The proton responsiveness in the extracellular domain of GLIC differs in the presence of the ELIC transmembrane domain

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Running title: A GLIC/ELIC chimera

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ABBREVIATIONS: GABA: γ-aminobutyric acid; GLIC: *Gloeobacter* ligand-gated ion channel; pLGIC pentameric ligand gated ion channel; ELIC *Erwinia* ligand-gated ion channel; GELIC GLIC-ELIC chimera; ECD: extracellular domain; TMD: transmembrane domain.

ABSTRACT

Prokaryotic homologues of Cys-loop receptors have proved useful in understanding their eukaryotic counterparts, but even the best studied of these, *Gloeobacter* ligand-gated ion channel (GLIC), is still not yet fully understood. GLIC is activated by protons with a pH_{50} between 5 and 6, implicating a histidine residue in its activation, but, although a histidine residue (His 11') in the pore-forming α-helix (M2) is known to be involved in gating, the His in the extracellular domain (ECD), His127, is not. Nevertheless there is evidence from a GLIC-glycine chimera for a proton sensitive residue or region in the GLIC extracellular domain. Here we create a novel chimeric receptor with the ECD of GLIC and the transmembrane domain of ELIC (GELIC). Expression of this receptor in oocytes reveals proton activation, although the pH_{50} (pH 6.7) differs from that of GLIC (pH 5.4). Exploration of protonatable residues in the ECD reveals 5 Asp residues (31,49,91,136 and 178) differ in their pK_as between the open and closed states of GLIC. Substitution of these residues with Ala or Asn shows somewhat similar effects for GLIC and GELIC in Asp91 mutants, but different effects for the others. Overall the data suggest that protonation of residues in the ECD is a requirement for channel opening in GELIC, but only plays a minor role in GLIC, where gating may be largely driven via protonation of the His residue in its pore.

KEY WORDS: Gloeobacter; Cys-loop receptor; pore block; antagonist; M2

The discovery of pentameric ligand gated ion channels (pLGIC) in prokaryotes (*1*) has considerably enhanced our understanding of the structure and function of these proteins. Homologues from *Erwinia* and *Gloeobacter*, known as ELIC and GLIC (*Erwinia* or *Gloeobacter* ligand-gated ion channel respectively), have been the most studied. Crystal structures of GLIC reveal a largely β-sheet extracellular domain (ECD) and α-helical transmembrane domain (TMD), as in other pLGIC, although GLIC lacks a Cys-loop and an intracellular domain (*2*,*3*). High resolution structures of GLIC have been determined for open, closed, and also partially closed (so-called locally closed) structures, which make it an excellent candidate for understanding the structural changes that occur during pore opening in the whole pLGIC family (*2*-*9*).

There is, however, a conundrum: GLIC is not activated by a small ligand, as are most pLGIC, but by protons, and it is still not clear which residues and/or regions of the protein are protonated, and how this links to pore opening. Most pLGIC have an orthosteric (agonist) binding site located in the ECD between adjacent subunits (*10*), but no such region has been functionally identified in GLIC. Nevertheless some compounds have been discovered that bind to the GLIC ECD, and these include caffeic acid, ketamine and cinnamic acid, all of which can inhibit GLIC function, demonstrating that there is a transduction pathway between this region of the protein and the pore (*11*) (*12*). The proton binding site, however, remains obscure. A His in the pore lining M2 region (11' His) was identified some years ago as a critical activation feature (*13*), and more recent data using non-canonical amino acids strongly support this residue as a, and possibly the, residue required for proton activation (*14*). In addition a chimeric protein, consisting of the ECD of ELIC and the TMD of GLIC, can be activated by protons, suggesting the GLIC activation site is in the TMD (*15*). However, studies on a chimeric receptor with the extracellular domain (ECD) of GLIC and a glycine receptor TMD, named Lily, revealed this protein is activated by protons, suggesting a proton activation site is located in the GLIC ECD (*16*) (*17*). Nevertheless the Lily pH_{50} (pH at 50% activation) is distinct to that of GLIC, suggesting there is some difference in the details of activation of Lily and GLIC. To further explore this GLIC activation site, we have created a GLIC-ELIC chimera, which we call GELIC. Here we probe some of the features of this protein in our quest to determine if the GLIC ECD has the same role in GLIC and chimeric proteins.

Materials and Methods

Cell culture and Oocyte Maintenance

Xenopus laevis oocyte-positive females were purchased from NASCO (Fort Atkinson, Wisconsin, USA) and maintained according to standard methods. Harvested stage V-VI *Xenopus* oocytes were washed in four changes of Ca^{2+}- free ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5), de-folliculated in 1.5 mg ml^{-1} collagenase Type 1A for approximately 2 h, washed again in four changes of ND96 (as above + 1.8 mM $CaCl_2$) and stored in ND96 containing 2.5 mM sodium pyruvate, 0.7 mM theophylline and 50 µg ml^{-1} gentamicin.

Receptor Expression

GELIC was constructed using the same domain boundaries/alterations as Lily (*17*). Site-directed mutagenesis was performed using the Stratagene QuikChange protocol. cRNA was transcribed *in vitro* from linearised 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 post-injection.

Electrophysiology

Using two electrode voltage clamp, *Xenopus* oocytes were clamped at -60 mV using an OC-725 amplifier (Warner Instruments, Hamden, CT, USA), Digidata 1322A and the Strathclyde Electrophysiology Software Package (Department of Physiology and Pharmacology, University of Strathclyde, UK; http://www.strath.ac.uk/Departments/ PhysPharm/). Currents were filtered at a frequency of 1 kHz. Micro-electrodes were fabricated from borosilicate glass (GC120TF-10, Harvard Apparatus, Kent, UK) using a one stage horizontal pull (P-87, Sutter Instrument Company, Novato, CA) and filled with 3 M KCl. Pipette resistances ranged from 1.0 – 2.0 MΩ. Oocytes were perfused with saline containing (in mM) NaCl (96), KCl (2), $MgCl_2$ (1) $CaCl_2$ (1.8) and MES or HEPES (10), pH 3.5 -8, at a rate of 4 ml min^{-1}. Drug application was via a simple gravity fed system calibrated to run at the same rate as the saline perfusion.

Analysis and curve fitting was performed using the 4 parameter equation in Prism (GraphPad Software, La Jolla, CA). Concentration-response data for each oocyte was normalised to the maximum current for that oocyte.

pK_a determination

The pK_a values in the open and closed structures of GLIC were obtained using the DEPTH online server based on the crystal structures of GLIC obtained at pHs 4.6 (PDB ID 3EAM) and 7 (4NPQ, chain A) (*18*) (*19*) (*20*) .

Results and Discussion

GELIC Activation

Current amplitude was tested using a range of pH concentrations. Receptors responded in a concentration dependent manner (Fig 1). Responses were small (up to 2 μA) compared to GLIC and ELIC (usually 10-30 μA) but at a similar level to those reported for Lily (*16*) (*17*). Concentration response curves revealed a pH_{50} of 6.7 ± 0.2 (n=8), which is significantly higher than values we obtained from GLIC (pH_{50} = 5.4 ± 0.2, n=5) but again similar to that of Lily (pH_{50} = 6.6). As ELIC is activated by small amines, including GABA (*21*), we also applied GABA, but no responses were observed up to 100 mM. This was as expected as the binding site for GABA is in the ELIC ECD.

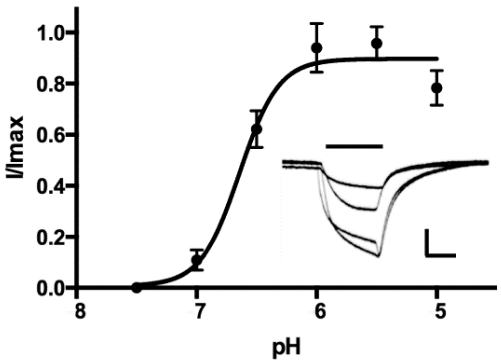

Figure 1: Function of GELIC: GELIC is activated by protons and a pH–response curve revealed a pH_{50} of 6.7. Data = mean ± SEM, n= 4. Inset = typical responses at pH 5.5, 6, 6.5 and 7; scale bars =150 nA and 30 s.

Picrotoxin inhibition of GELIC

There are a limited number of compounds that are known to inhibit the function of GLIC with high affinity (μM), and one of these is picrotoxin (PTX), which blocks the pore in many pLGIC including GLIC, where it has an IC_{50} of 3 μM (*22*). This is also one of the few compounds that inhibits both GLIC and ELIC (IC_{50} = 100 μM) (*23*). PTX inhibited GELIC responses in a concentration dependent manner (Fig 2), revealed by a concentration-

inhibition curve with a pIC_{50} of -3.37 ± .05, (IC_{50} = 430 μM). This is closer to the value obtained for ELIC than GLIC, consistent with PTX acting in the TMD, which is constituted from ELIC.

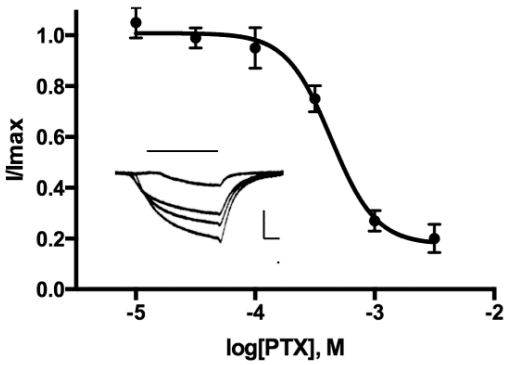

Figure 2: GELIC is inhibited by PTX. A concentration-inhibition curve at pH 6 reveals an IC_{50} of 430 μM. Data = mean ± SEM, n= 4-6. Inset = typical responses in the presence of 0, 0.1, 0.3 and 1mM PTX. Scale bars = 100 nA and 10 s.

Caffeic acid effects on GELIC

Previous data suggest that caffeic acid is one of a number of compounds that binds to the ECD of GLIC in a region close to the region that constitutes the orthosteric binding site of other pLGICs (*12*); we therefore tested caffeic acid on GELIC. The data (Fig 3) show that caffeic acid does inhibit GELIC and its IC_{50} (pIC_{50} = -4.34 ± 0.17, IC_{50}= 46 μM) is not significantly different (Students t-test, $p > 0.05$) to that of GLIC (pIC_{50} = -4.76 ± 0.12, IC_{50}= 17 μM). These data suggest that caffeic acid acts by binding to the same location in the ECD in GELIC as in GLIC, and it exerts its inhibitory effect in a similar manner in the two proteins.

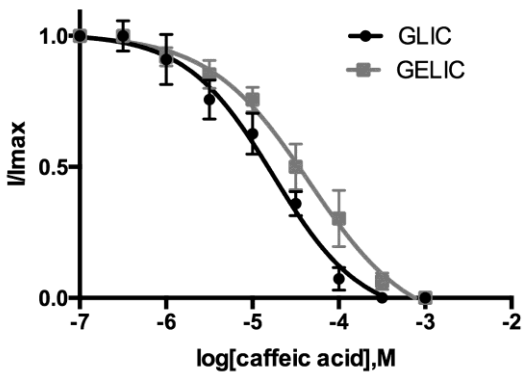

Figure 3: Caffeic acid similarly inhibits GLIC and GELIC. Concentration inhibition curves show similar IC_{50}s for GLIC (17 μM) and GELIC (46 μM) suggesting a similar mechanism of inhibition by caffeic acid in the two receptors. Data = mean ± SEM, n=6.

The role of charged residues in GLIC and GELIC

To clarify if GELIC and GLIC are activated by a similar mechanism, we examined residues in the ECD that could be involved in the activation process. As an initial screen of potentially important residues we determined the pK_a values of charged residues in the ECD that might contribute to channel opening, i.e. Asp, Glu, Lys and His, in open and closed structures using bioinformatics. The data revealed pK_a values for Asp vary between 2 (residue 115) and 6 (residue 91 in the open structure), with the majority between 2.5 and 4.5, consistent with the pK_a of the individual amino acid (3.7). Glu pK_as ranged from 4 to 7.6 (individual amino acid value= 4.3), while values for Lys were all in the range 9.5-11 (individual amino acid value = 10.3). There is only a single His in the ECD with pK_as of 6.8 and 6.6 (individual amino acid value = 6) in open and closed structures respectively. The pK_a values in the open and closed structures did not differ by more than 0.2 for any Glu or Lys residue, but there were differences of 0.6-2.8 for 5 Asp residues: 31, 49, 91, 136 and 178 (Fig

4). Four of these residues are located in regions of the ECD that contribute to the orthosteric binding pocket in other pLGICs, and the fifth is in the β1- β2 loop that is close to the TMD (Fig 5). These locations, combined with the pK_a data, indicate there is the potential for these Asp residues to play a role in the activation process.

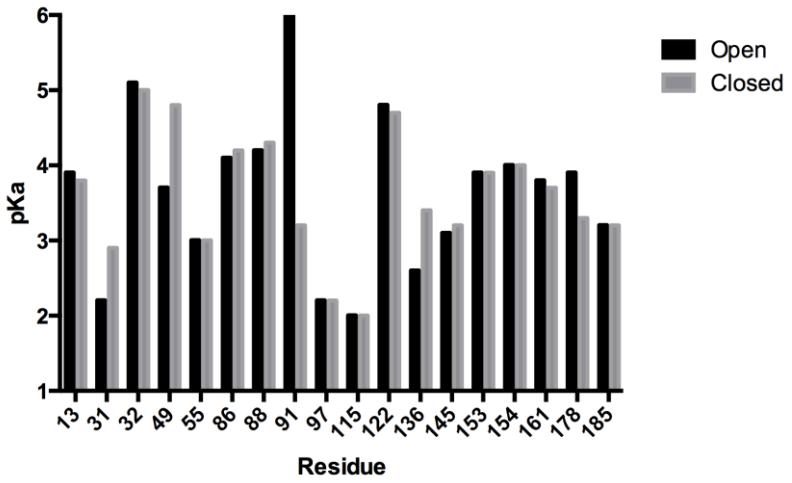

Figure 4: The pK_a values of Asp residues in the ECD of GLIC in open (at pH 4.6) and closed (at pH 7.0) structures.

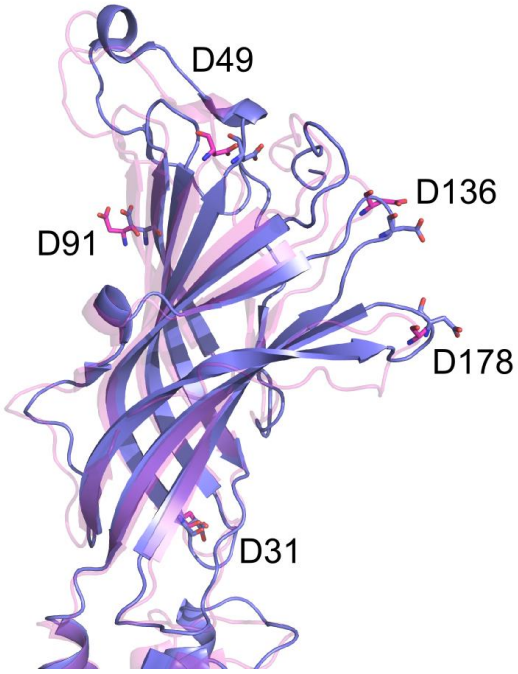

Fig 5: Structural alignment of open (blue) and closed (pink) forms of GLIC showing the locations of the Asp residues with different pK_as identified by DEPTH**.**

The roles of Asp 31, 49, 91, 136, and 178 in GLIC and GELIC

To explore the role of these residues we substituted each of them with Ala and Asn and examined functional parameters when they were expressed in oocytes; we also included an Asp residue that had no difference in pK_a as a control (Asp185). The data (Table 1; Fig 6) reveal that there is a considerable difference in the effects of altering Asp residues in the two proteins: Substitution of Asp31 or Asp49 in GLIC caused a small or no significant decrease in pH_{50} compared to WT, while in GELIC no function was observed when oocytes were challenged with pH up to 4. Substitution of Asp91 caused a decrease in pH_{50} compared to WT in both GLIC and GELIC but this was greater in GELIC. Substitutions of Asp136 or 178 with Asn caused no significant change in pH_{50} in GELIC, but the Ala substitutions resulted in abolition of function. Responses in GLIC with the same mutations differed: all were functional, with those at position 136 causing a decrease in pH_{50} compared to WT. Altering Asp185 had no effect in either GLIC or GELIC.

Table 1

	GLIC		**GELIC**	
Mutant	**pH_{50}**	**n_H**	**pH_{50}**	**n_H**
WT	5.4 ± 0.2	1.8 ± 0.3	6.7 ± 0.2	1.8 ± 0.3
D31A	4.8 ± 0.2*	1.3 ± 0.2	**NF**	
D31E	5.9 ± 0.2	1.8 ± 0.4	**NF**	
D31N	4.9 ± 0.1*	1.5 ± 0.4	**NF**	
D49A	5.1 ± 0.2	1.2 ± 0.5	**NF**	
D49E	5.8 ± 0.4	2.2 ± 0.1	6.9 ± 0.1	2.4 ± 0.3
D49N	5.2 ± 0.1	1.7 ± 0.4	**NF**	
D91A	5.1 ± 0.1*	1.5 ± 0.3	5.8 ± 0.1*	1.7 ± 0.2
D91N	4.8 ± 0.1*	1.6 ± 0.1	5.5 ± 0.2*	2.4 ± 0.6
D136A	4.2 ± 0.1*	2.5 ± 0.1	**NF**	
D136N	5.1 ± 0.1*	1.7 ± 0.2	6.6 ± 0.3	1.8 ± 0.3
D178A	5.2 ± 0.2	1.5 ± 0.4	**NF**	
D178N	5.1 ± 0.2	1.6 ± 0.3	6.9 ± 0.1	1.9 ± 0.4
D185A	5.1 ± 0.2	1.3 ± 0.3	7.1 ± 0.2	2.4 ± 0.2
D185N	5.2 ± 0.2	1.6 ± 0.3	6.6 ± 0.2	2.5 ± 0.3

Parameters obtained from concentration-response curves of GLIC and GELIC. NF = non functional when challenged with up to pH 4. Data = mean ± SEM, n=3-12; * significantly different to WT Students t-test , $p < 0.05$.

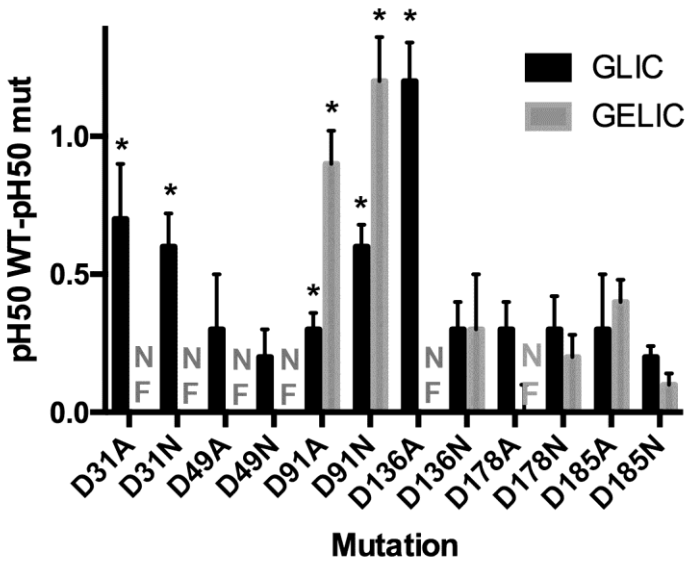

Fig 6: Comparison of pH_{50} values from mutant GLIC and GELIC compared to WT (from the data in Table 1). Many of the GELIC mutants were non functional (NF). Data = mean ± SEM, n=4-8. * = significantly different to 0 (i.e. no difference to WT) $p < 0.05$, Students t test.

These data suggest that the importance of some Asp residues is much greater in GELIC than in GLIC. The only Asp residue that has a similar effect in the two proteins is Asp91, where substitutions cause decreases in pH_{50} in both GLIC and GELIC. This residue is in a region known to be important for agonist activation in many pLGIC, being located in one of the so–called binding loops (loop E) which contribute to the orthosteric binding pocket. Caffeic acid, cinnamic acid, and various similar compounds which inhibit GLIC responses, have been shown to bind close to this region (*12*), demonstrating a transduction pathway exists from the GLIC 'orthosteric' site to the channel, as in other pLGIC; therefore residues in this region are good candidates to play a role in activation. The bioinformatics data (Fig 4) show Asp91 has the largest change in Asp pK_a values between open and closed GLIC structures, supporting a possible role for this residue. Previous studies (*24*) have shown that Asp91 is involved in an inter-subunit salt bridge with Arg179 in the open channel, (Fig 7), and used computational techniques to probe dynamic responses in receptor containing an Asp91 and two other mutations, which suggested a structural perturbation which can be transmitted via the β1- β2 loop or the pre M1 region, and thence to M2. Thus we conclude that there is evidence that Asp91 may be involved in GELIC and GLIC activation, although it is clearly not the sole activating residue as the receptors can still function when Asp91 is replaced by non charged residues.

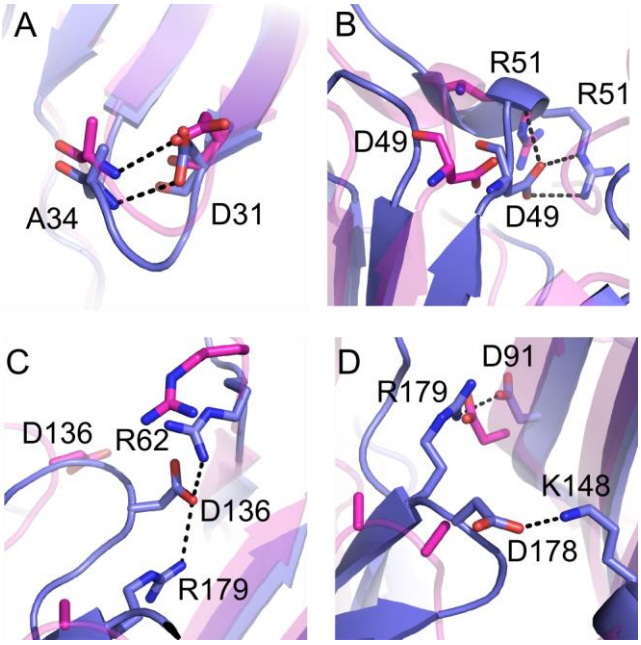

Fig 7: Interactions observed with Asp residues in the open (blue) but not the closed (pink) form of GLIC. (A) D31 forms a hydrogen bond with the main chain of A34. (B) D49 forms a salt bridge with R51 in the open structure only. (C) D136 forms salt bridges with R179 (loop C) and R62 across the interface. (D) D91 on the complementary face forms a salt bridge with R179 (loop C) and D178 (loop C) forms an intersubunit salt bridge with K148 on the complementary face. Note that side chains of D178 and R179 were not built in any of the chains in the 4NPQ structure.

Substitutions to the other Asp residues tested had very different effects in GLIC and GELIC. Neither Ala or Asn were tolerated in place of Asp31 in GELIC, whereas such substitutions caused only small changes (less than a pH unit) to the pH_{50} of GLIC. To determine if the lack of function in GELIC was due to lack of a negatively charged residue, we also tried Glu at this location: in GELIC these receptors were also non-functional, while in GLIC this substitution had no significant effect (Table 1). Asp31 forms part of the β1- β2 loop, which is considered an important structure in transducing binding information to the TMD in neurotransmitter–gated pLGICs. The structural data show Asp31 forms a hydrogen bond with the backbone of Ala34 (Fig 7A), which could be critical for the structural integrity of this loop, and hence perhaps the transduction properties. Thus these data suggest that a critical part of the transduction pathway in GELIC, but not in GLIC, involves the β1- β2 loop.

Asp49 is located close to the membrane in the ECD, which is some distance from Asp31, but substitution here with Ala and Asn had similar effects, with GELIC being sensitive to substitution while GLIC was not. Asp49 is part of loop D in the orthosteric binding site region, and so is a good candidate residue for activation if this is via the ECD. This could perhaps involve the formation of a salt bridge, which can be seen with Arg51 in the open but not the closed structure. Some support for this proposal comes from our experiments substituting Glu at this position, which resulted in a pH_{50} similar to WT in GELIC (Table 1), indicating a negatively charged amino acid residue in this location in the ECD is critical for function in GELIC. Thus these data suggest that a critical part of the transduction pathway in GELIC, but not in GLIC, involves loop D.

Asp136 and Asp 178 form part of loop C and both are predicted to form links with the adjacent subunit in the open state by hydrogen bonding with Arg62 and Arg179, or Lys148 respectively (Fig 7). GELIC is again more sensitive to substitution of these Asp residues, although Asn can effectively substitute for Asp at both positions, supporting the hypothesis that hydrogen bonds are required here for functional GELICs. Nevertheless Asp136Ala GLIC do show an increase in pH_{50}, suggesting this residue may have some importance in activation in this protein. Thus again the data show that Asp residues in loops of the ECD that are

known to play a role in pLGIC activation are more important in GELIC than GLIC, consistent with a major role of the ECD in activation in GELIC, but a lesser role in GLIC.

Conclusions

Our data show that GELIC, a GLIC-ELIC chimera, is functional, and can be activated by protons with a pH_{50} of 6.7. Proton activated responses can be blocked by picrotoxin with an IC_{50} of the same order of magnitude as ELIC, and caffeic acid with an IC_{50} similar to GLIC, indicating that the pore and orthosteric binding site region have features that would be expected in this chimera. Examination of protonatable residues revealed 5 Asps in the ECD that have different pK_as in open and closed structures, and mutating these residues shows they have different importance in GLIC and GELIC: GELIC is considerably more sensitive to substitutions than GLIC, with four of these Asp residues being critical for GELIC, but not GLIC, function. There is also of course the potential that the creation of chimeric receptors may alter global changes in the ECD that are required for channel opening, and, until we have more structural and functional information, we must be cautious in using such data to explain receptor activation. Nevertheless chimeras have proved to be powerful tools in understanding pLGICs, and here suggest that protonation of residues in the ECD is a requirement for channel opening in GELIC, but only plays a minor role in GLIC activation. We propose this is because GLIC activation is largely driven via protonation of the His residue in its pore (His11'), as suggested by Wang et al. (*13*) and Rienzo et al. (*14*). We speculate that GLIC activation via the ECD may only become significant in receptor chimeras where the transmembrane domain no longer has the ability to respond to protonation.

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