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Evidence that ethanol acts on a target in Loop 2 of the extracellular domain of α1 glycine receptors

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Abstract

Considerable evidence indicates that ethanol acts on specific residues in the transmembrane domains of glycine receptors (GlyRs). In this study, we tested the hypothesis that the extracellular domain is also a target for ethanol action by investigating the effect of cysteine substitutions at positions 52 (extracellular domain) and 267 (transmembrane domain) on responses to *n*-alcohols and propyl methanethiosulfonate (PMTS) in α1GlyRs expressed in *Xenopus* oocytes. In support of the hypothesis: (i) The A52C mutation changed ethanol sensitivity compared to WT GlyRs; (ii) PMTS produced irreversible alcohol-like potentiation in A52C GlyRs; and (iii) PMTS binding reduced the n-chain alcohol cutoff in A52C GlyRs. Further studies used PMTS binding to cysteines at positions 52 or 267 to block ethanol action at one site in order to determine its effect at other site(s). In these situations, ethanol caused negative modulation when acting at position 52 and positive modulation when acting at position 267. Collectively, these findings parallel the evidence that established the TM domain as a target for ethanol, suggest that positions 52 and 267 are part of the same alcohol pocket and indicate that the net effect of ethanol on GlyR function reflects the summation of its positive and negative modulatory effects on different targets.

Keywords: alcohol pocket, ethanol, glycine receptor, molecular model, propyl methanethiosulfonate, *Xenopus* oocyte.

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Glycine and $GABA_A$ receptors (GlyRs and $GABA_ARs$) are members of the cys-loop superfamily of ligand-gated ion channels (LGICs) (Ortells and Lunt 1995; Xiu *et al.* 2005). These LGICs have received considerable attention as putative sites of action causing the behavioral effects of ethanol (Dietrich *et al.* 1999; Harris 1999; Davies *et al.* 2003). Studies over the last decade identified several positions in the transmembrane (TM) domain that play a critical role in ethanol modulation of GlyRs and $GABA_ARs$ (Mihic *et al.* 1997; Wick *et al.* 1998; Ye *et al.* 1998; Yamakura *et al.* 1999; Ueno *et al.* 2000; Jenkins *et al.* 2001; Lobo *et al.* 2006). Chimeric studies suggested that serine-267 (S267) and alanine-288 (A288) in TM segments 2 and 3 of the α1GlyR subunit are important for allosteric modulation by ethanol (Mihic *et al.* 1997). Further work found that mutations at position 267 changed receptor sensitivity to ethanol, altered the qualitative ethanol response from potentiation to inhibition, and decreased the size of a putative alcohol pocket (Wick *et al.* 1998; Ye *et al.* 1998). These findings in GlyRs suggest that the TM domains play a role in alcohol modulation of receptor function and may form part of an alcohol pocket.

Subsequent studies tested the hypothesis that position 267 in the α1GlyR is a binding site for alcohols and anesthetics

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Abbreviations used: EC_X, effective concentration producing X% of the maximal current; $GABA_AR$, γ-aminobutyric acid type-A receptor; GlyR, glycine receptor; LGIC, ligand-gated ion channel; PMTS, propyl methanethiosulfonate; TM, transmembrane; WT, wildtype.

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by combining the use of anesthetic-like propyl methanethiosulfonate (PMTS) with the substituted cysteine accessibility method (Karlin and Akabas 1998; Mascia *et al.* 2000; Lobo *et al.* 2004). The authors proposed that PMTS would covalently bind to the substituted cysteine residue and change the normal effect of PMTS (reversible potentiation) to irreversible potentiation if the actions of PMTS and alcohols result from binding at this site. As predicted, exposing S267C GlyRs to PMTS caused irreversible potentiation and, thus, supported the notion that the actions of alcohols and anesthetics in these receptors are due to binding in the TM domains.

Recent experiments suggest that ethanol also acts on the extracellular domain. The initial evidence came from studies demonstrating that α1GlyRs are more sensitive to ethanol than are α2 GlyRs despite high sequence homology between α1 and α2 GlyRs (Mascia *et al.* 1996b). This work also found that an alanine to serine exchange at position 52 (A52S), one of the residues that differs from α1 and α2 GlyRs and located at the beginning of Loop 2 as defined by Sixma and colleagues (Brejc *et al.* 2001), could eliminate the difference in ethanol sensitivity between α1 and α2 GlyRs (Mascia *et al.* 1996b). More recent studies, which found that the A52S mutation eliminated the sensitivity of α1GlyRs to a direct ethanol antagonist, drew further attention to the extracellular domain as a target for ethanol action (Davies *et al.* 2004). Collectively, these studies suggest that there are multiple sites of ethanol action in α1GlyRs, with one site located in the TM domain (e.g., position 267) and another in the extracellular domain (e.g., position 52).

The present study tested the hypothesis that the extracellular domain is a target for ethanol action in α1GlyRs. This work also began to test the relationship between the extracellular and TM domains in mediating the actions of ethanol on this receptor. We accomplished this by investigating the effect of cysteine mutations at positions 52 and/or 267 on α1GlyR responses to glycine, alcohols, and PMTS.

Materials and Methods

Materials

Adult female *Xenopus laevis* frogs were obtained from Nasco (Fort Atkinson, WI, USA). Glycine, alcohols and alkanediols were purchased from Sigma (St Louis, MO, USA). PMTS was purchased from Toronto Research Chemicals Inc. (North York, Toronto, Canada). 100 mmol/L stock solutions of PMTS in dimethyl sulfoxide (DMSO), stored in aliquots at 4°C, were thawed and serially diluted with buffer (DMSO ≤ 0.3%) immediately prior to testing. DMSO at 0.3%, with or without glycine, had no appreciable effect on GlyR currents in WT or mutant receptors.

Mutagenesis and Expression of Human α1GlyR Subunit cDNA

Site-directed mutagenesis in the human GlyR α1 subunit was performed on cDNA subcloned into the pBK-CMV N/B 200 vector using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). Point mutations were verified by partial sequencing (DNA Core Facility, University of Southern California, USA). *Xenopus laevis* oocytes were isolated and injected with 1 ng of WT or mutant A52C, S267C, or A52C–S267C α1GlyR cDNA using procedures previously described (Davies *et al.* 2003, 2004). Injected oocytes were incubated at 18°C stored in Petri dishes with incubation medium (containing in mmol/L: KCl 2, NaCl 96, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 5, theophylline 0.6, pyruvic acid 2.5, 1% horse serum and 0.05 mg/mL gentamycin).

Electrophysiology

Electrophysiological measurements were made 1–10 days after injection as previously described (Davies *et al.* 2003, 2004). Briefly, oocytes expressing WT and mutant GlyRs were perfused in a 100 μL volume oocyte bath with modified Barth's saline (MBS) ± drugs at 2.0 mL/min. MBS contains in mmol/L: 83 NaCl, 1 KCl, 10 HEPES, 0.82 $MgSO_4$, 2.4 $NaHCO_3$, 0.91 $CaCl_2$ and 0.33 $Ca(NO_3)_2$ adjusted to pH 7.5. Oocytes were impaled with two electrodes backfilled with 3 M KCl with resistances of 0.5–3 MΩ and voltage clamped (–70 mV) (Warner Instruments, Model OC-725C, Hamden, CT, USA). Currents were continuously recorded with a strip-chart recorder (Barnstead/Thermolyne, Dubuque, IA, USA).

GlyR agonist activation

Oocytes expressing WT or mutant α1GlyRs were exposed to glycine (10–1000 μmol/L) for 30 s using 5–15 min washouts between applications to ensure complete resensitization (Mascia *et al.* 1996a,b; Davies *et al.* 2004). Responses were normalized to the maximal glycine response. Pilot experiments found that WT and cysteine mutant GlyR responses using a 1 min glycine application reached maximal response, which did not differ appreciably from results using 30 s applications. Therefore, we used the shorter application time to increase the efficiency and to minimize desensitization at the higher glycine concentrations.

GlyR agonist modulation

We used effective concentrations of agonist producing a specified percentage of the maximal current (EC_x) in each experiment to facilitate comparison of alcohol and PMTS effects across oocytes and receptor subtypes, while minimizing influence by differences in receptor expression levels (Davies *et al.* 2003, 2004). We used a glycine EC_2 for ethanol and hexanol experiments based on prior studies in WT GlyRs, which showed that alcohols robustly potentiate GlyR currents at low ($EC_{2–10}$), but not high (EC_{50+}) glycine ECs (Mascia *et al.* 1996b; Davies *et al.* 2004). We used a glycine EC_{10} with PMTS in order to facilitate comparison with prior studies in the TM domain (Mascia *et al.* 2000; Lobo *et al.* 2004). Preliminary studies from our laboratory in WT and mutant α1GlyRs (including A52C and S267C GlyRs) found that their responses to ethanol did not markedly differ when tested at ECs from 2–10.

Ethanol and hexanol

Oocytes were pre-incubated with ethanol (25–100 mmol/L) or hexanol (30–300 μmol/L) for 60 s before co-application of EC_2 glycine plus ethanol or hexanol for 30 s. Ethanol, hexanol and

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PMTS did not significantly affect holding currents of WT and A52C GlyRs in the absence of glycine. In contrast, these agents produced small direct effects in S267C and A52C–S267C GlyRs. Therefore, in the latter mutants, we measured peak height from the shifted baseline. The concentrations of ethanol tested in this study are commonly used in electrophysiological recordings of GlyRs and $GABA_A$Rs and roughly correspond to ethanol concentrations that cause behavioral intoxication (a blood alcohol concentration of 0.08% is approximately 17 mmol/L) and anesthesia (the minimum alveolar concentration or MAC for ethanol is 138 mmol/L) (Krasowski and Harrison 1999).

Single PMTS application

We used two protocols to determine the accessibility of position 52 to PMTS. *Protocol 1 (open-desensitized state)*: Oocytes were perfused with 30 μmol/L PMTS for 60 s before a 30 s coapplication with EC_{10} glycine to test for PMTS binding under conditions in which channels are open. *Protocol 2 (resting state)*: Oocytes were perfused with 30 μmol/L PMTS for 90 s in the absence of glycine to test for PMTS binding under conditions where the channels are closed. MBS was perfused for at least 10 min after PMTS exposure to ensure that unbound reagent completely washed out. The responses to EC_{10} glycine and/or PMTS were expressed as percent control of the initial glycine EC_{10} response.

Sequential PMTS applications

We extended Protocol 1 (open-desensitized state) to include a second PMTS exposure to test for PMTS saturation of cysteine residues at position 52 and/or 267. EC_{10} glycine and/or PMTS (30–300 μmol/L) were applied as follows, with MBS washout between applications: (i) Glycine, (ii) PMTS preincubation/PMTS + Glycine coapplication, (iii) Glycine, (iv) PMTS preincubation/PMTS + Glycine coapplication, and (v) Glycine.

Sequential PMTS–ethanol applications

The sequential protocol described above was modified to isolate the effects of ethanol on the extracellular and TM domains. This was accomplished by using saturating concentrations of PMTS (30–300 μmol/L depending on the mutant receptor tested) for the first application and 100 mmol/L ethanol substituted for the second PMTS application.

Alcohol cutoff

The *n*-chain alcohol cutoff refers to the failure to increase potency as a function of increasing the *n*-chain alcohol (*n*-alcohol) length and is thought to occur when the molecular volume of the alcohol exceeds the finite volume of a putative alcohol pocket (Pringle *et al.* 1981; Alifimoff *et al.* 1989; Wick *et al.* 1998). To investigate whether position 52 is part of an alcohol pocket, we tested the effects of octanol and decanol on A52C GlyRs, before and after PMTS exposure, using the general procedures described for sequential applications. We also tested the respective diols (1,8-octanediol; 1,10-decanediol) to minimize confounds from reduced solubility that occurs as the alcohol chain length increases (Peoples and Ren 2002). We limited the concentration and range of *n*-alcohols used in this study based on previous work that established alcohol cutoff in WT and S267C GlyRs (Wick *et al.* 1998; Mascia *et al.* 2000). We counterbalanced the order of drug presentations.

Molecular modeling

To help visualize a putative alcohol pocket that incorporated the current findings, we built a model of the α1GlyR by threading the human α1GlyR subunit primary sequence onto the backbone coordinates of a template, essentially as previously described (Trudell 2002; Trudell and Bertaccini 2004). In this case, the template was the cryo-electron micrograph of the nicotinic acetylcholine receptors (nAChR) - PDB ID 2BG9 (Unwin 2005). We used the Homology module of Insight 2005 L (Accelrys, San Diego, CA, USA). The alignment of the extracellular domain of GlyR with nAChR was as suggested by Sixma and coworkers (Brejc *et al.* 2001) and the alignment of the TM domain was as suggested by Bertaccini (Bertaccini and Trudell 2002). Loops and gaps in the threaded structure were generated with the same Homology module. The resulting α1GlyR structure was refined by optimizations to a gradient of 0.1 kcal/Å in which the backbone harmonic restraints were successively reduced from 100 to 10, 1, and 0 kcal/$Å^2$ using the Discover_3 module of Insight 2005 L.

Cavities in a single subunit of the resulting model were identified with the Binding Site Analysis module of Insight 2005 L using a 1 Å cubic grid and default settings except for a limit on the cavity aperture of 9 Å. The results are necessarily dependent on the choice of grid spacing. In addition to the final cubic grid spacing of 1 Å, cubic grid spacings of 0.5 to 1.5 Å per side were also used. The smallest grid spacing produced interconnected cavities that had dimensions too small to accommodate alcohols. Larger grid spacing produced many small, but discontinuous cavities. The ability of a cavity to accommodate an ethanol molecule is further modified by the dynamic motion of the side chains and the ability of the protein backbone to deform. Based on the current findings and previous work, we chose the output shown in the Results to illustrate the general nature and relative dimensions of the putative alcohol pocket. Although the volume of the cavity is a function of the choice of grid spacing, the Cα to Cα distance between A52 and S267 is taken from the cryo-electron micrograph of the nAChR (PDB ID 2BG9).

Data analysis

Data for each experiment were obtained from oocytes from at least two different frogs. Results are expressed as mean ± SEM. Where no error bars are shown, they are smaller than the symbols. We used Prism (GraphPAD Software, San Diego, CA, USA) to perform statistical analyses using one- or two-way repeated measures ANOVA and Bonferroni *post hoc* analyses. Concentration response data were analyzed using non-linear regression

$$[I = I_{\max}[\mathrm{A}]^{nH}/([\mathrm{A}]^{nH} + \mathrm{EC}_{50}^{nH})]$$

where I is the peak current recorded following application of a range of agonist concentrations, [A]; I_{max} is the estimated maximum current; EC_{50} is the glycine concentration required for a half-maximal response and n_H is the Hill slope.

Results

Glycine concentration response

We first tested the effect of cysteine mutations at positions 52 and/or 267 on α1GlyR glycine sensitivity and maximum

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current responses (Fig. 1). The wildtype (WT) GlyR EC_{50} and Hill slope for glycine agree with prior studies (Ryan *et al.* 1994; Saul *et al.* 1994; Mascia *et al.* 1996b; Davies *et al.* 2004). There were no significant differences between WT and A52C GlyRs in EC_{50} or Hill slope (Table 1). Therefore, the cysteine mutation at position 52 did not affect receptor agonist sensitivity. Likewise, there was not a significant difference between the maximal current amplitude (I_{max}) in WT and A52C GlyRs.

In contrast, the I_{max} of S267C and A52C–S267C GlyRs were significantly lower than for WT GlyRs. Moreover, there was a non-significant leftward shift in glycine sensitivity in S267C-containing mutant GlyRs. These changes are consistent with prior findings with S267C (Roberts *et al.* 2006) and S267Q GlyRs (Findlay *et al.* 2002, 2003), and suggest that mutations at position 267 can affect agonist responses by altering channel stability. The heterologous expression of GlyRs in *Xenopus* oocytes via nuclear cDNA injection does not provide the ability to measure channel kinetics or determine whether differences in I_{max} reflect changes in receptor expression or function. Regardless, all of the receptors appeared to respond normally to glycine measured via two-electrode voltage clamp.

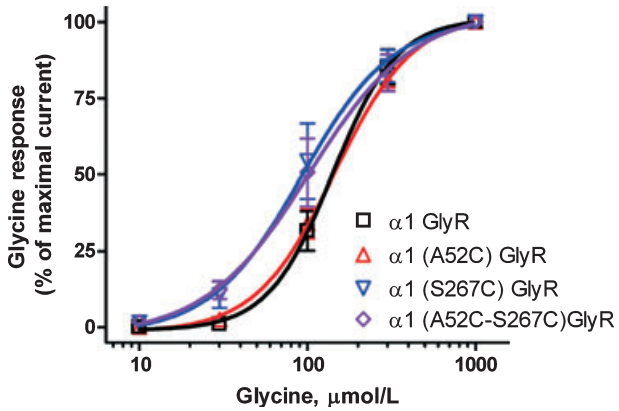

Fig. 1 There is no significant difference between wildtype and mutant α1GlyRs in sensitivity to glycine. The curves represent non-linear regression analysis of the glycine concentration responses in WT and mutant α1GlyRs from 4–5 different oocytes. Each data point represents the mean ± SEM.

Table 1 Summary of non-linear regression analysis results for the glycine concentration responses in wildtype and mutant α1GlyRs

Receptor	EC_{50} (μmol/L)	Hill Slope	I_{max} (μA)
WT	148 ± 24	2.6 ± 0.2	20.8 ± 1.6
A52C	148 ± 16	1.9 ± 0.1	17.7 ± 1.0
S267C	111 ± 26	2.0 ± 0.4	11.8 ± 1.9*
A52C–S267C	122 ± 32	1.8 ± 0.3	6.7 ± 1.0*

EC_{50}, Hill slope, and maximal current amplitude (I_{max}) are presented as Mean ± SEM from 4–5 different oocytes (as shown in Fig. 1). Statistical significance from WT α1GlyRs was assessed using one-way ANOVA with Bonferroni post test. (* $p < 0.05$ vs. WT).

Ethanol and hexanol concentration responses

We next tested the effect of cysteine mutations on ethanol sensitivity (Fig. 2). Ethanol (25–100 mmol/L) potentiated WT GlyR function in a concentration dependent manner, which was similar to previous reports (Davies *et al.* 2003, 2004; Mascia *et al.* 1996b). The A52C mutation right shifted the ethanol concentration response, which supports the notion that position 52 is important in mediating the actions of ethanol.

Substituting cysteine for serine at position 267 changed ethanol modulation of glycine in α1GlyRs from potentiation to inhibition (Fig. 2c). Ethanol inhibited S267C and A52C–S267C GlyR function in a concentration dependent manner. The ethanol inhibition in S267C GlyRs agrees with previous findings using 200 mmol/L ethanol in this mutant (Ye *et al.* 1998) and is consistent with the notion that the residue at position 267 is important for determining ethanol responses in GlyRs.

We tested hexanol at concentrations (30–300 μmol/L) that were functionally equivalent to those for ethanol. The effect of cysteine mutations on hexanol sensitivity paralleled the effects of ethanol (Fig. 2d). Since hexanol is approximately the same volume as PMTS, these results suggest that any differences between ethanol and PMTS effects on GlyRs in later studies do not reflect volume differences.

It is noteworthy that ethanol and hexanol, in the absence of glycine, produced small outward currents in S267C (data not shown) and A52C–S267C GlyRs (Fig. 2b). Prior studies found that mutation at the homologous position in $GABA_A$Rs also resulted in ethanol causing direct effects (Ueno *et al.* 2000). We explored possible mechanisms for the direct effects of ethanol in α1GlyRs using strychnine and picrotoxin on the mutant receptors in the absence of glycine (data not shown). As expected, the GlyR antagonist strychnine (10 μmol/L) did not alter the holding currents in S267C GlyRs. This finding suggests that there is no obvious contamination or receptor conformation change in S267C GlyRs that produces "pseudo-agonist" induced activation in the absence of glycine. In contrast, the channel blocker picrotoxin (100 μmol/L) caused outward currents in S267C GlyRs that were similar to the outward currents produced by ethanol and hexanol in the absence of glycine. The ability of picrotoxin to produce outward currents in S267C GlyRs suggests that there is a small tonic chloride conductance in receptors containing the cysteine mutation at position 267. Co-application of ethanol and picrotoxin did not further increase the outward currents elicited by picrotoxin. Taken together, these findings suggest that the direct effects of ethanol and hexanol arise through a reduction in the tonic chloride conductance. The decreased tonic conductance

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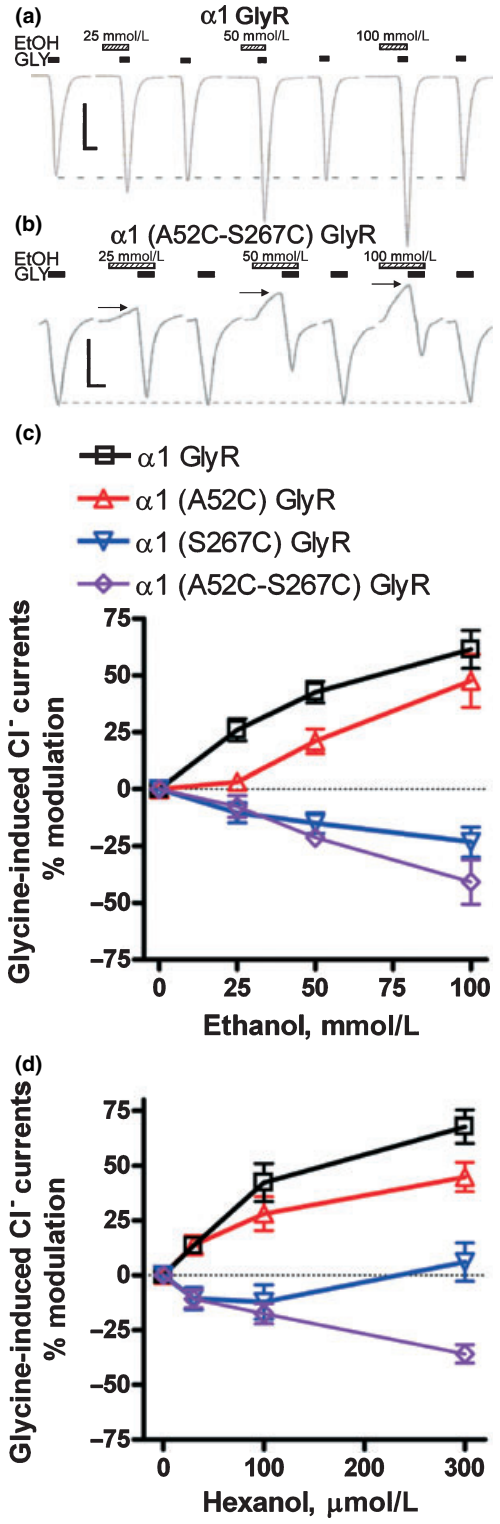

Fig. 2 Cysteine substitutions in α1GlyRs produce position-specific differences in ethanol and hexanol responses. Representative sequential tracings (from left to right) for the (a) potentiating (WT α1GlyR) or (b) inhibitory (α1 A52C–S267C GlyR) effects of ethanol. A52C GlyR tracings resemble the WT GlyR and S267C GlyR tracings resemble that shown for the A52C–S267C GlyR (data not shown). The arrows in (b) indicate the change in holding current in response to ethanol in A52C–S267C GlyRs and mark the point from which peak heights were measured for these mutant GlyRs. The horizontal bars above the tracing indicate time of glycine (lower, solid) and ethanol (upper, slanted line interior) applications (vertical scale bar = 100 nA; horizontal scale bar = 48 s). Ethanol concentrations are (from left to right) 25, 50, and 100 mmol/L. The dashed horizontal line represents the initial glycine EC_2 response. The EC_2 for oocytes in each receptor subtype occurred in the following ranges (in μmol/L): WT (15–40), A52C (20–35), S267C (10–20), and A52C–S267C (20–25). (c) Ethanol. Mean ± SEM percent ethanol modulation of the EC_2 glycine response for WT and mutant α1GlyRs (n = 4–8). ANOVA revealed significant main effect of mutation [$F_{3,58}$ = 61.34, p < 0.0001], a trend for the main effect of ethanol concentration [$F_{3,58}$ = 2.41, p = 0.077] and an interaction between main effects [$F_{9,58}$ = 12.35, p < 0.0001]. (d) Hexanol. Mean ± SEM percent hexanol modulation of the EC_2 glycine response for wildtype and mutant α1GlyRs (n = 4–6). ANOVA revealed significant main effects of mutation [$F_{3,58}$ = 61.45, p < 0.0001], hexanol concentration [$F_{3,58}$ = 11.64, p < 0.0001] and an interaction between the main effects [$F_{9,58}$ = 13.09, p < 0.0001].

produced by ethanol and hexanol in S267C GlyRs is likely the result of stabilizing the closed state of the receptor and is consistent with previous studies (Findlay *et al.* 2002, 2003).

Single PMTS application

We tested the hypothesis that position 52 of the α1GlyR is a target for ethanol capable of causing alcohol-like receptor modulation by using PMTS in cysteine substituted GlyRs. We predicted that PMTS would cause irreversible potentiation if PMTS binds to the substituted cysteine site and if the site is capable of causing alcohol-like receptor modulation (Karlin and Akabas 1998; Mascia *et al.* 2000; Lobo *et al.* 2004). We also evaluated whether receptor state affects the accessibility of PMTS to cysteines substituted at position 52.

The first experiment tested for PMTS binding under conditions in which channels are open (Protocol 1—PMTS in the presence of glycine). We found that exposure to 30 μmol/L PMTS with EC_{10} glycine significantly potentiated glycine responses in WT and A52C GlyRs (Fig. 3). Following washout, the responses to EC_{10} glycine in A52C GlyRs were significantly greater than the glycine responses prior to PMTS exposure. In contrast, the glycine response post-washout in WT GlyRs did not differ from the pre-PMTS response. These findings show that PMTS causes irreversible potentiation in A52C GlyRs, but not WT GlyRs. The absence of irreversible potentiation in WT GlyRs indicates that potentiation by PMTS in WT GlyRs does not result from it acting on and/or binding to naturally occurring cysteine residues.

We also tested for PMTS binding using a protocol in which channels are closed (Protocol 2—PMTS in the absence of glycine). The results with this protocol were not markedly different than the results using Protocol 1 when

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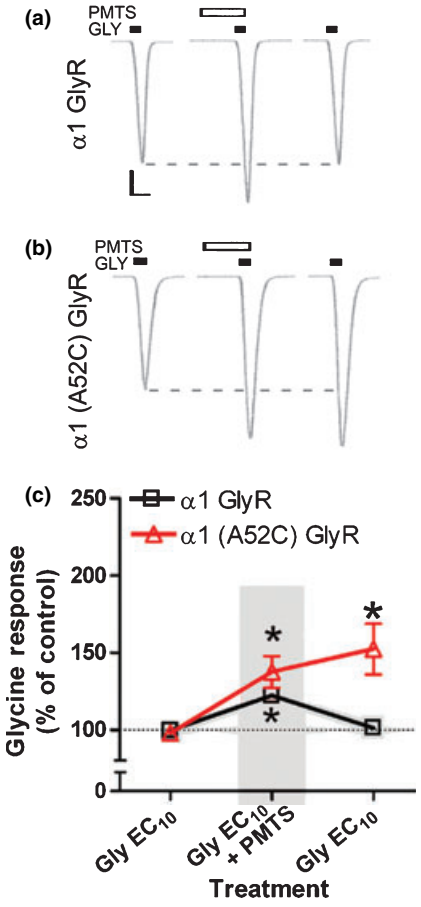

Fig. 3 PMTS exposure in the presence of glycine irreversibly potentiates A52C GlyRs. Representative sequential tracings (from left to right) for (a) WT and (b) A52C GlyRs. Horizontal bars above the tracing indicate time of glycine (lower, solid) and 30 μmol/L PMTS (upper, open) applications (vertical scale bar = 500 nA; horizontal scale bar = 48 s). The dashed horizontal line represents the initial glycine EC_{10} response. (c) Mean ± SEM percent control glycine response for WT and A52C GlyRs ($n = 5$). The gray shaded box indicates GlyR responses in the presence of PMTS. There was a significant main effect of treatment [$F_{2,12} = 17.98$, $p < 0.0001$], a trend for the main effect of mutation [$F_{1,12} = 5.57$, $p = 0.056$] and an interaction between main effects [$F_{2,12} = 11.15$, $p < 0.01$]. (* $p < 0.05$ vs. control glycine EC_{10} response).

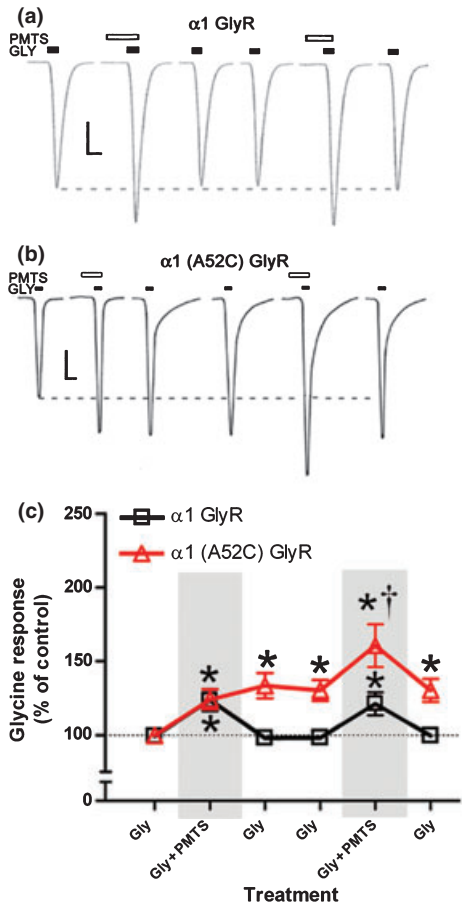

Fig. 4 A second exposure of A52C GlyRs to PMTS suggests multiple sites of PMTS action in α1GlyRs. Representative sequential tracings (from left to right) for (a) WT and (b) A52C GlyRs. Horizontal bars above the tracing indicate time of glycine (lower, solid) and 30 μmol/L PMTS (upper, open) applications (vertical scale bar = 500 nA; horizontal scale bar = 48 s). The dashed horizontal line represents the initial glycine EC_{10} response. (c) Mean ± SEM percent control glycine response for WT and A52C GlyRs ($n = 4$). The gray shaded boxes indicate GlyR responses in the presence of PMTS. There were significant main effects of treatment [$F_{5,35} = 17.79$, $p < 0.0001$], mutation [$F_{1,35} = 6.88$, $p < 0.05$] and an interaction between main effects [$F_{5,35} = 7.49$, $p < 0.0001$]. (* $p < 0.05$ vs. control glycine EC_{10} response, † $p < 0.05$ vs. residual effect of PMTS).

PMTS was tested in the presence of glycine (data not shown). As with Protocol 1, there was no irreversible potentiation in WT GlyRs. The degree of irreversible potentiation in A52C GlyR exposed to PMTS in the absence of glycine (125.3 ± 5%, $n = 5$) was significantly less than that seen with PMTS in the presence of glycine (145.9 ± 9%, $n = 5$, $p < 0.05$ by Student's *t*-test). This difference indicates that PMTS accessibility to position 52 is greater in the open state of the receptor than in the resting state of the receptor. Based on these findings, subsequent experiments tested PMTS in the presence of glycine.

Sequential PMTS applications

PMTS–PMTS (WT and A52C GlyRs)

To investigate whether 30 μmol/L PMTS exposure saturated the cysteines at position 52, we tested sequential PMTS applications in the presence of glycine in WT and A52C GlyRs (Fig. 4). The first and second PMTS applications each produced equivalent degrees of reversible potentiation in WT GlyRs. In contrast, the first and second PMTS applications to A52C GlyRs produced different responses. The first PMTS application to A52C GlyRs (Fig. 4c) produced irreversible potentiation that persisted at the same magnitude over several

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washouts and glycine applications. The second PMTS application to A52C GlyRs produced significant, reversible potentiation over and above the irreversible potentiation from the first PMTS exposure. The complete reversibility of the second PMTS application on A52C GlyRs suggests that the first exposure to 30 μmol/L PMTS saturated all cysteines at position 52. The reversible potentiation suggests that position 52 is not the only site in α1GlyRs that causes PMTS potentiation.

PMTS–PMTS concentration response

To investigate the possibility that PMTS acts on sites in the extracellular and TM domains, we tested the effects of sequential applications of a single PMTS concentration (30–300 μmol/L) to individual oocytes expressing WT or mutant GlyRs (Fig. 5). This protocol also further investigated the PMTS concentration necessary to saturate the substituted cysteines in these mutant GlyRs.

WT GlyRs

The first and second PMTS applications reversibly potentiated WT GlyR function in a concentration dependent manner (Fig. 5a). The degree of potentiation did not differ between the first and second PMTS applications.

A52C GlyRs

The first PMTS application potentiated A52C GlyR responses in a concentration dependent manner similar to WT (Fig. 5b). Subsequent tests with glycine revealed that the initial responses to high PMTS concentrations reflected both irreversible and reversible components and that the magnitude of the irreversible component was the same for all PMTS concentrations. This lack of a concentration response in the degree of irreversible PMTS potentiation confirms that 30 μmol/L PMTS saturates the cysteine substitutions at position 52. In contrast to the irreversible component, the reversible component of the PMTS response was concentration dependent. The response to a second PMTS application produced results similar to those seen following the first PMTS application. Together, these findings suggest that PMTS has multiple sites of action in GlyRs – one at position 52 (irreversible component) and at least one at another target (reversible component).

S267C GlyRs

The PMTS responses of S267C GlyRs were similar overall to those in A52C GlyRs, but there were some important distinctions (Fig. 5c). As in A52C GlyRs, the first PMTS application potentiated S267C GlyR responses in a concentration dependent manner. The degree of PMTS potentiation was several-fold greater in S267C GlyRs than in WT or A52C GlyRs, which suggests that the cysteine substitution at position 267 affects PMTS efficacy at this site. In contrast to A52C GlyRs, the degree of the irreversible component seen after washout was concentration dependent. Exposure to 300 μmol/L PMTS caused potentiation with both irreversible and reversible components. The response to a second PMTS application produced results similar to those seen following the first application. The second 300 μmol/L PMTS application did not increase the degree of irreversible potentiation, which indicates that binding to the cysteine substitutions at position 267 reached saturation with 300 μmol/L PMTS.

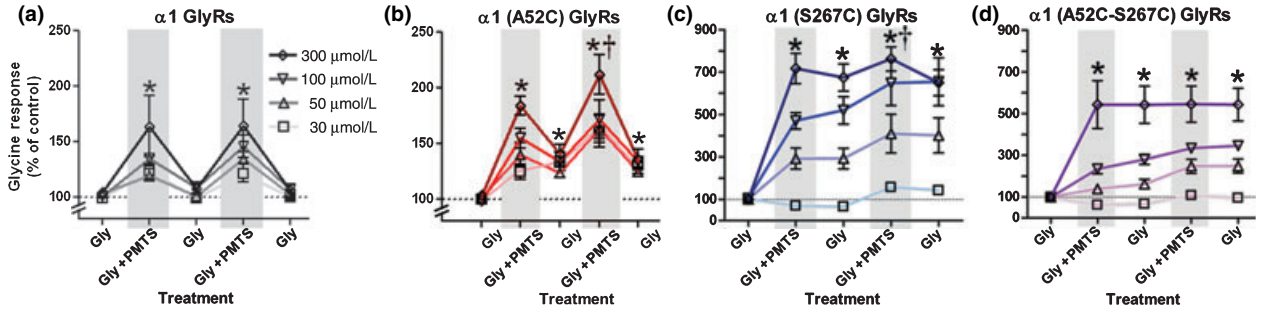

Fig. 5 Concurrent activation of positions 52 and 267 by PMTS is sufficient to explain all of the effects of PMTS on α1GlyRs. Mean ± SEM percent control glycine response for WT and mutant α1GlyRs (n = 4–10). Note that the graphs for (a) and (b) are presented on an expanded scale to facilitate interpretation of the PMTS concentration response in WT and A52C GlyRs. The gray shaded boxes indicate GlyR responses in the presence of PMTS. (a) WT GlyRs: There was a significant main effect of treatment [$F_{4,48}$ = 24.66, p < 0.0001], but there was not a significant main effect of PMTS concentration [$F_{3,48}$ = 2.064, p > 0.05] nor an interaction between main effects [$F_{12,48}$ = 1.45, p > 0.05]. (b) A52C GlyRs: There were significant main effects of treatment [$F_{4,64}$ = 68.31, p < 0.0001], a trend for the main effect of PMTS concentration [$F_{3,64}$ = 3.00, p = 0.061] and an interaction between main effects [$F_{12,64}$ = 3.23, p < 0.01]. (c) S267C GlyRs: There were significant main effects of treatment [$F_{4,80}$ = 70.21, p < 0.0001], PMTS concentration [$F_{3,80}$=20.2, p < 0.0001] and an interaction between main effects [$F_{12,80}$ = 14.72, p < 0.0001]. (d) A52C–S267C GlyRs: There were significant main effects of treatment [$F_{4,60}$ = 44.56, p < 0.0001], PMTS concentration [$F_{3,60}$ = 15.7, p < 0.0001] and an interaction between main effects [$F_{12,60}$ = 12.91, p < 0.0001]. (* p < 0.05 vs. control glycine EC_{10} response, † p < 0.05 vs. residual effect of PMTS; Statistical significance between treatment groups is indicated only for 300 μmol/L PMTS).

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Together, these findings further support the notion that PMTS has multiple sites of action in GlyRs – one at position 267 (irreversible component) and at least one at other targets including position 52 (reversible component).

A52C–S267C GlyRs

The overall responses of A52C–S267C GlyRs to PMTS were similar to A52C and S267C GlyRs, with one major difference (Fig. 5d). As with both single mutants, the first PMTS application potentiated A52C–S267C GlyR responses in a concentration dependent manner. PMTS potentiation in the double mutant, as with S267C GlyRs, was several-fold greater than in WT or A52C GlyRs. Like S267C GlyRs, the degree of irreversible potentiation seen after washout in the double mutant was concentration dependent. Unlike either of the single mutations, neither the first nor second PMTS application produced reversible potentiation at any concentration tested. The second 300 μmol/L PMTS application did not increase the degree of irreversible potentiation, which indicates that binding to the cysteine substitutions at positions 52 and 267 reached saturation with 300 μmol/L PMTS. These findings in WT and mutant GlyRs suggest that PMTS acts at positions 52 and 267. The lack of a reversible component to the PMTS potentiation in A52C–S267C GlyRs indicates that all of the effects of PMTS result from action at these two targets and suggested that the same might hold true for ethanol.

PMTS–ethanol

To isolate the effects of ethanol acting on positions 52 and/or 267, we tested the effects of sequential applications of PMTS followed by 100 mmol/L ethanol in WT and mutant GlyRs (Fig. 6). We reasoned that PMTS would bind to and saturate all cysteine substituted residues, produce maximal irreversible potentiation, and would effectively block further modulation through these bound sites. Subsequent ethanol applications would be active and produce reversible effects only if there were sites of action still available that were not bound or sterically hindered by PMTS binding to the substituted cysteine. The nature of the reversible response would reflect the effects of ethanol on the remaining sites.

WT GlyRs

The pattern of responses to sequential applications of PMTS followed by 100 mmol/L ethanol in WT GlyRs was essentially the same as found with sequential PMTS applications. PMTS and ethanol each reversibly potentiated WT GlyR function (Fig. 6a).

A52C GlyRs

PMTS exposure caused irreversible potentiation in A52C GlyRs (Fig. 6b). Subsequent ethanol exposure produced significant, reversible potentiation over and above the irreversible potentiation from the initial PMTS exposure. These findings in A52C GlyRs indicate that PMTS binding to cysteines substituted at position 52 does not prevent further modulation by ethanol and are consistent with the notion that ethanol causes potentiation by acting on other targets like position 267.

S267C GlyRs

PMTS exposure caused irreversible potentiation in S267C GlyRs (Fig. 6c). Subsequent 100 mmol/L ethanol exposure reversibly reduced the magnitude of the irreversible potentiation from the initial PMTS exposure (negative modulation). These findings in S267C GlyRs indicate that: (i) PMTS binding to cysteines substituted at position 267 does not prevent further modulation by ethanol; (ii) ethanol acts at sites other than those activated by PMTS binding at position 267; and (iii) ethanol acting on these other sites causes negative modulation of the receptor.

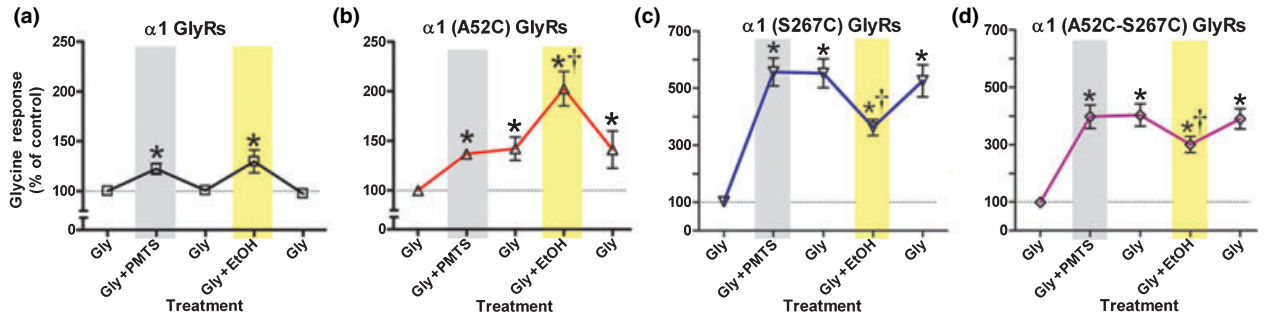

Fig. 6 PMTS binding to cysteines substituted at position 52 and/or 267 reveals position-specific negative and positive modulation by ethanol in α1GlyRs. Mean ± SEM percent control glycine response for (a.) WT and (b–d) mutant α1GlyRs (n = 4–9). The shaded boxes indicate GlyR responses in the presence of saturating concentrations of PMTS as shown in Fig. 5 (30 μmol/L for WT and A52C GlyRs, 300 μmol/L for S267C and A52C–S267C GlyRs) (gray) or 100 mmol/L ethanol (yellow). There were significant main effects of treatment [$F_{4,76}$ = 65.31, $p < 0.0001$], mutation [$F_{3,76}$ = 19.43, $p < 0.0001$] and an interaction between main effects [$F_{12,76}$ = 18.95, $p < 0.0001$]. (* $p < 0.05$ vs. control glycine EC_{10} response, † $p < 0.05$ vs. residual effect of PMTS).

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A52C–S267C GlyRs

PMTS exposure caused irreversible potentiation in A52C–S267C GlyRs (Fig. 6d). Subsequent ethanol exposure caused negative modulation. These results are similar to those with S267C GlyRs, but the magnitude of negative modulation by ethanol in the PMTS-exposed double mutant was significantly less than in PMTS-exposed S267C GlyRs (Fig. 6c vs d , $p < 0.05$ by Student's *t*-test). Therefore, blocking the negative modulation by ethanol acting on position 52 reduced the extent of negative modulation by ethanol, but did not eliminate it. The ability of ethanol to cause negative modulation when access to positions 52 and 267 is blocked or sterically hindered suggests that there is at least one other target for ethanol in GlyRs that can function independently from positions 52 and 267 to produce net negative modulation.

Alcohol cutoff

Prior work found that PMTS binding to cysteine substituted residues at position 267 reduced the alcohol cutoff in GlyRs from between decanol and dodecanol (Mascia *et al.* 1996a, 2000) to below octanol, but did not alter WT GlyR cutoff (Mascia *et al.* 2000). These findings were taken to support the hypothesis that residues in the TM domain of α1GlyRs represent part of an alcohol pocket. We hypothesized that position 52 in Loop 2 of the extracellular domain is also part of an alcohol pocket. If true, then PMTS binding to cysteines substituted at position 52 should also decrease the alcohol cutoff.

To test the notion that position 52 is part of an alcohol pocket, we studied the effects of *n*-alcohols on A52C GlyRs before and after PMTS exposure (Fig. 7). Octanol and decanol each produced significant reversible potentiation when applied prior to PMTS in A52C GlyRs (Fig. 7a). As expected, PMTS produced irreversible potentiation in A52C GlyRs when applied after the alcohols (data not shown). Following PMTS exposure, octanol, but not decanol, caused significant reversible potentiation in PMTS-exposed A52C GlyRs (Fig. 7a). This loss of decanol potency in PMTS-exposed A52C GlyRs indicates that PMTS binding to cysteines substituted at position 52 reduced the *n*-alcohol cutoff in these receptors.

We also tested alkanediols (diols) in order to investigate the possibility that the reduced decanol potency following PMTS exposure reflected its lower solubility compared to octanol (Peoples and Ren 2002). The responses by the diols paralleled those observed with *n*-alcohols of the same length (Fig. 7b). The higher concentrations of decanediol produced some potentiation when applied after PMTS, but the maximal level of potentiation plateaued at a significantly lower level than for decanediol applied before PMTS. Therefore, the diol findings are consistent with the notion that PMTS binding at position 52 reduces the size of an alcohol pocket.

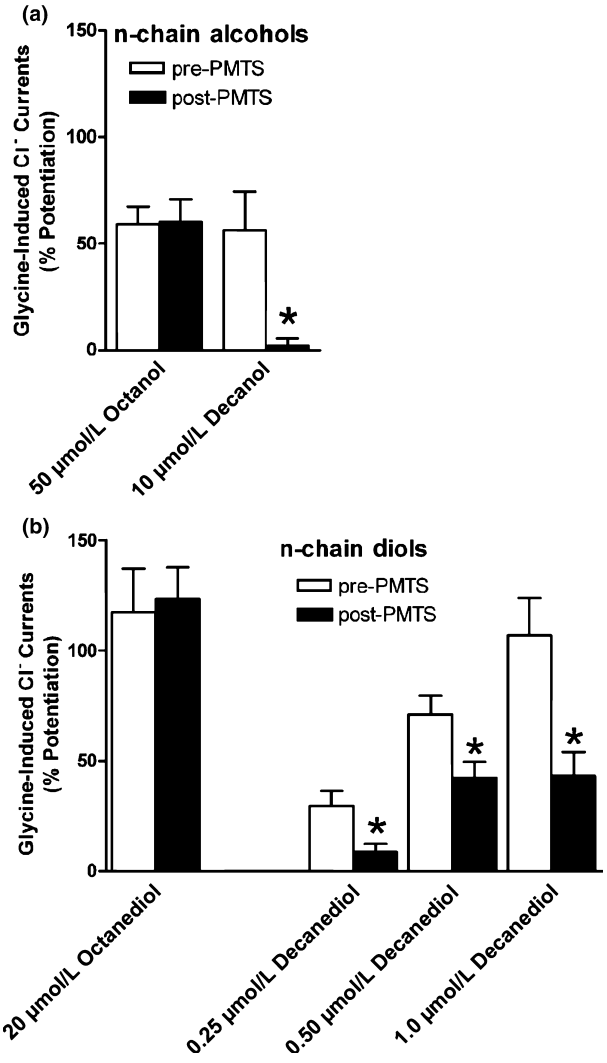

Fig. 7 PMTS exposure in α1 (A52C) GlyRs reduces the alcohol cutoff. (a) Alcohols and (b) Alkanediols. Mean ± SEM percent potentiation of EC_{10} glycine response before (white bars) and after (black bars) exposure to 30 µmol/L PMTS ($n = 4–6$). (* $p < 0.05$ Pre-PMTS vs. Post-PMTS by Student's *t*-test).

Molecular modeling

The Binding Site Analysis module of Insight 2005 L explored the α1GlyR subunit and found several cavities. However, not all the cavities found meet the criteria set by the experimental evidence. The largest cavity fulfills these criteria and is shown in Fig. 8. This pocket extends from the S267 residue in the TM domain, through the interface between the TM and extracellular domains, and ends at residue A52 in Loop 2 of the extracellular domain, with approximately 28Å separating the Cα atoms of A52 and S267. The interconnected sections of the pocket are large enough to accommodate ethanol and to allow its passage between these regions. The ability of the A52C–S267C double mutant to gate currents and bind PMTS is consistent

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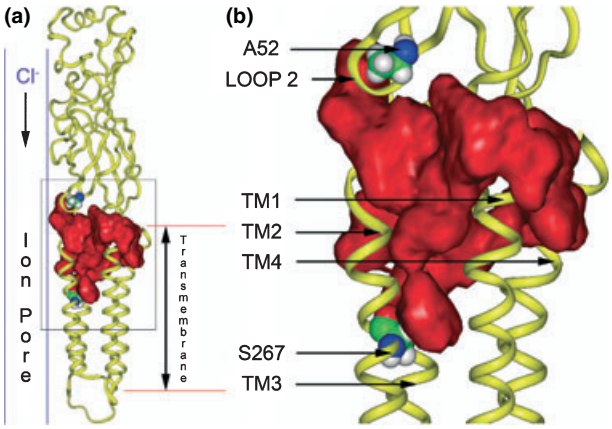

Fig. 8 Molecular model of an α1GlyR subunit with the alcohol pocket highlighted. (a) Full subunit. The backbone atoms of one α1GlyR subunit are shown as a yellow ribbon. Residues A52 and S267 are rendered as space-filling surfaces and atoms are colored red, black, white, and blue for oxygen, carbon, hydrogen, and nitrogen, respectively. The largest cavity found by the Binding Site Analysis module of Insight 2005 L is shown with a red surface. (b) Zoom view. The area enclosed by a rectangle in (a) is expanded to provide a view of the interface of the two domains. The Cα atoms of A52 and S267 are separated by approximately 28 Å.

with there being enough distance between positions 52 and 267 to prevent spontaneous disulfide linkage between the cysteine substitutions.

Discussion

The focus of the present investigation on the extracellular domain of α1GlyRs marks a departure from prior studies which implicate the TM domain as the initial target for ethanol action (Mihic *et al.* 1997; Wick *et al.* 1998; Ye *et al.* 1998; Yamakura *et al.* 1999; Ueno *et al.* 2000; Jenkins *et al.* 2001; Lobo *et al.* 2006). The limited studies to date in the extracellular domain found that mutations at position 52 in Loop 2 change ethanol sensitivity, alter sensitivity to an ethanol antagonist and can eliminate the subunit-dependent differences in ethanol sensitivity between α1 and α2 GlyRs (Mascia *et al.* 1996b; Davies *et al.* 2004). These initial findings support the notion that ethanol also acts on the extracellular domain.

The current investigation adds two key elements to the evidence supporting the extracellular domain as an ethanol target. First, this work employed cysteine mutagenesis at position 52 in α1GlyRs to show that PMTS binding to this site caused irreversible alcohol-like potentiation. These results demonstrate a cause-effect relationship between action on a site in the extracellular domain and alcohol-like GlyR modulation. Second, PMTS binding to cysteines substituted at position 52 in A52C GlyRs decreased the alcohol cutoff. The cutoff reduction suggests that the extracellular domain contributes to an alcohol pocket. Collectively, these findings with position 52 of the α1GlyR parallel findings that established the TM domain as a target for ethanol and indicate that ethanol acts on targets in both the TM and extracellular domains.

Our findings also suggest differences in the responses to ethanol at targets in the extracellular and TM domains in α1GlyRs. Ethanol potentiated WT and A52C GlyR function, but inhibited agonist action in S267C and A52C–S267C GlyRs. In contrast, PMTS caused potentiation in all receptors tested. The differences between PMTS and ethanol do not appear to reflect the differences in their volumes since hexanol results paralleled those of ethanol. Together, these findings with PMTS and ethanol suggest that the responses to anesthetics, *per se*, in WT GlyRs vary by agent (e.g., ethanol vs. PMTS), position (e.g., position 52 vs. 267), and possibly domain (e.g., extracellular vs. TM).

We tested these notions by using PMTS, in combination with cysteine mutations, to isolate PMTS and ethanol effects on putative sites of action in the extracellular and TM domains. We reasoned that PMTS binding to a cysteine-substituted residue would prevent further modulation at that site and, thus, would reveal the nature of the PMTS or ethanol effects through action on other targets that can function independently from the site to which PMTS binds. This approach is analogous to using specific receptor antagonists to help isolate and characterize the action of ligands at different sites.

From this perspective, our findings provide evidence that PMTS and ethanol cause different responses in α1GlyRs when their access to position 52 and/or 267 is blocked or sterically hindered. PMTS acting at either position 52 (extracellular domain) or 267 (TM domain) caused reversible potentiation when the other target was blocked. PMTS did not have an effect in the double mutant when access to both positions was blocked. Together, these findings indicate that: (i) PMTS causes potentiation when it acts on position 52 or 267; and (ii) PMTS acting on positions 52 and 267 accounts for all of the effects of PMTS on WT α1GlyR function.

In contrast to PMTS, the characteristics of the ethanol response depended on whether the initial PMTS exposure blocked access to position 52 and/or 267 in α1GlyRs. When position 52 was blocked, ethanol caused reversible potentiation, which suggests that ethanol acting on other targets (e.g., position 267) produces positive modulation. On the other hand, when position 267 was blocked, ethanol caused reversible inhibition, which suggests that ethanol acting on other targets (e.g., position 52) produces negative modulation. Interestingly, when positions 52 and 267 were both blocked, ethanol caused a small, but significant, amount of reversible negative modulation. This small amount of residual negative modulation in the double mutant GlyR indicates that positions 52 and 267 do not account for all of

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the effects of ethanol and that ethanol acting on the remaining site(s) causes negative modulation.

The aforementioned ethanol effects in PMTS-exposed WT and mutant GlyRs provide insight into the respective actions of ethanol on positions 52 and 267. Ethanol produced negative modulation when its action on position 267 was blocked by PMTS exposure in S267C GlyRs. The degree of negative modulation produced by ethanol decreased when both position 52 and 267 were blocked in the double mutant. These findings in S267C and A52C–S267C GlyRs suggest that ethanol acting on position 52 causes negative modulation. If true, then one would predict that blocking ethanol action on position 52 in A52C GlyRs would increase the degree of positive modulation versus WT GlyRs. The findings confirm this prediction and further support the conclusion that ethanol acting on position 52 produces negative modulation. Parallel evidence supports the notion that ethanol acting on position 267 produces positive modulation.

These ethanol findings in PMTS-exposed WT and mutant GlyRs are consistent with at least three functionally different targets for ethanol action in GlyRs: (i) Position 267 in the TM domain (ethanol-induced positive modulation); (ii) Position 52 in Loop 2 of the extracellular domain (ethanol-induced negative modulation); and (iii) One or more other sites (net ethanol-induced negative modulation) that may include residues near positions 52 and 267 not sterically hindered by PMTS binding. Collectively, these findings suggest that the net ethanol effect on WT GlyRs represents the summation of positive and negative modulatory effects on multiple targets.

These conclusions are unexpected. Therefore it is important to consider alternative explanations for the findings. For example, the proposed negative modulation could reflect conformational changes induced by mutation or by PMTS binding to cysteine substitutions at positions 52 and/or 267. These conformational changes in the receptor could alter interactions between these and other sites, which in turn, could change responses to allosteric modulators in a manner not consistent with responses at specific sites in WT GlyRs. The present and previous findings (Ye *et al.* 1998), which demonstrate that mutating positions 52 and 267 can alter the ethanol response of α1GlyRs in the presence and absence of glycine, are consistent with the latter possibility. However, the congruence of findings would be difficult to explain simply by mutation-induced conformational changes in receptor function. For example, the concept that ethanol acting on position 52 causes negative modulation is supported by a combination of findings in WT and mutant GlyRs, with different responses to ethanol and PMTS, that are unlikely to reflect coincidental changes in receptor function. Nevertheless, further studies are necessary to explore these and other possible alternative explanations to the present findings.

The contention that ethanol causes opposing actions on different targets within a receptor is supported by previous findings in the TM region of GlyRs (Ye *et al.* 1998). This prior study found an inverse linear relationship between the effects of ethanol on GlyR function and the molecular volume of amino acid substitutions at position 267, with a crossover from potentiation to inhibition at isoleucine. It was suggested that this switch in ethanol response reflected solely a crossover in the ethanol response at position 267. Subsequent studies with the S267Q mutation supported this hypothesis and suggested that the mutation changed ethanol from a positive to a negative allosteric modulator (Findlay *et al.* 2002, 2003). The present findings suggest that the crossover in ethanol effect with mutation of position 267 could result from changes in the summed response to ethanol action on multiple sites. For example, progressive increases in the molecular volume of the substitution at position 267 could reduce the degree of ethanol-induced positive modulation at a given concentration to the point where it reveals the negative modulation by ethanol acting on position 52 and/or other targets. Further research is necessary to investigate these scenarios. Regardless, the present findings add a new dimension to interpreting the relationship between structure and function in the actions of ethanol on GlyRs and other LGICs.

Prior studies support the concept that ethanol may have multiple targets with different responses in the same receptor protein. Propanethiol binding to cysteine substitutions revealed changes in *n*-alcohol modulation consistent with an excitatory site and an inhibitory site in the TM2 region of nAChRs (Borghese *et al.* 2003). In contrast to the present findings in GlyRs, the excitatory and inhibitory sites in nAChRs were on adjacent positions in the same domain. Similarly, recent studies with a TM mutation suggest positive and negative modulatory sites for isoflurane in $GABA_A$Rs (Hall *et al.* 2005). Moreover, the present findings with ethanol parallel the excitatory and inhibitory responses of GlyRs to low and high Zn^{++} concentrations, which are believed to result from its action at different sites on the receptor (Laube *et al.* 1995). Together, the evidence suggests that LGICs in general may have both positive and negative modulatory targets for ethanol and that these targets may reside in either or both the extracellular and TM domains.

The present study identifies a new question: Are the putative sites of ethanol action in the TM and extracellular domains of α1GlyRs part of a single alcohol pocket? We addressed this question by investigating the effects of PMTS binding to cysteines substituted at positions 52 (extracellular domain) and/or 267 (TM domain) on the alcohol cutoff. We postulated that at least one of these ethanol targets must be in the pocket that determines the WT GlyR cutoff. If each site is part of a separate pocket, then reaction of one site with PMTS should alter the WT cutoff, but reaction of the other site should not. Previous studies found that WT α1GlyRs

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have a cutoff between decanol and dodecanol and that PMTS binding to cysteines substituted at position 267 reduced the cutoff to below octanol (Mascia *et al.* 1996a, 2000). We found that PMTS binding to cysteines substituted at position 52 reduced the cutoff to between octanol and decanol. PMTS binding to cysteines substituted at both position 52 and 267 appeared to reduce the cutoff to below hexanol (sensitive to ethanol, but not PMTS as shown in Figs. 5d and 6d). Therefore, the cutoff findings indicate that neither positions 52 nor 267 belong to a separate pocket that can independently account for the WT cutoff.

The present findings do not eliminate the possibility that positions 52 and 267 are in separate pockets; however there is no experimental evidence to support this alternative. It is also possible that a pocket involving positions 52 and 267 could extend between subunits, but this possibility is not consistent with the orientation of position 267, in which the residue side chain faces into the subunit (Xu and Akabas 1996). Therefore, the available evidence supports the notion that position 52 of the extracellular domain and position 267 in the TM domain are part of the same alcohol pocket.

Collectively, the present study provides evidence that the extracellular and TM domains are targets for ethanol action in α1GlyRs and that positions 52 and 267 in the extracellular and TM domains, respectively, are part of a single alcohol pocket. Given that this pocket contains sites capable of producing ethanol effects, we describe the pocket as an ethanol "action pocket" to distinguish it from classical binding sites, which have higher affinity for their substrates. Taking these findings and previous work into consideration, molecular modeling of the α1GlyR revealed a cavity that extends approximately 28 Å from the Cα atoms of A52 to S267 that could function as this alcohol action pocket. The interconnected sections of the pocket are large enough to accommodate ethanol and to allow its passage between these regions. The proposed pocket could hold several ethanol molecules and fewer larger alcohols. The estimated distance between position 52 and 267 (28 Å) likely precludes action by one ethanol molecule at both sites simultaneously, and suggests that at low concentrations, ethanol can enter and move in the pocket without touching one of these action sites. As the concentration of ethanol increases, the probability that ethanol molecule(s) will be acting on one or more of these sites at a given moment increases. The net response to ethanol on receptor function will represent the summation of the actions of ethanol on these targets. This putative alcohol action pocket is consistent with the reduced electron density seen in cryo-electron micrographs of nAChRs (Unwin 2005) and with the intertwined loops in our previous molecular models (Trudell 2002; Trudell and Bertaccini 2004). Further study is necessary to investigate the model, to map the role of other extracellular domain residues (within and outside of Loop 2) in the actions of ethanol, to discover if other alcohol action pockets exist, and to investigate whether the present findings generalize to other LGICs.

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References

Alifimoff J. K., Firestone L. L. and Miller K. W. (1989) Anaesthetic potencies of primary alkanols: implications for the molecular dimensions of the anaesthetic site. *Br. J. Pharmacol.* **96**, 9–16.

Bertaccini E. and Trudell J. R. (2002) Predicting the transmembrane secondary structure of ligand-gated ion channels. *Protein Eng.* **15**, 443–453.

Borghese C. M., Henderson L. A., Bleck V., Trudell J. R. and Harris R. A. (2003) Sites of excitatory and inhibitory actions of alcohols on neuronal α2β4 nicotinic acetylcholine receptors. *J. Pharmacol. Exp. Ther.* **307**, 42–52.

Brejc K., van Dijk W. J., Klaassen R. V., Schuurmans M., van der Oost J., Smit A. B. and Sixma T. K. (2001) Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature* **411**, 269–276.

Davies D. L., Trudell J. R., Mihic S. J., Crawford D. K. and Alkana R. L. (2003) Ethanol potentiation of glycine receptors expressed in *Xenopus* oocytes antagonized by increased atmospheric pressure. *Alcohol. Clin. Exp. Res.* **27**, 1–13.

Davies D. L., Crawford D. K., Trudell J. R., Mihic S. J. and Alkana R. L. (2004) Multiple sites of ethanol action in α1 and α2 glycine receptors suggested by sensitivity to pressure antagonism. *J. Neurochem.* **89**, 1175–1185.

Dietrich R. A., Dunwiddie T. V., Harris R. A. and Erwin V. G. (1999) Mechanism of action of ethanol: initial central nervous system actions. *Pharmacol. Rev.* **41**, 489–537.

Findlay G. S., Wick M. J., Mascia M. P., Wallace D., Miller G. W., Harris R. A. and Blednov Y. A. (2002) Transgenic expression of a mutant glycine receptor decreases alcohol sensitivity of mice. *J. Pharmacol. Exp. Ther.* **300**, 526–534.

Findlay G. S., Phelan R., Roberts M. T., Homanics G. E., Bergeson S. E., Lopreato G. F., Mihic S. J., Blednov Y. A. and Harris R. A. (2003) Glycine Receptor Knock-In Mice and Hyperekplexia-Like Phenotypes: Comparisons with the Null Mutant. *J. Neurosci.* **23**, 8051–8059.

Hall A. C., Stevens R. J. N., Betts B. A., Yeung W. Y., Kelley J. C. and Harrison N. L. (2005) Subunit-dependent block by isoflurane of wild-type and mutant [alpha]1S270H GABAA receptor currents in Xenopus oocytes. *Neurosci. Lett.* **382**, 332–337.

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Harris R. A. (1999) Ethanol actions on multiple ion channels: which are important? *Alcohol. Clin. Exp. Res.* **23**, 1563–1570.

Jenkins A., Greenblatt E. P., Bertaccini E., Faulkner H. J., Light A., Meng X., Andreasen A., Viner A., Trudell J. R. and Harrison N. L. (2001) Measuring a general anesthetic binding cavity in the GABAa receptor. *J. Neurosci.* **21**, 1–4.

Karlin A. and Akabas M. H. (1998) Substituted-Cysteine Accessibility Method. *Methods Enzymol* **293**, 123–145.

Krasowski M. D. and Harrison N. L. (1999) General anaesthetic actions on ligand-gated ion channels. *Cell Mol. Life Sci.* **55**, 1278–1303.

Laube B., Kuhse J., Rundström N., Kirsch J., Schmieden V. and Betz H. (1995) Modulation by zinc ions of native rat and recombinant human inhibitory glycine receptors. *J. Physiol. (Lond)* **483**, 613–619.

Lobo I. A., Mascia M. P., Trudell J. R. and Harris R. A. (2004) Channel Gating of the Glycine Receptor Changes Accessibility to Residues Implicated in Receptor Potentiation by Alcohols and Anesthetics. *J. Biol. Chem.* **279**, 33919–33927.

Lobo I. A., Trudell J. R. and Harris R. A. (2006) Accessibility to residues in transmembrane segment four of the glycine receptor. *Neuropharmacology* **50**, 174–181.

Mascia M. P., Machu T. L. and Harris R. A. (1996a) Enhancement of homomeric glycine receptor function by long-chain alcohols and anaesthetics. *Br. J. Pharmacol.* **119**, 1331–1336.

Mascia M. P., Mihic S. J., Valenzuela C. F., Schofield P. R. and Harris R. A. (1996b) A single amino acid determines differences in ethanol actions on strychnine-sensitive glycine receptors. *Mol. Pharmacol.* **50**, 402–406.

Mascia M. P., Trudell J. R. and Harris R. A. (2000) Specific binding sites for alcohols and anesthetics on ligand-gated ion channels. *Proc. Natl Acad. Sci. USA* **97**, 9305–9310.

Mihic S. J., Ye Q., Wick M. J. *et al.* (1997) Sites of alcohol and volatile anaesthetic action on $GABA_A$ and glycine receptors. *Nature* **389**, 385–389.

Ortells M. O. and Lunt G. G. (1995) Evolutionary history of the ligand-gated ion-channel superfamily of receptors. *TINS* **18**, 121–127.

Peoples R. W. and Ren H. (2002) Inhibition of *N*-Methyl-D-aspartate receptors by staight-chain diols: Implications for the mechanism of the alcohol cutoff effect. *Mol. Pharmacol.* **61**, 169–176.

Pringle M. J., Brown K. B. and Miller K. W. (1981) Can the lipid theories of anesthesia account for the cutoff in anesthetic potency in homologous series of alcohols? *Mol. Pharmacol.* **19**, 49–55.

Roberts M. T., Phelan R., Erlichman B. S., Pillai R. N., Ma L., Lopreato G. F. and Mihic S. J. (2006) Occupancy of a single anesthetic binding pocket is sufficient to enhance glycine receptor function. *J. Biol. Chem.* **281**, 3305–3311.

Ryan S. G., Buckwalter M. S., Lynch J. W., Handford C. A., Segura L., Shiang R., Wasmuth J. J., Camper S. A., Schofield P. R. and O'Connel P. (1994) A missense mutation in the gene encoding the α1 subunit of the inhibitory glycine receptor in the spasmodic mouse. *Nature Genet.* **7**, 131–135.

Saul B., Schmieden V., Kling C., Mülgardt C., Gass P., Kuhse J. and Becker C.-M. (1994) Point mutation of glycine receptor α1 subunit in the spasmodic mouse affects agoinst responses. *FEBS Lett.* **350**, 71–76.

Trudell J. R. (2002) Unique assignment of inter-subunit association in $GABA_A$ α1β3γ2 receptors determined by molecular modeling. *Biochim. Biophys. Acta* **1565**, 91–96.

Trudell J. R. and Bertaccini E. (2004) Comparative modeling of a $GABA_A$ alpha1 receptor using three crystal structures as a template. *J. Mol. Graph Model* **23**, 39–49.

Ueno S., Lin A., Nikolaeva N., Trudell J. R., Mihic S. J., Harris R. A. and Harrison N. L. (2000) Tryptophan scanning mutagenesis in TM2 of the $GABA_A$ receptor α subunit: effects on channel gating and regulation by ethanol. *Br. J. Pharmacol.* **131**, 296–302.

Unwin N. (2005) Refined structure of the Nicotinic Acetylcholine Receptor at 4Å Resolution. *J. Mol. Biol.* **346**, 967–989.

Wick M. J., Mihic S. J., Ueno S., Mascia M. P., Trudell J. R., Brozowski S. J., Ye Q., Harrison N. L. and Harris R. A. (1998) Mutations of γ-aminobutyric acid and glycine receptors change alcohol cutoff: Evidence for an alcohol receptor? *Proc. Natl Acad. Sci. USA* **95**, 6504–6509.

Xiu X., Hanek A. P., Wang J., Lester H. A. and Dougherty D. A. (2005) A Unified View of the Role of Electrostatic Interactions in Modulating the Gating of Cys Loop Receptors. *J. Biol. Chem.* **280**, 41655–41666.

Xu M. and Akabas M. H. (1996) Identification of channel-lining residues in the M2 membrane-spanning segment of the $GABA_A$ receptor α1 subunit. *J. Gen. Physiol.* **107**, 195–205.

Yamakura T., Mihic S. J. and Harris R. A. (1999) Amino Acid Volume and Hydropathy of a Transmembrane Site Determine Glycine and Anesthetic Sensitivity of Glycine Receptors. *J. Biol. Chem.* **274**, 23006–23012.

Ye Q., Koltchine V. V., Mihic S. J., Mascia M. P., Wick M. J., Finn S. E., Harrison N. L. and Harris R. A. (1998) Enhancement of glycine receptor function by ethanol is inversely correlated with molecular volume at position α267. *J. Biol. Chem.* **273**, 3314–3319.

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