The TM2 6' Position of GABA_A Receptors Mediates Alcohol Inhibition

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Received September 21, 2011; accepted November 8, 2011

ABSTRACT

Ionotropic GABA_A receptors (GABA_ARs), which mediate inhibitory neurotransmission in the central nervous system, are implicated in the behavioral effects of alcohol and alcoholism. Site-directed mutagenesis studies support the presence of discrete molecular sites involved in alcohol enhancement and, more recently, inhibition of GABA_ARs. We used *Xenopus laevis* oocytes to investigate the 6' position in the second transmembrane region of GABA_ARs as a site influencing alcohol inhibition. We asked whether modification of the 6' position by substitution with larger residues or methanethiol labeling [using methyl methanethiosulfonate (MMTS)] of a substituted cysteine, reduced GABA action and/or blocked further inhibition by alcohols. Labeling of the 6' position in either $\alpha 2$ or $\beta 2$ subunits reduced responses to GABA. In addition, methanol and ethanol potentiation increased after MMTS labeling or substitution with

tryptophan or methionine, consistent with elimination of an inhibitory site for these alcohols. Specific alcohols, but not the anesthetic etomidate, competed with MMTS labeling at the 6' position. We verified a role for the 6' position in previously tested $\alpha 2\beta 2$ as well as more physiologically relevant $\alpha 2\beta 2\gamma 2$ s GABA_ARs. Finally, we built a novel molecular model based on the invertebrate glutamate-gated chloride channel receptor, a GABA_AR homolog, revealing that the 6' position residue faces the channel pore, and modification of this residue alters volume and polarity of the pore-facing cavity in this region. These results indicate that the 6' positions in both $\alpha 2$ and $\beta 2$ GABA_AR subunits mediate inhibition by short-chain alcohols, which is consistent with the presence of multiple counteracting sites of action for alcohols on ligand-gated ion channels.

Introduction

Ionotropic GABA_A receptors (GABA_ARs) are ligand-gated ion channels that mediate the majority of inhibitory neurotransmission in the central nervous system (Farrant and Kaila, 2007). They belong to the Cys-loop superfamily, which includes glycine receptors (GlyRs), nicotinic acetylcholine receptors (nAChRs), and serotonin receptors. Functional receptors in this family are composed of five subunits, each containing an N-terminal extracellular domain, four transmembrane (TM) helices (TM1–TM4), and a cytoplasmic loop between TM3 and TM4. The ion pore is formed by the TM2 regions of all five subunits. Each TM2 region is a helix with residues numbered from the cytoplasmic (0') to the extracellular (20') end (Miller,

1989). The human genome encodes at least 16 different GABA_AR subunits, with the most common stoichiometry being two α , two β , and one γ subunit. These subunits are arranged clockwise in the order $\alpha\beta\alpha\beta\gamma$, as viewed down the axis of the ion pore from the extracellular side (Trudell, 2002).

Behavioral and genetic studies implicate a variety of factors in manifestations of short- and long-term alcohol use (Harris et al., 2008). Particular attention has focused on GABA_ARs, which contribute to behavioral effects of ethanol, including tolerance, dependence, and withdrawal, are partially mediated through GABA_ARs (Chester and Cunningham, 2002; Boehm et al., 2004; Mehta and Ticku, 2005; Krystal et al., 2006; Lobo and Harris, 2008). Genetic studies in animals have provided evidence that the $\alpha 1$ and $\alpha 2$ subunits of GABA_ARs are required for specific behavioral actions of alcohol (Werner et al., 2006; Blednov et al., 2011). In addition, the GABRA2 gene, which encodes the $\alpha 2$ subunit, has been associated with alcohol use disorders in human populations (Enoch, 2008).

This work was supported by the National Institutes of Health National Institute of Alcohol Abuse and Alcoholism [Grants T32-AA007471, F32-AA019851, AA06399].

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

<http://dx.doi.org/10.1124/jpet.111.188037>.

ABBREVIATIONS: GABA_AR, GABA_A receptor; nAChR, nicotinic acetylcholine receptor; GlyR, glycine receptor; TM, transmembrane; DTT, dithiothreitol; MTSEA, 2-aminoethyl methanethiosulfonate; MTS, methanethiosulfonate; MMTS, methyl methanethiosulfonate; PMTS, propyl methanethiosulfonate; HMTS, hexyl methanethiosulfonate; EtOH, ethanol; MeOH, methanol; HeOH, hexanol; BuOH, butanol; EC₅₀, 50% of maximal response; GluCl, invertebrate glutamate-gated chloride channel receptor.

Molecular studies of alcohol action on GABA_ARs and related channels have suggested that alcohol acts at extracellular sites (Perkins et al., 2009), intracellular sites (Guzman et al., 2009), and transmembrane sites (Mihic et al., 1997; Mascia et al., 2000; Borghese et al., 2003; Crawford et al., 2007). Specifically, in GABA_ARs, residues α 2(Ser270) and β 1(Ser265) (both 15' positions on TM2) and α 2(Ala291) and β 1(Met286) (two equivalent positions on TM3) are necessary for the potentiating effects of alcohol and are implicated in an alcohol interaction pocket between TM2 and TM3 (Mihic et al., 1997; Mascia et al., 2000). However, alcohol action is likely to be complex and may involve interaction with multiple sites (Howard et al., 2011b). Indeed, GABA_ARs, GlyRs, and nAChRs show evidence of inhibitory as well as potentiating effects of alcohol. In GABA_ARs, mutation of the TM2 potentiating sites in the mutant α 2(S15'I) β 1(S15'I) resulted in ethanol inhibition that was of the same magnitude as ethanol potentiation of the wild type (Mihic et al., 1997), suggesting that blockage of the enhancing site had unmasked an inhibitory mechanism. Likewise, in GlyRs, blockage of an engineered cysteine at the TM2 site α 1(Ser15') by covalent labeling with methanethiosulfonate (MTS) reagents unmasked an inhibitory action of ethanol (Crawford et al., 2007). In nAChRs, similar experiments provided evidence of both enhancing and inhibitory sites on the α 2 subunit TM2 region (Borghese et al., 2003). These results suggest that the physiological effects of alcohol represent the summation of enhancing and inhibitory interactions.

Evidence supports the existence of an inhibitory site of alcohol action at the 6' position in TM2 of GABA_AR subunits. Substitution with tryptophan at residue Thr261 (Thr6') in the GABA_AR α 2 subunit enhanced alcohol potentiation (Ueno et al., 2000), suggesting that the tryptophan moiety blocked an alcohol inhibitory site and unmasked additional enhancement by alcohol. Furthermore, cross-linking studies have provided evidence that the 6' positions of GABA_AR α and β subunits interact (Horenstein et al., 2001; Shan et al., 2002; Yang et al., 2007) and may contribute to a combined inhibitory site for anesthetics (Rosen et al., 2007). In this study, we used site-directed mutagenesis and MTS labeling of GABA_ARs expressed in *Xenopus laevis* oocytes to test the hypothesis that 6' position residues in both α 2 β 2- and α 2 β 2 γ 2-containing GABA_ARs contribute to the inhibitory effects of alcohol.

Materials and Methods

Materials. Adult female *X. laevis* frogs were purchased from Xenopus Express, Inc. (Plant City, FL) or Nasco (Fort Atkinson, WI). GABA, methanol (MeOH), ethanol (EtOH), butanol (BuOH), hexanol (HeOH), and dithiothreitol (DTT) were purchased from Sigma-Aldrich (St. Louis, MO). MTS reagents (methyl and hexyl) were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada), and etomidate was purchased from Tocris Bioscience (Ellisville, MO). All chemicals used were of reagent grade.

Mutagenesis and Expression. Human α 2 and γ 2s GABA_AR subunits were contained in the pCIS2 vector, and the human β 2 subunit was in the pBK-CMV vector. The desired tryptophan (α 2 T261W), cysteine (α 2 T261C and β 2 T256C), or methionine (α 2 T261M) mutations were produced by using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). In brief, complementary mutagenic primers were designed at the site of desired mutation. Polymerase chain reaction was performed by us-

ing primers and wild-type cDNA. All mutations were verified by using double-stranded DNA sequencing. *X. laevis* oocytes were manually isolated and treated with collagenase (type IA, 0.5 mg/ml) for 10 min and placed in sterile modified Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 10 mM HEPES, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, and 0.91 mM CaCl₂, buffered to pH 7.5) supplemented with 10,000 units penicillin, 50 mg of gentamicin, 90 mg of theophylline, and 220 mg of sodium pyruvate per liter (incubation medium). Oocytes were injected with 50 nl of nuclease-free water containing wild-type or mutant α 2 β 2 or α 2 β 2 γ 2s at ratios of 1.5:1.5 or 1.5:1.5:4.5 ng, respectively. Oocytes were stored at 13°C in incubation medium until experiments were conducted.

Two-Electrode Voltage-Clamp Electrophysiology. Recordings were performed as described previously (Borghese et al., 2003). In brief, GABA-induced currents were recorded at room temperature 2 to 7 days after cDNA injection. Oocytes were placed in a chamber and continuously bathed with ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM CaCl₂·2H₂O, 1 mM MgCl₂·6H₂O, and 5 mM HEPES, buffered to pH 7.5). Whole-cell voltage clamp was maintained at -70 mV via glass electrodes (1.5 M Ω) filled with 3 M KCl by using a Warner Instruments (Hamden, CT) model OC-725 oocyte clamp. All drugs were prepared immediately before bath perfusion. Methanol, ethanol, butanol, and GABA were diluted directly into ND96 buffer. Hexanol, etomidate, and stock MTS reagents were prepared in dimethyl sulfoxide and then diluted into ND96 buffer. Methyl methanethiosulfonate (MMTS) and hexyl methanethiosulfonate (HMTS) were applied at concentrations of 0.5 and 0.05 mM, respectively.

GABA response curves were determined by application of increasing GABA concentrations in 20- or 30-s intervals with 5- to 15-min washes. For all experiments, the maximal response was determined by applying 1 mM GABA (or 7 mM for the methionine mutant), and a concentration that stably produced 5% of maximal response (EC₅) was used for all subsequent measurements. Modulation of GABA response by alcohols, anesthetics, or zinc was measured by a 1-min preapplication of modulator, followed by perfusion of modulator plus agonist for 30 s.

To investigate MTS reagent accessibility to the introduced cysteine residues, oocytes were perfused with MTS reagent for 1 min alone or followed by MTS plus EC₅ or EC₅₀ GABA for 30 s. Oocytes were then washed for 5 to 10 min in ND96 buffer. The current produced by EC₅ GABA immediately before MTS perfusion was normalized to 100%. MTS effects were determined by the equation $(I_{\text{after}}/I_{\text{before}} - 1) \times 100$, where I_{before} and I_{after} are current magnitudes induced by EC₅ or EC₅₀ agonist before and after application of the MTS reagent, respectively. MTS reagents [MMTS and 2-aminoethyl methanethiosulfonate (MTSEA)] produced equivalent results when coapplied with EC₅ as with EC₅₀ GABA, indicating that over the 30-s coapplication period a similar number of channels became accessible to modification under both conditions. The effect of MMTS on alcohol modulation was determined by the aforementioned alcohol protocol, followed by treatment with MMTS plus EC₅ or EC₅₀ GABA, remeasurement of EC₅, and repetition of the alcohol potentiation protocol. MTS reagents did not significantly alter baseline currents.

For experiments involving coapplication of MTS reagents with alcohols or anesthetics, EC₅ or EC₅₀ was determined as stated previously. Alcohol (methanol, ethanol, butanol, or hexanol) or etomidate was applied alone for 1 min, immediately followed by alcohol plus EC₅ agonist plus MTS for 30 s. The effect of coapplication of alcohol/anesthetic and MTS was determined by using the aforementioned equation.

Data Analysis. All values are represented as means $\pm$ S.E.M. from three or more independent experiments. Nonlinear regression analysis was performed to determine EC₅₀ and Hill coefficient values from GABA concentration response curves by using Prism 5.01 (GraphPad Software, Inc., San Diego, CA). All data were normalized to maximal response and fitted to the equation $I_A = I_{\text{max}}/[1 + (EC_{50}/A)^n]$, where I is the current at GABA concentration A , I_{max} is the maximal current, EC₅₀ is the GABA concentration that produces 50% of the maximal response, and n is the Hill coefficient. Statistical

significance between wild-type and mutant receptors was determined by using two-tailed paired Student's *t* test or a one-way analysis of variance with the Bonferroni post hoc test.

Molecular Modeling. A homology model of an $\alpha 2\beta 2\gamma 2$ GABA_AR was generated by aligning the relevant sequences of human GABA_AR subunits with the recently published sequence of an anion-selective glutamate receptor in the Cys-loop superfamily (GluCl_{cryst}; Protein Data Bank ID code 3RHW) (Hibbs and Gouaux, 2011). We first aligned each of the GABA_AR $\alpha 2$, $\beta 2$, and $\gamma 2$ s sequences with a single subunit in the homopentameric GluCl_{cryst}. Then we concatenated the GABA_AR sequences to form five subunits in the order $\alpha 2\beta 2\alpha 2\beta 2\gamma 2$ (Trudell, 2002). We used the Sequence Alignment module of Discovery Studio 2.5 (Accelrys, San Diego, CA) to produce an initial alignment. Then we manually aligned the sequences, based on the X-ray structure of GluCl_{cryst} to assure that the residues important to the present studies were correctly displayed. We submitted that alignment to the Modeler module of Discovery Studio to build five similar models with the additional restriction to form and optimize the known Cys-loop disulfide bonds. The GABA_AR sequences have inserts in several places compared with the sequence of GluCl. Typically these inserts are in loops that connect two β strands. We refined the structure of the loops in each of the five initial models. We calculated the total energy of each model by using the CHARMM force field and chose the one with the lowest (favorable) energy. To place the side chains of each amino acid in the best position relative to neighboring residues, we applied the Side Chain Refinement module of Discovery Studio on all residues of this model. To further improve the structure, this model was then optimized with CHARMM to a gradient of 0.0001 kcal/mol $\cdot$ Å in the presence of harmonic restraints on the backbone atoms of 10 kcal/Å² and the Accelrys version of the CHARMM force field. Then we relaxed the structure with 100,000 1-fs steps of molecular dynamics at 300 K with the same harmonic restraints on the backbone atoms. Finally, we reoptimized the model as described above.

To model local structural changes associated with cysteine substitution or MMTS labeling, cysteine or methionine was substituted at the 6' positions of both α subunits. Mutant models were then optimized with CHARMM as described above. The MMTS-labeled structure was approximated by replacing the C γ atom of the substituted methionine residues with sulfur by using the Build Structure module. Images were produced by using the Persistence of Vision Raytracer (<http://www.povray.org/>).

Results

The primary objective of this work was to determine whether the 6' position in TM2 of $\alpha 2$ and $\beta 2$ GABA_AR subunits influences the inhibitory effects of alcohols on the function of the receptor. We hypothesized that the increased molecular volume contributed by tryptophan or methionine substitutions, or the labeling of an introduced cysteine with MMTS, would block any inhibitory effect of alcohols, as well as mimic the presence of alcohols by reducing receptor function.

Tryptophan Substitution in the $\alpha 2$ GABA_AR 6' Position Unmasks Potentiation by Short-Chain Alcohols.

To extend previous evidence of 6' tryptophan substitutions blocking alcohol inhibition of $\alpha 2\beta 2$ GABA_ARs (Ueno et al., 2000), we examined the effect of *n*-alcohols and volatile anesthetics on GABA_ARs of the same composition ($\alpha 2\beta 2$) containing the tryptophan substitution $\alpha 2(T6'W)$. Because GABA_ARs are implicated in immobilizing effects of *n*-alcohols and volatile anesthetics (Quinlan et al., 1998), we initially tested alcohol and anesthetic concentrations previously shown to produce 50% immobility (Alifimoff et al., 1989). Compared with wild-type receptors, tryptophan mutants showed greater enhancement by methanol and ethanol, but

not by propanol, butanol, pentanol, hexanol, isoflurane, or chloroform (Fig. 1A). Consistent with previous findings (Ueno et al., 2000), tryptophan-mutant but not wild-type receptors were also sensitive to a lower concentration of ethanol (30 mM), approximately twice the legal threshold for intoxication (Harris et al., 2008), as well as an equivalently reduced concentration of methanol (89 mM) (Fig. 1, B–D). Also consistent with past studies (Ueno et al., 2000), the introduction of tryptophan at position 6' did not significantly affect GABA responses (Table 1).

Most GABA_ARs in brain are heteromers of α , β , and γ subunits (Möhler, 2006); therefore, to probe a more physiologically relevant model system, we tested alcohol modulation of $\alpha 2\beta 2\gamma 2$ s GABA_ARs. We confirmed incorporation of the γ subunit by first testing modulation by 1 μ M zinc, which potently inhibits $\alpha 2\beta 2$ but not $\alpha 2\beta 2\gamma 2$ s GABA_ARs (Hosie et al., 2003; Trudell et al., 2008). Oocytes injected with GABA_AR cDNA including the $\gamma 2$ s subunit exhibited less than 10% zinc inhibition (data not shown). In Fig. 2, sample traces show alcohol modulation of wild-type $\alpha 2\beta 2\gamma 2$ s and mutant $\alpha 2(T6'W)\beta 2\gamma 2$ s GABA_ARs. Compared with wild type, $\alpha 2(T6'W)\beta 2\gamma 2$ s GABA_ARs showed greater enhancement by high concentrations of both methanol and ethanol (Fig. 2). These receptors were also sensitive to the lower concentration of methanol tested and showed a trend toward sensitivity to the lower concentration of ethanol (Fig. 2, B and C). We noted that the $\alpha 2(T6'W)$ mutation in γ -containing receptors did enhance GABA sensitivity (Table 1), which was consistent with past evidence that the bulky tryptophan substitution at residues in the pore-lining TM2 helix can alter gating properties (Ueno et al., 2000).

MMTS Labeling Inhibits $\alpha 2(T6'C)\beta 2$ GABA_ARs. Cysteine mutagenesis and disulfide trapping are commonly used to probe protein structure/function relationships (Karlin and Akabas, 1998) and have been specifically applied to mimic anesthetic and alcohol interactions (Mascia et al., 2000). We predicted that an alcohol inhibition site should be accessible to small MTS reagents, and MTS labeling of an engineered cysteine residue in such a site should result in persistent inhibition. To test this prediction, we introduced cysteine at the 6' position of the $\alpha 2$ GABA_AR subunit, expressed wild-type or cysteine-mutant $\alpha 2$ subunits with $\beta 2$, and characterized the resulting channels before and after treatment with MMTS.

The $\alpha 2(T6'C)\beta 2$ mutant receptors were similar to wild type in maximal responses and GABA affinity (Table 1). Application and washout of 500 μ M MMTS in the presence of EC₅₀ GABA produced a significant and persistent decrease in GABA response in $\alpha 2(T6'C)\beta 2$ but not wild-type receptors (Figs. 3, A and B). Application and washout of 500 μ M MMTS in the presence of EC₅₀ GABA produced similar results (data not shown). When applied in the absence of GABA, MMTS did not alter subsequent GABA-induced currents for wild-type or cysteine-mutant receptors (Fig. 3C). Consistent with previous findings in $\alpha 1(T6'C)\beta 2\gamma 2$ receptors (Xu and Akabas, 1996), we also observed persistent modulation, in the positive direction, of $\alpha 2(T6'C)\beta 2$ receptors by the positively charged MTS reagent MTSEA (data not shown). Neither wild-type nor $\alpha 2(T6'C)\beta 2$ mutant GABA-elicited currents were altered by a 2-min application of 10 mM DTT before treatment with MTS reagents (data not shown), indicating that neither receptor type underwent spontaneous cross-linking.

Fig. 1. The effects of a tryptophan mutation on alcohol and anesthetic modulation in wild-type (black) and mutant (gray) $\alpha 2\beta 2$ GABA_ARs. **A**, substitution of tryptophan in $\alpha 2(T6'W)\beta 2$ GABA_ARs increased modulation of GABA-induced currents by MeOH (590 mM) and EtOH (200 mM), but not by *n*-alcohols propanol (PrOH; 73 mM), butanol (BuOH; 11 mM), pentanol (PeOH; 3 mM), or hexanol (HeOH; 570 μ M) or anesthetics isoflurane (Iso; 0.3 mM) or chloroform (Chloro; 1 mM). **B** and **C**, representative tracings show the effect of a tryptophan mutation on alcohol modulation in wild-type and mutant $\alpha 2\beta 2$ GABA_ARs expressed in oocytes. **B**, wild-type (left) and $\alpha 2(T6'W)\beta 2$ (right) currents before, during, and after application of 89 or 590 mM MeOH. **C**, wild-type (left) and $\alpha 2(T6'W)\beta 2$ (right) currents before, during, and after application of 30 or 200 mM EtOH. **D**, substitution of tryptophan similarly increased enhancement of GABA-induced currents by low and moderate methanol and ethanol concentrations. Bars represent means $\pm$ S.E.M. ($n = 3-10$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

MMTS Labeling or Methionine Substitution at the $\alpha 2$ 6' Position Unmask Alcohol Potentiation. We hypothesized that if MMTS labeling leads to occupation of a receptor site mediating alcohol inhibition, then MMTS should reduce alcohol inhibition. Previous studies have

shown that blocking alcohol potentiation with MMTS reagents resulted in channels that were inhibited by alcohol, presumably due to the unmasking of inhibitory effects (Borghese et al., 2003; Crawford et al., 2007). Here, we predicted the opposite phenomenon: labeling the 6' position with

TABLE 1

GABA concentration response relationships for wild-type and 6' mutant GABA_ARs

GABA concentration response curves were generated by measuring currents induced by increasing agonist concentrations (0.1 μ M–7 mM). Values are represented as the mean $\pm$ S.E.M. The EC₅₀ is the agonist concentration that elicits half of maximal response. Currents were normalized to the maximal current, and response curves were produced by using nonlinear regression to the Hill equation. *N* is the number of oocytes tested for each receptor.

Receptor	GABA EC ₅₀	Hill Coefficient	Maximal Response	<i>N</i>
	μ M		nA	
$\alpha 2\beta 2$	41 $\pm$ 15	0.8 $\pm$ 0.1	4759 $\pm$ 324	5
$\alpha 2(\text{T6}'\text{W})\beta 2$	11 $\pm$ 3	1.5 $\pm$ 0.7	4023 $\pm$ 500	6
$\alpha 2(\text{T6}'\text{C})\beta 2$	7 $\pm$ 3	1.6 $\pm$ 1.0	3221 $\pm$ 288	3
$\alpha 2(\text{T6}'\text{M})\beta 2$	88 $\pm$ 8*	0.9 $\pm$ 0.1	3836 $\pm$ 1385	3
$\alpha 2\beta 2(\text{T6}'\text{C})$	10 $\pm$ 3	2.0 $\pm$ 1.0	1884 $\pm$ 467*	3
$\alpha 2\beta 2\gamma 2s$	56 $\pm$ 11	1.4 $\pm$ 0.1	5889 $\pm$ 945	3
$\alpha 2(\text{T6}'\text{C})\beta 2\gamma 2s$	10 $\pm$ 7*	0.9 $\pm$ 0.3	5748 $\pm$ 2144	3
$\alpha 2\beta 2(\text{T6}'\text{C})\gamma 2s$	2 $\pm$ 1*	1.6 $\pm$ 0.3	5226 $\pm$ 1685	3
$\alpha 2(\text{T6}'\text{W})\beta 2\gamma 2s$	10 $\pm$ 1*	2.0 $\pm$ 1.6	6043 $\pm$ 810	3

* $p < 0.05$, significant effect of mutation compared with respective wild-type receptor.

MMTS should reduce inhibitory binding and thereby augment net alcohol potentiation, in parallel to the tryptophan substitution results shown above. Indeed, treatment by MMTS robustly enhanced potentiation of $\alpha 2(\text{T6}'\text{C})\beta 2$ receptors by either methanol (Fig. 4A) or ethanol (Fig. 4B). We noted that cysteine substitution initially reduced short-chain alcohol potentiation relative to wild type, without altering potentiation by the anesthetic etomidate (Fig. 4C); MMTS treatment restored modulation to approximately wild-type levels (Fig. 4, A and B). There was no change in wild-type receptor modulation by either alcohol after MMTS treatment (Fig. 4, A and B).

Given that MMTS labeling may not have been complete, possibly accounting for the lack of change in alcohol effects relative to wild type, we noted that the structure of a methanethiolated cysteine residue (Fig. 4D) is similar to that of a methionine residue (Fig. 4E). Therefore, we expect a substituted methionine to duplicate the effects of complete MMTS labeling. Indeed, compared with wild type, $\alpha 2(\text{T6}'\text{M})\beta 2$ mutant receptors showed enhanced potentiation by methanol and ethanol, but not by hexanol or etomidate (Fig. 4F). The methionine mutation also reduced receptor sensitivity to GABA (Table 1), which is consistent with occupation of an inhibitory site by the bulkier residue.

Alcohols Prevent MMTS Modification of $\alpha 2(\text{T6}'\text{C})\beta 2$ GABA_ARs. Our results indicate that the 6' position mediates inhibition by both alcohols and MMTS, possibly via a shared binding site. We hypothesized that if alcohols and MMTS reagents indeed interact with a shared site, then coapplication with alcohols could prevent MMTS action at the 6' position. Indeed, coapplication of an immobilizing concentration of methanol (590 mM) (Alifimoff et al., 1989) with MMTS and GABA prevented inhibition of receptor function (Fig. 5A). Conversely, an immobilizing concentration of butanol (11 mM) did not block the effects of MMTS (Fig. 5B); in fact, butanol concentrations as high as 33 mM failed to prevent inhibition by MMTS (data not shown). This result corresponds to our tryptophan mutant data indicating that inhibition via the 6' position is specific to short-chain alcohols (Fig. 1A); however, we also observed competition between MMTS and the longer-chain alcohol hexanol (Fig. 5C), as discussed in more detail below.

To further substantiate a direct interaction with the 6'

position, we asked whether etomidate could compete with MMTS labeling. Etomidate is thought to bind at the lipid-facing α - β interface at the extracellular end of the transmembrane domain (Li et al., 2009), distal to the internal pore-facing 6' positions; therefore, we expected it would not interfere with MMTS-induced persistent inhibition of GABA-activated currents. Indeed, whereas 1 μ M etomidate modulated $\alpha 2(\text{T6}'\text{C})\beta 2$ receptors to an equivalent degree as wild type (Fig. 4C), coapplication of the anesthetic did not interfere with MMTS inhibition (Fig. 5D).

Hexyl Reagents May Interact Silently with the $\alpha 2$ 6' Position. The changes we observed in alcohol modulation of tryptophan mutants (Fig. 1A) suggested that, whereas the 6' position mediates inhibitory effects of methanol and ethanol, it does not influence inhibition by longer-chain alcohols. To test the accessibility of the 6' position to longer-chain reagents, we treated wild-type and $\alpha 2(\text{T6}'\text{C})\beta 2$ GABA_ARs with HMTS. Indeed, HMTS applied alone (data not shown) or in the presence of agonist had no effect on GABA-induced currents in wild-type or mutant receptors (Fig. 6A).

To reconcile this result with our previous observation that hexanol competes with MMTS labeling (Fig. 5C), we hypothesized that hexanol and HMTS might interact with the 6' position silently, that is, without affecting the function of the receptor. To test this hypothesis, we treated mutant GABA_ARs successively with HMTS and MMTS. After HMTS treatment, MMTS failed to produce persistent inhibition of GABA responses (Fig. 6B), suggesting that HMTS had covalently blocked the 6' position. Wild-type responses were not significantly different from mutant responses in the presence of HMTS.

Cysteine Substituted in the $\beta 2$ GABA_AR 6' Position Shows Evidence of Spontaneous Cross-Linking. To investigate the contribution of β subunits to alcohol inhibition, we introduced cysteine at the 6' position of the $\beta 2$ subunit and coexpressed it with wild-type $\alpha 2$. Although the GABA EC₅₀ for $\alpha 2\beta 2(\text{T6}'\text{C})$ receptors was not different from wild type, the maximal response was reduced by more than 50% (Table 1). Successive applications of agonist caused escalating rundown, whereas perfusion with 10 mM DTT led to a 2-fold increase in agonist response (data not shown). We concluded that $\beta 2(\text{T6}'\text{C})$ subunits formed spontaneous cross-links, most likely between adjacent subunits, which could be at least partially reversed by DTT treatment. We noted that adjacent β subunits are found only in $\alpha\beta$ GABA_ARs, whereas in $\alpha\beta\gamma$ GABA_ARs the γ subunit separates the two β subunits (Trudell et al., 2008). These findings are consistent with previous reports in $\alpha 1\beta 1$ GABA_ARs that adjacent β subunits cross-link via engineered Thr6' cysteines in the closed state, whereas nonadjacent α subunits do not cross-link (Shan et al., 2002; Yang et al., 2007). Because of the formation of spontaneous disulfide bonds, we were unable to fully characterize alcohol and MTS reagent modulation of $\alpha 2\beta 2(\text{T6}'\text{C})$ GABA_ARs.

MMTS Labeling Inhibits $\alpha 2(\text{T6}'\text{C})\beta 2\gamma 2s$ and $\alpha 2\beta 2(\text{T6}'\text{C})\gamma 2s$ GABA_ARs. We also assessed MMTS effects on the more physiologically relevant γ -containing GABA_ARs (Möhler, 2006). Incorporation of the γ subunit was confirmed by an absence of zinc modulation as described above. In γ -containing GABA_ARs, cysteine substitution at the $\alpha 2(\text{Thr6}')$ position increased sensitivity to GABA. Conversely, similar to $\alpha 2\beta 2$ receptors, treatment of $\alpha 2(\text{T6}'\text{C})\beta 2\gamma 2s$ receptors with MMTS

Fig. 2. Representative tracings showing the effect of a tryptophan mutation on alcohol modulation in wild-type and mutant $\alpha 2\beta 2\gamma 2s$ GABA_ARs expressed in oocytes. **A**, wild-type (left) and $\alpha 2(T6'W)\beta 2\gamma 2s$ (right) currents before, during, and after application of 89 or 590 mM MeOH. **B**, wild-type (left) and $\alpha 2(T6'W)\beta 2\gamma 2s$ (right) currents before, during, and after application of 30 or 200 mM EtOH. **C**, in $\gamma 2s$ -containing receptors, substitution of tryptophan similarly increased enhancement of GABA-induced currents by both the methanol concentrations tested and by moderate concentrations of ethanol. Bars represent means $\pm$ S.E.M. ($n = 3-7$). *, $p < 0.05$; **, $p < 0.01$.

in the presence of EC₅ GABA reduced subsequent GABA-evoked currents (Fig. 7A, ■). MMTS treatment did not alter GABA responses in wild-type $\alpha 2\beta 2\gamma 2s$ receptors (Fig. 7A, ○).

It is noteworthy that incorporating the $\gamma 2s$ subunit allowed us to characterize receptors containing the $\beta 2(T6'C)$ mutant subunit in more detail: these receptors showed no evidence of spontaneous cross-linking, presumably because γ replaces one of the adjacent β subunits and eliminates the proximal disulfide bond partner (Cromer et al., 2002; Trudell, 2002). Similar to $\alpha 2(T6'C)$ -containing receptors, $\alpha 2\beta 2(T6'C)\gamma 2s$ mutants exhibited enhanced sensitivity to GABA, whereas a single application of 500 μ M MMTS and EC₅ GABA produced a long-lasting decrease in GABA-evoked currents that was not

observed in the wild type (Fig. 7B). Thus, occupation of the 6' position in either the α or β GABA_AR subunits inhibits channel function in physiologically relevant GABA_AR subtypes.

Molecular Modeling Reveals Structural Consequences of 6' Modification. To characterize the structural characteristics of the 6' position and effects of its substitution or labeling, we built a molecular model from the recent crystallographic structure of the GABA_AR homolog GluCl from *Caenorhabditis elegans* (Hibbs and Gouaux, 2011) with energy optimization before and after 100,000 1-fs steps of molecular dynamics simulation. Modeling of the $\alpha 2\beta 2\gamma 2s$ -containing GABA_AR on GluCl (Fig. 8A) oriented the 6' position residue extending into the channel pore approximately midway along the z -axis of the

Fig. 3. The effects of MMTS on GABA responses in wild-type and cysteine-mutant $\alpha 2\beta 2$ receptors. **A**, representative tracings from wild-type (left) and $\alpha 2(T6'C)\beta 2$ mutant (right) receptors before and after coapplication of MMTS and GABA. **B**, application and washout of $500 \mu M$ MMTS in the presence of EC_5 GABA (M + G) reduced all subsequent GABA responses in $\alpha 2(T6'C)\beta 2$ (■) but not wild-type (○) receptors. **C**, MMTS in the absence of GABA (M) had no effect on subsequent EC_5 GABA responses. In **B** and **C**, values represent means $\pm$ S.E.M. ($n = 5-7$). **, $p < 0.01$; ***, $p < 0.001$.

transmembrane domain; conversely, the 15' residue was nearer the extracellular end of the transmembrane domain, facing toward the neighboring TM3 helix (Fig. 8B). Based on this molecular dynamics result, we built additional models replacing the 6' position with cysteine or an MMTS-labeled cysteine. As expected, substitution with cysteine decreased side-chain volume and polarity in the proximal pore region, whereas MMTS labeling partially blocked the same region (Fig. 8C).

Discussion

Our results indicate that alcohols have inhibitory as well as enhancing effects on GABA_ARs, mediated by the 6' position in TM2. We demonstrate that a single amino acid substitution at this position is sufficient to alter the proposed inhibitory effect of short-chain alcohols on GABA_ARs. Replacement of the $\alpha 2(Thr6')$ position with the bulkier residues tryptophan or methionine selectively enhanced potentiation of receptor function by methanol and ethanol, which is consistent with blockage of an inhibitory site in this region. Conversely, substitution at the same site with cysteine, which is smaller than the wild-type threonine, selectively reduced potentiation by methanol or ethanol, which is consistent with the expansion of an inhibitory site to allow higher-affinity alcohol interactions. It is noteworthy that neither increased nor decreased volume at the 6' position affected modulation by long-chain alcohols or etomidate, indicating that the inhibitory mechanism mediated by this region is restricted to short-chain alcohols. This result is reminiscent of nAChRs, in which modification of the 16' position in TM2 selectively altered action of alcohols shorter than buta-

nol, whereas modification of the 17' position altered the action of alcohols longer than hexanol (Borghese et al., 2003).

In methionine-substituted $\alpha 2\beta 2$ receptors, as well as cysteine-substituted $\alpha 2\beta 2\gamma 2s$ receptors, GABA sensitivity varied in a manner consistent with occupation or evacuation of an inhibitory site, respectively; however, other substitutions showed opposite or insignificant changes in GABA potency, supporting past evidence that TM2 mutations may have a variety of effects on gating (Ueno et al., 2000), which may or may not be related to their effects on alcohol modulation.

Our experiments with MMTS provide more direct evidence for an inhibitory alcohol site at the 6' position. In parallel to the well characterized potentiating sites at which MTS labeling enhanced GABA_AR function (Mascia et al., 2000), MMTS labeling of engineered cysteines at the 6' position of $\alpha 2$ or $\beta 2$ subunits inhibited receptor function. Furthermore, whereas cysteine substitution at the 6' position reduced short-chain alcohol potentiation (consistent with an enhanced inhibitory effect of these modulators), MMTS labeling of this residue restored alcohol potentiation to wild-type levels. Thus, in parallel to recent studies of GlyRs (Crawford et al., 2007) and nAChRs (Borghese et al., 2003), MTS reagents can be valuable tools for discriminating between multiple sites of alcohol interaction: substitution and labeling of our target residue revealed an inhibitory effect previously masked by the potentiating effects of alcohols at other sites in the same receptor. Furthermore, in parallel to recent work on the 15' potentiating site (McCracken et al., 2010), we showed that coapplication of methanol blocked the inhibitory effect of MMTS on the 6' cysteine mutant. This result is consistent with direct

Fig. 4. The effects of MMTS labeling and methionine substitution on alcohol modulation in wild-type and mutant $\alpha 2\beta 2$ GABA_ARs. **A**, potentiation of $\alpha 2(T6'C)\beta 2$ receptors (gray bars) by 590 mM methanol was enhanced after MMTS labeling, whereas potentiation of wild-type receptors (black bars) was unchanged. In each pair, pre represents potentiation immediately before treatment with MMTS, and post represents potentiation after application and washout of MMTS + EC₅ GABA and measurement of new EC₅. **B**, potentiation of $\alpha 2(T6'C)\beta 2$ mutants by 200 mM ethanol was similarly enhanced by MMTS; columns are labeled as in **A**. **C**, potentiation by 590 mM MeOH and 200 mM EtOH, but not 1 μ M etomidate, decreased in $\alpha 2(T6'C)\beta 2$ receptors (gray bars) compared with wild-type receptors (black bars). In **A** to **C**, bars represent means $\pm$ S.E.M. ($n = 5-8$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. **D**, reaction scheme showing labeling by MMTS (**1**) of a cysteine-containing protein (**2**), yielding a rapidly decomposing sulfinic acid by-product (Bruce and Kenyon, 1982) (**3**) and a methanethiolated protein (**4**). **E**, chemical structure of methionine in a protein; note the similarity to **4**. **F**, methionine mutant $\alpha 2(T6'M)\beta 2$ (gray bars) showed increased potentiation by 590 mM MeOH and 200 mM EtOH, but not by 570 μ M HeOH or 1 μ M etomidate, relative to wild type (black bars). Bars represent means $\pm$ S.E.M. ($n = 3-10$). *, $p < 0.05$; **, $p < 0.01$.

competition for a 6'-proximal inhibitory binding site, although we cannot exclude the possibility that allosteric modification of the receptor renders the 6' site inaccessible to MMTS.

We were surprised to observe competition for MMTS label-

ing by hexanol, because our initial experiments indicated that the inhibitory effect of interaction at the 6' position was restricted to short-chain alcohols, and treatment with a longer-chain MTS reagent (HMTS) did not alter GABA-induced currents. However, HMTS treatment did block subsequent

Fig. 5. The effect of coapplication of alcohols or anesthetics with MMTS on wild-type (○) and cysteine mutant (■) $\alpha 2\beta 2$ GABA_ARs. A, preapplication of 590 mM MeOH for 1 min followed by coapplication with 500 μ M MMTS and EC₅ or EC₅₀ GABA for 30 s blocked the inhibitory action of MMTS. B, conversely, preapplication and coapplication with 11 mM BuOH allowed persistent MMTS inhibition of $\alpha 2(T6'C)\beta 2$ receptors, comparable with Fig. 3B. C, preapplication and coapplication with 570 μ M HeOH blocked MMTS inhibition. D, preapplication and coapplication with 1 μ M etomidate (ETOM) allowed MMTS inhibition. Values are represented as means $\pm$ S.E.M. ($n = 4$). *, $p < 0.05$; ** $p < 0.01$.

Fig. 6. The effects of HMTS on wild-type (○) and cysteine mutant (■) $\alpha 2\beta 2$ GABA_ARs. A, 50 μ M HMTS applied with EC₅ GABA (H) did not alter subsequent GABA-induced currents from wild-type or $\alpha 2(T6'C)\beta 2$ receptors. B, treatment with HMTS plus GABA (H) blocked subsequent inhibition by 500 μ M MMTS plus GABA (M). Values are represented as means $\pm$ S.E.M. ($n = 4$).

labeling by MMTS, indicating that the 6' position is indeed accessible to longer-chain reagents but that the interaction is silent (does not alter function). These findings highlight the need for caution in interpreting MTS reagent effects. Al-

though a change in function after MTS treatment generally implies that a functionally relevant cysteine residue is accessible to the reagent (at least in some functional states of the protein), a lack of change in function does not necessarily

Fig. 7. The effect of MMTS on wild-type (○) and cysteine mutant (■) γ -containing GABA_ARs. A, similar to $\alpha 2(T6'C)\beta 2$ GABA_ARs, treatment with 500 μ M MMTS plus EC₅ GABA (M) resulted in persistent inhibition of $\alpha 2(T6'C)\beta 2\gamma 2s$ but not wild-type receptors. B, MMTS treatment (M) also persistently inhibited $\alpha 2\beta 2(T6'C)\gamma 2s$. Values are represented as means $\pm$ S.E.M. ($n = 4-5$). **, $p < 0.01$; ***, $p < 0.001$.

Fig. 8. Predicted locations of alcohol interaction sites based on the GABA_AR homolog GluCl (Protein Data Bank ID 3RHU). A, sequence alignment of TM2 helices from each GABA_AR subunit with GluCl, highlighting residues critical to alcohol modulation at positions 6' (dashed box) and 15' (dotted box). B, transmembrane domain of an $\alpha 2\beta 2\gamma 2s$ GABA_AR homology model after molecular dynamics simulation, with residues at positions 6' (dashed circle) and 15' (dotted circle) in each TM2 helix displayed as spheres and colored by heteroatom (oxygen atoms, red; nitrogen atoms, blue). Subunits $\alpha 2$ (dark gray), $\beta 2$ (white), and $\gamma 2s$ (light gray) are labeled; transmembrane helices TM1 to TM4 are labeled in the lower $\alpha 2$ subunit. Model is shown from the extracellular side; for clarity, the N-terminal extracellular domain of each subunit is not shown. C, predicted effects of amino acid substitution or labeling at position 6'. Top, enlarged view of central pore as in B. Middle, equivalent view after cysteine substitution at Thr(6') in the α subunit. Bottom, cysteine residues labeled by MMTS. All 6' residues are colored by heteroatom as in B (sulfur atoms, yellow).

imply inaccessibility; in some cases, MTS labeling may simply have no functional consequence (Karlin and Akabas, 1998). Furthermore, labeling of $\alpha 2(T6'C)\beta 2$ receptors with the larger, positively charged MTS reagent MTSEA irreversibly potentiated GABA action both in our hands and in $\alpha 1(T6'C)\beta 2\gamma 2s$ receptors (Xu and Akabas, 1996). Thus, specific chemical properties of a bound modulator or labeling agent may modify energetic pathways between open, closed, and desensitized states in complex ways; in the case of MTS reagents, properties such as charge and volume may deter-

mine whether receptor function is inhibited (MMTS), enhanced (MTSEA), or not changed (HMTS).

We also noted that MMTS labeling of 6' position cysteines required coapplication of at least a low concentration of GABA. This labeling suggests that MMTS reactivity at the 6' position is sensitive to conformational changes produced by channel gating, raising the possibility that binding of small molecules in this region may be selective for open or desensitized states of the receptor. It is noteworthy that MTSEA labeling at the 6' position was observed in the presence and absence of GABA in our hands and others (Xu and Akabas, 1996), implicating either enhanced accessibility of this reagent to the closed state of the channel or its ability to act as an agonist and increase channel openings in the absence of GABA. Thus, the chemical properties of different MTS reagents may affect their sensitivity to protein conformational changes, sometimes with counterintuitive results: in this case, a larger reagent (MTSEA) could react where a smaller one (MMTS) did not.

Methionine substitution served as a valuable positive control for MMTS labeling in this study. The structural similarity between methionine and a methanethiolated cysteine was confirmed in a recent crystal structure of a protein labeled with MMTS, in which the labeled cysteine residue adopts a conformation interchangeable with that of a methionine residue (Brams et al., 2011). Previous work using methionine to mimic methanethiolation has allowed targeted "labeling" of engineered positions, ruling out nonspecific effects of MMTS on endogenous cysteines or other aspects of channel function (Howard et al., 2011a). This approach may be a valuable tool for controlling common variables in future MTS studies. It is noteworthy that whereas MMTS labeling restored alcohol potentiation approximately to wild-type levels, methionine substitution seemed to block alcohol inhibition even more effectively, enhancing alcohol potentiation relative to wild type. This result may reflect subtle differences between the structurally similar methionine and methanethiolated cysteine or indicate that MMTS labeling in our experimental protocol was not complete, whereas the methionine mutant necessarily contained the "labeled" residue in all α subunits in all receptors.

The relative participation of different GABA_AR subunits in alcohol modulation is a topic of continuing debate (Lobo and

Harris, 2008). An important contribution of our work is the consistency of the inhibitory site in a variety of GABA_AR subunit arrangements. We initially investigated GABA_ARs containing only $\alpha 2$ and $\beta 2$ subunits for the sake of consistency with previous studies (Ueno et al., 2000) and to simplify the number of different subunits contributing to the putative inhibitory site. Having tested a variety of $\alpha 2\beta 2$ mutants, we extended this work by showing that tryptophan substitution at $\alpha 2$ (Thr6') also reduces alcohol potentiation of γ -containing receptors, which are the predominant population in brain (Möhler, 2006). Furthermore, although many structure/function studies on alcohol modulation have focused on GABA_AR α subunits (Mascia et al., 2000), McCracken et al. (2010) recently demonstrated a critical role for the TM2 15' position in the GABA_AR $\beta 2$ subunit in receptor potentiation by alcohols and anesthetics. We also showed conservation of the inhibitory effect of MMTS labeling at the 6' position in $\alpha 2$ and $\beta 2$ subunits, again in the context of γ -containing receptors. These results support the contribution of 6' residues from multiple subunits, either to symmetrical inhibitory sites or a shared inhibitory region, in physiologically relevant GABA_ARs.

Although interpretation of structure/function data in GABA_ARs is challenging because of a lack of high-resolution structural data, the recent crystallographic determination of the structure of GluCl, a homolog of GABA_ARs from *C. elegans*, has provided a valuable new template for modeling (Hibbs and Gouaux, 2011). Our models of the $\alpha 2\beta 2\gamma 2s$ GABA_AR, the first to our knowledge based on the atomic-resolution eukaryotic homolog GluCl, revealed a pore-facing region dramatically influenced by modification of the $\alpha 2$ 6' position residue. Substitution at this position did not block the pore in any of the models, but did modify volume and polarity at one of the narrowest points in the conductive pathway. Conversely, the same models place the 15' residue, which is well characterized as an alcohol potentiating site (Mihic et al., 1997), nearer the extracellular end of the transmembrane domain facing toward the neighboring TM3 helix. Our data support an inhibitory action of short-chain alcohols mediated by the pore-facing 6' position, which may be independent of potentiating effects of alcohols at structurally distant positions, such as 15'. These results are consistent with previous studies supporting the existence of multiple interaction sites for alcohols on ligand-gated ion channels, in some cases with opposing functional consequences (Borghese et al., 2003; Crawford et al., 2007; Howard et al., 2011b). The biophysical basis for alcohol modulation continues to reveal startling complexity and will require further dissection at the molecular level to understand the interplay of low-affinity interactions that result in such dramatic effects on the brain and behavior.

Acknowledgments

We thank Cecilia Borghese, Mandy McCracken, and Lindsay McCracken for technical support and many helpful discussions.

Authorship Contributions

Participated in research design: Johnson, Howard, and Harris.

Conducted experiments: Johnson.

Performed data analysis: Johnson, Howard, and Trudell.

Wrote or contributed to the writing of the manuscript: Johnson, Howard, Trudell, and Harris.

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