Molecular Requirements for Ethanol Differential AllostERIC Modulation of Glycine Receptors Based on Selective Gβγ Modulation*

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It is now believed that the allosteric modulation produced by ethanol in glycine receptors (GlyRs) depends on alcohol binding to discrete sites within the protein structure. Thus, the differential ethanol sensitivity of diverse GlyR isoforms and mutants was explained by the presence of specific residues in putative alcohol pockets. Here, we demonstrate that ethanol sensitivity in two ligand-gated ion receptor members, the GlyR adult α1 and embryonic α2 subunits, can be modified through selective mutations that rescued or impaired Gβγ modulation. Even though both isoforms were able to physically interact with Gβγ, only the α1 GlyR was functionally modulated by Gβγ and pharmacological ethanol concentrations. Remarkably, the simultaneous switching of two transmembrane and a single extracellular residue in α2 GlyRs was enough to generate GlyRs modulated by Gβγ and low ethanol concentrations. Interestingly, although we found that these TM residues were different to those in the alcohol binding site, the extracellular residue was recently implicated in conformational changes important to generate a pre-open-activated state that precedes ion channel gating. Thus, these results support the idea that the differential ethanol sensitivity of these two GlyR isoforms rests on conformational changes in transmembrane and extracellular residues within the ion channel structure rather than in differences in alcohol binding pockets. Our results describe the molecular basis for the differential ethanol sensitivity of two ligand-gated ion receptor members based on selective Gβγ modulation and provide a new mechanistic framework for allosteric modulations of abuse drugs.

Glycine receptors (GlyRs)4 are members of the ligand-gated ion receptor (LGIC) superfamily, which includes the Cys-loop family composed of the inhibitory γ-aminobutyric acid receptors and GlyRs and the excitatory nicotinic acetylcholine (nAChR) and 5-hydroxytryptamine receptors. These ionotropic receptors mediate fast synaptic transmission in the central nervous system (1, 2). Specifically, inhibitory GlyRs are critical for the control of excitability in the mammalian spinal cord and brain stem, regulating important physiological functions such as pain transmission, respiratory rhythms, motor coordination, and neuronal development (3–7).

Like all Cys-loop receptors, GlyRs are heteropentameric complexes composed of α and β subunits, which can assemble to form homomeric (5α) or heteromeric (2α3β) channels. To date, molecular cloning studies have demonstrated four isoforms of the α GlyRs (α1–4) and one β isof orm. Homomeric and heteromeric receptors share most of the GlyR general features, including a high percentage of identity between α GlyRs (∼75%). Nevertheless, biochemical, immunocytochemical, and in situ hybridization studies have shown that the expression of the subunits is developmentally and regionally regulated (3, 4, 8). For example, the α2 subunit expression increases after birth, whereas expression of the α3 subunit appears mainly restricted to early developmental stages (3, 4, 8, 9). On the other hand, several studies have shown that α GlyR isoforms differ in physiological properties, such as conductance, apparent agonist affinity, desensitization, and channel kinetics (3, 4, 10, 11). For instance, single-channel studies showed that the opening probability of α2 GlyRs was very low after a fast application of glycine, suggesting that they cannot be activated by fast neurotransmitter release at synapses (11). Similarly, other electrophysiological studies have reported that α GlyR isoforms possess different sensitivities to allosteric regulators, such as neurosteroids, zinc ions, and ethanol (12–14). These studies, in agreement with others in cultured spinal neurons and hypoglossal motoneuron slices (15–16), showed that receptors comprising α1 are more sensitive to ethanol than those containing α2 subunits (12). Interestingly, this differential ethanol sensitivity was associated to alanine 52 in α1 GlyRs, as its replacement by its threonine or serine counterpart (threonine or serine) generated GlyRs with a lower ethanol sensitivity (12, 17). Based on these results and other studies with cysteine-modifying reagents (18), a pocket site for ethanol was suggested to exist near the extracellular loop 2 and Ala-52 residue in α2 GlyRs.
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Despite the existence of studies that investigated several aspects of GlyR subunit functions, our knowledge on intracellular signaling that might regulate these isoforms is limited. In this context, recent evidence reveals that the α1 GlyRs are modulated by G proteins through the Gβγ heterodimer (19). Noteworthy, it has recently been shown that the degree of GlyR-Gβγ functional interaction is critical for ethanol-induced potentiation of the glycine-activated current (20). However, it is currently unknown if Gβγ can bind and allosterically modulate other GlyR isoforms and if this can impact on their differential ethanol sensitivity.

In the present study we identified extracellular and transmembrane residues that control the Gβγ and ethanol modulation of α1 and α2 GlyRs. Our results show that despite both being capable of binding Gβγ, only α1 GlyRs were positively modulated by Gβγ and pharmacological ethanol concentrations. Remarkably, simultaneous switching of two residues in transmembrane domains 2 and 3 (TM2 and TM3) plus an extracellular amino acid localized in loop 2 can reversibly control the Gβγ modulation, generating receptors with high and low ethanol sensitivity, respectively. These results provide novel information about the relevance of Gβγ modulation and on the molecular basis for the differential sensitivity of LGICs to ethanol.

EXPERIMENTAL PROCEDURES

cDNA Constructs—Mutations were inserted using the QuikChange™ site-directed mutagenesis kit (Stratagene) in cDNA constructs encoding the rat GlyRs in a pCI vector (Promega). For the construction of chimeric receptors, an XbaI site was added in a conserved region within the TM3 domain, allowing us to combine DNA regions by standard subcloning. The cDNA encoding GlyR intracellular loops were first subcloned in the GST fusion vector pGEX-5X3 (GE Healthcare). Then, GST fusion proteins were generated in Escherichia coli BL21 using 10 mM isopropyl-1-thio-β-d-galactopyranoside. After 6 h the cells were collected and sonicated in lysis buffer (1X phosphate buffer (pH 7.4)) and were then permeabilized (0.3% Triton X-100, and protease inhibitor mixture set II) at 4 °C for 1 h. Then the beads were washed 5 times, and bound proteins were separated on 12% SDS-polyacrylamide gels. Bound Gβγ proteins were assayed using a brief (1–6 s) pulse of glycine every 60 s. The modulation of the glycine current by ethanol (Sigma) was assayed using a pulse of glycine (EC10) co-applied with ethanol to each receptor studied, without any pre-application. In all the experiments, a brief pulse of 1 mM glycine was performed at the end of the recording period to test that the glycine concentration corresponded to the actual EC10 in each single experiment. Cells that displayed responses <EC10 or >EC15 were discarded. For the Gβγ-induced tonic modulation, human Gβ3, and Gγ2 expression plasmids were cotransfected with the respective GlyR. To identify successfully transfected cells and reduce the expression variability of the Gβ3, Gγ2, dimers, a pIRE2-EGFP-Gβ3 plasmid was used as a positive marker. Stercine (1 mM) blocked all the current elicited by wild type, chimeric, and mutant glycine receptors. The methodology for single channel recordings in outside-out configuration has been previously published (19–21). Briefly, patch pipettes were coated with R6101 elastomer (Dow-Corning) and had tip resistances of 7–15 megohms after fire polishing. Cells were voltage-clamped at −50 mV, and the data were filtered (1-kHz low-pass 8-pole Butterworth) and acquired at 5–20 kHz using pClamp software (Axon Instruments, Inc.). Agonist and alcohol solutions were applied to cells using a stepper motor-driven rapid solution exchanger (Fast-Step, Warner Instrument Corp.) Cells were maintained in extracellular medium containing 150 mM NaCl, 5 KCl, 2 mM CaCl2, 10 mM HEPES, 10 mM glucose (pH 7.4). The intracellular recording solution contained 140 mM CsCl, 2 mM Mg ATP, 10 mM BAPTA, and 10 mM HEPES (pH 7.2).

Construction of Glutathione S-Transferase Fusion Proteins and GST Pulldown Assays—DNA fragments encoding wild type α GlyR intracellular loops were first subcloned in the GST fusion vector pGEX-5X3 (GE Healthcare). Then, GST fusion proteins were generated in Escherichia coli BL21 using 10 mM isopropyl-1-thio-β-d-galactopyranoside. After 6 h the cells were collected and sonicated in lysis buffer (1X phosphate buffer, 1% Triton X-100, and protease inhibitor mixture set II (Calbiochem)). Subsequently, proteins were purified using a glutathione resin (Novagen), and normalized amounts of GST fusion proteins were incubated with purified bovine Gβγ protein (Calbiochem). Incubations were done in 800 μl of binding buffer (200 mM NaCl, 10 mM EDTA, 10 mM Tris (pH 7.4), 0.1% Triton X-100, and protease inhibitor mixture set II) at 4 °C for 1 h. Then the beads were washed 5 times, and bound proteins were separated on 12% SDS-polyacrylamide gels. Bound Gβγ was detected using a GST antibody (Santa Cruz Biotechnology) and a chemiluminescence kit (PerkinElmer Life Sciences). Finally, the relative amounts of Gβγ were quantified by densitometry.

Immunofluorescence, Image Visualization, and Analysis—HEK293 cells were first fixed with 4% paraformaldehyde (0.1 M phosphate buffer (pH 7.4)) and were then permeabilized (0.3% Triton X-100) and blocked (10% normal horse serum). Subsequently, all night incubation with a monoclonal FLAG (Stratagene) and polyclonal hexahistidine antibodies (HisTag, United States Biological) was carried out. Epitope visualization was performed by incubating the sample with two secondary antibodies conjugated to FITC and Cy3 (1:600;...
Jackson ImmunoResearch Laboratories). Finally, the cells were fitted with coverslips using Fluorescence Mounting Medium (Dako Cytomation). For quantitative analysis, cells were chosen randomly for imaging using a Nikon confocal microscope (TE2000, Nikon). Single stacks of optical sections in the z axis were acquired, and dual color immunofluorescent images were captured in simultaneous two-channel mode. Colocalization was studied by superimposing both color channels. The cross-correlation coefficient ($r$) between both fluorescence channels was measured using computer software (Metamorph, Universal Imaging Corp.) starting from separate immunoreactivity to GlyR-His and $G_{\beta_1}$-FLAG in the same cell (22). The theoretical maximum for $r$ was 1 for identical images, and a value close to 0 implied a complete different localization of the labels. Subsequently, the obtained data were compiled, analyzed, and plotted.

**Molecular Modeling**—The GlyR model was constructed by homology using coordinates from the Torpedo nAChR at 4 Å resolution (23, 24) (PDB code 2BG9) and acetylcholine-binding protein structure (PDB code 1UV6) (25) using the software Modeler (26, 27). The models were relaxed by energy minimization using a Conjugate Gradient protocol in the software GROMACS (28). To optimize the H-Bond net, the models were processed by the server REMO (29). Electrostatic surface potentials were calculated using APBS software (30). The individual charges were assigned using pdb2pqr software (31) with the AMBER force field (32). The final images were generated with Pymol (33).

**Data Analysis**—Statistical analyses were performed using ANOVA and are expressed as the mean ± S.E.; values of $p < 0.05$ were considered statistically significant. For all the statistical analysis and plots, the Origin 6.0 (MicroCal) software was used. Normalized values were obtained by dividing the current amplitude obtained with time of GTPγS dialysis by the current at minute 1.

## RESULTS

**Effects of G Protein Activation and Ethanol Sensitivity in Wild Type Glycine Receptor Subunits**—GlyR subunit expression during development is highly regulated (4, 8, 9). Indeed, the $\alpha_2$ GlyR is the main subunit during embryogenesis and early postnatal life, whereas $\alpha_4$ GlyRs are present at adult stages. The presence of $\alpha_2$ GlyRs in immature neurons and its absence in 2–3-week-old neurons has been consistently shown by different groups in both in vitro and in vivo preparations from rat and mouse, which has led to the study of functional properties of these GlyR subunits in their native configuration (4, 8). To investigate their sensitivity to G protein activation, we examined cultured spinal neurons at different developmental stages in vitro using intracellular applications of a non-hydrolyzable GTP analog (Fig. 1A). Previous reports using neuronal and recombinant $\alpha_1$ GlyRs showed that the amplitude of the glycine-activated current was strongly enhanced after 15 min of intracellular dialysis with GTPγS, implying that $G_{\beta\gamma}$ enhances GlyR activity (19). Interestingly, this modulation was only found in older neurons (63 ± 13%, $n = 6$, 13–14 DIV) (Fig. 1A), indicating that the $\alpha_1$ subunit is necessary for the G protein $\beta\gamma$ modulation. To test this further, we next studied G protein allosteric modulation using HEK 293 cells transfected with $\alpha_1$ and $\alpha_2$ GlyR isoforms. After 15 min of whole-cell recording in the presence of intracellular GTPγS, only the glycine-evoked current elicited by $\alpha_1$ GlyRs was strongly modulated (77 ± 13%, $n = 11$) (Fig. 1, C and E), suggesting that $\alpha_2$ GlyRs lack some critical molecular characteristics for the $G_{\beta\gamma}$ modulation despite their high sequence homology. To further characterize this modulation, we examined if GTPγS overexpression tonically modulated these two GlyRs, as described for Ca$^{++}$, GIRK (G protein-gated inwardly rectifying potassium) channels, and $\alpha_1$ GlyRs (19, 34, 35). Previous studies using human $\alpha_1$ GlyRs showed that the concentration-response relationship was shifted to the left after $G_{\beta\gamma}$ dimers were coexpressed, reflected by a significant reduction in its EC$_{50}$ with respect to control cells (19). Similar to these results, rat $\alpha_1$ GlyRs were tonically modulated by GTPγS overexpression, showing a decrease in their EC$_{50}$ from 41 ± 1 to 26 ± 2 μM (−34 ± 6% of tonic modulation) (Fig. 1B, supplemental Table 1). On the other hand, $\alpha_2$ GlyRs did not show tonic modulation (−4 ± 7%). We next studied the ethanol sensitivity of these subunits using equipotent concentrations of glycine (EC$_{50}$) for each receptor and found that $\alpha_1$ GlyRs were more sensitive to ethanol than $\alpha_2$ subunits especially at low millimolar concentrations (Fig. 1, D–F). For example, the application of 100 μM ethanol potentiated the $\alpha_1$ GlyR glycine-activated current in 54 ± 7% ($n = 8$), whereas the enhancement of the current in $\alpha_2$ was only 9 ± 3% ($n = 7$). Thus, all this evidence indicates that these GlyR $\alpha$ isoforms are differentially modulated by GTPγS and ethanol despite their high homology. Recent studies have reported that GTPγS modulation is critical for ethanol effects on $\alpha_1$ GlyRs (20). Therefore, it is possible to suggest that the allosteric action of ethanol on GlyRs is determined by differential interaction with GTPγS heterodimers.

**Functional and Direct Protein Interaction between Glycine Receptor Subunits and G Protein $\beta\gamma$ Dimers**—Because the discovery of the first effector protein for GTPγS, an ever-increasing number of effectors have been reported (36, 37), including two members of the Cys-loop superfamily, GlyRs and nAChRs (19, 38). In both cases G protein $\beta\gamma$ subunits modulate these receptors in a phosphorylation-independent manner, generating an enhancement in the agonist-evoked current linked to an increased open channel probability. Additionally, in vitro experiments have shown a direct interaction between GTPγS and the large intracellular loop of $\alpha_1$ GlyRs and $\alpha_{2–4}$ nAChRs. Two basic amino acid motifs in the large intracellular loop of the human $\alpha_1$ GlyR subunit are essential for GTPγS binding (316RFRRK and 385KK), and these regions have been postulated to form an electronegative area that shapes the GTPγS interaction surface in a pentameric GlyR configuration. Supporting a causative role for GTPγS binding in ethanol potentiation of GlyRs, it was previously found that mutations in these sequences and reduction in the availability of free GTPγS altered the GTPγS binding and significantly attenuated the ethanol actions on recombinant and native GlyRs (20, 39).

To analyze the presence of these motifs within other GlyR subunits, the sequences of $\alpha_1$ and $\alpha_2$ GlyR intracellular loops were examined (Fig. 2A). The data showed that similar to the rat and human $\alpha_1$ GlyR subunits (39), rat $\alpha_2$ also presents...
these basic motifs. The expected structural homology in these two subunits is supported by structural modeling which shows that the intracellular regions important for \(G/\beta/\gamma\) modulation are predicted to be \(\alpha\)-helices, similar to those in the transmembrane regions (Fig. 2B) and the MA stretch of nAChR (23, 24). Furthermore, the electropositive surfaces for these motifs were conserved in \(\alpha_1\) and \(\alpha_2\) GlyRs.

To determine whether the \(\alpha_2\) GlyR intracellular loop is able to bind \(G/\beta/\gamma\) proteins \textit{in vitro}, we constructed GST fusion proteins encoding the TM3–4 loops. GST fusion proteins were first expressed and purified, and then \textit{in vitro} binding assays were performed using purified \(G/\beta/\gamma\) (Fig. 2C). In agreement with previous reports with human \(\alpha_1\) GlyRs (39), rat \(\alpha_1\) GlyR TM3–4 loop was able to bind \(G/\beta/\gamma\) as compared with GST. The GST fusion protein containing the \(\alpha_2\) intracellular loop also binds \(G/\beta/\gamma\), demonstrating the exis-

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**FIGURE 1. Effects of G protein activation and ethanol sensitivity of \(\alpha_1\) and \(\alpha_2\) GlyR subunits.**

A, the bar graph shows that only 13–14 DIV spinal neurons, which contain primarily \(\alpha_1\) GlyR subunits, are sensitive to G protein activation with GTP-\(\gamma\)S. B, shown are current traces obtained in transfected HEK cells expressing wild type \(\alpha_1\) and \(\alpha_2\) GlyRs, recorded at 1 and 15 min of whole cell recording using intracellular GTP-\(\gamma\)S. C, the graph summarizes the time course of the normalized glycine-evoked current elicited by \(\alpha_1\) and \(\alpha_2\) GlyRs during the dialysis with the non-hydrolyzable GTP analog. D, glycine concentration-response curves for \(\alpha_1\) and \(\alpha_2\) GlyRs in the absence (filled symbols) or presence of overexpressed \(G/\beta_1\), \(\gamma_2\) (open symbols). E, shown are examples of current traces in the presence or absence of 100 mM ethanol from wild type \(\alpha_1\) and \(\alpha_2\) GlyRs. F, shown are concentration-response curves for ethanol (1–200 mM) in \(\alpha_1\) and \(\alpha_2\) GlyRs using an equipotent glycine concentration (EC_{10}) for both receptors. Data are the means ± S.E. from 9–15 cells. Differences were significant \(p < 0.001\) (**), ANOVA.
tence of protein-protein interactions. To further confirm these data in a cellular context, we performed double immunofluorescent analysis in HEK 293 cells transfected with Gβγ and Gα2 subunits using hexahistidine and FLAG epitopes to identify the expressed GlyRs and Gα2 subunits, respectively. In agreement with the GST pulldown data, the cellular distribution of the GlyR isoforms and Gβγ dimers displayed a significant overlap in their expression patterns (Fig. 2D). The correlation analysis yielded high coefficient values, providing quantitative support for good colocalization between these GlyR isoforms and Gβγ. Although the spatial resolution of confocal microscopy is limited, the significant colocalization of the GlyR isoforms and Gβγ is consistent with a direct interaction in a cellular context.

**FIGURE 2.** Functional protein interaction between Gβγ and the α2 GlyR TM3–4 loop. A, shown is partial primary sequence alignment between the TM3–4 loops of α1 and α2 GlyR subunits. Note that the critical basic residues for Gβγ binding are conserved. B, shown are a ribbon diagram and electrostatic potential surface representations of a single GlyR α subunit modeled from the nAChR template. The right panel shows a detailed view of the motifs important for Gβγ modulation. Negative and positive charges are in red and blue, respectively. C, Gβγ binding to wild type GlyR subunits and total GST fusion protein amounts revealed using an antibody against GST. The arrow indicates Gβγ bound to a polyclonal anti-Gβγ antibody. The graph represents the relative amounts of bound Gβγ normalized with their corresponding loaded amount of GST fusion protein. The values were obtained from five different experiments. D, HEK 293 cells transfected with Gβγ-FLAG, Gγ2, and His-tagged α1 GlyRs were fixed and stained with antibodies against hexahistidine (green) and FLAG (red), which recognize tagged GlyRs and Gβγ, respectively. Images were merged to visualize colocalization. The graph summarizes the mean correlation coefficients (r) between GlyR subunits and Gβγ for each stained cell studied. E, shown is a schematic depiction of wild type and chimeric GlyRs used in this section. F, shown are current traces of chimeric α1α2 GlyRs-associated chloride currents in the presence of intracellular GTPγS or after the application of 100 mM ethanol. G, the bar graph summarizes the effects of non-hydrolyzable GTP analog dialysis (15 min) and 100 mM ethanol on the glycine-evoked current. Statistical analyses were significant (***, p < 0.001, ANOVA, versus α1 GlyRs).
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Altogether, these data demonstrate that the $\alpha_2$ GlyR intracellular loop is able to interact with G$\beta\gamma$. Thus, we next designed a chimeric approach to test the presence of functional G$\beta\gamma$ modulation in this sequence. These chimeric GlyRs between $\alpha_1$ and $\alpha_2$ subunits were generated combining the coding region downstream from the TM3–4 loop of one specific subunit with the region upstream of the TM3 end of another subunit, giving GlyRs with exchanged intracellular loops plus TM4 (Fig. 2E). The analysis of agonist concentration-response curves shows that the $\alpha_1$-$\alpha_2$ and $\alpha_2$-$\alpha_1$ exchanges did not significantly modify the receptor physiology (supplemental Table 1). Next, we used intracellular dialysis with GTP$\gamma$S to evaluate the G protein $\beta\gamma$ modulation of these constructs. We found that the exchange of the TM3–4 loop of the $\alpha_1$ subunit with the $\alpha_2$ counterpart did not affect the G$\beta\gamma$ allosteric modulation (Fig. 2, F–G). For example, the GTP$\gamma$S-mediated current enhancement in the $\alpha_1$-$\alpha_2$ GlyR was $85 \pm 17\%$ ($n = 7$), which was not significantly different from the wild type $\alpha_1$ GlyR. On the other hand, changing the TM3–4 loop of $\alpha_2$ subunits with the corresponding $\alpha_1$ region did not recover the G$\beta\gamma$ modulation despite the fact that the $\alpha_1$ GlyR intracellular loop possesses all the molecular elements required for a functional modulation by the G protein heterodimer. Subsequently, the effect of 100 mM ethanol was studied on these GlyRs using an equipotent concentration of glycine for each construct. The $\alpha_1$-$\alpha_2$ GlyR displayed a similar potentiation in comparison with the $\alpha_2$ GlyR (Fig. 2G), whereas the $\alpha_2$-$\alpha_1$ GlyRs remained insensitive to ethanol, in agreement with the results using GTP$\gamma$S.

Based on all these results, we conclude that changing the TM3–4 loop between the $\alpha_1$ and $\alpha_2$ receptor isoforms did not change the physiology, intracellular regulation, or ethanol pharmacology of the respective GlyRs. In addition, these results suggest that the absence of G$\beta\gamma$ functional modulation and low ethanol sensitivity displayed by $\alpha_2$ GlyRs is due to the lack of molecular features that allow specific conformational changes after G$\beta\gamma$ binding, which finally generates the allosteric modulation of the ion channel.

Two Transmembrane Residues Are Critical for the G$\beta\gamma$ Ethanol Allosteric Modulations of the GlyR $\alpha_2$ Subunit—It is well accepted that the transmembrane regions of the LGIC superfamily members are critical for correct ion channel function and regulation. In the Cys-loop pentameric conformation, each subunit contributes four transmembrane domains to form the ion channel, with TM2 domains shaping the central ion pore (2). Using mutagenesis and electrophysiology, several studies have determined the importance of TM domains for GlyR function (2–4). For example, residues Gly-254 and Ser-267 present in the TM2 domain of $\alpha_1$ GlyRs contribute to single channel conductance and ethanol potentiation, respectively (10, 40, 41). Due to the potential role on the allosteric effects of ethanol, it is possible that residues in TM domains besides intracellular amino acids could explain the differential alcohol sensitivity displayed by these GlyR isoforms. To analyze this hypothesis, we first performed an alignment of the $\alpha_2$ GlyR subunits upstream of the TM3–4 loop, focusing on the TM2–3 domains (Fig. 3A). These sequences displayed high homology profiles (>95%), with only two divergent residues at positions Gly-254 and Ser-296. Significantly, two critical residues involved in the ethanol and general anesthetic effects on GlyRs, Ser-267 and Ala-288 (40, 41), were fully conserved between $\alpha_1$ and $\alpha_2$ isoforms (Fig. 3A). Thus, these analyses suggest that these previously described residues cannot completely explain the differential ethanol sensitivity displayed by the GlyR isoforms, and we, therefore, focused our analyses toward the non-conserved TM amino acids. The primary sequences show that the $\alpha_1$ GlyR, sensitive to G$\beta\gamma$ and ethanol, has Gly-254 in the TM2 and Ser-296 in the TM3, whereas the $\alpha_2$ GlyR has two alanine residues in these positions (Fig. 3A). Despite these differences, our molecular modeling studies show that the $\alpha$-helix conformation proposed for the TM domains was well conserved, supporting the experimental data that showed functional ligand-gated ion channels.

To investigate the importance of these non-conserved residues in TM2 and TM3, mutant and chimeric $\alpha_1$ and $\alpha_2$ GlyRs were generated to swap these residues between the constructs (Fig. 3C). Mutations G254A and S296A in the $\alpha_1$ and $\alpha_1$-$\alpha_2$ GlyRs significantly attenuated the effect of intracellular GTP$\gamma$S (Fig. 3D). For instance, the GTP$\gamma$S-mediated current enhancement in the $\alpha_2$ GlyR, G254A/S296A GlyR was only $10 \pm 8\%$ ($n = 6$). Application of 100 mM ethanol to the double-mutated $\alpha_1$ GlyR also showed a significant decrease in the current potentiation ($18 \pm 2\%$ ($n = 7$)) (Fig. 3D). Interestingly, singly mutated $\alpha_1$ GlyRs demonstrated that Gly-254 and Ser-296 can abolish G protein and ethanol actions, indicating that they also participate in G$\beta\gamma$ and ethanol modulations. Therefore, we should be able to recover G$\beta\gamma$ and ethanol modulation through reverse mutations in the $\alpha_2$ GlyR, which we denominated A254G and A296S to conserve a nomenclature relative to $\alpha_1$ GlyRs. Our electrophysiological analysis revealed that the double-mutated $\alpha_2$ GlyR was not significantly modified, showing an unchanged apparent affinity for glycine (supplemental Table 1). Interestingly, the current elicited by the $\alpha_2$ A254G/A296S GlyR was still insensitive to activation of G proteins and 100 mM ethanol, displaying a $3 \pm 2\%$ ($n = 5$) and a $10 \pm 2\%$ ($n = 7$) of potentiation, respectively (Fig. 3D). This behavior was conserved even when the A254G and A296S mutations were incorporated in the $\alpha_1$-$\alpha_2$ GlyR, demonstrating that the presence of $\alpha_2$ GlyR TM and intracellular sequences was not enough to recover the G$\beta\gamma$ and ethanol modulation of $\alpha_2$ GlyRs. Therefore, we decided to explore regions upstream of the TM domains to determine the existence of other critical features that allow functional G protein regulation and high ethanol sensitivity.

Simultaneous Mutations in Transmembrane and Extracellular Residues within the $\alpha_2$ GlyR Subunit Generate Ligand-gated Ion Channels Modulated by G$\beta\gamma$ with High Ethanol Sensitivity—The proposed current structure of the LGIC superfamily members comprises an extracellular domain with several $\beta$-sheets containing the neurotransmitter binding sites and other regions that allow coupling of agonist binding to channel opening (2–4). Several electrophysiological and molecular modeling studies have postulated that loop 2 and loop 7 (the conserved “Cys-loop”) are critical for receptor activation because they transfer energy of ligand binding to the transmembrane regions responsible for opening the ion channel (2–4, 42–44). It has been recently shown that two residues, Glu-53 and Asp-57, in loop 2 are critical for the activation mechanism of the $\alpha_1$ GlyR.
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(44). Interestingly, a specific mutation (A52S) within the same region of the $\alpha_1$ GlyR has been previously linked to the spasmodyc mice phenotype (45) and ethanol sensitivity (12, 18). However, the same residue has also been directly implicated in glycine-evoked current after 15 min of dialysis with GTP/S-me-

$\alpha_1$, NMDAAPARLGLITTVLHMTQSSGRASLPKYSYVPA
$\alpha_2$, NMDAAPARLGLITTVLHMTQSSGRASLPKYSYVPA

Within loop 2 of the $\alpha_1$ GlyR was non-conserved due to the presence of a threonine residue within the $\alpha_2$ GlyR isoform (Fig. 4A). Molecular modeling studies show that the conformation suggested for these domains were similar between the $\alpha$ subunits, showing a close proximity between the $\beta$-turns of the extracellular regions and the extracellular region of the ion channel TM2–3 loop (2, 44) (Fig. 4B). However, the $\alpha_2$ GlyR loop 2 displays an extended $\beta$-strand structure that is also observed when the mutation A52T was introduced in the $\alpha_1$ GlyR. Thus, we investigated the importance of this position for the allosteric actions of G$\beta$y and ethanol on $\alpha_1$ and $\alpha_2$ GlyRs (Fig. 4C). As previously described (12, 17, 48), the A52T mutation significantly impaired the apparent affinity for glycine and ethanol sensitivity of the $\alpha_1$ subunit. Additionally, it also attenuated the G protein activation (Fig. 4, C–G). For instance, the GTP-S-mediated current enhancement in the $\alpha_1$ A52T GlyR was only 13 ± 2% ($n = 6$), whereas the ethanol potentiation induced by 100 mM was 16 ± 2% ($n = 6$). The results suggest that this single amino acid is a key element serving to explain the resistance of the $\alpha_2$ GlyRs to G$\beta$y and ethanol modulations. To explore this idea, we generated the reverse T52A mutation within the wild type $\alpha_2$ GlyR sequence, which we denominated as T52A in the $\alpha_2$ GlyR to conserve the nomenclature relative to $\alpha_1$ GlyRs. Contrary to the results obtained with the $\alpha_1$ A52T mutant, the analysis of the concentration-response curve of the reverse T52A in $\alpha_2$ showed a significant left-shift displacement in the apparent affinity for glycine, as previously described (48) (supplemental Table 1). Despite this change, this substitution did not restore the G protein modulation or the ethanol sensitivity (Fig. 4, E–G). However, this result is consistent with the absence of the critical TM elements (Gly-254 and Ser-296) for the G protein and alcohol regulation. Thus, these results strongly suggest that the full recovery of G$\beta$y and ethanol modulation in $\alpha_2$ GlyRs could be achieved through the simultaneous TM plus loop 2 reversal mutations. In agreement with this, the triple-mutated $\alpha_2$ T52A/A254G/A296S GlyR displays high G protein modulation, showing a 87 ± 7% ($n = 6$) enhancement in the glycine-activated current after intracellular dialysis with GTPyS (Fig. 4, E–G).

**FIGURE 3.** Two transmembrane residues are critical for functional $\alpha_1$ GlyR regulation by G$\beta$y and ethanol. A, shown is the primary sequence alignment between $\alpha_1$ and $\alpha_2$ GlyR subunits from the TM2 to TM3 region. The positions that correspond to Gly-254 and Ser-296 in wild type $\alpha_1$ GlyRs were the only non-conserved residues and are highlighted in orange. B, shown are molecular representations of single $\alpha_1$ (blue) and $\alpha_2$ GlyR (red) TM regions. The superposition of both structures demonstrates that the overall $\alpha$-helix structure is highly conserved. C, shown is a schematic representation of the chimeric and mutant GlyRs used to study the role of the non-conserved TM residues between $\alpha_1$ and $\alpha_2$ GlyRs. D, a bar graph summarizes the normalized glycine-evoked current after 15 min of dialysis with GTPyS and the sensitivity to 100 mM ethanol of wild type, chimeric, and mutant GlyRs studied. Note that TM mutations in $\alpha_1$ GlyRs abolished both G protein and ethanol effects, whereas reversal substitutions in $\alpha_2$ GlyRs did not display any significant change. Differences were significant (***, $p < 0.001$, ANOVA) between $\alpha_1$, GlyRs and all the TM mutants.
Noteworthy, these exchanges also generated a GlyR sensitive to pharmacological ethanol concentrations, displaying a 57 ± 7% (n = 6) of current potentiation with 100 mM ethanol (Fig. 4, D–G). This phenomenon was also reproduced when these three substitutions were included in the α2 α1 GlyR, demonstrating that the ethanol and Gβγ modulation of α2 GlyRs are controlled by contributions of TM2–3 and loop 2 that are unique in the α1 GlyR (Fig. 4G).

To confirm the high ethanol sensitivity at the single channel level, we performed outside-out recordings from membranes expressing wild type α1, α2, and the α2 T52A/A254G/A296S GlyRs. Application of 10 mM ethanol strongly modulated wild type α1 GlyRs, producing a significant enhancement of the open-channel probability (144 ± 19% above control, n = 5) without changes in the main conductance (92 ± 2 versus 93 ± 2 picosiemens in the presence of ethanol) (Fig. 5, A and B). On the other hand, α2 GlyRs were not significantly affected by ethanol (7 ± 2%, n = 5), in accordance with the results obtained by using the whole-cell configuration. Both ion channels displayed their previously reported features, with a higher main conductance (122 ± 4 picosiemens) and long openings for α2 GlyRs versus the presence of different levels of subconductance and long opening bursts for α1 GlyRs (Fig. 5, A and B, supplemental Table 2) (3, 10–11, 49). Interestingly, the α2 T52A/A254G/A296S GlyRs displayed a single channel profile similar to wild type α1 GlyRs, exhibiting similar open time distribution profiles with a main-channel conductance of 87 ± 2 picosiemens (Fig. 5, A and B, supplemental Table 2). Moreover, these GlyRs fully recovered the sensitivity to ethanol, displaying an important enhancement of the open-channel probability (153 ± 44%, n = 5) that was not significantly different from wild type α1 GlyRs. Further analysis indicated that both ethanol-sensitive receptors displayed a significant
increase in the mean open time during ethanol application, whereas the open time for α3 GlyRs remained unchanged (Fig. 5A). Throughout, it is interesting to note that the general activity profile of the α2 T52A/A254G/A296S GlyRs was not absolutely equivalent to the wild type α1 GlyRs, suggesting that only the G protein and ethanol sensitivity rather than the overall ion channel function was specifically influenced by these three mutations (Fig. 5A).

Altogether we identified key residues in extracellular and TM domains that fully explain the differential Gβγ and ethanol sensitivity of the α1 and α2 GlyRs. In addition, because extracellular, TM, and intracellular elements of the GlyR isoforms at the same time modulates the functional Gβγ modulation and ethanol sensitivity, it is possible to suggest the existence of a direct relationship between ethanol sensitivity and Gβγ modulation. In agreement with our previous evidence (20), we found a highly significant correlation between the sensitivity of the receptors to 100 mM ethanol and G protein activation ($r^2 = 0.9664, p < 0.0001$) plotting the wild type, chimeric, and mutated GlyRs (supplemental Fig. 1). Thus, these data provide additional evidence indicating that Gβγ signaling participates in the differential ethanol modulation of these GlyR isoforms.

**DISCUSSION**

The results shown here and others that we previously described (20) allow us to identify the molecular elements that explain the differential ethanol sensitivity of two receptors that belong to the Cys-loop superfamily based on the selective intracellular modulation through G protein βγ subunits. Interestingly, these requirements are found along the receptor, suggesting that the Gβγ and ethanol sensitivity lies on a series of subtle changes impacting the channel structure. The first of these elements consists of a direct interaction of the Gβγ dimer with the receptor through basic residues in the TM3–4 intracellular loop (20, 39). The data showed that α1 and α2 GlyRs bind Gβγ, but only the α1 GlyR conformation allowed an effective conversion of Gβγ binding into functional allosteric modulation. Two other resi-
Molecular Requirements for Modulation of Glycine Receptors

FIGURE 6. Molecular requirements for Gβγ and ethanol modulations of α1 and α2 GlyRs. In a resting state with glycine bound, G protein activation or pharmacological ethanol concentrations increase free Gβγ dimer availability, which subsequently interacts with α1 and α2 GlyRs through conserved basic residues within the TM3–4 loop. Intracellular Gβγ binding induces a conformational change in the TM domains, generating a GlyR with a Gβγ-activated conformation. The presence of the pivotal residues Gly-254 and Ser-296 in α1, GlyRs allow reaching this configuration. Previous to channel opening, the receptor should change its conformation toward a pre-opened or flipped state, which is believed to depend on residues that control the coupling of agonist binding to channel opening. The Ala-52 in α2 GlyRs has been previously shown to be critical for a facilitated transition from resting to flipped states, which is also a requirement for a functional Gβγ modulation. Thus, only the GlyRs with a Gβγ-activated TM configuration and suitable flipping rates can be modulated by Gβγ, resulting in receptors with high ethanol sensitivity.

The α1, GlyR TM domains were identified as key elements for a transmembrane configuration that will allow ion channel conformational changes after Gβγ binding. The data showed that the presence of Gly-254 in TM2 and Ser-296 in TM3 in addition to Gβγ binding was not enough to facilitate channel opening in α1 GlyRs, thus, directing our attention into sites that drive the coupling of agonist binding to channel gating described for the Cys-loop ion channels. In agreement with this idea, we determined that an extracellular residue present in the loop 2 of α1 GlyRs (Ala-52) is another critical feature for high sensitivity to Gβγ and ethanol. Interestingly, this particular residue has been postulated as a key factor for the GlyR function based on studies using the α1 GlyR A52S mutation present in the spasmodic mouse, which is the amino acid present in the wild type α2 GlyRs at that position (12, 17, 45). The functional characterization of this mutant showed low glycine apparent affinity, unchanged agonist binding, low ethanol sensitivity, and slow synaptic kinetics (45, 50). To explain these changes, recent single channel analysis postulated a mechanism in which the A52S mutation in the human α1 GlyR impairs the transition between a resting closed state and a pre-opened closed state (denominated “flipped” state) of the glycine-bound GlyR, without changes in the final transition from the flipped state to the opened state (i.e. channel “gating”) (47, 51). Particularly, Plested et al. (47) postulated that the most plausible effect of the A52S mutation on the receptor function was a 100-fold reduction on glycine affinity for the flipped conformation. Because Ala-52 is in a region thought to be involved in the transduction of agonist binding to channel gating (2, 43–44, 46, 47), its mutation appears to affect the conformational changes leading to channel opening. Furthermore, the sensitivity of a Cys-loop LGIC member can be recovered by specific mutations that are not related to a direct binding of alcohol within the ion channel structure. Also, the data suggest that transmembrane conformational changes within the ion channel structure after Gβγ binding and the isomerization rate to the pre-opened flipped state are core elements to explain the differential ethanol sensitivity of these two GlyR isoforms. It is important to note that our study postulates the pre-open flipped conformation as a requirement for the optimal intracellular regulation and ethanol sensitivity of the Cys-loop superfamily, which is complementary with the key role that this transition has to explain the partial agonism within the Cys-loop superfamily (51). Furthermore, these data confirm the critical role of Gβγ signaling as an important determinant for the ethanol sensitivity of the GlyRs, which also might be important to explain the diverse effects of ethanol on γ-aminobutyric acid receptors (52–54). Because several properties of the Cys-loop ion channels can be modified by the presence or absence of specific subunits in the pentameric structure, this study also raises the possibility that different subunit combinations within the Cys-loop family members could give receptors with differential Gβγ sensitivities based on specific transmembrane configurations and flipping rates, which will display highly variable ethanol sensitivities depending on signal transduction states. In summary, these data provide support for the hypothesis that a main determinant for some Cys-loop ion channels with different ethanol sensitivities arises from a selective Gβγ modulation. Thus, this mechanism provides a novel mechanism of action regarding the LGIC superfamily regulation by alcohol, which could help to understand the
complex nature of alcohol effects on the human nervous system.

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