Molecular Dynamics Simulation Links Conformation of a Pore-Flanking Region to Hyperekplexia-Related Dysfunction of the Inhibitory Glycine Receptor

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Summary

Inhibitory glycinergic receptors mediate rapid synaptic inhibition in mammalian spinal cord and brainstem. The previously identified hyperekplexia mutation GLRA1(P250T), located within the intracellular TM1-2 loop of the GlyR α1 subunit, results in altered receptor activation and desensitization. Here, elementary steps of ion channel function of α1(250) mutants were resolved and shown to correlate with hydropathy and molar volume of residue α1(250). Single-channel recordings and rapid activation kinetic studies using laser pulse photolysis showed reduced conductance but similar open probability of α1(P250T) mutant channels. Molecular dynamics simulation of a helix-turn-helix motif representing the intracellular TM1-2 domain revealed alterations in backbone conformation, indicating an increased flexibility in these mutants that paralleled changes in elementary steps of channel function. Thus, the architecture of the TM1-2 loop is a critical determinant of ion channel conductance and receptor desensitization.

Introduction

The strychnine-sensitive glycine receptor (GlyR), a member of the ligand-gated ion channel family, is the principal mediator of fast inhibitory synaptic transmission in the human brainstem and spinal cord [1–4]. Presently, four glycine binding α subunits (α1–4) and one structural β subunit of vertebrate GlyRs have been identified [5]. Despite considerable advances in the understanding of receptor function, comparatively little is known about the structure of ligand-gated ion channels. According to the classical topology model for acetylcholine receptor-type ion channels, five identical or homologous subunits assemble into a pentameric membrane complex [3]. For each subunit, the extracellular N-terminal domain comprises about 50% of the protein sequence, followed by four hydrophobic membrane-spanning segments (TM1–TM4). TM2 of each subunit points to the center of the complex, forming the inner lining of the central ion pore [5]. A large TM3-4 loop is thought to mediate intracellular interactions; the short C terminus is extracellular. To date, the X-ray structure of a molluscan acetylcholine binding protein (AChBP) represents the best model structure for the extracellular N-terminal domain of this ion channel superfamily [6]. This structure contains 8% of α helix, which is considerably less than found for the GlyR N-terminal domain by CD and FTIR spectroscopy (24%) [7]. Based on protease accessibility of reconstituted GlyRs, a different topology for parts of the N-terminal domain and TM1 was proposed, containing mainly β sheets in TM1 [8]. In contrast, a 4 Å electron microscopy structure of the nicotinic acetylcholine receptor from Torpedo californica [9] revealed considerable amounts of α helix in the protein. This structure shows that TM1 and TM2 are both α helical, connected by a short loop, thus forming a helix-turn-helix (HTH) motif, in agreement with the classical topology model of the GlyR. While secondary structure assignments of the N-terminal domain are diverse [7], the helical structure of the pore-lining TM2 has been demonstrated by NMR analysis for acetylcholine receptors [10] and GlyRs [11].

Mutations in the GLRA1 gene encoding the α1 subunit of the GlyR underlie the human neurological disorder, hyperekplexia (STHE, stiff baby syndrome, OMIM #138491) [12]. The mutant allele GLRA1(P250T), associated with a dominant form of hyperekplexia [13], encodes an amino acid exchange within the short intracellular TM1-2 loop. Proline 250 is highly conserved among ligand binding (α) subunits of the glycine/GABA receptor channel family, but absent from the corresponding position of cation channels, such as nicotinic acetylcholine or 5-HT3 receptors. The TM1-2 loop region has been recognized as part of the ion channel selectivity filter of acetylcholine-type ion channels [14]. Recombinant GlyR α1(P250T) channels disclosed reduced apparent agonist affinity as well as complete and rapid receptor desensitization. While individual channels could not be resolved, noise analysis suggested a reduced single-channel conductance [13]. Receptor desensitization and apparent ligand affinity were shown to be governed by the opposing action of hydrophobic volume versus charge in position α1(250) [15].

Here, analysis of elementary steps of GlyR channel function showed that the intracellular mutation α1(P250T) reduced channel conductance and stabilized the desensitized state, but had little effect on channel gating. Deletion of P250 resulted in a shift in EC50 but no dramatic changes in desensitization or conductance. Molecular dynamics (MD) simulations of the protein domain ranging from TM1 to TM2, using a simple helix-turn-helix motif based on published coordinates of this region, gave trends of backbone conformation and protein flexibility for the investigated α1(P250) mutants that correlated with ion channel desensitization and EC50 for glycine. Thus, MD simulation of a simple model system...
representing an excised receptor domain allowed the prediction of functional characteristics of the corresponding ion channel.

Results

Whole-Cell Current-Recording—Dose-Response Properties and Desensitization

Homomeric GlyR α1(wt) and α1(P250) mutant channels were expressed in HEK 293 cells, and glycine-mediated whole-cell currents recorded using rapid perfusion. Consistent with previous observations [13, 15], desensitization of whole-cell currents for wild-type and mutant receptors differed markedly. While wild-type and α1(P250A) currents desensitized to negligible extents (<5%, t\text{desens,sat.} > 25 s; Figure 1A), desensitization was dramatically increased for the mutants bearing large, hydrophobic side chains as well as for the hyperekplexia mutant α1(P250T) (desensitization 100%, t\text{desens,sat.} < 0.1 s; Figure 1A). Removal of the residue α1(P250) had no
Glycine Receptor Structure-Function Relationships

Table 1. Receptor Characteristics of GlyR α1(245–251) Mutants (Means ± SD)

<table>
<thead>
<tr>
<th>GlyR Variant</th>
<th>EC50 (nM)</th>
<th>kdesens (s⁻¹)</th>
<th>Fraction Isteady-state (%)</th>
<th>n Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1 wildtype</td>
<td>11 ± 1</td>
<td>&lt;0.04</td>
<td>&lt;5</td>
<td>8</td>
</tr>
<tr>
<td>α1(N245L)</td>
<td>438 ± 7</td>
<td>0.13 ± 0.08</td>
<td>75 ± 32</td>
<td>3</td>
</tr>
<tr>
<td>α1(M246L)</td>
<td>65 ± 11</td>
<td>0.32 ± 0.04</td>
<td>29 ± 7</td>
<td>3</td>
</tr>
<tr>
<td>α1(D247L)</td>
<td>7 ± 1</td>
<td>0.29 ± 0.04</td>
<td>82 ± 24</td>
<td>11</td>
</tr>
<tr>
<td>α1(A248L)</td>
<td>33 ± 4</td>
<td>0.81 ± 0.33</td>
<td>72 ± 39</td>
<td>4</td>
</tr>
<tr>
<td>α1(A249L)</td>
<td>51 ± 5</td>
<td>&lt;0.04</td>
<td>&lt;5</td>
<td>8</td>
</tr>
<tr>
<td>α1(P250L)</td>
<td>1840 ± 62</td>
<td>20 ± 4</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>α1(A251L)</td>
<td>452 ± 112</td>
<td>0.48 ± 0.09</td>
<td>90 ± 2</td>
<td>6</td>
</tr>
<tr>
<td>α1(P250T)</td>
<td>344 ± 116</td>
<td>0.07 ± 0.04</td>
<td>27 ± 25</td>
<td>3</td>
</tr>
</tbody>
</table>

*Fast phase observed in 2 of 5 cells; 3 of 5 cells were used for EC50 determination.

Dose-response data yielded an ~19-fold difference in EC50 values (wild-type: 11 ± 1 μM; α1(P250T); 192 ± 14 μM [15]). Hill coefficients nHill were 2.9 ± 0.5 for the wild-type and 2.2 ± 0.2 for the mutant [15] (Table 2). For the α1(ΔP250) receptors, a right-shift in dose-response curve was observed, the fitted constants were EC50 = 203 ± 9 μM, and nHill = 2.2 ± 0.1 (Figure 1B). The concentration dependence of maximum whole-cell currents (Figure 1B) was compatible with a 2-ligand model of receptor activation. We found neither improvement of fit nor significant alterations in the constants when a third ligand was included. This is reminiscent of a GlyR isoform expressed in embryonic mouse spinal neurons [16], and homomeric serotonin 5-HT3 receptors [17]. Dose-response data comprise information from two equilibria, ligand binding and gating. Here, we used an independent technique—laser pulse photolysis (lpp)—to evaluate the gating constant φ. Resensitization, defined as the period between consecutive saturating glycine applications after which a maximum whole-cell current response could again be elicited from GlyR transfected HEK cells, was practically spontaneous for the wild-type (τresens < 5 s, within the time resolution of the application system), but markedly slower for α1(P250T) mutant channels (τresens = 44 ± 3 s, not shown). Note that due to their large non-desensitizing current, wild-type receptors may not have desensitized completely during 10 s applications of glycine (τsteady-state > 95% of Isteady-state, while all of the P250T channels were fully desensitized (τsteady-state < 0) after ~2 s. Prolonged application of glycine (up to 60 s) to wild-type receptors confirmed the low extent and slow time course of desensitization. Strycrine inhibition of α1(P250T) currents was consistent with a competitive, or partially competitive inhibition mechanism [2] and indicated similar strycrine affinities for wild-type and P250T mutant receptors (not shown).

Leucine Scan: Positional Effects within the GlyR TM1-2 Loop

To test whether the differences in EC50 values and desensitization rates of GlyR α1(P250T) mutants were due to a specific position within the GlyR TM1-2 loop, a hydrophobic scan across this region was performed. Previously, we could show that leucine had the most pronounced effect on receptor desensitization and EC50 [15] (Table 1). Thus, TM1-2 loop residues (i.e., N245–A251) were individually replaced by leucine. All mutants gave functional homomeric receptor channels, as assessed by whole-cell current recordings at saturating concentrations of glycine (Figure 1C; Table 1). Distinct patterns were observed for EC50 (Figure 1D) and receptor desensitization (Figure 1E). Distribution of EC50 values was reminiscent of a U-shaped positional dependence, with residues close to the putative membrane entry being most sensitive to hydrophobic substitution (Figure 1D), in marked contrast to the positional dependence of receptor desensitization rates (Figure 1E). Note that the mutant A249L, adjacent to position α1(250), was completely non-desensitizing, while both, EC50, and kdesens, were highest for the P250L mutant. One possible explanation could be that two neighboring side chains in a polypeptide may not face in the same direction. It may, therefore, be assumed that desensitization is sensitive to the protein surface of the TM1-2 loop with the main determinant of desensitization being located at position α1(250).

Table 2. Constants Describing Activation and Gating of Recombinant Homomeric Glycine Receptors (Means ± SD)

<table>
<thead>
<tr>
<th>GlyR Variant</th>
<th>α1(wt)</th>
<th>α1(P250T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50 [nM]</td>
<td>11 ± 1</td>
<td>192 ± 14*</td>
</tr>
<tr>
<td>nHill</td>
<td>2.9 ± 0.5*</td>
<td>2.2 ± 0.2*</td>
</tr>
<tr>
<td>τresens [s]</td>
<td>&lt;5</td>
<td>44 ± 3</td>
</tr>
<tr>
<td>γ [ps]</td>
<td>69*</td>
<td>71 ± 10*</td>
</tr>
<tr>
<td>kdesens [s⁻¹]</td>
<td>1630 ± 40*</td>
<td>2200 ± 100*</td>
</tr>
<tr>
<td>kC [M⁻¹]</td>
<td>23 ± 2, 38 ± 30*</td>
<td>29 ± 9*</td>
</tr>
<tr>
<td>φ</td>
<td>0.014 ± 0.001*, 0.017 ± 0.013*</td>
<td>0.035 ± 0.015*</td>
</tr>
<tr>
<td>Pmax</td>
<td>0.99*</td>
<td>0.97*</td>
</tr>
</tbody>
</table>

*From dose-response curves.
†From [15].
‡Cell-attached patch, slope conductance of main state.
§From outside-out patches.
¶From laser-pulse photolysis.

Single-Channel Recordings—Conductance and Open State Dwell Time

Functional impairment of mutant receptor function may be due to (1) low protein expression, (2) reduced open probability, or (3) smaller single-channel conductance of mutant channels. To differentiate between these possibilities, we performed single-channel recording experi-
ments. Cell-attached recordings from HEK 293 cells showed similar bursting behavior (Figure 2A) and slope conductance for wild-type and α1(ΔP250) constructs, indicating that chloride permeation across these channels was not significantly different (α1(wt): γ_{up} = 64 ± 4 pS, α1(ΔP250): γ_{up} = 49 ± 6 pS, Figure 2B). In contrast, α1(P250T) channels showed a dramatic reduction in unitary conductance, yet single channels could be resolved (Figure 2A). The transition corresponding to the level closest to baseline was fit with a linear regression yielding a slope conductance of ~10 pS (Figure 2B).

Wild-type single channels recorded from outside-out patches produced a main conductance level of ~69 pS (Figure 2D), in agreement with cell-attached recordings (γ = 64 pS, Figure 2B). This value was lower than the reported maximum conductance of 86 pS [18], but was reproducibly observed as the predominant conductance level with our line of HEK 293 cells. Under the same experimental conditions, α1(P250T) channels were hard to resolve in outside-out patches, resulting only in a slightly skewed current histogram (Figure 2E). The calculated conductance for α1(P250T) channels of 7 pS agreed with the value of 10 pS obtained from cell-attached recordings (Figure 2B).

Main state dwell-time histograms were constructed from cell-attached single-channel traces (Figure 2A), which had to be extensively filtered due to the low conductance of α1(P250T) mutant channels. The open-state dwell time histograms could be fit by double-exponential time courses (Figure 2C), yielding time constants for the wild-type of α1(wt): τ_{fast} = 3.0 ± 1.3 ms (65%) and τ_{slow} = 28.8 ± 12.1 ms (35%). For mutant channels, the time constants were τ_{fast} = 4.1 ± 0.9 ms (82%) and τ_{slow} = 54.0 ± 29.7 ms (18%), indicating that the mutation had no detectable effect on the open-close equilibrium of GlyR channels. Open state dwell time constants for the wild-type agreed with reported values [19]. Similar open channel dwell times for α1(wild-type) and α1(P250T) mutant channels were a necessary, but not sufficient, indication of unaltered gating in α1(P250T) receptors.
Laser-Pulse Photolysis-Direct Measurement of $k_{op}$ and $k_{cl}$

As low conductances of mutant $\alpha 1(P250T)$ receptors precluded a detailed single-channel analysis, channel gating was investigated by an additional, independent method. To this end, laser-pulse photolysis (LPP) was used as a rapid kinetic technique, allowing direct determination of the rate constants $k_{op}$ and $k_{cl}$ [20]. Upon irradiation with a 340 nm laser pulse, free glycine was released within less than 25 μs from its biologically inert precursor, α-CNB-caged glycine [19, 21]. LPP current traces for wild-type [19] and $\alpha 1(P250T)$ mutant channels permitted an analysis of channel opening and closing rates (Figures 3A and 3B). Using the 2-ligand model of receptor activation, rate constants of $k_{op}$ and $k_{cl}$ were obtained from the intercept and the slope of the linear fit of the data according to equation 4 (Figure 3C). Constants for the wild-type were $k_{op} = 1630 \pm 40$ s$^{-1}$ and $k_{cl} = 23 \pm 2$ s$^{-1}$, in good agreement with previously reported values [19] (Table 2).

The LPP method does not require the release of saturating ligand concentrations, since the linear relationship given by equation 4 is valid over the entire concentration range [20]. The technique relies on the assumption that ligand binding occurs on a much faster time scale than channel-opening and closing, as previously shown for GlyRs, where the association rate for glycine of $10^5$ M$^{-1}$s$^{-1}$ exceeds the channel-opening rate of 2200 s$^{-1}$ by more than three orders of magnitude [19]. Nevertheless, ligand binding may become rate-limiting at low ligand concentrations. For the $\alpha 1(P250T)$ mutant, $k_{op}$ had to be extrapolated from measurements at low agonist concentration, since the technique has an upper limit of photorelease from caged glycine that was not saturating. While this might result in a higher error associated with numeric values of the rate constants, the principal results, namely that (1) $k_{cl}$ is largely unchanged between wild-type and mutant receptors, and (2) $k_{op} >> k_{cl}$ for both GlyR constructs, are valid despite the limited agonist concentration range available for $\alpha 1(P250T)$ channels.

Molecular Dynamics Simulation of the TM 1-2 Domain Structure

To identify alterations in the domain structure of the intracellular TM 1-2 loop that correlated with the observed changes in elementary steps of channel function, molecular dynamics (MD) simulations were carried out using a simple helix-turn-helix motif (Figure 4A). For TM2, coordinates from published NMR structures of model peptides of the nicotinic acetylcholine receptor [10] were used to generate the starting structures. This starting geometry was in good agreement with a recently published structure of the Torpedo nicotinic acetylcholine receptor at 4 Å resolution [9]. The entire motif, consisting of 64 residues, was placed in a simulated water box (Figure 4A) applying periodic boundary conditions; no explicit membrane fragment was included in the simulations. Energy minimization followed by MD equilibration and production was carried out for the wild-type and the mutants $\alpha 1(P250A/S/T/V/L/F)$. In each case, wild-type coordinates served as identical geometrical basis. After modifying the mutant residue at position 250, the systems were geometry optimized and subjected to MD equilibration and production phases. The
introduced residue types were chosen to reflect increasing hydrophobic volume in the critical position 250 [15]. Serine was included to detect potential effects of the hydroxyl function during the simulation.

To analyze the influence of the point mutations on the geometries of the different helix-turn-helix-motifs, we calculated for each mutant the average structure over each of the 200 snapshots sampled during the production phases of each molecular dynamics run. Each mutant average structure for the wild-type and $\alpha_1(P250A)$ was overlaid on the average wild-type geometry by a root mean square (RMS) fit of the $C_\alpha$ coordinates of the protein backbone residues L233–I244 and V253–T258. Fitting over all residues of the model system appeared inappropriate because the long helices were both kinked during all simulations. Interestingly, the areas denoted above, which are located adjacent to the loop, perfectly maintained their helical secondary structure during the simulation. In contrast, P250 mutants with increasing side chain hydrophobicity ($P250A/S/T/V/L$) showed a gradual outward movement of the peptide backbone within the TM1-2 loop, exposing the protein region around position 250 (Figure 4B, black arrow), consistent with an altered protein surface presented to the cell interior at the TM1-2 loop. The calculated RMS values (Figure 5A) indicated a threshold above which pronounced desensitization was observed in the GlyR mutants. Alanine and serine, both producing wild-type-like geometries of the different helix-turn-helix-motifs, were calculated for every mutant the average structure over each of the 200 snapshots sampled during the production phases of each molecular dynamics run. Each mutant average structure for the wild-type and $\alpha_1(P250)$, which was characterized by no or little desensitizing whole-cell current responses, gave an equilibrium loop conformation that differed from the wild-type (Figure 4B, gray arrow), but did not present the outward bulge that was observed in the hydrophobic mutants. Apparently, desensitization of mutant receptors in whole-cell current recordings coincided with a particular conformation observed in the MD simulations of the loop model system (Figure 4B).

The simulation results (Figure 4B) suggested that the equilibrium protein conformation of the two parallel helices of the helix-turn-helix motif was only slightly affected by structural disturbances in position $\alpha_1(P250)$, while most of the deviations were located within the
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Figure 5. Molecular Dynamics Simulations of GlyR α1(P250) Mutants

Correlation between RMS deviations of the indicated protein region and the hydropathy/volume index (HVI) for residue 250. Increasing values of HVI indicate an increasing hydrophobic surface presented by the residue, RMS deviations served as a measure for local protein flexibility, R is the correlation coefficient.

(A) RMS deviation of the region covering the loop TM1-2 (residues N245–R252) calculated after fitting the mutant average structures (Cα atoms only) of the residues V235–I244, T258 to the wild-type.

(B) RMS deviation versus hydropathy/volume index (HVI) for the cytoplasmic half of TM 2.

(C) Correlation of RMS deviation versus HVI for the TM 1-2 loop. Note that in (B) and (C) the mutant P250F was not included in the fit, due to inconsistent electrophysiological properties of the construct GlyR α1(P250F) [15].

(D) Correlation of desensitization rates [ln(kdes)], of GlyR α1(P250) mutants versus RMS deviation for the TM 1-2 loop.

(E) Correlation of desensitization rates [ln(kdes)], of GlyR α1(P250) mutants versus RMS deviation for the cytoplasmic half of helix 2.

TM1-2 loop (Figures 4B and 5). To confine the influence of amino acid substitutions on the averaged geometries, we turned to an alternative description of the orientation of TM 2 in relation to TM 1. This could be achieved by fitting the Cα atoms of residues V235–I244, representing TM 1, and T258. The latter residue prevented the loop and TM 2 from rotation upon the helix axis of TM 1, which would potentially complicate interpretation of the simulation results. Based on this fit, Figure 5A shows the RMS Cα differences of residues N245 to T258, representing the TM1-2 loop and the N terminus of helix 2, respectively (note that the deletion mutant, α1[ΔP250], could not be used in these calculations, due to the different number of amino acid residues). RMS values of the α1(P250A/S) mutants showed only small geometric deviations from the wild-type. The hydrophobic residues α1(P250V/L/F) caused increasing RMS deviations from the wild-type coordinates in the protein region around and including the TM 1-2 loop (Figures 4B and 5B-5D). These RMS differences correlated closely with the hydropathy/volume index, HVI, for residue 250, which had previously been shown to be an empiric predictor for ion channel properties in GlyR α1(250) mutants [15]. RMS deviations of the entire motif could be broken down into separate contributions of TM 2 and the short loop. The correlation between HVI for residue 250 and the resulting RMS deviation of the TM 1-2 loop was excellent (Figure 5C, R = 0.98), being much stronger than for the cytoplasmic side of TM 2 (Figure 5B, R = 0.73). Note that the mutant α1(P250F) did not fit into this model, despite its increased hydrophobic volume. This is consistent with previous observations, where whole-cell current responses for the construct GlyR α1(P250F) were rarely observed, compared to >95% success rates for all other subunits [15]. Apparently, the effects of the phenylalanine side chain are not compatible with a regular GlyR function, and cannot be fully accounted for by MD simulations. The hyperekplexia mutant α1(P250T) was characterized by increased RMS deviation of the TM 1-2 loop region, similar to that of the hydrophobic mutants (Figures 4B and 5). Presence or absence of hydroxyl groups had no significant effect on simulation results or channel function, as evident from the comparison of the alanine and serine mutants, indicating that...
specific hydrogen bonds or interactions with OH groups were not relevant. Rather, volume and hydrophobicity of residue α1(250), as reflected in the HVI, were the only determinants of backbone conformation. Given the ns time scale of the simulation, RMS deviation was taken as a measure for protein flexibility on the ms-s time scale of receptor desensitization. Indeed, correlation of \( \ln(k_{\text{on}}) \) of mutant receptor constructs versus RMS (Figures 5C and 5D) showed excellent correlation for the TM 1-2 loop (Figure 5E, \( R = 0.95 \)), while correlation for the cytoplasmic face of helix 2 was considerably weaker (Figure 5D, \( R = 0.84 \)). This indicated that desensitization was most sensitive to changes of protein flexibility in the TM 1-2 loop.

Discussion

The identification of protein domains that control ligand-gated ion permeation is a prerequisite to understand neurotransmitter receptor function and molecular pathology. Here, we investigated the intracellular hyperkplexia mutation α1(P250T) and related amino acid substitutions [13, 15] as a model for the dependence of elementary steps of GlyR activation and desensitization on subtle alterations of protein structure. Deviation of the TM1-2 loop from wild-type architecture was identified as an essential parameter of ion channel dysfunction. The functional impact could be correlated to structural changes observed by MD simulations of the excised protein motif.

In electrophysiological recordings, the apparent glycine affinity of α1(P250T) receptors was reduced by approximately one order of magnitude as compared to the wild-type. This is contrasted by radioligand binding studies [13, 15] and strychnine inhibition of glycine-induced whole-cell currents where both \( K_{\text{f}} \) for strychnine and equilibrium \( K_{\text{g}} \) for glycine were found to be unaffected by the mutation [15]. Discrepancies between equilibrium binding data and whole-cell current recordings suggest an involvement of elementary steps downstream of ligand binding, which are not accessible by equilibrium radioligand studies [22, 23]. Desensitization studies were consistent with the presence of distinct receptor states: wild-type receptors desensitized slowly, but resensitized rapidly, while the α1(P250T) mutant desensitized quickly and resensitized slowly. For the wild-type, desensitized and nondesensitized states appear to interconvert easily, suggesting similar thermodynamic stability. For α1(P250T) channels, rapid desensitization and slow current recovery indicated that the desensitized state was thermodynamically favored as compared to the wild-type, and both states would not interconvert in the continued presence of glycine. In addition to a lower conductance, the major functional consequence of the mutation α1(P250T) appeared to be a reduced ligand affinity of the nondesensitized receptor and a greatly increased stability of the inactivated state.

Analysis on the single-channel level indicated that the mutation α1(P250T) predominantly affected channel conductance, while the microscopic gating equilibrium, i.e., the transition between open and closed channel states, was essentially unchanged. This result was consistently obtained by three independent techniques: (1) concentration-dependence of wild-type and mutant whole-cell currents gave similar estimates for the gating constant \( \phi \), yielding an open probability near 1 for both constructs; (2) open-channel dwell times did not differ significantly between wild-type and mutant receptors; and (3) Laser-pulse photolysis using caged glycine [20] permitted a direct measurement of \( k_{\text{op}} \) and \( k_{\text{cl}} \), which were not significantly different for α1 wild-type and α1(P250T) mutant receptors, indicating an almost identical \( P_{\text{on}} \) (Table 2). This is in contrast to the hyperkplexia mutation α1(K276E), located in the extracellular TM2-3 loop, where the principal effect of the mutation is on gating, as evident from unchanged main state conductance and reduced open channel dwell times [24].

A hydrophobic scan of the intracellular region GlyR α1(245–251) yielded two key results: (1) receptor activation and desensitization were independent processes, which showed different sensitivity toward modification of individual residues within the TM1-2 loop; and (2) GlyR α1(250) is by far the most critical position with respect to both receptor EC50 and desensitization. Recently, Wotring et al. showed that deletion of residue P(290)-corresponding to GlyR α1(P250)-in the ρ1 GABA receptor slightly reduced ion selectivity; the double mutation ρ1(P290/A291E) resulted in a cation-selective GABA receptor [25]. It was concluded that alterations of effective charge near the cytoplasmic mouth of the channel pore affected ion selectivity [25]. Similar observations were reported for the GlyR α1(ΔP250) mutant, where removal of proline 250 resulted in slightly increased cation permeability [26], although these receptors were still found to be predominantly chloride selective. Our results for the α1(ΔP250) variant are in good agreement with reported data, indicating that excision of proline 250 predominantly affects pore size and selectivity rather than channel gating kinetics. Since the amino acid exchanges reported here left the effective charge of the TM1-2 loop unchanged (compare [25]), one should still expect all GlyR mutants studied here to be chloride channels. In a mutagenesis study of GABA 2 receptors, desensitization was shown to be sensitive to mutation of leucine 9c within TM 2 of α1β3γ2L receptors; introduction of hydroxylated residues resulted in prolonged channel openings, due to a decreased channel closing rate [27]. In this case, reduced hydrophobicity within TM 2 stabilized the open-channel state, thereby indirectly slowing macroscopic desensitization. Investigation of chimeric α1β3γ2L and α1β3γ2L GABA 2 receptors also implicated a region within TM2 in receptor desensitization [28]. Our experiments indicated that desensitization, but not the rates of channel opening and closing were affected by TM1-2 loop mutations. Thus, desensitization is probably not a homogenous process but is controlled by several domains of the receptor protein.

Structural effects of mutations in position α1(250) could be inferred by molecular dynamics simulations of the TM1-2 region assuming a helix-turn-helix motif. In a simple approach, coordinates for TM 2 from published NMR structures were used, and the motif was inserted into a water box. Energy minimization followed by extensive MD equilibration showed parallel trends for averaged backbone conformations and receptor current re-
responses [15]. Rapidly desensitizing mutants showed an increasing deviation from the wild-type peptide backbone, primarily within the TM 1-2 loop. The deletion mutant α1(P250) showed yet another backbone conformation, different from both the rapidly desensitizing mutant and the wild-type. This again paralleled channel properties of this mutant, which was non- or slowly desensitizing but had a reduced glycine affinity. Since the time scale of atom and molecule dynamics is much faster than the scale of protein interaction, the physiological consequence of GlyR α1(P250) mutations would be an increasingly hydrophobic protein surface at the TM 1-2 loop presented to the interior of the cell. Surprisingly, molecular modeling as well as functional analysis of the channel suggested that neither the particular conformation of proline, nor the presence of hydroxyl groups were of any consequence in this protein environment. Previous analysis of electrophysiological data showed that an index HVI, reflecting only hydrophobicity and volume of an amino acid side chain actually predicted ion channel function of mutant GlyRs [15]. Here, MD simulation also found HVI to be the critical parameter determining TM 1-2 loop conformation.

Desensitization, i.e., the transition to the liganded, closed-channel state, might be envisaged as a refolding of the receptor protein. Since MD simulations were performed on a picosecond time scale, and a receptor desensitization occurs on the order of milliseconds to seconds, RMS deviations should reflect local protein flexibility. Protein folding kinetics have been related to structural and orientational parameters of the polypeptide chain [20], observing logarithmic relationships [30]. Receptor desensitization at a saturating concentration of ligand will occur almost exclusively from the bili-ganded state (RL0), and can thus be described by the single rate constant kdes [23]. Accordingly, we investigated how changes in kdes related to alterations of protein flexibility of the GlyR TM 1-2 loop, as expressed by the relation of RMS deviation ∼ln(kdes). Indeed, RMS deviations in the TM 1-2 loop more closely correlated with changes in kdes than those of the cytoplasmic face GlyR mutants, including the deletion mutant α1(P250) were generated and their ion channel properties investigated using patch-clamp recording techniques. Increased hydrophobic volume within the loop region resulted in rapidly desensitizing, low-affinity receptor channels with position α1(250) being the most critical. Independently, molecular dynamics (MD) simulations of a helix-turn-helix motif representing the TM1-2 loop region of the receptor gave deviations in protein conformation consistent with increased backbone flexibility for mutant constructs, which showed an excellent correlation with functional characteristics obtained from patch-clamp studies. Thus, MD simulation of a simple model system allowed prediction of the corresponding GlyR ion channel properties. These results indicate that the short intracellular TM1-2 loop of the GlyR represents an independent regulatory domain whose structure-function relationship can now be unraveled.

Experimental Procedures

Site-Directed Mutagenesis

Single nucleotide exchanges corresponding to codons α1(245–251), as well as the P250 deletion were introduced by PCR-mediated site-directed mutagenesis as described [15]. All mutated clones were sequenced across the PCR-generated sequence to verify successful mutagenesis using the ABI sequencer system (ABI systems, Weiterstadt, Germany).

Whole-Cell Recording and Data Analysis

GlyR constructs were expressed in HEK293 cells as described [15]. Whole-cell recording experiments were performed using an EPC9 amplifier controlled by Pulse software (HEKA electronics, Lambrecht, Germany). Recording pipettes (2–5 MΩ) were pulled from borosilicate glass (World Precision Instruments, Berlin, Germany) using a Sutter P-97 horizontal puller. Ligand application was carried out using a U-tube that bathed the suspended cell in a laminar flow and allowed ligand equilibration over the cell with a time resolution of 10–30 ms. The external buffer consisted of NaCl 137 mM, KCl 5.4 mM, CaCl2 1.8 mM, MgCl2 1.0 mM, and HEPES 5.0 mM (pH adjusted to 7.2 with NaOH); the internal buffer was CsCl 120 mM, N-[2-(N-morpholino)ethanesulfonic acid] 10 mM, MgCl2 1.0 mM, and HEPES 10 mM (pH adjusted to 7.2 with CsOH). Current responses were measured at room temperature, at a holding potential of −60 mV unless otherwise noted. Strychnine inhibition measurements were performed under conditions of simultaneous application of agonist and inhibitor. For laser-pulse photolysis experiments, the HEPES concentration was raised to 30 mM to counter possible acidification of the solution upon dissolution of the caged compound.

Dose-response curves were constructed from the maximum current amplitudes and calculated by a nonlinear fitting routine using Microcal Origin (Additive, Friedrichsdorf, Germany). Dose-response data were normalized to the maximum response at saturating glycine concentration (2 mM). The Hill equation (equation 1) was used for analysis. As an additional check for consistency, data were ana-
lyzed according to a two-ligand model assuming independent binding sites [31].

\[ I_{\text{max}} = \frac{I_{\text{max0}}}{1 + \frac{[G]}{K_{\text{Gly}}}} \]  

(1)

Here, \( I_{\text{max}} \) is the maximum observed current, \( I_{\text{max0}} \) the current obtained at a given glycine concentration, \( K_{\text{Gly}} \) the glycine concentration evoking half-maximum current amplitudes, and [Gly] the glycine concentration. For desensitization analysis of whole-cell current \( HVI \) was computed as described [15]. Briefly, the mean open time of the current decay function plus a constant.

\[ l_{\text{obs}} = l_1 * e^{-t/l_1} + l_2 * e^{-t/l_2} + l_{\text{const}} \]  

(2)

\( l_{\text{obs}} \) is the observed current, \( l_1 \) is the fraction of current amplitude decaying with time constant \( \tau_1 \), \( l_2 \) is the fraction of current amplitude decaying with time constant \( \tau_2 \), and \( l_{\text{const}} \) is the nondesensitizing current fraction. Generally, desensitization could be described satisfactorily using a single exponential function.

**Single Channel Recording**

For cell-attached recordings, pipettes of 5–10 MΩ resistance were filled with external buffer containing 5 μM glycine for α1(wt), 100 μM for α1(P250), or 20 μM glycine for α1(P250T) constructs. For outside-out patches, pipettes were filled with intracellular buffer and the cell membrane was punctured using a 10–20 MΩ electrode. Data were recorded at a sampling rate of 3–10 kHz, stored in ASCII format, converted to pClamp format using Axoscope (Axon Instruments, Union City, CA), and analyzed using the programs Fetchan and Pestat from the pClamp 6 program suite (Axon Instruments); doublet events and multiple openings were discarded. Note that due to the low conductance of α1(P250T) channels, some noise remained after filtering, which was binned among the high-frequency transitions and may have increased the number of brief events in the dwell time histogram. The 2 ms component was, therefore, not considered in the analysis of open channel dwell times. Typical single-channel traces are given in Figure 2. The number of patches used for analysis were: α1(wt) cell-attached 5, outside-out 2; α1(P250T) cell-attached 7, outside-out 2; α1(P250): cell-attached 2.

**Laser-Pulse Photolysis**

An EPC7 amplifier (HEKA) and pClamp 6 (Axon Instruments) software were used for data acquisition. After storage on a computer hard disk, individual current traces were analyzed offline using Microcoulometric software. αCNB-caged glycine [21], kindly provided by Professor G.P. Hess (Cornell University, Ithaca, NY), was flown over intact Torpedo nicotinic acetylcholine receptor α subunit, for which the threedimensional structure was solved by NMR spectroscopy (PDB entry 1DX2), revealed sequence homology of the two TM 2 regions which justified the use of the nACh TM 2 coordinates for the generation of our HTH model. The TM 1 region of the HTH fragment was modeled as an ideal α helix. The backbone dihedral angles of the loop region were assumed in a way that both helix axes are almost parallel. To achieve optimal comparability of all simulations, this system always served as template for the generation of the point mutations at position P250, for which only the considered amino acid was replaced.

To validate our starting geometry for MD calculations, we extracted the TM1-loop–TM2 motif of the receptor an chain from coordinates (RCSB Database entry 1OED) of the Torpedo nicotinic acetylcholine receptor that had recently become available [9]. These coordinates were compared to our average mutant geometries by an RMS fit of the Cα coordinates of the model protein backbone residues L233–I244 and V253–T258 to the corresponding residues of the extracted receptor HTH-motif. Although within the starting geometry for our mutants, only TM2 was modeled according to experimental NMR data, the overall geometries of the MD averages were in excellent agreement with the structural data provided by electron-microscopic techniques. The RMSD values over the above residue range are smaller than 3 Å for all mutants. Considering the fact that the resolution of the receptor pore is only 4 Å, this comparison validates the geometry and assumptions of our model and justifies its use for the geometric and dynamic description of the TM1-2 loop motif under investigation.

**Molecular Dynamics Simulations**

All simulations were performed using the AMBER5 suite of programs and the previously established parm94 force field [35]. Each of the generated starting geometries was placed in a box of TIP3P water molecules, assuring that at least 10 Å were between the peptide and the edges of the box. To neutralize the overall system, two chloride ions were added according to the electrostatic potential of the peptide. The final system had a box size of 58 × 83 × 41 Å consisting of 64 amino acids, two chloride ions, and 4494 water molecules. For nonbonded interactions, a cutoff of 9 Å was used in all simulations with periodic boundary conditions. SHAKE was employed to keep all bonds involving hydrogen atoms rigid. The simulation time step was 1 fs with constant pressure conditions.

\[ k_{\text{on}} = k_{\text{off}} + k_{\text{cat}} \left[ \frac{L}{L + K_{d}} \right] \]  

(5)

The channel gating equilibrium constant \( \phi \) is then calculated as:

\[ \phi = \frac{k_{\text{on}}}{k_{\text{off}}} \]  

(6)

**Calculation of the Combined Hydropathy/Volume Index, HVI**

HVI was computed as described [15]. Briefly, the mean molar volume \( \text{vol(mean)} \) after [33] of 14 proteinogenic amino acid residues (G,A,S,P,T,V,I, L,F,C,Y,R,Q,N) was calculated to be 88.8 ml/mol. The volume index for each residue was:

\[ \text{vol(residue)} = \frac{\text{vol(mean)}}{10} \]

where values ranged from −4.5 to 3.5. These volume index values were added to the corresponding hydropathy indices after [34] without further weighting of the two indices. This procedure yielded a combined hydropathy/volume index, HVI, ranging from −10 (R) to 7 (F).

**Three-Dimensional Structure of the Helix-Turn-Helix Motif**

For the generation of the starting geometry of the wild-type P250, the amino acids F214 to V277 of the human GlyR α1 chain precursor sequence (SwissProt entry P23415, locus GRA1_HUMAN) were assumed to fold in a typical helix-turn-helix (HTH) motif (TM 1: 220–244; loop 1–2: 245–252; TM 2: 253–270). Alignment of the GRA1_HUMAN HTH fragment with the sequence of the TM 2 region of the Torpedo californica nicotinic acetylcholine receptor α subunit, for which the three-dimensional structure was solved by NMR spectroscopy (PDB entry 1DXZ), revealed sequence homology of the two TM 2 regions which justified the use of the nACh TM 2 coordinates for the generation of our HTH model. The TM 1 region of the HTH fragment was modeled as an ideal α helix. The backbone dihedral angles of the loop region were assumed in a way that both helix axes are almost parallel. To achieve optimal comparability of all simulations, this system always served as template for the generation of the point mutations at position P250, for which only the considered amino acid was replaced.

Validation of our starting geometry for MD calculations, we extracted the TM1-loop–TM2 motif of the receptor an chain from coordinates (RCSB Database entry 1OED) of the Torpedo nicotinic acetylcholine receptor that had recently become available [9]. These coordinates were compared to our average mutant geometries by an RMS fit of the Cα coordinates of the model protein backbone residues L233–I244 and V253–T258 to the corresponding residues of the extracted receptor HTH-motif. Although within the starting geometry for our mutants, only TM2 was modeled according to experimental NMR data, the overall geometries of the MD averages were in excellent agreement with the structural data provided by electron-microscopic techniques. The RMSD values over the above residue range are smaller than 3 Å for all mutants. Considering the fact that the resolution of the receptor pore is only 4 Å, this comparison validates the geometry and assumptions of our model and justifies its use for the geometric and dynamic description of the TM1-2 loop motif under investigation.
(coupling constant 0.2 ps) and coupling the temperature to a heat bath with a time constant of 0.2 ps.

Prior to the molecular dynamics (MD) simulations, all systems were geometry optimized by 5000 cycles of conjugate gradient minimization to relax all atoms without any geometrical restraints. The general procedure for all MD simulations of the protein fragment in the water box comprises (1) heating the system to 300 K and equilibration for 400 ps and (2) production phase for 400 ps for data acquisition. During equilibration, the temperature was raised to 300 K and the solvent density stabilized around 1 g/cm$^3$. During the production phase, 200 snapshots of the actual geometries were collected as a trajectory for calculating average geometries with the AMBER module carnal, and for simulation stability control.

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