Novel Regulatory Site within the TM3–4 Loop of Human Recombinant α3 Glycine Receptors Determines Channel Gating and Domain Structure*

Hans-Georg Breitinger†1, Carmen Villmann†, Nima Melzer§, Janine Rennert‡, Ulrike Breitinger†1, Stephan Schwarzinger†, and Cord-Michael Becker‡1,2

From the †Institut für Biochemie, Emil-Fischer-Zentrum, Friedrich-Alexander-Universität Erlangen-Nürnberg, D-91054 Erlangen, Germany and §Institut für Biopolymere, Universität Bayreuth, D-95440 Bayreuth, Germany

Glycine receptors are Cys loop ligand-gated ion channels that mediate fast inhibitory synaptic transmission in the mammalian central nervous system. The functionally distinct splice variants α3L and α3K of the human glycine receptor differ by a 15-amino acid insert within the long intracellular TM3–4 loop, a region of high intersubunit diversity. In a mutational study, effects of the insert on ion channel function and secondary structure of the TM3–4 loop were investigated. Whole cell current responses and protein surface expression data indicated that the major effect of mutations within the insert was on channel gating. Changes in channel gating correlated with the distribution of charged residues about the splice region. Analysis of complex molecular weight indicated that recombinant TM3–4 loops of α3L and α3K associated into oligomers of different stoichiometry. Secondary structure analysis suggested that the insert stabilized the overall fold of the large cytoplasmic domain of α3L subunits. The absence of the insert resulted in a channel that was still functional, but the TM3–4 cytoplasmic domain appeared not stably folded. Thus, our data identified the spliced insert within the large TM 3–4 loop of α3 Gly receptors as a novel regulatory motif that serves a 2-fold role: (i) the presence of the insert stabilizes the overall spatial structure of the domain, and (ii) the insert presents a control unit that regulates gating of the receptor ion channel.

Glycine receptors (GlyRs),3 together with γ-aminobutyric acid receptors, are the principal carriers of fast synaptic inhibition in the mammalian central nervous system. They share structural and functional homology with other members of the ligand gated ion channel family (1–3). To date, four ligand binding subunits (α1–4) capable of forming homomeric functional ion channels and one β subunit have been identified (1–4). α1 subunits, prevalent in spinal cord and brain stem, are associated

8 This work was supported by Deutsche Forschungsgemeinschaft Grants SFB 359-A13 (to H.-G. B. and C.-M. B.), SPP 1026, and BR 1507/4 and by the Fonds der Chemischen Industrie.
1 Present address: The German University in Cairo, Biochemistry Department, New Cairo City, Egypt.
2 To whom correspondence should be addressed: Fahrstrasse 17, D-91054 Erlangen, Germany. Fax: 49-9131-852-2485; E-mail: cmb@biochem.uni-erlangen.de.
3 The abbreviations used are: GlyR, glycine receptor; BES, 2-(bis(2-hydroxyethyl)amino)ethanesulfonic acid; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography; CD, circular dichroism.

EXPERIMENTAL PROCEDURES

Generation of GlyR α3 Mutants—Single nucleotide exchanges, as well as deletions and duplications within the alternatively spliced insert of GlyR α3L, were introduced by PCR-mediated site-directed mutagenesis using an overlap extension PCR approach (10). Mutagenesis primers (Amer-sham Biosciences) contained nucleotides specific for the amino acid exchange together with a silent restriction site for a rapid
check of mutated clones. 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 Taq polymerase in the supplied buffer (Roche Applied Science). PCR conditions were 5 min at 95 °C for denaturation, 5 min at 50 °C for annealing, 5 min at 72 °C for elongation in the first cycle, followed by 28 cycles of 1 min at 95 °C, 2 min 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 reintegrated into GlyRa3. All mutated clones were sequenced across the PCR-generated sequence to verify successful mutagenesis using the ABI sequencer system (ABI Systems). Note that deletion or duplication of a six-residue stretch within the insert left the hydroxylated residues and neighboring positive charges intact (see Fig. 1).

**Cell Culture and Transfection**—HEK 293 cells were grown in 80-cm², 225-cm³ Nunc flasks at 37 °C, 5% CO₂, in a water-saturated atmosphere. Minimum essential medium (Invitrogen), supplemented with penicillin (100 IU), streptomycin (100 mg/liter), l-glutamine, and 10% heat-inactivated fetal calf serum (Invitrogen) were used for experiments 1–3 days after transfection. These cells could be kept up to 5 days, although they routinely were used for experiments 1–3 days after transfection.

**Protein Characterization and Radioligand Binding**—For crude membrane preparations, the cells were harvested using ice-cold PBS (13.7 mM NaCl, 0.27 mM KCl, 0.43 mM Na₂HPO₄, 0.14 mM KH₂PO₄, pH 7.4) and collected by centrifugation (10 min, 1000 rpm). All of the subsequent steps were carried out on ice. The cell pellet was taken up in a 20-fold volume of 10 mM potassium phosphate buffer, pH 7.4, supplemented with protease inhibitors (Complete EDTA-free (Roche Applied Science), 5 mM EDTA, 5 mM EGTA), homogenized using a glass potter and an ultraturrax, and centrifuged (20 min, 35,000 × g). This step was repeated, and the membrane pellet was finally resuspended in a 5-fold volume of storage buffer (25 mM potassium phosphate, pH 7.4, 200 mM KCl, plus protease inhibitors) and stored in 400-μl aliquots. Total protein content was determined using the Lowry method. Western blot analysis from membrane preparations was carried out using the GlyR-pan-α monoclonal antibody mAb-4a, a cy5-coupled goat anti-mouse secondary antibody gaMlgG-Cy5 (Dianova) and a Storm 860 Fluorimager (Molecular Dynamics, Krefeld, Germany) for visualization.

**Surface Protein Biotinylation**—COS7 cells were transfected (10 μg of plasmid/10-cm dish) using DEAE-Dextran (10 mg/ml) 48 h before labeling the cells. Following three washing steps with ice-cold PBS, the cells were incubated 15 min with 1 mg/ml EZ-link TM sulfo-N-hydroxy-sulfosuccinimide-S-biotin (Pierce) in cold PBS (pH 8.0) with gentle agitation at 4 °C. The cells were again washed and incubated with quenching buffer (192 mM glycine, 25 mM Tris in PBS) for 10 min at 4 °C. The cells were collected using cold PBS and centrifuged for 10 min at 1000 × g, and cell pellets were homogenized in lysis buffer (1% Triton X-100 in Tris-buffered saline containing 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A, 2 mM EDTA, pH 8.0) and centrifuged. The supernatants were incubated with 50 μl of streptavidin-Sepharose beads (Sigma) for at least 2 h at 4 °C while rotating. The beads were pelleted by brief centrifugation, and aliquots of the supernatants were taken to represent the unbound, intracellular pool. Eluted proteins were boiled in 1× sample buffer for 5 min and loaded on a SDS gel.

**Electrophysiological Recordings and Data Analysis**—Whole cell currents were recorded using a HEKA EPC9 amplifier.
Regulation of α3 Glycine Receptors by TM3–4 Loop

(HEKA Electronics, Lambrecht, Germany) controlled by Pulse recording software (HEKA Electronics) on a personal computer. Recording pipettes were pulled from borosilicate glass (World Precision Instruments, Berlin, Germany) using a Sutter P-97 horizontal puller. Ligand application using a U-tube gave a time resolution of 10–30 ms. The external buffer consisted of 137 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 5.0 mM Hepes, pH adjusted to 7.2 with NaOH; the internal buffer was 120 mM CsCl, 20 mM N(Et)₄Cl, 1.0 mM CaCl₂, 2.0 mM MgCl₂, 11 mM EGTA, 10 mM Hepes, pH adjusted to 7.2 with CsOH. Current responses were measured at room temperature of 21–23 °C, and the holding potential was −60 mV. Responses to the saturating concentration of 2 mM glycine were used for normalization of dose-response data. Maximum current responses from each individual cell were also recorded and averaged for each subunit (see Table 1).

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 (Microcal Origin), dose-response data were first analyzed using the following Hill equation,

\[ I_{\text{glycine}} = \frac{[\text{Glycine}]_{\text{max}}}{[\text{Glycine}]_{\text{max}} + EC_{50}} \]  

(Eq. 2)

where \( I_{\text{glycine}} \) is the current amplitude at a given glycine concentration, \( I_{\text{sat}} \) is the current amplitude at saturating concentrations of glycine, \( EC_{50} \) is the glycine concentration producing half-maximal current responses, and \( n_{Hill} \) is the Hill coefficient. Currents from each individual cell were normalized to the maximum response at saturating glycine concentrations. For each construct, the mean maximum current at saturating glycine concentration (2 mM) was calculated from all cells that were used for analysis. Dose-response curves were then scaled to this mean current amplitude. \( EC_{50} \) values are listed in Table 1. Dose-response data were fit to the saturating concentration of 2 mM glycine were used for normalization of dose-response data. Maximum current responses from each individual cell were also recorded and averaged for each subunit (see Table 1).

EC₅₀ was related to the \( K_d \) value obtained from the two-ligand model as follows: a glycine concentration of \( EC_{50} \) gives a half-maximum response; thus, with \([\text{Glycine}]/(\Phi + 2)^{1/2} - 1 \)  

(Eq. 5)

one obtains

\[ EC_{50} = \frac{K_d}{\frac{1}{\Phi} + 1} \]  

(Eq. 6)

Dose-response curves were then fitted using this equation. A kinetic model that requires binding of two ligands to independent sites prior to channel opening and considers channel gating (13–15) was used for subsequent analysis of dose-response data. The average maximum current at saturating glycine concentrations (2 mM) was used to scale current response data for each construct; dose-response data were then fit to Equation 3.

\[ I_{\text{glycine}} = \frac{I_{\text{max}}}{1 + \left(\frac{K_d}{I_{\text{sat}}} + 1\right)^2} \]  

(Eq. 3)

Here, \( I_{\text{max}} \) is the theoretical maximum current that would be observed if all channels were open simultaneously, \( I_{\text{glycine}} \) is the current obtained at a given glycine concentration, \( K_d \) is the dissociation constant of glycine from the receptor, \( I_{\text{sat}} \) is the concentration of ligand (i.e. glycine), and \( \Phi^{-1} = k_{op}/k_{cl} \) is the gating constant. The solid lines through the dose-response data (see Fig. 3, B and C) were computed using this model.
chromatography using a Perseptive BioCAD HPLC apparatus and a PD10 column (PerSeptive Biosystems, Freiburg, Germany). The purified polypeptide was lyophilized and taken up in 10 mM Na\(^+\) phosphate buffer, pH 8.0. Amino acid analysis was performed on a Merck-Hitachi L-6200 system, equipped with a F-1050 fluorometer and a D-2500 integrator. The concentration of synthetic a3L polypeptide was determined after acid hydrolysis in 6 M HCl at 155 °C for 1 h and precolumn derivatization with o-phthalaldehyde/3-mercaptopropionic acid (17). CD spectra were normalized to the protein concentration and normalized spectra deconvoluted using the CDSSTR algorithm and the Reference set 7 at Dichroweb (18, 19). The values for helix 1 and helix 2 as well as sheet 1 and sheet 2 were added to obtain total helix and total sheet content (20). The concentration of the synthetic a3L polypeptide was 0.0452 mg/ml. Thermal denaturation was carried out by a heating the sample from 30 to 80 °C and cooling back to 30 °C at a rate of 2 K/min.

RESULTS

Design and Expression of GlyR a3 Constructs—The large cytoplasmic TM3–4 loop is a region of high sequence diversity between different glycine receptor subunits (Fig. 1A). For the long splice variant of the human a3 GlyR subunit, a3L, secondary structure prediction algorithms suggest an extension of a cytosolic a-helix, covering the alternatively spliced exon. In the homologous cytosolic region, the propensity to attain an a-helical fold appears to be less pronounced in a3K. To identify the influence of alterations of the spliced 15-residue insert within the TM3–4 loop, glycine receptor a3 constructs were generated using site-directed mutagenesis. The three hydroxyl-bearing residues Thr\(^{358}\), Tyr\(^{367}\), and Ser\(^{370}\) were replaced by their closest hydrocarbon analogs, covering all possible permutations (Fig. 1B). Because the length of the spliced protein region itself may be a determinant of receptor function, the 15-amino acid insert was further shortened or elongated by removal (a3L\(^{\Delta 6}\)) or duplication (a3L\(^{+6}\)) of the 6-amino acid segment 359EAFALE364 (Fig. 1B). This segment was chosen to leave the minimum phosphorylation consensus motif (+X/-O-H) intact in both constructs. A cluster of six positive charges, which precedes the alternatively spliced region (7), has been shown to be critical for endoplasmic reticulum trafficking and membrane insertion of recombinant a1 GlyR (21). This cluster was not modified in our study.

Western blot analysis revealed expression of all constructs in HEK 293 cells (Fig. 2A). Quantification of receptor antigen content in HEK 293 membranes using a dot blot receptor assay (12) indicated similar expression efficiency for all subunits (Fig. 2B). Relative amounts of GlyR antigen (arbitrary units) for the different constructs varied between 2.7 ± 0.5 (a3L\(^{367F}\)) and 6.1 ± 1.9 (a3L), i.e. by a factor of 2.3 (Fig. 2B). Because crude membrane preparations may also contain endoplasmic reticulum membranes, surface protein was determined taking advantage of the high affinity between biotin and streptavidin.

To determine the fraction of recombinant a3L subunit variants expressed at the plasma membrane in COS7 cells, surface proteins were labeled by biotinylation using sulfo-N-hydroxysulfosuccinimide-S-S-coupled biotin and subjected to subsequent precipitation. Plasma membrane integration and intracellular protein accumulation levels were analyzed by immunoblotting of biotinylated precipitates and supernatants using the pan-a antibody, mAb-4a (Fig. 2C). The supernatants represent the unbiotinylated intracellular pool (Fig. 2C, lower panel). No differences were detectable between extra- and intracellular fractions from cells transfected with a3L, a3K, a3L\(^{\Delta 6}\), a3L\(^{+6}\), and a3L\(^{T358A/S370A}\) mutants as compared with cells carrying the wild type a1 subunit protein (Fig. 2C, upper panel). The glycine receptor-specific antibody revealed double bands in the surface fraction, most likely because of different degrees of glycosylation; the ratio of these double bands, however, was similar for all of the constructs tested. These observations indicated that alterations in the spliced insert within the TM3–4 loop did not affect receptor biosynthesis or surface expression in the recombinant system.

Radioligand Binding—To assess whether TM3–4 mutations had an effect on equilibrium antagonist binding, displacement of \([\text{H}]\text{strychnine}\) by cold strychnine was examined (Fig. 2D). The tested constructs showed \(K_d\) values for strychnine ranging from 5.3 ± 0.4 nM (a3K) to 30.1 ± 2.2 nM (a3L\(^{\Delta 6}\)). It should be noted that although these constants varied ~6-fold, they did not show any trend, in contrast to current dose-response data. In fact, the two subunits that differed most in their current response characteristics (a3L\(^{\Delta 6}\) and a3L\(^{T358A/S370A}\)) had quite similar \(K_d\) values in the ligand displacement test (Table 1). Thus, radioligand studies supported the hypothesis that ligand binding properties of GlyR a3 constructs were not notably affected by the modifications of the TM3–4 loop under study (Fig. 2D and Table 1). Although equilibrium radioligand binding gives only limited information about ligand affinity of the resting receptor, these results nevertheless suggested that mutations in the TM3–4 loop had no significant effect on the ligand-binding pocket of a3 glycine receptors.

Whole Cell Current Recordings—In whole cell current recordings from HEK 293 cells transfected with GlyR a3 constructs (Fig. 3, A and B), a clear correlation between maximum current amplitudes and EC\(_{50}\) values was observed (Fig. 3D), where high sensitivity (low EC\(_{50}\)) was associated with large current amplitudes (Fig. 3C and Table 1). The responses could be classified into three groups (Fig. 3, A and B): (i) high affinity, high \(I_{\text{max}}\) (a3L\(^{\Delta 6}\)); (ii) intermediate affinity and \(I_{\text{max}}\) (a3L, a3K, a3L\(^{+6}\), a3L\(^{T358A/Y367F/S370A}\), a3L\(^{T358A}, a3L\(^{T358A/F370A}\), and a3LY367F/S370A); and (iii) low affinity and \(I_{\text{max}}\) (a3L\(^{T358A/Y367F}\), and a3L\(^{T358A/S370A}\)). The extremes for EC\(_{50}\) and \(I_{\text{max}}\) were 3.9 ± 0.5 \(\mu\)M (a3L\(^{\Delta 6}\)) and 157 ± 23 \(\mu\)M (a3L\(^{T358A/Y367F}\)) and 360 ± 30 pA (a3L\(^{T358A/S370A}\) and 3870 ± 400 pA (a3L\(^{\Delta 6}\)//), respectively. Thus, the EC\(_{50}\) and \(I_{\text{max}}\) values observed differed by factors of 40 and 11, respectively, compared with <2.3-fold variability of protein expression levels. Furthermore, those constructs that differed most in their electrophysiological properties showed similar protein expression in HEK 293 cells (Fig. 2B). Current responses of both the high activity mutant (a3L\(^{\Delta 6}\)), as well as the low activity mutants (a3L\(^{T358A}\), a3L\(^{T358A/Y367F}\), and a3L\(^{T358A/S370A}\)), differed markedly from the wild types (a3L and a3K).

Dose-response data (Fig. 3) were fit to a two-ligand model of receptor activation to estimate which of the constants describ-
Regulation of α3 Glycine Receptors by TM3–4 Loop

FIGURE 1. GlyR α-subunit sequences and constructs. A, alignment of various receptors in the TM3–4 loop region. The alternatively spliced region is indicated. Note the large sequence divergence in the region of the insert. Lower panel, Jpred (34) secondary structure predictions for α3L (middle row) and α3K (bottom row). Use of several other prediction routines from the EXPASY server gave similar results. The α-helical content of α3L according to the prediction is ~38% (35 of 92 residues), in good agreement with secondary structure analysis by CD spectroscopy. B, glycine receptor α3 variants used in the study; the one-letter codes for amino acids are used. The alternatively spliced 15-residue segment (positions 358–372) is indicated by **bold letters**, and flanking sequences are shown in gray. Deleted residues within the spliced segment are shown as **bold dashes**, and flanking sequences are aligned on either side of the insert. Mutated residues are highlighted.

whereas the value for the gating constant Φ = \( k_{cl}/k_{op} \) was found to vary between 0.007 and 5.38, i.e. over 750-fold (Table 1). The EC₅₀ values calculated using constants obtained from the two-
Regulation of α3 Glycine Receptors by TM3–4 Loop

The data are given as the means ± S.D. The entire assay was performed three times using duplicate determinations for each data point (6 measurements/subunit).

### TABLE 1

Dose-response properties of GlyR α3 mutants

<table>
<thead>
<tr>
<th>Subunit</th>
<th>EC_{50}</th>
<th>nH</th>
<th>I_{max}</th>
<th>Calculated EC_{50}</th>
<th>K_{D}</th>
<th>Φ</th>
<th>I_{max}</th>
<th>Number of cells</th>
<th>K_{D} ([^3]H Stry)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α3L</td>
<td>35 ± 4</td>
<td>1.8 ± 0.3</td>
<td>2410 ± 410</td>
<td>40</td>
<td>56 ± 14</td>
<td>0.23 ± 0.11</td>
<td>11118 ± 890</td>
<td>dpm_{nonspec} = 613 ± 45, α3K (dpm_{max} = 20711 ± 910, dpm_{nonspec} = 1524 ± 230), α3L (dpm_{max} = 18626 ± 674, dpm_{nonspec} = 1224 ± 68), and α3L_{f} (dpm_{max} = 22714 ± 1427, dpm_{nonspec} = 1546 ± 105)</td>
<td></td>
</tr>
<tr>
<td>α3K</td>
<td>24 ± 2</td>
<td>2.8 ± 0.4</td>
<td>2088 ± 240</td>
<td>30</td>
<td>43 ± 9</td>
<td>0.23 ± 0.09</td>
<td>130 ± 12</td>
<td>3.11</td>
<td>1.93 ± 1589</td>
</tr>
<tr>
<td>α3L_{f}</td>
<td>3.9 ± 0.5</td>
<td>1.8 ± 0.4</td>
<td>3870 ± 400</td>
<td>3.6</td>
<td>39 ± 16</td>
<td>0.007 ± 0.005</td>
<td>3938 ± 452</td>
<td>5</td>
<td>140 ± 12, 145 ± 6</td>
</tr>
<tr>
<td>α3L_{a}</td>
<td>26 ± 1</td>
<td>4.7 ± 0.7</td>
<td>1850 ± 150</td>
<td>23</td>
<td>19 ± 3</td>
<td>0.30 ± 0.06</td>
<td>2540 ± 148</td>
<td>6</td>
<td>30.1 ± 2.2</td>
</tr>
<tr>
<td>α3L_{a}</td>
<td>86 ± 10</td>
<td>1.3 ± 0.3</td>
<td>570 ± 50</td>
<td>91</td>
<td>33 ± 7</td>
<td>3.24 ± 0.34</td>
<td>1161 ± 132</td>
<td>6</td>
<td>5.3 ± 0.4</td>
</tr>
<tr>
<td>α3L_{a}</td>
<td>8.9 ± 0.4</td>
<td>1.6 ± 0.4</td>
<td>1790 ± 220</td>
<td>8.1</td>
<td>7 ± 2</td>
<td>0.67 ± 0.04</td>
<td>2896 ± 205</td>
<td>5</td>
<td>27.5 ± 4.5</td>
</tr>
<tr>
<td>α3L_{a}</td>
<td>28 ± 4</td>
<td>1.2 ± 0.2</td>
<td>1350 ± 220</td>
<td>17</td>
<td>16 ± 3</td>
<td>1.04 ± 0.50</td>
<td>2738 ± 142</td>
<td>5</td>
<td>27.8 ± 5.9</td>
</tr>
<tr>
<td>α3L_{a}</td>
<td>157 ± 23</td>
<td>1.1 ± 0.2</td>
<td>680 ± 120</td>
<td>148</td>
<td>51 ± 12</td>
<td>5.38 ± 1.93</td>
<td>1589 ± 145</td>
<td>6</td>
<td>20.2 ± 1.7</td>
</tr>
<tr>
<td>α3L_{a}</td>
<td>89 ± 7</td>
<td>1.8 ± 0.3</td>
<td>360 ± 30</td>
<td>93</td>
<td>53 ± 16</td>
<td>3.11 ± 0.30</td>
<td>1215 ± 72</td>
<td>5</td>
<td>27.8 ± 5.9</td>
</tr>
<tr>
<td>α3L_{a}</td>
<td>16 ± 2</td>
<td>1.6 ± 0.3</td>
<td>1370 ± 140</td>
<td>23</td>
<td>11 ± 2</td>
<td>1.40 ± 0.90</td>
<td>3340 ± 145</td>
<td>8</td>
<td>30.1 ± 2.2</td>
</tr>
<tr>
<td>α3L_{a}</td>
<td>51 ± 5</td>
<td>1.6 ± 0.3</td>
<td>2020 ± 110</td>
<td>54</td>
<td>52 ± 12</td>
<td>0.55 ± 0.10</td>
<td>2451 ± 275</td>
<td>5</td>
<td>12.2 ± 1.7</td>
</tr>
</tbody>
</table>

*Data from dose-response curves.
*Data from two-ligand model.
*Data for whole cell recording.
*Data from the radioligand binding assay.
The TM3–4 loop of α3L was synthesized chemically, yielding a stable polypeptide of correct mass (Fig. 4E). The CD spectrum (Fig. 4F) indicated a secondary structure similar to the native TM3–4 loop obtained from recombinant expression. Secondary structure analysis indicated ~41% of α-helix and 25% of β-sheet (Fig. 4G), in good agreement with the values found for the native protein. Thermal unfolding (Fig. 4F, inset) gave a melting temperature for the fold of 58 °C, with a predominant loss of helical structures. Data from the synthetic protein confirmed the presence of stable secondary structures in the TM3–4 loop of the α3L loop polypeptide.

DISCUSSION

Ligand-gated ion channels of the Cys loop receptor type share both pentameric architecture of the protein complex as well as transmembrane topology of subunits (2, 3, 23, 24). Amino acid sequence homology between mammalian GlyR α subunit orthologs ranges from 80 to 98%, whereas intersubunit homology between α and β sequences is on the order of 60% (2). The cytoplasmic TM3–4 loop is the region of highest sequence diversity between GlyR α subunits, as well as other members of the Cys loop receptor superfamily (25). This domain carries motifs for phosphorylation (26), ubiquitination (27), as well as putative Ca\(^{2+}\)-dependent intracellular factors (28), and a basic cluster thought to ensure correct transmembrane topology (21). Two natural splice variants of the human GlyR α3 subunit differ in desensitization kinetics (8); hydroxyl functions within this insert are relevant, but not exclusive, determinants of receptor kinetics (9). Here, the impact of the 15-residue insert on channel function was probed by removal of its hydroxyl groups and varying its length. Comparison of ion channel properties using patch-clamp recording techniques and secondary structure analysis of isolated TM3–4 loops identified a novel regulatory motif within this cytoplasmic domain of GlyR α3 subunits.

GlyR α3L Mutations Affect Ion Channel Function—Although cell surface protein expression levels were largely unaffected, whole cell current responses of recombinant α3 GlyRs revealed a striking sensitivity of ion channel function to alterations within the 15-residue insert in α3L. Maximum current amplitudes (I\(_{\text{max}}\)) and EC\(_{50}\) were found to differ 11- and 40-fold, respectively, despite almost identical protein expression. Based on current responses, GlyR α3 mutants could be classified into three groups: (i) one high sensitivity mutant (α3L\(^{256}\)), characterized by large I\(_{\text{max}}\) and small EC\(_{50}\); (ii) constructs of intermediate I\(_{\text{max}}\) and EC\(_{50}\) values (α3L, α3K, α3L\(^{256}\), α3L\(^{259}\)/Y367F/S370A, α3L\(^{259}\)/Y367F, α3L\(^{259}\)/Y367F/S370A, and α3L\(^{259}\)/Y367F/S370A); and (iii) low sensitivity mutants (α3L\(^{259}\), α3L\(^{259}\)/Y367F, and α3L\(^{259}\)/Y367F/S370A), displaying low I\(_{\text{max}}\) and large EC\(_{50}\) values. Subunit expression was not different between the various α3 constructs, as determined by dot-blot assay as well as surface protein expression by biotinylation. Thus, because receptor density was excluded as a cause for the variations in I\(_{\text{max}}\), differences in whole cell current responses could be attributed to alterations in (i) ligand affinity,
Regulation of α3 Glycine Receptors by TM3–4 Loop

(ii) single channel conductance, or (iii) ion channel gating. The value of EC_{50} is composed of two equilibria, namely ligand binding and gating (13), whereas I_{max} depends on the number of receptors on the cell surface, as well as channel conductance and gating. We tested, therefore, to which extent other steps of receptor desensitization (9, 15, 32) and ion permeation (33). Similar effects have been observed with γ-aminobutyric acid, type A receptor mutants (34), also belonging to the Cys loop family of amino acid gated anion channels.

Ligand binding appeared least sensitive to alterations within the large intracellular loop, yet desensitization and channel gating were both affected by changes within the TM3–4 loop. Removal of all OH groups of the spliced insert resulted in altered receptor desensitization (9), whereas the results described here identified changes in channel gating caused by structure modifications within the insert.

Mutations in the GlyR α3L Splice Insert Affect Protein Surface Polarity—How can the gating differences observed for GlyR α3 mutants be attributed to changes in protein structure? An alignment of the amino acid sequences around the alternatively spliced region resulted in a reduced duration of channel openings. Alterations in channel gating were also demonstrated for recombinant glycine receptors from zebrafish, where a soluble Ca^{2+}-binding factor was proposed to mediate these effects (28). The marked difference in open channel dwell times between cell-attached and outside-out recordings would be consistent with an intracellular mode of ion channel regulation. In the glycine receptor α1 subunit, mutations within the TM1–2 loop resulted in altered receptor desensitization (9, 15, 32) and ion permeation (33). Similar effects have been observed with γ-aminobutyric acid, type A receptor mutants (34), also belonging to the Cys loop family of amino acid gated anion channels.

The concomitant changes of EC_{50} and I_{max} were consistent with modification of ion channel gating (14) as the main effect of the mutations. Indeed, when dose-response data were analyzed using a two-ligand model of receptor activation (29), the I_{max} and K_D values were found to differ 3.4- and 8-fold, respectively. In contrast, the value for the gating constant Φ varied ~770-fold (Table 1), consistent with variations channel gating.

The hyperekplexia mutants GlyR α1(K276E) (30) and GlyR α1-(Q266H) (31), located within the TM2–3 loop and TM2, respectively, impaired channel gating, leading to a reduced duration of channel openings. Alterations in channel gating were also demonstrated for recombinant glycine receptors from zebrafish, where a soluble Ca^{2+}-binding factor was proposed to mediate these effects (28). The marked difference in open channel dwell times between cell-attached and outside-out recordings would be consistent with an intracellular mode of ion channel regulation. In the glycine receptor α1 subunit, mutations within the TM1–2 loop resulted in altered receptor desensitization (9, 15, 32) and ion permeation (33). Similar effects have been observed with γ-aminobutyric acid, type A receptor mutants (34), also belonging to the Cys loop family of amino acid gated anion channels.

Ligand binding appeared least sensitive to alterations within the large intracellular loop, yet desensitization and channel gating were both affected by changes within the TM3–4 loop. Removal of all OH groups of the spliced insert resulted in altered receptor desensitization (9), whereas the results described here identified changes in channel gating caused by structure modifications within the insert.
hydrophobic residues over the entire region is similar for all constructs, but the net charge of each segment is different between mutants. The high efficacy mutation, α3LΔ6, is characterized by a (+)-(−)-(−) sequence of charged blocks, whereas intermediate and low efficacy mutants display the sequence (+)-(−)-(−)−. This sequence of net charges is also retained in the short splice variant, α3K (Fig. 5). Furthermore, Thr358 is absent in the low efficacy mutants with the exception of the triple mutant α3L T358A/Y367F/S370A. Taken together, an extended stretch of positive charges after the alternative splice site and the presence of a hydroxyl group were associated with efficient channel gating, whereas insertion of a short segment appears 2-fold: (i) the insert also stabilizes the fold of the TM3–4 loop of α3L glycine receptors, affected single channel conductances, but the major contribution to conductance still was mediated through the channel-lining TM2 domain (43). Overall, it appears that both charges and structural alterations within the cytoplasmic TM3–4 domain are indeed contributing but not exclusive determinants of ion channel function.

Secondary structure analysis and structure prediction using bioinformatics tools consistently indicated that the spliced insert in α3 GlyRs determines the folding of large parts of the intracellular TM3–4 domain. Alterations of critical positions within the insert as well as insert size confirmed a correlation between the structure of the insert and the function of the corresponding ion channel.

Thus, the role of the spliced insert in human α3 GlyRs appeared 2-fold: (i) the insert also stabilizes the fold of the TM3–4 domain, and (ii) the insert is a determinant of ion channel desensitization (8) and gating. Further studies are needed to identify the molecular partners and mechanisms of ion channel regulation. Yet, GlyR function is remote-controlled by intracellular protein domains that, although physically distant from the receptor ion pore, appear to contribute to ion permeation and channel gating.

Acknowledgments—Helpful discussions with Drs. H. Sticht, P. Broges-towski, K. Becker, and C. Kluck and cell culture maintenance by Rosa Weber are gratefully acknowledged. We thank Dr. E. Hannappel for help with amino acid analysis of the synthesized proteins, Dr. Finn Bauer for help with CD spectroscopy, and P. Wenzeler for performing protein synthesis.
Regulation of α3 Glycine Receptors by TM3–4 Loop