Charaterisation of the Effects of Charged Residues in the Intracellular Loop on Ion Permeation in α1 Glycine Receptor-Channels

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Running title: M3-M4 loop residues influence the γ of α1 glycine receptors

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The Cys-loop receptor-channels mediate fast synaptic transmission in the nervous system. The M2-demarcated transmembrane pore is an important determinant of their ion permeation properties. Portals within the intracellular domain are also part of the permeation pathway in cationic Cys-loop receptors, with charged residues in a helical MA-stretch partially lining these openings profoundly affecting channel conductance. It is unknown if analogous portals contribute to the permeation pathway in anionic Cys-loop receptors. We therefore investigated the influence of charged residues within the proposed MA-stretch on functional properties of the homomeric glycine α1 receptor. Up to eight basic residues in the MA-stretch were concurrently mutated to a negatively charged glutamate, and wild-type and mutant subunits were expressed in HEK-293 cells. Mutation of all eight residues produced a non-functional receptor. The greatest reduction in conductance at negative membrane potentials (from 92.2 ± 2.8 to 60.0 ± 2.2 pS) was observed with glutamate present at the 377, 378, 385 and 386 positions (the 4E subunit). Inclusion of additional glutamate residues within this subunit did not decrease conductance further. Neutralising these residues (the 4A subunit) caused a modest decrease in conductance (80.5 ± 2.3 pS). Outward conductance at positive potentials was not markedly affected. Anion to cation selectivity, and concentration-response relationships, were unaffected by the 4A or 4E mutations. Our results identify basic residues affecting conductance in the glycine receptor, suggesting that portals are part of the extended permeation pathway, but that the M2-demarcated channel pore is the dominant determinant of permeation properties in glycine receptors.

Rapid and co-ordinated communication between neurons at synapses in the central nervous system (CNS) is critical for the maintenance of normal brain function. Key to this process is the Cys-loop superfamily of ligand-gated ion channels (LGICs), which includes the cation-selective nicotinic acetylcholine (ACh) and 5-hydroxytryptamine type 3 (5-HT3), and the anion-selective glycine and γ-aminobutyric acid type A (GABA_A) receptors. LGICs convert the chemical messages conveyed by neurotransmitters into excitatory, or inhibitory, electrical signals via the selective conduction of ions. The polarity of the most permeant ion (ion-charge selectivity) and the rate at which the ions permeate (the single channel conductance, γ) are major determinants of how this synaptic signal affects neuronal excitability.

Cys-loop LGICs are composed of five protein subunits arranged around a central ion conducting channel pore. Each of the five subunits consist of a large extracellular domain harbouring the neurotransmitter binding site, four membrane spanning domains (M1-M4) and, in mammalian LGICs at least, a large intracellular
loop connecting M3-M4. The electron microscopic images of the *Torpedo marmorata* nicotinic ACh receptor (1-3) provide our most complete picture of the structure of the ion permeation pathway of mammalian LGICs. Ions entering the wide external vestibule are funnelled through a narrow transmembrane pore lined by residues within the α-helical second transmembrane (M2) domain. This M2-demarcated channel pore extends into an intracellular vestibule perforated by five openings, or portals, with a narrow diameter (8Å in the closed *Torpedo* nicotinic ACh receptor; (3)), comparable to that of the open M2 pore. Such portals provide the only pathway for the passage of ions into the cytoplasm and are lined partially by residues from an amphipathic helix within the intracellular M3-M4 loop known as the membrane-associated helix (MA-stretch).

Traditionally, the determinants of LGIC ion conductance and selectivity were thought to be limited to the M2 domain that lines the integral channel pore and immediate flanking sequences (reviewed in 4, 5). Cumulating recent evidence, however, for cation-selective LGICs also indicates a key contribution from the M3-M4 loop and/or MA-stretch residues in cation conductance (6-11). The homomeric 5-HT3A receptor, for example, has a very low γ (0.4 – 1.0 pS; (12)) relative to most other LGICs. Co-expression of 5-HT3A and 5-HT3B subunits produces an increased γ, and a series of 5-HT3A/3B receptor chimeras and mutant 5-HT3A receptors revealed that the major determinants of homomeric 5-HT3A receptor sub-pS γ were located within the MA-stretch. In particular, replacement of three positively-charged arginine residues (R432, R436, R440 in the human 5-HT3A subunit sequence) by their human 5-HT3B counterparts (QDA, respectively) increased γ to about 35-40pS, with R436D exerting the greatest influence (6-8, 11). The R436 residue is also an important determinant of the relative Ca2+ permeability of the 5-HT3A receptor (13). Complete deletion of the 115-residue 5-HT3A M3-M4 loop (and replacement with a 7 residue loop from a bacterial LGIC homologue) produced no further increase in γ, suggesting that residues within the MA-stretch were solely responsible for the low γ of the 5-HT3A receptor (9).

Subsequent mutagenesis work indicates the impact of the M3-M4 loop region on γ is a conserved feature of the ion conduction properties of nicotinic ACh receptors (8, 10). Glutamate to arginine mutations to invert residue charge at analogous positions in the α4 and β2 nicotinic ACh subunit MA-stretch can reduce the γ of the α4β2 nicotinic ACh receptor from about 30 pS to 12 pS (8). The α7 nicotinic ACh receptor subunit M3-M4 loop also presents a high-conductance phenotype and only neutral or negatively charged residues within homologous regions of its MA-stretch (10, 14). Thus, critical residues in the MA-stretch lining the portals and affecting conductance appear to be conserved amongst mammalian cationic Cys-loop LGICs.

Whether such a structural determinant of conductance extends to the anionic LGICs is unknown. Although the α1 glycine receptor subunit contains a large number of basic residues in the region of the intracellular M3-M4 loop putatively corresponding to the cationic MA-stretch (3), earlier studies of anionic Cys-loop LGICs suggested that these receptors lacked the MA-stretch (15-17). Nevertheless, the high conductance phenotype of the α1 glycine receptor homomers (~90 pS) is consistent with some of these positively-charged basic residues framing putative intracellular portals and supporting high anion flux rates.

In this study, we directly investigated the influence of charged residues within the proposed MA-stretch on functional properties of the α1 glycine receptor homomeric channels, a high conductance anionic Cys-loop LGIC with well characterised ion permeation properties (4, 18). Our results identify particular sets of basic residues affecting conductance, suggesting the portals are indeed features of the extended glycine receptor permeation pathway. However, the modest magnitude of their effect on conductance, and the lack of effect on ion-charge selectivity, suggests that the M2-demarcated channel pore remains the dominant determinant of α1 glycine receptor permeation properties, where the strongest interactions with permeant ions occur.

**EXPERIMENTAL PROCEDURES**

*Molecular biology and transient transfection of HEK-293 cells* - cDNAs encoding
human wild type (WT) and mutant α1 glycine receptor subunits were cloned into pcDNA1.1. Point mutations were introduced using standard molecular biological techniques and all constructs were sequenced to confirm fidelity. Transient transfection of HEK-293 cells with cDNA was performed using polyethyleneimine (jetPEI; Polyplus transfection). All WT and mutant subunits were expressed as homomeric receptors. HEK-293 cells were co-transfected with the expression marker, CD4 surface antigen, and CD4-antibody-coated polystyrene beads were used to identify successfully transfected cells (Dynabeads M-450, Dynal A.S.). Cells were used for electrophysiological experiments 1-3 days after transfection. Cells were sub-cultured twice-weekly and incubated in a medium comprised of Modified Eagle’s medium and 10% calf serum. Cells were maintained at 37°C in an atmosphere of 5% CO2 (100% relative humidity).

Electrophysiological recordings – Standard whole-cell patch-clamp recordings were used to initially assess functional glycine receptor expression and subsequently to characterise the concentration-response properties and anion to cation selectivity (PCl/PNa) of the WT and 4A and 4E mutant glycine receptors (see Table 1 for a description of the mutations). The outside-out patch-clamp configuration was used to record single glycine receptor channel currents and to quantify γ. Experiments were performed at a room temperature of 21 ± 2°C. All patch pipettes were made using thick-walled Clark borosilicate glass (Warner Instruments) and pulled using a horizontal electrode puller (P-87, Sutter Instruments Co.). They were fire-polished to give final resistances of 3-5 MΩ for macroscopic (whole-cell) recordings and 5-10 MΩ for single-channel recordings. The tips of pipettes used for single-channel recordings were also coated with Sylgard (Dow Corning). Currents were recorded with PClamp 9.0 or 10.2 software and an Axopatch 1D or 200B amplifier (Molecular Devices Corp.). For single-channel recordings data were filtered and digitized with a Digidata 1440A or 1200B (Molecular Devices Corp.) at 5 kHz and 20 kHz, respectively. Macroscopic currents were filtered and digitized at 1 kHz and 5 kHz, respectively. All recordings were stored onto the hard drive of a PC (under MS Windows XP) for subsequent offline analysis. For macroscopic current recordings the series resistances ranged from between 5-12 MΩ and was electronically compensated by ~80%.

Composition of solutions used - The standard external bath solution contained (in mM): NaCl 145, CaCl2 2.0, MgCl2 2.0, glucose 10 and HEPES 10 (pH 7.4 adjusted with ~5 mM NaOH). The standard internal solution used to fill the patch electrodes comprised (in mM): KCl 145, CaCl2 2, MgCl2 2, EGTA 5 and HEPES 10 (pH 7.4 adjusted with ~15 mM KOH). These standard solutions gave rise to a calculated liquid junction potential of -4.7 mV, using the Windows version of JPCalc (19) which was accounted for when quantifying γ values. These standard solutions were used for all measurements of γ and for the macroscopic concentration-response relationships. For quantifying relative anion – cation selectivity (PCl/PNa) a number of simpler solutions were used. The control 1 NaCl external solution contained (in mM) NaCl 145, glucose 10 and HEPES 10. The control “symmetrical” internal solution contained: (in mM) NaCl 145, CaCl2 2, EGTA 5 and HEPES 10. For the 0.5 external NaCl dilution solution, 75 mM NaCl was replaced with an iso-osmotic concentration of sucrose (136 mM). For the 0.25 external NaCl dilution solution, 112.5 mM NaCl was replaced with an iso-osmotic concentration of sucrose (189 mM). These external solutions were nominally divalent cation free (no added divalent ions). The pH of all these solutions was adjusted to 7.4 with a known concentration of NaOH (~ 5mM). The liquid junction potentials (VJp) arising from these three solutions were 0.4, -3.1 and -6.6 mV for the 1, 0.5 and 0.25 NaCl solutions, respectively, and were accounted for when calculating permeability ratios.

Glycine was dissolved in the bath solution and applied via a gravity-fed bath perfusion system. For whole-cell recordings, glycine and the external dilution solutions were applied using a Y-tube perfusion tube positioned close to the cell being recorded from to give an exchange time, measured using open pipette responses, of 10-50 ms.

Data analysis – Continuous sections of data recorded from outside-out patches in which unitary events predominated were selected for analysis of single-channel conductance using pClamp9 or 10.2. Selected traces were digitally
refiltered at 1.5 or 2 kHz for subsequent construction of all-point histograms, such additional filtering did not alter the peaks of clearly resolved histograms but did make distinct peaks easier to identify. All-points amplitude histograms were generated from continuous periods of stable recordings using a bin size of 0.05 pA and a range of 200 bins (i.e. 10 pA). Gaussian distributions were fitted to the histograms using a Levenberg-Marquardt least squares minimization procedure with up to 8000 iterations. The closed amplitude peak was fitted separately from the open amplitude peak(s). The number of Gaussian distributions required to adequately fit the open amplitude peaks was increased until there was no further improvement in the fits. Single-channel conductance (γ) values were determined from the relationship \( \gamma = i / (V_m - V_{rev}) \), in which \( i \) is the current amplitude of single channel events, \( V_m \) is the corrected membrane potential (\( V_p - V_{LJP} \)), and \( V_{rev} \) the reversal potential, which was approximated as zero for the standard solutions with almost symmetrical Cl- concentrations.

Macroscopic current amplitudes in response to different glycine concentrations (1 to 1000 μM) were measured manually as the difference between the current peak and the baseline current just prior to glycine application. Only data from experiments in which the full range of different concentrations was tested and that showed two to three stable initial control responses, and a similar control response following completion of the experiment, were chosen for further concentration-response analysis. The relationship between current amplitude and glycine concentration in each experiment was fit to the Hill equation (Eq. 1) to obtain the maximal current response (\( I_{max} \)), the concentration giving a response 50% of the maximum (\( EC_{50} \)) and the Hill slope (\( n_H \)). These values were then averaged for each glycine receptor construct, as indicated in the results. For presentation of the group concentration-response curve in Fig 2, the currents from each experiment were normalized to the \( I_{max} \), and these normalized mean values were also fit to Eq. 1.

\[
\text{Eq. 1. } I_{\text{glycine}} = I_{\text{max}} / (1 + EC_{50}n_H/[\text{glycine}]^{n_H})
\]

For quantification of \( P_{Cl} / P_{Na} \), macroscopic current-voltage (I-V) relationships in the symmetrical and external dilution solutions were recorded by applying steps, in 10 mV intervals, from 0 mV to between -30 mV and +30 mV, for a duration of 100 ms. The current response was averaged for the duration of the voltage pulse excluding uncompensated capacitive current transients. Control I-V curves were similarly obtained prior to, and following, glycine application, averaged, and then subtracted from those in the presence of glycine (1 mM). For all I-V curves, the data points were fitted to quadratic or third order polynomials and the reversal potential read directly from the I-V curves. The reversal potentials in each experiment were plotted against the external Cl- activity, and the data were fit with the Goldmann-Hodgkin-Katz (GHK) equation (Eq. 2) to obtain the ion-charge selectivity ratios (\( P_{Cl} / P_{Na} \)). The \( P_{Cl} / P_{Na} \) was calculated for each experiment and then averaged. Ion activities, rather than concentrations, were used for all calculations and determined by graphing and interpolating published activity coefficients (20).

\[
\text{Eq. 2. } V_{rev} = (RT/F) \ln \left\{ \frac{(a_{Na}^o + (P_{Cl} / P_{Na}) a_{Cl}^i)}{(a_{Na}^i + (P_{Cl} / P_{Na}) a_{Cl}^o)} \right\}
\]

Where \( V_{rev} \) is the potential at which the current is zero, \( R \) is the molar gas constant, \( T \) is temperature in Kelvin, \( F \) is Faraday’s constant, \( P_{ion} \) is the permeability of the ion and \( a_{ion} \) is the activity of the ion in the extracellular (') or intracellular (i') solutions.

Data are presented as the mean ± S.E.M. Data sets were compared using one-way analysis of variance (ANOVA) with a post hoc Tukey’s test. P<0.05 was considered to be significant.

### RESULTS

**Basic residues within the putative MA-stretch are important for receptor γ**. MA-stretch residues of the cation-selective 5-HT\(_3\)A and α4β2 nicotinic ACh receptors have been shown to affect γ by both steric and electrostatic influences (7, 8). In particular, a series of positively charged arginine residues have been identified as key determinants of the sub picoSiemen γ of 5-HT\(_3\)A receptors (6). Unlike 5-HT\(_3\)A receptors, α1 glycine receptors have a robust γ (~90 pS).
Alignment of the proposed MA-stretch of the α1 glycine receptor subunit with that of the 5-HT3A subunit and other related subunits revealed that this region contains a number of positively charged residues (Figure 1). We therefore investigated the influence of positively charged residues on the anion flux of α1 glycine receptors. We mutated the eight positively charged lysine (K) or arginine (R) residues within the putative MA-stretch to negatively charged glutamate residues (E). We undertook cumulative mutations, concurrently mutating from between four and up to all eight of the lysine or arginine residues to glutamate, focusing on the region aligned with the series of specific positively charged arginine residues shown to hinder cation flux of 5-HT3A receptors (6,7) (Figure 1A). The specific mutations and their notation are shown in Figures 1C and in Table 1.

Mutant subunits were expressed in HEK-293 cells and single-channel currents were recorded from excised outside-out membrane patches. The dominant conductance levels obtained from multiple Gaussian fits to all-point amplitude histograms are reported. Subconductance states were observed in both WT and mutant receptors, but the dominant states reported here were typically also of the highest γ. Application of glycine (1-10 μM) to outside-out patches resulted in clear single-channel responses at -65 mV for glycine receptor mutants with up to seven R/K to E mutations (producing the 4E to 7E subunits). Mutation of all eight positively charged residues to glutamate (producing the 8E subunit) resulted in a non-functional receptor (n = 22). This suggests that either the replacement of R392 by glutamate is not tolerated or that the combined effect of all eight mutations is not tolerated. It is notable that an arginine residue is highly conserved at the homologous position across all glycine and most GABA_A receptor subunits. The introduction of four through to seven glutamate residues into the proposed MA-stretch of the α1 glycine receptor resulted in a significantly reduced γ compared to wild-type, from ~92 pS to between ~81 and ~60 pS, as summarized in Table 1. The largest decrease in γ (to ~60 pS) occurred for sets of multiple mutations comprising four, six, or seven introduced glutamates. Glutamate mutations to two pairs of positively charged residues within the putative MA-stretch (R377E, K378E, K385E, K386E; the 4E mutant) were the minimum mutations that resulted in this decreased γ and we conducted further experiments on these residues (see below).

*Mutations to charged portal residues do not affect macroscopic concentration-response curves.* Before commencing further detailed studies on ion permeation properties of the mutant 4E receptors, we examined their macroscopic concentration-response relationships using whole-cell recordings. Point mutations to regions within the MA-stretch of the nicotinic ACh receptor have been shown to affect channel gating (21) and our mutants enabled us to examine for the first time in glycine receptors whether charged residues in the putative MA-stretch affected glycine potency. Furthermore, it was important to examine single-channel conductance properties at equivalent concentrations of glycine. Sample macroscopic currents are shown in Figure 2A for the WT, 4A and 4E receptors illustrating no major changes in current kinetics. As shown in Figure 2B and Table 2, the concentration-response curves for the WT, 4E and 4A (R377A, K378A, K385A, K386A) glycine receptor constructs essentially overlapped, with no significant differences in the averaged EC_50 or Hill coefficients.

*Residue charge in the MA-stretch is a determinant of receptor γ.* We further quantified the γ properties of the 4E mutant glycine receptor relative to the WT receptor by analysing single-channel current-voltage (i-V) relationships between -65 mV and +55 mV and quantified inward and outward chord conductances. The WT glycine receptor displayed an inwardly rectifying i-V curve, as previously described (22), with mean γ values of 73 pS at +55 mV and 92 pS at -65 mV (Figures 3A, 3D). We quantified the extent of rectification by dividing the outward current γ (at +55 mV) by the inward current γ (at -55 mV) to obtain a rectification index of 0.8. The 4E mutant...
glycine receptor gave a reduced inward $\gamma$ at negative membrane potentials (~62 pS at -65 mV), but only a very modest reduction in outward current $\gamma$, resulting in a rectification index of 1.0 (Figures 3C, 3D). To distinguish between effects of changes in charge or steric characteristics at these residue positions, we also mutated the R/K residues to alanine (A), a simple amino acid whose side-chain consists of a methyl group, lacking both bulk as well as charge. The $i$-$V$ relationship for this 4A mutant glycine receptor showed a small reduction in inward $\gamma$ (to ~80 pS at -65 mV) and outward $\gamma$, resulting in a rectification index of 0.9 (Figures 3B, 3D). Hence the effect of the 4E mutations was predominantly to decrease inward $\gamma$, by changing the side-chain charge at these residue positions. Inverting the charge resulted in a conversion of the WT single-channel current inwardly rectifying $i$-$V$ to a linear $i$-$V$ relationship for the 4E glycine receptor.

**Fig. 3 near here**

Residues in the $\alpha_1$ glycine receptor putative MA-stretch have no impact on ion-charge-selectivity. The above results reveal that positive charges on critical residues within the putative MA-helix influence inward current. To determine if these charged residues interact with permeant ions to the extent that they can also affect ion selectivity, dilution potential measurements were made for the WT, 4A and 4E glycine receptors. Dilution of the initially symmetrical external NaCl solution results in a shift of the Cl$^\text{-}$ electrochemical potential to positive membrane potentials and a shift in the Na$^+$ electrochemical potential to negative membrane potentials. As shown in Figure 4A-4C, the reversal potentials for all three glycine receptors shifted to positive membrane potentials as the external NaCl was diluted, indicating all three were predominantly anion selective. Anion to cation ($P_{Cl} / P_{Na}$) permeability ratios were quantified in each experiment by fitting the $V_{rev}$ values as a function of the external Cl$^\text{-}$ activity to the GHK equation (Eq. 2; Figure 4D). There was no significant difference in the averaged $P_{Cl} / P_{Na}$ values for WT, 4E and 4A receptors.

**Fig. 4 near here**

The above dilution potential experiments were conducted in external and internal solutions nominally free of divalent cations. Divalent cations can themselves influence permeation properties, for example, external divalent cations can increase the glycine receptor $P_{Cl} / P_{Na}$ ratio (Sugiharto et al., under review). We replicated the results in external solutions containing 2 mM of CaCl$_2$ and MgCl$_2$ and there were also no changes in $P_{Cl} / P_{Na}$ between WT (24.6 ± 0.6, n = 8, ref. 23), 4A (25.1 ± 1.7, n = 7) and 4E (25.5 ± 1.4, n = 6) glycine receptors.

**DISCUSSION**

The role of the intracellular M3-M4 loop in Cys-loop receptor function is yet to be fully understood, partly as this region exhibits the greatest degree of sequence variation among receptor subunits and a complete structural model of this loop is lacking. To date, much research in this area focuses on phosphorylation sites located within the loop and the role of specific residues in receptor kinetics, expression and attachment of the receptor to the cell cytoskeleton (24, 25, 26).

More recently, studies of the cation-selective 5-HT$_3$A and $\alpha_4\beta_2$ nicotinic ACh receptors have identified a series of residues at the C-terminal end of the M3-M4 loop that play a key role in determining receptor $\gamma$ (6, 8). These residues form part of an $\alpha$-helix thought to partially line a series of portals that lie beneath the M2-lined transmembrane channel pore (3). These portals form an obligatory pathway for ion flux, with both steric and electrostatic characteristics of these MA-stretch residues having an impact on $\gamma$ in cationic LGICs (7). Whether a similar structure - intracellular portals framed by residues in a MA-stretch - exists in anionic LGICs is not known, yet it is important to establish if this is indeed a conserved structural and functional feature amongst the Cys-loop LGIC superfamily. Furthermore, determining this is necessary for a complete understanding of the molecular determinants of ion permeation, particularly since the M2 domain has been considered as the primary determinant of ion permeation properties in the Cys-loop LGICs (4). It was recently reported that deletion of the entire M3-M4 loop of the homomeric $\rho1$ GABAA receptor (and replacement by the seven residue bacterial Cys-loop LGIC...
sequence) resulted in no apparent change of ion-charge selectivity, although the precise permeability ratios were not quantified (9). The effects of more specific mutations on ion-charge selectivity, or the effects of mutations to the putative MA-stretch, on single-channel conductance, have not been examined for any anionic LGIC.

The current study investigated the role of charged residues in the putative MA-stretch at the C-terminal end of the α1 glycine subunit M3-M4 loop on receptor γ, rectification ion-charge selectivity and additionally agonist potency. Given that critical charged residues in and adjacent to the M2 domain of all the Cys-loop LGICs have marked affects on these permeation parameters, and charged residues in the MA-stretch of 5-HT3A and αβ2 nicotinic ACh receptors influence γ, we focussed on the positively charged (basic) residues within this region of the α1 glycine receptor. We proposed that positive charge in the putative MA-stretch facilitates the passage of negative ions through the channel, particularly as they flow out of the cell at negative membrane potentials. Concurrent mutation of seven of these positive lysine, or arginine, residues to the negative glutamate residue did indeed reduce γ at negative potentials, by about 30%. A similar reduction was also seen with concurrent mutations of four, five, or six residues that all contained mutations to two sets of adjacent positively charged residues, specifically R377, K378, K385 and K386. These residues may align reasonably closely to a series of three, well-conserved arginine residues responsible for the subpicrosiemen γ of 5-HT3A receptors (27). Hence we conclude that intracellular portals partially lined by charged M3-M4 loop residues that can influence conductance, are a conserved feature amongst both anionic and cationic LGICs.

Further investigation of the effects of these four residues on ion permeation indicated that the side chain charge was the critical element responsible for the reduced γ. Replacement with a small, uncharged, alanine had smaller effects on γ than inversion of charge by mutation to glutamate. The volume of arginine (111.5 Å), lysine (108.5 Å) and glutamate (90.4 Å) are similar, and larger than that of alanine (60.6 Å) (28). The modest change in γ observed with the introduction of alanine residues into the MA-stretch suggests that the steric properties of residues at the positions investigated are not major determinants of ion conduction. Rather, the electrostatic properties of these residues have the greatest impact on γ. Indeed, inward current flow (corresponding to Cl\(^-\) efflux) was predominantly reduced, with small, or negligible, effects on outward current flow. It is difficult to envisage how a steric influence could produce effects upon γ that depend upon the direction of anion flux.

We interpret such conductance data by proposing that γ is determined by the rate of ion flux across a dominant selectivity filter at the internal end of the M2 delineated pore. Positive charges surrounding the intracellular portals would electrostatically attract anions to an intracellular vestibule allowing a greater concentration of anions in this region which, in the presence of a negative membrane potential, would increase the inward current (outward flux of Cl\(^-\)). The outward current was less affected by the portal charges.

Consistent with our previous proposal that the M2 domain is the dominant determinant of ion permeation, was the fact that the 4E mutation had no effect on ion-charge selectivity of the glycine receptor. We propose that ions do not interact as strongly with charged residues lining the portals as they do with charged residues at the dominant selectivity filter at the intracellular end of M2. This may be a result of different relative diameters of the M2 pore and the intracellular portals, with the latter being wider for the glycine receptor. The proposed diameter of the intracellular portals in the closed state of the Torpedo nicotinic ACh receptor portals is comparable to that proposed for the M2 pore of that channel (3). It will be interesting to determine if the diameters of the portals of anionic LGICs are similar to those of Torpedo, or if the portals of the open state of LGICs are wider than those in the closed state. Very recently it was shown that mutation of the three MA-stretch arginines that are critical for the 5-HT3A receptor conductance, also had no effect on ion-charge selectivity in those channels (13).

In conclusion, our results indicate that intracellular portals lined by charges that electrostatically influence γ are conserved across the Cys-loop LGIC superfamily. Biophysical and molecular models of permeation through these channels need to be developed to incorporate this
extended permeation pathway. However, the lack of effects on ion charge selectivity and the changes in rectification that we observed are consistent with an M2-delineated selectivity filter being the dominant determinant of ion permeation properties in glycine receptors. Whether a similar scenario occurs for other anion selective Cys-loop LGICs remains to be determined.

REFERENCES


**FOOTNOTES**

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The abbreviations used are: LGICs, ligand-gated ion channels; ACh, acetylcholine; 5-HT3, 5-hydroxytryptamine type 3; GABA<sub>A</sub>, γ-aminobutyric acid type A; HEK, human embryonic kidney; MA, membrane associated; $P_{Cl}/P_{Na}$, anion to cation selectivity; WT, wild-type; γ, single channel conductance; $i-V$, single-channel current-voltage relationship; $I-V$, macroscopic current-voltage relationship; $I_{\text{max}}$, maximum current response; $EC_{50}$, the concentration giving a response 50% of the maximum; $n_H$, the Hill slope; GHK, Goldman-Hodgkin-Katz.
FIGURE LEGENDS

Figure 1. Charged residues in the putative MA-stretch of the α1 glycine receptor influence channel conductance. A, An alignment of residues within the MA-stretch of cationic LGIC subunits (Torpedo nicotinic ACh receptor α-subunit, human α4, α7 and β2 nicotinic ACh and human 5-HT3A and 5-HT3B subunits). Charged residues known to influence cation conductance in 5-HT3 subunits are identified by shaded boxes. B, An alignment of residues within the proposed MA-stretch of human anionic LGIC subunits. Positively charged residues in the α1 glycine receptor subunit are identified by shaded boxes. C, Schematic diagram indicating location of the putative α1 glycine receptor MA-stretch relative to the M3-M4 loop and the transmembrane domains, indicated as grey cylinders. Residues mutated in this study are indicated.

Figure 2. Charged residues in the putative MA-stretch do not influence the potency of glycine. A, Representative macroscopic currents at a pipette potential of -40 mV for WT, 4A and 4E glycine receptors in response to 1 mM glycine, applied during the period indicated by the black bar. B, Macroscopic concentration-response curves for WT, 4A and 4E glycine receptors. Data points were averaged from peak whole cell currents in response to increasing concentrations of glycine, all recorded at a pipette potential of -40 mV and normalised to the maximum current in each experiment obtained from fits of the data in each cell to Equation 1 (n = 5 or 6 for each mutant). The curve represents the best fit of Equation 1 to the averaged data (r^2 > 0.98). Concentration-response parameters averaged from fitting Equation 1 to data from each experiment, are shown in Table 2. The EC_{50} values (mean ± SEM) were 33.8 ± 1.7 μM (WT); 38.5 ± 2.1 μM (4E) and 34.2 ± 2.4 μM (4A).

Figure 3. Portal charges electrostatically influence single-channel conductance. A-C, Sample single-channel currents recorded from outside-out membrane patches in symmetrical solutions at pipette potentials between -60 mV and +60 mV, for WT (A), 4A (B) and 4E (C) glycine receptors. D, Mean single-channel current (i) plotted against membrane potential (V_m) for WT, 4A and 4E glycine receptors, as indicated. Error bars represent SEM, curves represent quadratic polynomials fit to the data (r^2 > 0.98).

Figure 4. Ion-charge selectivity ratios are unaffected by portal mutations which reduce conductance. A-C, Sample macroscopic current-voltage (I-V) relationships for WT (A), 4A (B) and 4E (C) glycine receptors under symmetrical NaCl concentrations (1 NaCl, upper row) and after dilution of the external NaCl by 50% (0.5 NaCl, centre row) and by 75% (0.25 NaCl, lower row). Mean V_{rev} values (1 NaCl) and changes in V_{rev} values (0.5 NaCl, 0.25 NaCl) are shown (n = 7 in each case). The inset in each panel shows the currents in the presence of glycine and in response to 100 ms voltage steps to pipette potentials between -30 mV and +30 mV (from 0 mV), in intervals of 10 mV. The scale bars refer to currents at all three external NaCl concentrations. In the upper row (1 NaCl) both the initial control I-V and that obtained after returning the external solution to the 1 NaCl following the NaCl dilutions are illustrated. D, Plots of mean changes in V_{rev} for WT (upper panel), 4A (middle panel) and 4E (lower panel) glycine receptors against external Cl^{-} activity. The solid lines show optimal fits to the GHK equation (Eq. 2), the dotted lines show the Cl^{-} equilibrium potential as a function of external Cl^{-} activity. The mean P_{Cl} / P_{Na} ion charge selectivity ratios, averaged from each experiment, are also shown (n = 7 in each case).
TABLES

Table 1. Mutation of positively charged residues in the MA-stretch of α1 glycine receptors reduces single-channel conductance, γ.
The indicated lysine (K), or arginine (R), residues were mutated to glutamate (4E – 7E) or alanine (4A). γ was measured in outside-out patches held at a membrane potential of -65 mV in response to 1-10 μM glycine. Values represent mean ± S.E.M. * significantly different from WT (P<0.05), # significantly different from 4E (P<0.05).

<table>
<thead>
<tr>
<th>GlyR constructs</th>
<th>Substituted residue(s)</th>
<th>γ (pS) (n)</th>
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<tr>
<td>WT</td>
<td>Nil</td>
<td>92.2 ± 2.8</td>
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<tr>
<td>4A</td>
<td>K386, K385, K378, R377</td>
<td>80.5 ± 2.3*#</td>
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<tr>
<td>4E</td>
<td>K386, K385, K378, R377</td>
<td>60.0 ± 2.2*</td>
</tr>
<tr>
<td>5Ea</td>
<td>K386, K385, K378, R377, K371</td>
<td>67.7 ±3.5*</td>
</tr>
<tr>
<td>5Eb</td>
<td>K389, K386, K385, K378, R377</td>
<td>69.9 ± 3.0*</td>
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<tr>
<td>5Ec</td>
<td>K386, K385, R383, K378, R377</td>
<td>65.3 ± 2.5*</td>
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<tr>
<td>6E</td>
<td>K389, K386, K385, K378, R377, K371</td>
<td>59.9 ± 1.5*</td>
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<tr>
<td>7E</td>
<td>K389, K386, K385, R383, K378, R377, K371</td>
<td>60.0 ± 1.9*</td>
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Table 2. The potency of glycine at 4A, or 4E, glycine receptors does not differ from that of WT glycine receptors.
Glycine concentration-response parameters were obtained using whole-cell current recordings at a holding potential of -40 mV. Values represent mean ± S.E.M of I<sub>max</sub>, EC<sub>50</sub> and n<sub>H</sub> derived from fits of the Hill equation (Equation 1) to the data from each experiment.

<table>
<thead>
<tr>
<th></th>
<th>WT (n=6)</th>
<th>4E (n=5)</th>
<th>4A (n=6)</th>
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<tr>
<td>I&lt;sub&gt;max&lt;/sub&gt; (nA)</td>
<td>3.24 ± 0.60</td>
<td>8.55 ± 3.03</td>
<td>2.81 ± 0.99</td>
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<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (μM)</td>
<td>33.8 ± 1.7</td>
<td>38.5 ± 2.1</td>
<td>34.2 ± 2.4</td>
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<tr>
<td>n&lt;sub&gt;H&lt;/sub&gt;</td>
<td>1.8 ± 0.1</td>
<td>2.1 ± 0.1</td>
<td>1.9 ± 0.2</td>
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FIGURE 1

A.

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B.

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<td>F 409</td>
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C.

[Diagram of M3 and M4 helices with labeled residues R392, K389, K386, K385, R383, K378, R377, and K371.]
FIGURE 2

A.

WT  4A  4E

B.

Response (% of max)

[ glycine ] (μM)

WT  4A  4E
FIGURE 3

A. WT

B. 4A

C. 4E

D.

\[ i \text{ (pA)} \]

\[ V_m \text{ (mV)} \]

\[ \triangle \text{ WT (n=3)} \]

\[ \bullet \text{ 4A (n=6)} \]

\[ \square \text{ 4E (n=5)} \]
FIGURE 4

A. WT

1 NaCl

\( \Delta V_{\text{rev}} = 11.6 \pm 0.7 \text{ mV} \)

0.5 NaCl

\( \Delta V_{\text{rev}} = 10.7 \pm 0.5 \text{ mV} \)

0.25 NaCl

\( \Delta V_{\text{rev}} = 22.6 \pm 1.5 \text{ mV} \)

B. 4A

1 NaCl

\( \Delta V_{\text{rev}} = 11.5 \pm 0.7 \text{ mV} \)

0.5 NaCl

\( \Delta V_{\text{rev}} = 10.7 \pm 0.5 \text{ mV} \)

0.25 NaCl

\( \Delta V_{\text{rev}} = 22.8 \pm 1.4 \text{ mV} \)

C. 4E

1 NaCl

\( \Delta V_{\text{rev}} = 11.5 \pm 0.7 \text{ mV} \)

0.5 NaCl

\( \Delta V_{\text{rev}} = 10.7 \pm 0.5 \text{ mV} \)

0.25 NaCl

\( \Delta V_{\text{rev}} = 22.8 \pm 1.4 \text{ mV} \)

D. WT

\( P_{\text{Cl}}/P_{\text{Na}} = 7.5 \pm 0.2 \)

\( P_{\text{Cl}}/P_{\text{Na}} = 7.0 \pm 0.5 \)

\( P_{\text{Cl}}/P_{\text{Na}} = 7.6 \pm 0.3 \)
Characterization of the effects of charged residues in the intracellular loop on ion permeation in α1 glycine receptor-channels
Jane E. Carland, Michelle A. Cooper, Silas Sugiharto, Hyo-Jin Jeong, Trevor M. Lewis, Peter H. Barry, John A. Peters, Jeremy J. Lambert and Andrew J. Moorhouse

J. Biol. Chem. published online December 2, 2008

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