Opposing Effects of Molecular Volume and Charge at the Hyperekplexia Site α1(P250) Govern Glycine Receptor Activation and Desensitization*

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Allelic variants of the glycine receptor α1 subunit gene GLRA1 underlie the human neurological disorder hyperekplexia. Among these, the subunit variant α1(P250T) is characterized by an amino acid substitution within the cytoplasmic TM1–2 loop. To identify structural elements at position α1(P250) that govern receptor function, homomeric mutant receptor channels were subjected to electrophysiological analysis after recombinant expression in HEK293 cells. Wild-type α1(P250) channels were non-desensitizing with an EC50 for glycine of 8 μM, whereas bulky hydrophobic side chains of the channel variants α1(P250V/L/F) showed rapid desensitization (τdes, 50–250 ms) and EC50 values of 400–1800 μM. Small side chains (P250G/A/S) gave rise to wild-type-like channels. Effects of volume were counteracted by charge: α1(P250E/R) were non-desensitizing; EC50 was ~70 μM. The mutants α1(P250C/Y) displayed intermediate channel properties (EC50, 42/70 μM; τdes, 3300/2800 ms, respectively). The isotropic forces volume and hydropathy were sufficient to account for the observed effects of residue α1(P250) on receptor function. Indeed, channel behavior was best predicted by a combined hydropathy/volume index describing the hydrophobic surface of individual amino acids. These observations characterize the short intracellular TM1–2 loop as a regulatory domain for channel activation and a crucial mediator of glycine receptor desensitization.

The strychnine-sensitive glycine receptor is a ligand-gated ion channel mediating fast inhibitory synaptic transmission in mammalian spinal cord and brain stem. Currently, four ligand-binding subunits (α1–4) have been identified, all of which are able to form functional homomeric receptor channels. A structural β subunit, which contributes to synaptic anchoring of receptor complexes, does not form functional channels on its own (1–5). Mutant alleles of the glycine receptor α1 subunit gene (GLRA1) have been shown to underlie the hereditary human motor disorder hyperekplexia (startle disease, stiff baby syndrome, OMIM 138491). This disease is characterized by excessive startle reactions, increased muscle tone, and myoclonia. Hyperekplexia mutations cluster within the pore-lining transmembrane segment TM2 and its flanking regions, including TM1 (2, 4, 5).

Similar to other ligand-gated ion channels of the acetylcholine receptor superfamily, glycine receptor subunits comprise distinct functional domains: ligand binding determinants have been localized to segments of the extracellular N-terminal region and the short extracellular TM2–3 loop. Channel gating, i.e. the transition between the fully liganded closed and open states, has been associated with an intramolecular movement of TM2 (4). Based on the effects of hyperekplexia mutations on channel function, the short loops flanking TM2, i.e. the intracellular TM1–2 and the extracellular TM2–3 loop, have been postulated to act as hinges controlling TM2 positioning and movement (4, 6–8). In a detailed mechanistic study, Lewis et al. (7) have characterized the TM2–3 missense mutant α1(K276E), which exerts pronounced effects on channel gating. This mutation drives the channel to a predominantly closed form, leaving ligand affinity and channel conductance largely unaffected. In contrast, the amino acid exchange P250T, situated within the intracellular TM1–2 loop, gives rise to channels that desensitized rapidly and completely in the continued presence of glycine (8). The mutation α1(P250T) exerts almost no effect on the open-close equilibrium of the channel, whereas apparent kinetic affinity for glycine and single-channel conductance are reduced (8).

Here, position α1(P250), situated within the short intracellular TM1–2 loop, was characterized as a crucial determinant of ion channel function. Molar volume was identified as the principal chemical parameter governing receptor function: bulky, hydrophobic residues gave rise to receptors displaying up to 200-fold reduced apparent affinity for glycine. In contrast to the wild-type channel, desensitization for these mutants was rapid and complete. Increased polarity at position α1(P250) produced opposite effects, favoring wild-type-like receptor properties. For each mutant, channel behavior was best predicted by a combined hydropathy/volume index describing the molecular properties of individual amino acid side chains.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Single-nucleotide exchanges corresponding to codon α1(250) were introduced by PCR-mediated mutagenesis using an overlap extension PCR approach (9). Mutagenesis primers (Amersham Pharmacia Biotech, Freiburg, Germany) contained nucleotides specific for the amino acid exchange together with a silent restriction site for a rapid check of mutated clones (Fig. 1). PCRs were set up as follows: 1 ng of template DNA; 50 μM each dATP, dCTP, dGTP, and dTTP; 100 pmol of each primer, and 2 units of high-fidelity

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**Taq** polymerase was in the supplied buffer (Roche Molecular Biochemicals, Mannheim, Germany). PCR conditions were 5 min at 95 °C for denaturation, 5 min at 50 °C for annealing, and 5 min at 72 °C for elongation in the first cycle, followed by 28 cycles of 1 min at 95 °C, 2 min at 50 °C, and 2.5 min at 72 °C. The last cycle ended with a 10-min 72 °C amplification step. The final fragments were cut with restriction enzymes as close as possible to the mutated site to minimize the PCR-generated sequence and reinserted into GlyR α1. All mutated clones were sequenced across the PCR-generated sequence to verify successful mutagenesis using the ABI sequencer system (ABI Systems, Weiterstadt, Germany).

**Membrane Preparation, Protein Analysis, and Radioligand Binding Assays**—HEK293 cells were cultured and transfected, and crude membrane fractions were prepared as described previously (10). Western blot analysis using the monoclonal antibody MAb4a was carried out as described previously (11) using the Cy5-coupled goat anti-mouse antibody (Dianova, Hamburg, Germany) and a Storm 860 FluorImager (Molecular Dynamics, Krefeld, Germany) for visualization.

Specific radioligand binding to membrane fractions was determined by a filtration assay (11) using 50 μg of total protein per individual measurement. For glycine displacement, [*3H]*strychnine (PerkinElmer Life Sciences, Zaventem, Belgium; specific activity, 47.8 mCi/mmol) was used at a final concentration of 19.2 nM. Using Origin (Microcal, Northampton, MA), data were fit to the equation below.

\[
dpm_{\text{spec}} = \left( \frac{dpm_{\text{obs}} - dpm_{\text{unspec}}}{K_0 + [\text{glycine}] + K_0} \right) + dpm_{\text{unspec}} \quad \text{(Eq. 1)}
\]

Here, \(dpm_{\text{spec}}\) represents specific binding expressed as scintillator counts (decays per minute), \(dpm_{\text{obs}}\) is the observed radioactivity, and \(dpm_{\text{unspec}}\) is unspecific binding; [glycine] and [Str] are the concentrations of glycine (varied) and [*3H]*strychnine, respectively; \(K_0\) and \(K_0\) are the equilibrium dissociation constants for strychnine (11 nM) and glycine, respectively. \(K_0\) was determined for GlyR α1(wt), P250F, and P250L subunits by displacement of unlabeled strychnine with 9.6 nM [*3H]*strychnine as described. The same equation was used, replacing [glycine] with [cold strychnine], and \(K_0\) and \(K_0\) was determined by a filtration assay (11) using 50 μg of total protein per individual measurement. For glycine displacement, [*3H]*strychnine (PerkinElmer Life Sciences, Zaventem, Belgium; specific activity, 47.8 mCi/mmol) was used at a final concentration of 19.2 nM. Using Origin (Microcal, Northampton, MA), data were fit to the equation below.

**Electrophysiological Recordings and Data Analysis—Whole-cell recording experiments were performed using a HEKA EPC9 amplifier (HEKA Electronics, Lambrecht, Germany) controlled by Pulse software (HEKA).** Recording pipettes were pulled from borosilicate glass (World Precision Instruments, Berlin, Germany) using a Sutter P-97 horizontal puller (Sutter, Novato, CA). Ligand was applied using a U-tube (12) that bathed the suspended cell in a laminar flow of solution, giving a bath of 50 μM glycine. Using a HEKA EPC9 amplifier controlled by Pulse software (HEKA Electronics, Lambrecht, Germany) and a Polygraph (DataQ Instruments, Cleveland, OH), data were collected with a time resolution for equilibration of 10–30 ms (13, 14). Given the extremely fast desensitization of mutants P250F and P250L, an underestimation of their EC\(_{50}\) and \(t_{\text{decay}}\) values at near-saturating ligand concentrations cannot be excluded. The external buffer consisted of 137 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 1.0 mM MgCl\(_2\), and 5.0 mM Hepes (pH adjusted to 7.2 with NaOH); the internal buffer was 120 mM CsCl, 20 mM NaCl, 1.0 mM CaCl\(_2\), 2.0 mM MgCl\(_2\), 11 mM EGTA, and 10 mM Hepes (pH adjusted to 7.2 with CsOH). Current responses were measured at room temperature (21 °C–23 °C) at a holding potential of −60 mV.

Dose-response curves were constructed from the peak current amplitudes obtained with at least seven appropriately spaced glycine concentrations in the range 5–10,000 μM glycine. Using a nonlinear algorithm, dose-response data were fitted to the Hill equation shown below.

\[
I_{\text{glycine}} = \frac{[\text{Glycine}]^{[\text{Hill}]} \cdot EC_{\text{glycine}}^{[\text{Hill}]}}{[\text{Glycine}]^{[\text{Hill}]} + EC_{\text{glycine}}^{[\text{Hill}]} + I_{\text{const}}} \quad \text{(Eq. 2)}
\]

\(I_{\text{glycine}}\) is the current amplitude at a given glycine concentration, \(I_{\text{const}}\) is the current amplitude at saturating concentrations of glycine, \(EC_{\text{glycine}}\) is the glycine concentration producing half-maximal current responses, and \(n_{\text{Hill}}\) is the Hill coefficient. Currents from each individual cell were normalized to the maximum response at saturating glycine concentrations.

For desensitization analysis, the decaying current phase was fitted to a single exponential decay function plus a constant as shown by the following equation.

\[
I_{\text{obs}} = I_1 \times \exp(-t/t_1) + I_2 \times \exp(-t/t_2) + I_{\text{const}} \quad \text{(Eq. 3)}
\]

\(I_{\text{obs}}\) is the observed current, \(I_1\) is the fraction of current amplitude decaying with time constant \(t_1\), \(I_2\) is the fraction of current amplitude decaying with time constant \(t_2\), and \(I_{\text{const}}\) is the nontdesensitizing current fraction. Generally, only a single exponential function was required to fit current desensitization.

To establish a combined index reflecting the effects of both molar volume and charge of residue 250, the mean molar volume (vol(mean)) of all investigated residues was calculated (86.8 ml/mol) based on data by Zamyatnin (15). The index was then computed as shown below.

\[
\text{Values ranged from} \quad -4.5 \to 3.5. \quad \frac{\text{vol(residue)} - \text{vol(mean)}}{10} \quad \text{(Eq. 4)}
\]

**Generation and Expression of GlyR α1(P250) Mutant Subunits**—To identify molecular determinants that underlie the altered receptor channel function resulting from the hyperekplexia allele GLRA1(P250T) (8), a series of GlyR α1(250) point mutants was generated using an overlap extension PCR protocol for site-directed mutagenesis (Fig. 1). After transfection of HEK293 cells with wild-type and mutant GlyR α1 subunits, Western blot analysis using the monoclonal antibody MAb4a verified significant levels of expression with all newly generated mutants (Fig. 2). All subunits gave whole-cell current responses to glycine stimulation (Fig. 3).

**Chemical Architecture of Amino Acid Position a1(250) and Receptor Channel Function**—Whole-cell currents were recorded from single detached HEK293 cells using a cell-flow device for ligand application. The time resolution for equilibration of a single cell with ligand was 10–30 ms (14, 17), which was fast enough to separate the individual steps of receptor activation from the slower process of desensitization. In our system, homomeric α1 wild-type GlyRs showed practically no desensitization (<5%) during a 10-s application of glycine, and whole-cell currents returned to baseline after removal of glycine (Fig. 3).

Dose-response curves for GlyR α1 mutants are presented in Fig. 4. Comparison of the channel properties of mutant receptors revealed significant differences in both desensitization behavior and apparent ligand affinities in whole-cell current.
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(iii) Constructs exhibiting intermediate channel properties included P250C and P250Y. EC50 values were increased approximately 5-fold, and desensitization was pronounced, albeit slow.

(iv) A group of nondesensitizing constructs displaying a 5–8-fold reduced ligand affinity included P250E/R/Q.

Aliphatic amino acids do not differ significantly with respect to the molar properties of their side chains such as polarity, dielectric constant, hydrophilicity/polarity, and so forth. Thus, the aliphatic series A-P-V-I-L-F permitted the isolated analysis of the molecular parameter side chain volume. Indeed, the EC50 values determined for mutants P250A/P/V/I/L correlated with molecular volume (15), and desensitization rates and EC50 values changed in parallel (Fig. 5; Table I). No such correlation was observed for amino acids containing heteroatoms. With the exception of α1(P250T) (EC50 = 192 μM; Table I), EC50 values were below 100 μM. Due to the limits of time resolution of the application system, tmax and tdes values observed for the rapidly desensitizing mutants P250I/L might even be underestimated at higher glycine concentrations (e.g. [glycine] > EC70).

A comparison of whole-cell currents recorded from the mutants P250Y and P250F showed that tyrosine caused a pronounced but slow desensitization (τdes = 2.8 s), whereas desensitization induced by phenylalanine was rapid (Table I; Fig. 4). For P250F, only three cells produced significant current responses to 2000 μM glycine, with desensitization being rapid and complete. Still, the ligand concentration used may have been non saturating. Introduction of cysteine, the thiol homologue of serine, into position 250 also resulted in distinct desensitization (τdes = 3.3 s), most likely due to increased bulk. Desensitization for P250T, however, was significantly faster than that for P250C, despite similar side chain volumes (Table I; Fig. 4).

Based on the observation that the volume effect of aliphatic side chains was countered by polarity, an attempt was made to establish an index combining both parameters. An index for relative molecular volumes was generated based on data by Zamyatnin (15), ranging from -4.5 (G) to 3.5 (Y). To account for polarity, hydropathy index values according to Engelman et al. (16) were added without further weighting of these index scales. This procedure yielded a combined HVI ranging from -10 (R) to 7 (F). As shown in Fig. 5 (inset), HVI was predictive for receptor desensitization, because desensitization was only observed for α1(250) amino acid residues characterized by HVI values ≥ 0. Negative HVI values corresponded to nondesensitizing channel behavior (Table I).

Consistent with similar HVI values for residues with hydrocarbon side chains and their hydroxylated counterparts, comparison of aliphatic and hydroxylated α1(250) residues showed that aliphatic OH-groups were without detectable effect on receptor function. Small α1(250) residues like alanine and serine both gave rise to wild type-like channels. Thr enine and valine also produced almost identical receptor channels (P250T, τdes = 0.12 s and EC50 = 192 μM; P250V, τdes = 0.25 s and EC50 = 391 μM). Equilibrium Radioligand Binding—Apparent affinities from kinetic studies do not allow an isolated characterization of the ligand binding step of receptor activation. Whereas equilibrium ligand binding studies may favor the desensitized receptor state, they nevertheless permit a comparison of relative ligand affinities between receptor mutants. Using [3H]strychnine displacement by unlabeled strychnine, antagonist binding was found to be unaffected by the chemical nature of residue α1(250): equilibrium Kdis values were 11 nM for the wild type, 9 nM for P250F, and 14 nM for P250L (Fig. 6A). Likewise, glycine-displaceable [3H]strychnine binding for wild-type and P250A/
CV/FIL subunits (Fig. 6B) yielded $K_i$ values ranging from 25 to 98 μM. In contrast to pronounced alterations of apparent ligand affinities (EC$_{50}$) observed in electrophysiological studies, no significant differences in equilibrium agonist affinities were found. These results indicate that the molecular structure of the ligand binding region is not significantly altered by the amino acid exchanges studied here. Apparently, the intracellular mutation site had no effect on the extracellular process of ligand reception, consistent with findings from an alanine scan of the same region (6).

**DISCUSSION**

Strychnine-sensitive GlyRs are the principal carriers of inhibitory synaptic transmission in mammalian spinal cord and brain stem. The human neurological disorder hyperekplexia has been associated with mutant alleles of GLRA1, the gene...
encoding the GlyR α1 subunit (1, 2, 4). Among these, the mutation GlyR α1(P250T), which causes an amino acid exchange within the short cytoplasmic loop linking transmembrane segments 1 and 2, results in dramatically increased desensitization and reduced single-channel conductance (8). Here, we identified molecular parameters of position α1(250) that govern GlyR channel function.

**Effects of Residue 250 Chemical Structure on GlyR Channel Function**—After verifying that recombinant expression was not detectably affected by amino acid exchanges in position 250, the functional effects of amino acid substitutions in position α1(250) of the human GlyR were studied on recombinant receptors using patch-clamp techniques. This led to the identification of the four subunit categories below.

(i) Small side chains (P/G/A/S) gave rise to wild-type-like channels, showing nondesensitizing current responses and a high apparent glycine affinity. The wild-type proline is in fact the bulkiest of these residues.

(ii) Bulky aliphatic residues produced a dramatically reduced apparent ligand affinity and desensitized rapidly. The extent of functional impairment correlated with side chain volumes of A/P/V/I/L/F and hydroxylated residue T.

(iii) Intermediate constructs (C/Y) showed slightly reduced apparent ligand affinity in whole-cell current measurements and slow but distinct desensitization. Because both side chains are expected to be partially ionized at physiological pH, the effects on receptor function may be attributed to their electrostatic properties. This suggests that side chain volume is not an exclusive determinant of receptor channel function but is counteracted by polarity or charge.

(iv) Polar residues (Q/E/R) were nondesensitizing and displayed moderately reduced apparent ligand affinities (Table II). Similar to P250CY, the presence of a full charge or a polar center in these mutants counteracted the effect of bulk, favoring nondesensitizing, high-affinity channels.

Among the aliphatic set of subunits, rapid desensitization corresponded to reduced apparent ligand affinity as observed in current recordings, an effect not paralleled by alterations in equilibrium ligand binding affinities. Apparently, increased side chain volume favored the rapidly desensitizing, low-affinity receptor form. This resembles the modulation of GlyRs by aliphatic alcohols and anesthetics, where the molar volume of a single amino acid residue was found to influence drug responses (18–20), and the efficacies of various agonists in the

![Figure 5](image)

**FIG. 5. Effect of molecular volume of residue α1(250) on GlyR current responses.** EC₅₀ values are plotted versus molecular volume (15) of amino acid 250. One-letter codes of residue 250 are indicated in the graph. Excluding glycine (○), a linear regression for amino acids with aliphatic side chains (■) yielded a correlation coefficient of r = 0.88. For amino acids with other functional groups (○), no correlation between molar volume and EC₅₀ was found (r = 0.01). Note that no mathematical relationship is implied by the curves. The inset shows a plot of EC₅₀ versus HVI, the combined hydrophobicity/molar volume index (see “Experimental Procedures”). Δ and ■ represent negative and positive HVI values, respectively.

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Strychnine Kᵣ (nM)</th>
<th>Glycine Kᵣ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1 wt</td>
<td>11 ± 5</td>
<td>68 ± 7</td>
</tr>
<tr>
<td>α1(P250A)</td>
<td>n.d.*</td>
<td>75 ± 16</td>
</tr>
<tr>
<td>α1(P250C)</td>
<td>n.d.</td>
<td>31 ± 6</td>
</tr>
<tr>
<td>α1(P250F)</td>
<td>9 ± 4</td>
<td>24 ± 4</td>
</tr>
<tr>
<td>α1(P250V)</td>
<td>n.d.</td>
<td>98 ± 18</td>
</tr>
<tr>
<td>α1(P250L)</td>
<td>14 ± 8</td>
<td>66 ± 8</td>
</tr>
</tbody>
</table>

* n.d., not determined.

With all the other subunit mutants analyzed here, an amino acid substitution affected the change of more than one chemical parameter. Our observations indicate that molecular volume and polarity exerted opposing effects on receptor function: increased polarity opposed the effects of volume. This was most evident from those amino acids that carry either a fully charged (R and E), or a polar (C, Y, and N) side chain (note that the pKᵣ for sulfhydryl groups is around 7, so partial deprotonation of this group can be expected, rendering the side chain of cysteine more polar than that of threonine). Given similar side chain volumes, increased polarity thus favored wild-type-like channel properties.

When the effects of bulk and polarity in position α1(250) were described by a combined HVI, receptor desensitization was observed only for amino acids displaying an HVI > 0, whereas negative HVI values were indicative of nondesensitizing channel variants. Indeed, preponderance of HVI values showed that the two isotropic forces volume and hydropathy were sufficient to account for the observed effects of residue α1(250) on recombinant GlyR function. It should be emphasized that the definition of the volume and hydropathy index, and thus the zero point of HVI, was arbitrary. Still, this index shows that the combined effects of volume and hydropathy (polarity) are sufficient to describe the effects of residue 250 side chain structure on receptor function.

**Structural Consequences of GlyR α1(250) Mutants**—The amino acid bond introduced into the protein backbone by proline usually favors kinks or narrow turns in polypeptide chains, breaking α-helical formations. However, as shown in Fig. 5, the

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molecular volume of proline alone, and not its conformation, was sufficient to account for the observed functional properties. The functional characteristics of GlyR α1(250) mutants were (i) unaltered equilibrium ligand binding, (ii) unaltered channel gating (close-open transition), and (iii) a change in the signal transduction process. This suggests that the amino acid ex-

Fig. 6. Equilibrium ligand binding experiments. A, [3H]strychnine (9.6 nM) displacement by unlabeled strychnine. (■), concentration-dependent binding data used to compute $K_D$; (○), maximum binding (no unlabeled strychnine; data points drawn on the y axis) and nonspecific binding (1 μM unlabeled strychnine). B, [3H]strychnine (19.2 nM) displacement by unlabeled glycine. (■), data points used to calculate glycine $K_D$; (○), control data points. Control data points were not used for analysis.
changes induced a subtle reorientation of the receptor protein, which did not extend to the extracellular ligand binding pocket. In structural terms, the tether holding TM1 and TM2 is altered so that the shape of the ion pore is changed, leaving the gating movement of TM2 unaffected. This model is consistent with a reduced single-channel conductance observed for the α1(P250T) mutant (8).

Receptor desensitization was greatly favored by increased hydrophobic volume at the hyperekplexia locus α1(250). The effects of these mutations on the overall spatial structure of the receptor protein seem minor yet are sufficient to affect single-channel conductance and the internal signal transduction process in the sequential R-T-E-D model of ligand-gated receptor function (Reception of ligand-Transduction of signal within the protein-Effect (channel opening/closing)-Desensitization). Structurally, increased steric demand could disturb the geometry of the intracellular hinge that defines the position of TM2, thereby driving the channel-forming part of the receptor to the closed, desensitized state. Alternatively, the increased hydrophobic surface may promote interactions with other intracellular domains of receptor polypeptides. In this case, the structural transition underlying desensitization may include an intrinsic block of the open channel, effected by motile elements within the cytosolic TM3–4 loop (21). Tools for the investigation of elementary steps of ligand-gated ion channel function are presently available, whereas identification of the structural origin of receptor desensitization still requires further investigation.

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REFERENCES