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Crotonic Acid Blocks the *Gloeobacter* Ligand-Gated Ion Channel (GLIC) via the Extracellular Domain

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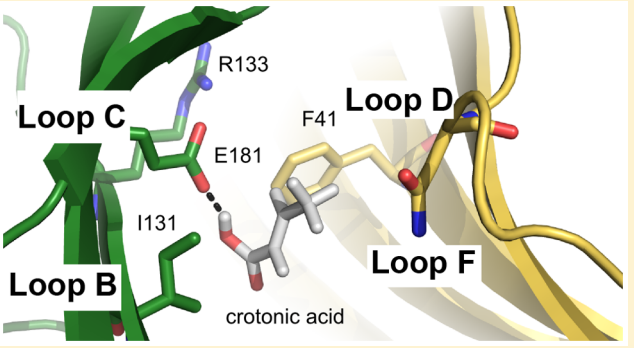
ABSTRACT: Cys-loop receptors play important roles in signal transduction in multicellular organisms, but similar proteins exist in prokaryotes, the best studied of which is the *Gloeobacter* ligand-gated ion channel (GLIC). GLIC is activated by protons with 50% activation (pH_{50}) at pH 5.5, and while a histidine residue in its pore-forming α-helix (M2) is known to be involved in gating, there is also evidence of a proton-sensitive region in the extracellular domain. However, this proton-sensitive region does not appear to be located in the region of GLIC equivalent to the agonist binding site in related proteins. Here we explore functional effects of a range of compounds that could bind to this site and show that some GABA analogues, the most potent of which is crotonic acid, inhibit GLIC function. Mutagenesis and docking studies suggest crotonic acid can bind to this region of the protein and, when bound, can allosterically inhibit GLIC function. These data therefore suggest that there is a transduction pathway from the orthosteric binding site to the pore in GLIC, as exists in related eukaryotic ligand-gated ion channels, and thus provide further evidence that this prokaryotic receptor is a good model for understanding this family of proteins.



Cys-loop receptors are responsible for the transmission of fast synaptic impulses in nervous systems and neuromuscular junctions, and thus, it was surprising when homologous proteins were identified in prokaryotes.[1] One such homologue was found in the cyanobacterium *Gloeobacter violaceus* and is known as GLIC (*Gloeobacter* ligand-gated ion channel). Crystal structures of GLIC reveal a structure similar to that of Cys-loop receptors, i.e., a largely β-sheet extracellular domain (ECD) and an α-helical transmembrane domain (TMD), although GLIC lacks a Cys-loop and an extended intracellular domain. Many high-resolution structures of GLIC have been determined in the presence of a range of compounds, which, given the scarcity of such structures for eukaryotic pentameric ligand-gated ion channels (pLGICs), makes it an attractive template for understanding the structural changes that occur during pore opening in the whole pLGIC family.[2−6]

GLIC, however, is not activated by a small molecule like most pLGICs, but by protons, and it is still not clear which residues and/or regions of the protein are altered by protonation to trigger pore opening. Wang et al.[7] identified a His in the pore-lining M2 region (11′ His) as a critical feature in the proton-gated opening, and this hypothesis is supported by studies using noncanonical amino acids.[8] Nevertheless, studies of a chimeric receptor with a GLIC-ECD and α1 glycine receptor-TMD revealed this protein (Lily) is activated by protons, suggesting the GLIC activation site is located in the ECD.[9] There is, however, no evidence to suggest that activation occurs via the region equivalent to the agonist (orthosteric) binding site in pLGICs, so the question of whether data from GLIC can be extrapolated to its eukaryotic relatives remains, as does the question of whether its distinct mechanism of action means that it is not a good model for understanding these allosteric proteins. One route to alleviating concern about this problem is to identify ligands that bind to the orthosteric binding site and modify the function of GLIC, i.e., to demonstrate that there is a similar transduction pathway in GLIC and other pLGICs. Several compounds that bind to the ECD in GLIC have been identified (including ketamine, acetic acid, and cinnamic acid derivatives[10−12]), and at least some of these inhibit GLIC function; however, structural data have shown they bind a little below where might be expected if they were binding in the orthosteric binding site. Here we have explored the effect of a range of other compounds, including various GABA analogues (Figure 1), which could potentially bind in this site; the effect of the most potent of these, crotonic acid, is explored in GLIC using docking studies and a range of mutant receptors. The data obtained support our hypothesis that compounds can bind in the GLIC orthosteric binding site and, when bound, inhibit the function of GLIC, supporting a conserved transduction mechanism.

MATERIALS AND METHODS

Oocyte Maintenance. *Xenopus laevis* oocyte-positive females were purchased from NASCO (Fort Atkinson, WI)

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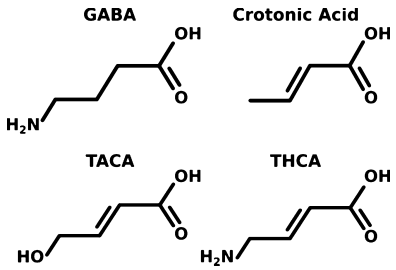

Figure 1. Chemical structures of GABA and the GABA analogues used in this study.

and maintained according to standard methods. Harvested stage V and VI *Xenopus* oocytes were washed in four changes of Ca^{2+}-free ND96 [96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES (pH 7.5)], defolliculated in 1.5 mg mL^{-1} collagenase type 1A for approximately 2 h, washed again in four changes of ND96 (as described above with 1.8 mM $CaCl_2$), and stored in ND96 containing 2.5 mM sodium pyruvate, 0.7 mM theophylline, and 50 mM gentamicin.

Receptor Expression. A codon-optimized version of GLIC, fused to the signal sequence of the α7 nACh receptor subunit and kindly provided by P-J Corringer, was cloned into pGEMHE for oocyte expression. Mutant receptors were created using QuikChange mutagenesis (Agilent, Santa Clara, CA). cRNA was transcribed *in vitro* from the linearized pGEMHE cDNA template using the mMessage mMachine T7 kit (Ambion, Austin, TX). Stage V and VI oocytes were injected with 50 nL of ~400 ng μL^{-1} cRNA, and currents were recorded 1−4 days postinjection.

Electrophysiology. Using a two-electrode voltage clamp, *Xenopus* oocytes were clamped at −60 mV using an OC-725 amplifier (Warner Instruments, Hamden, CT), Digidata 1322A, and the Strathclyde Electrophysiology Software Package (Department of Physiology and Pharmacology, University of Strathclyde, Glasgow, U.K.). Currents were filtered at a frequency of 1 kHz. Microelectrodes were fabricated from borosilicate glass (GC120TF-10, Harvard Apparatus, Kent, U.K.) using a one-stage horizontal pull (P-87, Sutter Instrument Co., Novato, CA) and filled with 3 M KCl. Pipette resistances ranged from 1.0 to 2.0 MΩ. Oocytes were perfused with saline containing 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 10 mM MES or HEPES (pH 3.5−8) at a constant rate of 12−15 mL min^{-1}. Drug was applied via a simple gravity-fed system calibrated to run at the same rate as the saline perfusion.

Analysis and curve fitting were performed using the four-parameter equation in Prism (GraphPad Software, La Jolla, CA). Concentration−response data for each oocyte were normalized to the maximal current for that oocyte.

Docking. A three-dimensional structure of crotonic acid was created *de novo* using Chem3D Ultra 7.0 (CambridgeSoft, PerkinElmer, Waltham, MA) and energy-minimized using the MM2 force field. Crotonic acid was docked into GLIC (Protein Data Bank entry 4QH1, with acetates removed) using GOLD version 5.3 [The Cambridge Crystallographic Data Centre (CCDC), Cambridge, U.K.]. The docking sphere was constrained to a 10 Å radius surrounding the C_α atom of E181 to cover the predicted ligand binding pocket. Ten genetic algorithm runs were performed on each docking exercise using default parameters. All protein figures were visualized using PyMOL version 1.3 (DeLano Scientific, Palo Alto, CA).

RESULTS AND DISCUSSION

Identifying Compounds That Bind to the ECD of GLIC. GLIC does not have a "classic" orthosteric binding site in the ECD between adjacent subunits, but the structural data indicate there is a cavity in this region that might be able to accommodate a ligand. We therefore explored the effects of a range of small compounds: glycine, alanine, 5-HT, acetylcholine, taurine, GABA, glutamate, glutamine, muscimol, indole acetic acid, aminophosphonic acid, crotonic acid, *trans*-4-aminocrotonic acid (TACA), and *trans*-4-hydroxycrotonic acid (THCA). None activated the receptor at concentrations of ≤10 mM, but crotonic acid, THCA, and TACA at 300 μM inhibited pH 5.5-induced GLIC responses by 55, 40, and 6%, respectively (Figure 2). We tested these using a single pH as

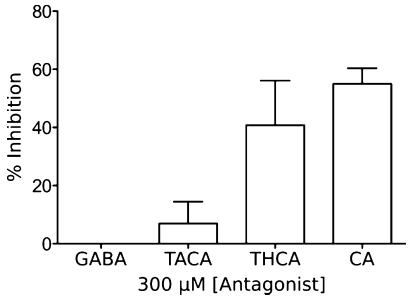

Figure 2. Inhibition of GLIC by GABA analogues. TACA, THCA, and crotonic acid (CA) at 300 μM inhibit pH 5.5-induced responses of GLIC to different levels. GABA (also at 300 μM) is ineffective. Data are means ± sem (n = 4−6).

the percentage of the ionized compound differs at different pHs (e.g., for crotonic acid at pH 6 it is 6% while at pH 5.5 it is 17%), and it is not yet known whether the ionized form, the nonionized form, or both are responsible for the inhibition of GLIC responses.

Site of Action of Crotonic Acid. We believe that crotonic acid inhibition is likely to be mediated via the ECD, but it is also possible it could act by blocking the channel pore. Inhibition of pH 5.5-induced responses in GLIC yielded an inhibition response curve with an IC_{50} of 620 ± 70 μM (n = 4) (Figure 3). This is not too dissimilar to the IC_{50} of picrotoxin (100 μM[13]), so to determine whether crotonic acid binds at the pore of GLIC, we introduced a Thr to Ala substitution at the 2′ pore-lining residues. T2′A GLIC had a pH_{50} of 5.5 ± 0.3, similar to that of the wild type, and crotonic acid inhibition revealed an IC_{50} of 380 ± 50 μM (n = 4), which is not

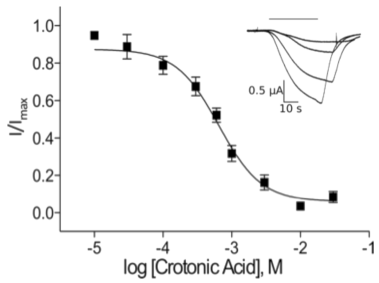

Figure 3. Crotonic acid inhibition of GLIC. A concentration inhibition curve reveals an IC_{50} of 620 μM. Data are means ± sem (n = 4). Inset = typical responses in the presence of 0, 0.1, 1, and 10 mM crotonic acid.

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significantly different from that of the wild type (Student's t test; $p > 0.05$). We also created receptors with −2′, 6′, and 9′ Ala substitutions, but these were nonfunctional (<200 nA current elicited at pH 4). However, as residue 2′ is a key residue in the binding of picrotoxin, and also other known Cys-loop receptor pore blockers, the lack of a change in crotonic acid potency indicates that crotonic acid inhibition is not mediated by blocking the channel pore.

To probe the possible binding of crotonic acid to the region equivalent to the orthosteric binding site (subsequently termed the GLIC orthosteric binding site), we created a single-residue mutation in each of the regions that contribute to the GLIC orthosteric binding site (six regions of the ECD, loops A−F, contribute to the orthosteric site in other pLGICs) and one other location, to act as a control. Each residue was substituted with Ala, and we then determined if there was a change in crotonic acid inhibition. This involved first determining the pH_{50} of the mutant receptors (to ensure effects at pH 5.5 were not due to a change in pH_{50}) and then testing the inhibition by crotonic acid. Figure 4A shows the pH_{50} values of each of the mutant receptors and reveals that substitution of loop A residue N80 and loop F residue N152 with Ala resulted in decreases in pH_{50}; crotonic acid inhibition at pH 5.5 could not therefore be accurately compared with that of other receptors, and these mutations were not considered further.

In the remaining receptors, there was a reduced level of inhibition by crotonic acid in GLIC containing Ala substitutions of loop B residues I131 and R133 and loop D residue F41 (Figure 4B). These residues align with residues critical for ligand binding in other pLGICs; e.g., the Glu equivalent of I131 in ELIC is involved in an electrostatic interaction with the quaternary amine of ACh[14] and is responsible for coupling ligand binding to channel gating in the $GABA_A$ receptor.[15] R133 in GLIC aligns with the loop B residue involved in cation−π interactions in many pLGICs, including the 5-HT_3 receptor W183,[16] nACh receptor α1 W149,[17] GABAρ receptor Y198,[18] glycine receptor F159,[19] and *Erwinia* ligand-gated ion channel (ELIC) F133.[14,20]

To identify possible interactions between these residues and crotonic acid, we docked crotonic acid into the GLIC orthosteric binding site. These data (Figure 5) show that crotonic acid is surrounded by F41, I131, N152, E181, and R133; it could form hydrophobic interactions with I131 and F41 and a hydrogen bond with E181. This location at least partly overlaps with the locations of cinnamic acid, caffeic acid, and ketamine in GLIC,[10,11] all of which are located slightly below what would usually be defined as the orthosteric binding site; e.g., they all interact with N152. Crotonic acid is a little higher in the binding pocket, but not as high as has been observed for some agonists in other pLGICs, e.g., GABA in ELIC.[18] It should be noted, however, that to date there have been very few published structures of pLGICs with agonists in their orthosteric binding pockets, so it is not yet clear how far this pocket could extend. Our functional data broadly support our docking data, although it is surprising that E181A does not result in a change in crotonic acid potency. This suggests that interactions with other residues may be more important, which may also be the case for caffeic acid, which again is predicted to hydrogen bond with E181, but no change was reported in caffeic acid potency in E181A-containing receptors.[11]

Use of Cysteine Modification To Probe Potential Crotonic Acid Binding Residues. To further probe residues that may be involved in crotonic acid binding, we created the appropriate Cys mutants and modified them with MTSEA-biotin, a sulfhydryl modifying reagent that is larger and more stable than many of the others in common use, and therefore especially useful for probing potential binding sites. The Cys substitutions in GLIC were mostly well tolerated, resulting in pH_{50} values similar to that of wild-type GLIC. The exception was F41C, for which pH_{50} was reduced to 4.7, precluding its use in testing crotonic acid inhibition at pH 5.5.

Treatment of Cys mutation-containing receptors with MTSEA-biotin had no effect on the pH_{50} values of Y22C, S93C, R133C, and E181C. There was also no effect on wild-type GLIC, which contains a Cys at position 26; thus, either this is not accessible to MTSEA-biotin, or modification has no effect on function. However, the pH_{50} was decreased in I131C-containing GLIC (Figure 6). This mutant was therefore also not tested further.

Inhibition by crotonic acid was then examined pre- and post-MTSEA biotin treatment and showed that there were differences for receptors modified at R133 and E181, the

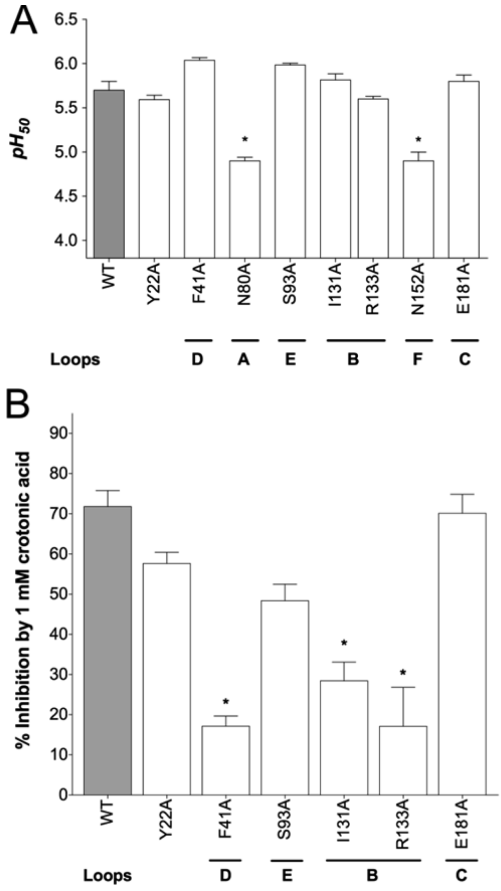

Figure 4. (A) pH_{50} of GLIC and a range of mutants. Substitution of selected residues with Ala in each of the binding loops A−F revealed a change in pH_{50} in N80A- and N123A-containing receptors. Y22A was a control. Data are means ± sem (n = 4−10). Asterisks denote values statistically different from that of the wild type (WT) (one-way analysis of variance with Dunnett's post test; $p < 0.05$). (B) Inhibition of GLIC (pH 5.5)-induced responses by 1 mM crotonic acid. Crotonic acid caused decreased levels of inhibition in F41A-, I131A-, and R133A-containing receptors compared to that of the wild type. N80A and N152A could not be tested as they have different pH_{50} values. Data are means ± sem (n = 3−13). Asterisks denote values statistically different from that of the wild type (one-way analysis of variance with Dunnett's post test; $p < 0.05$).

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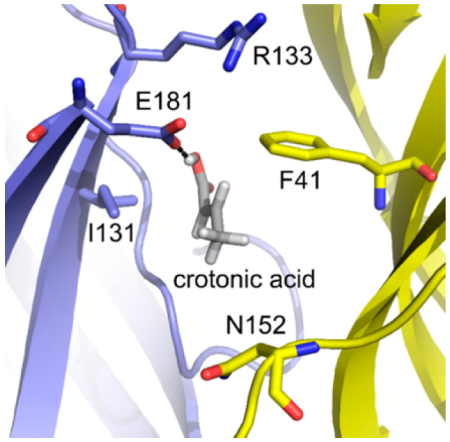

Figure 5. Docking of crotonic acid to the region equivalent to the pLGIC orthosteric binding site in GLIC. Crotonic acid could form a hydrogen bond with E181, and there is the potential for hydrophobic interactions with I131 and F41.

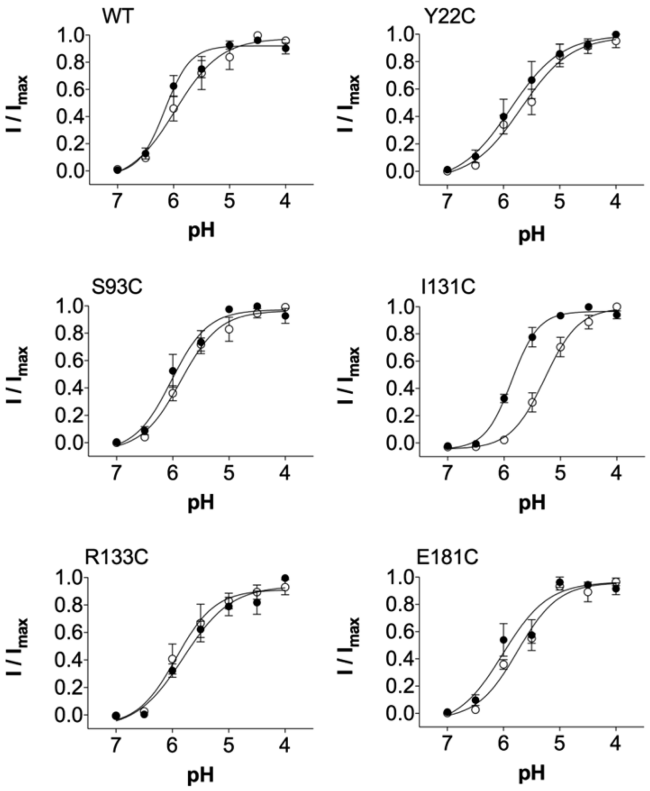

Figure 6. Effect of treatment with MTSEA-biotin on pH–response relationships in GLIC Cys mutants. Filled and empty circles represent data points before and after treatment with MTSEA-biotin, respectively. Data are means ± sem (n = 4).

specificity of which was confirmed by reversal of the effect when MTSEA-biotin was removed using dithiothreitol (DTT) (Table 1 and Figure 7). For R133C-modified receptors, the extent of inhibition was reduced following treatment; this is consistent with the model, as MTSEA-biotin attached to position 133 could prevent crotonic acid from reaching its binding site. Conversely, for E181C-modified receptors, inhibition was enhanced following treatment; we speculate that this is due to a subtle structural change that facilitates access to crotonic acid. Thus, overall, the data support our

Table 1. Inhibition by Crotonic Acid before and after Modification by MTS

	% inhibition by 1 mM crotonic acid			
	pre-MTS	post-MTS	post-DTT	n
wild type	76 ± 6	73 ± 5	ND[b]	9
Y22C	38 ± 7	28 ± 2	ND[b]	3
S93C	71 ± 5	79 ± 5	ND[b]	5
I131C	59 ± 5	63 ± 4	ND[b]	6
R133C	30 ± 4	18 ± 5[a]	28 ± 3	9
E181C	41 ± 4	58 ± 4[a]	40 ± 5	11

[a]Significantly different from that of pre-MTS (p < 0.05; Student's t test). R133C- and E181C-containing mutants were also tested following treatment with DTT to remove MTS. [b]Not determined.

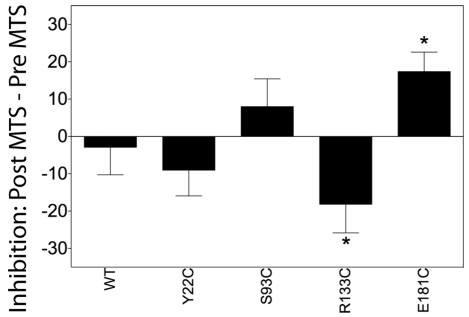

Figure 7. Difference in the percent inhibition of pH 5.5-induced responses by crotonic acid following MTSEA-biotin treatment of Cys mutant receptors. R133C showed a reduced level of inhibition, while modification of E181C enhanced inhibition. The other mutants and wild type showed no significant effects. Data are means ± sem (n = 3–11). An asterisk denotes a significantly different value (Student's t test; p < 0.05). The data from which this figure was derived are listed in Table 1.

hypothesis that crotonic acid can bind to the GLIC orthosteric binding site and inhibit GLIC function.

CONCLUSIONS

In conclusion, we show that some GABA analogues can inhibit GLIC function, and exploration of one of these, crotonic acid, suggests it exerts its inhibitory effects by interacting at a region equivalent to the orthosteric binding site. These data support our hypothesis that the region in GLIC equivalent to the pLGIC orthosteric binding site can communicate with the GLIC pore, suggesting conservation of the transduction mechanism between eukaryotic and prokaryotic pLGICs. Thus, our data contribute to a range of previous studies that suggest data from GLIC, the structurally best understood pLGIC, can reasonably be extrapolated to understand the structure and function of all other members of this family.

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Notes

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■ ABBREVIATIONS

nACh, nicotinic acetylcholine; AChBP, acetylcholine binding protein; ECD, extracellular domain; ELIC, *Erwinia* ligand-gated ion channel; GABA, γ-aminobutyric acid; GLIC, *Gloeobacter* ligand-gated ion channel; pLGIC, pentameric ligand-gated ion channel; sem, standard error of the mean; TACA, *trans*-4-aminocrotonic acid; THCA, *trans*-4-hydroxycrotonic acid; TMD, transmembrane domain.

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