Inhibition of the Ethanol-induced Potentiation of α1 Glycine Receptor by a Small Peptide That Interferes with Gβγ Binding*

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Loreto San Martin1, Fabian Cerda1, Veronica Jimenez5, Jorge Fuentealba4, Braulio Muñoz4, Luis G. Aguayo1, and Leonardo Guzman1†

From the 1Department of Physiology, Faculty of Biological Sciences and 4Department of Organic Chemistry, University of Concepcion, 403901 Concepcion, Chile

Background: Gβγ interaction with GlyR is an important determinant in ethanol potentiation of this channel.

Results: A small peptide, RQHC7, can inhibit ethanol potentiation of GlyR currents.

Conclusion: Results with RQHC7 indicate that ethanol mediated potentiation of GlyR is in part by Gβγ activation.

Significance: Molecular interaction between Gβγ and GlyR could be used as a target for pharmacological modification of ethanol effects.

Previous studies indicate that ethanol can modulate glycine receptors (GlyR), in part, through Gβγ interaction with basic residues in the intracellular loop. In this study, we show that a seven-amino acid peptide (RQHC7), which has the primary structure of a motif in the large intracellular loop of GlyR (GlyR-IL), was able to inhibit the ethanol-elicted potentiation of this channel from 47 ± 2 to 16 ± 4%, without interfering with the effect of Gβγ on GIRK (G protein activated inwardly rectifying potassium channel) activation. RQHC7 displayed a concentration-dependent effect on ethanol action in evoked and synaptic currents. A fragment of GlyR-IL without the basic amino acids did not interact with Gβγ or inhibit ethanol potentiation of GlyR. In silico analysis using docking and molecular dynamics allowed to identify a region of ~350Å2 involving aspartic acids 186, 228, and 246 in Gβγ where we propose that RQHC7 binds and exerts its blocking action on the effect of ethanol in GlyR.

The glycine receptor (GlyR)3 is a ligand-gated ion channel (LGIC) that has critical inhibitory functions in the spinal cord, brain stem, and some upper brain regions. Its activation by the neurotransmitter glycine increases its permeability to chloride ions (Cl−) (1), producing a fast inhibition of action potential generation in the postsynaptic membrane. GlyRs are involved in several important physiological processes such as motor and respiratory rhythm regulation and pain perception respiratory rhythm regulation and pain perception (1), being ethanol, due to its biomedical relevance, one of the most important glycinergic modulators. To its biomedical relevance, ethanol is one of the most important glycinergic modulators. In addition, it is well accepted that this drug of abuse has a potent action on inhibitory channels, such as GlyR and γ-aminobutyric acid receptors, where it induces a robust potentiation of their channel activities. Along with the N-methyl d-aspartate (NMDA) and 5-hydroxytryptamine type 3 receptors (2), these LGICs offer the most current molecular explanation for the well known depressing effects that this drug induces on animal behavior.

As a common feature of a member of the Cys-loop family of LGIC, GlyR have a quaternary structure composed of five subunits arranged around a central pore. Four α (α1–4) and only one β subunit have been described. Each subunit possesses an extracellular N-terminal domain, four transmembrane domains (TM 1–4) and a large intracellular loop (IL) between TM 3 and 4 (1). Although this cytoplasmic domain is a common feature in the LGIC family, its primary sequence is poorly conserved. In addition, the structural and functional properties of this intracellular domain are not well understood. Nevertheless, it was reported that it is important for ion permeation in the Torpedo acetylcholine receptor (3). In addition, the GlyR-IL has been found to have a role in G protein modulation (4–6), sorting (7, 8), synaptic clustering (9), and receptor phosphorylation (10–12).

G proteins are important intracellular regulators of many signal transduction pathways, and their function is regulated by the guanine nucleotide exchange activity of activated receptors and other proteins, such as RGS (regulators of G protein signaling), GDI (GDP dissociation inhibitor) and GAP (GTPase-activating protein). Therefore, in the activated state, both Ga and Gβγ subunits are able to modulate multiple effector proteins (13). G protein-regulated pathways are recognized as one of the most important in terms of hormonal cell signaling mechanisms (14), and the pharmacological modification of these pathways has been a central focus of research. In this context, the use of small peptides to induce molecular interference of protein-protein interactions has been of importance in the study of molecular events in signal transduction pathways (15). However, not much is known regarding a potential pharmaco-

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† To whom correspondence should be addressed: Dept. of Physiology, Faculty of Biological Sciences; University of Concepcion, Concepcion Casilla 160-C, Chile. Tel.: 56-41-2661229; Fax: 56-41-2245975; E-mail: josesguzman@udec.cl.

‡ The abbreviations used are: GlyR, glycine receptor; IL, intracellular loop, Gβγ, βγ dimer of G protein; LGIC, ligand-gated ion channel; TM, transmembrane; GIRK, G protein activated inwardly rectifying potassium channel.

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logical modulation of ion channels and ethanol modulation using small peptides (20). Furthermore, this strategy has been applied to the inhibition of Gβγ-mediated activation of adenyl cyclase, GIRK channels (16), and phospholipase C (17–19).

In this report, we show that a seven-amino acid peptide (termed RQHC7γ) is capable of interfering with Gβγ binding to the GlyR-IL and inhibiting ethanol potentiation. In terms of selectivity, the peptide did not have effects on another Gβγ effector, the activation of GIRK through a GABAγ agonist. Moreover, the potentiation of the synaptic activity induced by ethanol was inhibited in the presence of RQHC7γ. Finally, the use of in silico techniques predict that this peptide binds with high affinity to a site in Gβγ where this protein would interact with GlyR.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Constructions**—Expression vectors for GABAβ1 and GABAβ2 were kindly provided by Dr. Andres Couve (University of Chile). The GIRK1 plasmid was used as the template for GST fusion protein constructions using PCR products designed for the insertion in the pGEX-5X3 vector (GE Healthcare). The plasmid encoding GIRK1, and GIRK4 were kindly provided by Dr. Stephen Ikeda (National Institutes of Health). All RQH peptides (RQH, RQHα1, RQHα2, RQHγ, RQHγ1, RQHγ2) were obtained from 13–14 days mouse embryos (strain C57BL/6). Whole-cell recordings were performed using a holding potential of −60 mV. Patch electrodes were filled with the following: 140 mM KCl, 10 mM BAHA, 10 mM HEPES (pH 7.4), 4 mM MgCl2, 2 mM ATP, and 0.5 mM GTP, with or without 200 μM RQH peptides. The external solution contained the following: 150 mM NaCl, 5.4 mM KCl, 2.0 mM CaCl2, 1.0 mM MgCl2, 10 mM HEPES (pH 7.4), and 10 mM glucose. In the case of GIRK channel activity, KCl concentration was 120 and 30 mM in the internal and external solutions, respectively. Baclofen (10 μM) was applied in short pulses (4–5 s) every 2 min during 8 min. For the recording of ethanol-mediated potentiation of GlyR, a previously described methodology was used (6, 21). Ethanol (100 mM) was co-applied with glycine (15 μM), and the results were expressed as percentage of potentiation at 15 min. Although potentiation can be detected with 10 mM ethanol (25), we utilized 100 mM in most experiments to facilitate the statistical analysis. Synaptic activity was recorded after 13–16 days of in vitro culture. 6-cyano-7-nitroquinoxaline-2,3-dione (4 μM), bicuculline (4 μM), and tetrodotoxin (100 nM) were applied to the external solution to isolate miniature glycnergic events.

**Docking and Molecular Dynamics**—An α helix representation of the peptide was created based on the phi and psi angles calculated for crystallized proteins stored in the Protein Data Bank. The structure was minimized using molecular mechanics by the steepest descent method on CHARMM force field implemented in NAMD software. Docking and clustering were performed as described in Guzman et al. (20). Briefly, docking was performed with Zdock to obtain 2000 complex structures, which were grouped in 20 clusters by ClusPro (version 1.0). The structure for Gβγ was obtained from Protein Data Bank (1TGB). For molecular dynamics, the representative complex of the most populated cluster was solvated in a cubic water box (90.3 × 75.4 × 70.7 Å) using water model TIP3P. These calculations were performed in NAMD (22) considering a CHARMM force field, 20-ns total time, integration time of 2 fs, 12 Å cut-off, and 10000 minimization previous steps using the steepest descendent method. The final structure of molecular dynamics was taken to perform a free energy calculation using FastContact and distance measures by VMD software (23). Root mean square deviation was computed first by aligning only the interacting residue with the average positions for each of the atoms involved (data not shown). 3000 frames were considered for this calculation. Then, deviation was calculated from distances based on changes of side chain atom positions through time by VMD software (24).

**Data Analysis**—Statistical analyses were performed using analysis of variance, and the results are expressed as the arithmetic mean ± S.E. Values of p < 0.05 were considered statistically significant. Origin (MicroCal, version 6.0) software was used for all statistical analyses.

**RESULTS**

Regulation by signaling pathways has been described for several members of LGICs, including the effects of ethanol and G proteins on GlyRs (6, 21, 25). In the case of GlyR, it has been previously described that the GlyR-IL contains motifs that are important for G protein- and ethanol-mediated potentiation. For example, it was found that a stretch of residues located in the N-terminal region of this domain is important for the interaction with Gβγ (20). In addition, electrophysiological studies demonstrated that both GTPγS and ethanol potentiation of GlyR were affected by the mutation of residues 316–320 pres-
ent in this domain (21). The importance of these residues was also shown by direct in vitro interaction between the GlyR-IL and Gβγ using GST pulldown experiments (20).

In the present study, we used the region from amino acids 309 to 325 in the GlyR-IL to generate a series of peptides (Fig. 1). Subsequently, to study the capacity of these peptides at inhibiting the binding between Gβγ and GlyR-IL, we used an in vitro assay that utilizes the GST-GlyR-IL fusion protein, Gβγ and the small peptides. The previously studied RQH peptide (that has the entire 309–325 region) was included as a positive control for inhibition of the interaction between Gβγ and GST-IL (20). The data showed that both RQHC10 and RQHC7 diminished the binding of Gβγ to the GlyR-IL to 28 ± 18 and 36 ± 21% of control, respectively. However, the data show that RQHN did not affect the interaction of Gβγ with GST-IL (Fig. 2). These results demonstrate that there is a region in the IL that interacts with Gβγ under in vitro conditions and correlates well with previous functional studies (21).

To obtain information about the region of Gβγ that might be involved in the interaction with RQHC7, in silico studies were performed. Previous modeling assays determined that the RQHC7 structure is most likely a helix (20). In addition, docking, clustering, and molecular dynamics were done to determine the interaction nature of RQHC7 and Gβγ. The results of these analyses identified a region of ~350 Å² in Gβγ characterized by the presence of three acidic residues, Asp-186, -228, and -246, which might serve as the binding region to the peptide, RQHC7, which might serve as the binding region to the peptide, RQHC7. These results agree with the previous functional studies (21).

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3.2 Å, Fig. 3C). Free energy calculations and atomic distances between these interacting residues further supported the presence of a binding region. To have an idea about deviations in atom positions of interacting amino acids, Arg-3, -6, -7 (in RQHC7) and Asp-186, -228, and -246 (from Gβγ) were considered for further molecular dynamic studies. The results were analyzed using a root mean square deviation (Fig. 3D), which showed that deviations in the position of side chains were <2 Å, which supports the notion that electrostatic interactions maintain the shape of the interface between RQHC7 and Gβγ.

It was recently shown that ethanol potentiation of GlyR is affected by Gβγ via interaction with the GlyR-IL (21). Because RQHC7 was able to inhibit the binding of Gβγ to the GlyR-IL, we decided to study whether this peptide was able to inhibit the ethanol potentiation of the GlyR. Patch clamp assays were performed in cultured spinal cord neurons expressing the α1β subunits (26), and the small peptides (at 200 μM) were included into the recording pipette to allow them to diffuse into the cytoplasm. Glycine (15 μM, at EC15) and ethanol (100 mM) were extracellularly perfused to the cells to activate GlyR. After 15 min of internal solution dialysis, glycinergic responses were recorded in the absence and presence of ethanol (Fig. 4). In the control condition, ethanol potentiated the GlyR current to 47 ± 2% above control, in agreement with previous reports (6, 20, 21). However, use of RQH, RQHC10, and RQHC7 peptides reduced the potentiation to 19 ± 4%, 17 ± 1%, and 16 ± 4% of control, respectively. On the other hand, RQHN, a peptide that lacks the basic motif (Fig. 1), did not inhibit this effect showing that RQHC7 is the shorter peptide containing the motif to inter-
To inhibit ethanol potentiation (Fig. 4). Furthermore, the RQHC7 peptide inhibited the ethanol potentiation in a concentration-dependent manner where 20, 100, and 200 \( \mu \)M of the peptide in the internal solution reduced the ethanol potentiation to 45 ± 5, 29 ± 1, and 16 ± 4% above control, respectively (Fig. 5B). In addition, the inhibitory effect of RQHC7 was also detected using a lower ethanol concentration of 50 mM, which potentiated the current to 29 ± 4% above control versus 13 ± 1% with intracellular diffusion of RQHC7 (Fig. 5C).

Previous results from our laboratory showed that RQH also interfered with the signaling of G\( \beta\)\( \gamma \) in GIRK channels after stimulation of the GABAB receptor (20). In the present study, we reduced that peptide to a core of seven amino acids to generate a more specific effect on GlyR in comparison with GIRK. This possibility was studied in HEK cells that were transfected with both GABA\( \beta \) receptors and GIRK channels (Fig. 6). In this experiment, a specific agonist, baclofen, activates GABA\( \beta \) receptors leading to activation of a G\( \gamma \) protein. The G\( \beta\)\( \gamma \) dimer of this protein opens the GIRK channel. In this experiment, baclofen was applied by external perfusion and 200 \( \mu \)M RQH, or RQHC7, were applied in the internal solution during 8 min. The data show that the potassium current was inhibited by RQH to 41 ± 1% of the initial current. On the other hand, RQHC7 was unable to inhibit the GIRK current suggesting that this seven-amino acid motif induced a more specific inhibition of G\( \beta\)\( \gamma \) modulation to GlyR in comparison with RQH.

The above results were performed inducing an evoked Cl\( ^{-} \) current by application of exogenous glycine. To establish if RQHC7 had effects on ethanol alteration of synaptic parameters, we recorded glycineric inhibitory post-synaptic currents (mIPSC) pharmacologically isolated in cultured spinal cord neurons. Among the parameters of the synaptic currents that were analyzed, the decay time constant was associated to the time that the single channels were maintained opened after synaptic stimulation. The analysis of this glycineric activity showed that the decay time constant of the synaptic currents was increased by ethanol in a 42 ± 9% (from 12 ± 0.9 to 17 ± 1.6
Thus, residues from Arg-309 to Leu-315 are not critical for the interaction of G protein with the GlyR-IL, which is in agreement with previous mutational studies on these residues (21). We demonstrated that RQHC7 (residues 316 to 322 of the 17-amino acid RQH peptide, besides blocking the effect of RQH, was also able to bind Gβγ causing an inhibition with GlyR binding. In agreement with this idea, in silico studies suggest that binding of the RQHC7 peptide was in a region that presented several acidic residues, which agrees with previous studies using RQH (20). Interestingly, free energy calculation obtained for RQHC7 was very similar to the one computed for SIGK (Protein Data Bank code 1XHM), another previously described Gβγ interacting peptide (18) (−24 kcal/mol and −28 kcal/mol, respectively). Furthermore, the identified region of Gβγ is functionally relevant because it contains motifs that interact with other effectors, namely GIRK, adenylyl cyclase, phospholipase C, and the α subunit of G proteins (28).

In previous reports, it has been shown that the α1 GlyR subunit is potentiated by ethanol at a concentration of 10 mM and above (21, 25). In this report, we used 50 and 100 mM of ethanol inducing potentiations of 29 and 45% above control, respectively (Fig. 5). Furthermore, it has been previously demonstrated that ethanol potentiation was inhibited by the use of Gβγ scavengers (21). The peptides RQH, RQHC10 and RQHC7 derived from the IL sequence also interfered with the ethanol effect on GlyR, suggesting that the molecular mechanism likely involves its binding and blocking of the Gβγ region that interacts with the GlyR-IL. Interestingly, inhibition of the ethanol effect by RQHC7 was also elicited at a pharmacologically relevant concentration (50 mM), creating the possibility of a potential target for ethanol toxicity.

Gβγ is a critical signaling modulator that has many important targets such as , phospholipase C, calcium, and potassium channels (29). It is recognized that the binding region of these Gβγ targets share a common region in one interface of Gβγ, and this close molecular proximity might hinder the design of a specific ligand that interfere in the Gβγ-GlyR protein-protein interaction. For instance, a previous study demonstrated that the 17-amino acid RQH peptide, besides blocking the effect of

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**FIGURE 4. Blockade of ethanol potentiation on GlyR activity by small peptides.** A, traces are evoked Cl− currents recorded in the absence and presence of ethanol and small peptides. Both glycine (EC50, 15 μM) and ethanol (100 mM) were applied through a perfusion pipette. Small peptides (200 μM) were applied intracellularly through the recording pipette for 15 min (effect of RQHC7 is indistinguishable from RQH, and is not shown). B, the values represent the percent potentiation induced after 15 min of intracellular perfusion compared with the effect at 1 min. ***, p < 0.005.**
ethanol on GlyR, also interfered with the Gβγ-dependent activation of GIRK channels (20). In the present study, supporting the idea that molecular selectivity can be achieved, we found that the shorter peptide RQHC7 did not interfere with this effect of Gβγ on GIRK channels, confirming that the seven-amino acid peptide (RQHC7) displayed an improved specificity.

It is likely that, in part, the effects of ethanol in motor control are mediated by the potentiation of glycinergic transmission in spinal cord and brainstem synapses (30, 31). In our hands, ethanol can affect a population of spinal neuron synapses containing mature α1 GlyRs. We analyzed the parameters of frequency, amplitude, rise time and decay time constant of the mIPSCs. The frequency of the synaptic events reflects a presynaptic measure of vesicular release. On the other hand, it is believed that the other parameters depend on the properties of the postsynaptic receptors such as the number and kinetic properties (32, 33). We showed that ethanol induces an increase in decay time constant consistent with a postsynaptic action, and this effect was reverted almost completely by the intracellular perfusion of the RQHC7 peptide. We postulate that these data represent the intracellular interference of Gβγ signaling that ultimately causes the inhibition of the ethanol synaptic potentiation of the glycinergic currents.

Peptides have been used as functional probes to study GPCR signaling pathways to elucidate their molecular dynamics, structural-functional organization, complex formation, and their role in the control of signaling. This was the case for peptides derived from the intracellular domain of dopamine (D1-DR, D2-DR), serotonin (5-HT1A-R, 5-HT1B-R), and acetylcholine (m3-MChR, m3-MChR) receptors, that selectively bind and are able to activate their respective G proteins, resembling the activity of the activated receptor (35–38). Furthermore, pep-

**FIGURE 5.** Concentration-dependent inhibition of ethanol potentiation by RQHC7. Both glycine and ethanol were applied through a perfusion pipette. Glycine was used at a concentration equivalent to an EC15 (15 μM) and ethanol at 100 mM and 50 mM as indicated. A, evoked Cl− currents were recorded in the absence and presence of ethanol (100 mM) and RQHC7 at three concentrations. B, the percentage of ethanol potentiation was calculated with respect to the current obtained in the absence of ethanol. C, effects of RQHC7 on potentiation produced by 50 mM ethanol. RQHC7 peptide (200 μM) was applied intracellularly through the recording pipette into spinal cord neurons. The percentage of ethanol potentiation was calculated with respect to the current obtained in the absence of ethanol and peptides. Analysis of variance and t-student statistical methods were applied. ***, p < 0.005; **, p < 0.01.**
tides derived from α2 and β2 adrenergic receptors stimulate Gβγ and Gαo, respectively, and are believed to have potential pharmacological actions (37, 39–41).

Gβγ signaling is recognized to be important in several physiopathological conditions such as heart failure (42), tumorigenesis (43), cell migration and invasion (44), pharmacological tolerance and dependence (45), inflammatory processes (46), and several endocrine pathologies (47, 48), among others. One of the first attempts at blocking the function of Gβγ with selective peptides was developed for adenylyl cyclase. Chen and co-workers (16) inhibited the Gβγ-stimulated activity of a type 2, phospholipase C, β-adrenergic receptor kinase, and GIRK with a small peptide (QEHA) derived from the C2 domain of this adenylyl cyclase. Similar results were obtained with peptides derived from GIRK (50). Furthermore, Smrcka and co-workers (17, 18, 34, 51, 52) used a phage peptide display technique for screening peptides that bind to Gβγ. From these studies, the existence of a Gβγ scavenger that can have a potential use in heart failure and be a synergistic aid to β-adrenergic receptor blockers is now recognized (49). Interestingly, the concentrations of RQHC7 (20 to 200 μM) used in this report are comparable with the ones used for the QEHA peptide (300 μM) (16) and is close to the same order of magnitude as SIRK (5 to 10 μM) (51). Future studies will aim to identify small molecule to

FIGURE 6. RQHC7 does not interfere with the activation of GIRK channels. A, HEK cells expressing GABAB receptor and GIRK channels were stimulated with baclofen (10 μM) to record the Gβγ-gated potassium current in the presence and absence of RQH and RQHC7 peptides intracellularly applied for 8 min. B, quantification of the potassium current after 8 min of peptide diffusion as a percentage of the initial response. *, p < 0.05.

FIGURE 7. Ethanol enhancement of the decay time constant of the synaptic GlyR events was inhibited by RQHC7. A, isolated glycinergic mIPSC from synaptic activity of cultured spinal neurons recorded in the absence (black traces) and presence of ethanol (100 mM, gray traces) and RQH C7 and RQH N peptides (200 μM each, intracellularly applied). In the control condition, 115 event were considered per each cell registered (n = 5), 116 when we used RQHC7 peptide (n = 4), and 45 when we used RQH N peptide (n = 5). B, percentage variation in decay time constant between no ethanol and ethanol conditions (as shown in A). In the absence of peptides, ethanol induces an increment in decay time constant (no peptide bar) with respect to control condition. In presence of intracellular RQHC7 (200 μM), ethanol induces only a small increase (RQHC7 bar). On the other hand, in presence of intracellular RQH N (200 μM), ethanol increases the decay time constant to a similar magnitude as the control condition (RQH N bar). *, p < 0.05.
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circumvent the difficulties of working with peptides as therapeu tic molecules.

Finally, we propose that the use of Gγy-blocking peptides to interfere with pharmacologically altered glycinergic synapses might prove to be a novel approach to modify alcohol consumption and its associated health problems.

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