Recent studies suggest that alcohols, volatile anesthetics, and inhaled drugs of abuse, which enhance γ-aminobutyric acid, type A, and glycine receptor-activated ion channel function, may share common or overlapping molecular sites of action on these receptors. To investigate this possibility, these compounds were applied singly and in combination to wild-type glycine α1 receptors expressed in *Xenopus laevis* oocytes. Data obtained from concentration-response curves of the volatile anesthetic enfurane constructed in the presence and absence of ethanol, chloroform, or toluene were consistent with competition for a common binding pocket on these receptors. A mutant glycine receptor, insensitive to the enhancing effects of ethanol but not anesthetics or inhalants, demonstrated antagonism of anesthetic and inhalant effects on this receptor. Although ethanol (25–200 mM) had no effect on its own in this receptor, it was able to inhibit reversibly the enhancing effect of enfurane, toluene, and chloroform in a concentration-dependent manner. These data suggest the existence of overlapping molecular sites of action for ethanol, inhalants, and volatile anesthetics on glycine receptors and illustrate the feasibility of pharmacological antagonism of the effects of volatile anesthetics.

Ethanol, volatile anesthetics, and inhaled drugs of abuse are central nervous system depressants. Although it was once thought that they produced their effects *in vivo* by the nonspecific disordering of cell membranes, it is now generally accepted that these compounds instead exert their actions on specific proteins (1–3). Among these protein targets are the glycine receptors (Gly-Rs),1 responsible for the majority of inhibitory neurotransmission in the brain stem and spinal cord. Gly-Rs are members of a superfamily of ligand-gated ion channels that are sensitive to many neuromodulators, such as GABA, glycine, collagenase, ENF, CHCl3, EtOH, TOL, and TCE; and prevented further enhancement by the anesthetics enfurane or isoflurane (7). In the present study, we demonstrate a reversible binding site interaction through the simultaneous administration of two positive allosteric modulators on Gly-Rs (6, 7), and chemically diverse compounds, such as α-alcohols, anesthetics, and inhalants, may even share a common binding site on these ligand-gated ion channels (3, 7–8). Mutations affecting alcohol and volatile anesthetic actions on receptors may hinder the physical interactions of these compounds with their binding sites or may instead interfere with the abilities of alcohols and anesthetics to transduce their signals to modulate channel function after the compounds have bound.

A conventional investigation of binding sites on receptors would include radioligand competition studies. Unfortunately the low potencies (μM to mM) of alcohols, inhalants, and volatile anesthetics, coupled with the current lack of an anesthetic site antagonist, make these studies unfeasible (9). Recently, an indirect experimental approach by Mascia et al. (7) addressed the anesthetic-binding site/transduction site issue by substituting cysteine for single amino acids believed to line the anesthetic-binding pocket on homomeric α1 Gly-Rs: the amino acids serine 267 and alanine 288. Propanethiol or propyl methanethiosulfonate covalently cross-linked to the receptor at the substituted cysteine at Ser-267 irreversibly enhanced Gly-R currents and prevented further enhancement by the anesthetics enfurane or isoflurane (7). In the present study, we demonstrate a reversible binding site interaction through the simultaneous administration of two positive allosteric modulators on wild-type and mutant α1 Gly-Rs. Specifically, we tested the hypothesis that ethanol (EtOH), the volatile anesthetics enfurane (ENF) and chloroform (CHCl3), and the inhaled drugs of abuse toluene (TOL) and 1,1,1-trichloroethane (TCE) share common or overlapping binding sites on these receptors. Some of this work has been presented previously in abstract form (10).

**EXPERIMENTAL PROCEDURES**

Materials—Penicillin, streptomycin, gentamicin, 3-aminobenzoic acid ethyl ester, GABA, glycine, collagenase, ENF, CHCl3, EtOH, TOL, and TCE were purchased from Sigma. All other chemicals used were of reagent grade. Frogs of the species *Xenopus laevis* were obtained from Xenopus Express (Homosassa, FL).

Oocyte Isolation and cDNA Nuclear Injection—The oocyte isolation and cDNA injection procedures were performed as described previously (3). Oocytes were injected with cDNAs (1.5 ng/30 nl) of wild-type or mutated human glycine receptor α1-subunits subtype A. A conventional investigation of binding sites on receptors would include radioligand competition studies. Unfortunately the low potencies (μM to mM) of alcohols, inhalants, and volatile anesthetics, coupled with the current lack of an anesthetic site antagonist, make these studies unfeasible (9). Recently, an indirect experimental approach by Mascia et al. (7) addressed the anesthetic-binding site/transduction site issue by substituting cysteine for single amino acids believed to line the anesthetic-binding pocket on homomeric α1 Gly-Rs: the amino acids serine 267 and alanine 288. Propanethiol or propyl methanethiosulfonate covalently cross-linked to the receptor at the substituted cysteine at Ser-267 irreversibly enhanced Gly-R currents and prevented further enhancement by the anesthetics enfurane or isoflurane (7). In the present study, we demonstrate a reversible binding site interaction through the simultaneous administration of two positive allosteric modulators on Gly-Rs (6, 7), and chemically diverse compounds, such as α-alcohols, anesthetics, and inhalants, may even share a common binding site on these ligand-gated ion channels (3, 7–8). Mutations affecting alcohol and volatile anesthetic actions on receptors may hinder the physical interactions of these compounds with their binding sites or may instead interfere with the abilities of alcohols and anesthetics to transduce their signals to modulate channel function after the compounds have bound.

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**Results**

The concentrations of glycine receptor enhancement, described by the following biphasic sigmoidal Equation 1,

\[
Y = \frac{A + \frac{D}{1 + e^{X - B}}}{1 + e^{X - F}}
\]  
*(Eq. 1)*

where \(Y\) is the current; \(X\) is the concentration of drug; \(A\) and \(D\) are maximal possible currents; \(B\) and \(E\) are \(EC_{10}\) values, and \(C\) and \(F\) are slope constants.

**Statistics**—Statistics were performed on data obtained from oocytes, by one-way or two-way analysis of variance and appropriate post hoc tests. Within-subject designs were employed whenever feasible. Data are presented as mean ± S.E.; \(n\) values refer to the number of different oocytes from which data were obtained. Statistical significance was defined as \(p < 0.05\) on paired \(t\) tests, Tukey’s or Dunnett’s post hoc test. All groups of data were collected using oocytes obtained from at least two different frogs. The theoretical curves presented in Fig. 9 were created using the following biphasic sigmoidal Equation 1, thus suggesting that a second separate site for chloroform does not exist.

Alcohols and volatile anesthetics produce leftward shifts of glycine concentration-response curves, and as a result, percent enhancement of receptor function by alcohols and anesthetics decreases when higher agonist concentrations are used. Alcohols and anesthetics have minimal effects on peak current amplitudes when maximally effective glycine concentrations are used. We tested whether the apparent competition observed between ENF and the other compounds could be due to the current produced by ENF already being maximal, thus preventing any further enhancement by a second modulator.

First we identified the concentration of glycine (EC\(_{10}\) glycine) that closely approximated the size of the current elicited by EC\(_{10}\) glycine + 1.74 mM ENF. We then tested the ability of 100 mM ETOL to enhance currents of that size, whether elicited by a low concentration of glycine in the presence of ENF or by a higher EC\(_{10}\) concentration of glycine in the absence of ENF. Fig. 4 illustrates that 100 mM ETOL potentiated currents elicited by EC\(_{EQ}\) glycine but not currents elicited by EC\(_{10}\) glycine in the presence of 1.74 mM ENF. Similar results were obtained.
with 0.56 mM TCE in the presence of 1.0 mM ENF (data not shown).

We next tested to determine if competition for a modulator-binding site could be observed in a Gly-R mutated at a residue at or near the putative modulator-binding pocket. When the serine residue at amino acid 267 is mutated to an isoleucine (S267I), mutant Gly-Rs are created that retain their sensitivities to ENF and CHCl3 but are insensitive to EtOH (6). We tested the hypothesis that EtOH was still binding to its putative binding site, despite having no efficacy on S267I Gly-Rs. This could be demonstrated by its antagonism of the effects of other modulators that still had enhancing effects on S267I Gly-Rs (Fig. 5). EtOH (200 mM), while having essentially no effect on its own, was able to antagonize the potentiation produced by 0.42 mM TOL (p < 0.001, n = 5, paired t test), 0.25 mM CHCl3 (p < 0.001, n = 6), and 0.5 mM ENF (p = 0.002, n = 6). EtOH antagonized the enhancing effects of 0.25 mM CHCl3 in a concentration-dependent manner (F(4,16) = 37.1, p < 0.0001), even at EtOH concentrations as low as 25 mM (Fig. 6). No significant enhancing or inhibiting effects of 25–200 mM EtOH were noted on S267I Gly-R when it was co-applied with EC50 glycine for a further 30 s. Representative current tracings are provided above each graph. Data are presented as the mean ± S.E. of six oocytes.

**DISCUSSION**

The serine residue at position 267 of the α1-subunit, proposed to compose part of the alcohol and anesthetic-binding pocket in Gly-Rs, is located near the extracellular terminus of the second of four membrane-spanning regions on the protein. A series of single amino acid mutations made at Ser-267 α1 Gly-R alter the enhancing effects of ethanol (6), volatile anesthetics (8, 13), and inhaled drugs of abuse (3) on GABAA and glycine receptors. An excellent linear correlation between the effects of the ether ENF and the halogenated alkane TCE across a panel of Ser267 mutant receptors is consistent with a common binding site despite the dissimilarities in their chemical structures (3). Mutations in the glycine α1-subunit at positions 267 and 288 (in TM3) change alcohol cut off, defined as the largest chain n-alcohol capable of enhancing receptor function, which could be interpreted as a change in the size of the alcohol-binding pocket (14). GABA_A α3β1 (S2651)-receptors (the equivalent amino acid as serine 267 in the α1 glycine receptor) lose sensitivity to isoflurane but not its isomer ENF (8). Finally, irreversible enhancement of Gly-R function is observed as a consequence of cross-linking propyl methanethiosulfonate to S267C mutant Gly-Rs; this also blocks further enhancement by enflurane and isoflurane (7). A substantial body of data thus implicates this region of the glycine and GABA_A receptors as being critical for mediating the functional effects of alcohols and volatile anesthetics. Molecular sites for propofol and other intravenous anesthetics are located elsewhere on the protein (15), as are the sites for benzodiazepines on the GABA_A receptors (16).
As with any ligand-receptor interaction, the effect that an allosteric modulator has on a ligand-gated ion channel depends on both the compound and the receptor. Compounds that bind at a receptor site may be classified as agonists, antagonists, and inverse agonists, depending on their efficacies, which can be positive, zero, or negative, respectively. Modulation of receptor function can be altered by either changing the structure of the drug or by changing a relevant portion of the receptor. For example, after residue 142 on the GABAA receptor \( \gamma \) subunit is conservatively mutated, benzodiazepine receptor antagonists and inverse agonists function as partial agonists (17). Mutation of \( \alpha_1 \) Gly-R Ser-267 to isoleucine (S267I) results in a receptor that demonstrates responses to glycine similar to those of wild-type receptors and retains sensitivity to the enhancing effects of enflurane but not ethanol (6). By mutating \( \alpha_1 \) Gly-R Ser-267 to isoleucine, we altered modulator efficacy, changing ethanol to an antagonist capable of antagonizing the enhancing effects of enflurane. At the concentrations tested, ethanol does not significantly decrease currents evoked by glycine in S267I \( \alpha_1 \) Gly-R, strongly suggesting that ethanol is binding to a modulator site common to anesthetics and inhalants, but possesses minimal efficacy, rather than binding elsewhere to favor the stabilization of a closed conformation of the chloride channel.

The ethanol effects on ENF and CHCl3 concentration-response curves (Fig. 8, A and B) do not initially suggest a competitive interaction between ethanol and the volatile anesthetics in the S267I mutant receptors. Ethanol produces a rightward shift at low concentrations of ENF and CHCl3, but the curves fail to reach the same maximum, which would be expected if the interaction were competitive. Despite the shapes of these concentration-response curves, we believe competitive antagonism may be occurring at a common alcohol/anesthetic-binding site. There are two possible explanations for the shapes of the observed concentration-response curves. First, ethanol could be binding to a site distinct from that of ENF and CHCl3 and decreasing the affinities of ENF and CHCl3 to bind. Al-
though the shapes of the curves favor this possibility at low concentrations of ENF and CHCl₃, it is difficult to reconcile the effects seen at the high end of the ENF curve. The second possibility is that there are two separate sites for ENF on these receptors, the first a pocket at Ser-267 where EtOH can compete, and the second a low affinity inhibitory site. This second site has long been postulated based on the non-sigmoidal nature of volatile anesthetic concentration-response curves, including decreasing enhancing effects at high anesthetic concentrations as well as rebound currents that are observed upon washout of drug (18). We created a mutant (S267F α₁ Gly-R) that illustrates the dissociation between the enhancing effects of volatile anesthetics and their inhibiting effects (Fig. 8). S267F α₁ Gly-R show no signs of the enhancing effects of low concentrations of enflurane but do exhibit inhibition of Gly-R function at higher concentrations of ENF. A hypothetical composite ENF concentration-response curve is presented in Fig. 9, illustrating the potential contribution from a second inhibitory binding site for ENF, based on data presented here and observed by others (18). At low anesthetic concentrations the enhancing effects predominate, but when higher concentrations are achieved, inhibition predominates. In the S267I receptors, EtOH antagonism of ENF binding to the higher affinity enhancing site but not the lower affinity inhibitory site could make competitive antagonism appear to be non-competitive. Regardless of the mechanism responsible, our observations suggest the possibility that compounds acting as ethanol and volatile anesthetic receptor antagonists may exist. Currently, no known compound competitively antagonizes anesthetics from their sites of action on wild-type ligand-gated ion channels, although saturable and displaceable binding of volatile anesthetics has been demonstrated on bovine serum albumin (19), on Ca²⁺-ATPase (20), and in rat brain synaptosomes (21). The ethanol antagonism of anesthetic effects we demonstrate in S267I α₁ Gly-R is a promising indication that receptor antagonists might be designed for alcohol-, inhalant-, and anes-

![Figure 6](image6.png)

**Fig. 6.** Ethanol antagonizes chloroform enhancement of Gly-R currents in a concentration-dependent manner. Representative tracings illustrate that ethanol antagonism of chloroform enhancement of S267I α₁ Gly-R function occurs in a concentration-dependent manner. A presents data as the mean ± S.E. of five oocytes. All concentrations of ethanol tested antagonized the enhancing effects of chloroform on S267I α₁ receptors. Compounds were pre-applied to oocytes for 60 s before being co-applied with EC₁₀ glycine for a further 30 s.

![Figure 7](image7.png)

**Fig. 7.** Ethanol antagonism of chloroform enhancement of S267I α₁ Gly-R function is reversible. Top panel, ethanol blockade of a glycine + CHCl₃ current is fully reversible after allowing for a 700-s washout period. Bottom panel, 3-min applications of low and high concentrations of glycine in the absence and presence of 100 mM EtOH demonstrate minimal EtOH effects on peak height, desensitization rates, and steady-state currents in S267I α₁ Gly-R.

![Figure 8](image8.png)

**Fig. 8.** Ethanol effects on chloroform and enflurane concentration-response curves in S267I α₁ Gly-Rs. Ethanol antagonizes the enhancement of Gly-R function produced by a range of CHCl₃ (A) and ENF (B) concentrations. Inhibition of Gly-R function is observed when higher concentrations of anesthetics are applied. We tested whether inhibition of receptor function at these higher concentrations was independent of the enhancing effects observed at lower concentrations. A Gly-R bearing a phenylalanine residue at position 267 (S267F) was resistant to the enhancing effects of ENF. This S267F Gly-R still displayed the inhibition of receptor function by higher concentrations of ENF (C). Data are presented as the mean ± S.E. of 4–8 oocytes.

![Antagonism of Anesthetic Effects on Glycine Receptors](image9.png)

**Antagonism of Anesthetic Effects on Glycine Receptors**

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thetic-binding sites on wild-type GABA<sub>A</sub> and glycine receptors. Mascia et al. (7) demonstrated that the Cys-267<sub>a1</sub> Gly-R residue could be irreversibly labeled with an anesthetic compound resulting in persistent enhancement of receptor function. The current study extends this work to show that compounds that are not covalently bound to this alcohol/anesthetic site can also be used to demonstrate competition for binding to this site.

In summary, ethanol, volatile anesthetics, and inhaled drugs of abuse appear to compete for a fixed number of binding sites as evidenced by sub-additivity of enhancement of ligand-evoked currents of recombinant Gly-Rs expressed in X. laevis oocytes. EtOH antagonized the enhancement produced by several of these compounds on a mutated receptor previously shown to be insensitive to EtOH. Finally, our results suggest that it may be feasible to consider the possibility that compounds could be designed specifically to antagonize alcohol, inhalant, and volatile anesthetic effects on ligand-gated ion channels. Such discoveries could lead to improved treatments for acute alcohol, anesthetic, or inhalant overdose, or even new pharmacotherapeutic approaches for alcoholism.

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