Identification of intracellular and extracellular domains mediating signal transduction in the inhibitory glycine receptor chloride channel

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Fast synaptic neurotransmission is mediated by transmitter-activated conformational changes in ligand-gated ion channel receptors, culminating in opening of the integral ion channel pore. Human hereditary hyperekplexia, or startle disease, is caused by mutations in both the intracellular or extracellular loops flanking the pore-lining M2 domain of the glycine receptor α1 subunit. These flanking domains are designated the M1–M2 loop and the M2–M3 loop respectively. We show that four startle disease mutations and six additional alanine substitution mutations distributed throughout both loops result in uncoupling of the ligand binding sites from the channel activation gate. We therefore conclude that the M1–M2 and M2–M3 loops act in parallel to activate the channel. Their locations strongly suggest that they act as hinges governing allosteric control of the M2 domain. As the members of the ligand-gated ion channel superfamily share a common structure, this signal transduction model may apply to all members of this superfamily. Keywords: β-alanine/glycine/ligand-gated ion channel superfamily/signal transduction/taurine

Introduction

Fast synaptic neurotransmission in the mammalian central nervous system is mediated by members of the ligand-gated ion channel (LGIC) superfamily, which includes receptors for acetylcholine, GABA, serotonin and glycine (Grenningloh et al., 1987; Schofield et al., 1987; Maricq et al., 1991). These channels, which are located in the post-synaptic membrane, are opened by pre-synaptically released neurotransmitter and permit an ionic flux which changes the membrane potential. Receptors of this family consist of five similar membrane-spanning subunits arranged in a ring to form a central ion-conducting pore. The subunits of the different receptor types each comprise a large N-terminal extracellular domain, which contains the ligand binding sites and four putative membrane spanning domains (M1–M4), the second of which forms the channel-lining α-helix (reviewed in Devillers-Thiery et al., 1993; Karlin and Akabas, 1995). Since the ligand binding domain is distant from the M2 region (Valenzuela et al., 1994; Unwin, 1995), channel activation requires long-range allosteric interactions. Although the functional molecular architecture of the ligand binding region and the ion channel pore has been intensively investigated, little is known about the transduction mechanism linking these domains.

Analysis of mutations underlying the human hereditary disorder hyperekplexia (or startle disease) initially identified one residue involved in gating of the glycine receptor (GlyR) chloride channel. Two separate point mutations at the same base pair in the gene encoding the human GlyR α1 subunit resulted in the arginine at position 271 (R271) being substituted by either a leucine (R271L) or a glutamine (R271Q) (Shiang et al., 1993). R271 lies at the external vestibule of the ion channel pore and forms part of the extracellular loop linking the second and third membrane-spanning domains (the M2–M3 loop). The physiological effect of these mutations is to reduce the glycine efficacy and single channel conductance of recombinant homomeric and heteromeric GlyRs (Langosch et al., 1994; Rajendra et al., 1994, 1995a). In addition, both mutations converted β-alanine and taurine from full agonists into competitive antagonists without dramatically affecting their binding affinities (Laube et al., 1995; Rajendra et al., 1995a). As these results are consistent with an uncoupling of the agonist binding process from the channel activation gate, it was concluded that R271 formed a crucial component of the allosteric signal transduction pathway (Langosch et al., 1994; Rajendra et al., 1994, 1995a; Lynch et al., 1995).

Additional startle disease mutations have recently been identified in the M2–M3 loop (Y279C, Shiang et al., 1995; K276E, Elmslie et al., 1996) and in the intracellular M1–M2 loop (I244N, Rees et al., 1995). We show here that the Y279C and K276E mutations, like the R271L and R271Q mutations, convert taurine and β-alanine into competitive antagonists with little disruption to their binding affinities. The I244N mutation also results in a dramatic reduction in taurine efficacy. These results demonstrate that the startle disease mutations may act by disrupting the allosteric transduction (or gating) process between ligand binding and channel activation and raise the possibility that the M1–M2 loop and the M2–M3 loop act as the structural components mediating this process.

To investigate this possibility, we constructed sequential alanine substitution mutants of all residues in the M1–M2 intracellular loop and the M2–M3 extracellular loop and for each mutant GlyR measured the binding affinities of taurine and β-alanine and their agonist efficacies relative to glycine. Since numerous mutations in both domains did indeed reduce or eliminate the taurine and β-alanine efficacies with minimal change to their binding affinities, we conclude that both domains act in parallel to activate the channel. The locations of these domains suggest they act as hinges to allow the M2 domain to move with
Activation of glycine-gated ion channel receptors

Fig. 1. Examples of agonist-activated currents (A–D) and averaged dose–response curves (E) in cells expressing GlyRs incorporating naturally occurring human startle disease mutations. (A) Currents activated by 100 mM glycine, β-alanine and taurine for the I244N, K276E and Y279C mutant GlyRs. (B) Complete inhibition by taurine of currents activated by a half-maximal concentration (2 mM) of glycine for the L276E mutant GlyR. (C) Partial inhibition by taurine of currents activated by a half-maximal concentration (500 μM) of glycine for the I244N mutant GlyR. (D) Examples of glycine dose–response curves for I244N, and K276E and Y279C mutant GlyRs. Numbers above each trace indicate glycine concentration in mM. (E) Averaged dose–response curves for currents activated by glycine (upper panel), β-alanine (centre) and taurine (lower panel) for wild-type GlyRs (○) and for GlyRs expressing human startle disease mutations where β-alanine and taurine have a reduced efficacy (open symbols) or no efficacy (shaded symbols). In this and all subsequent figures, the glycine dose–response curves are normalized to 1 and the β-alanine and taurine dose–response curves are normalized to the maximal glycine-activated current for each mutant GlyR. The data for the R271L and R271Q mutant GlyRs in (E) and Figure 4 are reproduced from Rajendra et al. (1995a).

Results

Human startle disease mutations act by disrupting transduction

All experiments described in this report were performed on human α1 homomeric GlyRs recombinantly expressed in mammalian HEK 293 cells. Taurine and β-alanine act as full agonists of human α1 GlyRs when expressed in this system (Pribilla et al., 1992; Rajendra et al., 1995a,b), although they act as partial agonists of the same GlyRs when expressed in Xenopus oocytes (Schmieden et al., 1992; Laube et al., 1995).

We sought to investigate the mechanism by which the following recently identified startle disease mutations disrupt the function of the human α1 GlyR homomer: I244N (Rees et al., 1994), K276E (Elmslie et al., 1996) and Y279C (Shiang et al., 1995). For each mutant GlyR, we examined the agonist efficacies of taurine and β-alanine relative to glycine, the concentration of each agonist required for half-maximal current activation (EC50) and, in mutant GlyRs where β-alanine and taurine exhibited partial or no agonist efficacy, the concentration required for half-maximal inhibition of glycine-gated currents (IC50). Sample electrophysiological responses from cells expressing these mutant GlyRs are shown in Figure 1A–D, averaged dose–response curves are shown in Figure 1E and the results of all electrophysiological measurements are summarized in Table I. The binding properties of the competitive antagonist [1H]strychnine were also examined with the dissociation constant (Kd), number of expressed receptors (Bmax) and inhibition constants (KI) for glycine, β-alanine and taurine displacement of bound [1H]strychnine being determined for wild-type and mutant GlyRs. Averaged results from all binding experiments are summar-
Table I. Functional properties of mutant GlyRs as determined by electrophysiological recording

<table>
<thead>
<tr>
<th>GlyR</th>
<th>Phenotype</th>
<th>Glycine activation</th>
<th>β-alanine activation</th>
<th>Taurine activation</th>
<th>β-alanine inhibition</th>
<th>Taurine inhibition</th>
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<tr>
<td></td>
<td></td>
<td>EC50 (μM)</td>
<td>IC50 (μM)</td>
<td>Max IgG/Igly</td>
<td>Max IgG/Igly</td>
<td>IC50 (μM)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>wild-type</td>
<td>18 ± 2</td>
<td>10.0 ± 2.5</td>
<td>52 ± 4</td>
<td>0.99 ± 0.01</td>
<td>153 ± 43</td>
</tr>
<tr>
<td>Disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I244N</td>
<td>partial</td>
<td>274 ± 44</td>
<td>0.41 ± 0.09</td>
<td>396.52</td>
<td>0.85 ± 0.03</td>
<td>2210 ± 460</td>
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<tr>
<td>R271L</td>
<td>complete</td>
<td>6700 ± 1500</td>
<td>5.7 ± 2.8</td>
<td>a</td>
<td>0 ± 0</td>
<td>a</td>
</tr>
<tr>
<td>R271Q</td>
<td>complete</td>
<td>12000 ± 600</td>
<td>1.8 ± 0.6</td>
<td>a</td>
<td>0 ± 0</td>
<td>a</td>
</tr>
<tr>
<td>R276E</td>
<td>complete</td>
<td>1820 ± 68</td>
<td>1.1 ± 0.7</td>
<td>a</td>
<td>0 ± 0</td>
<td>a</td>
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<tr>
<td>R279C</td>
<td>complete</td>
<td>1310 ± 380</td>
<td>0.19 ± 0.03</td>
<td>a</td>
<td>0 ± 0</td>
<td>a</td>
</tr>
<tr>
<td>M1-M2 loop</td>
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</tr>
<tr>
<td>W243A</td>
<td>partial</td>
<td>114 ± 9</td>
<td>1.47 ± 0.40</td>
<td>2100 ± 570</td>
<td>0.82 ± 0.05</td>
<td>2940 ± 490</td>
</tr>
<tr>
<td>I244A</td>
<td>partial</td>
<td>719 ± 141</td>
<td>1.23 ± 0.45</td>
<td>300 ± 360</td>
<td>0.54 ± 0.03</td>
<td>t</td>
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<tr>
<td>N245A</td>
<td>wild-type</td>
<td>20.17</td>
<td>3.7 ± 1.1</td>
<td>69 ± 9</td>
<td>0.96 ± 0.02</td>
<td>131 ± 26</td>
</tr>
<tr>
<td>N246A</td>
<td>wild-type</td>
<td>123 ± 15</td>
<td>2.34 ± 0.41</td>
<td>880 ± 230</td>
<td>0.92 ± 0.03</td>
<td>810 ± 90</td>
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<td>D247A</td>
<td>wild-type</td>
<td>19 ± 4</td>
<td>2.21 ± 0.41</td>
<td>60 ± 17</td>
<td>0.98 ± 0.01</td>
<td>146 ± 24</td>
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<tr>
<td>P520A</td>
<td>wild-type</td>
<td>27 ± 5</td>
<td>2.89 ± 0.56</td>
<td>37, 42</td>
<td>1.02 ± 0.04</td>
<td>83, 89</td>
</tr>
<tr>
<td>M2-M3 loop</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>S270A</td>
<td>wild-type</td>
<td>22 ± 3</td>
<td>2.99 ± 0.82</td>
<td>42 ± 4</td>
<td>0.99 ± 0.03</td>
<td>153 ± 42</td>
</tr>
<tr>
<td>R271L</td>
<td>complete</td>
<td>3170 ± 500</td>
<td>9.5 ± 1.1</td>
<td>a</td>
<td>0.03 ± 0.01</td>
<td>t</td>
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<tr>
<td>S273A</td>
<td>wild-type</td>
<td>27 ± 4</td>
<td>4.43 ± 0.63</td>
<td>51 ± 4</td>
<td>1.01 ± 0.05</td>
<td>221 ± 11</td>
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<tr>
<td>L274A</td>
<td>partial</td>
<td>465 ± 100</td>
<td>3.41 ± 0.51</td>
<td>2100 ± 450</td>
<td>0.75 ± 0.04</td>
<td>12500 ± 3600</td>
</tr>
<tr>
<td>P275A</td>
<td>wild-type</td>
<td>27 ± 4</td>
<td>2.47 ± 0.62</td>
<td>50 ± 8</td>
<td>1.00 ± 0.01</td>
<td>66 ± 2.9</td>
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<td>K276A</td>
<td>partial</td>
<td>1000 ± 180</td>
<td>3.06 ± 0.80</td>
<td>a</td>
<td>0.06 ± 0.03</td>
<td>a</td>
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<tr>
<td>V277A</td>
<td>partial</td>
<td>1920 ± 450</td>
<td>2.83 ± 0.51</td>
<td>a</td>
<td>0 ± 0</td>
<td>a</td>
</tr>
<tr>
<td>S278A</td>
<td>partial</td>
<td>104 ± 15</td>
<td>3.63 ± 0.32</td>
<td>1240 ± 380</td>
<td>0.84 ± 0.03</td>
<td>2830 ± 910</td>
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<tr>
<td>Y279A</td>
<td>partial</td>
<td>1400, 1930</td>
<td>0.035, 0.04</td>
<td>a</td>
<td>0.0</td>
<td>a</td>
</tr>
<tr>
<td>V280A</td>
<td>wild-type</td>
<td>21 ± 4</td>
<td>1.45 ± 0.54</td>
<td>55 ± 9</td>
<td>0.96 ± 0.04</td>
<td>130 ± 25</td>
</tr>
<tr>
<td>K281A</td>
<td>wild-type</td>
<td>40 ± 4</td>
<td>3.89 ± 0.85</td>
<td>177 ± 6</td>
<td>0.97 ± 0.02</td>
<td>442 ± 285</td>
</tr>
<tr>
<td>I283A</td>
<td>wild-type</td>
<td>24 ± 8</td>
<td>2.88 ± 0.47</td>
<td>48 ± 8</td>
<td>1.01 ± 0.01</td>
<td>98 ± 23</td>
</tr>
<tr>
<td>R271L</td>
<td>partial</td>
<td>480 ± 130</td>
<td>6.5 ± 0.8</td>
<td>810 ± 100</td>
<td>0.46 ± 0.09</td>
<td>1480 ± 380</td>
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<tr>
<td>R271Q</td>
<td>partial</td>
<td>840 ± 150</td>
<td>10.2 ± 1.1</td>
<td>760 ± 70</td>
<td>0.36 ± 0.07</td>
<td>2740 ± 730</td>
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<td>R271H</td>
<td>partial</td>
<td>340 ± 70</td>
<td>8.9 ± 1.3</td>
<td>1200 ± 220</td>
<td>0.29 ± 0.06</td>
<td>2600, 5800</td>
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</table>

Values are given as mean ± SE in cases where n > 3. In cases where n < 3, individual values are shown. Min IgG/Igly and Min IgG/Igly represent the mean proportional glycine-gated currents remaining in the presence of 100 mM β-alanine or taurine respectively. Hill coefficients are not displayed as there was no significant difference in Hill coefficient values between mutant and wild-type GlyRs. In both Tables I and II data for R271L and R271Q mutant GlyRs only are reproduced from Rajendra et al. (1995a).

aParameters that could not be quantified as currents were either non-existent or too small to measure.
bNot determined.
residues (R271, K276 and Y279) are distributed throughout the M2–M3 loop, they raise the possibility that this entire loop may form an important structural component of the allosteric signal transduction mechanism.

In the I244N mutant GlyR, taurine was converted into a partial agonist, although β-alanine remained virtually a full agonist (Figure 1A and E and Table I). Taurine was also converted into a partial antagonist of glycine-gated currents (Figure 1C) and, as expected, β-alanine did not display detectable antagonism (n = 3; data not shown). In the I244N mutant GlyR, the average EC₅₀ values of glycine, β-alanine and taurine were increased by a factor of 10–15 over their respective wild-type values (Figure 1E and Table I). We were unable to detect significant [³H]strychnine binding in this mutant GlyR, although in electrophysiological experiments we confirmed that 1 μM strychnine did completely inhibit currents activated by a half-saturating concentration (200 μM) of glycine (n = 2; data not shown). Since cells expressing recombinant GlyRs containing the I244N mutation were characterized by extremely small peak currents (Iₘₐₓ, see Table I), it would appear that this mutation greatly impaired the efficiency of GlyR expression while leaving the strychnine binding site essentially intact. This interpretation is supported by the observation that when I244 was mutated to the more conservative alanine, strychnine binding was regained but Bₘₐₓ was significantly reduced (42%) relative to the wild-type value (Table II).

Since the β-alanine and taurine efficacies and glycine EC₅₀ values of the I244N mutant GlyR lie between those of the wild-type GlyR and the K276E and Y279C mutated GlyRs, the I244N mutation may be considered to partially disrupt the agonist transduction mechanism and is consequently designated as having a ‘partial’ disruption phenotype. These properties imply that the M1–M2 loop, like the M2–M3 loop, is important for transducing agonist binding into channel activation.

### Residues in the M1–M2 and M2–M3 loops control agonist efficacy

To investigate further the possibility that the M1–M2 and M2–M3 loops may act in parallel to mediate activation of the channel, we sought to examine the contribution of all residues in both loops to this process. As the various startle disease mutations are not conservative, they may have imposed additional functions on mutated residues.GlyRs containing the I244N mutation were characterized by extremely small peak currents (Iₘₐₓ, see Table I), it would appear that this mutation greatly impaired the efficiency of GlyR expression while leaving the strychnine binding site essentially intact. This interpretation is supported by the observation that when I244 was mutated to the more conservative alanine, strychnine binding was regained but Bₘₐₓ was significantly reduced (42%) relative to the wild-type value (Table II).

Since the β-alanine and taurine efficacies and glycine EC₅₀ values of the I244N mutant GlyR lie between those of the wild-type GlyR and the K276E and Y279C mutated GlyRs, the I244N mutation may be considered to partially disrupt the agonist transduction mechanism and is consequently designated as having a ‘partial’ disruption phenotype. These properties imply that the M1–M2 loop, like the M2–M3 loop, is important for transducing agonist binding into channel activation.
mutants of all non-alanine residues in the M1–M2 intracellular loop from W243 to R252 and in the M2–M3 extracellular region from S270 to I283. Each mutant GlyR was investigated using similar electrophysiological and radioligand binding procedures as previously described. The averaged results of all electrophysiological measurements are summarized in Table I and averaged results from all radioligand binding studies are summarized in Table II. With the exception of R252A, all mutations resulted in expressed GlyRs which displayed glycine-gated currents and [3H]strychnine binding. Cells transfected with cDNA encoding the R252A mutant GlyR displayed no glycine-gated currents (n = 25 cells) and no significant [3H]strychnine binding (n = 3), suggesting that the R252A mutation precluded expression of functional GlyRs. This is consistent with the previous observation that other mutations of this residue (R252Q and R252E) prevented GlyR protein from being inserted into the cell membrane (Langosch et al., 1993). As discussed above, the I244N, I244A and W243A mutant GlyRs were also characterized by reduced \( B_{\text{max}} \) values, suggesting that these residues are also important for receptor expression.

Several GlyRs mutated in the M1–M2 loop (N245A, D247A and P250A) and in the M2–M3 loop (S270A, S273A, P275A, V280A and I283A) exhibited phenotypes that were indistinguishable from wild-type GlyRs in both their electrophysiological properties and their ligand binding affinities (Tables I and II). We conclude that such mutated residues do not form part of the agonist transduction pathway. However, four other mutations in the M2–M3 loop (R271A, K276A, V277A and Y279A) essentially abolished the agonist efficacies of \( \beta \)-alanine and taurine (Table I). For the V277A mutant GlyR, examples of taurine inhibition of currents activated by a half-saturating (2 mM) concentration of glycine in this mutant GlyR are shown in Figure 2C. Other characteristics of the V277A mutant GlyR include a large (107-fold) increase in the glycine EC\(_50\) with respect to the wild-type value (Table I and Figure 2D and E), an 8-fold increase in the glycine \( K_i \) and smaller (2-fold) increases in the \( K_i \) values for \( \beta \)-alanine and taurine (Table II). Very similar electrophysiological and pharmacological properties were shared by the R271A,
Activation of glycine-gated ion channel receptors

Fig. 3. Examples of agonist-activated currents (A–C) and averaged dose–response curves (D) for GlyRs incorporating alanine substitution mutations in the M1–M2 loop. (A) Currents activated by 100 mM glycine, β-alanine and taurine for I244A and D247A mutant GlyRs. (B) Partial inhibition by taurine of currents activated by a half-maximal concentration (1 mM) of glycine for the I244A mutant GlyR. Due to the rapid desensitization, glycine was co-applied with the indicated concentration of taurine. (C) Onset and recovery of desensitization for the I244A mutant GlyR. A half-maximal concentration (1 mM) of glycine was applied for 2 s in order to desensitize ≥90% of channels, then re-applied for 5 s commencing at the times indicated by the dots. (D) Examples of glycine dose–response curves for D247A and I244A mutant GlyRs. Numbers above indicate glycine concentration in mM. (E) Averaged dose–response curves for wild-type GlyRs ( ● ) and selected mutant GlyRs (open symbols) activated by glycine (upper panel), β-alanine (centre) and taurine (lower panel).

K276A and Y279A mutant GlyRs (Tables I and II). As these mutant GlyRs clearly exhibit the ‘complete’ disruption phenotype, we conclude that V277 in addition to R271, K276 and Y279 comprise crucial elements of the allosteric transduction mechanism.

Three additional mutations within the M2–M3 loop resulted in the conversion of β-alanine and taurine from full agonists into partial agonists. The mutations which conferred this characteristic were L274A, S278A and K281A (Table I). An example of the reduced agonist efficacy of taurine and β-alanine in the L274A mutant GlyR is shown in Figure 2A. As expected for competitive partial agonists, taurine and β-alanine also displayed properties of partial antagonists (Table I). The latter mutant exhibited a wild-type phenotype (Table I) and is included for comparison. An example of partial antagonism by taurine of glycine-activated currents in the I244A mutant GlyR is shown in Figure 3B. The L274A, S278A and K281A mutant GlyRs were also characterized by less pronounced (2- and 47-fold) increases in their respective glycine EC_{50} values relative to the wild-type value (see Table I and Figure 2D and E). Taurine and β-alanine EC_{50} values were also dramatically increased over their respective wild-type values (Figure 2E and Table I). The binding affinities for glycine, β-alanine and taurine in these mutant GlyRs were intermediate between those of wild-type GlyRs and mutant GlyRs displaying the complete disruption phenotype (Table II). The L274A, S278A and K281A mutant GlyRs may therefore be classified as having a ‘partial’ disruption phenotype.

Three mutations in the M1–M2 loop (W243A, I244A and M246A) also reduced the agonist efficacies of β-alanine and taurine. Sample recordings from cells expressing GlyRs mutated in the M1–M2 loop are displayed in Figure 3. Figure 3A shows examples of saturating agonist responses for the I244A and D247A mutant GlyRs. The latter mutant exhibited a wild-type phenotype (Table I) and is included for comparison. An example of partial antagonism by taurine of glycine-gated currents in the I244A mutant GlyR is shown in Figure 3B. The same mutation also resulted in a dramatically reduced sensitivity (40-fold) to activation by glycine (Figure 3D and E). Averaged dose–response curves for all M1–M2 loop mutations which resulted in conversion of taurine and β-alanine into partial agonists are shown in Figure 2E. In terms of their electrophysiological properties, the W243A,
I244A and M246A mutant GlyRs exhibit the ‘partial’ disruption phenotype and therefore also form crucial elements of the allosteric signal transduction mechanism. However, apart from their reduced $B_{\text{max}}$ values, the radioligand binding properties of these intracellular mutant GlyRs are indistinguishable from the wild-type GlyR in their $K_d$ values for strychnine or in their $K_i$ values for glycine, β-alanine and taurine (Table II).

### Increased desensitization rate in W243A and I244A mutant GlyRs

An unexpected finding was that the W243A and I244A mutations also conferred a dramatically increased desensitization rate. Following a 2 s application of a saturating concentration (1 mM) of glycine, wild-type GlyR currents declined to an average of 89 ± 3% (± SEM, $n = 14$) of their peak values. In contrast, after a 2 s application of 100 mM glycine, currents in W243A and I244A mutant GlyRs had decreased to 11 ± 5% and 10 ± 2% ($n = 11$ for both) respectively of their peak values. This difference is highly significant ($P < 0.0001$, unpaired t-test) for both mutants with respect to the wild-type value. The I244N mutant GlyR also displayed a significantly more rapid rate of desensitization than the wild-type GlyR (mean 77 ± 3%, $n = 10$, $P < 0.025$), but the desensitization rates of all other mutant GlyRs were not significantly different to the wild-type GlyR desensitization rate. Examples of strongly desensitizing responses for the I244A mutant GlyR can be seen in Figure 3. As displayed in Figure 3C, recovery from desensitization was also rapid, with a half-recovery time of ~2 s ($n = 2$) for the I244A mutant GlyR.

### Progressive disruption to transduction caused by mutations at R271

To determine whether modifications to the side chain group on a single residue may result in a continuum of phenotypes from partial to complete disruption, we examined a series of mutations to R271. This residue was investigated as it has already been characterized via three mutations to differing side chain groups (R271L, R271Q and R271A). In an attempt to characterize the side chain characteristics required for minimal disruption to the transduction process, we constructed and examined the following mutations: R271K, R271H and R271N. The conservative R271K substitution retains the positive charge and large side chain. The R271H substitution conserves the positive charge but not the large side chain. On the other hand, the R271N substitution is uncharged and polar and, like the structurally similar R271Q substitution, may be expected to result in a complete disruption phenotype. Averaged dose–response curves for glycine, β-alanine and taurine are shown for all R271 mutant GlyRs in Figure 4. Surprisingly, these experiments revealed that the R271K, R271H and R271N mutant GlyRs each exhibited the partial disruption phenotype, in contrast to those of the R271L, R271Q and R271A mutant GlyRs, which each exhibited the complete disruption phenotype. As expected for mutant GlyRs with the partial disruption phenotype, the radioligand binding properties of the R271K, R271H and R271N mutant GlyRs were also intermediate between those of the wild-type GlyR and the R271L, R271Q and R271A mutant GlyRs (Table II). Hence, mutations to the same residue can result in a continuum of phenotypes from partially disrupted to completely disrupted. Although we were unable to distinguish an obvious correlation between the structural and chemical properties of the side chain and the resulting phenotype, these results demonstrate that residues identified by partial disruption phenotypes contribute to the transduction process.

### Discussion

#### The M1–M2 and M2–M3 loops form allosteric signal transduction domains

Alanine substitution mutations to a total of four out of 11 possible residues in the M2–M3 loop resulted in the complete disruption phenotype. This phenotype is characterized by a complete loss of agonist efficacy for β-alanine and taurine, dramatic increases (≥55-fold) in the glycine EC$_{50}$, the conversion of β-alanine and taurine into complete antagonists of glycine-gated currents and large increases in the $K_i$ values for glycine relative to those for β-alanine and taurine. In addition, mutations to a further six out of a possible 14 residues throughout the M1–M2 and M2–M3 loops resulted in a partial disruption phenotype. This phenotype is characterized by the conversion of taurine and β-alanine from full agonists into partial...
Activation of glycine-gated ion channel receptors

Fig. 5. A model for signal transduction for members of the ligand-gated ion channel superfamily. (A) The first three membrane-spanning domains of the human GlyR α1 subunit are illustrated showing a summary of results obtained in this study. Residues attached by stems represent the startle disease mutations examined in this study. Lightly shaded residues indicate those startle disease mutations or alanine substitution mutations which resulted in β-alanine and taurine being converted into partial agonists. Darkly shaded residues indicate those mutations which resulted in a complete loss of efficacy for these ligands. Thus, the lightly and darkly shaded residues represent "partial" disruption and "complete" disruption phenotypes respectively. Residues outlined in bold indicate those where mutations induced dramatically increased rates of desensitization. (B) Alignment of the M2–M3 signal transduction domains from various members of the ligand-gated ion channel superfamily. Circles indicate residues that convert agonists into antagonists, squares indicate residues that redistribute single channel conductances and diamonds indicate residues that alter allosteric enhancement or Hill coefficients. See text for details.

agonists with partial antagonist efficacy, less dramatic increases (2- to 47-fold) in the glycine EC50 and relatively smaller increases in the Ki values for glycine. Figure 5A illustrates the locations of the mutations which cause both the complete disruption phenotype (darkly shaded residues) and the partial disruption phenotype (lightly shaded residues). In addition, mutated residues which cause an increased desensitization rate are outlined in bold.

Since glycine, β-alanine and taurine share overlapping binding sites (Schmieden et al., 1992, 1993), any mutation which converts β-alanine and taurine from agonists into antagonists without changing their binding affinities may act by simply disconnecting their binding sites from the channel activation gate (Rajendra et al., 1995a). An alternative proposal is that the R271L and R271Q mutations selectively disrupt a common agonist recognition subsite and thereby unmask antagonist subsites for β-alanine and taurine (Laube et al., 1995; Schmieden and Betz, 1995). It is difficult to differentiate between these models by analysing mutations of a single residue, as there is no functional difference between an uncoupled competitive agonist site and an antagonist site. However, as the antagonist subsite model (Laube et al., 1995; Schmieden and Betz, 1995) would require a highly specific lesion to disrupt the agonist recognition site selectively in a common ligand binding pocket, it is clearly incompatible with the involvement of numerous intracellular and extra-cellular residues, as observed in the present study (Figure 5A). Hence, our results strongly support the idea that mutations which convert taurine and β-alanine from agonists into antagonists act in parallel to transduce the ligand binding signal into channel activation in GlyR. Furthermore, since all members of the ligand-gated ion channel superfamily share considerable sequence and structural homology (Devillers-Thiery et al., 1993; Karlin and Akabas, 1995),
this model is likely to apply to all members of this superfamily. A variety of experimental evidence from various members of the ligand-gated ion channel superfamily supports the involvement of residues in the M2–M3 loop in mediating allosteric transformations. In the GlyR α1 subunit, we have demonstrated that the R271L and R271Q mutations convert the competitive antagonist picrotoxin into an allosterically acting potentiator, suggesting a convergence of allosteric pathways (Lynch et al., 1995). Furthermore, in R271L and R271Q mutant GlyRs, single channel conductance states are redistributed towards lower unitary levels (Langosch et al., 1994; Rajendra et al., 1995a) without radically changing the anion–cation selectivity ratio or the current–voltage relationship (Rajendra et al., 1994). These mutations may therefore prevent allosteric transitions to one or more conducting conformations. A similar interpretation may also apply to the E297S mutation in the GlyR β subunit (Bormann et al., 1993) and to the K→M substitution at position +21 of the Xenopus nicotinic acetylcholine receptor (nAChR) γ subunit (Murray et al., 1995), which are both homologous to A273 of the GlyR α1 subunit and both also result in significant changes to unitary channel conductances. In both the α7 and β4 nAChRs, the aspartic acid residues at the positions corresponding to Y279 and V280 respectively in the GlyR α1 subunit were recently shown to disrupt the coupling between agonist binding and channel activation (Campos-Caro et al., 1996). In the GABA A receptor (GABA A R) p1 subunit, the R316A mutation (homologous to position K276 in the GlyR α1 subunit) increased the Hill coefficient for GABA activation, suggesting a modification to the allosteric coupling mechanism (Kusana et al., 1994). Collectively, these results, which are summarized in Figure 5B, support the notion of a common signal transduction mechanism applying to all ligand-gated ion channel receptors.

Based on comparison of electron diffraction images of nAChR in the open and closed states, Unwin (1995) proposed a structural model for the activation of members of this receptor superfamily. The model postulates that in the closed state the M2 domains are kinked inwards to constrict the channel pore and upon activation by acetylcholine are twisted sideways to create an open passage. This rotation is clearly a crucial step in the transduction process and implies that the intracellular and extracellular loops flanking the M2 domain act as hinges to permit this rotation. Consistent with this model, the simplest explanation of our results is that the M1–M2 and the M2–M3 loops act as the hinges and that mutations that disrupt transduction act by preventing this rotation from taking place. However, since other structural rearrangements are also associated with channel activation (Karlin and Akabas, 1995; Unwin, 1995), further signal transduction domains may also exist.

The M2–M3 loop may interact with the glycine binding site
Mutant GlyRs expressing the complete disruption phenotype are also characterized by dramatic increases in the glycine K i values (Table II). An increase in K i is frequently taken as evidence that the mutated residue forms part of a binding domain. However, due to the principle of reciprocity between transduction and binding processes, binding experiments alone cannot unequivocally define whether a residue comprises a binding site or a transduction site (Colquhoun and Farrant, 1993). We have demonstrated that the M1–M2 loop mutations which result in partial disruption phenotypes have K i values for glycine, β-alanine, and taurine which are not significantly different to wild-type values (Table II). However, M2–M3 loop mutations with similar partial disruption phenotypes generally have dramatically increased glycine K i values (Table II), suggesting that the M2–M3 loop interacts more directly with the glycine binding site. Furthermore, the relatively larger increases in glycine K i values, compared with those for taurine and β-alanine, imply that the glycine binding site is more directly linked to the M2–M3 loop than are the binding sites for taurine and β-alanine. In this respect, we propose that R271 forms the point of closest functional contact between the M2–M3 loop and the glycine binding site, as mutations to this residue are characterized by much larger increases in glycine K i values than are observed at any other residue (Table II).

Desensitization
Mutations to two residues (W243A and I244A) in the intracellular M1–M2 loop not only partially disrupt the transduction process but also result in a greatly increased rate of desensitization onset and recovery (Figure 3). If such a property is conferred by a simple side chain substitution, then a similar property could also be imposed by allosteric interactions converging on this residue. A major determinant of desensitization in the related nAChRs is the phosphorylation state (Raymond et al., 1993; Levitan, 1994). Like these receptors, the GlyR α1 subunit contains functional phosphorylation sites in the intracellular M3–M4 loop (Vaello et al., 1994), but it is as yet unclear as to whether phosphorylation modulates the desensitization rate of this receptor. It is, however, tempting to speculate that phosphorylation sites, or other intracellular modulatory sites, may control the desensitization rate by interacting with the channel gating mechanism via residues in the M1–M2 loop.

A common human startle disease phenotype
Inherited startle syndromes in humans and a variety of animal species are characterized by a reduced glycineic tone (Rajendra and Schofield, 1995). Molecular analysis of human startle disease pedigrees has so far identified a total of six mutations, each of which maps to the GlyR α1 subunit (R271L and R271Q, Shiang et al., 1993; I244N, Rees et al., 1994; Y279C, Shiang et al., 1995; K276E, Elmslie et al., 1996; Q266H, Milani et al., 1996). The disease phenotype conferred by the R271L and R271Q mutations was characterized by both a reduced glycine efficacy and a reduced single channel conductance (Langosch et al., 1994; Rajendra et al., 1994, 1995a). In the present study, we investigated the I244N, Y279C and K276E startle mutations and found in each case that the glycine sensitivity was profoundly decreased (Table I). In addition, the K276E and Y279C mutations resulted in dramatically diminished I max values (11 and 2% respectively of the wild-type value) without corresponding decrements in the [3H]strychnine B max values (Table II), strongly suggesting that these mutations also lowered either the
single channel conductance or the channel open probability, or both. However, in the I244N mutant GlyR, the sharp reduction in \( I_{\text{max}} \) was accompanied by a complete loss of \([\text{H}]\)strychnine binding. Since strychnine-inhibitable glycine-gated currents were readily observed in these mutant GlyRs, the most likely explanation for the lack of strychnine binding was a drastically decreased efficiency of expression of functional GlyRs. It remains to be determined whether reduced GlyR expression also occurs in vivo.

In summary, decreased receptor glycine efficacies caused by disruptions to the allosteric transduction mechanism can account for the phenotypes conferred by all human startle disease mutations studied so far. Decreased chloride conductances and receptor expression efficiencies may also contribute to the phenotype caused by some mutations.

**Conclusion**

The major finding of this study is that numerous residues in both the M1–M2 and M2–M3 loops are involved in the transduction process between ligand binding and channel activation. This conclusion is supported by a variety of experimental evidence from other members of the LGIC superfamily, reviewed above, as well as the structural model of Unwin (1995). The Unwin model postulates that in the closed state the M2 domains are kinked inwards to constrict the channel pore and upon activation are twisted sideways to create an open passage. Consistent with this model, the simplest explanation of our results is that the M1–M2 and M2–M3 loops act as the hinges to facilitate this rotation and that mutations which disrupt signal transduction act by preventing this rotation from taking place. Whereas agonists interact with the external M2–M3 loop to activate the channel, other intracellular sites may interact with the M1–M2 loop to modulate channel desensitization. Given their highly conserved structures, it is likely that all ligand-gated ion channel receptors share this mechanism for channel activation. A second major finding is that human startle disease mutations reduce or eliminate the agonist efficacies for taurine and \( \beta \)-alanine without dramatically affecting their ligand binding affinities. This indicates that these mutations act in a common manner to disrupt the allosteric transduction process linking agonist binding to channel activation.

**Materials and methods**

**Mutagenesis and expression of human GlyR α1 subunit cDNA**

All procedures have recently been described in detail (Rajendra et al., 1995a). Briefly, mutations were constructed to human GlyR α1 cDNA in the pcIS2 expression vector using oligonucleotide-directed PCR and confirmed by sequencing the cDNA clones. Mutant plasmid constructs were transiently transfected into the human embryonic kidney 293 cell line using the modified calcium phosphate precipitation method of Chen and Okayama (1987). Patch-clamp studies and \([\text{H}]\)strychnine binding assays were conducted at least 48 h after transfection.

**Electrophysiology**

Glycine-gated currents were measured using whole-cell recording (Hamill et al., 1981) at a holding potential of −60 mV. Cells were continually superfused with a bathing solution containing 140 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM HEPES, 10 mM glucose, pH 7.4 with NaOH. Patch pipettes had tip resistances of 1–4 MΩ when filled with the standard intracellular solution containing 145 mM CsCl, 2 mM CaCl\(_2\), 2 mM MgCl\(_2\), 10 mM HEPES, 10 mM EGTA, pH 7.4 with CsOH. The empirical Hill equation, fitted by a non-linear least squares algorithm, was used to calculate the apparent 50% effective concentrations for activation (EC\(_{50}\)) and for inhibition (IC\(_{50}\)) under conditions as described in Results. Hill coefficients were also calculated but were excluded from Tables I and II for brevity. There was, however, no consistent trend between the respective Hill coefficients and any other parameter measured in this study.

**\([\text{H}]\)Strychnine binding assays**

Transfected cells or crude membrane preparations were incubated with \([\text{H}]\)strychnine (1–50 nM, 23 Ci/mM; New England Nuclear) with and without 10 μM cold strychnine to determine non-specific binding or with 20 nM \([\text{H}]\)strychnine and \(0–10^{-7}\) M glycine, \(\beta\)-alanine or taurine to generate agonist competition curves. After incubation to equilibrium at 4°C for 60 min, cells were collected by rapid filtration onto Whatman GF/B filter paper and crude membranes were collected by centrifugation at 13 000 r.p.m. for 5 min. The amount of radioactive strychnine remaining bound to either filters or to resuspended pellets was determined. The \( K_d \) and \( B_{\text{max}} \) for the \([\text{H}]\)strychnine saturation isotherms and the IC\(_{50}\) and IC\(_{\text{max}}\) values for glycine, \(\beta\)-alanine and taurine displacement of bound \([\text{H}]\)strychnine were estimated using the Iplot program (Graphpad Software, San Diego, CA).

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**References**


