

The Basic Property of Lys385 Is Important for Potentiation of the Human $\alpha 1$ Glycine Receptor by Ethanol

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ABSTRACT

Ethanol alters the function of several members of the Cys-loop ligand-gated ion channel superfamily. Recent studies have shown that the sensitivity of the $\alpha 1$ glycine receptor (GlyR) to ethanol can be affected by the state of G protein activation mediated by the interaction of G $\beta\gamma$ with intracellular amino acids in the GlyR. Here, we evaluated the physicochemical property of Lys385 that contributes to ethanol modulation by using mutagenesis, patch-clamp, and biochemical techniques. A conserved substitution (K385R) did not affect either the apparent glycine EC₅₀ (40 ± 1 versus $41 \pm 0.5 \mu\text{M}$) or the ethanol-induced potentiation (53 ± 5 versus $46 \pm 5\%$) of the human $\alpha 1$ GlyR. On the other hand, replacement of this residue with glutamic acid (K385E), an acidic

amino acid, reduced the potentiation of the GlyR to $10 \pm 1\%$. Furthermore, mutations with a hydrophobic leucine (K385L), a hydrogen bond donor glutamine (K385Q), or a neutral residue (K385A) also reduced ethanol modulation. Finally, substitution by a large and hydrophobic residue (K385F) and deletion of 385 (Lys385₋) reduced ethanol modulation to 10 ± 4 and $17 \pm 0.4\%$, respectively. Experiments using dynamic cysteine substitution with a methanethiosulfonate reagent and homology modeling indicate that the basic property and the position of Lys385, probably because of its interaction with G $\beta\gamma$, is critical for ethanol potentiation of the receptor.

Introduction

Glycine is the principal inhibitory neurotransmitter present in the spinal cord and brain stem, and it has significant action in motor and respiratory control (Legendre, 2001; Lynch, 2004). It activates homomeric and heteromeric glycine receptors (GlyRs), which are composed of five subunits (α_{1-4} and β). GlyRs, such as GABA_A receptors, 5-hydroxytryptamine type 3 (5-HT₃) receptors, and nicotinic acetylcholine receptors (nAChRs), belong to the Cys-loop ligand-gated ion channel superfamily (Cys-loop LGICs) (Lester et al., 2004). It is noteworthy that these receptors share several structural similarities, such as the presence of a large N-ter-

minal extracellular domain that contains the ligand binding site and four transmembrane (TM) segments (TM₁₋₄) with the pore formed by TM2 and one large intracellular loop (IL) connecting TM₃ and TM₄ that participate in intracellular modulations (Lester et al., 2004; Unwin, 2005).

Similar to other members of the family, the function of GlyRs can be altered by several modulators and intracellular signals, such as neurosteroids (α -xalone), Zn²⁺, protein kinase C, cAMP-dependent protein kinase (Legendre, 2001; Lynch, 2004), and G $\beta\gamma$ (Ahmadi et al., 2002; Yevenes et al., 2006). Furthermore, general anesthetics and alcohols are potent modulators of this inhibitory receptor (Aguayo and Pancetti, 1994; Yamakura et al., 1999; Harris et al., 2008). It is believed that the effect of ethanol on the GlyR is relevant because of its implications in human health, including alterations in motor control, respiration, and cardiovascular depression.

Several studies have intended to elucidate the mechanisms by which ethanol can affect LGICs such as GlyRs (Harris et al., 2008; Yevenes et al., 2008), 5-HT₃ receptors (Lovinger and White, 1991), nAChRs (Godden et al., 2001), GABA_A receptors (Mihic et al., 1997), and N-methyl-D-aspartate receptors (Ren et al., 2008). In the case of GlyRs, for example,

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ABBREVIATIONS: GlyR, glycine receptor; IL, intracellular loop; LGIC, ligand-gated ion channel; nAChR, nicotinic acetylcholine receptor; 5-HT₃, 5-hydroxytryptamine type 3; TM, transmembrane; HEK, human embryonic kidney; PDB, Protein Data Bank; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; SCAM, substituted-cysteine accessibility method; WT, wild type; MTSEA, methanethiosulfonate ethylammonium; ESP, electrostatic surface potential.

the presence of binding sites in TM regions (Mihic et al., 1997; Ye et al., 1998; Yamakura et al., 1999) and the activation of G $\beta\gamma$ proteins (Yevenes et al., 2008) have been postulated to be important for ethanol sensitivity. In addition, a critical residue in loop 2 of the extracellular domain, Ala52, was reported to modulate ethanol sensitivity on the receptor (Perkins et al., 2008).

It was previously shown that several residues in the IL play roles on the physiological and pharmacological properties of various LGICs. For example, it was reported that the IL regulates nAChR and GlyR channel gating via G $\beta\gamma$ binding (Fischer et al., 2005; Yevenes et al., 2006). In addition, a single residue (Arg436) in the IL of the human 5-HT_{3A} receptor was found to regulate channel conductance (Deeb et al., 2007; Carland et al., 2009). Furthermore, another study showed that the most important residue controlling the actions of G $\beta\gamma$ and ethanol on GlyRs was Lys385 (Yevenes et al., 2008). Therefore, in the present study we characterized which physicochemical property of K385 is correlated to ethanol-induced modulation of this receptor by using site-directed mutagenesis, patch clamp, cysteine-recognizing MTS reagents, and structural modeling.

Materials and Methods

cDNA Constructs. The cDNA construct encoding the human glycine receptor $\alpha 1$ subunit with a C-terminal hexahistidyl tag sub-

cloned in a pCI vector (Promega, Madison, WI) for expression in HEK 293 cells has been described previously (Yevenes et al., 2006). Mutations were inserted by using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). All constructions were confirmed by full sequencing. The glycine receptor amino acids were numbered according to their positions in the mature protein sequence.

Cell Culture and Transfection. HEK 293 cells were cultured by using standard methodologies. HEK 293 cells were transfected by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with 2 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, and recordings were made after 18 to 24 h.

Electrophysiology. Whole-cell recordings were performed as described previously (Yevenes et al., 2006). A holding potential of -60 mV was used. Patch electrodes were filled with 140 mM CsCl, 10 mM 1,2-bis(*O*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid, 10 mM HEPES, pH 7.4, 4 mM MgCl₂, 2 mM ATP, and 0.5 mM GTP. The external solution contained 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. For G protein activation experiments, GTP γ S (0.5 mM; Sigma, St. Louis, MO) was added directly to the internal solution, replacing GTP. The amplitude of the glycine current was assayed by using a brief (1–6 s) pulse of glycine every 60 s. The modulation of the glycine current by 100 mM ethanol (Sigma) was assayed by using a short application of glycine (EC₂₀) coapplied with ethanol to each receptor studied without any preapplication. Although the effect of ethanol was evident at 10 mM (Aguayo et al., 1996), in this study we used 100

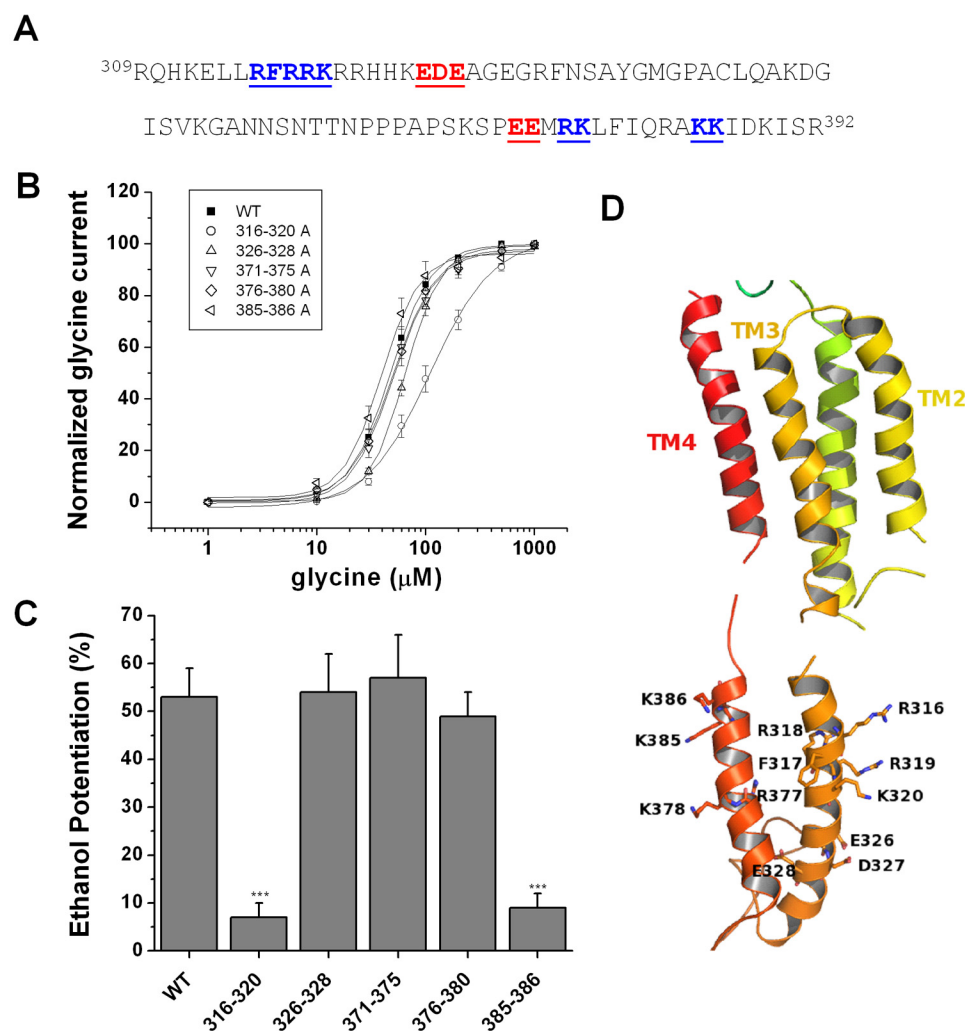


Fig. 1. Intracellular residues in the intracellular loop of $\alpha 1$ GlyR important for ethanol-induced potentiation. **A**, primary sequence of the large IL between TM3 and TM4. The red and blue colors indicate the positive or negative charged residues, respectively. **B**, the graph summarizes concentration-response relationships for WT and 316–320Ala, 326–328Ala, 371–375Ala, 376–380Ala, and 385–386Ala mutations. **C**, effects of mutations of basic and acidic residues on GlyR potentiation induced by 100 mM ethanol. ***, $p \leq 0.001$. Each bar represents the mean \pm S.E.M. obtained from at least five cells. **D**, homology ribbon model for the IL where the residues colored in **A** are indicated.

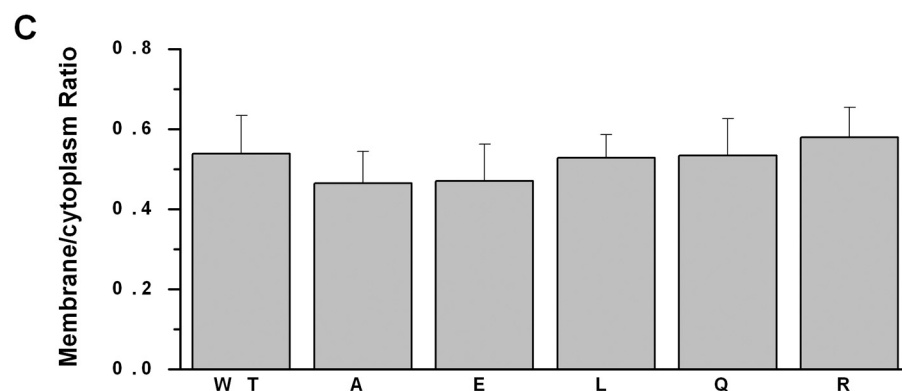
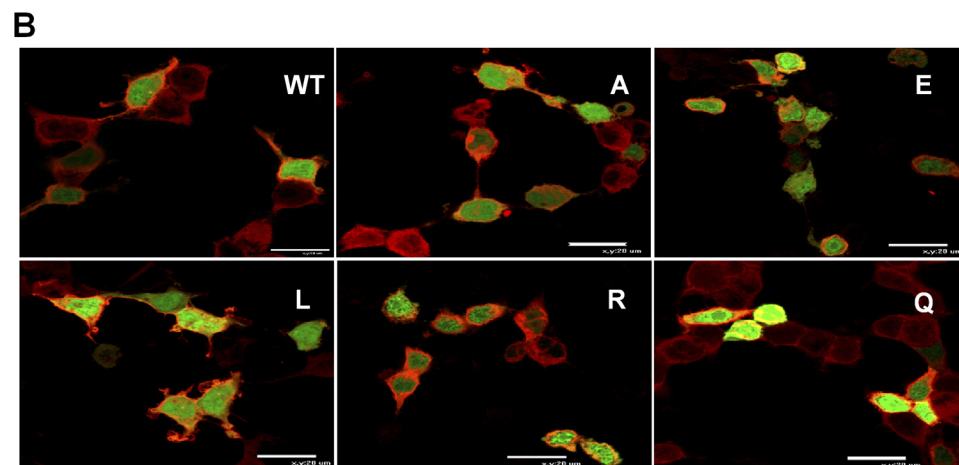
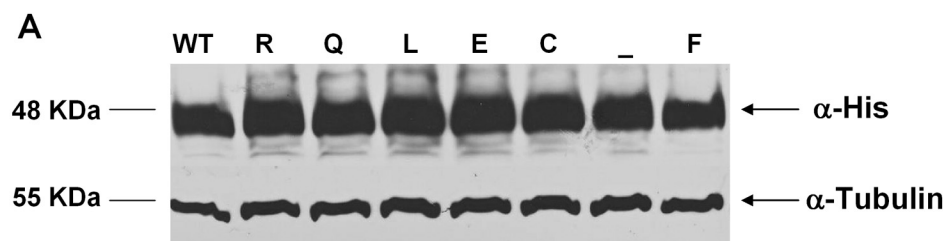
TABLE 1

Electrophysiological parameters of WT and Lys385 mutations of $\alpha 1$ GlyR

Shown is a summary of the values for glycine sensitivity (EC_{50}), Hill coefficient (n_H), and maximal currents (I_{max}) for WT and the different mutations of $\alpha 1$ GlyR. The EC_{50} , n_H , and I_{max} values were measured from the glycine concentration-response relationships by using 1 to 1000 μM . K385H[±] corresponds to values obtained at pH 6.0. Each value represents the mean \pm S.E.M.

Receptor	No. of Cells	EC_{50}	n_H	I_{max}
		μM		pA
WT	10	40 ± 1	2.4 ± 0.1	2771 ± 563
316-320Ala	15	$110 \pm 8^*$	$1.5 \pm 0.1^*$	$1787 \pm 237^*$
326-328Ala	16	68 ± 1	$2.5 \pm 0.0.1$	3622 ± 401
371-375Ala	6	53 ± 3	2.2 ± 0.2	4560 ± 695
376-380Ala	5	50 ± 2	2.1 ± 0.2	3630 ± 755
385-386Ala	9	38 ± 2	2.5 ± 0.3	4162 ± 654
K385R	4	41 ± 0.5	2.4 ± 0.1	2947 ± 687
K385Q	5	23 ± 0.5	2.9 ± 0.2	2952 ± 178
K385L	8	23 ± 0.4	2.5 ± 0.1	3155 ± 460
K385E	6	$11 \pm 0.4^*$	2.0 ± 0.1	3273 ± 847
K385F	5	$98 \pm 1.5^*$	2.5 ± 0.1	2868 ± 1228
K385A	7	23 ± 0.5	2.4 ± 0.1	2855 ± 616
K385C	7	$16 \pm 1^*$	$1.7 \pm 0.1^*$	$1129 \pm 185^*$
K385H	7	18 ± 0.5	2.6 ± 0.1	2491 ± 341
K385H [±]	4	42 ± 0.5	2.5 ± 0.1	3664 ± 957

* $P \leq 0.05$ with respect to WT.



mM to facilitate statistical analysis. In all of 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 EC_{20} in each single experiment. Cells having responses $< EC_{50}$ or $> EC_{20}$ were discarded. Strychnine (1 μM) blocked all of the current elicited by wild-type and mutant glycine receptors (data not shown). For substituted-cysteine accessibility method (SCAM) studies, stock solutions of MTS reagents (200 mM) obtained from Toronto Research Chemicals Inc. (North York, ON, Canada) were stored at $-20^{\circ}C$. Before each experiment, MTS reagents were diluted in the electrode solution for whole-cell experiments to yield a final concentration of 200 μM .

Immunofluorescence, Image Visualization, and Analysis. HEK 293 cells were first fixed with 4% paraformaldehyde (0.1 M phosphate buffer, pH 7.4) and then permeabilized (0.3% Triton X-100) and blocked with 10% horse serum. Subsequently, all night incubation with a polyclonal hexa-histidine antibody (His Tag; US Biological, Swampscott, MA) was carried out. Epitope visualization was performed by incubating the sample with secondary antibodies conjugated to fluorescein isothiocyanate and Cy5 (1:600; Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Finally, the cells were coverslipped by using Fluorescence Mounting Medium (Dako North America, Inc., Carpinteria, CA). For quantitative analysis, cells were chosen randomly for imaging using Nikon confocal

Fig. 2. Expression and localization of Lys385 mutants overexpressed in HEK cells. A, the Western blots were obtained from HEK cell lysates expressing the WT and mutant versions of $\alpha 1$ GlyR. Each letter corresponds to the substitution (R, K385R; Q, K385Q; L, K385L; E, K385E; C, K385C; F, K385F), and _ represents deletion. B, confocal microscopy obtained in HEK cells transfected with the $\alpha 1$ GlyR WT and single point mutations in Lys385. A, Ala; E, Glu; L, Leu; R, Arg; Q, Gln. The red signal corresponds to GlyR detected with an anti-His antibody, and the green color corresponds to green fluorescent protein used for the identification of transfected cells for patch clamp recordings. The bar corresponds to 20 μm . C, the graph summarizes the fluorescence ratio between membrane (periphery) and cytoplasm (center) associated to GlyR WT and mutant expression using an anti-His antibody.

microscopy (TE2000; Nikon, Melville, NY). Single stacks of optical sections in the z-axis were acquired, and dual-color immunofluorescent images were captured in simultaneous two-channel mode. The ratio of receptor fluorescence intensities obtained in the periphery and cytoplasm in transfected HEK cells was analyzed as a way of indirectly evaluating receptor membrane expression of the mutants by using an ImageJ software plugin (National Institutes of Health, Bethesda, MD).

Western Blot. HEK cells cultured in 35-mm wells were lysed with standard solution. The amount of protein was quantified by using microBSA (Thermo Fisher Scientific, Waltham, MA). Two micrograms of total protein was charged per lane in a polyacrylamide denaturing 12% gel and run for 2 h. The proteins were transferred to a nitrocellulose membrane for immunodetection by using a primary antibody against His Tag (US Biological; 1:500, 4°C overnight) and a secondary antibody against IgG coupled to horseradish peroxidase (1:1000; room temperature; 2 h). Signal detection was measured by using a Luminescence kit (Thermo Fisher Scientific).

Molecular Modeling. The GlyR model was constructed by homology using coordinates from the Torpedo nAChR at 4-Å resolution (Unwin, 2005) (PDB ID code 2BG9) and acetylcholine-binding protein structure (PDB ID code 1UV6) (Celie et al., 2004) using the software Modeler (Eswar et al., 2006). The models were relaxed by energy minimization using a Conjugate Gradient protocol with GROMACS (Hess et al., 2008). To optimize the H-Bond net, the models were processed by the server REMO (<http://zhanglab.cmb.med.umich.edu/REMO/>; Li and Zhang, 2009).

Intracellular Loop Modeling. Because of the lack of sequence identity with a protein having a defined structure that allows building a protein model by “homology modeling,” we decided to model this region by a “folding recognition” technique. For this, we analyzed the intracellular loop sequence in the web server for folding recognition, 3D-Jury (Ginalski et al., 2003). We obtained a template in the SecG subunit of the preprotein translocase (PDB ID code 3DIN). Using Modeler and the E chain of 3DIN as a template, we developed the 3D model of the GlyR intracellular loop for WT and mutants.

Electrostatic Potential Surface. Using APBS (Baker et al., 2001) together with the input files generated by PDB2PQR (Dolinsky et al., 2007), the electrostatic potential surface was generated. To evaluate the difference between wild type and mutants on electrostatic potential surfaces, we used the server VisualDEP (<http://152.74.15.29>). All the images were generated with the software PyMol (for reference see Yevenes et al., 2008) and retouched with GIMP 2.6.10 (<http://www.gimp.org/>). To determine the partial charge distribution in the MTS-modified cysteine residue, Ghemical 2.99 (<http://www.brothersoft.com/gchemical-349099.html>) and MPQC/6-31** (<http://www.mpqc.org/>) as quantum mechanic engines were used (Hassinen and Peräkylä, 2001).

Data Analysis. Statistical analyses were performed by using analysis of variance and are expressed as arithmetic mean \pm S.E.M. Values of $P \leq 0.05$ were considered statistically significant. For all the statistical analysis and plots, Origin 6.0 software (MicroCal LLC, Northampton, MA) was used. Normalized values were obtained by dividing the current amplitude obtained with the time of GTP γ S dialysis by the current at minute one.

Results

Several studies have shown that positively charged residues are important for the modulation exerted by G $\beta\gamma$ in several effectors, such as phospholipase C β and G protein-coupled inwardly rectifying potassium channel (Touhara et al., 1995; Ford et al., 1998; Cantí et al., 1999; Barr et al., 2000). Clusters of charged residues present in the IL of the GlyR were analyzed to determine whether their mutations modified the extent of G $\beta\gamma$ -dependent ethanol-induced po-

tentiation in human $\alpha 1$ GlyRs. Five clusters of charged residues exist in the IL (316–320, 326–328, 374–375, 377–378, and 385–386) (Fig. 1A), and all the mutations of these residues to alanine were able to form functional membrane receptors whose properties are shown in Fig. 1B and Table 1. Whereas ethanol potentiated the WT GlyR by $53 \pm 5\%$, alanine substitutions in the 316 to 320 and 385 to 386 clusters significantly decreased the effect of ethanol to 7 ± 3 and $9 \pm 3\%$, respectively. On the other hand, alanine substitutions in the acidic 326 to 328 cluster, or in positions 374 to 378, did not alter the potentiation by ethanol (Fig. 1C). After these results, we focused on the Lys385 residue because it was reported that it displayed the largest contribution to ethanol potentiation (Yevenes et al., 2008). The localization of Lys385 in the intracellular loop is relevant because it has been suggested that the sensitivity to ethanol depends on protein-protein interactions between G $\beta\gamma$ and GlyRs (Yevenes et al., 2008). This potential interaction would implicate complementary charges with G $\beta\gamma$ and secondary structure requirements (Yevenes et al., 2008). In addition, it is known that the IL in other LGICs participates in ion channel function and receptor intracellular modulation (Fi-

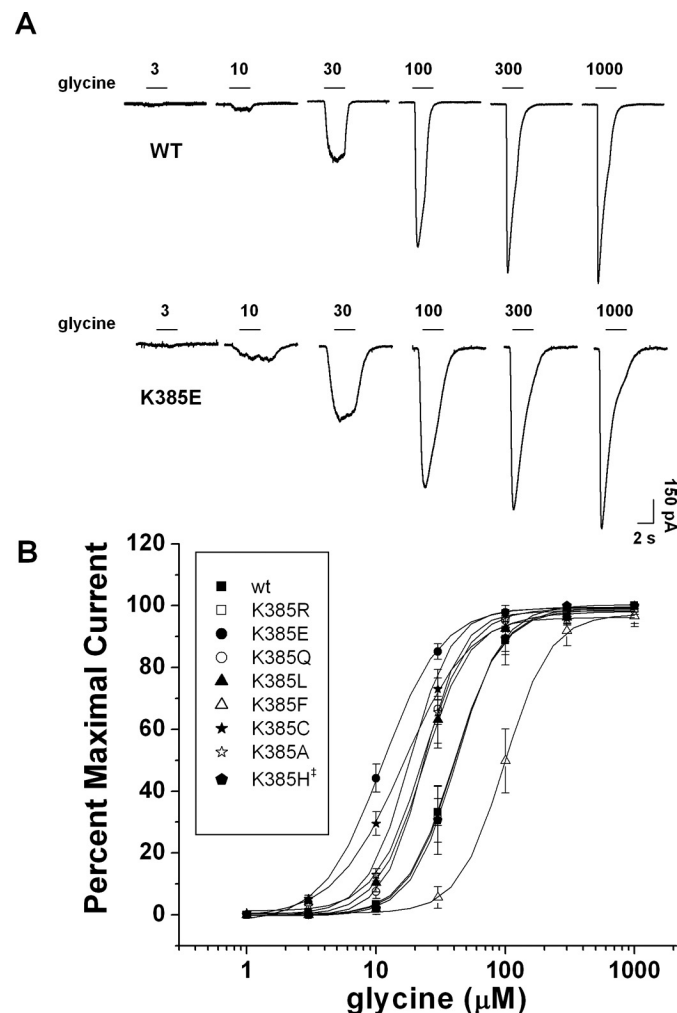


Fig. 3. Macroscopic electrophysiological properties of WT and mutant GlyRs recorded in HEK 293 cells. A, the current traces correspond to WT and the K385E mutation using several glycine concentrations in micromolar. B, the graph summarizes concentration-response relationships for WT and K385R, K385E, K385Q, K385A, K385H[±], K385L, K385F, and K385C mutations.

scher et al., 2005; Deeb et al., 2007). Using homology models for LGICs (Unwin, 2005; Yevenes et al., 2006, 2008) and a “threading” approach to predict structure in the less characterized IL, we constructed a model for this region. The results show that Lys385 is facing outward (Fig. 1D) and forms a common region with this basic cluster facing the other basic region of the IL (positions 316–320).

Expression and Localization of Lys385 Mutants. We decided to use a mutagenesis approach that would not significantly alter the α -helix structure proposed to exist in this region (Anderson et al., 2005; Unwin, 2005; Livesey et al., 2008). In addition, previous studies have shown that modifications in basic residues present in the IL can affect the correct topology and alter the accurate folding of the receptor

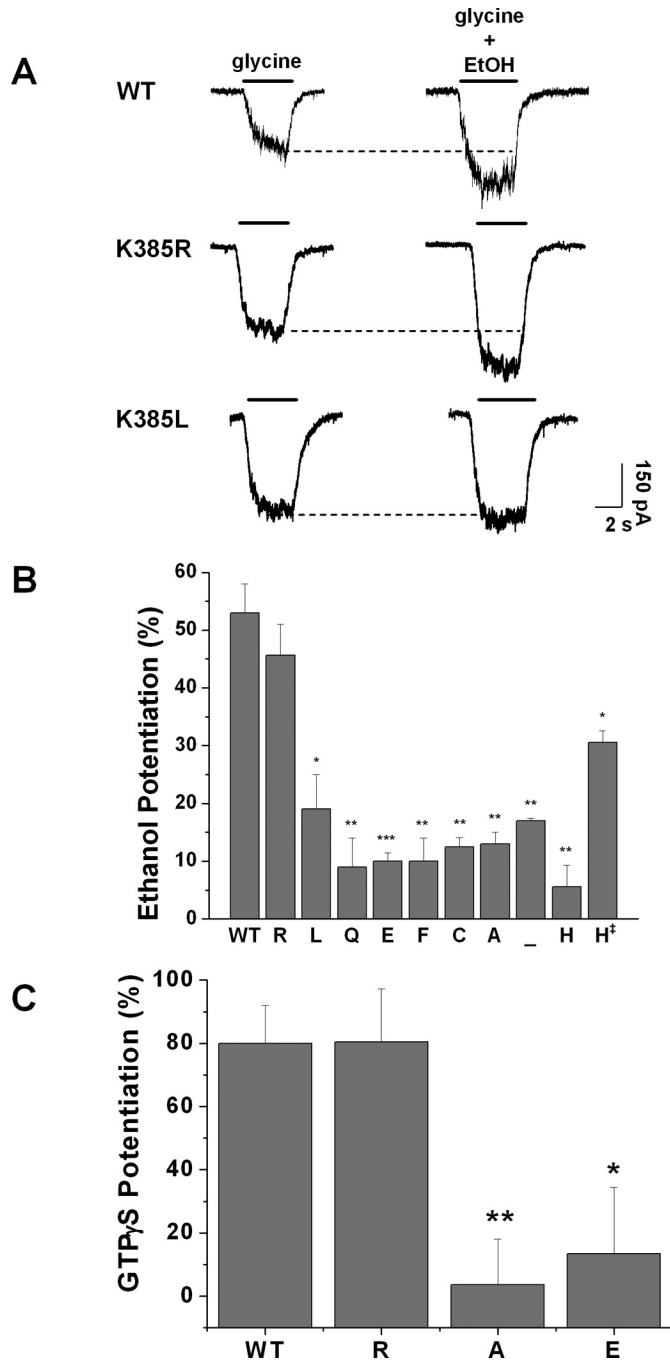


Fig. 4. The presence of a basic residue in 385 determines ethanol potentiation in $\alpha 1$ GlyR. **A**, the current traces show the effects of ethanol (100 mM) in WT, K385R, and K385L mutations recorded at an equipotent concentration of glycine (EC_{20}). **B**, the graph illustrates the ethanol-induced potentiation (100 mM) on WT and the 385 mutants. R, K385R; L, K385L; Q, K385RQ; E, K385E; F, K385F; C, K385C; A, K385A. H and H⁺ are the unprotonated and protonated forms of histidine residue, respectively. **C**, the graph shows the percentage of potentiation obtained in the presence of 500 μ M intracellular GTP γ S at 15 min in WT, K385R (R), K385A (A), and K385E (E). *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

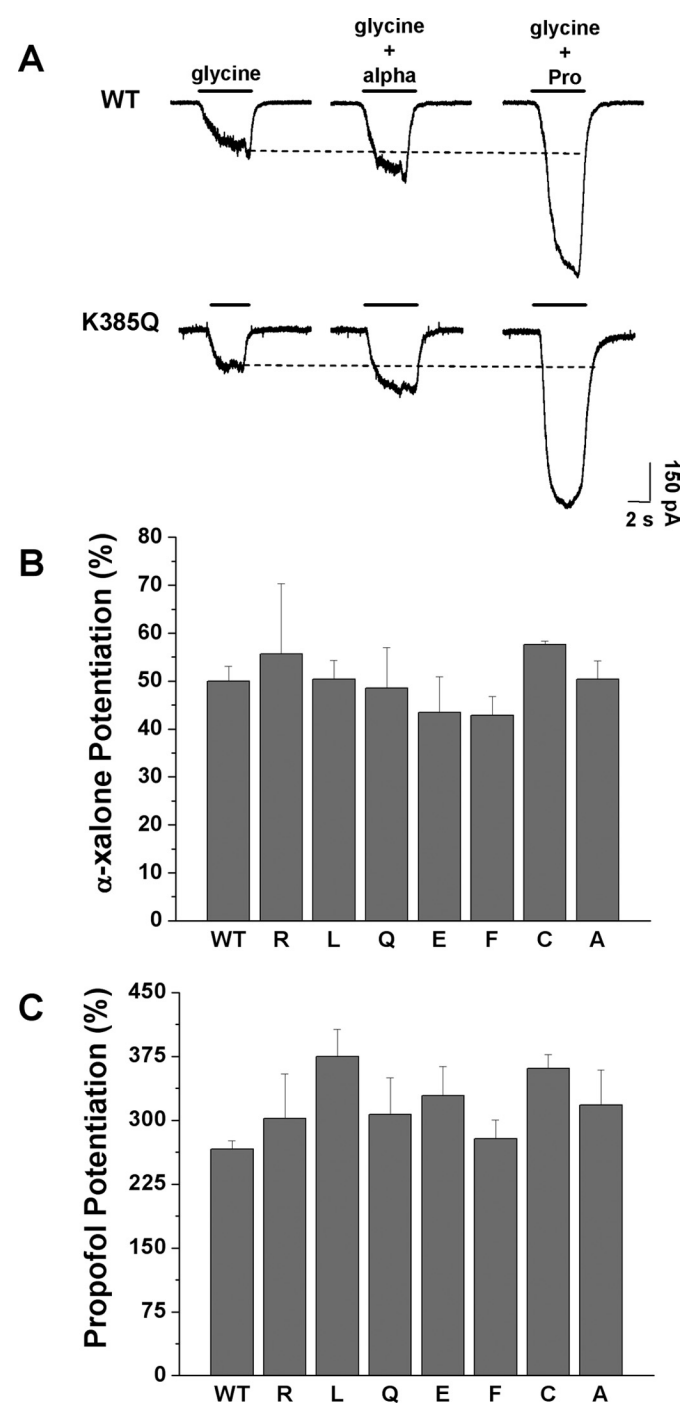


Fig. 5. Substitutions of residues with distinct physicochemical properties in 385 did not affect propofol and α -xalone modulations. **A**, current traces in the absence and presence of α -xalone (50 μ M) and propofol (30 μ M) in WT and the K385Q mutation at EC_{20} glycine. **B**, the graph summarizes the percentage potentiation of WT and mutants in the presence of α -xalone. **C**, the graph summarizes the percentage of potentiation of WT and mutants in presence of propofol. R, K385R; L, K385L; Q, K385RQ; E, K385E; F, K385F; C, K385C; A, K385A.

in the membrane (Sadtler et al., 2003). This mutagenesis approach ranks substitutions to preserve the appropriate secondary structure in the domain of interest while altering a single physicochemical property of Lys385 (Anderson et al., 2005). Western blot analysis done after 24 h post-transfection, using tubulin as a housekeeping control, showed that WT and mutants (Arg, Leu, Gln, Glu, Phe, Cys, and deletion) were expressed to very similar levels (Fig. 2A). Additional confocal microscopy analysis using 0.2- μ m sections revealed that the expressed receptors (Fig. 2B, shown in red) were located mainly in the cell periphery, possibly associated with the cellular membrane (Fig. 2, B and C).

Sensitivity of Lys385 Mutants to Glycine. Previous studies showed that the effects of ethanol on $\alpha 1$ GlyRs highly depend on glycine concentration, starting at 10 mM (Aguayo et al., 1996; Ye et al., 1998). Therefore, before testing the effect of ethanol on the distinct Lys385 mutations, we evaluated basic electrophysiological properties by using the whole-cell patch-clamp technique with various concentrations of glycine. Macroscopic parameters for the Cl^- current, such as EC_{50} , Hill coefficient (n_H), and maximum current (I_{max}), were compared in HEK 293 cells expressing the WT and mutant receptors. Figure 3A illustrates representative traces of currents recorded for WT and K385E receptors in the presence of increasing glycine concentrations. The data show that the shape of the current was very similar in both WT and the K385E mutant. Full concentration-response curves for substituted receptors (K385R, K385Q, K385L, K385E, K385F, K385H $^+$, K385A, and K385C) are shown in Fig. 3B. Data for the values of EC_{50} , n_H , and I_{max} for WT and mutants are given in Table 1, and these results show that the conserved substitution (K385R) displayed a concentration-response curve with similar characteristics to WT (Fig. 3B, \square and \blacksquare , respectively). The K385H mutant was studied at pH 6.0 and 7.4 to evaluate whether its level of protonation was able to alter the receptor physiological properties. It is note-

worthy that the EC_{50} was 42 ± 1 at 6.0 and 18 ± 1 at 7.4 (Table 1). On the other hand, K385E (Fig. 3B, \bullet) and K385C (Fig. 3B, \star) substitutions shifted the curve to the left, reducing the EC_{50} values with respect to WT. The K385F mutant (Fig. 3B, \triangle), on the other hand, showed the largest increase in the EC_{50} of the series, and this is in correlation with its position in the amino acid priority substitution rank (Anderson et al., 2005). In summary, all Lys385 mutants were expressed in the cell membrane and were functional.

Effects of Substitutions in Lys385 to Ethanol Sensitivity. Representative traces of the Cl^- current activated by using an equipotent concentration of glycine (EC_{20}) for WT, K385R, and K385L mutations in the presence or absence of 100 mM ethanol are shown in Fig. 4A. The data show that only the K385R substitution maintained the WT ethanol sensitivity, whereas all of the other substitutions abolished this potentiation (Fig. 4B). Additional studies with the protonated K385H mutation (H $^+$; pH 6.0) showed that ethanol was able to potentiate the current, although to a lower degree ($31 \pm 2\%$) than the WT. K385H was insensitive to ethanol at pH 7.4 (H), suggesting that the positive charge is important for the ethanol modulation (Fig. 4B). Finally, in agreement with previous studies showing that ethanol requires G $\beta\gamma$ protein activation to potentiate the WT GlyR (Yevenes et al., 2008, 2010), we found that the current elicited by activation of K385R was positively modulated by intracellular GTP γ S, whereas that in K385E was insensitive to modulation by G $\beta\gamma$ (Fig. 4C).

Specificity of the Mutations for Ethanol Sensitivity. Previous studies have shown that mutations in a single residue can affect the receptor sensitivity to different modulators (Mihic et al., 1997). Therefore, we tested whether modifications in the physicochemical property at position 385 could render the mutants insensitive to neurosteroids and general anesthetics. Figure 5 shows that α -xalone and propofol produced the same degree of potentiation in WT and mutant receptors (Fig. 5).

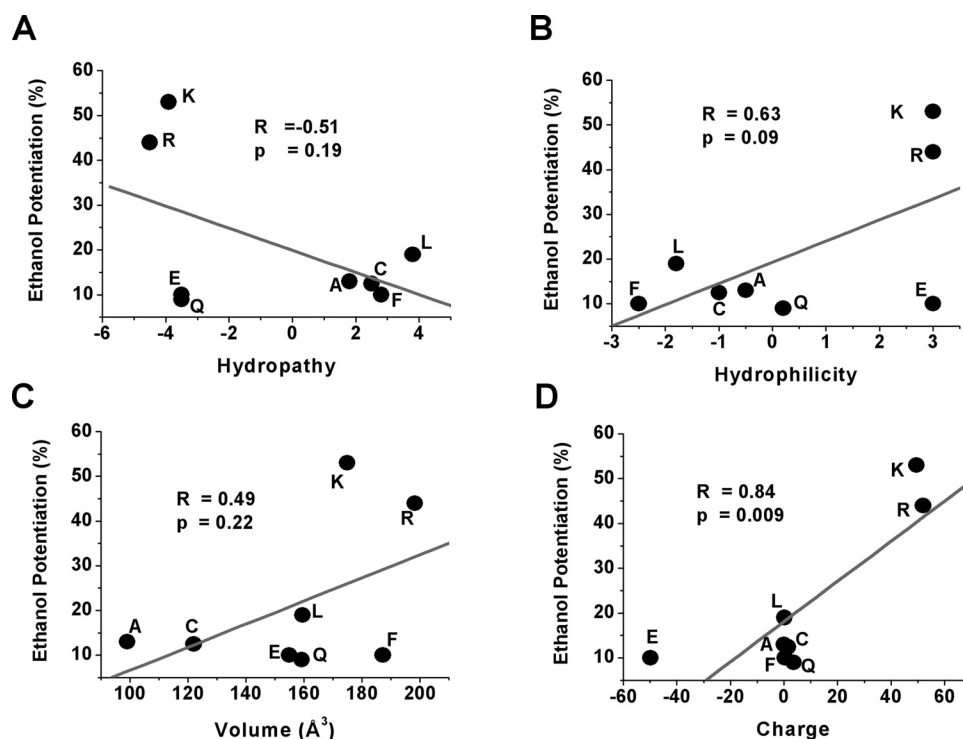


Fig. 6. The effects of ethanol depend on the basic property of the residue present at position 385. The graphs illustrate the relationships between the different physicochemical properties of the residues substituted in position 385 and the effect of 100 mM ethanol. A, hydropathy. B, hydrophilicity. C, volume. D, charge. Note that only polarity was highly correlated to ethanol modulation ($p < 0.009$). R was 0.84. R, Arg; L, Leu; Q, Gln; E, Glu; F, Phe; C, Cys; A, Ala.

Correlation between Ethanol Sensitivity and Physicochemical Properties. Next, we examined whether the presence of potentiation was correlated to one or more physicochemical properties of the substitutions to establish correlations that could help to understand the chemical nature of ethanol sensitivity in the GlyR. To construct these correlations, the values for volume, hydrophilicity, hydropathy, and charge for each of the seven substitutions were plotted against the potentiation of the receptor by ethanol (Ye et al., 1998; Ren et al., 2008). The analyses showed that the residue charge was the only property statistically related to the potentiation of $\alpha 1$ subunits by ethanol ($p = 0.009$; $r = 0.84$). The other properties did not show any significant correlation with the degree of current potentiation (Fig. 6).

Evaluation of the Influence of Lys385 on the Surface Electrostatic Potential. To analyze the contribution of Lys385 on the surface electrostatic potential, we analyzed the predicted three-dimensional model of the IL by using APBS analysis (Baker et al., 2001) and VisualDep (<http://152.74.15.29>). The region containing Lys385 showed a highly positive electrostatic potential (Fig. 7, A and B, shown in blue inside the circle). On the other hand, the mutant K385E displayed a shift in the surface electrostatic potential, indicating that the nature of the amino acid in this position is important for the region polarity (Fig. 7, C and D, shown in red). The K385F mutation showed a similar pattern to that of K385E, which is interesting because the nonlocalized electrons in the phenylalanine ring add a negative charge to the region (Fig. 7, E and F). These analyses, together with the

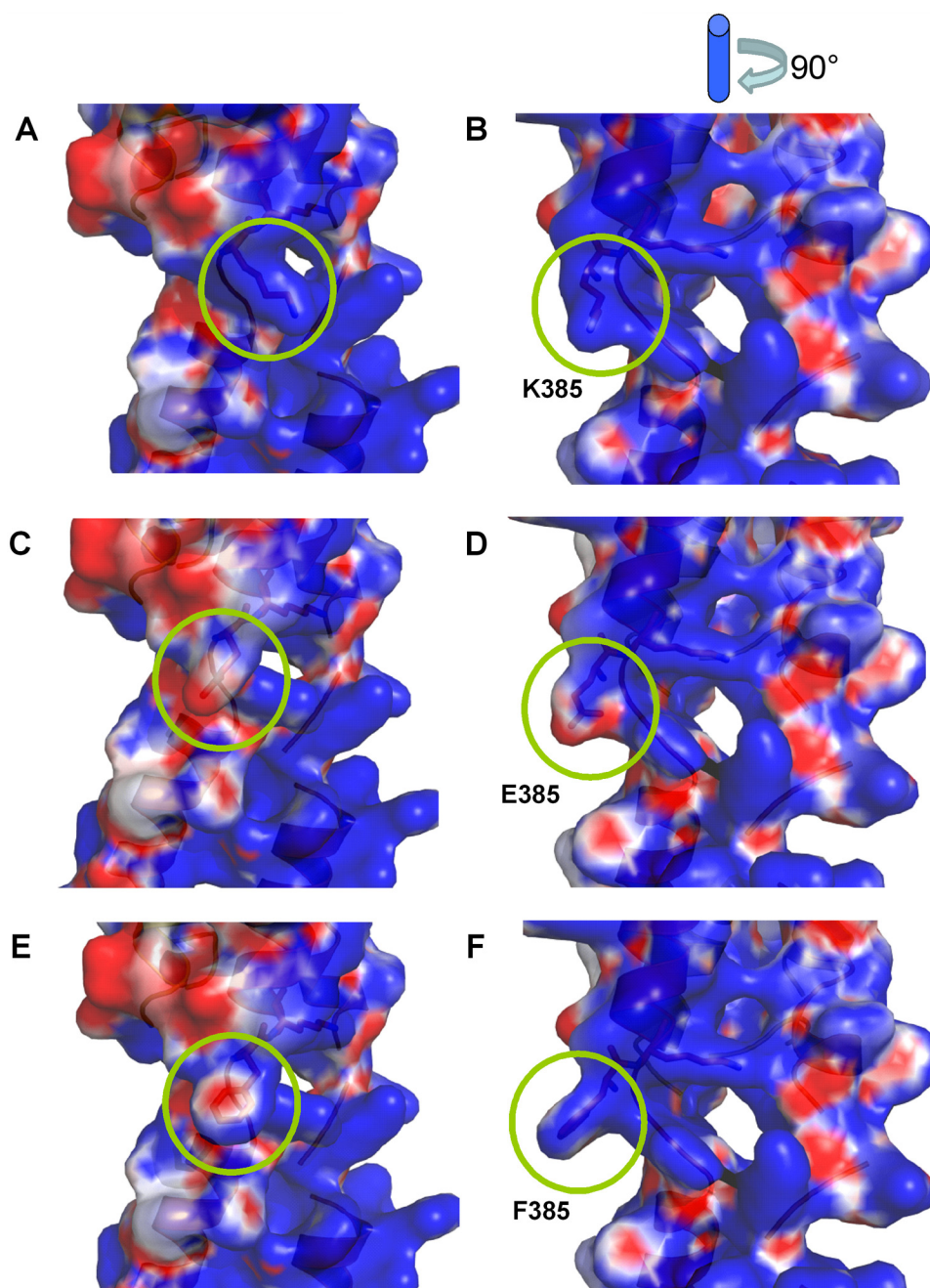


Fig. 7. Effects of substitutions at 385 on the region electrostatic surface potential. A and B, electrostatic surface potential (ESP) representation showing a positive region (blue) surrounding the residue Lys385 (green circle) in two different views. C and D, the mutation K385E induced a large decrease in the positive ESP in the region surrounding position 385. E and F, mutation K385F shows a decrease in the positive ESP and suggests a structural modification caused by the presence of a phenylalanine residue in the region surrounding position 385. Red and blue represents negative and positive regions, respectively.

experimental results, suggest that the charge of this amino acid is critical for determining the sensitivity to ethanol.

Dynamic Cysteine Modification. To evaluate whether a protein residue is exposed to a hydrophilic environment, cysteine substitution and chemical reagents that are able to covalently transfer radical groups to the cysteine are used (Deeb et al., 2007). In this study, a reagent with a basic group (MTSEA) was used in the cysteine-substituted mutation. The K385C mutation was characterized by an EC_{50} shifted toward the left ($15 \pm 0.4 \mu\text{M}$; Fig. 8A, \square) with respect to the WT (Fig. 8A, \circ). This shift is advantageous because we can monitor whether the MTS reagent, intracellularly applied, can modify this parameter as expected by the addition of a positive charge. From a structural view no large changes in volume are predicted with respect to lysine (147 versus 145 \AA^3). When the reagent was applied to WT GlyRs (Fig. 8A, \bullet), no significant changes on the current were detected, which is interesting because the WT receptor has a cysteine in position 344 that could potentially interfere with the interpretation of the results. Figure 8A shows that 15 min of application of the positively charged reagent MTSEA to the K385C

mutant shifted the EC_{50} toward the right ($32 \pm 4 \mu\text{M}$), which is consistent with a partial rescue of function by the positive charge (\blacksquare). We then evaluated the response of the K385C mutant to ethanol (100 mM) and $\text{GTP}\gamma\text{S}$ (0.5 mM) after intracellular application of MTSEA for 15 min (Fig. 8B). Unlike the WT GlyR (Fig. 8B, white bars), ethanol and $\text{GTP}\gamma\text{S}$ were unable to potentiate the current (6 ± 0.7 and $5 \pm 1.4\%$, respectively) after the treatment, indicating that although MTSEA is transferring a positive charge to K385C, the receptor remained insensitive to ethanol and $\text{GTP}\gamma\text{S}$.

Discussion

The present study examined which physicochemical property of Lys385, located in the IL of GlyR, was correlated with ethanol modulation of this inhibitory receptor. Our results are consistent with the idea that positive charges in the IL need complementary negative charges in $\text{G}\beta$ for interaction and functional modulation.

Importance of the IL for LGIC Functions. LGICs are macromolecules found in neuronal membranes, and they play critical roles in cell signaling (i.e., excitability, neurotransmitter release). They selectively recognize the neurotransmitter that binds to extracellular domains and subsequently undergoes restricted transmembrane rearrangement that allows channel opening. Ion channels show different properties regarding channel conductance and gating kinetics, and only recently it became apparent that intracellular domains are able to regulate the functions of LGICs. For example, it was shown that Arg436 in the "MA" stretch of the intracellular loop of the 5-HT_{3A} receptor regulates channel conductance (Livesey et al., 2008). For example, replacing three arginines with QDA enhanced single-channel conductance. In $\alpha 4\beta 2$ nACh receptors, similarly, changing residues in homologous positions to 5-HT_{3A} receptor (Glu584, Phe588, and Glu592) with positive amino acids caused a reduction in channel conductance (Peters et al., 2010). Regarding GlyRs, it was shown that replacement of all basic amino acids present in the IL to negatively charged residues produced a strong impact on the GlyR, rendering it non-functional. In addition, simultaneous mutations of arginine and lysine at positions 377, 378, 385, and 386 to glutamate reduced the conductance of the channel, showing their importance in channel function (Carland et al., 2009). Furthermore, the correct assembly of GlyR seems to depend on the presence of the RFRKR cluster in the intracellular loop (Sadler et al., 2003).

Other studies have shown that the IL in $\alpha 1$ GlyRs interacts with $\text{G}\beta\gamma$ via two intracellular motifs (Yevenes et al., 2006). Furthermore, it was found that the Lys385 mutation caused the biggest impact on G protein modulation. Similar attenuations in ethanol actions were found with these mutations, supporting the existence of a high correlation between these two modulations (Yevenes et al., 2008). In agreement, the present study showed that this basic residue was the most important for ethanol action on GlyRs (Fig. 1B). The presence of basic residues in the IL of GlyR and their interaction with $\text{G}\beta\gamma$ are in agreement with previous studies that showed that lysine and arginine were important for modulation of voltage-gated calcium channels, phospholipase C, and adrenergic receptor kinase (Touhara et al., 1995; Cantí et al., 1999; Barr et al., 2000).

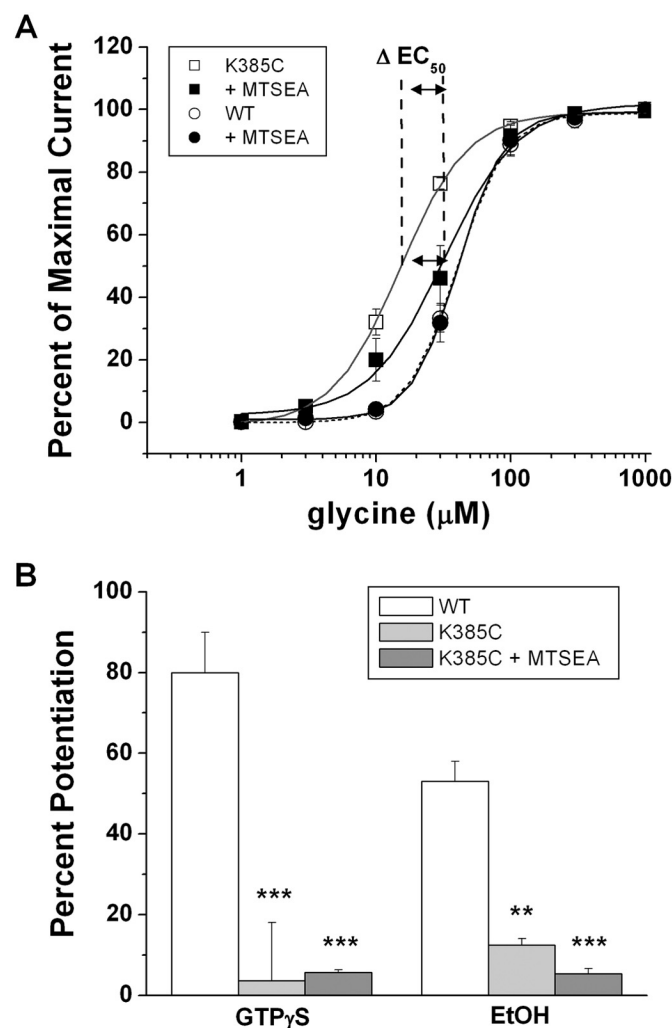


Fig. 8. SCAM modifications of 385. A, concentration-response curves of WT $\alpha 1$ GlyR (circles) and K385C mutant (squares) in the absence (open symbols) or presence (filled symbols) of MTSEA. B, the graph summarizes the percentage of potentiation of WT (white bars), K385C (light gray bars), and K385C in the presence of MTSEA (dark gray bars) using $\text{GTP}\gamma\text{S}$ (500 μM) and ethanol (EtOH) (100 mM). **, $p \leq 0.01$; ***, $p \leq 0.001$.

A Positive Charge in the 385 Residue Is Important for Ethanol Action on GlyRs. Building from a previous study (Yevenes et al., 2008), the present analyses indicate that the basic property of Lys385, reproduced in Arg385 and His385, is relevant for ethanol-induced modulation. All of the other mutations (K385E, K385Q, K385L, K385F, K385C, and K385A) were less sensitive to the allosteric actions of ethanol. The data also showed that mutants sensitive to ethanol were potentiated by G protein activation (WT and K385R) and mutants insensitive to ethanol (K385A and K385E) were insensitive to G $\beta\gamma$ modulation. These data are in good agreement with previous studies that examined a large number ($n = 20$) of chimeric and mutant GlyRs and showed a high correlation between GTP γ S and ethanol modulations (Yevenes et al., 2008, 2010).

The correlation analysis between physicochemical properties (i.e., polarity, charge, hydropathy, hydrophilicity, and volume) and ethanol modulation showed that only charge was highly correlated to ethanol modulation of the GlyR. Furthermore, analysis of electrostatic potential and predicted structure of the region neighboring Lys385 showed

that the substitution altered only charge distribution in the region (Fig. 7), without changes on the α -helix secondary structure. Taken together, these results show a critical role for the basic charge of Lys385, providing charge distribution for interaction with G $\beta\gamma$, leading to ethanol modulation of GlyRs.

The importance of charge in Lys385 is distinct to the molecular volume property reported for residues 267 and 288 in GlyRs (Ye et al., 1998; Yamakura et al., 1999) associated to the binding pocket for both ethanol and anesthetics. Other studies in nAChR showed a significant correlation between molecular volume and n -alcohol potentiation actions, supporting the idea of a site for alcohol in the receptor (Godden et al., 2001). Studies in *N*-methyl-D-aspartate receptors have shown that two residues, Phe637 and Met823, were involved in ethanol sensitivity, and the molecular volume and hydrophobicity of these two residues participate on this interaction (Ren et al., 2008). A similar type of analyses in ATP-gated P2X purinoceptor 4 receptors identified two residues (Asp331 and Met 336) whose hydropathy and polarity influenced ethanol inhibitory actions (Popova et al., 2010). Our hypothesis

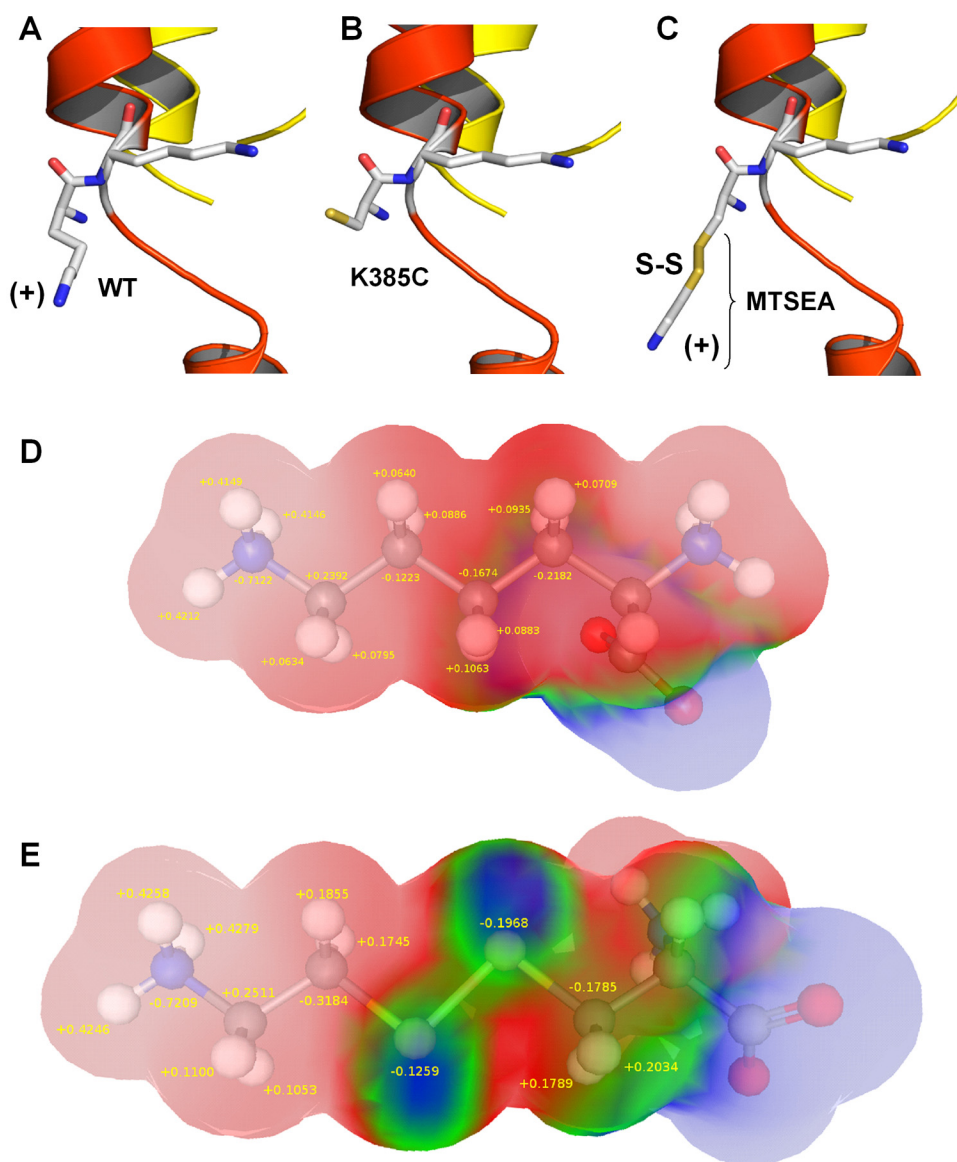


Fig. 9. Structural differences between WT and K385C-MTSEA-modified receptors. A, a stick model of Lys385 and Lys386 in WT $\alpha 1$ GlyR. The protein is shown as a ribbon. B, a representation of the mutant K385C. The cysteine residue allows for chemical modification. C, chemical modification of K385C with MTSEA gives the residue an electropositive (MTSEA) character, but with a different charge distribution and structure to lysine. D, the charge distribution present in the lateral chain of lysine. The Van der Waals surface is represented and colored according to atomic charge (blue, negative; red, positive). It can be noticed that the lateral chain is positive because of the sum of all partial charges. E, the charge distribution of the lateral chain in the MTS-modified cysteine. The disulfide bond presents a negative (blue) charge unlike the natural residue.

proposing that G $\beta\gamma$ interacts directly with the IL of the GlyR should depend on classic protein-protein interactions, regulated by hydrophobic, hydrogen bonding, and electrostatic forces (for review see Leckband, 2000), and this agrees with the complex interaction of G $\beta\gamma$ with several effectors (Ford et al., 1998). Studies in residues in loop 2 of the GlyR ectodomain, which together with the interface to TM2 is believed to serve as a binding site for ethanol (Crawford et al., 2007), showed that polarity at position 52 plays a key role in determining sensitivity to ethanol (Perkins et al., 2008).

We recently postulated that the molecular requirements needed to modulate the GlyR by ethanol are positioned along the structure of GlyR (Yevenes et al., 2010), reconciling results from different laboratories in terms of identifying regions important for ethanol actions (Mihic et al., 1997; Crawford et al., 2007). For example, Ala52 in loop 2 of $\alpha 1$ might transfer binding energy into conformational changes to open the ion channel affecting residues in TM2 (Gly254) and TM3 (Ser296). Finally, basic intracellular residues are required to bind G $\beta\gamma$, thereby facilitating channel opening (i.e., increase in open probability).

A Positive MTS Reagent Affected GlyR Activation but Not Its Modulation by Ethanol. The K385C mutation, in addition to being resistant to ethanol, showed differences in activation with respect to WT (Fig. 3; Table 1). Therefore, we wanted to rescue WT properties by using the SCAM in this mutation. This reaction involves the sulfur atom from the reagent and the cysteine amino acid, which generate a disulfide bridge, thereby modifying the substituted residue (Deeb et al., 2007). For instance, the MTSEA reagent possesses a basic radical group ($-S-CH_2-CH_2-NH_3^+$) similar to the lysine residue (Fig. 9, C and E). Concentration-response curves obtained after application of the reagent showed no differences when the WT GlyR was treated with MTSEA, indicating that Cys344 was not accessible to the reagent. It is noteworthy that, in agreement with aqueous intracellular accessibility, MTSEA significantly modified the properties of the K385C mutant receptor, producing a shift in the curve in the direction of the WT receptor, with EC_{50} values of 32 ± 4 and 40 ± 1 μ M, respectively. Thus, the addition of a basic group to the K385C mutant was sufficient to partly recover the phenotype of the GlyR. On the other hand, the MTSEA-treated K385C mutant receptor remained insensitive to GTP γ S and ethanol (Fig. 8B). This lack of modulation can be explained because, despite the gain of a positive charge in the conserved α -helix region, the resulting disulfide bond generated an alteration in charge distribution on the modified residue (Fig. 9). This is probably because of the nature of the unnatural amino acid formed with the reagent because the added positive charge would be somewhat dissipated by the electronegative environment produced by the disulfide bond. This in turn should modify the pattern of the positive charge in the amine added in the lateral peptide chain. The data suggest that ethanol sensitivity in GlyRs depends on precise electrostatic interactions between G $\beta\gamma$ and the GlyR IL. Future experiments with crystallized proteins will contribute to elucidating a more precise molecular interaction between G β and Lys385.

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Authorship Contributions

Participated in research design: Castro and Aguayo.

Conducted experiments: Castro, Figueroa, and San Martin.

Performed data analysis: Castro.

Wrote or contributed to the writing of the manuscript: Castro, Yevenes, and Aguayo.

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