT), glycine (Gly), and GABA. Mutations leading to channel dysfunctions have been associated with a number of pathological conditions, including Alzheimer and Parkinson disease, schizophrenia, epilepsies, myasthenia gravis, and congenital myasthenic syndromes, and have also been implicated in nicotine addiction and lung cancers (5).

Several diverse sources have contributed toward our current structural understanding of the LGIC family. In particular, the most comprehensive view of the entire channel comes from a 4-Å-resolution structural model of nicotinic acetylcholine receptor (nAChR) generated using cryo-electron microscopy data from the electric organ of *Torpedo marmorata* (9). Because this structure is in the absence of a ligand, it likely represents the closed conformation. In addition, high-resolution structures of the acetylcholine binding protein (AChBP), a homologue of the ligand-binding domain of nAChR, in the presence of different agonist and antagonist have yielded a detailed description of the conformational changes associated with ligand binding (10–12). Further, recent discovery of bacterial homologues of the LGIC family of channels (13) have paved way to determination of high-resolution crystal structures of channels in multiple conformational states and have provided a valuable structural framework for eukaryotic LGIC function (14–16). Although these channels lack certain conserved features found in the eukaryotic LGIC, including the large intracellular domain and the disulfide-bonded cysteine pair in the Cys loop, there is remarkable conservation of the overall fold in the 8-sandwich extracellular domain (ECD) and in the helical transmembrane domain (TMD). When expressed in eukaryotic cells, the *Gloeobacter violaceus* homologue (GLIC) was found to be activated by protons (17) and the *Erwinia chrysanthemi* homologue (ELIC) by primary amines (18). The crystal structures of ELIC in the absence of a ligand (15) and GLIC at an acidic pH (14, 16) reveal distinct conformational changes in the ECD and TMD and are thereby proposed to represent the closed and open conformations, respectively. Furthermore, the recently solved crystal structure of the invertebrate glutamate-activated chloride channel (GluCL) in the activated conformation revealed the binding site for positive modulator ivermectin with the overall conformation of the channel similar to GLIC (19).

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\(^{2}\)The abbreviations used are: LGIC, ligand-gated ion channel; ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; AChBP, acetylcholine binding protein; ECD, extracellular domain; TMD, transmembrane domain; GLIC, *Gloeobacter violaceus* ligand-gated ion channel; ELIC, *Erwinia chrysanthemi* homologue; NIEDDA, Ni(II) ethylenediaminediacetic acid.
Activation and Desensitization Gating in GLIC

Besides structural homology, GLIC displays ionic selectivities typical of nAChRs with a similar permeability for Na⁺ and K⁺ but no permeability for Cl⁻ ions (17). Analogous to findings in nAChR, GLIC is blocked and modulated by long- and short-chain alcohols, general anesthetics, and other clinically relevant drugs (20–22). GLIC structures with propofol and desflurane reveal putative binding sites that are in agreement with functional measurements in nAChR (20). A functional chimera of GLIC-ECD with glycine receptor TMD yields anionic channels that are activated by pH (23), whereas a chimera of GLIC with the ICD of serotonin receptor functions as a pH-activated channel inhibited by chaperone protein RIC-3 (24). In both cases, the chimeras retain the functional properties of the individual domains, suggesting that the pathway for allosteric communication between the ECD-TMD-IMD underlying ligand binding and channel gating are essentially conserved across prokaryotic and eukaryotic channels.

Understanding the molecular nature of the allosteric mechanism that couples agonist binding at the ECD to channel gating within the TMD is at the heart of the LGIC field. However, drawing direct insights from bacterial homologues has been complicated by the overall uncertainty in assigning functional states to the conformational states depicted by the ELIC and GLIC crystal structures. The structures of ELIC and GLIC are proposed to be the closed and the open conformation (14, 15) on the basis of functional measurements in heterologous expression systems where channels were shown to display non-decaying macroscopic currents in response to pH changes (14, 16, 17, 22, 25). However, two groups report that GLIC desensitizes, albeit with very different kinetics (26, 27). A systematic characterization of GLIC gating would therefore be essential to determine how its functioning compares to the eukaryotic LGIC and also to directly correlate crystallographic structural snapshots with functional conformational states of the channel.

In this study we address the conformational states of GLIC by directly measuring the functional properties of the purified and reconstituted channels in proteoliposomes through patch-clamp recordings. Our findings reveal several fundamental properties of GLIC gating and modulation by pH, voltage, permeant ions, blockers, and membrane lipid composition. We also investigate the structural correlates of the underlying conformational transition by site-directed spin labeling and EPR spectroscopy of residues comprising of loop C (connecting strands β9 and β10 in the ECD). These findings provide insights into the functioning of GLIC and thereby aid in pairing up structural data with functional measurements.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—The GLIC gene cloned into a modified pET26b vector was expressed as a fusion construct with N-terminal maltose binding protein as described previously (14, 16). Briefly, BL21 (DE3) Escherichia coli cells transformed with the construct were grown in Terrific Broth medium containing 50 μg/ml kanamycin at 37 °C to an A₆₀₀ of 1.0. Cells were induced with 0.2 mM isopropyl 1-thio-β-D-galactopyranoside overnight at 20 °C. Membranes were prepared by homogenizing the cells in 150 mM NaCl, 20 mM Tris base (pH 7.4) (buffer A) with protease inhibitors and centrifuged at 100,000 × g for 1 h. Membranes were solubilized in buffer A using 40 mM dodecyl-β-D-maltoside (Anatrace) at 4 °C. GLIC was purified by binding to amylose resin and eluting with 20 mM maltose. The maltose binding protein tag was cleaved with human rhino virus (HRV) 3C protease (GE Healthcare), and the GLIC protein was separated using size exclusion chromatography on a Superdex 200 10/300 column (GE Healthcare).

**Fluorescence Studies**—To determine whether GLIC aggregated on the membrane, the native cysteine (Cys-26) was purified and labeled at a 10:1 (label/channel) molar ratio with either fluorescein-maleimide or tetramethylrhodamine-maleimide (Molecular Probes). The labeled proteins were mixed in equal proportion and reconstituted into preformed liposomes. We tested the time course of aggregation in three lipid systems: asolectin, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine: 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (sodium salt) (3:1 molar ratio), and E. coli polar extract. The protein-to-lipid reconstitution ratio was 1.750 molar, which is three times that used for EPR studies. Fluorescence measurements were made on a Horiba FluoroMax-3 spectrophotometer. The excitation wavelength was set at 480 nm, and the emission spectra were collected from 460 nm to 660 nm. An increase in the amplitude of rhodamine emission (measured at 565 nm) because of FRET was used as an indicator of lateral aggregation of GLIC on the membrane.

**Membrane Reconstitution and Electrophysiology**—Electrophysiological measurements were made by patch clamp recordings in channel-reconstituted liposomes prepared as described earlier (28–30). Purified protein was reconstituted into preformed asolectin vesicles by diluting in 150 mM NaCl, 10 mM HEPES (pH 7.0) (reconstitution buffer). Detergent was removed by incubating the proteoliposome suspension with biobeads (Bio-Rad). The suspension was centrifuged at 100,000 × g for 1 h, and the pellet was resuspended in reconstitution buffer. A drop of the proteoliposome was placed on a glass slide and dried overnight in a desiccator at 4 °C. The sample was then rehydrated with 20 μl of buffer, which yielded giant liposomes. GLIC was reconstituted in 1:10000 protein:lipid (molar ratio) for macroscopic currents. Currents were measured using an inside-out patch clamp of proteoliposomes in symmetrical NaCl unless stated otherwise. All experiments were performed at room temperature. Recording pipettes were pulled from thin-walled borosilicate glass and heat-polished to a resistance of 1.5–2 MΩ and filled with 150 mM NaCl, 10 mM HEPES (pH 8.0). A low pH was obtained using 10 mM sodium citrate buffer. Currents were elicited in response to pH jumps using an RCS-200 fast solution exchanger (switch time, 2 ms) fed by gravity (Biologic). Currents were measured using Axopatch 200B digitized at a 10-kHz sampling frequency and were analyzed using clampfit 10.2.

**Site-directed Spin Labeling and EPR Spectroscopy**—The native Cys (Cys-26) was mutated to Ser, and single Cys mutants in loop C were generated using the Cys-free construct (C26S) as the template. Gel filtration chromatography was used to ensure that the mutants did not change the hydrodynamic behavior of the channel. Purified protein was labeled with a methanethiosulfonate spin probe (1-oxyl-2,2,5,5-tetramethylpyrrolidin-3-yl) methyl methanethiosulfonate (Toronto Research) at a 10:1...
label:protein molar ratio and reconstituted at a 1:3000 protein:lipid (molar ratio) in a mixture of asolectin. It is likely that there is a variable degree of labeling for different positions on the basis of the location and exposure of the side chains. However, the spin labels at each of these positions in loop C are beyond the range of distance (> 20 Å) to cause dipolar broadening in a continuous-wave EPR spectroscopic measurement, and, therefore, the number of labels on the pentamer do not affect the spectral properties. Continuous-wave EPR measurements were performed at room temperature on a Bruker EMX X-band spectrometer equipped with a dielectric resonator and a gas-permeable TPX plastic capillary. First derivative absorption spectra were recorded at an incident microwave power of 2.0 milliwatt, a modulation frequency of 100 kHz, and a modulation amplitude of 1.0 G. The spectra were normalized to the number of spin in the sample. The line shape of EPR spectra reflects the mobility of the spin probe at a particular position and is strongly correlated to the location and tertiary fold of the protein (31). The solvent accessibility of the spin label is accessed by the collisional frequency to molecular oxygen and NiEDDA (100 mM) (32–34). The accessibility parameter (II) 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 (32).

RESULTS

Membrane Orientation in Proteoliposomes and pH-dependent Activation of GLIC

GLIC was expressed and purified as described previously (14, 16) and reconstituted in preformed liposomes. We used FRET to determine whether GLIC aggregated in reconstituted vesicles, i.e. whether independent GLIC molecules came together within a distance < 50 Å on the membrane. This technique allows us to find the most suitable membrane composition that maintains a monodisperse population of channels. The native Cys (Cys-26) in the ECD of GLIC was labeled with fluorescein-maleimide and tetramethylrhodamine-maleimide (35–37) and reconstituted in E. coli polar lipids, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine:1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (sodium salt) (3:1), or asolectin (Fig. 1A). Fluorescence measurements show that GLIC reconstituted in asolectin showed no FRET (Fig. 1A), and even freezing/thawing the sample (conditions known to promote aggregation) produced minimal change in the FRET levels (Fig. 1A, right panel). Therefore, asolectin was chosen for reconstitution for both functional and dynamic measurements.

To characterize functional properties of purified GLIC, we carried out patch clamp recordings from inside-out patches in reconstituted proteoliposomes. Upon jumps to acidic pH using a rapid solution exchanger, GLIC was seen to activate within milliseconds (~10 ms) and, in the continued presence of a low pH, to slowly desensitize within seconds (1–3 s) (Fig. 1B). Although channels on the membrane were sensitive to changes in the bath pH, there was no effect of pH change in the pipette solution (data not shown), suggesting that GLIC was predominantly oriented in one direction so that in inside-out patches the extracellular domain faced the bath/solution exchanger.

We measured the pH dependence of activation by eliciting currents at various pH values. Fig. 2A shows representative macroscopic current traces of GLIC in symmetrical 150 mM Na⁺ solutions at ~50 mV holding potential. The amplitude of the peak current decreased progressively as the activating pH was made more basic so that there were very small currents beyond pH 4.0. A plot of peak amplitudes with respect to pH, fit to a Hill equation, yields a pH 50 of 2.90 ± 0.07 with a positive cooperativity of nH 1.2 ± 0.2 (Fig. 2A, inset). The rising phase of the macroscopic is sigmoidal with a significant pH dependence (Fig. 2B). Deactivation measured after applying a short pulse of pH 2.5 is also sigmoidal (Fig. 2C). GLIC activates and deactivates with a time constant (time required for the current to reach half its maximal value) of 11 ± 1.6 and 37 ± 14 ms, respectively. In comparison, GLIC deactivates much faster than desensitization (~10-fold).
Activation and Desensitization Gating in GLIC

To measure the effect of pH on desensitization, currents were elicited in response to pH jumps at −50 mV. The time constant of desensitization was measured by a monoexponential fit to the macroscopic current decay (Fig. 3A, left panel). GLIC desensitization increased with decreasing pH so that τ changed from 1.6 ± 0.8 s at pH 2.0 to 10.8 ± 1.2 s for pH 3.25 (Fig. 3A, right panel). For currents measured at a more basic pH, the current decay was longer than 10 s. We then asked whether channels also tend to desensitize in the pH range where we could not elicit measurable macroscopic currents. We first applied a pH 2.5 control pulse to estimate the number of channels that allowed the currents to reach its steady-state value. This was followed by a variable interpulse interval at pH 8.0. The number of channels that recovered during this interval of time in pH 8.0 was estimated by a second pulse to pH 2.5. The fractional recovery (Ipeak/Imax) plotted as a function of interpulse interval and fit to monoexponential yields a τ recovery of 0.3 ± 0.02 s (Fig. 4B). Overall, these results show that GLIC desensitization is favored under conditions of enhanced channel opening, whereas recovery from desensitization is favored during deactivation. In comparison to measurements in heterologous expression systems (14, 16, 25, 27, 38), GLIC gating in proteoliposomes is more rapid. Further, the pH50 of activation in our membrane reconstituted system is shifted by approximately two units from measurements in eukaryotic cells. We believe that this variation might arise because of differences in membrane lipid compositions, particularly because ionizable residues within the transmembrane segment have been shown to alter pH dependence of the channel (38).

Modulation of Desensitization

Voltage-dependence—We studied the effect of voltage on desensitization kinetics of GLIC by eliciting currents in response to pH jumps from 8.0 to 2.5 at different membrane potentials (Fig. 5A). For both depolarizing and hyperpolarizing potentials, channels were seen to desensitize much more rapidly at larger voltages. However, the key difference is the amplitude of steady-state current (a reflection of recovery from desensitization), which increases as the membrane is made more depolarized. Therefore, entry into desensitization is favored at hyperpolarizing potential, whereas recovery is enhanced at depolarized potentials. A plot of Isteady-state/Ipeak versus voltage fit to a Boltzmann function yields a z value of 1.2 ± 0.19 and v1/2 of desensitization of 20.4 ± 5.6 mV (Fig. 5B).

Permeant Ions—Crystal structures of GLIC in the presence of various permeant ions reveal distinct binding sites for monovalent and divalent cations (Fig. 6A) (39). Divalent ions were found at the intracellular pore entry, whereas monovalent cations were further within the pore. We measured the effect of both monovalent and divalent permeant ions on desensitization using bi-ionic conditions. Inward currents were measured with 150 mM external concentration of monovalent cations or 75 mM divalent cation and 145 N-methyl glucamine (NMG)/5 mM Na+ in the pipette (Fig. 6B). There is no difference in the amplitude of peak currents with different permeant ions, consistent with GLIC being non-selective across monovalent cations.

cally no change in the amplitude of the current, but as the duration increased, more and more channels were lost, suggesting that channels desensitized during the prepulse (Fig. 3B, right panel). Therefore, GLIC desensitizes even in the proton concentration range that does not evoke a measurable ionic response.

At steady-state GLIC shows very little current, revealing a slow recovery from desensitization in the ligand-bound state and that the predominant mode of recovery is through deactivation of the channel (with increase in extracellular-bound pH). To study the time course of recovery during deactivation of the channel, we used a double pH pulse protocol (Fig. 4A) where channels were first switched from pH 8.0 to 2.5 for a duration that allowed the currents to reach its steady-state value. This was followed by a variable interpulse interval at pH 8.0. The number of channels that recovered during this interval of time in pH 8.0 was estimated by a second pulse to pH 2.5. The fractional recovery (Ipeak/Imax) plotted as a function of interpulse interval and fit to monoexponential yields a τ recovery of 0.3 ± 0.02 s (Fig. 4B). Overall, these results show that GLIC desensitization is favored under conditions of enhanced channel opening, whereas recovery from desensitization is favored during deactivation. In comparison to measurements in heterologous expression systems (14, 16, 25, 27, 38), GLIC gating in proteoliposomes is more rapid. Further, the pH50 of activation in our membrane reconstituted system is shifted by approximately two units from measurements in eukaryotic cells. We believe that this variation might arise because of differences in membrane lipid compositions, particularly because ionizable residues within the transmembrane segment have been shown to alter pH dependence of the channel (38).
Besides, there is also no difference in desensitization between various monovalent permeant ions. Further, we find that GLIC has a larger conductance for \( \text{Ca}^{2+} \) and that there is also a marginal decrease in desensitization (3301 ± 565 ms for \( \text{Na}^+ \) versus 4902 ± 683 ms for \( \text{Na}^+ / \text{Cd}^{2+} \)). Also, GLIC currents are blocked by \( \text{Cd}^{2+} \), and as compared with \( \text{Ca}^{2+} \), desensitization is slower (3301 ± 565 ms for \( \text{Na}^+ \) versus 4902 ± 683 ms for \( \text{Na}^+ / \text{Cd}^{2+} \)) (Fig. 6C). These findings show that monovalent cations do not alter desensitization, whereas divalents decrease desensitization, with the pore blockers causing a much stronger effect.

**Blockers**—GLIC currents are blocked by lidocaine, and crystal structures reveal a binding site between the 9' and 6' residues in the pore lining M2 helix (Fig. 7A). To test whether lidocaine binding affects desensitization in GLIC, we measured currents in the presence of varying lidocaine concentrations (Fig. 7B). In addition to a decrease in the amplitude of current, there is also a significant increase in the decay time of the current peak. To further investigate the effect of lidocaine in slowing desensitization, we applied a saturating concentration of lidocaine pulse (for varying durations) at the peak of the macroscopic current and evaluated the fraction of non-desensitized channels with a wash-off pulse (Fig. 7C). The loss of channels to desensitization was much smaller in the presence of lidocaine (dotted red lines) than in its absence (solid red trace). These results reveal that lidocaine binding indeed slows the overall desensitization rate of GLIC.

**Membrane Composition**—nAChR channels are well known to be extensively modulated by membrane lipid composition. The transitions between closed to desensitized conformation are abrogated in the absence of negative lipids and cholesterol (40). We wanted to test the effect of cholesterol on GLIC desensitization (because membrane vesicles already include negative lipids). We reconstituted GLIC in asolectin membranes with varying cholesterol concentrations (0–30 mol %). Although there was no measurable change in the rise time of activation, the desensitization increased remarkably with increase in the cholesterol content of the membrane (Fig. 8A). A plot of the time constant as a function of cholesterol levels reveals a linear correlation (Fig. 8B).

**Structural Correlates of the Desensitization Mechanism**

To determine whether the structural changes accompanying GLIC desensitization show similarity to molecular events in LGIC desensitization, we specifically probed loop C (connecting strands \( \beta 9 \) and \( \beta 10 \) in the ECD) (Fig. 9A). Loop C lies in the vicinity of the agonist-binding site in the LGIC, and the crystal structures of AChBP reveal distinct conformational changes in the agonist- and antagonist-bound states (10, 12). Six cysteine mutants covering loop C were created on a cysteine-free background. Individual mutants were purified, spin-labeled, and reconstituted in asolectin membranes and studied by EPR spectroscopy under conditions of pH 8.0 and pH 2.5. Because these
measurements are carried out at a steady state, the conformational state of GLIC at pH 8.0 and 2.5 corresponds to the closed and the activated desensitized state, respectively. Our analyses were centered on two types of dynamic EPR structural information: first, estimations of the spin probe motional freedom from the inverse of central line width of the first derivative absorption spectra ($1/\Delta H_0$). For a nitroxide spin probe in a motionally restricted environment, the line width is larger (and, hence, has a smaller $1/\Delta H_0$) compared with that of a spin probe in a location with high freedom of movement. Second,
the solvent accessibility (O2, NiEDDA) of the spin probe was evaluated by collisional relaxation methods. Fig. 9A shows the line shapes of spectra for residues in loop C in the closed (pH 8.0, black) and activated-desensitized conformation (pH 2.5, red). In the closed conformation (at pH 8.0), loop C residues reveal a wide range of spectral mobilities, from the immobile line shape of position 177 to the very mobile line shape at positions 178 and 179. At every position, the spectra contain two major resolved components, arising from relatively immobile (blue arrow) and mobile components (purple arrow), respectively. Such multicomponent spectra are indeed expected of spin labels at positions that are involved in tertiary interactions (31), as exemplified by the location of loop C in the subunit interface. Under the steady state of pH 2.5, in which GLIC is predominantly in its desensitized conformation, residues 175, 178, and 179 show remarkably immobile spectra, whereas residue 176 shows a small increase in mobility and residues 177 and 180 are mobile.
Activation and Desensitization Gating in GLIC

FIGURE 9. Conformational changes in loop C underlying desensitization. A, location of loop C in the crystal structure of GLIC. Comparison of the X-band continuous-wave EPR spectra of spin-labeled mutants in loop C in the closed (black, pH 8.0) and desensitized conformations (red, pH 2.5). The blue and purple arrowheads shows the location of immobile and mobile components of the spectra. B, residue-specific environmental parameters in the closed (black) and desensitized (red) states. Mobility $\Delta H_{\text{mob}}$ (top panel), $O_2$ accessibility $\Pi_{O_2}$ (center panel), and NiEDDA accessibility (INiEDDA, bottom panel).

remain essentially unaltered (Fig. 9A and supplemental Fig. 1). More specifically, for residues 175, 178 and 179, there is a significant change in the relative populations of the two components, namely an increase in the immobile component and a corresponding decrease in the mobile component (shown at higher resolution in the inset). These changes clearly highlight a structural transition in loop C during the closed to activated desensitized conformational transition. Analysis of the complete spectral data set for loop C is shown in Fig. 9B with probe mobility ($\Delta H_{\text{mob}}^{-1}$, top panel), $O_2$ accessibility ($\Pi_{O_2}$, center panel), and NiEDDA accessibility (INiEDDA, bottom panel). The pattern of mobilities across loop C at pH 8.0 also mirror variation in $O_2$ and NiEDDA collisional rates, i.e. residues that are very mobile are also solvent-exposed. The residues that undergo conformational change to a more constrained environment in the desensitized state also show a decrease in the $O_2$ accessibility. NiEDDA accessibility at pH 2.5 was not measured because of instability of the complex at a very acidic pH (42). Although our study precludes us from describing individual native side chain movements, the overall loop C motion during desensitization points toward greater immobilization.

DISCUSSION

Desensitization mechanisms in LGIC (46) are thought to play an important role in shaping synaptic transmission (47, 48) and, thereby, govern neural networks associated with memory and the learning process. More recently, their underlying role in non-neuronal cell function, such as cell growth control and modulation of immune response, has begun to emerge (49). Abnormalities in desensitization mediate several disease conditions, such as autosomal dominant nocturnal frontal lobe epilepsy and congenital myasthenic syndrome, and are also implicated in nicotine addiction and lung cancer (50–55). LGIC desensitization is modulated by a range of endogenous and exogenous factors, including lipids, $Ca^{2+}$, neurotransmitters, neuropeptides, and a several classes of drugs (reviewed in Ref. 56). In particular, positive allosteric modulators of nAChR that potentiate channel function by slowing desensitization are being developed as a possible therapeutic strategy in the treatment of Alzheimer disease, schizophrenia, depression, and pain (49, 57). However, the mechanism of how agonists and allosteric ligands modulate structural changes that lead to channel opening and desensitization remains enigmatic.

The overall structural similarity, along with comparable drug sensitivity, makes GLIC an attractive system to serve as a structural archetype for the eukaryotic LGIC, although there is considerable discrepancy surrounding channel properties at the functional level. GLIC activation in oocytes and HEK cells reveal slow opening kinetics and currents that do not decay over an extended period of time. Both properties significantly differ from eukaryotic channels in that they activate and desensitize quickly (58–60). Patch clamp recordings from purified and reconstituted GLIC revealed several differences from previous functional measurements. Specifically, GLIC currents show rapid activation and deactivation in response to pH changes, and in the sustained presence of protons currents decay within seconds because of desensitization. In general, the activation and deactivation kinetics of GLIC are ~10-fold slower than some of the nAChR channels, whereas the time constant of desensitization is ~50-fold slower (46). However, these results are not surprising because bacterial homologues of voltage-gated channels have also been reported to display relatively slower kinetics in comparison to their eukaryotic counterparts (61, 62).

Does GLIC desensitization bear any resemblance to the mechanism in eukaryotic LGIC? GLIC desensitization shows a strong dependence on the activating stimulus, and even very low concentrations of the agonist desensitizes the channel without evoking an ionic response. The rate of desensitization
increases with hyperpolarization and is modulated by external divalent cations. These properties reveal a striking similarity to the mechanism in nAChR (46, 59, 63–67).

Lidocaine blocks GLIC currents in a voltage-dependent manner. Crystal structures of GLIC in the presence of a lidocaine analog reveal a binding site close to the 9′ position in M2. The 9′ position is highly conserved across the LGIC family, and mutating the 9′ residue in several LGIC members affects desensitization (63, 68, 69). Polar substitutions at this position slows desensitization and removes the block by lidocaine analog QX-222 (63). We find that lidocaine not only blocks GLIC but also slows desensitization, suggestive of a “foot-in-the-door” type of mechanism seen for quaternary ammonium compounds in several channels (70). In a way this result was somewhat surprising in that lidocaine has been shown to enhance desensitization of nAChR (71). Differences in the pore architecture might underlie a variation in the allosteric effects of the blocker. It is also interesting to note that the GLIC structure in the putative open state reveals bound detergent molecules (dodecyl-β-D-maltoside) within the pore closely interacting with the 9′ position (16). Our lidocaine findings support the idea that hydrophobic interactions near the 9′ position might stabilize the open conformation of GLIC (22).

Findings from early studies involving functional reconstitution of nAChR revealed the requirement of both cholesterol and anionic lipids (such as phosphatidic acid) to retain optimal flux and desensitization properties (40, 72–74). It has been argued that cholesterol affects structural and dynamic properties of the channel by directly interacting with the channel rather than by altering the physical environment of the lipid bilayer (75). Molecular dynamic simulations predict that binding of cholesterol to both deeply buried and lipid-protein interfacial sites stabilizes the nAChR structure and supports contact between the ECD and the TMD required for channel activation (76). Characterization of the effect of cholesterol on the single-channel properties of nAChR show that open channel duration and open probability decreased linearly with an increase in membrane cholesterol content (77). Further, depleting cholesterol in the plasma membrane of rat hippocampal neurons through acute treatment with drugs that scavenge cholesterol and inhibit its synthesis led to dramatic decreases in the desensitization rates of α7-nAChR (78). In a similar way, GLIC reconstituted in asolectin membranes showed robust currents that desensitized much more rapidly as the cholesterol content in the membrane was increased. Further work needs to be carried out to determine whether this property is related to membrane fluidity or through direct effects of cholesterol binding. Although bacterial membranes lack cholesterol, cyanobacteria such as *G. violaceus* do synthesize sterol-like compounds (haponoids) that modulate membrane rigidity and interactions with protein (79). Also, it has been recently shown that prokaryotic inward rectifying (Kir) channels display direct modulation by cholesterol in a way parallel to higher homologues (80, 81), suggesting the presence of evolutionarily conserved motifs.

Finally, are the structural changes underlying closed-to-open-to-desensitized transitions similar in these prokaryotic and eukaryotic channels? Gating conformational changes in LGIC that link ligand-binding at the ECD to channel opening and desensitization are highly debated. Nevertheless, a general consensus has emerged that agonist binding induces the closure of loop C from an uncapped to a capped conformation (10, 12, 82, 83), which is conveyed to the pore lining M2, resulting in channel opening. The loop C motion has been widely studied in AChBP using a number of techniques, including x-ray crystallography (10, 12, 84, 85), deuterium-hydrogen exchange (83), fluorescence spectroscopy (86–88), NMR spectroscopy (89), and molecular dynamic simulations (86). Collective findings from these studies point to an agonist-mediated loop C movement toward a restricted conformation with decreased solvent accessibility.

Although it is not clear whether charged residues in the analogous ligand-binding pocket of GLIC indeed govern the pH-dependent channel activation, conformational changes monitored by EPR spectroscopy reveal clear immobilization of residues in loop C during activation/desensitization. A more sterically constrained and less solvent-accessible conformation of loop C is consistent with agonist-induced closure of this region.

In summary, GLIC activates and desensitizes in a manner analogous to members of the LGIC family; however, with relatively slower kinetics. GLIC shares many common modulators of desensitization. Structural transitions associated with GLIC desensitization parallel those implicated during ligand binding and desensitization in higher homologues. The ability to trap channels in a known functional conformation now allows for a thorough structural characterization of these end states. Overall, GLIC is a good model to address some of the key questions pertaining to LGIC gating mechanisms and modulations by allosteric ligands.

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