Structural basis for alcohol modulation of a pentameric ligand-gated ion channel

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Despite its long history of use and abuse in human culture, the molecular basis for alcohol action in the brain is poorly understood. The recent determination of the atomic-scale structure of GLIC, a prokaryotic member of the pentameric ligand-gated ion channel (pLGIC) family, provides a unique opportunity to characterize the structural basis for modulation of these channels, many of which are alcohol targets in brain. We observed that GLIC recapitulates bimodal modulation by n-alkyl alcohols, similar to some eukaryotic pLGICs: methanol and ethanol weakly potentiated proton-activated currents in GLIC, whereas n-alcohols larger than ethanol inhibited them. Mapping of residues important to alcohol modulation of ionotropic receptors for glycine, γ-aminobutyric acid, and acetylcholine onto GLIC revealed their proximity to transmembrane cavities that may accommodate one or more alcohol molecules. Site-directed mutations in the pore-lining M2 helix allowed the identification of four residues that influence alcohol potentiation, with the direction of their effects reflecting α-helical structure. At one of the potentiation-enhancing residues, decreased side chain volume converted GLIC into a highly selective channel, comparable to its eukaryotic relatives. Modulation of four residues (at the 16′ and 17′ positions in M2) mediating inhibitory and excitatory interactions of n-alcohols with a pLGIC family member.

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unique opportunities to characterize ion channel modulation via a simplified model system. The similarity of GLIC to eukaryotic pLGICs was recently confirmed by the crystal structure of the Caenorhabditis elegans glutamate-gated chloride channel α (GluCl) (29). GLIC-mediated currents can be recorded in heterologous expression systems (30), and its homomeric assembly and limited intracellular domain restrict possible mechanisms of modulation. Furthermore, GLIC crystal structures contain ordered water and detergent molecules in close proximity to the M2 helices (25, 26), possibly mimicking interactions with alcohol. Recent functional (31) and structural (32) characterizations of GLIC inhibition by general anesthetics, as well as the GlyR-like allosteric modulation of a GLIC/GlyR chimera described in the accompanying article (33), further support the relevance of this prokaryotic channel to pLGIC modulation. We proposed that GLIC comprises a simplified, structurally accessible model system for structure and function of pLGICs, including modulation by n-alcohols.

Results and Discussion

**GLIC Exhibits Differential Modulation by n-Alcohols.** Despite structural and functional similarities between GLIC and eukaryotic pLGICs, modulation of GLIC by alcohols has not been previously demonstrated. An anesthetic concentration (34) of methanol (590 mM) did not directly activate GLIC during preapplication at neutral pH; however, in the presence of methanol, proton-gated GLIC currents elicited at pH 5.5 (pEC10 = 2) were potentiated approximately twofold (Fig. 1A). An anesthetic concentration of hexanol (570 μM) similarly had no direct effect on oocyte currents but instead inhibited proton-gated GLIC currents at pH 5.5 by more than 50% (Fig. 1B).

Both potentiation and inhibition by n-alcohols were more pronounced at lower levels of channel activation (Fig. 1C). At pH 4.5, where GLIC conducts more than 50% of its maximal current, modulation by either alcohol was negligible. Neither potentiation nor inhibition by n-alcohols was use-dependent, because the quantity of modulation did not change with successive activations in the prolonged presence of alcohol (Fig. 1A and B). Both these features are consistent with alcohol actions on mammalian pLGICs (7, 35).

The profile of n-alcohol modulation of GLIC is reminiscent of eukaryotic pLGICs. In particular, nAChRs exhibit potentiation by short-chain and inhibition by long-chain n-alcohols (9). To characterize the pattern of chain length-dependent modulation in GLIC, we measured concentration-dependent modulation of proton-gated GLIC currents at pH 5.5 by a range of n-alcohols. High concentrations of both methanol and ethanol potentiated GLIC, with negligible modulation below 590 mM (Fig. 2A). Conversely, n-alcohols larger than ethanol inhibited GLIC, their potency increasing with chain length from propanol to nonanol (Fig. 2B). Decanol had similar potency to nonanol (Fig. 2B), suggesting that it could still be accommodated by the inhibitory site but that its increased volume did not enhance binding. By one common definition, the largest alcohol exhibiting increased potency compared with shorter-chain alcohols constitutes the alcohol cutoff (4), in this case nine carbons—a profile similar to nAChRs (36), GlyRs (7), and GABA_A Rs (8).

Fig. 1. Modulation by n-alcohols of GLIC currents in Xenopus laevis oocytes. Current traces show successive GLIC activations by pH 5.5 (pEC10) in the presence and absence of (A) 580 mM methanol or (B) 570 μM hexanol. (Scale bars, 2 μA, 5 min). (C) Modulation by either short-chain n-alcohols (590 mM methanol, gray) or long-chain n-alcohols (36 μM octanol, black) was more pronounced at higher pH (lower level of activation). Errors are SEM, n = 2–30.

Fig. 2. Profile of GLIC modulation by n-alcohols. (A) GLIC currents were potentiated by high concentrations of methanol or ethanol. Because it was not possible to collect data for complete concentration response relationships, curves represent linear regression fits. (B) GLIC currents were inhibited by n-alcohols larger than ethanol in a dose-dependent manner. Potency of inhibition by long-chain n-alcohols (propanol and larger) increased with chain length up to nonanol. Curves represent nonlinear regression fits as described in SI Methods. In A and B, errors are SEM, n = 2–18. (C) Region of GLIC (Protein Data Bank ID 3EAM) (25) transmembrane domain surrounding M2 residues previously implicated in alcohol modulation of eukaryotic pLGICs, shown as spheres: L(17′) (L241, yellow) and I(16′) (I240, green), homologous to the L263 “excitatory site” and L262 “inhibitory site” in M2 of the n2 nAChR (21); and N(15′) (N239, pink), homologous to S267 in the α1 GlyR (19). Position F(14′) (F238, orange), demonstrated in this study to strongly influence alcohol modulation of GLIC, is also shown. Cavities (black) neighboring the implicated residues were calculated using the Hollow script as described in SI Methods. Upper: Two GLIC subunits (gray, white) viewed from the plane of the membrane in the channel pore; cavity regions corresponding to the pore lumen are removed for clarity. Transmembrane helices M1–M4 are labeled in one subunit. Lower: Full pentameric channel transmembrane domain and cavities, viewed from the extracellular side. (D) Views as in C. Upper: Solvent-excluded surfaces surrounding cavities defined in C with polar (blue), nonpolar (red), and intermediate (purple) regions colored by residue hydrophobicity. For clarity, M2 helices are not shown. Lower: Internal cavities as in C with intrasubunit (red) and intersubunit (blue) regions colored independently of linking tunnels and pore lumen (purple). Transmembrane helices are labeled in one subunit.
Residues Implicated in Alcohol Modulation Border Multiple Internal Cavities. We used the known crystal structure of GLIC to model residues implicated in alcohol action. Alcohol has been proposed to occupy water-filled amphiphilic cavities in proteins (37). Furthermore, ion channel residues associated with alcohol modulation are proposed to neighbor regions critical to gating, thus converting the limited binding energy of one or more alcohol molecules into changes in the gating energy landscape as efficiently as possible (38). Therefore, we asked whether amino acid residues homologous to those that affect alcohol modulation in pLGICs might border solvent-accessible cavities and/or influence ion channel gating in GLIC.

We focused on the GLIC transmembrane domain, which aligns in a straightforward fashion with those of several pLGICs (39) (Fig. S1). The resulting alignments place amino acid residues involved in alcohol modulation around cavities at the extracellular end of the transmembrane domain (Fig. 2C), a region considered critical for channel gating (17). Each GLIC subunit is associated with one intrasubunit and one intersubunit cavity, connected to one another via narrow channels through a pore-facing linking tunnel (Fig. 2D) (32). The intersubunit cavity is primarily hydrophobic (Fig. 2D); it is predicted to open onto the hydrophobic core of the lipid bilayer and is partially occupied in the crystal structure by lipids (25). It is also occupied by anesthetic inhibitors in two recently co-crystallized structures (32). The intersubunit cavity and linking tunnel are more hydrophilic (Fig. 2D) and are occupied by multiple crystallographically resolved water molecules (25, 26). The intersubunit cavity is accessible to the lipid bilayer, whereas the linking tunnel opens onto the pore lumen (Fig. 2 C and D). When modeled onto the GLIC channel, most M2 residues involved in alcohol modulation cluster around the water-filled intersubunit cavity and linking tunnel (Fig. 2C). Indeed, a cavity homologous to the intersubunit-linking tunnel region was occupied by ethanol in a recent 2-μs molecular dynamics simulation in the α1 GlyR (40).

Specific Mutations in the Pore-Lining Helix Modify Alcohol Potentiation. To identify specific residues involved in alcohol modulation of GLIC, and potentially of eukaryotic pLGICs, we performed cysteine-scanning mutagenesis on the M2 helix of GLIC and measured the effect of individual cysteine substitutions on gating and alcohol modulation. We focused on the C-terminal end of the M2 helix, near the extracellular domain, because of past evidence for alcohol modulation sites in this region (Fig. 2C). All mutants tested produced functional channels in oocytes except L(8′)C and H(11′)C. Mutant I(9′)C showed evidence of rapid disulfide bond formation (Fig. S24, Top) and could not be characterized fully. Modulation by various n-alcohols was tested for all other mutants at their respective EC_{10} activation levels, as in previous studies of alcohol modulation in GlyRs and GABA_{A}Rs (19).

Potentiation by high concentrations of ethanol was unchanged in all but four of the mutants tested. Most dramatically, cysteine substitution at F(14′) and L(17′) markedly enhanced potentiation (Fig. 3A, Upper). Indeed, the F(14′)C mutation enhanced potentiation by both methanol and ethanol relative to wild type and produced potentiation rather than inhibition by propanol and butanol (Fig. 3D). This mutant was potentiated by ethanol at concentrations as low as 20 mM (Fig. 3E), approximately the blood alcohol concentration considered legally intoxicating in the United States (1). Thus, a single substitution at the 14′ position converts GLIC into a channel that is modulated by pharmacologically relevant concentrations of ethanol in a manner similar to eukaryotic pLGICs (1). Sequence alignments reveal that several ethanol-sensitive pLGICs contain smaller residues at 14′ (Fig. S1), suggesting that side chain volume at this position may influence alcohol sensitivity throughout this protein family.

To determine the physicochemical requirements for the ethanol effect observed in F(14′)C, we tested alternative substitutions at the 14′ position. Decreasing hydrophobicity by substituting tyrosine at this position did not change ethanol potentiation; on the other hand, decreasing side chain volume by substituting alanine enhanced ethanol potentiation to an even greater degree than cysteine (Fig. 3F). This alternative substitution also removes the possibility for disulfide bond formation, indicating that covalent cross-linking is not required for the enhancement of ethanol effects observed in F(14′)C. Also consistent with cysteine substitution, alanine substitution dramatically shifted the chain-length cutoff for potentiation: mutant F(14′)A was potentiated by alcohols as large as hexanol, whereas longer-chain alcohols had similar effects as on wild-type GLIC (Fig. S3A and SI Discussion). Similar to wild-type GLIC, the F(14′)A mutant exhibited greater ethanol potentiation at lower levels of channel activation (Fig. S3B), although an anesthetic concentration (200 mM) of ethanol did potentiate proton-evoked responses in the mutant at all levels of activation tested. The evident enhancement of maximal proton responses by ethanol (Fig. S3C) was further reminiscent of nAChRs (21).
In contrast to the positions at which cysteine enhanced ethanol potentiation (14' and 17'), which are oriented toward the inter-subunit interface in the GLIC crystal structure, substitutions at A(13') and I(16'), which face the channel pore (Fig. S2B), removed or reversed potentiation by ethanol (Fig. 3A and Fig. S2D, Upper). The pattern of ethanol-enhancing and -suppressing residues was thus consistent with the α-helical structure of the M2 region. Notably, the 17' and 16' positions correspond to residues that mediate excitatory and inhibitory effects of n-alcohols, respectively, in the nAChR (21). Inhibition by 11 mM butanol (approximately IC₅₀ for wild-type GLIC) was unchanged in all mutants except F(14'C) (Fig. 3A and Fig. S2D, Lower). Evaluation of longer-chain alcohol effects on this mutant revealed inhibition by 36 μM (≈IC₅₀) octanol that was indistinguishable from wild-type GLIC (Fig. 3D). The consistency of long-chain alcohol effects in all M2 mutants suggests that mechanisms of potentiation and inhibition are independent, as has been proposed for both nAChRs (41) and GABAARs (42).

Mutations in the M2 helix, as expected, also altered pH responses of GLIC; sample curves in Fig. 3B show examples of both enhanced and inhibited proton gating. Given that alcohol modulation varies with the level of channel activation (Fig. 1C), we corrected for variations in gating by calculating the pEC₁₀ for each mutant and using this pH to activate the channel in all modulation experiments. There was no correlation between pEC₁₀ and ethanol potentiation (Fig. 3C), indicating that variations in these two channel properties were independent.

**Labeling of Specific M2 Residues Mimics Alcohol Potentiation.** Labeling of introduced cysteines with MTS reagents has been used previously to mimic alcohol binding to specific residues in pLGICs (6). If an alcohol binding site contains an exposed cysteine side chain, covalent labeling with an MTS reagent should result in persistent modification that mimics modulation by an n-alcohol, while reducing or blocking further alcohol modulation (6) (SI Discussion). Given the low potency of ethanol for potentiation of wild-type GLIC, we chose the smallest MTS reagent (6) (SI Discussion) for modulation of wild-type GLIC. Although methyl MTS itself is not a potent alcohol agonist, labeling of introduced cysteines with MTS reagents has been used previously to mimic alcohol binding to specific residues in GlyRs and GABAARs, 15' (19), does not influence alcohol modulation (Fig. 3A) or mediate current enhancement after methyl MTS labeling (Fig. 4A) in GLIC; however, this residue is one register below position 18', at which methyl MTS labeling both mimics (Fig. 4A and D) and blocks subsequent (Fig. 4E) alcohol potentiation. Given that several...
residues at the extracellular end of M2 are smaller in GlyRs and GABA\textsubscript{A}Rs than in GLIC (Fig. S1), the cavity that borders the 18\textsuperscript{th} position is likely to penetrate deeper into the subunit in the eukaryotic channels, potentially accessing the 15\textsuperscript{th} position directly below. Recent evidence for enhanced alcohol potentiation in a GLIC chimera containing the α1 GlyR transmembrane domain (33) supports the principle that local changes in this domain substantially influence alcohol modulation.

Transmembrane Cavities Implicated in Alcohol Potentiation of GLIC.

To model the structural basis for alcohol potentiation in GLIC, we performed molecular dynamics simulations of both wild-type GLIC and the potentiation-enhanced mutant F(14)A. Early in the 200-ns simulation, the 14\textsuperscript{th} substitution caused a structural change with a dramatic increase in the kink angle of the M2 helix (Fig. S4A), around the level of the hydrophobic gate at the 9\textsuperscript{th} position (43). Over the second half of the simulation the kink angle was consistently larger in the F(14)A mutant than in the wild-type channel (Fig. 5B). This increased kink angle bent the C-terminal end of the M2 helix away from the pore lumen, increasing the diameter of the pore and spreading the subunits apart from one another above the level of the kink (Fig. 5A). Thus, for example, at the end of 200-ns simulation, C\textalpha{} atoms of neighboring I(16\textsuperscript{th}) residues are separated by 10.0 ± 0.4 Å in wild-type GLIC but by 11.3 ± 0.6 Å in F(14)A. Conversely, the enhanced kink angle in the F(14)A mutant compressed the pore constriction at the level of I(9), obstructing the ion conduction pathway at this residue (Fig. 5A); this obstruction may account for the reduced pH sensitivity of F(14)A mutants relative to wild-type GLIC (Fig. 5B). It should be noted that the functional state of the GLIC crystal structure is currently controversial; some recent studies suggest it represents a desensitized state (27, 28), whereas its close structural alignment with the open-state structure of GluCl supports its open conducting conformation (29). It remains to be determined how close simulations such as ours may be to a conductive state.

The increased separation between subunits in F(14)A was associated with changes in the transmembrane cavities (Fig. 5 C and D and Fig. S4). Whereas the intrasubunit cavity was of similar volume and variability in both wild-type (335 ± 52 Å\textsuperscript{3}) and F(14)A mutant (369 ± 75 Å\textsuperscript{3}) simulations (Fig. 5 C and D; red), the so-called linking tunnel between the intra- and inter-subunit cavities (32) was substantially larger and more variable throughout the simulation for the F(14)A mutant (316 ± 98 Å\textsuperscript{3}) relative to wild-type GLIC (117 ± 33 Å\textsuperscript{3}) (Fig. 5 C and D; purple). The intersubunit cavity also displayed substantial mobility but was too variable to permit characterization as a discrete cavity throughout the simulations. Generally, the central channel cavities (consisting of the pore lumen and contiguous linking tunnel region) formed a “T” shape when viewed from the plane of the membrane in the channel pore (Fig. S4A). Conversely, because of the increased kink angle of the M2 helix and widening and deepening of the linking tunnels, the same central cavity formed an irregular “Y” shape in the F(14)A mutant (Fig. S4B).

The enlargement of the linking tunnel cavity in the F(14)A mutant offers an indirect explanation for the pharmacology of enhancement. It is notable that the F(14)C mutant was not itself enhanced by methyl MTS treatment, suggesting that this residue may not be fully accessible or that its labeling is not sufficient to mimic alcohol potentiation. Whether or not the 14\textsuperscript{th} position is directly accessible to solvent, our simulation data demonstrate the dramatic influence of this residue on the conformation of the C-terminal portion of M2 and thus the volume of nearby cavities, particularly the linking tunnel. The deeper, wider linking tunnel in the mutant could accommodate more alcohol molecules and/or bind them with greater affinity; furthermore, it could accommodate larger alcohols than in the wild type, suggesting a structural basis for the potentiating effects of longer-chain alcohols on 14\textsuperscript{th}-substituted mutants (Fig. 3D and Fig. S2C). Small residue substitutions at L(17), one register above F(14) (Fig. 5C), could cause similar changes, potentially accounting for the enhanced ethanol potentiation of mutant L(17) (Fig. 3A). Furthermore, the 17\textsuperscript{th} and 18\textsuperscript{th} positions, at which methyl MTS labeling mimicked alcohol potentiation (Fig. 4), were located at the periphery of the linking tunnel (Fig. 5C and Fig. S4); methyl MTS modification of either residue could orient the methanethiolate label similarly to an alcohol molecule. Recent simulations of the closely related GlyR indicate that occupation of this cavity could stabilize the open state of the channel (40). Conversely, other regions of the protein—including the intrasubunit cavity that was recently implicated in inhibition by volatile anesthetics (32)—were relatively unaltered in the F(14)A mutant (Fig. 5C and D), consistent with the existence of an independent mechanism for long-chain alcohol inhibition.

Conclusions

We demonstrated GLIC to be an alcohol-sensitive channel with a profile of modulation similar to some eukaryotic pLGICs. We were able to tune potentiation of GLIC by short-chain alcohols.
with specific mutations in the pore-lining helix, without influencing an evidently independent mechanism of inhibition by long-chain alcohols. We implicated specific residues at the extracellular end of M2 in alcohol potentiation, consistent with past studies of eukaryotic pLGICs. Mutagenesis at one of these positions (14) was sufficient to convert GLIC into a potently ethanol-sensitive channel, providing a useful model system for future structural and functional characterization. Furthermore, whereas past studies have conflicted over the relative roles of putative intrasubunit and intersubunit cavities in alcohol modulation of pLGICs, our mutagenesis and molecular modeling suggest a model in which short-chain alcohols occupy a pore-facing pocket that includes the linking tunnel. This cavity primarily occupies the intersubunit interface but borders on residues previously implicated in an intra-subunit alcohol binding region (44); hence, past evidence for both intra- and intersubunit sites of action may in fact implicate the same cavity. These results further establish GLIC as a valuable model system for structural and functional studies of pLGICs.

Methods

Oocytes Electrophysiology. Oocyte electrophysiology, including molecular biology, oocyte preparation, two-electrode voltage clamp recordings, treatment with alcohols and MTS reagents, and statistics were performed as previously described (20, 30). Specifications are provided in SI Methods.

Modeling and Molecular Dynamics. Chemical structures were prepared using MarvinSketch 5.3.7 (ChemAxon), and protein structures were represented using the University of California, San Francisco Chimera package (45). Cavities were calculated using the Hollow script (46) or Fpocket package (47) as described in SI Methods. Molecular models of wild-type GLIC and the F(14)A mutant were built from PDB ID 3EAM with protonation states similar to those described by Bocquet et al. (25). The mutant was optimized using the ROSETTA program (48). Each model was inserted in a lipid bilayer, relaxed, and finally simulated for 200 ns as described in detail in SI Methods. The M2 kink angle was computed with a custom VMD (49) script.

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**Supporting Information**

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**SI Methods**

**Molecular Biology.** Expression plasmids containing wild-type GLIC in the PMT1 vector were prepared as previously described (1). Mutagenesis was carried out using the QuikChange II site-directed mutagenesis kit (Agilent) using commercially made mutagenic primers (IDT). Mutant cDNA was confirmed by automated fluorescent DNA sequencing.

**Xenopus laevis Oocyte Preparation.** Extraction of ovarian tissue from *Xenopus laevis* frogs was in accordance with the National Institutes of Health guide for the care and use of laboratory animals. Tissue was placed in Modified Barth’s Solution (MBS) containing 88 mM NaCl, 10 mM Heps, 2.4 mM NaHCO3, 1 mM KCl, 0.91 mM CaCl2, 0.82 mM MgSO4, and 0.33 mM Ca(NO3)2, adjusted to pH 7.5. After manual isolation with forceps, oocytes were treated with collagenase type 1A solution containing 0.5 mg/mL collagenase, 83 mM NaCl, 5 mM Heps, 2 mM KCl, and 1 mM MgCl2, adjusted to pH 7.5, for 10 min. Oocyte nuclei were injected via the animal pole with GLIC wild-type or mutant cDNA using a microdispenser (Drummond Scientific). Injected oocytes were singly stored at 13 °C in MBS supplemented with 220 μg/mL sodium pyruvate, 90 μg/L theophylline, 50 μg/L gentamicin, 10 μg/L streptomycin, and 10,000 U penicillin for 2-17 d.

**Oocyte Electrophysiology.** Oocytes were placed in chambers (∼100 μL volume) and perfused (2.0 mL/min) with Ringer’s buffer (123 mM NaCl, 10 mM Heps, 2 mM KCl, 2 mM MgSO4, and 2 mM CaCl2) using a peristaltic pump through 18-gauge Teflon and Viton tubing (Cole-Parmer). Oocytes were impaled with two glass electrodes filled with 3 M KCl and clamped at −70 V using an OC-725C oocyte clamp (Warriner Instruments). Currents were digitized and continuously plotted using the PowerLab 4/30 data acquisition system (AD Instruments). To activate GLIC currents, low-pH Ringer s buffer was applied in which Hepes was neutralized with protons, 10 mM NaCl, and 2 mM MgSO4, adjusted to pH 7.5, for 10 min.

**Modeling and Molecular Dynamics.** Sequence alignments were performed using the ClustalW2 Multiple Sequence Alignment algorithm (3). Cavities in the static GLIC crystal structure (Fig. 2 C and D, main text) were calculated using the Hollow script (4) with a grid spacing of 0.5 Å. Density maps were built from pseudoatoms at 4-Å resolution.

For molecular dynamics simulations, wild-type GLIC was represented by Protein Data Bank ID 3EAM, with hydrogen atoms built to similar protonation states as depicted by Bocquet et al. (5). The GLIC F(14′)A mutant was built using the rotamer library SCWRL (6) to introduce the mutation and rebuild side chains of the mutant and four closest neighbors in the sequence. The ROSETTA refinement program (7) was used to relax the structure, with protonation identical to wild-type. Each model was inserted into a pure dioleoylphosphatidylcholine (DOPC) bilayer by deleting overlapping lipid molecules, keeping 306 DOPC lipids, after which roughly 34,000 waters were added. To neutralize the net charge and achieve physiological ion concentration of ∼100 mM, 61 and 86 water molecules were replaced by Na+ and Cl− ions, respectively. Simulations were performed using Gromacs 4.5.3 (8) with the Amber03 force field (9) for protein and ions, TIP3P (10) parameters for water, and the Berger force field for DOPC (11). All bonds were constrained using the LINCS algorithm allowing a time step of 2.5 fs. Particle mesh Ewald electrostatics was used with a 10-Å cutoff for non-bonded interactions and neighbor lists updated every 10 steps. Three baths (protein, water, and ion, membrane) were coupled to a temperature of 310 K using velocity rescaling with a time constant of τT = 0.1 ps. The x,y dimensions were scaled isotropically with a Berendsen weak barostat and the z dimension independently to a reference pressure of 1 bar, τP = 1 ps and compressibility of 4.5 × 10−5 bar−1. The system was minimized for 10,000 steps with steepest descent. It was relaxed with position restraints of 1,000 kJ mol−1 nm−2 on the protein, 10 ns with backbone restraints, and finally 20 ns with Cα restraints. Production runs of 200 ns were performed without any restraints.

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<td>Methanethiosulfonate (MTS) Treatment</td>
<td>Methyl MTS was purchased from Toronto Research Chemicals Inc, diluted to 500 mM in DMSO, and stored as aliquots at 4 °C. Experimental solutions were prepared by diluting a stock aliquot 1:1,000 in Ringer’s buffer and sonicating for 1 min.</td>
<td>To quantify methyl MTS effects, oocytes were treated for 2 min with pEC10 buffer, followed by 5-min washout; 1-min treatment with 500 μM methyl MTS; 5 more minutes washout; and 2 more minutes pEC10 buffer. Percent current enhancement was calculated as [(Rc − Rfl)/Rfl] × 100, where Rfl represents the peak pEC10 response after MTS treatment and washout, and R0 represents the pre-MTS pEC10 response.</td>
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The M2 kink angle was computed within VMD (12) using a custom script, calculating the angle between the two principal axes of inertia of the top and bottom part of M2. The bottom part of M2 was defined by Cα of residues 221–238 and the top part by Cα of residues 238–245. Average cavity volumes over the course of molecular dynamics simulations (Fig. 5 C and D, main text) were computed in three steps using mpocket, a module of the Fpocket package (13). First, mpocket was used to compute all cavities over the course of the simulation. Second, grids were extracted for intrasubunit and linking tunnel cavities present in at least 20% of the trajectory frames. Onto those 10 cavity grids (5 intrasubunit plus 5 linking tunnel), the largest cavity subspace of each type was selected and superimposed on the other 4 cavities of the same type. Third, average volumes were calculated for each cavity within the previously defined grids. All parameters were according to Fpocket defaults, except the volume calculation, for which we used 10,000 Monte Carlo iterations instead of 2,500. Density maps were built from Fpocket output coordinates at 3.2 Å resolution.

SI Discussion

Small Substitutions at GLIC F(14) Increases the Cutoff for Potentiation.

The F(14)C mutation enhanced potentiation by both methanol and ethanol relative to wild-type, and produced potentiation rather than inhibition by propanol and butanol (Fig. 3D, main text). We characterized the chain-length cutoff for potentiation in the F(14)A mutant in greater detail, measuring concentration response curves for alcohols containing two to eight carbons (Fig. S24). Ethanol, propanol, and butanol potentiated the alanine mutant with increasing potency, whereas pentanol and hexanol potentiated with potency similar to that of butanol. Heptanol was entirely inhibitory at the concentrations tested, and octanol inhibition of the mutant was superimposable with that of wild-type GLIC, further supporting the presence of an inhibitory mechanism independent of the changes in the 14′ mutant.

MTS Labeling Can Mimic Alcohol Binding.

Labeling of introduced cysteines with MTS reagents has been used previously to mimic alcohol binding to specific residues in pLGICs (14). In the presence of water and at physiological pH, an alkyl MTS reagent reacts efficiently with an exposed cysteine to yield an alkanylthiolated product (15) that is structurally similar to the original cysteine with an n-alcohol bound (Fig. 4B, main text). Washing out the excess MTS reagent removes all nonspecific interactions with the receptor or lipid membrane, such that any changes observed after treatment and washout should represent specific interaction with the engineered cysteine.

The degree of methyl MTS enhancement of 17′ and 18′ position cysteine mutants was greater than that observed with the maximal concentration of methanol tolerated by the oocyte, consistent with the suggestion that saturating alcohol potentiation occurs at a concentration higher than we were able to test. Notably, current enhancement by methyl MTS was similar to the maximal degree of methanol potentiation observed in the F(14′)C mutant (Fig. 3B, main text), which approaches saturation at much lower concentrations compared with wild type. Thus, the direction and magnitude of MTS effects in the reactive mutants mimic potentiation by short-chain alcohols.

Consistent with early publications using MTS reagents to mimic alcohol modulation of pLGICs (14, 16), we applied methyl MTS in the absence of ligand, that is, in the presumed closed state of the channel. In some recent studies, MTS reagents have also been applied in the presence of ligand, permitting labeling in the presumed open state of the channel (17). It is possible that some of the cysteine mutants tested, although unaffected by MTS treatment at neutral pH (in the closed state), might be affected in the open state. However, the low reactivity of MTS reagents at low pH (18) rendered such experiments impractical. The future discovery of alternative activating ligands for GLIC would facilitate more complete characterization of channel function in this and other contexts.

Methionine Substitution Recapitulates Methyl MTS Labeling. If labeling of an ion channel residue indeed substitutes for alcohol binding, then it should also reduce or ablate further alcohol potentiation at an equivalent level of activation (pEC50). Although we did observe such an effect in some oocytes, experimental limitations including rundown of GLIC currents at prolonged time points made consistent recalculation of pH responses and alcohol modulation impractical. As an alternative approach, we noted that the structure of a methanethiolated cysteine residue (Fig. 4B, main text) is similar to that of a methionine residue (Fig. 4C, main text). Indeed, the recent crystal structure of a pLGIC extracellular domain homolog labeled with methyl MTS reveals that the labeled cysteine residue adopts a conformation interchangeable with that of a methionine residue (19). Therefore, a substituted methionine can approximate labeling of a substituted cysteine.

Using this logic, we compared cysteine vs. methionine substitutions at positions (17′ and 18′) that showed evidence of labeling by methyl MTS. Although gating was enhanced by methionine vs. cysteine substitutions at these residues (Fig. 4D, main text), ethanol potentiation was completely abolished in the L(17′)M and V(18′)M mutants (Fig. 4E, Upper, main text). Thus, modification of either position 17′ or 18′ with a methyl MTS-labeled or structurally equivalent residue substitutes for alcohol potentiation. In fact, occupation of both the 17′ and 18′ positions with the larger methionine side chain resulted in moderate inhibition as opposed to potentiation by ethanol (Fig. 4E, Upper, main text), suggesting that ethanol may interact with an independent inhibitory site whose effects are unmasked by blocking of these residues. On the other hand, methionine vs. cysteine substitutions at both the 17′ and 18′ positions failed to alter inhibition by butanol (Fig. 4E, Lower, main text), further substantiating an independent mechanism for long-chain alcohol inhibition.


Fig. S1. Sequence alignment of GLIC M2 helix with eukaryotic pLGICs containing residues implicated in alcohol modulation, particularly 15′ (pink) in GlyRs and GABAARs (1), and 16′ (green) and 17′ (yellow) in nAChRs (2). Additional highlighted residues include the 14′ position (orange), critical for enhancing ethanol potentiation in GLIC, and the 18′ position (brown) at which, along with 17′, methyl MTS labeling mimics alcohol potentiation. Numbers in top row indicate key residues in prime notation. Symbols in bottom row indicate sequence conservation, where “::” indicates highly conserved substitutions and “.” indicates weakly conserved substitutions (3).

Effects of pore-lining residue substitutions. (A) Current traces for GLIC mutant channels activated by pEC_{10} solution prior and subsequent to 1-min treatment with 10 mM DTT. In a symmetrical pentamer, a pore-facing residue from one subunit may be in close proximity to equivalent residues from neighboring subunits; therefore, cysteines at these positions might form intersubunit disulfide bonds that thus indirectly inhibit alcohol modulation. Indeed, cysteine substitution at the pore-facing 9′ residue yielded only small currents that decreased further upon successive channel activations; treatment with 10 mM DTT dramatically enhanced these currents (Top). These data indicate that the I(9′)C mutant undergoes intersubunit cross-linking that inhibits channel function, consistent with the role of the 9′ position as a constriction point critical to channel gating (1). Indeed, substitution of I(9′) with alanine was previously shown to slow channel deactivation (2) and corresponds to the canonical L(9′) gating site in the nAChR (3). Some spontaneous cross-linking was also evident in the L(16′)C mutant (Bottom). Conversely, all other measurable mutants conducted robust currents that were not substantially altered by treatment with DTT, as shown by the representative trace of A(13′)C (Middle). Scale bars represent 0.5 μA, 1 min. (B) Region of GLIC (Protein Data Bank ID 3EAM) showing three TMD helices (gray) viewed from the plane of the membrane in the channel pore. Pore-lining residues I(16′), A(13′), and I(9′) are represented as green spheres. These residues make hydrophobic contacts with detergent molecules included in the crystallization conditions, suggesting that alcohols might interact with these residues in a similar manner; three of these detergents are shown, with carbon (yellow) and oxygen (red) atoms colored independently. (C) Activation curves for wild-type GLIC (crosses) and mutants in which I(16′) was mutated to methionine (diamonds), alanine (squares, dotted curve), glutamine (triangles, dotted curve), and cysteine (circles); curves fitted as in Fig. 3D (main text). Substitutions at the 16′ position had wide-ranging effects on channel gating. Substitution with glutamine, which occupies nearly 90% of the side chain volume of isoleucine (4) but is substantially more polar, weakened proton gating, reducing pEC_{10} by ≈0.5 pH units. Substitution with cysteine or alanine, which are smaller and more polar than isoleucine (5), weakened proton gating, reducing pEC_{10} by approximately 0.5 pH units. The effects on gating and alcohol modulation of alanine substitution at the 16′ position were similar to cysteine, indicating that the changes observed in the cysteine mutant cannot be attributed solely to the spontaneous cross-linking demonstrated in A. (D) Modulation by 600 mM ethanol (Upper) or 11 mM butanol (Lower) of mutants with various substitutions at I(16′)′ in order of increasing polarity (significance vs. wild-type, Dunnett’s multiple comparison test, analysis of variance). Along with A(13′)C (Fig. 3A, main text), all substitutions tested at the 16′ position reduced ethanol potentiation, in some cases resulting in channels that were potently inhibited by ethanol. Consistent with other M2 mutants (Fig. 3C, main text), no correlation between shifts in gating and alcohol modulation was evident, as mutations that enhanced, inhibited, or had no effect on channel gating as shown in C all removed ethanol potentiation. The lack of correlation between physicochemical properties, pH gating, and ethanol potentiation of pore-facing residue mutants supports a model in which these positions are critical to potentiation by short-chain alcohols, such that substitution with any other residue weakens ethanol potentiation and may unmask independent inhibitory effects. The absence of significant changes in butanol inhibition in the 13′ (Fig. 3A, main text) and 16′ position mutants further supports the existence of such an independent inhibitory mechanism. In C and D, errors are SEM, n = 2–7. **P < 0.001.

Fig. S3. Enhanced alcohol potentiation of GLIC F(14)A. (A) Modulation of mutant F(14)A (circles, solid lines) by alcohols containing two to eight carbons. This mutant was robustly potentiated by alcohols as large as hexanol but was inhibited by octanol with a profile similar to that of wild type; solid curve for octanol represents nonlinear regression fit as in Fig. 2B (main text). Dotted lines represent fitted wild-type modulation curves as in Fig. 2A and B (main text). (B) Modulation of wild-type GLIC (600 mM ethanol, white) and mutant F(14)A (200 mM ethanol, black) was more pronounced at lower levels of proton activation (higher pH), although ethanol potentiated mutant currents under all conditions tested. (C) Proton response curves for GLIC mutant F(14)A in the absence (gray) and presence (black) of 200 mM ethanol. Curves represent nonlinear regression fits as described in SI Methods, normalized to the fitted maximal activation without ethanol. In A and B, vertical scale bars are broken to display both wild-type and mutant responses clearly. Errors are SEM, n = 2–27.

Fig. S4. Transmembrane cavities during wild-type and F(14)A simulations. (A) Region of GLIC surrounding key M2 positions after 200-ns molecular dynamics simulation. Upper: M2 helices, viewed from the plane of the membrane in the channel pore, with the "T"-shaped central cavity (purple) calculated as in Fig. 2C (main text). Residues at positions 14′ (orange), 17′ (yellow), and 18′ (brown) are shown as sticks. Lower: Full pentameric channel transmembrane domain and cavities viewed from the extracellular side, with residues shown as spheres. Intrasubunit (red) and linking tunnel (purple) cavities excluding the channel pore were calculated as in Fig. 5C (main text). (B) Equivalent views as in A of GLIC F(14)A mutant. Upper: "Y"-shaped central cavity in purple.