Analysis of hyperekplexia mutations identifies transmembrane domain rearrangements that mediate glycine receptor activation

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*Running title: Glycine receptor activation mechanism

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Background: Understanding how Q226E and V280M produce tonic activation may reveal how glycine receptors activate.

Results: Q226E generates an attraction between first and second transmembrane domains. V280M separates first and third transmembrane domains.

Conclusion: We propose either movement can initiate activation.

Significance: Comparison with X-ray structures of bacterial Cys-loop receptors suggests these activation mechanisms apply broadly across the receptor family.

ABSTRACT

Pentameric ligand-gated ion channels (pLGICs) mediate numerous physiological processes and are therapeutic targets for a wide range of clinical indications. Elucidating the structural differences between their closed and open states may help in designing improved drugs that bias receptors towards the desired conformational state. We recently showed that two new hyperekplexia mutations, Q226E and V280M, induced spontaneous activity in α1 glycine receptors. Q226, located near the top of TM1, is closely apposed to R271 at the top of TM2 in the neighbouring subunit. Using mutant cycle analysis we inferred that Q226E induces activation via an enhanced electrostatic attraction to R271. This would tilt the top of TM2 towards TM1 and hence away from the pore axis to open the channel. We also concluded that the increased side chain volume of V280M, in the TM2-TM3 loop, exerts a steric repulsion against I225 at the top of TM1 in the neighbouring subunit. We infer that this steric repulsion would tilt the top of TM3 radially outwards against the stationary TM1 and thus provide space for TM2 to relax away from the pore axis to create an open channel. Because the transmembrane domain movements inferred from this functional analysis are consistent with the structural differences evident in the X-ray atomic structures of closed and open state bacterial pLGICs, we propose that the model of pLGIC activation as outlined here may be broadly applicable across the eukaryotic pLGIC receptor family.

Glycine receptors (GlyRs), which belong to the family of pentameric ligand-gated ion channel (pLGIC) receptors, mediate inhibitory neurotransmission in the spinal cord, brainstem and retina (1,2). In humans, four α GlyR subunits (α1 – α4) and a single β subunit are known (3). GlyRs express as α homomers or as αβ heteromers. However, synaptic GlyRs are thought to exist predominantly as αβ heteromers as the β subunit is required for synaptic clustering via the cytoplasmic scaffolding protein, gephyrin (4,5). Each GlyR subunit contains an extracellular domain (ECD) harbouring a ligand-binding site and a transmembrane domain (TMD) formed by four membrane-spanning α-helices (termed TM1 – TM4) that are connected by flexible loops (6-9). The TM2 domains line the central ion-conducting pore with the other domains arranged concentrically around it providing an interface between the hydrophilic pore and the hydrophobic membrane.

Several pLGIC receptors, notably the nicotinic acetylcholine receptor and the GABA type-A receptor (GABA₆R), have long been important therapeutic targets, and the GlyR has recently emerged as a potential target for indications including inflammatory pain sensitisation (10-12), opioid-induced breathing depression (13), tinnitus (14) and temporal lobe epilepsy (15). It is important to resolve the mechanisms by which the structure of pLGIC receptors changes between the
closed and open states in order to design new drugs that bias the receptor towards the desired conformation.

Human hereditary hyperekplexia (or startle disease) is most commonly caused by missense or nonsense mutations that disrupt the function of the α1 GlyR subunit (16,17). Analysis of the effects of hyperekplexia mutations have provided several important insights into GlyR structure and function (2). We recently characterized two new hyperekplexia mutations, Q226E and V280M, that each resulted in spontaneous activity in both α1 homomeric and α1β heteromeric GlyRs (18). Q226 is located near the top of TM1 whereas V280 is located in the extracellular TM2-TM3 loop. Both sites are thus located near the ECD-TMD interface where agonist-induced conformational changes are transduced, via the movement of TM2 α-helices, into channel opening (6-9). In this study, we employed a variety of functional approaches to elucidate the molecular mechanisms by which the Q226E and V280M mutations induce spontaneous channel openings with the aim of characterizing TMD movements underlying channel activation.

**EXPERIMENTAL PROCEDURES**

**Molecular biology** - Mutations were introduced into the human α1 GlyR using the QuikChange site-directed mutagenesis kit (Stratagene, UK) and confirmed by direct sequencing of the entire transgene-coding region.

**Cell culture** - HEK AD293 cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10 % Fetal Bovine Serum, 30 U/ml penicillin and 30 μg/ml streptomycin, transiently transfected via the calcium-phosphate method with wild type and mutated cDNAs and used in experiments two to three days after transfection.

**Fluorescence-based imaging** - Experiments were performed on HEK AD293 cells and the α1 GlyR and the YFP-I152L plasmid DNA were co-transfected in equal amounts. When the transfection was terminated 16 h later by rinsing with fresh culture medium, cells were plated into the wells of a 384-well plate. Within the following 24 – 32 h, the cell culture medium was replaced by an extracellular control solution (140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, and 10 mM glucose, pH 7.4). Cells were imaged using YFP-I152L fluorescence quench as an indicator of the anion influx rate (19). Fluorescence images of each well were obtained twice: once before and once after the application of a sodium iodide solution (140 mM NaI, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, and 10 mM glucose, pH 7.4) containing defined concentrations of glycine. Mean percentage quench values represent data averaged from three experiments carried out on different plates. Each experimental value was an average of the percentage quench of all fluorescent cells in three wells on the same plate, with each well containing > 200 cells.

**Electrophysiology** - Glycine-gated currents were measured in HEK AD293 cells transfected as described above by whole-cell patch-clamp electrophysiology at -40 mV. Alternatively, single-channel currents were recorded from outside-out excised patches. During experiments, cells were continually superfused with the extracellular control solution as detailed above. Patch pipettes were pulled to a tip resistance of 1 – 4 MΩ (whole-cell) or 6 – 12 MΩ (outside-out) when filled with a standard internal solution (145 mM CsCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, 10 mM EGTA, pH 7.4). Using an Axon MultiClamp 700B amplifier (Molecular Devices), whole-cell currents were filtered at 1 kHz and digitized at 2 kHz whereas single-channel currents were filtered at 2 kHz and digitized at 5 kHz. Voltage-clamp fluorometry experiments were performed as previously described (20). Briefly, oocytes were removed from the ovaries of *Xenopus laevis* frogs, incubated in OR-2 (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl2, 5 mM HEPES, pH 7.4) containing 1.5 mg/ml collagenase for 2 h at room temperature on a shaker and injected with 10 ng of RNA generated form wild type or mutated human α1 GlyR. All constructs contained the functionally silent C41A mutation (21). Oocytes were cultured for 2 – 3 days at 18 °C in ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 5 mM HEPES, pH 7.4) containing 275 mg/l sodium pyruvate, 110 mg/l theophylline and 0.1 % (v/v) gentamicin. For labelling, oocytes were incubated with 10 μM sulforhodamine methanethiosulfonate (MTSR) diluted in ND96 for 1 min on ice. 3 mM KCl was used as internal solution and recordings were performed at -40 mV.

**Statistical analysis** - EC50, nH and Imax or Fmax values for glycine-induced activation of current and fluorescence were obtained using the Hill equation fitted with a non-linear least squares algorithm (SigmaPlot 12.0). All results are expressed as mean ± S.E.M. of three or more independent experiments and statistical analysis was performed using one way ANOVA followed by post-hoc test or Student’s paired t-test, as appropriate, with significance at P < 0.05.

**Western blotting** – HEK AD293 cells transfected as described above were lyzed two days after transfection with a sample buffer containing 2 % Sodium Dodecyl Sulfate (w/v), 10 % glycerol (v/v).
and 0.1 M Tris, pH 6.8. To break potential disulfide bonds, whole-cell lysates were treated with 100 mM dithiothreitol (DTT). After sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and Western blotting, proteins were detected with rabbit anti-GlyR α1 primary antibody (Millipore, 1:3000) and subsequently with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, 1:50000). All experiments were replicated at least three times.

RESULTS

Identification of residues interacting with Q226 and V280 - The crystal structure of the C. elegans α glutamate-gated chloride channel receptor (GluCIR) has recently been determined in the open state (22). As it shares a 34% amino acid sequence identity with the α1 GlyR, it provides the highest homology structural template available for identifying candidate residues that may interact with Q226 and V280. The α GluCIR residues Q219 and N264 (which correspond to Q226 and R271 in the α1 GlyR) are closely apposed across the subunit interface, where their α carbon (Cα) atoms are separated by a distance of 8.3 Å (Fig. 1A). Residues corresponding to both Q226 and R271 are highly conserved in GABA_ARs and GlyRs (Fig. 1C) and their close apposition suggests that an energetic interaction, possibly an H-bond, may exist between them in the open state. The GluCIR structure reveals that residues L218 and I273 (which correspond to I225 and V280 in the α1 GlyR) are also apposed across the subunit interface, with Cα–Cα separation of 8.9 Å (Fig. 1B). These residues are also highly conserved in anionic pLGICs (Fig. 1C), with their chemical properties suggesting the existence of a hydrophobic interaction between them.

To determine whether the relative orientations of these residue pairs are altered as the channel opens, we compared the distances between their respective Cα atoms in ELIC and GLIC, which are bacterial pLGICs crystallized in closed and open state conformations, respectively (23-25). The locations of the ELIC and GLIC residues corresponding to I225, Q226, R271 and V280 in the α1 GlyR are shown in Fig. 1D. In ELIC, the Cα–Cα separation of residues corresponding to Q226 and R271 is 10.0 Å. In GLIC, this distance is reduced to 7.5 Å, similar to GluCIR (8.3 Å), suggesting that Q226 and R271 move closer together as the channel opens. On the other hand, the Cα–Cα separation of residues corresponding to I225 and V280 is 7.6 Å in ELIC, 9.0 Å in GLIC and 8.9 Å in GluCIR, suggesting their respective domains move apart when the channel opens. As agonist binding signals are transmitted to the gate via movements applied to the external end of the TM α-helices (8,9), we propose that the TMD reorientations as depicted in Fig. 1D may be responsible for initiating channel opening. This in turn prompts us to hypothesize that the Q226E mutation induces spontaneous activity via an increased electrostatic attraction to R271 thus stabilizing the domains in a closely apposed conformation which would mimic the open state. We further hypothesize that the increased side chain volume of the V280M hyperekplexia mutation produces spontaneous activity by sterically repelling I225 and thus also mimicking the open state conformational change.

Evidence for a direct interaction between Q226 and R271 - To test whether Q226 and R271 physically interact, the following three mutations were made: Q226R, R271Q and Q226R/R271Q. The R271Q mutation, which occurs naturally in human hyperekplexia, is known to drastically reduce both glycine sensitivity and single channel conductance (26-29). If the Q226R mutation rescues the effects of R271Q by restoring side chain complementarity, we would propose a physical interaction exists between the two residues. To assess the functional properties of these mutants, HEK AD293 cells were transfected with each construct in turn and glycine dose-response relationships were quantitated by whole-cell patch-clamp electrophysiology. Fig. 2A shows sample dose-response recordings for the wild type and the three mutated constructs and in Fig. 2B the normalized mean current amplitudes are plotted against the applied glycine concentration for each construct. The mean glycine EC50 values are presented in Fig. 2C and all averaged curve fit parameters are summarized in Table 1. The glycine EC50 values for receptors incorporating Q226R or Q226R/R271Q were near 1.5 mM whereas the EC50 value for R271Q GlyRs was near 12 mM, as previously shown (27). These results indicate that Q226R partially compensates for the reduced glycine sensitivity of R271Q. Further, GlyRs incorporating R271Q exhibited dramatically reduced maximal current amplitudes that were also compensated by the Q226R mutation (Fig. 2D, Table 1). As the R271Q mutation reduces the dominant single channel conductance state from around 90 to 15 pS (26,28), single channel recordings of Q226R and Q226R/R271Q receptors were performed to evaluate whether the Q226R mutation can also compensate for the reduced conductance. Single channel current-voltage relationships for receptors containing Q226R or
Q226R/R271Q mutations revealed unitary conductances similar to those of α1 wild type GlyRs (Fig. 2E, F). As there was no significant difference in the single channel conductance of receptors containing the wild type, Q226R or Q226R/R271Q subunits, we conclude that an arginine at Q226 can compensate for the drastically reduced conductance of the R271Q receptor. This compensation of both the glycine sensitivity and the single channel conductance strongly suggests that these two residues lie in close proximity. To calculate the coupling energy between them, a mutant cycle analysis was performed as described previously (30,31) using the equation:

$$\Delta G = -RT \ln \left( \frac{EC_{50,ww} \times EC_{50,mm}}{EC_{50,wm} \times EC_{50,mw}} \right)$$

where $\Delta G$ is the coupling energy, $R$ is the universal gas constant, $T$ is temperature (K), $EC_{50,ww}$ is the wild type EC50 value, $EC_{50,mm}$ is the double mutant EC50 value, and $EC_{50,wm}$ and $EC_{50,mw}$ are the two single mutant EC50 values. This equation predicts the coupling energy between Q226 and R271 to be 13.2 kJmol⁻¹. If the N264 residue in the GluClR structure was substituted by an arginine, the distance between the polar oxygen group of Q219 and the positively charged nitrogen group of R264 would be 7 Å. In comparison with interactions as assayed in other proteins (32), a coupling energy of 13.2 kJmol⁻¹ for residues lying 7 Å apart is high indicating a strong attractive interaction between Q226 and R271.

We next generated the Q226C, R271C and Q226C/R271C mutant GlyRs to determine whether Q226 and R271 lie sufficiently close together for disulfide trap to occur. Two cysteine residues can form a disulfide bridge if their Cα-Cα separation distance is not more than 6.5 Å (33). Although the Cα-Cα separation of 8.3 Å (based on the GluClR structure) is too large for crosslinking to occur, random thermal motions or glycine-mediated gating motions may be of sufficient magnitude for disulfide trap to occur. Of course, the mean Cα-Cα separation in α1 GlyRs may also be reduced. The first step was to quantitate the glycine EC50 values of the Q226C, R271C and Q226C/R271C receptors. As shown in Fig. 3A, B, and summarized in Table 1, the glycine EC50 values of all three mutated receptors were increased relative to the wild type receptor. Interestingly, the glycine sensitivity for Q226C/R271C receptors was less reduced than for receptors containing the single mutations Q226C or R271C. Using the above equation, the coupling energy between the respective residues was calculated to be 15.3 kJmol⁻¹ confirming the strong energetic interaction as reported above.

To probe for disulfide bond formation, we investigated the effects of an oxidizing and a reducing agent on the double mutant Q226C/R271C receptor. First, an EC50 (1.5 mM) glycine concentration was applied several times to establish a stable baseline. Cells were then perfused with 2 mM DTT for 1 min followed by two glycine EC50 applications separated by 30 s. Following that, 0.3 % H₂O₂ was applied for 1 min also followed by two glycine EC50 applications. A sample experiment is shown in Fig. 3C (upper trace). Although the initial DTT application significantly increased current to 116 ± 5 % of control (n = 6 cells; P < 0.05 by paired t-test), neither a subsequent H₂O₂ application (111 ± 8 %) nor a second DTT application (114 ± 9 %) exerted a significant effect on current magnitude. As we propose that Q226 and R271 are closer in the open state, the effect of oxidizing and reducing agents on the current amplitude was also tested in the presence of EC50 glycine. Therefore the oxidizing agent H₂O₂ was applied together with an EC50 glycine to simulate the open state in which crosslinking might occur. However, as shown in Fig. 3C (lower trace), no significant effect relative to the initial control current was observed after the application of DTT (91 ± 6 %, n = 5 cells) and H₂O₂ containing EC50 glycine (93 ± 5 %) as well as after a second application of DTT (101 ± 13 %). From this we conclude that either no crosslinks were formed or that the applied agents could not access a pre-formed disulfide bond. We thus employed Western blotting to probe directly for dimer formation. Surprisingly, both the Q226C and R271C single mutant GlyRs formed subunit dimers that were reduced by DTT (Fig. 3D). The double mutant Q226C/R271C GlyR formed not only dimers but also tetrameric or pentameric aggregates that were relatively resistant to reduction by DTT. As discussed in detail below, these data do not provide unequivocal evidence for dimer formation as assembled Q226C/R271C GlyRs.

**Evidence for a direct interaction between V280 and I225** - The V280M mutant GlyR also exhibits a high level of spontaneous channel activity (18). As valine and methionine are both hydrophobic but methionine is larger, we hypothesized that the spontaneous activity arises from a steric repulsion between V280 and I225 of the adjacent subunit (Fig. 1). To test whether the level of spontaneous activity is dependent on the volume of the side chain, we mutated V280 in turn to the hydrophobic amino acids, alanine, leucine and tryptophan. The side chain volume in water per residue is 78.9 Å³
for valine, 103.2 Å³ for methionine, 28.9 Å³ for alanine, 107.0 Å³ for leucine and 167.3 Å³ for tryptophan. HEK AD293 cells were transfected with each mutant construct in turn and a yellow fluorescent protein anion influx assay (19) was employed to quantify the relative magnitudes of spontaneous anion influx in each mutated receptor. The fluorescence assay was necessary as the high level of spontaneous activity in V280L and V280W mutant GlyRs meant that it was difficult to achieve stable electrophysiological recordings. In these experiments, cells were bathed initially in NaCl solution and the percentage reduction in quench was quantitated upon replacement of the control NaCl solution with NaI solution plus or minus glycine. Because YFP-I152L is quenched by iodide but not by chloride ions (19), potent quench by iodide in the absence of glycine indicates spontaneously active channels. In Fig. 4A, the fluorescence quench was plotted against the applied glycine concentration. For wild type receptors, the glycine EC₅₀ and the maximal fluorescence quench values (expressed as a percentage of control fluorescence) were 16.9 ± 3.0 μM and 48 ± 1 %, respectively, whereas the corresponding values for V280A receptors were 5.6 ± 1.7 μM and 40 ± 1 %, respectively. The V280A GlyR showed no evidence of spontaneous activity, and the glycine sensitivity of these receptors was similar to that previously demonstrated using electrophysiology (34). We thus infer that a small hydrophobic substitution at position 280 does not induce spontaneous activity. In contrast, receptors incorporating V280M, V280L or V280W mutations exhibited a strong quench upon addition of glycine-free NaI solution with a magnitude that was proportional to side chain volume (Fig. 4B). We hence conclude that the level of spontaneous activity is proportional to side chain volume at position 280.

To test whether I225 and V280 interact, we generated the single mutant I225C and V280C receptors and the double-mutant I225C/V280C receptor. Using whole-cell patch-clamp electrophysiology, we found that the glycine sensitivity of receptors incorporating I225C or V280C mutations was modestly increased (Fig. 5A-C, Table 1). In contrast, the glycine sensitivity of I225C/V280C receptors was significantly reduced resulting in a coupling energy of -7.7 kJmol⁻¹ between the two side chains. As the Cα–Cα separation of residues corresponding to I225 and V280 is 9 Å in the GluCIR structure, this energy value can be considered as very high relative to the coupling energy for other residue pairs (32).

To determine whether any of the cysteine mutant GlyRs exhibited spontaneous channel activity, we quantitated the relative magnitudes of the current inhibited by 100 μM picrotoxin as a percentage of the current activated by saturating (10 mM) glycine in the same cell. A sample experiment for the V280C mutant GlyR, revealing a large spontaneous current, is shown in Fig. 5D. The results averaged for the I225C, V280C and I225C/V280C mutant GlyRs are summarized in Fig. 5E. We propose the large leak current in the V280C mutant GlyR can be explained by the disruption of a hydrophobic bond that helps to maintain the close separation between these residues in the closed state.

We next investigated the possibility of disulfide bond formation between I225C and V280C. We applied DTT and H₂O₂ as previously described while monitoring the magnitude of 100 μM glycine-gated currents. The mean current magnitude changes expressed as percentages of the original control current magnitude were as follows: after first DTT application: 106 ± 16 %, after subsequent H₂O₂ application: 94 ± 27 %, after second DTT application: 97 ± 31 %. All results were averaged from the same six cells and revealed no significant differences in current magnitude (P > 0.05 by paired t-test). A Western blot also showed no evidence for dimer formation. We thus infer that I225C and V280C interact energetically but do not form a disulfide bond (data not shown).

**Effects of Q226E and V280M on TM2-TM3 loop conformation - Glycine-induced conformational changes are transmitted from the glycine-binding site to the gate via conformational changes in the TM2-TM3 domain (8,9,34). To determine the effect of Q226E and V280M mutations on TM2-TM3 conformation and vice versa, we next employed K276C receptors. K276 is located in the TM2-TM3 loop and it has previously been shown that cysteines introduced at the position corresponding to K276 in GABAₐR β2 subunits are able to efficiently crosslink neighbouring β2 subunits in α1β2GABAₐRs (35). Our first approach involved determining whether K276C residues could also crosslink in α1 GlyRs, and if so, whether these crosslinks could be disrupted by the Q226E or V280M mutations. We thus generated the K276C single mutant GlyR and the Q226E/K276C and K276C/V280M double mutant GlyRs. These constructs were transfected into HEK AD293 cells and whole-cell glycine dose-response relationships were determined. For receptors containing K276C, the glycine sensitivity was dramatically reduced (Fig. 6A, Table 1) as previously described (21). In contrast, for receptors containing Q226E/K276C or K276C/V280M, the
sensitivity to glycine was only modestly reduced to 500 µM and 90 µM, respectively. Glycine dose-response recordings were also performed after the application of 2 mM DTT for 1 min, however, no significant changes in the glycine sensitivity were detected (Fig. 6A, Table 1). Interestingly, the current amplitude for K276C receptors was significantly increased after the application of DTT (Table 1). We also probed the three receptors for spontaneous channel activity using the method as described above (Fig. 5D, E). No inhibitory effect of picrotoxin was observed in Q226E/K276C GlyRs (n = 4 cells). In contrast picrotoxin inhibited 2.5 ± 0.6% (n = 4 cells) of the saturating glycine-activatable current in K276C/V280M GlyRs.

To probe for disulfide bond formation, 2 mM DTT and 0.3 % H₂O₂ were applied alternately for 1 min each, as described above, while the magnitude of currents activated by EC₅₀ glycine (3000 µM for K276C, 500 µM for Q226E/K276C and 90 µM for K276C/V280M) was monitored. In K276C mutant GlyRs, DTT significantly increased current magnitude whereas H₂O₂ rapidly and irreversibly reduced current magnitude (Fig. 6B, C), consistent with the formation and breakage of crosslinks between adjacent subunits. However, the Q226E/K276C and K276C/V280M receptors showed no significant response to either DTT or H₂O₂ treatment, suggesting either that crosslink formation did not occur or that crosslinks had formed spontaneously and could not be reduced by DTT.

To resolve between these possibilities, we performed a Western blot analysis on the wild type and all three mutated constructs (Fig. 6D). Compared to α1 wild type receptors that did not form dimers, all mutant receptors exhibited a similar incidence of dimer formation that was reversed by DTT. Our results are consistent with a model whereby the reduction of crosslinks in K276C GlyRs enhances TM2-TM3 loop flexibility and leads to more efficacious glycine-induced activation. In contrast, crosslink reduction does not enhance glycine-induced current magnitudes in either of the double mutant GlyRs. We infer that the Q226E and V280M mutations position the TM α-helices into a conformation that is already highly conductive to efficacious glycine-induced activation. In such a scenario, breakage of the disulfide bonds would exert no additional enhancement of glycine efficacy and thus exert no effect on glycine-gated current magnitudes.

The effects of Q226E and V280M mutations on conformational changes near R271 were quantitated using voltage-clamp fluorometry (36). In this technique, a sulfhydryl-tagged fluorophore (often a rhodamine derivative) is covalently attached to a cysteine introduced into a receptor domain of interest. Because rhodamine fluorescence exhibits an increase in quantum efficiency as the hydrophobicity of its environment is increased, glycine-induced changes in fluorescence intensity can be interpreted as local conformational changes. These experiments were carried out in Xenopus oocytes as the detection of small glycine-induced fluorescence changes is not routinely possible in HEK AD293 cell-expressed receptors.

Previously, we reported that MTSR (sulforhodamine methanethiosulfonate), when covalently attached to R271C in TM2 of the homomeric α1 GlyR, exhibited an increase in fluorescence intensity upon glycine binding (20). As the glycine concentration-response relationships for current and fluorescence were overlapping, we concluded that conformational changes at the channel gate (as reported by the current response) were tightly coupled with conformational changes near R271 (as reported by the fluorescence response). As we sought to investigate the effect of the Q226E and V280M mutations on these glycine-induced fluorescence changes, we compared current and fluorescence responses in the R271C single mutant GlyR and the Q226E/R271C and R271C/V280M double mutant GlyRs. Averaged glycine current dose-response relationships for unlabeled and MTSR-labeled R271C mutant GlyRs are shown in Fig. 7A, with mean glycine EC₅₀, nᵣ and Iₘ₅ₒ/Fₘ₅ₒ values summarized in Table 2. As previously demonstrated, the glycine fluorescence dose-response in the MTSR-labeled GlyR overlapped with the current dose-response.

This experimental approach was repeated on Q226E/R271C and R271C/V280M mutant GlyRs. The glycine EC₅₀ for unlabeled Q226E/R271C GlyRs was near 1700 µM (Fig. 7B, Table 2). In MTSR-labeled Q226E/R271C GlyRs the current EC₅₀ was near 600 µM and the fluorescence EC₅₀ was significantly higher at around 1000 µM. For R271C/V280M receptors, the glycine EC₅₀ was dramatically reduced due to the presence of V280M. The glycine EC₅₀ for unlabeled receptors was 12 µM and for MTSR-labeled receptors the current EC₅₀ was 2 µM and the fluorescence EC₅₀ was significantly higher at 6 µM (Fig. 6C, Table 2). These results indicate that the hyperekplexia mutations uncouple conformational changes at the channel gate from conformational changes occurring near to R271.

Finally, the effect of the pore blocker picrotoxin was tested on all three receptors to confirm whether spontaneous activity was present in the double mutant GlyRs. For R271C/V280M GlyRs, the application of 100 µM picrotoxin blocked a leak
current which corresponded to 68 ± 21 % of the saturating (30 mM) glycine-activatable current. However, no significant change in baseline current was detected for receptors containing R271C or Q226E/R271C GlyRs (n = 4 for each receptor). For all three receptors, no change in fluorescence was detected upon the application of picrotoxin suggesting that the environment of residue R271 does not change when picrotoxin blocks the pore.

**DISCUSSION**

*The interaction between Q226 and R271* - As the Cα-Cα separation of Q226 and R271 is predicted to reduce from 10 to ~8 Å as the channel opens (Fig. 1), we hypothesized that the spontaneous activity induced by Q226E was due to an enhanced electrostatic attraction to R271. To test this, we introduced a positive charge at Q226 via the Q226R mutation. Separately, we also eliminated the positive charge at R271 via the R271Q mutation. Both mutations dramatically reduced glycine sensitivity, consistent with our hypothesis. However, the double Q226R/R271Q mutant GlyR largely restored glycine sensitivity, with mutant cycle analysis demonstrating a strong energetic attraction between the two residues. This result provides strong support for our hypothesis.

The positive charge at R271 enhances the GlyR single channel conductance via a direct electrostatic interaction with the permeating anions (37), thus explaining why the R271Q human hyperekplexia mutation drastically reduces single channel conductance (26,28). This reduction in conductance was entirely reversed by inserting the positive charge at Q226 in the Q226R/R271Q double mutant GlyR (Fig. 2E-F). Although this does not provide evidence for an energetic interaction between Q226 and R271, it does suggest that the two residues are located in sufficiently close proximity that the removal of a positive charge from one site can be compensated by the addition of a positive charge to the other.

Our attempt to demonstrate proximity between Q226 and R271 via cysteine trapping yielded equivocal results. Although we demonstrated a strong energetic coupling between Q226C and R271C via mutant cycle analysis, we found that oxidizing and reducing agents had no significant effect on glycine-activated current magnitudes. Western blotting showed that Q226C/R271C double mutant GlyRs could indeed form dimer complexes, as predicted. However, because single mutant Q226C or R271C GlyRs could also form dimers, and Q226C/R271C GlyRs could form pentameric or tetrameric subunit aggregates, we could not rule out the possibility of other mechanisms contributing to subunit aggregation.

We conclude that GlyRs can be activated directly by increasing an electrostatic attraction between Q226 at the top of TM1 and R271 at the top of TM2 of the neighbouring subunit. In wild type GlyRs, the formation of an H-bond between Q226 and R271 may help stabilize the glycine-induced open state. When taken together with the crystal structure information (Fig. 1), we propose this enhanced attraction would tilt the outer end of TM2 away from the pore opening, resulting in pore opening.

*The interaction between V280 and I225* - Comparison of closed and open state pLGIC structures suggests that the Cα-Cα separation of V280 and I225 increases from 7.6 to ~9 Å as the channel opens (Fig. 1). We therefore hypothesized that the spontaneous activity in the V280M mutant GlyR was due to the larger methionine side chain exerting a steric repulsion from I225. This would increase the separation of TM1 and TM3, and thus mimic the closed to open state structural change. To test this hypothesis, we introduced progressively larger hydrophobic side chains at V280 and monitored both glycine sensitivity and the mean level of spontaneous channel activity. We found that larger side chains did indeed induce higher levels of spontaneous activity, consistent with our hypothesis. Although our attempts to demonstrate proximity between V280 and I225 via cysteine trapping were unsuccessful, we did demonstrate a significant energetic coupling between V280C and I225C via mutant cycle analysis. In this case the individual cysteine mutations had little effect on the glycine EC_{50} value, but the double cysteine mutant receptor exhibited dramatically reduced glycine sensitivity. This suggests that the double cysteine substitution (I225C/V280C) may have increased the space available for the respective domain backbones to move closer together, stabilizing the closed state. The V280C mutant GlyR exhibited a leak current that was larger than may be expected due to its sidechain volume (Fig. 5D). This can be explained by the disruption of a putative hydrophobic bond that helps to maintain the close apposition between V280 and I225 in the closed state.

A comparison of GLIC versus ELIC structures reveals that during channel opening, the top of the TM1 does not move although the top of TM3 moves radially outwards from the pore in parallel with TM2 (7,23,25). When taken in conjunction with our functional evidence, we propose that increasing the side chain volume at V280 tilts the top of TM3 radially outwards against the stationary
TM1 of the adjacent subunit, thus providing space for TM2 to relax away from the pore axis to create an open channel. Consistent with this mechanism, ivermectin is also thought to open the GluClR by directly spreading TM1 and TM3 (22).

Effects of mutations on subunits dimerized via K276C crosslinks - Two findings prompted us to conclude that GlyR α1 subunits dimerize via disulfide bonds between their respective K276C residues. First, current magnitude in K276C mutant GlyRs was dramatically increased by a reducing agent and irreversibly reduced by an oxidizing agent. Second, dimer formation was directly demonstrated by Western blotting. These results are consistent with a previous study on α1β2 GABA_A Rs where β2 subunits were shown to dimerize via crosslinks between TM2-TM3 loop residues corresponding to K276C (35). Ca−Ca separation must usually be < 6 Å for disulfide bond formation to occur (33). In the ELIC, GLIC and α GluClR crystal structures, the Ca−Ca separation of residues corresponding to K276 in adjacent subunits is 20 - 24 Å (22-25). Given this large distance, dimerization via K276C crosslinks provides evidence for exceptionally large thermal motions in the TM2-TM3 loop of α1 subunits. We cannot rule out the possibility of dimer formation between non-adjacent subunits, but this would implicate an even greater Ca−Ca separation.

Our results are consistent with a model whereby the reduction of crosslinks in K276C GlyRs enhances TM2-TM3 loop flexibility and thereby leads to more efficacious glycine-induced activation. Given that a maximum of two dimer pairs can exist per receptor, it is clear that at least one (and possibly both) crosslinks must be reduced for maximally efficacious receptor activation. However, the reduction of K276C crosslinks did not enhance glycine-induced current magnitudes in Q226E/K276C or K276C/V280M double mutant GlyRs. We therefore infer that Q226E or V280M mutations configure the GlyR TMD into a conformation conducive to maximally efficacious glycine-induced activation. In such a scenario, the increased flexibility in the TM2-TM3 loop induced by reduction of the disulfide bonds would exert no additional effect on glycine-gated current magnitudes.

Conclusion - We have described how the hyperekplexia mutations, Q226E and V280M, induce spontaneous GlyR activation. The mechanism we propose is consistent with the structural differences evident in the X-ray atomic structures of closed and open state pLGICs suggesting that it may be broadly applicable across the eukaryotic pLGIC receptor family. In addition, a specific prediction of our study is that an H-bond between Q226 in TM1 and R271 in TM2 in the neighbouring subunit is necessary for stabilizing the GlyR in the open state.

REFERENCES

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**FOOTNOTES**

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The abbreviations used are: Cα, α-carbon atom; DTT, dithiotreitol; ECD, extracellular domain; GABAαR, GABA type-A receptor; GluClR, glutamate-gated chloride channel receptor; GlyR, glycine receptor; MTSR, methanethiosulfonate rhodamine; pLGIC, pentameric ligand-gated ion channel; TM, transmembrane; TMD, transmembrane domain.

**FIGURE LEGENDS**

Fig. 1. Location of residues hypothesized to interact with the α1 GlyR residues, Q226 and V280. A. Model of the *C. elegans* α GluClR (PDB 3RIF) showing two neighbouring subunits (coloured light and dark grey, respectively), with residues homologous to α1 GlyR Q226 and R271 coloured light and dark purple, respectively. The lower panel shows the view towards the membrane from the extracellular space with the ECD removed. B. Same structure and orientations as in A, but with residues homologous to α1 GlyR I225 and V280 coloured light and dark blue, respectively. C. Multiple sequence alignment of the TMD regions indicated pLGIC receptors with Q226 and V280 and their interacting residues coloured as in panels A and B. D. Model structures of ELIC (left, PDB 2VLO) and GLIC (right, PDB 3EAM) showing two neighbouring subunits (coloured light and dark grey, respectively) viewed towards the membrane from the extracellular space with the ECD removed. As in panels A and B, residues homologous to α1 GlyR Q226, R271, I225 and V280 are coloured light and dark purple and light and dark blue, respectively.

Fig. 2. Electrophysiological characterization of Q226R, R271Q and Q226R/R271Q mutant α1 GlyRs. A. Examples of currents activated by indicated glycine concentrations for each receptor type. In this and subsequent figures, thin horizontal bars indicate the duration of glycine applications and numbers represent glycine concentration in μM. B. Averaged normalized glycine dose-response relationships for the four receptors. C. Mean glycine EC50 values. ***P < 0.001 relative to α1 GlyR via one way ANOVA followed by Dunnett’s post-hoc test. D. Mean maximal glycine-mediated current amplitudes. **P < 0.01 relative to α1 GlyR via one way ANOVA followed by Dunnett’s post-hoc test. E. Averaged single channel current-voltage relationships for indicated receptors recorded in outside-out patches. The wild type, Q226R and Q226R/R271Q GlyRs exhibited mean single channel conductance values of 92 ± 4 pS (n = 3), 99 ± 4 pS (n = 6) and 98 ± 3 pS (n = 6), respectively. These values were not significantly different to each other using one way ANOVA followed by Tukey’s post-hoc test. The R271Q current-voltage relationship, shown previously

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to be 15 pS (26,28), is indicated as a dashed line. F. Sample single channel activations in wild type, Q226R and Q226R/R271Q GlyRs recorded at -80 mV. Channel openings are downward, with dashed lines denoting the main open conductance level. Channel amplitude histograms are also displayed. In this analysis we only included sections of recording in which the channel exhibited a stable transition from one conductance level to another. We did not include sections of record containing unresolved channel openings. As the histograms reveal an absence of stable openings at subconductance levels, we infer that the brief openings of reduced magnitude were mostly unresolved larger amplitude events curtailed by filtering.

Fig. 3. Electrophysiological and biochemical characterisation of Q226C, R271C and Q226C/R271C mutant α1 GlyRs. A. Averaged normalized glycine dose-response relationships for the four receptors. B. Mean glycine EC<sub>50</sub> values. ** P < 0.01, *** P < 0.001 relative to α1 GlyR via one way ANOVA followed by Dunnett’s post-hoc test. C. Examples of current traces activated by EC<sub>50</sub> glycine (1.5 mM) in Q226C/R271C GlyRs,.The first two traces in each row were recorded from a naïve cell. Subsequent traces were recorded following 1 min applications of 2 mM DTT or 0.3% H<sub>2</sub>O<sub>2</sub> (together with EC<sub>50</sub> glycine for the lower trace) as indicated. Averaged results are presented in the text. D. Western blot of wild type, Q226C, R271C and Q226C/R271C mutant α1 GlyRs in the absence and presence of 100 mM DTT as indicated. A protein preparation from untransfected cells is included as a control. Numbers on the left represent size in kD. Similar results were obtained in blots performed on three separate protein preparations.

Fig. 4. Functional characterisation of α1 GlyRs incorporating mutations at V280. A. Glycine-dependence of fluorescent quench for the wild type, V280M, V280A, V280L and V280W mutant GlyRs using the YFP-I152L anion influx assay. The percentage quench is equal to (1 - final fluorescence/control fluorescence) X 100%. All displayed data points represent the average quench from three experiments with three wells each and > 200 cells per well. B. Mean percentage quench in the absence of glycine for the indicated receptors. **** P < 0.001, ***** P < 0.0001 relative to α1 GlyR via one way ANOVA followed by Dunnett’s post-hoc test.

Fig. 5. Electrophysiological characterisation of I225C, V280C and I225C/V280C mutant α1 GlyRs. A. Examples of currents activated by indicated glycine concentrations for each receptor type. B. Averaged normalized glycine dose-response relationships for wild type and indicated mutant receptors. C. Mean glycine EC<sub>50</sub> values. **** P < 0.0001 relative to α1 GlyR via one way ANOVA followed by Dunnett’s post-hoc test. D. Sample trace for V280C mutant α1 GlyRs showing the maximal glycine-induced current amplitude and the inhibition of the leak current by 100 μM picrotoxin. E. Magnitude of picrotoxin-inhibited current expressed as a percentage to the maximal glycine-gated current amplitude. * P < 0.05, *** P < 0.001 relative to α1 GlyR via one way ANOVA followed by Dunnett’s post-hoc test.

Fig. 6. Effects of Q226E and V280M on GlyR dimerization via K276C crosslinks. A. Averaged normalized glycine dose-response relationships for wild type and indicated mutant receptors before and after the application of 2 mM DTT. B. Sample current recordings of K276C, Q226E/K276C and K276C/V280M GlyRs activated by EC<sub>50</sub> glycine (3000 μM, 500 μM and 90 μM, respectively). The first two traces were recorded from a naïve cell. Subsequent traces were recorded following 1 min applications of 2 mM DTT or 0.3% H<sub>2</sub>O<sub>2</sub> as indicated. C. Normalized glycine EC<sub>50</sub> current amplitudes of K276C, Q226E/K276C and K276C/V280M GlyRs before and after the application of DTT and H<sub>2</sub>O<sub>2</sub>. The normalized currents are represented in percentages. P-values were calculated relative to the control current with a paired t-test: * P < 0.05. B. Western blot of wild type, Q226C, R271C and Q226C/R271C mutant α1 GlyRs in the absence and presence of 100 mM DTT as indicated. A protein preparation from untransfected cells is included as a control. Numbers on the left represent size in kD. Similar results were obtained in blots performed on three separate protein preparations.

Fig. 7. Effects of Q226E and V280M on conformational changes near TM2 as determined by voltage-clamp fluorometry. A-C. Averaged normalized glycine dose-response relationships for both current (black triangles) and fluorescence (red triangles) at MTSR-labeled R271C, Q226E/R271C and R271C/V280M GlyRs using voltage-clamp fluorometry. Current dose-response relationships for unlabeled receptors are also shown (black circles). Mean parameters of best fit to individual dose-response relations are presented in Table 2. D. Examples of simultaneous current (black) and fluorescence (red) recordings from oocytes expressing labeled R271C GlyRs (upper traces) and R271C/V280M GlyRs (lower traces).
Table 1. Properties of wild type and indicated mutant α1 GlyRs using whole-cell patch-clamp electrophysiology

<table>
<thead>
<tr>
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<tr>
<td></td>
<td>EC_{50} (µM)</td>
<td>n_H</td>
<td>I_{max} (nA)</td>
<td>n</td>
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<tr>
<td>α1 %</td>
<td>64 ± 8</td>
<td>3.8 ± 0.3</td>
<td>19 ± 3</td>
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<tr>
<td>α1-Q226R</td>
<td>1705 ± 334</td>
<td>1.3 ± 0.2****</td>
<td>15 ± 5</td>
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<tr>
<td>α1-R271Q</td>
<td>12100 ± 4592***</td>
<td>1.2 ± 0.1****</td>
<td>2 ± 1**</td>
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<tr>
<td>α1-Q226R/R271Q</td>
<td>1547 ± 145</td>
<td>2.1 ± 0.2***</td>
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<tr>
<td>α1-Q226C</td>
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<td>1.1 ± 0.1****</td>
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<tr>
<td>α1-R271C</td>
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<td>1.0 ± 0.1****</td>
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<td>α1-Q226C/R271C</td>
<td>1657 ± 206</td>
<td>0.9 ± 0.0****</td>
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<td>α1-I225C</td>
<td>54 ± 13</td>
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<td>27 ± 5</td>
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<tr>
<td>α1-V280C</td>
<td>26 ± 8</td>
<td>1.6 ± 0.5**</td>
<td>18 ± 4</td>
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<tr>
<td>α1-I225C/V280C</td>
<td>496 ± 41****</td>
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<tr>
<td>Before DTT</td>
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<td>α1-Q226E/K276C</td>
<td>565 ± 30</td>
<td>1.6 ± 0.1****</td>
<td>3 ± 1**</td>
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<td>α1-K276C/V280M</td>
<td>90 ± 10</td>
<td>1.7 ± 0.2****</td>
<td>9 ± 2</td>
<td>5</td>
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<tr>
<td>After DTT</td>
<td></td>
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<td></td>
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<tr>
<td>α1-K276C</td>
<td>4530 ± 1362****</td>
<td>1.1 ± 0.1****</td>
<td>7 ± 3*</td>
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<td>α1-Q226E/K276C</td>
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<td>α1-K276C/V280M</td>
<td>144 ± 20</td>
<td>1.1 ± 0.1****</td>
<td>9 ± 2</td>
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</tbody>
</table>

* P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 relative to wild type α1 GlyR via one way ANOVA followed by Dunnett’s post-hoc test.

% Results for the wild type α1 GlyR were reproduced from (18).
Table 2. Properties of wild type and indicated mutant α1 GlyRs using voltage-clamp fluorometry.

<table>
<thead>
<tr>
<th></th>
<th>Before MTSR labeling</th>
<th>After MTSR labeling</th>
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<tr>
<td></td>
<td>EC_{50} (µM)</td>
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<tr>
<td>α1-R271C</td>
<td>6314 ± 336</td>
<td>1.7 ± 0.0</td>
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<tr>
<td>α1-Q226E/R271C</td>
<td>1712 ± 206***</td>
<td>1.2 ± 0.1*</td>
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<tr>
<td>α1-R271C/V280M</td>
<td>12 ± 0****</td>
<td>2.2 ± 0.1*</td>
</tr>
<tr>
<td>α1-R271C</td>
<td>977 ± 128</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>α1-R271C/V280M</td>
<td>6 ± 1***</td>
<td>1.6 ± 0.1</td>
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* P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 relative to R271C mutant α1 GlyR via one way ANOVA followed by Dunnett’s post-hoc test.
Figure 1

A GluCl - open state

B GluCl - open state

C

<table>
<thead>
<tr>
<th>M1</th>
<th>M2</th>
<th>M3</th>
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<td>HLERQMGYYL</td>
<td>IQMYIPSLLIVILSWISFWINMDAAPARVGLGITTVLTMTTQSSGS</td>
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<tr>
<td>GLRA2_HUMAN</td>
<td>HLERQMGYYL</td>
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<td>TLRRQVGFYMMGVYAPTLLIVVLSWLSFWINPDASAARVPLGIFSVLSLASECTTLAAELPK</td>
<td>V-SYKAID18M1AVC</td>
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<td>IQTYMPSILITILSWVSFWINYDASAARVALGITTVLTMTTQSSGS</td>
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<td>IQTYIPCTLIVVLSWVSFWINKDAVPARTSLGITTVLTMTTLSTIA</td>
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<td>GBRP_HUMAN</td>
<td>ELRRNVLYFILETYVPSTFLVVLSWVSFWISLDSVPAPTCIOVTLILSTMTTQSSGS</td>
<td>TSLPNTNCFIKAID18M1AVC</td>
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<td>ACNA7_HUMAN</td>
<td>TMRRRTLYYGLNLLIPCVLISALALLVFLLPADS-GEKISLGITVLLSLTVFMLLVAEIMPATSDS</td>
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<td>5HT3A_HUMAN</td>
<td>VRRRPFLYVSSLSSPLFPAQPISGQGQVPLPQDS-GERVSFKTLLLGGVFLIVVLWSLNNNAI12TLPGLQGFYVVC</td>
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<td>GLCL_CRTS</td>
<td>QLRRKFSFYLLQLYIPSCMLLTLSWVSFWIFGRTAIPVRVLQTVILMTMTQAGINSQLPFF-SY1H18M1AVGAC</td>
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<td>GLIC_GLOVI</td>
<td>RISQYFFSYIPNI1LMLFLFLF1SWATAFMETSYSY--AVNYLVSTILASHIAFN1LVETVLWFT-FYM1TTGAT1FPNI</td>
<td></td>
</tr>
</tbody>
</table>

D

ELIC - closed state

GLIC - open state
Figure 2

A

\begin{center}
\includegraphics[width=\textwidth]{figure2A.png}
\end{center}

B

\begin{center}
\includegraphics[width=\textwidth]{figure2B.png}
\end{center}

C

\begin{center}
\includegraphics[width=\textwidth]{figure2C.png}
\end{center}

D

\begin{center}
\includegraphics[width=\textwidth]{figure2D.png}
\end{center}

E

\begin{center}
\includegraphics[width=\textwidth]{figure2E.png}
\end{center}

F

\begin{center}
\includegraphics[width=\textwidth]{figure2F.png}
\end{center}
Figure 3

A

\[ I_{\text{Lmax}} \]

\[ 0 \quad 0.2 \quad 0.4 \quad 0.6 \quad 0.8 \quad 1.0 \quad 1.2 \]

\[ 0 \quad 100 \quad 1000 \quad 10000 \quad 100000 \quad 1000000 \]

[Choline] (\mu M)

- el
- al-Q226C
- al-R271C
- al-Q226C/R271C

B

\[ E_{\text{C(min)}} (\mu M) \]

\[ 0 \quad 2000 \quad 4000 \quad 6000 \quad 8000 \quad 10000 \quad 12000 \]

- al
- al-Q226C
- al-R271C
- al-Q226C/R271C

C

el-Q226C/R271C

DTT

H2O2

open state

closed state

DTT

D

DTT

H2O2

DTT

- + - + - + - +

2 nA

5 s

DTT
Figure 4

A

Quench (%) vs. [Glycine] (µM)

- α1
- α1-V280M
- α1-V280L
- α1-V280A
- α1-V280W

B

Quench (%)

Bar graph showing quench without glycine (%)

- α1
- α1-V280M
- α1-V280L
- α1-V280A
- α1-V280W

***
Figure 5

A

\[ a1,4223C \]

0.1 1 10 100 1000 10000 30000

0.1 1 10 100 1000 10000 30000

\[ a1-V280C \]

\[ a1-4223C/V280C \]

D

10 mM GLY

100 μM PTX

PTX inhibition

0.5 nA

5 s

B

\[ G0 \]

[0, 0.1, 10, 100, 1000, 10000, 100000]

\[ IC_{50}(\mu M) \]

C

\[ IC_{50}(\mu M) \]

D

PTX inhibition (%)
Figure 6

A

\[ \frac{I}{I_{\text{max}}} \] vs [Glycine] (µM)

- a1-K276C before DTT
- a1-K276C after DTT
- a1-Q226E/K276C before DTT
- a1-Q226E/K276C after DTT
- a1-K276C/V280M before DTT
- a1-K276C/V280M after DTT

B

\[ \text{H}_2\text{O}_2 \]

1 nA

5 s

C

\[ \text{H}_{\text{cat}} \]

Control

DTT

\[ \text{H}_2\text{O}_2 \]
Figure 7

A

\[ \text{[Glycine] (µM)} \]

B

\[ \text{[Glycine] (µM)} \]

C

\[ \text{[Glycine] (µM)} \]

D

\[ \text{[Glycine] (µM)} \]