

Glycine and γ -Aminobutyric Acid_A Receptor Function Is Enhanced by Inhaled Drugs of Abuse

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ABSTRACT

Inhalable solvents possess significant abuse liability and produce many of the neurobehavioral effects typically associated with central nervous system-depressant agents, including motor incoordination, anxiolysis, and the elicitation of signs of physical dependence on withdrawal. We tested the hypothesis that the commonly abused solvents toluene, 1,1,1-trichloroethane (TCE), and trichloroethylene (TCY) affect ligand-gated ion channel activity, as do other classes of central nervous system-depressive agents. TCE and toluene, like ethanol, reversibly enhanced γ -aminobutyric acid (GABA)_A receptor-mediated synaptic currents in rat hippocampal slices. All three inhalants significantly and reversibly enhanced neurotransmitter-activated currents at $\alpha 1\beta 1$ GABA_A and $\alpha 1$ glycine receptors ex-

pressed in *Xenopus* oocytes. We previously identified specific amino acids of glycine and GABA_A receptor subunits mediating alcohol and volatile anesthetic enhancement of receptor function. Toluene, TCE, and TCY were tested on several glycine receptor mutants, some of which were insensitive to ethanol and/or enflurane. Toluene and TCY enhancement of glycine receptor function was seen in all these mutants. However, the potentiating effects of TCE were abolished in three mutants and enhanced in two, a pattern more akin to that seen with enflurane than ethanol. These data suggest that inhaled drugs of abuse affect ligand-gated ion channels, and that the molecular sites of action of these compounds may overlap with those of ethanol and the volatile anesthetics.

Inhaled agents rank fourth behind alcohol, marijuana, and tobacco in incidence of abuse in the United States. Commonly abused inhalants include toluene (TOL), 1,1,1-trichloroethane (TCE), trichloroethylene (TCY), xylene, and hexane. Inhalation of these substances produces short-term feelings of euphoria accompanied by muscular incoordination and altered sensorium and behavior (for a review, see Evans and Balster, 1991). Several studies have demonstrated the reinforcing effects of TOL and other organic inhalants in animals (Weiss et al., 1979; Evans and Balster, 1991), whereas others have noted similarities in the biological actions of inhalants with those of classic central nervous system (CNS) depressants such as ethanol and the barbiturates (Bowen et al., 1996a). For example, inhaled agents have anxiolytic (Bowen et al., 1996b) and anticonvulsant (Wood et al., 1984) properties, impair motor coordination (Moser and Balster, 1985) and elicit signs of physical dependence on withdrawal (Evans and Balster, 1991, 1993). Furthermore, like other CNS-de-

pressant agents, inhalants have biphasic effects on spontaneous locomotor activity in rodents, with increased activity seen at lower doses and diminished locomotion seen at higher doses (Gause et al., 1985; Kjellstrand et al., 1985). Symptoms of toxicity after chronic use are numerous and include white matter changes (Rosenberg et al., 1988), peripheral neuropathy, cerebellar degeneration, dementia (Lolin, 1989), and damage to the heart, lungs, kidneys, and liver (Marjot and McLeod, 1989).

Despite their significant incidence of abuse, the mechanisms of inhalant action remain poorly understood. The common CNS-depressant effects produced by ethanol, the volatile anesthetic agents, and inhaled drugs of abuse suggest the possibility that these compounds have overlapping mechanisms of action. Previous work has established neurotransmitter-activated ion channels as primary sites of action of alcohols and volatile anesthetic agents. Ethanol has inhibitory actions at *N*-methyl-D-aspartate (NMDA) receptors (Lovinger, 1997) and enhances γ -aminobutyric acid (GABA)_A and glycine receptor (Gly-R; Mihic, 1999) function. Pharmacologically relevant concentrations of volatile anesthetic agents also potentiate GABA_A- and Gly-R-mediated currents (Franks and Lieb, 1994). However, only very recently have

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ABBREVIATIONS: TOL, toluene; CNS, central nervous system; GABA, γ -aminobutyric acid; Gly-R, glycine receptor; IPSC, inhibitory postsynaptic current; MBS, modified Barth's saline; NMDA, *N*-methyl-D-aspartate; TCE, 1,1,1-trichloroethane; TCY, trichloroethylene; TM2, transmembrane domain 2.

inhalant effects on ligand-gated ion channel function begun to be investigated. Cruz et al. (1998) recently found that TOL, like ethanol, inhibits NMDA receptor-mediated currents in a concentration-dependent manner. We examined the effects of TOL, TCY, and TCE on two members of a superfamily of ligand-gated ion channels: the Gly-Rs and GABA_A receptors. Our previous work showed that the mutation of specific amino acids of Gly-R and GABA_A receptor subunits rendered the resulting receptors insensitive to the enhancing effects of alcohol and volatile anesthetic agents (Mihic et al., 1997). We tested the hypothesis that inhalant drugs of abuse share common mechanisms of action with ethanol and volatile anesthetic agents by determining whether receptor subunit mutations resulting in anesthetic or alcohol insensitivity also resulted in insensitivity to inhaled drugs of abuse. Some of this work has previously been presented in abstract form (Beckstead et al., 1999).

Experimental Procedures

Materials. Penicillin, streptomycin, gentamycin, 3-aminobenzoic acid ethyl ester, GABA, glycine, collagenase, TOL, TCE, and TCY were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of reagent grade. *Xenopus laevis* were obtained from Xenopus Express (Homosassa, FL).

Oocyte Isolation and cDNA Nuclear Injection. Oocytes were isolated from *X. laevis* housed at 17–19°C on a 12-h light/dark cycle and fed Nasco frog brittle thrice weekly. On the day of surgery, they were anesthetized by immersion for 45 min in 0.3% w/v 3-aminobenzoic acid ethyl ester. A 1-cm incision was made in the abdominal wall, and a piece of ovary was removed and placed in isolation media (containing 108 mM NaCl, 1 mM EDTA, 2 mM KCl, and 10 mM HEPES at pH 7.5) for the extraction of oocytes. The thecal and epithelial layers of stage V and VI oocytes were removed with forceps under a light microscope. The follicular layer was excised by a 10-min immersion of oocytes in 0.5 mg/ml collagenase in collagenase buffer (83 mM NaCl, 2 mM KCl, 1 mM MgCl₂, and 5 mM HEPES, pH 7.5). Isolated oocytes were placed in modified Barth's saline (MBS) consisting of 88 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.82 mM MgSO₄, 2.4 mM NaHCO₃, 0.91 mM CaCl₂, and 0.33 mM Ca(NO₃)₂ at pH 7.5 and injected with cDNAs (1.5 ng/30 nl) of normal or mutated human glycine α 1 or GABA α 1 β 1 or α 1 β 2 γ 2L subunits. All subunit cDNAs were subcloned into the pBCKMV vector, which had been modified by removal of the *lac* promoter and the *lacZ* ATG (Mihic et al., 1997). Injected oocytes were placed singly into 96-well microtiter plates (Costar, Cambridge, MA) containing incubation media (MBS plus 2 mM Na pyruvate, 0.5 mM theophylline, 10 U/ml penicillin, 10 mg/l streptomycin, and 50 mg/l gentamycin, sterilized by passage through a 0.22- μ m filter). Oocytes usually expressed receptors in 1 to 2 days, and electrophysiological measurements were made 1 to 7 days after cDNA injection.

Oocyte Electrophysiological Recording. Animal poles of oocytes were impaled with two high-resistance (0.5–10 M Ω) glass electrodes filled with 3 M KCl, and oocytes were voltage-clamped at –70 mV using a Warner Instruments OC-725C oocyte clamp (Hamden, CT). The concentration of agonist producing 10% of a maximal effect (EC₁₀) was determined for each oocyte and used in studies that tested the effects of inhalant drugs of abuse. Washout periods (5–15 min) were allowed between drug applications to ensure complete resensitization of receptors. When inhaled agents were tested, receptors were preincubated with inhalant (TCE, TCY, or TOL) for 60 s before addition of the agonist/inhalant solution for an additional 30 s. Oocytes were perfused with MBS at a rate of 2 ml/min using a Masterflex USA peristalsis pump (Cole-Parmer Instrument Co., Vernon Hills, IL), which connected drug-containing vials to the perfusion chamber through 18-gauge polyethylene tubing. Changes in the

clamping current were recorded on a strip-chart recorder (Cole-Parmer Instrument Co.), and the peak currents were measured and used in data analysis. To determine inhalant effects on steady-state receptor-mediated currents, agonist solutions were applied until maximal desensitization occurred and then switched immediately to an agonist/inhalant solution for 60 s. Oocyte bath concentrations of inhalants were determined by gas chromatography as described by Eger et al. (1999).

Hippocampal Slice Electrophysiology. Transverse hippocampal slices were prepared from 4- to 6-week-old male Sprague-Dawley rats as described previously (Weiner et al., 1997). Whole-cell patch recordings were made from hippocampal CA1 pyramidal neurons at room temperature (22–24°C). Slices were superfused with an artificial cerebrospinal fluid comprised of 124 mM NaCl, 3.3 mM KCl, 2.4 mM MgCl₂, 2.5 mM CaCl₂, 1.2 mM NaH₂PO₄, 10 mM D-glucose, and 25.9 mM NaHCO₃, saturated with 95% O₂, 5% CO₂. The patch pipette solution contained 130 mM cesium-gluconate, 10 mM CsCl₂, 5 mM QX-314, 1 mM EGTA, 100 μ M CaCl₂, 2 mM Mg-ATP, 200 μ M Tris-GTP, and 10 mM HEPES. The pH of this solution was 7.25 (adjusted with CsOH), the osmolality was 285 \pm 5 mOsm, and this solution was kept on ice until immediately before use.

Pharmacologically isolated GABA_A inhibitory postsynaptic currents (IPSCs) were evoked in the presence of the glutamate receptor blockers 6,7-dinitroquinoxaline-2,3-dione (20 μ M) and DL-(–)-2-amino-5-phosphonopivalic acid (50 μ M) at a holding potential of –50 mV. Synaptic stimulation was delivered every 20 s using a concentric bipolar electrode (FHC, Bowdoinham, ME). GABA_A receptor-mediated IPSCs were collected in the continuous voltage-clamp mode using an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA), stored on a hard drive, and analyzed online and offline using pClamp 7.0 software (Axon Instruments). All drugs were applied directly to the artificial cerebrospinal fluid via calibrated syringe pumps (Razel, Stanford, CT). Drug effects were quantified as the percentage change in time-current area relative to the mean of control and washout values. Statistical analyses were carried out using a paired *t* test with a minimum level of significance of *P* < .05.

Statistical Analysis. Statistical analyses were performed on data obtained from oocytes by one-way or two-way ANOVA and appropriate post-hoc tests, as indicated. Data are presented as mean \pm S.E., and *n* values refer to the number of different oocytes from which data were obtained. Statistical significance was defined as *P* < .05 on Tukey's or Dunnett's post-hoc test. All groups of data were collected using oocytes obtained from at least two different frogs. Concentration-response relationships were fitted to sigmoidal curves (SPSS Sigma Plot 4.01) to determine ligand EC₅₀ values and Hill coefficients.

Results

TCE was tested for its effects on bicuculline-sensitive rat hippocampal GABA_A receptor responses elicited by presynaptic stimulation. Bath application of TCE reversibly enhanced the area of pharmacologically isolated GABA_A receptor-mediated IPSCs in hippocampal CA1 neurons by 70.8 \pm 19.9% (*t*₈ = 4, *P* < .004; Fig. 1A). This potentiation became evident a few minutes after the administration of TCE to hippocampal slices; the relatively slow onset was due to the time required for TCE to permeate into slices (Fig. 1B). Accounting for loss of TCE through tubing and through evaporation from the bath, the final concentration of TCE at the brain slice was determined to be 0.28 mM. Toluene, at a concentration of 0.32 mM at the slice, increased the area of GABA_A receptor-mediated IPSCs by 38.3 \pm 8.4% (*t*₁₂ = 5.1, *P* < .0003).

GABA_A α 1 β 1 recombinant receptors were expressed in *X. laevis* oocytes, and their sensitivities to TOL, TCY, and TCE

were determined. Stock solutions of 1 mM concentration of each of these compounds were prepared. Accounting for compound loss through tubing, the final concentrations of these inhalants in the oocyte bath were 0.42 mM TOL, 0.39 mM TCY, and 0.56 mM TCE. Each of these compounds reversibly enhanced the effects of an EC₁₀ value of GABA as illustrated in the tracings in Fig. 2A. Statistically significant enhancement of receptor function was obtained with all three compounds tested, with TOL producing the greatest potentiation of peak currents (Fig. 2B). These concentrations of the inhaled agents had no effects on receptors when administered in the absence of agonist (i.e., during the 1-min preincubation). TOL, TCY, and TCE also reversibly potentiated the effects of EC₁₀ glycine on homomeric glycine α 1 receptors (Fig. 2, C and D). Mean enhancement of Gly-R function ranged from approximately 60 to 90%, depending on the compound. As also observed in oocytes expressing GABA_A receptors, bath application of inhalants in the absence of glycine had no effect on baseline currents. We next tested whether inhalants also enhanced steady-state currents. After the application of a higher concentration of GABA (30 μ M) to α 1 β 2 γ 2L receptors, desensitization was observed, plateauing to steady-state levels that were minimally affected by inhalants (Fig. 3A). Although TOL was able to enhance the peak currents produced by 30 μ M GABA, it had almost no effect on steady-state currents (Fig. 3B). Inhalants shifted GABA and glycine concentration-response curves to the left and had no effects at the highest agonist concentrations tested (Fig. 4, A and B). TOL (0.42 mM) produced a sinistral shift of the GABA concentration-response curve ($F_{1,42} = 6.71$, $P < .02$), and TCE (0.7 mM) similarly shifted the glycine concentration-response curve ($F_{1,36} = 20.2$, $P < .0001$). To eliminate the possibility that the observed effects of inhalants were due to leaching of plasticizers from perfusion tubing, we also applied glycine with and without inhalants to oocytes by gravity flow via a glass Pasteur pipette directly into the oocyte bath. Enhancement of Gly-R function by in-

halants was still seen using this perfusion technique (data not shown).

We next tested the hypothesis that inhaled drugs of abuse could interact at the same molecular sites on Gly-Rs as do volatile anesthetics. Enflurane concentration-response curves were generated in the absence or presence of 0.56 mM TCE (Fig. 5). TCE clearly had an effect in the absence of enflurane, but the TCE enhancement of receptor function decreased as the enflurane concentration was increased. A repeated measure ANOVA demonstrated a significant overall effect of TCE ($F_{1,6} = 31.1$, $P < .001$), as well as a difference in the slopes of the enflurane and TCE-plus-enflurane concentration-response curves ($F_{3,18} = 7.7$, $P < .002$), indicating that TCE and enflurane effects were not merely additive.

A number of single amino acid mutations were made in the Gly-R α 1 subunit, and the resulting homomeric receptors were examined for changes in their sensitivities to inhaled drugs of abuse. The α 1 subunit was mutated at position 267 from the wild-type serine residue (S267) to one of six different amino acids: glutamine (S267Q), phenylalanine (S267F), glycine (S267G), aspartate (S267D), glutamate (S267E), or methionine (S267M). Most of these mutations produced little or no effect on the enhancement of Gly-R function by 0.42 mM

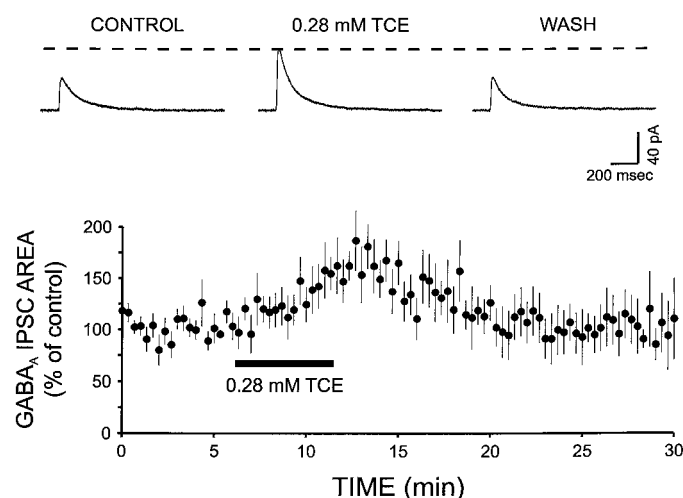


Fig. 1. Time course of TCE potentiation of GABA_A receptor-mediated IPSCs. Tracings demonstrate that TCE reversibly increased the peak height and area of GABA-mediated synaptic responses. The graph illustrates the effect of a 5-min application of 0.28 mM TCE on the area of GABA_A receptor-mediated IPSCs recorded from rat hippocampal pyramidal neurons. Each point represents the mean IPSC area \pm S.E. recorded from nine cells. Traces above the graph are averages of four to six IPSCs recorded from a representative cell before, during, and after TCE application.

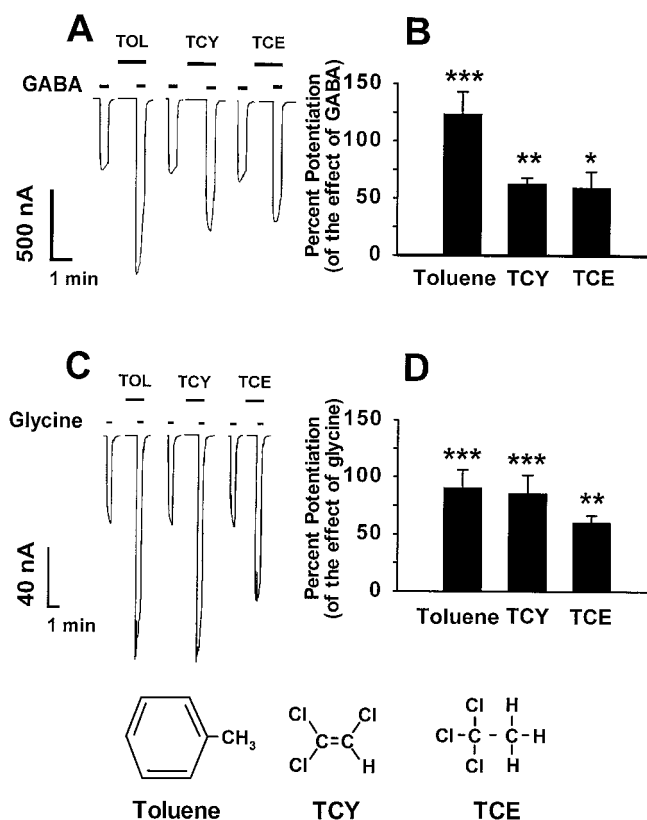


Fig. 2. TOL (0.42 mM), TCY (0.39 mM), and 1,1,1-trichloroethane (0.56 mM) significantly and reversibly enhance α 1 β 1 GABA_A- and α 1 Gly-R-mediated currents. Oocytes were preincubated with inhalant for 60 s before the 30-s incubation with inhalant plus GABA or glycine. Representative tracings are shown in A and C. Horizontal lines above the tracings indicate when inhalants or GABA/glycine were applied. For each oocyte, the concentration of agonist producing 10% of a maximal response (B, GABA, $1.3 \pm 0.7 \mu$ M; D, glycine, $43.6 \pm 3.4 \mu$ M) was first determined and that concentration used when the receptor was tested for enhancement of receptor function by inhalant. Data are presented as mean \pm S.E. obtained from five or six oocytes. * $P < .05$, ** $P < .01$, *** $P < .001$, Dunnett's test versus control.

TOL, although TOL sensitivity of the S267F mutant was significantly ($P < .01$) decreased compared with wild-type receptors (Fig. 6A).

Mutation of residue 267 of the Gly-R $\alpha 1$ subunit had more pronounced effects on TCY enhancement of Gly-R function. TCY had a significantly greater effect on the S267D ($P < .001$) and S267E ($P < .05$) mutants (Fig. 6B) than on wild-type Gly-Rs. TCY actions on the other mutants were similar to those on the wild-type receptor, although the S267M mutant showed a trend toward enhanced potentiation.

The mutation of amino acid residue 267 of the Gly-R $\alpha 1$ subunit markedly affected TCE enhancement of receptor function (Fig. 6C). In contrast to its effects on the wild-type

receptor, TCE had almost no effect in the S267Q ($P < .01$), S267F ($P < .001$), and S267M ($P < .001$) mutants. Conversely, significantly greater effects of TCE were seen in the S267D ($P < .001$) and S267E ($P < .001$) mutants compared with the wild-type receptor. With all three compounds tested, the mutation of Ser267 to glycine had no effect on inhalant actions at the Gly-R, suggesting that the hydroxyl side chain of the serine residue is not required for inhalant potentiation of the effect of glycine.

TCE concentration-response curves were generated on the wild-type, S267M, and S267Q $\alpha 1$ Gly-R. Representative tracings of results are provided in Fig. 7A. Although clear concentration-dependent enhancement was observed in the wild-type receptors, no effect was seen in the two mutants, except for inhibition of the S267Q $\alpha 1$ Gly-R mutant at the highest TCE concentration tested (Fig. 7B). Two-way ANOVA showed a significant effect of mutation ($F_{1,24} = 56.6$, $P < .0001$), a significant effect of TCE concentration ($F_{3,24} = 5.1$, $P < .008$), and a significant interaction between concentration and mutation ($F_{3,24} = 13.1$, $P < .001$) in a comparison of the wild-type and S267Q receptors. The glycine EC_{10} values did not differ between the wild-type and S267Q receptors: $84.3 \pm 4.2 \mu\text{M}$ for wild-type receptors and $77.5 \pm 4.2 \mu\text{M}$ for the S267Q mutant ($t_6 = 1.15$, $P > .29$). The glycine EC_{10} values for the other S267 mutants tested were S267M, $45 \pm 4.7 \mu\text{M}$; S267G, $13.3 \pm 3 \mu\text{M}$; S267D, $208 \pm 18.2 \mu\text{M}$; S267E, $208 \pm 10.6 \mu\text{M}$; and S267F, $8.3 \pm 0.7 \mu\text{M}$.

TCE, ethanol (Ye et al., 1998), and enflurane (S. J. Mihic, unpublished observations) enhancements of Gly-R function in wild-type receptors and the six S267 mutants were compared. Linear regression analysis of the effects of TCE compared with those of ethanol on the wild-type and mutated receptors (Fig. 8A) showed a poor correlation between these two compounds ($r^2 = 0.26$, $P > .24$). However, an excellent correlation ($r^2 = 0.98$, $P < .0001$) was observed between the effects of TCE and enflurane (Fig. 8B).

Discussion

For most of the 1900s, it was widely believed that ethanol and the volatile anesthetics exerted their effects *in vivo* in a

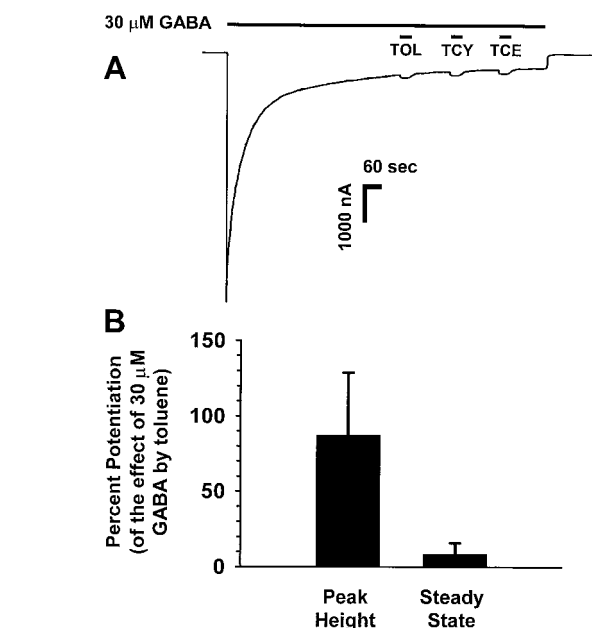


Fig. 3. TOL (0.42 mM), TCY (0.39 mM), and TCE (0.56 mM) minimally affect steady-state GABA_A receptor-mediated currents. Oocytes expressing $\alpha 1\beta 2\gamma 2\text{L}$ receptors were perfused with 30 μM GABA for approximately 10 min or until maximal receptor desensitization was observed. Oocytes were then perfused with 30 μM GABA plus inhalant for 60 s and then again with 30 μM GABA alone. This tracing is representative of the typical response observed under these experimental conditions (A). Although TOL minimally affected steady-state currents, it did enhance peak currents observed after coapplication with 30 μM GABA for 30 s (B).

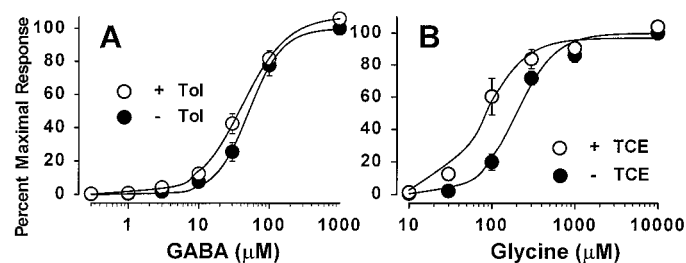


Fig. 4. Inhalant drugs of abuse produce sinistral shifts of agonist concentration-response curves. GABA and glycine concentration-response curves were generated in the absence (●) or presence (○) of inhalants. TOL (0.42 mM) significantly shifted the GABA concentration-response curve to the left (A), decreasing the GABA EC_{50} to 42 from 52 μM . TCE (0.7 mM) shifted the glycine concentration to the left (B), lowering the glycine EC_{50} from 199 to 81 μM . Toluene decreased the GABA Hill coefficient from 1.83 to 1.36, and TCE shifted the glycine Hill coefficient from 1.82 to 1.70. All data points were expressed as a percentage of the current produced by either 1 mM GABA or 10 mM glycine. Statistics are provided in the text. Data are expressed as mean \pm S.E. obtained from four oocytes.

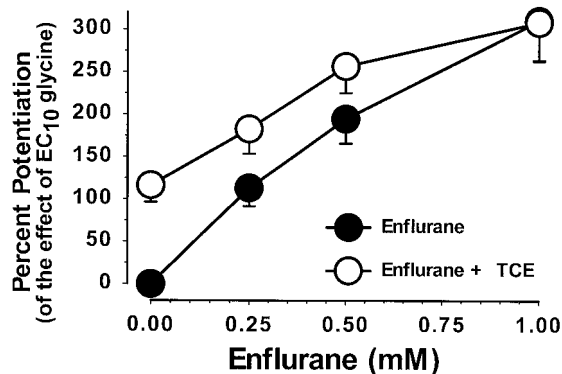


Fig. 5. TCE enhancement of Gly-R function is antagonized by the concomitant administration of enflurane. Enflurane concentration-response curves were generated in the absence (●) or presence (○) of 0.56 mM TCE. Enhancement of Gly-R function by TCE was greatest at the lowest enflurane concentrations tested and was absent when it was coapplied with 1 mM enflurane. Data are expressed as the percentage enhancement of the current produced by the application of glycine (EC_{10}) alone. Statistics are provided in the text, and data obtained from seven oocytes are expressed as mean \pm S.E.

nonspecific manner, through the disordering of cell membrane lipids. The Meyer-Overton correlation of anesthetic potency with lipophilicity and the observation that agents with markedly dissimilar structures could all produce the same behavioral end point made it appear unlikely that volatile anesthetic agents had discrete protein binding sites. By analogy, inhaled drugs of abuse, which also vary greatly in their chemical structures, were also thought to nonspecifically influence neuronal function (Balster, 1998). The demonstration that pharmacologically relevant concentrations of volatile anesthetic agents and alcohols affect the functioning of lipid-free proteins such as firefly luciferase (Franks and Lieb, 1984) promoted a shift in research focus toward protein sites of anesthetic action. Among these protein sites, the ligand-gated ion channels, particularly the GABA_A, glycine, and glutamate receptors, are now believed to play major roles in mediating the in vivo effects of ethanol and the volatile anesthetic agents (Franks and Lieb, 1994; Harris et al., 1995; Lovinger, 1997; Mihic, 1999). This work has recently been extended to the identification of specific amino acids of glycine and GABA_A receptor subunits that confer receptor sen-

sitivity to the enhancing actions of alcohols and volatile anesthetic agents (Mihic et al., 1997). In the present report, we tested the hypotheses that inhaled drugs of abuse also affect GABA_A and Gly-R function and that their molecular sites of action on these receptors may be similar to those of ethanol and the volatile anesthetic agents.

Our findings show that TOL and TCE enhance bicuculline-sensitive GABA-mediated synaptic currents (Weiner et al., 1997) in rat CA1 hippocampal neurons. The potentiation of GABA_A receptor function by other modulators has been found previously to profoundly affect behavior. For example, ethanol enhancement of GABA_A receptor function is linked to a loss of righting reflex (Martz et al., 1983) and the regulation of alcohol consumption (Nowak et al., 1998). Furthermore, the GABA_A receptor is also implicated in the discriminative stimulus effects of ethanol (Hodge and Cox, 1998). The spectrum of pharmacological effects common to ethanol and inhaled agents (Bowen et al., 1996a; Balster, 1998) indicates that some actions of inhalants may also result from potentiation of GABA_A receptor function in vivo. For example, Bowen et al. (1999) recently showed that mice trained to discriminate diazepam will also respond to TCE or methoxyflurane but not to TOL. Mice trained to discriminate ethanol from water will generalize to TOL and TCE, as well as to halothane and oxazepam (Rees et al., 1987). In addition, mice trained to discriminate TOL will generalize to methohexital and oxazepam (Knisely et al., 1990). Finally, the administration of the GABA_A receptor modulators midazolam, pentobarbital, and ethanol attenuated signs of physical dependence elicited after TCE withdrawal in mice (Evans

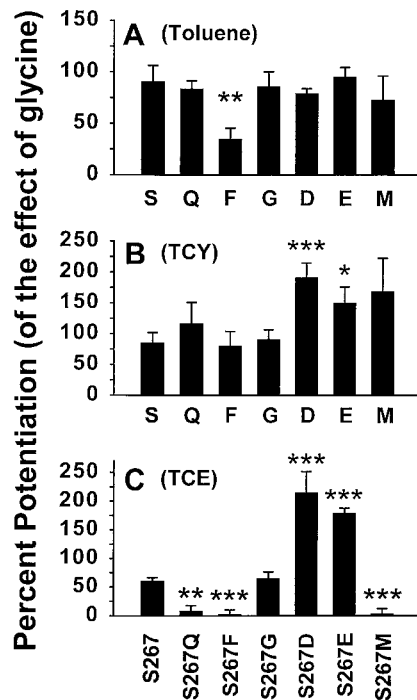


Fig. 6. Single amino acid mutations affect inhalant modulation of Gly-R function. The Gly-R $\alpha 1$ subunit was mutated at position 267 in TM2 from the wild-type serine residue (S267) to one of six different amino acids: glutamine (S267Q), phenylalanine (S267F), glycine (S267G), aspartate (S267D), glutamate (S267E), or methionine (S267M). Most of these mutations had little or no effect on the enhancement of Gly-R function by 0.42 mM TOL (A), although TOL sensitivity of the S267F mutant was significantly ($P < .01$) decreased compared with wild-type receptors expressed in oocytes. TCY had a significantly greater effect on the S267D ($P < .001$) and S267E ($P < .05$) mutants (B) than on wild-type Gly-Rs. TCY actions on the other mutants were similar to those on the wild-type receptor, although the S267M mutant showed a trend toward enhanced potentiation. The mutation of amino acid residue 267 of the Gly-R $\alpha 1$ subunit markedly affected TCE enhancement of receptor function (C). In contrast to its effects on the wild-type receptor, TCE had almost no effect in the S267Q, S267F, and S267M mutants. Conversely, significantly greater effects of TCE were seen in the S267D and S267E mutants compared with the wild-type receptor. Data are expressed as mean \pm S.E. obtained from four to six oocytes. * $P < .05$, ** $P < .01$, *** $P < .001$, Tukey's test versus wild type.

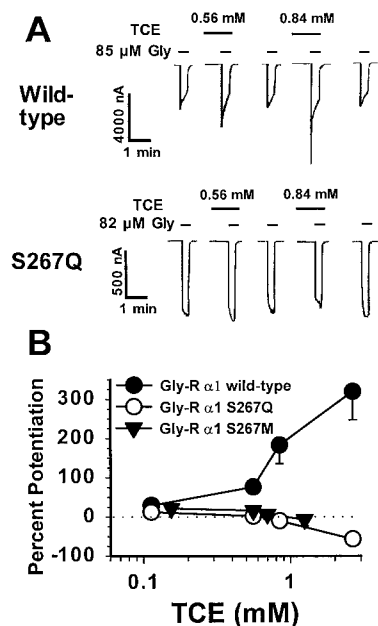


Fig. 7. Gly-R $\alpha 1$ S267Q and S267M mutations alter the effects of TCE on Gly-R function. Representative tracings of oocyte responses to glycine with or without TCE on wild-type $\alpha 1$ Gly-Rs are displayed (A, top). A, bottom, representative tracings of TCE effects on the Gly-R $\alpha 1$ S267Q mutant. Horizontal lines above the tracings indicate when TCE (0.56 mM or 0.84 mM) and glycine were applied. The S267Q mutation results in a receptor with a normal response to glycine but insensitivity to the enhancing effects of TCE. The wild-type and S267Q and S267M receptors were tested using a concentration of glycine producing 10% of a maximally effective response, in the absence or presence of 0.11 to 2.64 mM TCE (B). Data are presented as mean \pm S.E. of 4 to 10 oocytes.

and Balster, 1993). Substantial behavioral evidence has thus been obtained suggesting that the GABA_A receptor is a major biochemical site of inhalant action in vivo. Although additional possible mechanisms of action are not ruled out by these studies, enhancement of inhibitory neurotransmission is consistent with the sedation, incoordination, and dysphoria observed after inhalant use.

TOL, TCY, and TCE significantly increased ligand-gated currents in GABA_A α 1 β 1 and glycine α 1 receptors expressed in *X. laevis* oocytes at concentrations (~200–900 μ M) that others have found to occur in vivo (Naalsund, 1986; Kishi et al., 1988; You et al., 1994). No currents were elicited in the absence of neurotransmitter, implying that inhalants act as allosteric modulators at these ligand-gated ion channels and that the concentrations of inhalants tested did not compromise oocyte cell membrane integrity. The GABA_A and Gly-Rs are thus the second and third classes of ligand-gated ion channels shown to be sensitive to inhaled drugs of abuse. Toluene inhibits the function of heteromeric NMDA receptors composed of the NR1+2A, NR1+2B, or NR1+2C subunits, expressed in oocytes (Cruz et al., 1998). These findings are similar to those made previously in studies of the actions of alcohols and volatile anesthetic agents: GABA and Gly-R enhancement and NMDA receptor inhibition (Harris et al., 1995). Interestingly, receptors composed of GluR1, GluR1+2, or GluR6 subunits were insensitive to TOL (Cruz et al., 1998), although these receptors are sensitive to ethanol (Dildy-Mayfield and Harris, 1995).

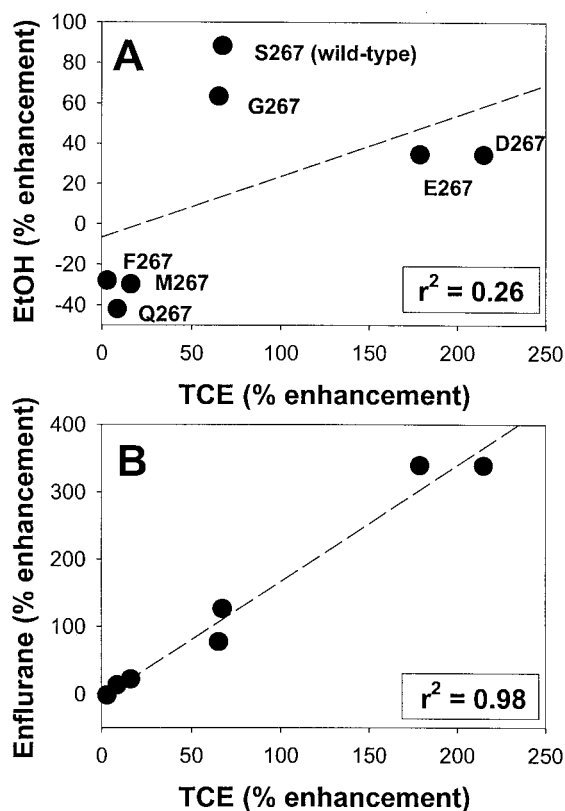


Fig. 8. TCE enhancement of Gly-R function was compared with that of two other CNS-depressive agents using a panel of S267 α 1 subunit mutants. The effects of TCE correlated poorly with the potentiating effects of ethanol (A) but exhibited an excellent correlation with those of the volatile anesthetic agent enflurane (B). Mutants are identified in A. Statistics are presented in the text.

We previously discovered that mutation of the Ser267 residue in transmembrane domain 2 (TM2) of the Gly-R α 1 subunit abolishes the modulatory actions of ethanol and enflurane (Mihic et al., 1997). The amino acid residue at position 267 clearly also determines TCE and, to a lesser extent, TCY and TOL actions at the Gly-R. Based on cysteine substitution studies performed on the GABA α 1 (Xu and Akabas, 1996) and nicotinic receptor α (Akabas et al., 1994) subunits, we predicted that the Gly-R α 1 S267 residue occupies a portion of the helical second transmembrane domain facing away from the ion channel pore and toward other transmembrane domains. This suggests the possibility that residues such as S267 in TM2, in conjunction with amino acid residues in other transmembrane domains, may form alcohol and anesthetic binding pockets (Wick et al., 1998). Because TCE modulation of Gly-R function is highly dependent on the amino acid residue occupying position 267 in the Gly-R α 1 subunit, TCE could also be physically interacting with that site to alter channel function. Alternatively, TCE and other inhalants may be interacting with the Gly-R at another more distant site, and the S267 residue instead plays a role in the transduction of a signal that affects ion channel opening.

We tested whether TCE could still enhance Gly-R function when it was coapplied with the volatile anesthetic enflurane. If these two compounds were interacting with a discrete site or sites on Gly-Rs, one might expect competition for binding to these sites to occur. This would be reflected in decreased TCE enhancement of Gly-R function as the enflurane concentration was increased. This was observed and there was, in fact, no TCE-induced enhancement observed when it was coapplied with 1 mM enflurane. It could be argued that 1 mM enflurane enhanced the effect of glycine to the extent that the current could not be increased any further by TCE. This is not the case. Enflurane enhanced the effect of glycine to 40% of that produced by a maximally effective concentration of glycine (i.e., EC₁₀ glycine plus 1 mM enflurane had the same effect as EC₄₀ glycine applied alone). However, in Fig. 4B, it can be seen that TCE still enhances an EC₄₀ response to glycine. This suggests that perhaps TCE had no effect in the presence of 1 mM enflurane because both compounds were competing for a limited number of sites on the Gly-R at which they could enhance receptor function. Saturable and displaceable binding of volatile anesthetic agents has previously been reported in rat brain synaptosomes (el-Maghrabi et al., 1992) and to BSA (Dubois et al., 1993) and Ca²⁺-ATPase (Lopez and Kosk-Kosicka, 1998).

The proximity of the S267 residue to the channel activation gate in TM2 may help explain how compounds such as inhalants and volatile anesthetic agents, with relatively low potencies, can so markedly affect the functioning of these multimeric proteins. That mutations at amino acid 267 would differentially affect TCE, TCY and TOL enhancement of Gly-R function is not unexpected. We previously demonstrated that the mutation of Ser267 to isoleucine abolished ethanol but not enflurane enhancement of Gly-R function; however, mutation to tyrosine eliminated the potentiating effects of enflurane (Mihic et al., 1997). It may be that TCY and TOL enhancement of Gly-R function may be abolished by other mutations at S267. Some of the S267 mutants displayed altered sensitivity to glycine. For example, the S267G and S267F mutants were more sensitive to glycine than wild-type receptors. However, this increased glycine sensitiv-

ity did not appear to be important in determining TCE sensitivity; the S267G receptors retained sensitivity to TCE, whereas the S267F receptors were insensitive. Similarly, the wild-type and S267Q receptors shared a similar sensitivity to glycine but differed markedly in TCE enhancement of receptor function. It might be argued that the lack of inhalant enhancement of receptor function in the S267Q mutant could be due to a concomitant enhancement of receptor function as well as desensitization in that mutant, which would tend to offset each other. This does not appear to be the case; the S267Q tracings in Fig. 7A show minimal desensitization in either the absence or presence of TCE.

In summary, inhaled drugs of abuse enhanced GABA-mediated synaptic currents in hippocampal neurons, as well as ligand-evoked currents of recombinant glycine and GABA_A receptors expressed in *X. laevis* oocytes. Although wild-type Gly-R function was enhanced by all three inhalants tested, the mutagenesis studies demonstrated a greater degree of inhalant specificity than might have been expected; although none of the mutants produced were insensitive to TOL or TCY, three were almost completely insensitive to TCE. Our results suggest that some of the behavioral consequences after the administration of inhaled drugs of abuse may be due to their actions on GABA_A and Gly-Rs.

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