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Activated G Protein α_s Subunits Increase the Ethanol Sensitivity of Human Glycine Receptors

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ABSTRACT

It is well known that ethanol modulates the function of the Cys loop ligand-gated ion channels, which include the inhibitory glycine receptors (GlyRs). Previous studies have consistently shown that transmembrane and extracellular sites are essential for ethanol actions in GlyRs. In addition, recent evidence has shown that the ethanol modulation of GlyRs is also affected by G protein activation through $G\beta\gamma$ subunits. However, more specific roles of G protein α subunits on ethanol actions are unknown. Here, we show that the allosteric effect of ethanol on the human α_1 GlyR is selectively enhanced by the expression of $G\alpha_s$ Q-L. For example, constitutively active $G\alpha_s$, but not $G\alpha_q$ or $G\alpha_i$, was able to displace the alcohol sensitivity of GlyRs toward low millimolar concentrations (17 ± 4 versus 48 ± 5% at 100 mM). Experiments under conditions that increased cAMP and protein kinase A (PKA)-mediated signaling, on the contrary, did not produce the same enhancement in sensitivity, suggesting that the $G\alpha_s$ Q-L effect was not dependent on cAMP/PKA-dependent signaling. On the other hand, the effect of $G\alpha_s$ Q-L was blocked by a $G\beta\gamma$ scavenger (9 ± 3% of control). Furthermore, two mutant receptors previously shown to have impaired interactions with $G\beta\gamma$ were not affected by $G\alpha_s$ Q-L, suggesting that $G\beta\gamma$ is needed for enhancing ethanol sensitivity. These results support the conclusion that activated $G\alpha_s$ can facilitate the $G\beta\gamma$ interaction with GlyRs in presence of ethanol, independent of increases in cAMP signaling. Thus, these data indicate that the activated form of $G\alpha_s$ is able to positively influence the effect of ethanol on a type of inhibitory receptor important for motor control, pain, and respiration.

Introduction

Ethanol is the most widely abused drug. Its consumption at intoxicating doses produces major modifications in motor, sensorial, and cognitive functions. The underlying mechanisms probably involve a wide variety of cellular effectors. A large body of evidence has demonstrated that ethanol can allosterically modulate the activity of several ligand-gated ion channels (LGICs), including members of the Cys loop family, composed of nicotinic acetylcholine, serotonin, GABA ($GABA_AR$) and glycine (GlyR) receptors (for review, see Perkins et al., 2010). Because these receptors mediate fast synaptic transmission in the mammalian central nervous system, the effects of ethanol on these membrane proteins might largely explain the strong alterations on human behavior after excessive drinking.

Inhibitory GlyRs are critical for the control of neuronal network excitability through a selective increase in Cl^- ion conductance, which is able to hyperpolarize the cell membrane (Aguayo et al., 2004; Lynch, 2004). GlyRs are composed of five subunits in a pentameric quaternary structure arranged around a central pore. Each subunit possesses four transmembrane (TM) domains and a large intracellular loop between TM3 and TM4 responsible for intracellular signal transduction modulation (Lynch, 2004). Previous studies in different cell types have consistently demonstrated that millimolar concentrations of ethanol can modulate the glycine-activated current (Aguayo et al., 1996; Mihic et al., 1997; Eggers et al., 2000; Lynch, 2004; Crawford et al., 2007; Perkins et al., 2010), although the molecular mechanisms involved are still not completely understood. Nevertheless, based on mutagenesis studies, it has been proposed that specific amino acids in the TM2–TM3 domains form discrete binding sites for ethanol, which also bind general anesthetics (Mihic et al., 1997; Harris et al., 2008). In addition, a residue in the extracellular domain was reported to contribute to

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ABBREVIATIONS: LGIC, ligand-gated ion channel; $GABA_AR$, GABA receptor; GlyR, glycine receptor; TM, transmembrane; GPCR, G protein-coupled receptor; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; PKA, protein kinase A; HEK, human embryonic kidney; GFP, green fluorescent protein; ANOVA, analysis of variance; ct-GRK, carboxyl-terminal G protein-coupled receptor kinase.

ethanol potentiation of GlyRs (Perkins et al., 2010), possibly by linking ligand binding to channel opening (Yévenes et al., 2010) or by configuring an ethanol acceptor site (Crawford et al., 2007). Furthermore, other studies determined that the molecular volume and hydrophobicity of S267 in TM2 also contributed to GlyR ethanol sensitivity (Yamakura et al., 1999) and alcohol binding (Mascia et al., 2000). On the other hand, it was also shown that ethanol modulates ion channel activity through modifications of intracellular signal transduction pathways. For instance, the sensitivity of GlyR and $GABA_A$ to ethanol was affected by G protein activation and protein kinases (Aguayo et al., 1996; Freund and Palmer, 1997; Mascia et al., 1998; Jiang and Ye, 2003; Zhu and Ye, 2005; Qi et al., 2007). Of interest, it was reported that ethanol can indeed affect specific intracellular transduction pathways (Yao et al., 2002; Morrow et al., 2004; Ron and Jurd, 2005). More recent studies have shown that the ethanol-mediated potentiation of GlyRs was affected by a molecular interaction between intracellular residues in the receptor with G protein βγ heterodimers (Yévenes et al., 2006, 2008).

G protein-coupled receptors (GPCRs) are transmembrane proteins that mediate most intracellular actions through pathways involving activation of G proteins. After GPCR activation by ligands, heterotrimeric G proteins modulate the activity of many effectors, cycling between an inactive GDP-bound and an active GTP-bound conformation (Hamm, 1998; Oldham and Hamm, 2008). This critical cycle allows the fine tuning of protein-protein interactions between Gα-GTP and Gβγ with their targets. Despite the critical importance of the active Gα subunits for G protein-mediated signaling, their relevance for ethanol effects on LGICs has not been directly investigated.

Previous studies have examined the role of signal transduction pathways on the sensitivity of GlyRs and $GABA_A$Rs to ethanol using intracellular application of G protein or kinase modulators via the patch pipette. Although these approaches have generated valuable information, the outcomes are restricted by the concentration and the specificity of the selected modulators. As an alternate approach to characterize the potential role of Gα subunits on the ethanol sensitivity of GlyRs, in the present study we used constitutively active mutant forms of three main Gα subunits, which are denominated Gα Q-L mutants due to a glutamine to leucine mutation in the GTP-binding site. The $G\alpha_s$ Q-L, for example, is a constitutively active mutant capable of generating high levels of cAMP through the stimulation of adenylyl cyclase in the absence of GPCR activation (Masters et al., 1989). Thus, these mutants can be used to investigate the role of a specific signal transduction pathway on a given effector, without potential nonselective interferences that might arise when more chemically based approaches are used.

This study shows that active $G\alpha_s$, possibly found during stressful conditions or under the influence of β_2-adrenergic pharmacotherapy, can selectively enhance the ethanol sensitivity of GlyRs through a Gβγ-linked mechanism. Thus, our results show that activated $G\alpha_s$ enhances the sensitivity of GlyRs to ethanol and suggests that other targets could also be affected in the same fashion by this drug of abuse.

Materials and Methods

cDNA Constructs. The cDNA encoding human α_1 glycine receptor subunits in the pCIS2 vector was obtained from N. L. Harrison (Columbia University, New York, NY). Mutant 316–320A and 385–386A GlyRs were described previously (Yévenes et al., 2006). Activated $G\alpha_s$, $G\alpha_{i2}$, and $G\alpha_q$ in pcDNA3.1 vectors (Invitrogen, Carlsbad, CA) were purchased from Missouri S&T cDNA Resource Center (Rolla, MO). The mutant Q87/R196 PKA catalytic α subunit (PKA Cα Q-R) cDNA was kindly provided by Dr. Stanley McKnight (University of Washington, Seattle, WA) and has been described previously (Orellana and McKnight, 1992; Orellana et al., 1993). To monitor optimal kinase expression in our experiments, a hexahistidine-tagged version of this mutant PKA in the pcDNA3.1 vector (Invitrogen) was designed and constructed. The EYFP-ct-GRK2 expression vector was described previously (Kammermeier and Ikeda, 1999).

Cell Culture and Transfection. HEK293 cells were cultured using standard methodologies. HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen) with 2 μg of DNA for each plasmid studied per well. Expression of GFP was used as a marker of positively transfected cells and recordings were made after 18 to 36 h.

Electrophysiology. Whole-cell recordings were performed as described previously (Yévenes et al., 2008). A holding potential of −60 mV was used. Patch electrodes were filled with 140 mM CsCl, 10 mM BAPTA, 10 mM HEPES, 4 mM $MgCl_2$, 2 mM ATP, and 0.5 mM GTP (pH 7.4). The external solution contained 150 mM NaCl, 10 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose (pH 7.4). The amplitude of the glycine current was assayed using a brief pulse (1–2 s) of glycine. The modulation of the glycine current by ethanol (Sigma-Aldrich, St. Louis, MO) was assayed using a pulse of 30 μM glycine coapplied with ethanol in each condition studied, without any preapplication. A brief pulse of 1 mM glycine was applied at the end of the recording period to test whether the maximal current amplitude remained stable after the recording (≥5 min). If the amplitude changed by more than 10%, the cell was discarded. To reduce the expression variability, a bicistronic vector carrying the $G\alpha_s$ Q-L gene together with enhanced GFP (pIRES2-$G\alpha_s$ Q-L) was used in most of the experiments. Otherwise, all the additional receptors and intracellular proteins were transfected at a 5:1 ratio related to the GlyR amount.

Measurement of Relative Changes in cAMP Levels. To investigate the relative amounts of cAMP under our experimental conditions, we used the dual luciferase reporter assay system (Promega, Madison, WI) following the manufacturer's protocol (Romo et al., 2008). In brief, HEK293 cells were grown in 24-well plates (80% confluent) and transfected with the plasmids pCRE-Luc and pRL-SV40 for cAMP-dependent firefly and *Renilla reniformis* luciferase activity, respectively. Expression of *R. reniformis* luciferase provides an internal control for transfection to normalize the cAMP-dependent firefly luciferase expression. In a set of experiments, a plasmid encoding human β_2-adrenergic receptor in the pCDNA3.1 vector (Invitrogen) was incorporated. For all the control reactions, the GlyR encoding plasmid was also transfected to mimic the cellular conditions attained in cells used to do the electrophysiological recordings. One day after transfection, firefly and *R. reniformis* luciferase activity were measured sequentially from a single lysate in a Wallac 1420 VICTOR^3 Luminometer (PerkinElmer Life and Analytical Sciences, Waltham, MA) using the dual luciferase assay system (Promega). Each single experiment was performed in triplicate, whereas each dataset was reproduced at least three times.

Immunofluorescence. 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). Then, all-night incubation with polyclonal $G\alpha_s$ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and polyclonal hexahistidine antibodies (His-Tag; US Biological, Swampscott, MA) was performed. Epitope visualization was performed by incubating the sam-

ple with two secondary antibodies conjugated to Cy3 (1:600; Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Finally, the cells were coverslipped using Fluorescence Mounting Medium (Dako North America, Inc., Carpinteria, CA) and were chosen randomly for imaging using a Nikon confocal microscopy (TE2000; Nikon, Melville, NY).

Data Analysis. Statistical analyses were performed using ANOVA and are expressed as arithmetic mean ± S.E.M.; values of $P < 0.05$ were considered statistically significant. In all the figures, the data points plotted as control and $G\alpha_s$ Q-L represent the pooled average of all the individual experiments. These data points were obtained from experiments performed in parallel for every condition. No significant differences were found between the averages obtained under the same experimental conditions (e.g., control or $G\alpha_s$ Q-L) from different batches of cells. For all the statistical analysis and plots, Origin 6.0 (OriginLab Corp., Northampton, MA) software was used.

Results

Effects of Constitutively Active G Protein α Subunits on Ethanol Sensitivity of Wild-Type α_1 Glycine Receptors. The $G\alpha_s$ pathway and PKA phosphorylation have been previously implicated in ethanol effects on GlyRs (Aguayo et al., 1996); therefore, we began our study by analyzing the ethanol sensitivity of GlyRs after the overexpression of the $G\alpha_s$ Q-L mutant. In agreement with previous results in control HEK cells (Aguayo et al., 1996; Ye et al., 1998), the glycine (~EC_{50}, 30 μM)-activated current elicited by activation of α_1 GlyRs was consistently potentiated by ethanol, displaying a threshold effect at a concentration between 1 and 10 mM (Fig. 1, A–C). For instance, whereas 1 mM ethanol did not produce a significant effect (2 ± 2%) on the current amplitude, 100 mM potentiated the glycine-evoked current above 17 ± 4% at EC_{50}. Of interest, the ethanol sensitivity was significantly enhanced after the expression of $G\alpha_s$ Q-L (Fig. 1, A and B). The current traces show that after QL overexpression, the application of 1 mM ethanol already was able to potentiate the current amplitude. The graph illustrates that this low concentration of ethanol increased the current amplitude by 25 ± 3%, reaching near 48 ± 5% potentiation with 100 mM (Fig. 1B). The data also show that under this condition the threshold for the ethanol effect was reduced to approximately 0.5 mM ethanol. To examine whether the $G\alpha_s$ Q-L effects correspond to a phenomenon specific for the $G\alpha_s$, we performed experiments expressing the constitutive active mutants $G\alpha_{i2}$ Q-L and $G\alpha_q$ Q-L. Contrary to $G\alpha_s$ Q-L, none of these activated Gα mutants modified the effect of 100 mM ethanol in GlyRs (Fig. 1, A–C), indicating that activated $G\alpha_s$ selectively increases the sensitivity of GlyR to alcohol. In other experiments, we found that both homomeric α_1 and $\alpha_1\beta$ heteromeric GlyRs were significantly potentiated by ethanol when $G\alpha_s$ Q-L was overexpressed in the cells. For example, the data in Fig. 2A show that active $G\alpha_s$ significantly enhanced the ethanol sensitivity in both receptor configurations ($P < 0.001$, ANOVA).

The effect of overexpression of the active forms of Gα on the sensitivity of GlyR to glycine was studied because the potentiation caused by ethanol was previously shown to be dependent on the concentration of glycine (Aguayo et al., 1996; Ye et al., 1998). The data from these experiments showed that $G\alpha_s$ Q-L did not alter the apparent affinity of GlyR, suggesting that the effects of ethanol were not related to changes on

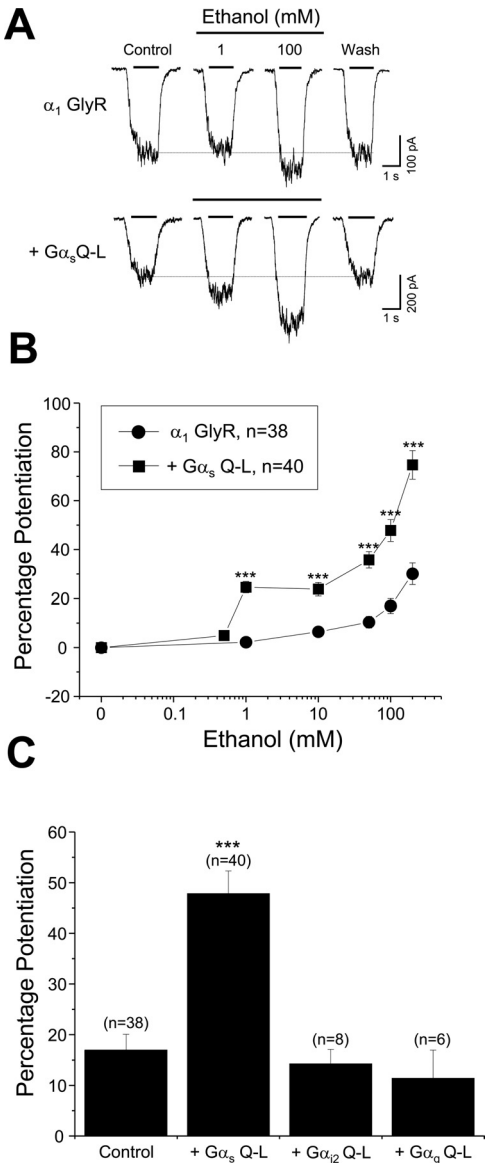

Fig. 1. Constitutively active $G\alpha_s$ enhances the sensitivity of human α_1 GlyRs to ethanol. A, current traces obtained in transfected HEK cells expressing wild-type α_1 GlyRs with and without $G\alpha_s$ Q-L in the presence of different ethanol concentrations. B, concentration-response curves to ethanol (0.5–200 mM) in control (●, $n = 38$) and $G\alpha_s$ Q-L-expressing cells (■, $n = 40$). C, graph summarizing the potentiation induced by 100 mM ethanol of the normalized glycine-evoked current elicited in cells cotransfected with either $G\alpha_{i2}$ Q-L ($n = 8$) or $G\alpha_q$ Q-L ($n = 6$) mutants. Note that only $G\alpha_s$ Q-L enhanced the ethanol sensitivity. All data are presented as means ± S.E.M. The data points plotted as control and $G\alpha_s$ Q-L represent the pooled average of all the individual experiments. ***, differences were significant at $P < 0.001$, ANOVA.

receptor affinity for the agonist (Fig. 2, B and C). For instance, the calculated EC_{50} values were not significantly different compared with the controls [α_1 GlyRs = 30 ± 2 μM ($n = 18$), $G\alpha_s$ Q-L = 33 ± 1 μM ($n = 16$), $G\alpha_{i2}$ Q-L = 30 ± 1 μM ($n = 7$), and $G\alpha_q$ Q-L = 28 ± 3 μM ($n = 6$)]. In addition, the current records showed that the time course of the response was not affected by the overexpression of $G\alpha_s$ Q-L. The normalized amplitude of the glycine-activated currents in control cells expressing only α_1 GlyRs or in cells cotransfected with $G\alpha_s$ Q-L was very stable over 15 min of recording [α_1 GlyRs = 97 ± 1% ($n = 9$) and $G\alpha_s$ Q-L = 102 ± 4% ($n =$

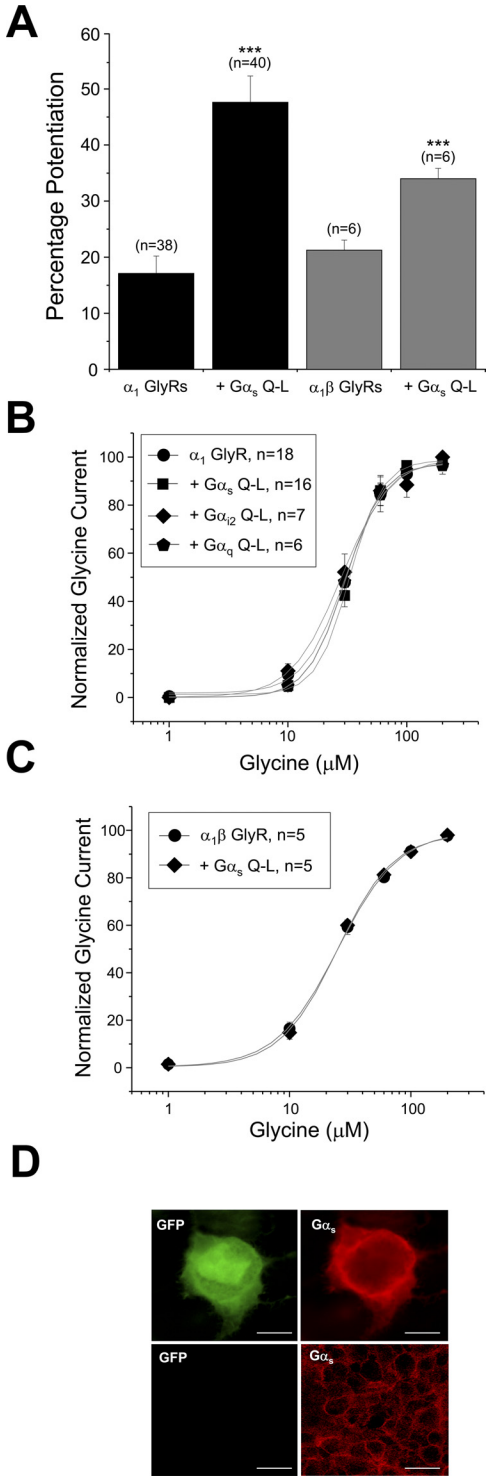

Fig. 2. $G\alpha_s$ Q-L overexpression in HEK 293 cells enhances the ethanol sensitivity of homomeric and heteromeric GlyRs. A, bar graph summarizing the potentiation elicited by 100 mM ethanol of the normalized glycine-evoked current elicited in homomeric α_1 (■) or $\alpha_1\beta$ heteromeric (▨) GlyRs cotransfected with $G\alpha_s$ Q-L in parallel experiments. ***, differences were significant at $P < 0.001$, ANOVA. B, data obtained in the absence (circles) or presence of $G\alpha_s$ Q-L (■), $G\alpha_{i2}$ Q-L (♦), or $G\alpha_q$ Q-L (⬟) show normal receptor activation. C, glycine concentration-response curves for heteromeric $\alpha_1\beta$ GlyRs in the absence (●) or presence of $G\alpha_s$ Q-L (♦). D, confocal microscopy images show HEK293 cells transfected with GFP and $G\alpha_s$ Q-L stained with a polyclonal antibody against $G\alpha_s$ (top panel, red). The bottom panels show endogenous $G\alpha_s$ expression in nontransfected cells. Scale bar, 10 μm (top panel); 50 μm (bottom panel).

7)]. All these results indicate that the properties of GlyR were unchanged by overexpression of $G\alpha_s$ Q-L. Using confocal microscopy, we found high levels of $G\alpha_s$ expression in cells transfected with the plasmids, assuring that the cells that were GFP-positive did indeed have high levels of activated Gα (Fig. 2D).

Chronic Increases in cAMP Levels or PKA Activity Did Not Alter the Sensitivity of GlyRs to Ethanol. The expression of $G\alpha_s$ Q-L generates a sustained chronic increase in cAMP levels in the absence of GPCR activation (Masters et al., 1989). Thus, we thought that the increase in ethanol sensitivity of GlyRs could result from a sustained activation of PKA, a known GlyR intracellular modulator (Song and Huang, 1990; Lynch, 2004). Therefore, to explore the potential role of cAMP and PKA on the ethanol sensitivity of GlyRs, we decided to perform electrophysiological recordings in cells with high levels of cAMP mimicking the cellular conditions generated by $G\alpha_s$ Q-L expression. Before performing the physiological experiment, we confirmed that $G\alpha_s$ Q-L indeed increased the cellular content of cAMP (Masters et al., 1989). Results obtained using a dual luciferase reporter system showed that the HEK293 cells displayed high relative cAMP levels (46 ± 7-fold over control) after expression of β_2-adrenergic receptors and chronic stimulation with the agonist isoproterenol (50 μM), which were not significantly different from the enhancement obtained with $G\alpha_s$ Q-L (Fig. 3A). Electrophysiological analysis of α_1 GlyRs expressed in cells under this high chronic cAMP condition showed that the EC_{50} for glycine was unchanged (36 ± 2 μM, $n = 7$). Likewise, the sensitivity of the GlyR to ethanol was not altered (Fig. 3, B and C), indicating that high levels of cAMP did not increase the ethanol sensitivity like that found with $G\alpha_s$QL. Nevertheless, to obtain an independent confirmation for the latter result, we directly examined the PKA role on GlyR ethanol sensitivity using overexpression of the catalytic PKA α subunit (which has been used to promote cAMP/PKA-dependent events (Mellon et al., 1989). Here, the PKA Cα mutant, denominated PKA Cα Q-R, which has two mutations that confer a higher constitutive activity and resistance for the regulatory PKA subunit inhibition, was overexpressed (Orellana and McKnight, 1992; Orellana et al., 1993). We first checked the kinase overexpression using a hexahistidine-tagged PKA Cα Q-R followed by immunocytochemistry. These experiments showed good levels of protein kinase expression after the cells were cotransfected with enhanced GFP (Fig. 4A). Similar to the results with chronic activation of cAMP (Fig. 3), the electrophysiological experiments showed that the ethanol potentiation of the glycine-evoked currents was not modified by PKA Cα Q-R expression (Fig. 4, B and C). Additional experiments showed that the EC_{50} for glycine was unchanged (32 ± 2 μM, $n = 8$).

Taken together, these results indicate that increases in cAMP and PKA activity are not involved in the regulation of ethanol effects on GlyRs, suggesting that the enhancement of alcohol sensitivity promoted by $G\alpha_s$ Q-L expression could be explained by other mechanisms that do not involve cAMP- or PKA-related signaling.

G Protein βγ Subunits Mediate the $G\alpha_s$ Q-L Increase in GlyR Sensitivity to Ethanol. The previous data suggest that constitutively active mutant $G\alpha_s$ increases the sensitivity of GlyRs to ethanol by cAMP- and PKA-independent mechanisms. Thus, it is possible to suggest that the increase

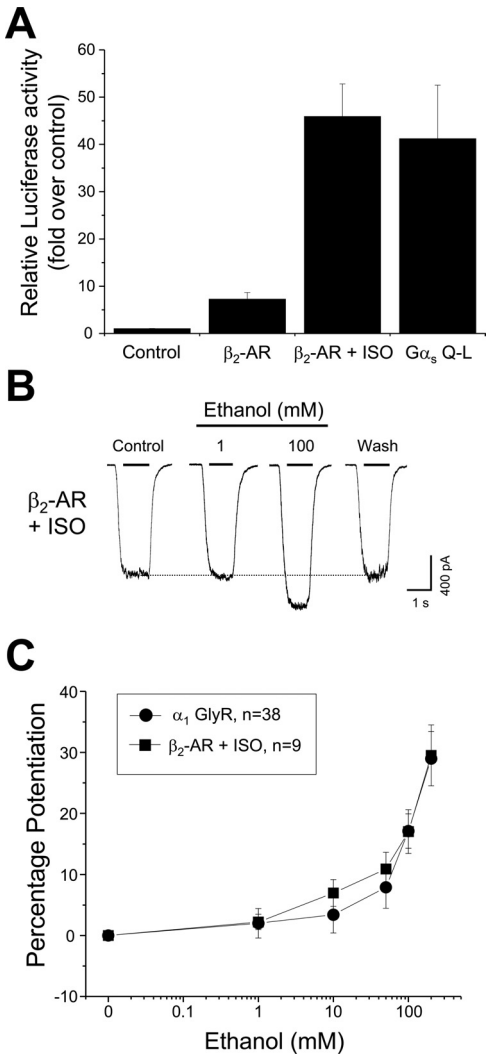

Fig. 3. Ethanol effects on GlyRs in cells with high cAMP levels. A, HEK293 cells were transfected with either β_2-adrenergic receptors (β_2-AR) or $G\alpha_s$ Q-L, and the increase in cAMP was detected with the reporters pCRE-Luc and pRL-SV40 as described under *Materials and Methods*. The bars are the means ± S.E.M. from four experiments. B, examples of whole-cell recordings from α_1 GlyRs in the presence or absence of ethanol in cells stimulated by overexpression of β_2-adrenergic receptors in the presence of isoproterenol (10 μM, 18–36 h). C, concentration-response curves for ethanol (1–200 mM) in controls (●) and after stimulation of β_2-AR with isoproterenol (■). Differences were not significant. ISO, isoproterenol.

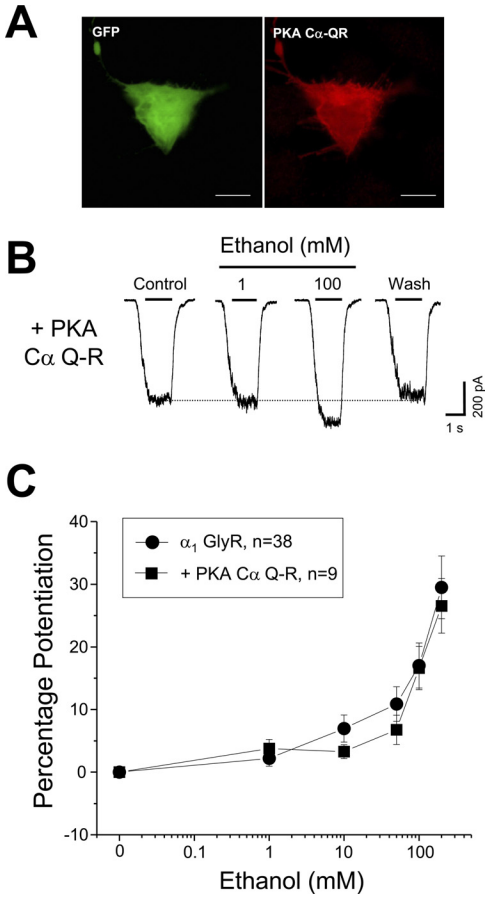

Fig. 4. PKA activity did not alter the ethanol sensitivity of GlyRs. A, the images show transfected HEK293 cells stained with antibodies against the hexahistidine epitope (red) that recognize tagged PKA Cα Q-R. GFP expression is shown in green (scale bar, 10 μm). B, glycine-activated responses in the presence of ethanol recorded in cells that expressed the constitutively active PKA Cα Q-R. C, concentration-response curves for ethanol (1–200 mM) in control (circles) and PKA Cα Q-R-expressing cells (■). Differences were not significant.

in sensitivity to ethanol produced by $G\alpha_s$ Q-L is via a membrane-delimited mechanism, independent of a diffusible second messenger and dependent on the Gβγ dimer (Yévenes et al., 2008; Guzman et al., 2009). Thus, we examined whether the latter pathway is involved in the shift on alcohol sensitivity promoted by $G\alpha_s$ Q-L. A commonly used strategy to explore the participation of Gβγ is the overexpression of "Gβγ sequesters," which are high-affinity proteins that bind the heterodimer and block their effects in a variety of cellular effectors (Ikeda, 1996; Kammermeier and Ikeda, 1999). Previous studies have identified the carboxyl-terminal region of the β-adrenergic receptor kinases (ct-β-adrenergic kinase or ct-G protein receptor kinase) as a specific sequester of Gβγ (Daaka et al., 1997; Kammermeier and Ikeda, 1999). Therefore, we studied the ethanol sensitivity of GlyRs after coexpression of $G\alpha_s$ Q-L and ct-GRK2. Of note, the Gβγ sequester significantly inhibited the effect of this active form of $G\alpha_s$ on ethanol sensitivity (Fig. 5, A and B). The potentiation of the glycine-evoked current with 100 mM ethanol was 9 ± 3% (n = 14) in the ct-GRK2-expressing cells, which was lower but not significantly different from the control condition (17 ± 3%, n = 38). These results suggest the presence of a Gβγ-driven, rather than a cAMP-PKA-mediated mechanism explaining the effect of active $G\alpha_s$ on ethanol sensitivity. Thus, we examined whether $G\alpha_s$ Q-L was able to enhance ethanol potentiation of two mutated GlyRs, which contained selective intracellular mutations in key basic residues that are critical for Gβγ modulation and ethanol effects (Yévenes et al., 2006, 2008). These mutated GlyRs were previously denominated 316–320A and 385–386A due to consecutive alanine substitutions in the corresponding residues ^{316}RFRRK^{320} and ^{385}KK^{386} of the human wild-type GlyR (Sadtler et al., 2003). The $G\alpha_s$ Q-L expression in HEK cells did not modify the ethanol sensitivity of these two mutant GlyRs (Fig. 5, C and D), supporting the idea that the mechanism by which $G\alpha_s$ Q-L enhances the ethanol potentiation in GlyRs requires the interaction of free Gβγ dimers with the large intracellular loop of the ion channel.

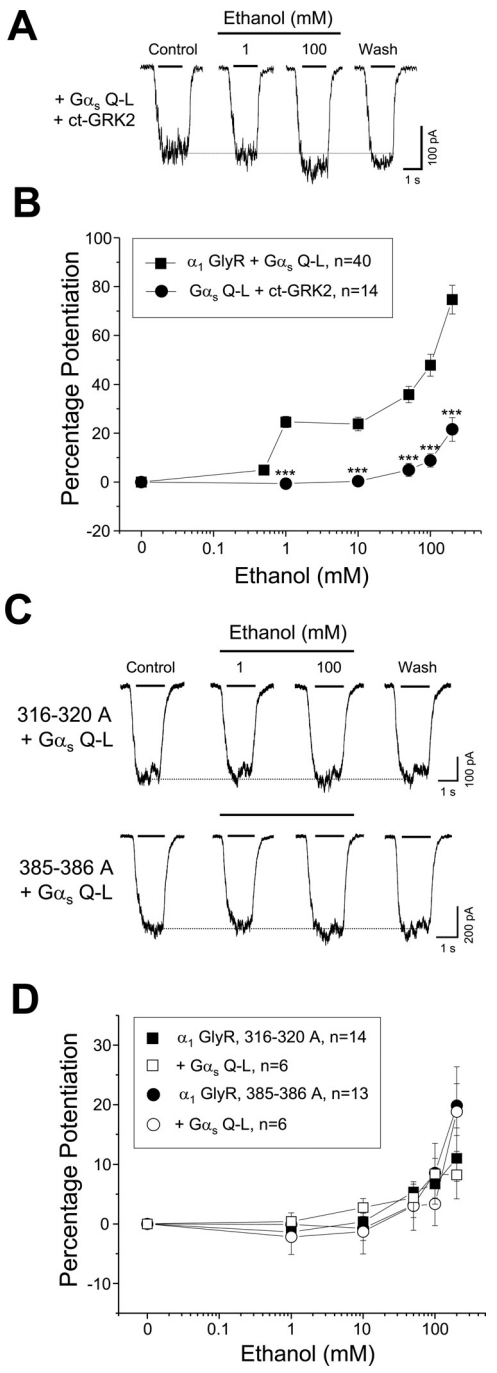

Fig. 5. The increase in ethanol sensitivity produced by activated $G\alpha_s$ depends on $\beta\gamma$ subunits. A, whole-cell recordings obtained in HEK293 cells that coexpressed $G\alpha_s$ Q-L and the $G\beta\gamma$ sequester ct-GRK2. Current traces during the application of 1 and 100 mM ethanol are shown. B, concentration response curves for ethanol (1–200 mM) show that the shift in the ethanol sensitivity promoted by $G\alpha_s$ Q-L (■) is blocked by expression of the $G\beta\gamma$ sequester (●). Differences were significant (***, $P < 0.001$, ANOVA). C, traces are Cl^- currents induced in two α_1 GlyRs with intracellular mutations in cells overexpressing $G\alpha_s$ Q-L during the application of different ethanol concentrations. D, graph shows that the expression of the constitutively active $G\alpha_s$ did not modify the ethanol sensitivity of either 316–320A (■) or 385–386A (●) mutated GlyRs with respect to cells expressing $G\alpha_s$ Q-L (○ and □). Differences were not significant.

Discussion

In the present study, we found that overexpression of an activated form of $G\alpha_s$ shifted the sensitivity of the GlyR toward lower concentrations of ethanol. This effect was blocked by reducing the activity of $G\beta\gamma$, suggesting that activated $G\alpha_s$ increases the availability of the dimer to interact with GlyRs.

Effects of Ethanol on G Protein Activation. Most ubiquitous cellular signal transduction pathways are associated to stimulation of GPCRs and subsequent activation of heterotrimeric G proteins (Hamm, 1998; Oldham and Hamm, 2008). Previous studies have shown that ethanol alters several cellular functions through modifications in G protein-associated signaling. For instance, it was reported that pharmacological ethanol concentrations (10–100 mM) affected $G\alpha_s$- and $G\alpha_q$-mediated intracellular pathways, causing changes in PKA and protein kinase C activities, respectively (Gonzales et al., 1986; Bode and Molinoff, 1988; Hoffman and Tabakoff, 1990; Yao et al., 2002; Morrow et al., 2004; Ron and Jurd, 2005). These findings are relevant because G proteins control diverse functions, including cell division, differentiation, and, in the case of the central nervous system, excitability regulation, synaptic transmission, and disease. Thus, it seems possible that ethanol might affect a number of cellular functions by altering G proteins. One of these functions can be associated with ion channels responsible for the control of neuronal excitability. In the present study, therefore, we used constitutively active mutant forms of $G\alpha$ subunits to study specific G protein pathways. These mutant proteins are unable to hydrolyze GTP, causing, in the case of $G\alpha_s$ Q-L, an elevation in intracellular cAMP through a sustained stimulation of adenylyl cyclase in the absence of GPCR activation (Masters et al., 1989), as shown in Fig. 3A. In addition, because the GTP-bound $G\alpha$ has a low affinity for $G\beta\gamma$, it is expected that the dimer will be available to interact with distinct effectors, such as ion channels (i.e., GlyRs). The present data support the latter possibility.

G Protein Activation Modifies Actions of Ethanol on Inhibitory LGICs. Ethanol affects the function of GlyRs by sites located in extracellular, transmembrane, and intracellular domains (Lynch, 2004; Perkins et al., 2010). In addition, previous studies have reported that the actions of ethanol on ion channels are affected by cell signaling associated with G proteins. For instance, potentiation of the glycine-activated current produced by ethanol is attenuated by protein kinase C inhibitors and guanosine 5′-*O*-(2-thiodiphosphate) in recombinant and neuronal systems (Aguayo et al., 1996; Mascia et al., 1998; Jiang and Ye, 2003; Zhu and Ye, 2005). In addition, although the inhibition of PKA was unable to alter ethanol effects on GlyRs (Aguayo et al., 1996; Mascia et al., 1998), chronic incubation with cholera toxin, which specifically affects the $G\alpha_s$ pathway, strongly abolished the alcohol potentiation in spinal neurons (Aguayo et al., 1996). Furthermore, the sensitivity of $GABA_A$Rs to ethanol was affected by G protein activation and protein kinases (Weiner et al., 1994; Freund and Palmer, 1997; Aguayo et al., 2002). Thus, this experimental evidence suggests that G proteins and associated kinases have critical roles in the control of ethanol effects on GlyRs and $GABA_A$Rs.

In the present study, we found that activated $G\alpha_s$, but not $G\alpha_q$ or $G\alpha_{i2}$, subunits affect the ethanol sensitivity of human α_1 GlyRs. Furthermore, the effect of activated $G\alpha_s$ occurred through a $G\beta\gamma$-linked mechanism, which was not reproduced by increases in cAMP and PKA-associated signaling. These results are in line with our previous studies showing that ethanol actions are affected by the interaction of $G\beta\gamma$ with

the intracellular loop of the ion channel (Yévenes et al., 2008; Guzman et al., 2009). If we take into account these observations, it appears that the activated form of $G\alpha_s$ shifts the ethanol sensitivity, facilitating the functional interaction between $G\beta\gamma$ and the GlyR. This enhanced interaction is probably produced by an increase in free $G\beta\gamma$ unable to bind to the excess GTP-bound $G\alpha_s$. At this point, the presence of a direct interaction between activated $G\alpha_s$ and GlyRs emerges as a potential chance to fine-tune the $G\beta\gamma$ interaction in a membrane-delimited context, especially considering previous reports that show direct interactions between $G\alpha$ subunits and ion channels such as nicotinic acetylcholine receptors and G protein-coupled inwardly rectifying potassium channels (Peleg et al., 2002; Fischer et al., 2005). However, our results cannot eliminate other possibilities such as the facilitation of the $G\beta\gamma$-GlyR interaction as a consequence of cytoskeleton modifications induced by activated $G\alpha_s$, which also is independent of cAMP and PKA (Yu et al., 2009).

Significance of the Present Findings for Ethanol Intoxication. We found that the overexpression of a constitutively active $G\alpha_s$ mutant ($G\alpha_s$ Q-L) shifted the ethanol sensitivity of GlyRs toward lower ethanol concentrations (1–10 mM). In this study, we used a higher concentration of glycine than was used in other previous studies (Mihic et al., 1997; Ye et al., 1998; Jiang and Ye, 2003; Crawford et al., 2007; Yévenes et al., 2008), because the effect of ethanol became much more evident already at 1 to 10 mM, which is close to the concentration achieved during low to moderate alcohol intake. At the glycine concentration used in the study, the receptors should have a higher saturation with the agonist, somewhat closer to a synaptic condition. Although our results do not define a mechanism by which activated $G\alpha_s$ subunits modulate the sensitivity of GlyR to alcohol, they show a new role for $G\alpha_s$ in GlyR pharmacology, which can help to reconcile the controversial results obtained by studying $GABA_A$ receptors, which at times can be more or less sensitive to ethanol (Aguayo et al., 2002; Weiner and Valenzuela, 2006). In addition, this study confirms the role of $G\beta\gamma$ interaction for the ethanol sensitivity of GlyRs (Yévenes et al., 2008; Guzman et al., 2009). Because GlyR potentiation by ethanol might be related to acute intoxication, sleep obstructive apnea, ethanol intake, and possibly alcoholism (Gibson and Berger, 2000; Molander et al., 2005), these data could also be useful for the pharmacological control of ethanol effects on GlyRs in vivo. The present results show that the effects of ethanol and $G\alpha_s$ Q-L were similar in homomeric α_1 and heteromeric $\alpha_1\beta$ receptors, and this implies that the sensitivity to ethanol of both synaptic and extrasynaptic GlyRs could be enhanced by G protein activation. The significance of homomeric receptors in relation to ethanol consumption is interesting because brain regions without significant glycinergic inputs, such as the nucleus accumbens and ventral tegmental area (Molander et al., 2005; Wang et al., 2005), are extrasynaptic in nature. Although the subunit composition of these GlyRs is still not clear and despite the fact that our study only examined α_1-containing GlyRs, our results suggest that G protein activation might also increase the ethanol sensitivity of other GlyR subunits (e.g., α_2 and α_3). Thus, the increase in neuronal inhibition resulting from the higher sensitivity of these receptors to ethanol should produce a strong reduction in excitability, shifting the toxic effects of ethanol toward lower concentrations. Future experiments designed to investigate these ideas will clarify the specific roles of activated $G\alpha_s$ and $G\beta\gamma$ on the alcohol sensitivity of GlyRs. Finally, it is possible to propose that during disorders that induce modifications in $G\alpha_s$-associated pathways (i.e., stress or antiasthmatic therapy with β_2 agonists) or altered levels of GTP-bound G proteins, the depressing effects of ethanol on the central nervous system might be enhanced because of the presence of higher levels of activated $G\alpha_s$.

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Authorship Contributions

Participated in research design: Yévenes, Moraga-Cid, Romo, and Aguayo.

Conducted experiments: Yévenes, Moraga-Cid, and Romo.

Performed data analysis: Yévenes, Moraga-Cid, and Romo.

Wrote or contributed to the writing of the manuscript: Yévenes and Aguayo.

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