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A Single Amino Acid Determines Differences in Ethanol Actions on Strychnine-Sensitive Glycine Receptors

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SUMMARY

Effects of ethanol on strychnine-sensitive glycine receptors were studied in *Xenopus laevis* oocytes expressing α_1 wild-type, α_2, or mutant α_1(A52S) homomeric glycine receptors. This α_1(A52S) mutant, in which a serine residue substitutes for alanine at amino acid 52, is responsible for the spasmodic phenotype in mice and alters the ability of glycine to activate the receptor. Pharmacologically relevant concentrations of ethanol (10–200 mM) reversibly potentiated the glycine receptor function in all receptors. Ethanol potentiation depended on the glycine concentration used, with decreased potentiation observed at higher glycine concentrations. Homomeric α_1 glycine receptors were more sensitive to the effects of ethanol than were α_2 or the mutant α_1(A52S) receptors. No differences were found in ethanol sensitivity between α_2 and the mutant α_1(A52S) receptors. The α_2 subunit has a threonine residue, a conservative substitution for serine, at amino acid 52. The general anesthetic propofol was also tested in homomeric α_1, α_2, or the mutant α_1(A52S) receptors. Propofol, at unaesthetic concentrations (1–5 μM), reversibly potentiated the glycine receptor function in a concentration-dependent manner and to an equal extent in the three subunits tested. These data suggest that the mutation of an alanine to serine at amino acid 52 of the α subunit is responsible for the difference in ethanol sensitivity seen in homomeric receptors composed of α_1 and α_2 subunits.

Neuronal inhibition in the spinal cord and brainstem is primarily mediated by glycine through the activation of a ligand-gated receptor linked to an integral Cl^- channel. This strychnine-sensitive glycine receptor is also found in supraspinal regions such as hippocampus, cortex, and cerebellum (1). The inhibitory glycine receptor, which shares sequence homology with the $GABA_A$ receptor (2), is composed of α and β subunits, with the α subunit containing the binding sites for agonists and antagonists (3, 4). Glycine receptor α subunits form functional homomeric receptors in *Xenopus laevis* oocytes and cultured mammalian cells, with properties similar to those of native receptors (1, 5). Four different subtypes of α subunits have been cloned (α_{1-4}), but only one β subunit has been found (5, 6). The α subtypes of the glycine receptor show distinct pharmacological properties (1, 7) and differing developmental expression (8). The α_1 and α_2 subunits are the most abundant α glycine receptor subunits in the central nervous system (8). During developmental progression from fetus to neonate, α_2 and β subunits predominate. During this period, most glycine receptors are homomeric α_2 receptors (9). Within 2–3 weeks after birth, the α_2 glycine receptor subunit is almost completely replaced by α_1, and the adult receptor is primarily of the heteromeric α_1 plus β form (10).

A number of recent studies using behavioral, biochemical, and electrophysiological techniques have indicated that the $GABA_A$ receptor/Cl^- channel complex is an important site of action of ethanol *in vivo* (11, 12). The depressant effect of ethanol may be due to an enhancement of inhibitory neurotransmission, which is in part mediated by GABA. However, very few studies have examined the actions of ethanol on glycine receptors. A recent report provides behavioral evidence showing that glycine and the glycine precursor serine are able to enhance the central depressant effects of ethanol, measured as loss of the righting reflex in mice (13). This behavioral action was blocked by strychnine, suggesting that glycine can enhance ethanol effects by acting on the strychnine-sensitive glycine receptor. Electrophysiological studies show that ethanol positively modulates the glycine receptor in embryonic spinal neurons of mouse and chick in a concentration-dependent manner (14, 15). Furthermore, ethanol is able to potentiate glycine-activated Cl^- uptake into synaptoneurosomes prepared from whole rat brain (16).

Because there are no published reports of the effects of ethanol on recombinant glycine receptors, we undertook the current study to evaluate a wide range of ethanol concentra-

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ABBREVIATIONS: GABA, γ-aminobutyric acid; MBS, modified Barth's saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

tions on glycine receptor subunits expressed in *X. laevis* oocytes. Electrophysiological studies of cultured embryonic spinal cord neurons have reported differences in ethanol sensitivity among individual cells (14). Because of the replacement of α_2 subunits with α_1 subunits during development, we were interested in determining whether these subunits differ in ethanol sensitivity and could potentially account for some of the variability in ethanol actions.

Experimental Procedure

Materials. Adult female *X. laevis* oocytes were obtained from Xenopus I (Ann Arbor, MI), glycine was from BioRad Laboratories (Hercules, CA), ethanol was from Aaper Alcohol and Chemical Co. (Shelbyville, KY), and propofol was from Aldrich Chemical (Milwaukee, WI). All other reagents were of reagent grade. Human α_1, α_2, and the mutant α_1(A52S) glycine receptor subunits cDNAs (5) were cloned into the mammalian expression vector PCIS 2 (17). Mutagenesis of alanine to serine was performed according to Ryan *et al.* (18).

Oocyte preparation, microinjection, and electrophysiological recording. Preparation of the oocytes and microinjection of the cDNA were performed as described previously (19). Isolated oocytes were placed in MBS (containing 88 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 0.91 mM $CaCl_2$, and 0.33 mM $Ca(NO_3)_2$, adjusted to pH 7.5). Glycine receptor subunit cDNAs [wild-type α_1 or α_2 or mutant α_1(A52S), 0.4 ng/30 nl] were injected into the animal poles of oocytes according to the "blind" method of Colman (20). The injected oocytes were cultured at 15–19° in sterile MBS supplemented with 10 mg/liter streptomycin, 10,000 units/liter penicillin, 50 mg/liter gentamicin, 90 mg/liter theophylline, and 220 mg/liter pyruvate.

Oocytes were used for recording on days 1–4 after injection. Oocytes were placed in a rectangular chamber (~100-μl volume) and perfused (2 ml/min) with MBS with or without drugs using a roller pump (Cole-Parmer Instrument, Chicago, IL) through 18-gauge polyethylene tubing (Clay Adams Co., Parsippany, NJ) that delivered the drug solutions to the recording chamber. The animal poles of oocytes were impaled with two glass electrodes (0.5–10 MΩ) filled with 3 M KCl and voltage-clamped at −50 to −70 mV using an Axoclamp 2A amplifier (Burlingame, CA). A strip-chart recorder (Cole-Parmer Instrument) continuously plotted the clamping currents. Glycine was dissolved in MBS and, in most experiments, applied for 20 sec. Oocytes were perfused with ethanol for 2 min or with propofol for 5 min before coapplication of glycine. A 5-min washout period was allowed between drug applications. When glycine was applied through a micropipette using a Picospritzer II (General Valve, Fairfield, NJ), data were acquired through an ADAC 4801 A/D board (Woburn, MA) using an IBM 386-compatible computer. Current sampling occurred every 5 msec, and data were acquired and analyzed using the Strathclyde Electrophysiology Software program WCP (ver. 1.2e). In these experiments, ethanol was applied by bath perfusion.

Statistical analysis. Statistical analyses were performed using the two-way analysis of variance and Fisher's post-hoc test with the SOLO program and an IBM-compatible computer. Nonlinear regression analysis of the concentration-response curves was performed using GraphPAD software (San Diego, CA). The threshold concentration of ethanol required to enhance glycine responses was determined by the signed rank test. SeqAid program files, used for secondary structure protein determination, can be obtained through the Internet.[1]

Results

Glycine concentration-response curves were determined in *X. laevis* oocytes expressing either α_1, α_2, or the mutant α_1(A52S) glycine receptor subunits (Fig. 1). The glycine EC_{50} was significantly lower in the α_1 receptors (155 μM) than in the α_2 (257 μM) or the mutant α_1(A52S) (478 μM) receptors [$F(2,155) = 52$, $p < 0.0001$]. The Hill coefficients were 1.6, 2.8, and 2.1 for the α_1, α_2, and α_1(A52S) receptors, respectively.

A complete ethanol (5–200 mM) concentration-response curve was next performed on *X. laevis* oocytes expressing homomeric α_1 or α_2 glycine receptor subunits or a mutant α_1 glycine receptor subunit in which a serine replaced an alanine in position 52, α_1(A52S) (Fig. 2). Because we found a difference in glycine affinity between subunits, we performed all subsequent experiments using the same effective glycine concentration (i.e., a concentration of glycine that produced a peak current that was 2% of the maximal current observed). Ethanol potentiated glycine receptor function in homomeric receptors composed of α_1, α_2, or α_1(A52S) subunits. The effect of ethanol increased linearly with concentration. The threshold concentration for statistically significant ethanol enhancement was 5 mM for α_1 and 10 mM for α_2 (Fig. 2, *top*). Analysis of all concentrations of ethanol tested showed a significantly greater effect of ethanol on α_1 than on α_2 receptors [$F(1,169) = 24$, $p < 0.0001$] (Fig. 2, *bottom*). The substitution of A52S in the mutant α_1 receptor significantly decreased its ethanol sensitivity compared with the wild-type α_1 receptor (Fisher's post-hoc test, $p < 0.0001$). There was no significant difference in ethanol effect between α_1(A52S) and α_2 receptors. The magnitude of the potentiation induced by ethanol was dependent on the concentration of glycine used; greater potentiation was found using low concentrations of glycine (Fig. 3).

To test the generality of the subunit differences documented for ethanol, the lipid-soluble alcohol propofol was tested with the three α subunits. Propofol potentiated glycine receptor function to an equal extent in all the homomeric glycine subunits tested (Fig. 4).

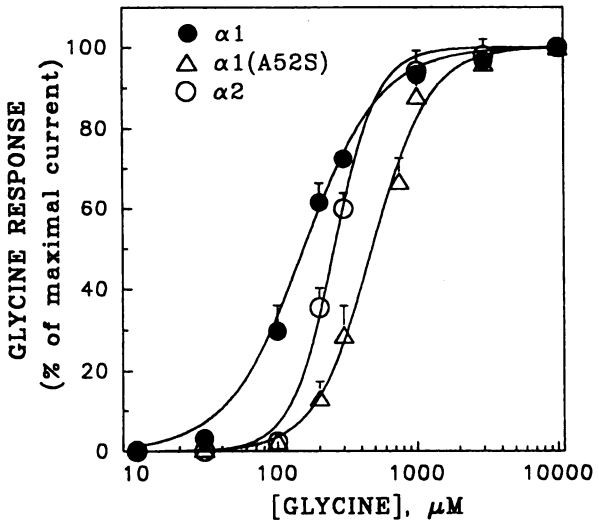

Fig. 1. Concentration-response curves for glycine (10–10,000 μM) activated Cl^- currents in *X. laevis* oocytes expressing α_1, α_2, or the mutant α_1(A52S) glycine receptor subunits. Glycine was applied for 20 sec, and peak currents were measured. Ordinate values are expressed as the percentage of maximal current observed using 10 mM glycine. The glycine EC_{50} values for α_1, α_2, and α_1(A52S) were 155 ± 19, 257 ± 14, and 478 ± 16 μM, respectively. Hill coefficients were 1.6 for α_1, 2.8 for α_2, and 2.1 for α_1(A52S). Values are mean ± standard error from five oocytes. See text for statistical analysis.

[1] ftp://iubio.bio.indiana.edu/molbio/ibmpc/sqaid381.exe

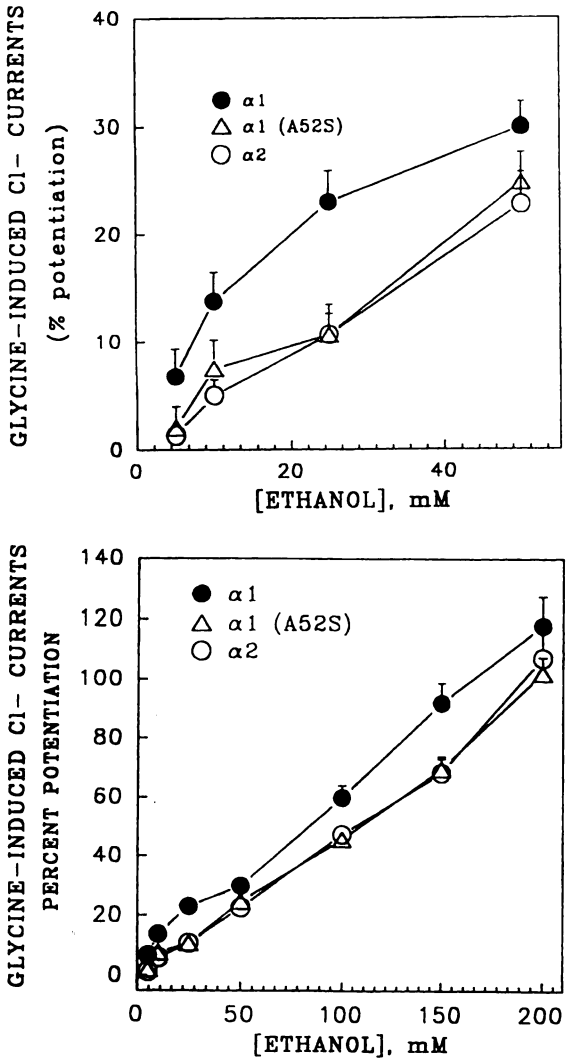

Fig. 2. Ethanol potentiation of currents evoked by glycine in *X. laevis* oocytes expressing homomeric α_1, α_2, or the mutant α_1(A52S) subunits. *Top*, expanded scale displays effects of low concentrations of ethanol (5–50 mM) on glycine receptor subunits. *Bottom*, results from all concentrations of ethanol tested. Ethanol was bath-applied for 2 min before being coapplied with an EC_2 concentration of glycine for 20 sec. The EC_2 concentration of glycine was determined for each oocyte. The concentration-dependent potentiation by ethanol (5–200 mM) was significantly greater in α_1 than in α_2 or the mutant α_1(A52S) receptors. See text for statistical analysis. No difference in ethanol sensitivity was found between α_2 and the mutant α_1(A52S) receptors. Values are mean ± standard error of 9–13 oocytes.

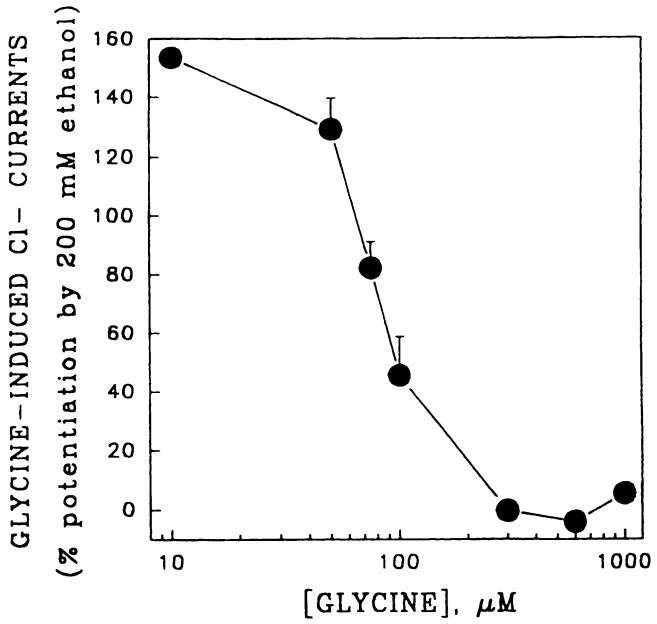

Fig. 3. Ethanol (200 mM) enhancement of glycinergic currents decreased as the glycine concentration was raised. Values are mean ± standard error of three oocytes expressing homomeric α_2 receptors.

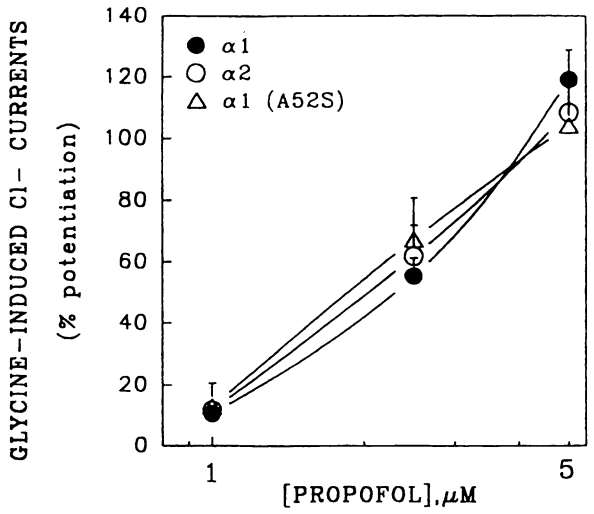

Fig. 4. Propofol potentiated, in a concentration-dependent manner, currents evoked by glycine in *X. laevis* oocytes expressing homomeric α_1, α_2, or the mutant α_1(A52S) subunits. Propofol (1–5 μM) was bath-applied for 5 min before being coapplied with an EC_2 concentration of glycine for 20 sec. No differences in propofol sensitivity were found among the three homomeric receptors tested. Values are mean ± standard error of five oocytes.

To obtain better time resolution of glycine action and to study the effects of ethanol on brief pulses of glycine, such as those that occur during synaptic transmission, a solution of 50 μM glycine was focally applied for 20 msec (this was the duration of the drug pulse) to *X. laevis* oocytes expressing homomeric α_1 glycine receptors. Pharmacologically relevant concentrations of ethanol (25–200 mM) that were bath applied potentiated the responses induced by 50 μM glycine (Fig. 5, *top*). This enhancing effect was reversed after a 5-min washout of ethanol. Ethanol potentiation of the glycine receptor function was seen as an increase in the area under the curve as well as the peak response. For example, a 100 mM concentration of ethanol increased the peak height by 32% and the area under the curve by 45%. Desensitization of glycine responses was modest under these conditions, and 100 mM ethanol, coapplied with 50 μM glycine, did not seem to affect the receptor desensitization (Fig. 5, *bottom*).

Discussion

Our results demonstrate that ethanol enhances the function of human homomeric glycine receptors expressed in *X. laevis* oocytes. Although ethanol affected glycine receptor function with all subunits tested, we found a difference in ethanol sensitivity among the subunits, with the α_1 wild-type receptor being more sensitive to the effects of ethanol than α_2. In addition, the difference in ethanol sensitivity between

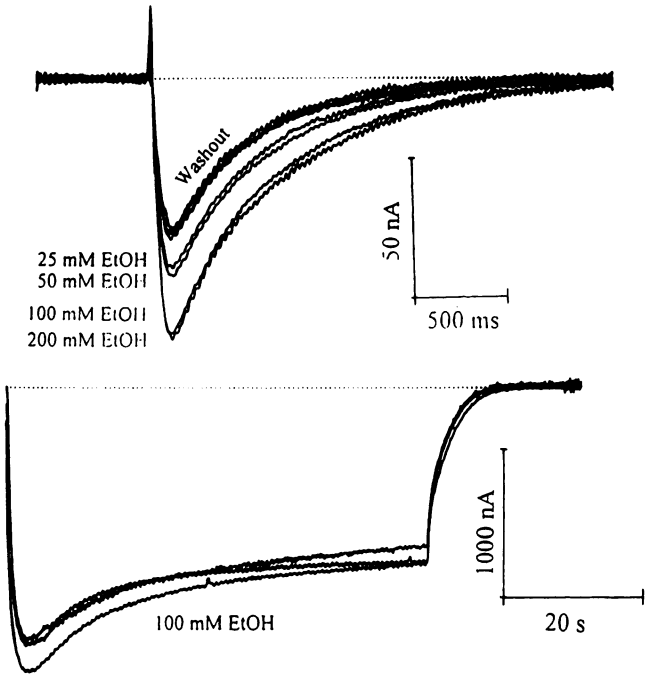

Fig. 5. *Top*, sample tracings of the potentiation of glycine currents induced by ethanol (*EtOH*) in *X. laevis* oocytes expressing the α_1 glycine receptor subunit. Ethanol (25–200 mM) was bath-applied for 2 min before a 20-msec pulse of glycine (50 μM) was focally applied. The potentiation was reversed after a 5-min washout of ethanol. Data were sampled every 3 msec. *Bottom*, sample tracing of the effect of ethanol on the desensitization of the α_1 glycine receptor. Ethanol (100 mM) was bath-applied for 2 min before glycine (50 μM) was applied with a picospritzer for 1 min. Eight minutes were allowed between applications of glycine. Data were sampled every 40 msec.

the α_1 and α_2 subunits could be attributed to a change in a single amino acid.

Previous studies have indicated a role for the strychnine-sensitive glycine receptor in ethanol actions. Behavioral studies have shown that glycine, like GABA, enhances the central depressant effects of ethanol, assessed as a loss of righting reflex. Intracerebroventricular administration of glycine to mice caused a dose-dependent prolongation of the duration of loss of righting reflex (13). Strychnine, but not the $GABA_A$ receptor antagonist bicuculline, was able to antagonize this effect of glycine in a dose-dependent manner. Ethanol was able to enhance glycine-mediated Cl^- flux into synaptoneurosomes prepared from the whole brains of young rats using a Cl^--sensitive fluorescent technique (16). This sort of interaction was also seen at the electrophysiological level; Celentano *et al.* (15), using whole-cell voltage-clamp techniques, showed that 50 mM ethanol produced an increase in the sensitivity to glycine in embryonic chick spinal neurons. Furthermore, Aguayo and Pancetti (14) demonstrated that ethanol potentiated glycine-activated Cl^- currents in mice embryonic spinal neurons. However, these investigators found that one population of neurons responded only to high concentrations of ethanol (100–450 mM), whereas another population responded to concentrations of 1–10 mM as well as to higher concentrations (14). Our results suggest that these two populations may represent cells expressing primarily α_1 or α_2 glycine subunits, respectively. However, the difference in ethanol sensitivity between α_1 and α_2 glycine receptors that we report in this study was not sufficiently large to explain, alone, the much larger differences found by Aguayo and Pancetti in neurons (14). During development, the neonate glycine receptors exist primarily in a homo-oligomeric form composed of α_2 subunits (9). Homo-oligomeric α_2 glycine receptors are replaced 2–3 weeks after birth by receptors containing the α_1 subunit (8, 10). Adult glycine receptors are predominantly heteromeric, composed of α plus β subunits. However, receptors with high conductance levels, characteristic of homo-oligomeric channels, are also found in the adult (10). Our studies were performed using homo-oligomeric α glycine receptors. We attempted to coexpress α and β subunits in *X. laevis* oocytes, but we have not been able to demonstrate the presence of heteromeric receptors. As noted by others (6), *X. laevis* oocytes do not efficiently assemble α and β subunits.

Because previous studies have shown a large range in the glycine EC_{50} values for homomeric receptors expressed in *X. laevis* oocytes (21), the first step in our studies was to compare the glycine concentration-response curves of homo-oligomeric α_1, α_2, and mutant α_1(A52S) glycine receptors. Rundstrom *et al.* (22) reported a difference between the α_1 and α_2 glycine receptor subunits in the EC_{50} for glycine, with the α_1 subunit being more sensitive than α_2. However, other studies did not show any differences in EC_{50} between α_1 and α_2 (5). A recent report (23) showed that the alanine-to-serine exchange at position 52 in the α_1 glycine receptor subunit expressed in *X. laevis* oocytes was responsible for a reduced response to glycine and glycine agonists in comparison with the α_1 wild-type. Similar results were found by Ryan *et al.* (18) using human embryonic kidney 293 cells. In agreement with those reports, we also found a 3-fold increase in the EC_{50} for glycine in the α_1(A52S) receptor compared with the α_1 wild-type.

Ethanol, at concentrations as low as 5 mM, potentiated the glycinergic responses of homomeric α_1 receptors, which were more sensitive to the effects of ethanol than α_2 or the mutant α_1(A52S). This alanine-to-serine mutation in position 52 of the α_1 glycine receptor subunit is responsible for the spasmodic phenotype, an inherited startle syndrome, in mice (9, 18). Furthermore, this A52S substitution in α_1 glycine receptor subunits is characterized by normal glycine binding properties but by an increased glycine EC_{50} when examined in either human embryonic kidney 293 cells or oocytes (18, 23). This suggests that A52S defines part of a site involved in receptor activation rather than receptor binding. The actions of ethanol demonstrated by our study are consistent with the potentiation by ethanol of the glycine response through enhancement of receptor activation. The α_1 glycine receptor subunit exhibits a high degree of homology with the α_2 subunit (76% amino acid identity) (5). Although most differences are in the cytoplasmic region between the third and the fourth transmembrane domains, the amino acid responsible for the differences in ethanol sensitivity is in the amino-terminal extracellular domain. Replacement of the hydrophobic amino acid alanine with the polar uncharged amino acid serine reduced ethanol sensitivity. At this position, the α_2 subunit contains a threonine that is a conservative substitution for serine. Thus, a single amino acid change significantly affects the sensitivity of glycine receptor subunits to ethanol. However, it also possible that glycine receptors composed of homomeric α_1 subunits reach the 2% of maximal activation at a lower level of occupancy than α_2 or α_1(A52S) glycine

receptors. Thus, ethanol may interact identically with all of the receptors tested but show an apparent difference in sensitivity due to different occupancy by an equieffective concentration of glycine. However, differences among the subunits were not found for high concentrations of ethanol or for propofol, and these results are difficult to reconcile with differential occupancy of the glycine receptor.

Our results raise several questions and possibilities. One question is whether a single mechanism can account for the effects of low (5–20 mM) and high (50–200 mM) concentrations of ethanol on both α_1 and α_2 subunits. The concentration-response curves suggest a separate low concentration effect that is greater for α_1 than for α_2, whereas the highest concentration (200 mM) had similar effects on both subunits. This may be analogous to the $GABA_A$ receptor for which the γ_{2L} subunit seems to be required for potentiation by low concentrations of ethanol (24) but all subunit combinations are affected by high concentrations (25). For the glycine receptor, the low concentration effect is reduced by mutation of Ala52, indicating the involvement of the amino-terminal domain. This is in contrast to the $GABA_A$ receptor for which a serine in the intracellular loop between transmembrane segments 3 and 4 is the key determinant of the differences between the alcohol sensitivity of the γ_{2S} and γ_{2L} subunits (26). Because an increase in the polarity of Ala52 by the addition of a hydroxyl group (to give serine) reduces the action of ethanol, it is tempting to propose that Ala52 is part of a hydrophobic pocket that binds ethanol. Following the methods of Garnier *et al.* (27) for the prediction of the secondary structure of proteins, we found that in the wild-type α_1 glycine receptor subunit, residue 52 lies in a region that corresponds to an α helix. However, the same region in the mutant α_1(A52S) and α_2 subunits is predicted to contain an extended β sheet. It is possible that this modification in the secondary structure of the α glycine receptor subunit, due to the single amino acid exchange, allows low concentrations of ethanol to interact more effectively with the receptor protein.

It is important to note that mutation of Ala52 reduces, but does not eliminate, the effects of ethanol. A possible explanation for this finding is that the mutation may change the affinity of ethanol for binding sites in the amino-terminal region. Thus, the ethanol concentration-response curve is merely shifted to the right by mutation of Ala52. Alternatively, ethanol may have multiple sites of action on the receptor, and the mutation may eliminate one of the sites but not affect others.

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