Evidence for α -Helices in the Large Intracellular Domain Mediating Modulation of the $\alpha 1$ -Glycine Receptor by Ethanol and $G\beta\gamma$ [§]

Carlos F. Burgos, Patricio A. Castro,¹ Trinidad Mariqueo, Marta Bunster, Leonardo Guzmán, and Luis G. Aguayo

Laboratory of Neurophysiology, Department of Physiology (C.F.B., P.A.C., T.M., L.G.A.), Laboratory of Molecular Neurobiology, Department of Physiology (L.G.), Laboratory of Molecular Biophysics, Department of Biochemistry and Molecular Biology (M.B.), and Ph.D. program in Pharmacology (T.M.), University of Concepción, Concepción, Chile

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ABSTRACT

The $\alpha 1$ -subunit containing glycine receptors (GlyRs) is potentiated by ethanol, in part, by intracellular $G\beta\gamma$ actions. Previous studies have suggested that molecular requirements in the large intracellular domain are involved; however, the lack of structural data about this region has made it difficult to describe a detailed mechanism. Using circular dichroism and molecular modeling, we generated a full model of the $\alpha 1$ -GlyR, which includes the large intracellular domain and provides new information on structural requirements for allosteric modulation by ethanol and $G\beta\gamma$. The data strongly suggest the existence of an α -helical conformation in the regions near transmembrane (TM)-3 and TM4 of the large intracellular domain. The secondary structure in the N-terminal

region of the large intracellular domain near TM3 appeared critical for ethanol action, and this was tested using the homologous domain of the $\gamma 2$ -subunit of the GABA_A receptor predicted to have little helical conformation. This region of $\gamma 2$ was able to bind $G\beta\gamma$ and form a functional channel when combined with $\alpha 1$ -GlyR, but it was not sensitive to ethanol. Mutations in the N- and C-terminal regions introduced to replace corresponding amino acids of the $\alpha 1$ -GlyR sequence restored the ability to be modulated by ethanol and $G\beta\gamma$. Recovery of the sensitivity to ethanol was associated with the existence of a helical conformation similar to $\alpha 1$ -GlyR, thus being an essential secondary structural requirement for GlyR modulation by ethanol and G protein.

Introduction

Glycine receptors (GlyRs) are members of the ligand-gated ion channel receptor (LGIC) superfamily, which includes the Cys-loop family composed of the inhibitory GlyRs and its close relatives, the GABA_A receptors (GABA_AR), the excitatory nicotinic acetylcholine receptors, and serotonin type 3 receptors (Lester et al., 2004; Lynch, 2004). The $\alpha 1$ -subunit containing GlyRs are found mainly in the spinal cord and brainstem neurons, where they participate in motor control, coordination of spinal reflexes, respiratory rhythms, and neuronal development (Legendre, 2001; Lynch, 2004). GABA_AR, on the other hand, are found in synaptic and nonsynaptic areas in upper brain regions (Cherubini and Conti, 2001).

GlyRs are pentameric receptors composed of $\alpha 1$ –4- and β -subunits, which can assemble to form homomeric or heteromeric

channels. Each subunit is composed of four hydrophobic transmembrane domains (TM1–4), where TM2 contributes to the ion channel formation. The large extracellular aminoterminal domain provides the neurotransmitter binding site (Speranskiy et al., 2007; Pless and Lynch, 2009). Finally, the large intracellular loop domain (LIL) between TM3 and TM4 is important for more recently found regulation of channel activity (Deeb et al., 2007; Carland et al., 2009; O'Toole and Jenkins, 2011; Castro et al., 2012) and is the most variable region of these receptor subunits. Additionally, it presents consensus sites for phosphorylation, modulation by intracellular mechanisms, and interaction with cytosolic and cytoskeletal proteins (Moss and Smart, 2001; Sine and Engel, 2006).

Although ethanol is one of the most frequently used drugs of abuse, the mechanisms of action in the central nervous system are not fully elucidated; however, it is recognized that GlyRs are important molecular targets (Aguayo and Pancetti, 1994; Harris, 1999; Ye et al., 2001). A number of studies have been directed at elucidating the site and mechanism of ethanol action in the GlyRs. To date, the effects of ethanol have been determined to be dependent on sites in 1) TM regions with participation of residues A254, S267, and S288 in the $\alpha 1$ sequence (TM2–TM3) (Crawford et al., 2007); 2) extracellular

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C.F.B. and P.A.C. contributed equally to this work.

¹Current affiliation: Department of Physiology & Membrane Biology & Shriners Hospital for Children Northern California, University of California, Davis School of Medicine, Sacramento, California.

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ABBREVIATIONS: BAPTA, 2-[2-[2-[bis(carboxylatomethyl)amino]phenoxy]ethoxy]-N-(2-oxoethyl)anilino]acetate; CT, C-terminal; GABA_AR, GABA_A receptor; CD, circular dichroism; GluCl, glutamate-gated chloride channel; GlyR, glycine receptor; GST, glutathione-S-transferase; IR, intermediate or central region; LGIC, ligand-gated ion channel receptor; LIL, large intracellular loop domain; NT, N-terminal; TM, transmembrane domain; UniProt, Universal Protein Resource.

domains involving A52 (Perkins et al., 2008); and 3) the large intracellular loop domain of GlyRs (Harris et al., 2008; Yevenes et al., 2008). For the last of these, the α 1-subunit of the GlyR is modulated by $G\beta\gamma$ through two motifs rich in basic residues (³¹⁶RFKRR³²⁰ and ³⁸⁵KK³⁸⁶) in the LIL (Yevenes et al., 2006), and this protein-protein interaction appears to be an important regulator, even though it is indirect, for ethanol-induced potentiation (Yevenes et al., 2008). A recent study using a chimeric construct of α 1- α 2-GlyR showed that in addition to these motifs, three residues outside the LIL—A52, S25, and G296—are critical for ethanol modulation in the α 1-subunit. Specifically, these mutations caused the loss of ethanol potentiation and suggested that these residues are important for agonist induced conformational changes before channel opening (Yevenes et al., 2010).

Despite the importance of the ethanol effect in GlyRs, there are still limited structural data on the events leading to the potentiation of glycinergic currents by ethanol. Recently, some binding sites for ethanol in TMs have been studied using structures in crystallized channels, such as glutamate-gated chloride channel (GluCl) (Hibbs and Gouaux, 2011) or prokaryotic *Gloeobacter* ligand-gated ion channel (GLIC)/*Erwinia chrysanthemi* ligand-gated ion channel (ELIC) (Bocquet et al., 2009; Howard et al., 2011; Corringer et al., 2012; Murail et al., 2012; Olsen et al., 2014). However, these protein structures lack the LIL, hindering a more detailed analysis of the events that lead to potentiation of GlyR by low concentrations (<100 mM) of ethanol through activation of $G\beta\gamma$. Therefore, using secondary structure prediction, circular dichroism (CD), and chimeric receptors, we examined the relationship between the secondary structure of the LIL of α 1-GlyR subunits and its sensitivity to ethanol. The results support the existence of a helical conformation in the N-terminal region of the LIL in α 1-GlyR subunits that is associated with the ability of ethanol to potentiate GlyR through activation of $G\beta\gamma$. This α -helix corresponds to a novel, necessary structural requirement for modulation by ethanol. Additionally, these data provide structural information about the LIL and its association with mechanisms of channel modulation, which is relevant because lack of the LIL in prokaryotic LGICs hampers potentiation by low concentrations of ethanol (Howard et al., 2011; Sauguet et al., 2013).

Materials and Methods

Secondary Structure Predictions. All predictions were made using the coding sequences of α 1 [United Protein Resource (*UniProt*) P07727], α 2 (*UniProt*: P22771), α 3-GlyR (*UniProt*: P24524), and γ 2-GABA_AR (*UniProt*: P18508) subunits from *Rattus norvegicus*. Three programs that incorporate different parameters in their prediction methods were used: PSIPRED (Department of Computer Science, Bioinformatics Group, University College of London, London, UK) (Buchan et al., 2013), JUFO (Meiler Lab, Vanderbilt University, Nashville, TN) (Leman et al., 2013), and Jpred3 (Cole et al., 2008). After all secondary structure predictions were finished with each of the programs used, we combined them to create a consensus secondary structure where each structural element (helix, β -sheet, random) was confirmed by at least two of the prediction servers used.

Protein Expression of the LILs. DNA constructs encoding the complete LIL of the α 1-GlyR subunit (amino acids 309–392), three truncated intracellular loops corresponding to the N-terminal (NT; amino acids 309–325), an intermediate or central region (IR; amino acids 326–381), and C-terminal (CT; amino acids 356–392), and the full sequence of LIL of the γ 2-GABA_AR subunit (amino acids 315–411) were cloned into the pGEX-5X-3 vector (GE Healthcare Life Sciences, Pittsburgh, PA). Glutathione-S-transferase (GST) fusion proteins were

expressed in *Escherichia coli* BL21 cells and harvested by centrifugation, followed by resuspension in lysis buffer (1× phosphate buffer, 1% Triton X-100, and protease inhibitor mixture set II) (Calbiochem, San Diego, CA), treatment with lysozyme (0.1 mg/ml, 30 minutes, 0°C), and sonication on ice collecting the supernatant. Expressed α 1-GlyR and truncated proteins were purified by chromatography in a fast protein liquid chromatography system with a GSTrap FF column (GE Healthcare Life Sciences), and γ 2 protein was purified with a glutathione resin (Novagen; Merck Biosciences, Whitehouse Station, NJ), all according to the manufacturer's protocol, using 50 mM Tris-HCl and 10 mM reduced glutathione (pH 8.0) for elution. Finally, proteins were dialyzed with 10 mM sodium phosphate buffer, pH 7.0.

Circular Dichroism of GST-Fusion Proteins. Measurements of CD were performed on a Jasco-J815 spectropolarimeter (Jasco Products Company, Oklahoma City, OK) in a 0.1-cm path-length quartz cell. Direct CD spectra for the LIL of α 1-GlyR subunits, truncated proteins, and the LIL of γ 2-GABA_AR were recorded in the spectral range of 200–250 nm at 0.5-nm intervals. Ten individual scans at 20°C were taken and averaged. All spectra were baseline-corrected by subtracting buffer runs and GST contribution. Protein concentrations were determined by measuring the absorbance at 280 nm, with an extinction coefficient calculated from the amino acid sequences using ProtParam (<http://web.expasy.org/protparam/>). The content analyses of secondary structures were performed from the CD spectra using the K2D3 server (Louis-Jeune et al., 2012).

GST Pull-Down. GST fusion proteins containing the LIL of the α 1-GlyR subunit and the γ 2-subunit of the GABA_AR were expressed in the *E. coli* BL21 strain by induction with isopropyl β -D-thiogalactopyranoside as described previously (Yevenes et al., 2006). Briefly, the expressed proteins were purified using a glutathione resin (Novagen). Normalized amounts of GST fusion proteins attached to glutathione resins were incubated with $G\beta\gamma$ proteins (Calbiochem). After washing, the presence of $G\beta$ was detected by SDS-PAGE (12% acrylamide). For Western blot analysis, the proteins were transferred to a nitrocellulose membrane, followed by immunodetection with an anti- $G\beta$ antibody (1:200; Santa Cruz Biotechnology, Inc., Dallas, TX). Signal detection was measured using a chemiluminescence kit (PerkinElmer Life Sciences, Waltham, MA) and quantified by densitometry.

Molecular Modeling. Models of the α 1-GlyR, γ 2-GABA_AR, and chimera α 1-LIL γ 2-subunits were constructed by homology using coordinates from the *Caenorhabditis elegans* GluCl at 3.35Å resolution (Hibbs and Gouaux, 2011) (PDB ID 3RIF) using the software Modeller 9v10 (Eswar et al., 2006). Because of the lack of sequence identity with a protein having a defined structure, the large intracellular domains of α 1- and γ 2-subunits were predicted by ab initio technique using QUARK (Denver, CO) (Xu and Zhang, 2012). In the next step, large intracellular domains were combined with their respective subunit models using Modeller to generate complete models of α 1 and γ 2. Finally, the models were relaxed by energy minimization using a conjugate gradient protocol in the software MacroModel (version 9.9, 2012, Schrödinger, LLC, New York, NY). All images presented were created with PyMOL (Schrödinger, LLC).

Construction of Chimera α 1-LIL- γ 2. The cDNA constructs encoding the rat glycine receptor α 1-subunit subcloned in a pCI vector (Promega Corporation, Madison, WI) have been described previously (Yevenes et al., 2010). For the construction of chimera α 1-LIL- γ 2, an XbaI site was added in the TM3 domain of the α 1-GlyR to replace the large intracellular domain up to the C terminus with the homologous region of the γ 2-GABA_AR subunit. Mutations were inserted in the TM4 and C-terminal to correct the sequence and convert it into an α 1-GlyR using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All the constructions were confirmed by full sequencing. The glycine and GABA_AR amino acids were numbered according to their position in the mature protein sequence.

Cell Culture and Transfection. HEK293 cells were cultured using standard methods. HEK293 cells were transfected using Xfect (Clontech Laboratories, Inc., Mountain View, CA) with 1 μ g of DNA for each plasmid studied per well (35 mm). Expression of green fluorescent

protein was used as a marker of positively transfected cells with a cotransfection ratio 2:1 of plasmids. Recordings were made after 18–24 hours.

Electrophysiology. Whole-cell recordings were performed as previously described (Castro et al., 2012). A holding potential of -60 mV was used. Patch electrodes were filled with (in mM): 140 CsCl, 10 BAPTA (2-[2-[2-[2-bis(carboxylatomethyl)amino]phenoxy]ethoxy]-N-(2-oxoethyl)anilino]acetate), 10 HEPES, pH 7.4, 4 MgCl₂, 2 ATP, and 0.5 GTP. The external solution contained (in mM): 150 NaCl, 5.4 KCl, 2.0 CaCl₂, 1.0 MgCl₂, 10 HEPES (pH 7.4), and 10 glucose. The resulting glycine activated current was measured at a range of concentrations (10–3000 μ M). With this, and using a logistic model, we built a concentration-response curve to obtain the values of EC₅₀ and Hill coefficient. The amplitude of the maximal current was not analyzed because of the variability in the number of receptors expressed in each cell after transient transfection. For G protein activation experiments, GTP in the internal solution was replaced by GTP γ S (0.5 mM; Sigma-Aldrich, St. Louis, MO). Normalized values were obtained by dividing the current amplitude obtained with a time of 15 minutes of GTP γ S dialysis by the current at minute 1. The sensitivity of recombinant and chimeric receptors to ethanol were assayed using a pulse of glycine (EC₂₀) coapplied with 100 mM ethanol (Sigma-Aldrich) to each receptor studied, without any preapplication. Although ethanol potentiates GlyRs starting at a concentration of 10 mM (Aguayo and Pancetti, 1994; Aguayo et al., 1996), in this study, we used 100 mM to compare the results with other previous studies and to make the statistical analysis more robust. 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 EC₂₀ in each single experiment. Cells having responses $<EC_5$ or $>EC_{20}$ were discarded. Strychnine (1 μ M) blocked all the currents elicited by wild-type and chimeric glycine receptors.

Data Analysis. Statistical analyses were performed using analysis of variance and are expressed as the arithmetic mean $\pm$ S.E.M.; values of $P < 0.05$ were considered statistically significant. For all the statistical analysis and plots, the Origin 9.0 (MicroCal, Northampton, MA) software was used.

Results

Analysis of the Secondary Structure of the α 1-GlyR with Molecular Modeling and CD Spectroscopy. In the absence of available structures for the GlyR LIL or other members of LGICs, we performed secondary structure predictions from the sequence of α 1-subunits, focused primarily on the LIL (Supplemental Fig. 1A). Analysis of the LIL of α 1-, α 2-, and α 3-subunits showed two regions with a high probability to form a α -helix structure near the TM3 (N terminus) and TM4 (C terminus). The N-terminal helix includes the cluster of basic amino acids ³¹⁶RFKRR³²⁰ described previously as essential for modulation by ethanol and G $\beta\gamma$ (Yevenes et al., 2008). On the other hand, the amino acids ³⁸⁵KK³⁸⁶ were found to be adjacent, but not encompassed, in the helical structure predicted in the C-terminal portion of the LIL.

With the aim of constructing a full model of the α 1-GlyR subunit containing the LIL, we predicted the LIL by ab initio using Quark (Xu and Zhang, 2012) and combined it with the structure of the GluCl (Hibbs and Gouaux, 2011). The model of α 1 confirmed the secondary structure predictions, with the presence of an α -helix structure in the LIL of α 1 (Fig. 1A). It is interesting that the orientation of the LIL appears flexible and slight variations are expected, especially in the angle between the LIL and the TM3–TM4 domains. It is possible that this flexibility is due to the presence of random regions connecting the α -helices predicted near the TMs, and this

conformation might allow the formation of a channel pore and to facilitate its interactions with regulatory cytoplasmic proteins (Fig. 1B).

To confirm the presence of the helical conformation predicted by in silico analysis of the α 1-GlyR, we examined the secondary structure of the LIL using CD spectroscopy. For this, four GST fusion proteins were constructed consisting of the full LIL of α 1 (GlyR-LIL), the N terminus of the LIL, including residues 309–325 (GlyR-NT), an intermediate or central region between residues 326–381 (GlyR-IR), and the C-terminal region from residue 356–392 (GlyR-CT) (Fig. 2A). All proteins were analyzed by SDS-PAGE to confirm correct expression and purification steps (Supplemental Fig. 2). The CD spectra (Fig. 2B) for α 1 confirmed that its secondary structure presents helical conformations (27%), together with β -sheet (22%), and the remaining exhibiting a random structure, in agreement with the expected flexibility of the LIL (Fig. 2C). Constructs that included only regions near the TM domains 3 and 4 showed an even higher content of helical structure. For example, GlyR-NT exhibited 34% and GlyR-CT 31% of α -helix, supporting the conclusion that this secondary structure is present at the boundaries of the LIL. The central segment of the LIL displayed the greatest proportion of random structure (GlyR-IR, 54%) probably linked to the flexibility required for its interaction with other cytoplasmic proteins. Finally, the percentage of secondary structure calculated from circular dichroism spectra were used to validate the model of the α 1 GlyR, and we found a good correlation between our model and CD analysis (32% α -helix in the LIL of the α 1 model located in the N- and C-terminal regions).

Protein Interaction between the LIL of α 1-GlyR and γ 2-GABA_AR Subunits with G $\beta\gamma$. The secondary structure prediction analysis showed that, similar to α 1, the α 2-subunit

Fig. 1. Full model of the α 1-GlyR subunit. (A) Representation of the α 1 monomer including the large intracellular domain determined by ab initio technique. The other regions were modeled using the template from the GLUC crystallographic data (PDB ID 3RIF). Two helical structures are present in the LIL, and basic amino acids described as important for modulation of GlyR by ethanol and G $\beta\gamma$ are indicated for reference (black arrows). The relative position of the membrane is shown by the dotted line. The rainbow colors represent the N- (blue) to C- (red) terminal progression of the protein. (B) Pentameric assembly of the GlyR composed of five identical α 1-subunits. (Left) Side vision of the full channel highlighting the basic amino acid clusters 316–320 (magenta) located in the α -helix near TM3, and 385–386 (red) in the proximity to TM4 after the second helix predicted in the LIL C-terminal. (Right) Top view with subunits distinguished by different colors showing monomer distribution and pore formation; bottom view of the GlyR presenting the orientation of both helices and localization of the basic amino acid clusters (magenta and red). The segment between residues 334 and 370 corresponding to random conformation was removed.

Fig. 2. Secondary structure of the LIL of the α 1-subunit determined with circular dichroism. (A) Schematic representation of the LIL of α 1-GlyR showing the full sequence and the three truncated proteins used. (B) CD spectra data from the LIL of α 1 (green) and truncated proteins GlyR-NT (magenta), GlyR-IR (red), and GlyR-CT (blue) and the LIL of γ 2 (gray) expressed as GST fusion proteins. (C) Secondary structure calculated from CD spectra of full α 1-LIL, truncated proteins and γ 2-LIL shown as a percentage of helix, β -sheet, and random conformation.

of the GlyR presented a high level of helix content (Supplemental Fig. 1, B and C) that was previously found to be important for the modulation by ethanol and $G\beta\gamma$ dimers. For instance, a previous study showed the ability of the LIL in α 1- and α 2-GlyR to biochemically interact with $G\beta\gamma$ (Yevenes et al., 2010), and this is supported by a high similarity in their sequence, presenting basic clusters at amino acids 316–320 and 385–386 (Fig. 3A). The chimera between α 1 and the LIL of α 2 interacted with $G\beta\gamma$ and was significantly potentiated by ethanol (Yevenes et al., 2010), which could be attributed to the similar structure in the LILs. To test whether a new chimera with a prediction of low or no secondary structure content in the corresponding LIL region was still sensitive to ethanol and $G\beta\gamma$ modulation, we constructed a α 1- γ 2-GABA_AR chimera. We chose γ 2 because, in addition to the low full-sequence similarity (35%) with α 1-GlyR, it had a similar LIL size (86/87 amino acids in α 1/ γ 2). Furthermore, the LIL of γ 2 showed a mainly random structure calculated from circular dichroism data (80%) with a reduced percentage of helical conformation (6%) (Fig. 2, B and C), also in agreement with our initial prediction (Supplemental Fig. 1D). Moreover, the LIL of γ 2 has a basic amino acid cluster near TM3 (residues 328–333) with a lysine at position 401 corresponding to lysine 386 in the α 1-GlyR that was described as essential for ethanol potentiation (Fig. 3A). Interestingly, the γ 2-subunit was described as a potential target for ethanol effects in the central nervous system with a synaptic distribution (Mihic, 1999; Loh and Ball, 2000; Olsen and Sieghart, 2009).

Using a GST pull-down assay, we found that the LIL of γ 2 was able to interact with $G\beta\gamma$ to a lower degree than the α 1-GlyR ($4\% \pm 10\%$ versus $90\% \pm 13\%$) (Fig. 3, B and C). This indicates that the LIL of γ 2-GABA_AR binds to $G\beta\gamma$ but does

not necessarily mean that it is directly associated to the modulation of the receptor. Therefore, functional experiments using a chimeric receptor were required to elucidate this aspect.

Modulation of Chimeric Receptor α 1-LIL- γ 2 by G Protein and Ethanol. We constructed a chimeric α 1-LIL- γ 2 receptor where the LIL of α 1-GlyR was replaced by the corresponding LIL of γ 2-GABA_AR (Fig. 4A). We then examined whether this chimera was modulated by ethanol and $G\beta\gamma$ to confirm the importance of the helical conformation in the LIL for allosteric modulation. Remarkably, this chimera produced a highly normal homomeric receptor, with an EC_{50} of $56 \pm 4 \mu M$ and a Hill coefficient (n_H) of 1.5 ± 0.1 (compared with an EC_{50} of 40 ± 1 and an n_H of 2.4 ± 0.1 for α 1 GlyR), indicating that the receptor was correctly expressed and localized in the cell membrane (Fig. 4B). Experiments using intracellular GTP γ S to activate G proteins showed that the chimera was insensitive to modulation by $G\beta\gamma$, and no significant change in the amplitude of the glycine evoked current was detected after dialysis of GTP γ S for 15 minutes (Fig. 4, C and D). Furthermore, ethanol had no significant effect on the activity of the chimeric receptor tested at a concentration of 100 mM ($15\% \pm 4\%$) (Fig. 4, E and F).

These results suggest that the lack of modulation of the chimera might be due to structural differences between α 1-GlyR and α 1-LIL- γ 2. Therefore, we constructed a predictive model that might account for these differences. Examination of the resulting model revealed important differences in the secondary structures of the LILs. In particular, the helical conformation present in the LIL of α 1 was absent in the LIL of γ 2 (Supplemental Fig. 3), and this is in agreement with the results previously described in secondary structure predictions of both subunits based on sequence analysis (Supplemental Fig. 1). Thus, this

Fig. 3. GST pull-down of the LIL of α 1-GlyR, γ 2-GABA_AR and G β γ . (A) Sequence alignments of the LILs of α -GlyRs and the γ 2-GABA_AR subunit. Bold letters show conserved residues and gray letters represent non-conserved amino acids. Basic residues near TM3 and TM4 are shown in red squares. (B) Western blot shows the interaction of α 1-LIL GlyR and γ 2-LIL GABA_AR with G β γ dimer by GST pull-down assays. Arrows indicate G β detected using a polyclonal anti-G β antibody and GST revealed by an anti-GST antibody, respectively. (C) Quantification of G β binding to the two LIL domains showing the relative amounts of bound G β normalized with their respective loaded GST fusion protein. * $P < 0.05$; ** $P < 0.01$. Data are mean $\pm$ S.E.M., $n = 4$.

difference in secondary structure could be implicated in the insensitivity of the chimera to GTP γ S and ethanol. To test this possibility, we mutated these regions to match the sequence found in α 1-GlyR previously reported to be important for allosteric modulation by ethanol.

Recovery of G Protein and Ethanol Modulation in the Chimera α 1-LIL- γ 2. To learn about the reason for the insensitivity of the chimeric receptor α 1-LIL- γ 2, we performed a sequence analysis of γ 2 and found a significant difference in the region near the TM3 domain (Fig. 5A). In particular, the sequence of γ 2 shows a proline at position 326 in the LIL, a residue that can prevent the formation of helical structures as described in the α 1-GlyR subunit. Also, the C terminus of γ 2-LIL lacked the equivalent lysine 385, recognized as important for modulation by ethanol, and presents an alanine 400 in this position (Castro et al., 2012). Therefore, we mutated the segment near the TM3 (³⁰⁹NRKPS³¹³) in the chimera α 1-LIL- γ 2 to an α 1-GlyR sequence (³⁰⁹RQHKE³¹³), together with the mutation A385K (Fig. 5A). The electrophysiologic properties of this mutated chimera denoted as α 1-LIL- γ 2 Δ LIL were similar to α 1-GlyR, displaying an EC₅₀ of $64 \pm 5 \mu\text{M}$ and an n_H of 2.8 ± 0.4 (Fig. 5B). Furthermore, the predicted model in silico showed these mutations allowed the formation of a helical structure in the regions near TM3 and -4, similar to that found in the LIL of the α 1-GlyR subunit (Fig. 5C). Interestingly, this modified chimera was potentiated by the dialysis of GTP γ S with an increase in the current evoked by glycine by $35\% \pm 5\%$ (Fig. 6A). Also, this receptor was sensitive to ethanol and displayed $42\% \pm 1\%$ potentiation of the glycine-evoked current in the presence of 100 mM ethanol (Fig. 6, C and D).

Taken together, our results confirm a structural requirement for the modulation of the chimera α 1-LIL- γ 2 Δ LIL, transforming an insensitive receptor into one capable of being potentiated by ethanol through G β γ . This change in sensitivity is associated with the recovery of the helical structure in the LIL, specifically in the regions near the transmembrane domains.

Discussion

The main aim of the present study was to provide information about the structural requirements of the LIL of GlyRs for ethanol-induced modulation. The present data support the existence of a helical structure in the intracellular domain of the α 1-GlyR that is critical for interaction with G β γ and potentiation by ethanol.

It is well accepted that inhibitory GlyRs are modular proteins formed by the N-terminal extracellular domain that contains the agonist binding site, TM domains forming the pore structure and the large intracellular loop domain connecting the TM3 and -4 (Thompson et al., 2010). It is now accepted that charged residues in the LIL have roles in ion channel conductance (Deeb et al., 2007; Gee et al., 2007; O'Toole and Jenkins, 2011). For example, mutations of positive amino acids in the C-terminal region of the LIL decreased the conductance of the channel (Carland et al., 2009). In addition, receptor desensitization (Hu et al., 2006), ion selectivity (Livesey et al., 2008; Peters et al., 2010), receptor assembly, targeting, and trafficking (Boyd et al., 2002; Melzer et al., 2010) are affected by modifications in the LIL. It was also shown that the LIL is involved in the modulation of GlyR by ethanol, via an interaction

Fig. 4. Characterization of the chimeric receptor $\alpha 1$ -LIL- $\gamma 2$ sensitivity to G protein and ethanol modulations. (A) Schematic representation shows the generation of the chimera $\alpha 1$ -LIL- $\gamma 2$. (B) Glycine concentration-response relationship for the $\alpha 1$ -GlyR and the chimera $\alpha 1$ -LIL- $\gamma 2$. (C) Representative glycine-evoked current traces at the beginning (1 minute) and after 15 minutes of intracellular dialysis of the nonhydrolyzable analog GTP- γ -S in HEK cells expressing the chimera $\alpha 1$ -LIL- $\gamma 2$. The concentration of glycine was 15 μ M (EC_{10-20}). (D) Effects of GTP- γ -S on $\alpha 1$ -GlyR and chimera $\alpha 1$ -LIL- $\gamma 2$ after 15 minutes of dialysis in terms of the percentage of potentiation of evoked currents in relation to the initial evoked current. (E) Representative glycine-evoked current traces (15 μ M) in the absence and presence of 100 mM ethanol in $\alpha 1$ -LIL- $\gamma 2$. (F) Effects of 100 mM ethanol on $\alpha 1$ -GlyR and chimera $\alpha 1$ -LIL- $\gamma 2$ in terms of percent of potentiation by 100 mM ethanol of 15 μ M glycine-evoked currents. ** $P < 0.001$. Data are mean $\pm$ S.E.M. ($n = 6$).

between basic amino acids with G $\beta\gamma$ dimers (Yevenes et al., 2008). Despite this wide range of modulatory actions, the LIL is not essential for the formation of a functional channel, as evidenced using truncated and chimeric receptors (Perán et al., 2006; Jansen et al., 2008; Goyal et al., 2011).

Because of its high flexibility and complex protein expression, there is no information yet on the atomic structure of LGICs, including the complete LIL (Bocquet et al., 2009; Hilf and Dutzler, 2009; Hibbs and Gouaux, 2011). This lack of information has hindered understanding of the mechanisms involved in the intracellular modulation of these receptors. To circumvent this problem, we performed predictive analysis of the secondary structure together with CD confirming the presence of α -helix structures (27%) in the full-length LIL, particularly near the TM domains (Figs. 2 and 3; Supplemental Fig. 1). Interestingly, this helical content was slightly lower than previously reported for $\alpha 3$ -GlyR, suggesting a high degree of structure conservation between α -subunits of GlyR (Breitinger et al., 2009). Also, a potential helical structure in

Fig. 5. Characterization of chimeric receptor $\alpha 1$ -LIL- $\gamma 2$ Δ LIL containing homologous replacements of amino acids in regions near TM3 and -4. (A) Mutations in the chimera $\alpha 1$ -LIL- $\gamma 2$ Δ LIL construct to include the residues present in the $\alpha 1$ -GlyR subunit. The $\alpha 1$ -LIL- $\gamma 2$ Δ LIL construct includes the cluster 309–313 RQHKE (blue) and the 385–386 basic cluster KK (red). (B) Glycine concentration-response curves for $\alpha 1$ -GlyR, chimera $\alpha 1$ -LIL- $\gamma 2$ and modified chimera $\alpha 1$ -LIL- $\gamma 2$ Δ LIL. (C) Homology model of chimera $\alpha 1$ -LIL- $\gamma 2$ Δ LIL having a recovery of helical structure similar to that observed in the model of the $\alpha 1$ -GlyR subunit.

the N-terminal LIL region was predicted in silico in pharmacologic studies of ethanol using small synthetic peptides derived from these regions (Guzman et al., 2009; San Martin et al., 2012). The presence of these helical structures is likely to serve for protein-protein interactions of the type previously proposed for other proteins (Bonacci et al., 2005; Everitt et al., 2009; Chin et al., 2013; Shim et al., 2013; Alexander et al., 2014) and antagonized by small peptides having helical structures

Fig. 6. Modulation of chimeric receptor $\alpha 1$ -LIL- $\gamma 2$ Δ LIL by $G\beta\gamma$ and ethanol. (A) Glycine-evoked current traces at the beginning (1 minute) and after 15 minutes of intracellular dialysis of the nonhydrolyzable analog GTP γ S in HEK cells expressing the chimera $\alpha 1$ -LIL- $\gamma 2$ Δ LIL. The concentration of glycine was 15 μ M (EC_{10-20}). (B) Effects of GTP γ S on chimera $\alpha 1$ -LIL- $\gamma 2$ and its modified version $\alpha 1$ -LIL- $\gamma 2$ Δ LIL after 15 minutes of dialysis as the percentage of potentiation of evoked currents with respect to the initial current amplitude. (C) Glycine-evoked current traces (15 μ M) in the absence and presence of 100 mM ethanol recorded in HEK cells expressing the chimera $\alpha 1$ -LIL- $\gamma 2$ Δ LIL. (D) Effects of 100 mM ethanol on chimera $\alpha 1$ -LIL- $\gamma 2$ and its modified version $\alpha 1$ -LIL- $\gamma 2$ Δ LIL shown as percent potentiation by 100 mM ethanol of 15 μ M glycine-evoked currents. ** $P < 0.001$. Data are mean $\pm$ S.E.M. from at least four cells.

(Tudan et al., 2002; Everitt et al., 2009; Guzman et al., 2009; San Martin et al., 2012; Zhang et al., 2014). For instance, several structural studies of protein- $G\beta\gamma$ interaction, including complexes between $G\beta\gamma$ and SIGK peptide (Davis et al., 2005; Lin and Smrcka, 2011), GRK2 (Tesmer et al., 2010; Thal et al., 2011) and phosducin (Loew et al., 1998; Gaudet et al., 1999), have reported participation of helical structures like those proposed in this study.

Furthermore, using a chimera made of $\alpha 1$ -GlyR and the LIL from $\gamma 2$ -GABA $_A$ R, a sequence almost without α -helices (Fig. 2C), we obtained new data that support our conclusion that the presence of this type of conformation in the N-terminal part of the LIL region in the $\alpha 1$ -GlyRs is required for ethanol modulation (Fig. 6). Likewise, a similar structure was predicted in the C-terminal part of the LIL region. Thus, this type of chimeric receptor approach adds new information to identify residues that are critical for ethanol actions in TM domains in GlyRs and GABA $_A$ Rs (Mihic et al., 1997). Moreover, another recent study used a chimera between $\alpha 1$ -GlyR and δ -GABA $_A$ R to identify residues in the extracellular loop 2 of GlyRs that generated a “super ethanol sensitive” conformation (Perkins et al., 2009).

Based on GST pull-down studies, we determined that the LIL of the $\gamma 2$ subunit was able to bind reasonably well to the $G\beta\gamma$ dimer (Fig. 3B). The ability to bind $G\beta\gamma$ is not directly associated to modulation of receptor activity, for example, the $\alpha 2$ -GlyR subunit binds $G\beta\gamma$, but it is not potentiated by G protein or ethanol (Yevenes et al., 2010). In this sense, the chimera $\alpha 1$ -LIL- $\gamma 2$ was not modulated by G protein and ethanol, suggesting

the existence of other structural differences (Fig. 4, C–F). This result was somewhat surprising because a previous study with the chimera $\alpha 1$ - $\alpha 2$ -GlyR where the LIL of the $\alpha 1$ -subunit was replaced by the corresponding one of $\alpha 2$ showed that this receptor was highly sensitive to ethanol modulation indicating a modular receptor function (Yevenes et al., 2010). Interestingly, the chimera $\alpha 1$ -LIL- $\gamma 2$ presented no large differences in properties to the $\alpha 1$ -GlyR ($EC_{50} = 56 \pm 4 \mu$ M versus $EC_{50} = 40 \pm 1 \mu$ M) (Fig. 4B), which could explain the insensitivity to ethanol and G protein because of reduced gating toward an open state. Additional analysis showed that mutations in the chimera $\alpha 1$ -LIL- $\gamma 2$ to corresponding residues found in $\alpha 1$ -GlyR resulted in a phenotype similar to that of $\alpha 1$ -GlyR ($EC_{50} = 49 \pm 1 \mu$ M), including modulation by GTP γ S ($35\% \pm 5\%$) and ethanol ($41\% \pm 2\%$) (Figs. 5 and 6). Thus, these sites mutated in the LIL of $\gamma 2$ produced an α -helix next to the TM regions that is important for the phenotype. These new results illustrate the requirement of several properties for ethanol modulation in the $\alpha 1$ -GlyR subunit, which are also present in the chimera $\alpha 1$ -LIL- $\gamma 2$ Δ LIL and correspond to the presence of a basic cluster in the LIL N-terminal region, two lysines in the LIL C-terminal region (Yevenes et al., 2008), and the presence of helical structures near the TM3 and TM4 regions. Additionally, the present results validate a complete model of the $\alpha 1$ -GlyR that demonstrates a high degree of helical structure in the LIL that might be necessary for interaction with helical structures in the “hot spot” in $G\beta\gamma$ (Figs. 2 and 3) (Davis et al., 2005; Smrcka, 2008).

In conclusion, the present study suggests that the amino acids located in the large intracellular loop near TM3 and TM4 induce a conformation in the LIL that allows the receptor to be modulated by ethanol and $G\beta\gamma$. This conformation comprises an α -helix structure and indicates that this feature is critical for ethanol potentiation via $G\beta\gamma$.

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Authorship Contributions

Participated in research design: Burgos, Castro, Aguayo.
Conducted experiments: Burgos, Castro, Mariqueo.
Contributed new reagents or analytic tools: Bunster.
Performed data analysis: Burgos, Castro, Aguayo.
Wrote or contributed to the writing of the manuscript: Burgos, Castro, Guzmán, Aguayo.

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Address correspondence to: Dr. Luis G. Aguayo, Department of Physiology, University of Concepción, P. O. Box 160-C, Concepción, Chile. E-mail: laguayo@udec.cl