

Functional and Structural Analysis of the GABA_A Receptor α_1 Subunit during Channel Gating and Alcohol Modulation*

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The substituted cysteine accessibility method has proven useful for investigating structural changes of the γ -aminobutyric acid type A (GABA_A) receptor during channel gating and allosteric modulation. In the present study, the surface accessibility and reaction rate of propyl- and hexyl-methanethiosulfonate to cysteine residues introduced into the third transmembrane segment of the GABA_A receptor α_1 subunit were examined. GABA-induced currents in *Xenopus* oocytes expressing wild type and cysteine mutant GABA_A receptors were recorded before and after application of methanethiosulfonate (MTS) reagents in the resting, GABA- or alcohol-bound (ethanol or hexanol) states. Our results indicate that a water-filled cavity exists around the Ala²⁹¹ and Tyr²⁹⁴ residues of the third transmembrane segment, in agreement with previous results. Furthermore, our data indicate that a conformational change produced by alcohols (200 mM ethanol or 0.5 mM hexanol) exposure induces the water cavity around the A291C and Y294C residues to extend deeper, causing the A295C and F296C residues to become accessible to the MTS reagents. In addition, exposure of the A291C, Y294C, F296C, and V297C mutants to MTS reagents in the presence of GABA had significant effects on their GABA-induced currents, indicating that the water cavity around A291C and Y294C residues expanded to F296C and V297C by a structural movement caused by GABA binding. Our data show that GABA_A receptor is a dynamic protein during alcohol modulation and channel gating.

Several neurotransmitter receptors and ion channels in brain are sites for alcohol action (1). The γ -aminobutyric acid type A (GABA_A)¹ receptor, which mediates the majority of inhibitory synaptic transmission in the brain, has been implicated as a target of alcohols and anesthetics (2, 3). Recent studies using heterologous expression systems have showed that clinically relevant concentrations of alcohols potentiate GABA_A receptors (4–8).

The GABA_A receptor is the major inhibitory neurotransmitter-gated ion channel protein in the mammalian brain. Each subunit has a large N-terminal extracellular domain that is involved in agonist binding, four transmembrane domains, a large intracellular loop between TM3 and TM4, and a short extracellular C terminus (9, 10). Several families and subtypes of GABA_A receptor subunits (six α , three β , three γ , one δ , one ϵ , one π , and one θ subunits) have been cloned to date (11), but the stoichiometry and subtype of most GABA_A receptors in the brain are believed to be two α , two β , and one γ subunits (12–14).

The substituted cysteine accessibility method (SCAM) has been used to investigate structure and conformational changes of ion channel domains in different functional states (15–21). To investigate structural dynamics of extracellular binding sites or TM of ion channel during gating and modulation, a single cysteine residue is introduced into the functional domain and probed with a water-soluble, sulfhydryl-specific reagent. If the substituted cysteine reacts with the reagent to cause the function of the channel to be irreversibly changed, the cysteine is assumed to be exposed at the water-accessible protein surface (15–18).

Previous SCAM results suggest that channel gating and allosteric modulation by diazepam or propofol induce conformation changes in the TM3 of GABA_A receptor α_1 subunit and that water-accessible cavities form around the TM3 segment. This indicates that structural movement by GABA, diazepam, or propofol binding might allow sulfhydryl-specific reagents to penetrate into the interior of the TM domain (15–17), and several amino acids in the TM2 and TM3 segments are critical for potentiating glycine and GABA_A receptors by volatile anesthetics and alcohols (4). A binding cavity for alcohols may exist in a crevice near the extracellular ends of the TM2 or TM3 regions of GABA_A and glycine receptors (7, 22, 23). However, the structure and function of the TM3 of GABA_A receptor during alcohol binding remains unknown. In the present study, we asked how the structure of the alcohol-bound GABA_A receptor differs from the resting or GABA-bound states.

We used the SCAM to investigate the structure and conformational changes of the TM3 region of GABA_A receptor α_1 subunit during channel gating and alcohol modulation. We propose that a water-filled cavity exists around the extracellular side of TM3 and that the cavity deepens to the middle region of TM3 during channel gating and alcohol binding. Furthermore, based on results of functional accessibility of PMTS and HMMS, our data suggest that the extracellular side of TM3 is more flexible or dynamic compared with cytosolic side of TM3. Thus, structural rearrangement of the existing water-filled cavities around the extracellular side of TM3 during channel gating and alcohol binding may have an important role in both forming the binding sites for alcohols and in the mech-

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¹ The abbreviations used are: GABA_A, γ -aminobutyric acid type A; TM, transmembrane segment; SCAM, substituted cysteine accessibility method; MTS, methanethiosulfonate; PMTS, propyl-methanethiosulfonate; HMMS, hexyl-methanethiosulfonate; MBS, modified Barth's solution.

anism of allosteric modulation of the GABA_A receptor by alcohols.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression—pGEMHE plasmids encoding wild type and 16 cysteine mutants from Ala²⁹¹ to Val³⁰⁷ in TM3 of the rat GABA_A α_1 subunit were described previously (15). cRNAs were synthesized from pCIS2 plasmid encoding human β_2 GABA_A receptor subunit and pGEMHE plasmids encoding rat α_1 or γ_{2s} GABA_A receptor subunits by using a T7 RNA polymerase kit (Stratagene, La Jolla, CA). *Xenopus laevis* oocytes were isolated and injected with the cRNAs (10 ng/50 nl) encoding wild type or mutant α_1 , wild type β_2 , and γ_{2s} subunits combinations in a 1:1:1 ratio of diethylpyrocarbonate-treated water (15). We introduced one additional mutation (I290C) using site-directed mutagenesis in pGEMHE plasmid encoding the rat GABA receptor α_1 subunit and a QuikChange site-directed mutagenesis kit (Stratagene). This mutation was verified by double-stranded DNA sequencing.

Electrophysiological Analysis—GABA-induced currents were recorded from oocytes 2–5 days after cRNA injection using a two-electrode voltage clamp (23). The oocytes were perfused at 1.8 ml/min with modified Barth's solution (MBS; 88 mM NaCl, 1.0 mM KCl, 2.4 mM NaHCO₃, 10 mM HEPES, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, 10 mg/liter streptomycin, 10,000 units/liter penicillin, 50 mg/liter gentamicin, 90 mg/liter theophylline, and 220 mg/liter pyruvate, pH 7.5). GABA in MBS was applied for 20–25 s. The holding potential was –70 mV.

GABA, ethanol, and hexanol were obtained from Sigma. MTS reagents (PMTS and HMTS) were obtained from Toronto Research Chemicals (North York, Canada). Solutions of GABA, ethanol, or hexanol were prepared in MBS. Stock solutions of PMTS and HMTS were first prepared in Me₂SO and then diluted in MBS to a final Me₂SO concentration not exceeding 0.05%. The solutions of alcohols or MTS reagents were freshly prepared immediately before use.

To investigate the surface accessibility of MTS reagents to GABA_A receptors, GABA control currents were first determined by using two different GABA concentrations corresponding to the EC_{5–10} and EC₅₀ values. These currents were determined to be stable after change of <5% in the GABA-induced currents on more than two consecutive applications. The EC_{5–10} or EC₅₀ are concentrations of agonist that evoke 5–10 or 50% of the maximal current (obtained by application of 1 mM GABA). Second, oocytes expressing wild type or mutant GABA_A receptors were perfused 90 s with PMTS or HMTS solutions in the resting state (0.5 mM MTS alone), GABA-bound state (0.5 mM MTS reagents in 1 mM GABA), or alcohol-bound state (0.5 mM MTS reagents in 200 mM ethanol or 0.5 mM hexanol). Following washing (10–15 min) with MBS, the GABA control currents (EC_{5–10} and EC₅₀) were redetermined. The effect of MTS reagents was calculated as follows: The percentage of change = $(I_{\text{after}}/I_{\text{before}}) - 1 \times 100$, where I_{before} and I_{after} indicate the values of the two control currents induced by EC_{5–10} or EC₅₀ GABA concentrations before and after the application of the MTS reagent.

To determine the percentage of potentiation on GABA (EC_{5–10}) response by alcohols (200 mM ethanol or 0.5 mM hexanol), the oocytes were perfused with alcohol for 1 min to allow for complete equilibration with alcohols before a 20-s coapplication with GABA (EC_{5–10}). The solutions were freshly prepared immediately before use.

Measurement of Reaction Rates—The MTS reaction rates with the introduced cysteines were determined by the effect of sequential brief applications of MTS in the resting, alcohol-bound, or GABA-bound states (21, 24, 25). After stabilization of EC_{5–10} or EC₅₀ GABA-induced currents on more than two consecutive applications, HMTS alone (for resting state), HMTS with 1 mM GABA (for GABA-bound state), or HMTS with 200 mM ethanol or 0.5 mM hexanol (for alcohol-bound states) solutions were applied for 20 s. 0.25–0.5 mM HMTS solution was used. All of the MTS and alcohol solutions were prepared immediately before use. After washing (10–15 min) with MBS, the EC_{5–10} and EC₅₀ GABA-induced currents were remeasured. To see the effects of alcohols on reaction rates in alcohol-bound states, 200 mM ethanol, or 0.5 mM hexanol solutions in MBS were preincubated for 60 s like the above alcohol potentiation experiments. This procedure was repeated until the GABA-induced response no longer changed. The normalized GABA-induced currents to the initial current were fitted to one phase exponential function using GraphPad Prism3 software (San Diego, CA) to calculate the first order rate constants (21, 24). To determine the second order rate constant (M⁻¹ s⁻¹), the first order rate constants obtained from the single exponential fitting were divided by the concentration of MTS used (26). The normalized current was plotted as a function of the

TABLE I
GABA EC₅₀, Hill coefficients, and maximum currents for the wild type and mutant GABA_A receptors in oocyte

The values are presented as the means \pm S.E. from four to eight oocytes (A300C receptor is not included because of little current response to GABA).

Mutant	EC ₅₀	Hill coefficient	I _{max}
	μM		nA
$\alpha_1\beta_2\gamma_{2s}$	44.9 \pm 4.6	1.34 \pm 0.07	3954 \pm 435
α_1 (I290C) $\beta_2\gamma_{2s}$	53.5 \pm 2.0	1.27 \pm 0.12	1793 \pm 172
α_1 (A291C) $\beta_2\gamma_{2s}$	35.8 \pm 3.5	1.14 \pm 0.12	1443 \pm 342
α_1 (V292C) $\beta_2\gamma_{2s}$	54.7 \pm 7.3	1.38 \pm 0.11	3294 \pm 435
α_1 (Y294C) $\beta_2\gamma_{2s}$	11.6 \pm 3.4	1.17 \pm 0.16	1700 \pm 275
α_1 (A295C) $\beta_2\gamma_{2s}$	123 \pm 10	1.50 \pm 0.17	1406 \pm 160
α_1 (F296C) $\beta_2\gamma_{2s}$	111 \pm 10	1.26 \pm 0.11	1180 \pm 327
α_1 (V297C) $\beta_2\gamma_{2s}$	95.9 \pm 18	1.15 \pm 0.24	3250 \pm 327
α_1 (F298C) $\beta_2\gamma_{2s}$	117 \pm 4.3	1.45 \pm 0.10	1333 \pm 153
α_1 (S299C) $\beta_2\gamma_{2s}$	27.9 \pm 6.4	0.94 \pm 0.09	2523 \pm 329
α_1 (L301C) $\beta_2\gamma_{2s}$	208 \pm 22	1.74 \pm 0.09	820 \pm 75
α_1 (I302C) $\beta_2\gamma_{2s}$	32.9 \pm 11	1.44 \pm 0.51	2594 \pm 363
α_1 (E303C) $\beta_2\gamma_{2s}$	24.4 \pm 1.8	1.03 \pm 0.08	1851 \pm 254
α_1 (F304C) $\beta_2\gamma_{2s}$	17.2 \pm 3.2	1.34 \pm 0.40	1736 \pm 397
α_1 (A305C) $\beta_2\gamma_{2s}$	23.8 \pm 3.3	1.41 \pm 0.23	1614 \pm 112
α_1 (T306C) $\beta_2\gamma_{2s}$	96.2 \pm 12	1.49 \pm 0.10	2438 \pm 413
α_1 (V307C) $\beta_2\gamma_{2s}$	78.1 \pm 8.6	1.43 \pm 0.09	2730 \pm 456

cumulative duration of HMTS application and fitted with a single exponential function using GraphPad Prism3 software (San Diego, CA).

Data Analysis—All of the values are presented as the means \pm S.E. of the mean (S.E.) from four or more independent experiments. Nonlinear regression analysis was performed to determine EC₅₀ and Hill coefficient values from GABA concentration–response curves by using GraphPad Prism3 software (San Diego, CA). The data were normalized to the maximal current in each oocyte and fitted according to the equation of the form: $I = I_{\text{max}}/[1 + (EC_{50}/A)^n]$, where I is the current, I_{max} is the maximal current recorded in a given oocyte, EC₅₀ is the GABA concentration for half-maximal current response, A is the GABA concentration, and n is the Hill coefficient.

Statistical significance of the difference between each mutant and wild type were also analyzed by one-way analysis of variance with the Dunnett's post hoc test with $p < 0.05$ representing significance using this software.

RESULTS

Expression and Functional Characterization of Cysteine Mutants—GABA_A receptors composed of only α and β subunits are able to bind GABA or alcohols and transduce their effects in the recombinant system (4, 6). However, the $\alpha\beta\gamma$ subunit (particularly $\alpha_1\beta_2\gamma_{2s}$) composition of GABA_A receptor are the prevalent combination in the mammalian brain (9, 14). Therefore, we used $\alpha_1\beta_2\gamma_{2s}$ combination for wild type and mutant GABA_A receptors expressed in *Xenopus* oocytes in this study. To assess whether cysteine mutations affected GABA_A receptor function, each individual cysteine mutant α_1 subunits were coexpressed with wild type β_2 and γ_{2s} subunits in oocytes. Then the GABA-induced currents (I_{GABA}) and alcohol potentiation of I_{GABA} were determined (Tables I and II). Most of the mutants showed GABA-induced currents similar to wild type, except for the A300C mutant, which showed little current response to 1 mM GABA (<200 nA). In general, cysteine substitutions were well tolerated within the region Ile²⁹⁰–Val³⁰⁷ of TM3. The mutations produced some changes in GABA sensitivity, with the most sensitive (Y294C) and the least sensitive (L301C) having GABA EC₅₀ values about 4-fold different from wild type receptors. For wild type receptors, 200 mM ethanol or 0.5 mM hexanol potentiated GABA EC_{5–10}-induced currents by 66 \pm 5 and 70 \pm 7%, respectively (Table II). These concentrations approximately correspond to the anesthetic concentration *in vivo* (27, 28). Ethanol and hexanol altered GABA EC_{5–10}-induced currents of all other cysteine mutants by amounts ranging from –8 \pm 4 to 67 \pm 8% and 35 \pm 4 to 93 \pm 4%, respectively (Table II).

TABLE II
Percentage potentiation of currents induced by GABA (EC₅₋₁₀)
by 200 mM ethanol or 0.5 mM hexanol for wild type and mutant
GABA_A receptors

The values are presented as the means ± S.E. from five to eight oocytes (A300C receptor is not included because of little current response to GABA).

Mutant	Ethanol (200 mM)	Hexanol (0.5 mM)
α ₁ β ₂ γ _{2s}	67 ± 5.3	70 ± 2.9
α ₁ (I290C)β ₂ γ _{2s}	58 ± 8.5	71 ± 9.8
α ₁ (A291C)β ₂ γ _{2s}	13 ± 4.2	47 ± 3.3
α ₁ (V292C)β ₂ γ _{2s}	52 ± 5.0	71 ± 3.2
α ₁ (Y294C)β ₂ γ _{2s}	-8 ± 1.7	54 ± 5.9
α ₁ (A295C)β ₂ γ _{2s}	20 ± 3.8	75 ± 14.4
α ₁ (F296C)β ₂ γ _{2s}	20 ± 1.1	55 ± 4.5
α ₁ (V297C)β ₂ γ _{2s}	67 ± 8.1	72 ± 3.2
α ₁ (F298C)β ₂ γ _{2s}	23 ± 6.4	93 ± 3.7
α ₁ (S299C)β ₂ γ _{2s}	17 ± 3.1	35 ± 4.3
α ₁ (L301C)β ₂ γ _{2s}	23 ± 6.4	82 ± 2.5
α ₁ (I302C)β ₂ γ _{2s}	22 ± 2.4	59 ± 7.6
α ₁ (E303C)β ₂ γ _{2s}	47 ± 2.9	75 ± 2.3
α ₁ (F304C)β ₂ γ _{2s}	63 ± 9.0	75 ± 10.0
α ₁ (A305C)β ₂ γ _{2s}	36 ± 2.1	63 ± 7.6
α ₁ (T306C)β ₂ γ _{2s}	59 ± 8.0	84 ± 3.3
α ₁ (V307C)β ₂ γ _{2s}	60 ± 8.3	90 ± 8.8

Reaction of Introduced Cysteines with MTS Reagents in the Resting State—We next examined the surface accessibility of uncharged propyl- and hexyl-methanethiosulfonate (PMTS and HMTS) as sulfhydryl-specific reagents to covalently label cysteines introduced into the TM3 domain. The wild type or mutant α₁ subunits were coexpressed with wild type β₂ and γ_{2s} subunits in *Xenopus* oocytes. For PMTS and HMTS, we first determined GABA-induced currents in *Xenopus* oocytes expressing wild type GABA_A receptors before and after treatment with MTS compounds. Exposure of wild type GABA_A receptors to MTS compounds had no significant effects on GABA-induced currents (Figs. 1 and 2). Additionally, application (1.5 min) of MTS compounds in the presence of GABA (1 mM), ethanol (200 mM), or hexanol (0.5 mM) did not affect GABA-induced currents in wild type GABA_A receptor (Figs. 1–3). Thus, endogenous cysteine residues were inaccessible for reaction with the MTS compounds, or reaction with the MTS compounds had no functional effects in wild type GABA_A receptors under these conditions (resting, GABA- or alcohol-bound states). Next, we asked whether conformational changes induced by agonist (GABA) or modulators (ethanol or hexanol) in the mutant receptors alter the accessibility of MTS reagents, PMTS and HMTS, to introduced cysteine in TM3. First, GABA-induced currents were recorded before and after treatment with MTS reagents without GABA or alcohols (resting state). The GABA-induced currents from A291C and Y294C mutants receptors were significantly changed after applying MTS reagents to these mutant receptors in the resting state (Figs. 1 and 2). Interestingly, the Y294C mutant showed opposite results from PMTS and HMTS treatments: PMTS inhibited GABA-induced currents, but HMTS potentiated GABA-induced currents at Y294C (Fig. 3). The results from A291C and Y294C mutants were consistent with previous findings that a water pocket exists around these amino acids (15, 22). Application of MTS reagents in the resting states did not affect the function of any other receptors (Figs. 1 and 2). This implies that these mutant receptors in the resting states are not accessible to MTS reagents, or reaction of the MTS reagents had no significant effects on mutant receptors.

Reaction of Introduced Cysteines with MTS Reagents in the GABA-bound State—Next, we asked whether application of MTS reagents in the presence of GABA (1 mM) affects GABA-induced currents of the mutant GABA_A receptors. Application of MTS reagents with 1 mM GABA significantly changed the GABA EC₅₋₁₀- or EC₅₀-induced currents of the A291C, Y294C,

F296C, and V297C mutants (Figs. 1 and 2). Y294C or F296C mutants had no significant effects of PMTS in the GABA-bound state but showed significant effects of HMTS in the GABA-bound state (Figs. 1 and 2). The A291C and Y294C mutants were also affected by MTS reagents in the resting state. Treatment with PMTS or HMTS in the presence of GABA significantly inhibited GABA-induced currents at A291C, F296C, and V297C mutants (Figs. 1–3). Results of the Y294C mutant were similar to results obtained in the resting state. PMTS with GABA also inhibited GABA-induced currents, but HMTS with GABA potentiated GABA-induced currents at Y294C mutant (Figs. 1 and 2). The other mutants were not significantly affected by MTS reagents in this condition.

Reaction of Introduced Cysteines with MTS Reagents in the Alcohol-bound States—Finally, GABA-induced currents in oocytes expressing mutant GABA_A receptors were determined before and after treatment of MTS reagents with ethanol or hexanol to test whether there are changes of accessibility of MTS reagents in the alcohol-bound state. GABA-induced currents of A291C, Y294C, A295C, and F296C mutant receptors were significantly changed after applying MTS reagents with alcohols (Figs. 1 and 2). A295C or F296C mutants had no significant effects of PMTS in the ethanol- or hexanol-bound states but showed significant effects of HMTS in the ethanol- and hexanol-bound states (Figs. 1 and 2). MTS reagents applied with alcohol had no significant effect on all other mutants tested (Figs. 1 and 2). A291C and Y294C mutants were already reactive to MTS reagents in the resting and GABA-bound states. Treatment with PMTS or HMTS in the presence of ethanol or hexanol significantly inhibited GABA-induced currents at A291C, A295C, and F296C mutants. For the Y294C mutant, PMTS with ethanol or hexanol also inhibited GABA-induced currents, and HMTS with ethanol or hexanol potentiated the currents (Figs. 1 and 2).

Reaction Rates of HMTS with Accessible Cysteine Mutants in the Resting, Alcohol-bound, or GABA-bound States—The reaction rates of MTS reagents with an introduced cysteine side chain are determined by the access pathway to the cysteine and by the local environment of sulfhydryl group of the substituted cysteine residue (18, 24, 25). To investigate the local environment of reactive substituted cysteines, reaction rates of the accessible mutants were determined. First, A291C and Y294C mutants are accessible to HMTS in the resting state, and we asked whether the physical environments around A291C or Y294C residues in the resting state are different from environments in the alcohol-bound or GABA-bound states. To accomplish this, we measured the rate of reactions of HMTS to A291C and Y294C mutants in the absence (Fig. 4) and presence of alcohols and GABA. Rate constants of A291C mutant were similar for resting, alcohol-bound and GABA-bound states (Table III). Y294C in the GABA-bound state reacted significantly faster with HMTS than in the resting or alcohol-bound states (Table III). This indicates that GABA-induced conformational changes around Y294C residue caused increased interaction between HMTS molecules and Y294C residue. The rate constant of F296C mutant in presence of GABA was similar to rates in the presence of ethanol or hexanol (Table III). Additionally, the A295C and V297C mutants show specific state-dependent accessibility of MTS reagents in the presence of alcohols and GABA, respectively (Table III).

DISCUSSION

Accessibility of MTS Reagents and Structural Rearrangements—With SCAM, it is assumed that the cysteine residues that react with MTS reagents are exposed at a water-accessible protein surface because water is required to ionize cysteine residues. MTS reagents react with ionized thiolate groups

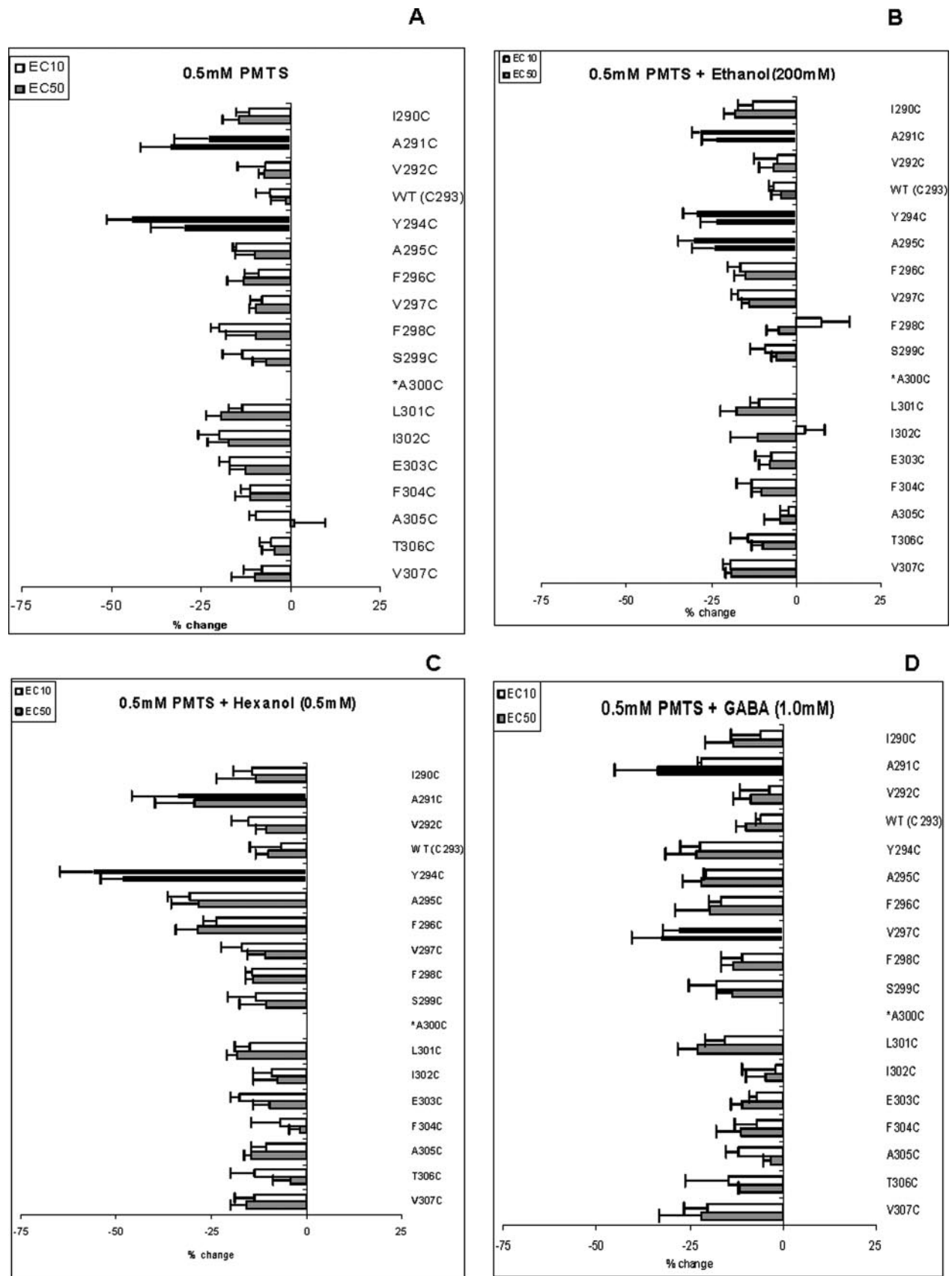


FIG. 1. Effects of PMTS on GABA-induced currents of wild type and mutant GABA_A receptors in the resting state (A), ethanol-bound state (B), hexanol-bound state (C), or GABA-bound state (D). For GABA-induced currents, the GABA EC₅₋₁₀ (top bar, white) and EC₅₀ (lower bar, gray) were applied on wild type (WT) and mutant GABA_A receptors. Black bars indicate effects that are significantly different statistically from the effect on wild type in each condition by a one-way analysis of variance, using the Dunnett's post hoc test. The percentage of change was calculated as $\{(I_{\text{after}}/I_{\text{before}}) - 1\} \times 100$, where I_{before} and I_{after} indicate the values of the two GABA-induced currents before and after the application of the sulfhydryl reagent (0.5 mM applied for 90 s). All of the values are presented as the means \pm S.E. from three to eight oocytes. *, A300C receptor is not included because of little current response to GABA.

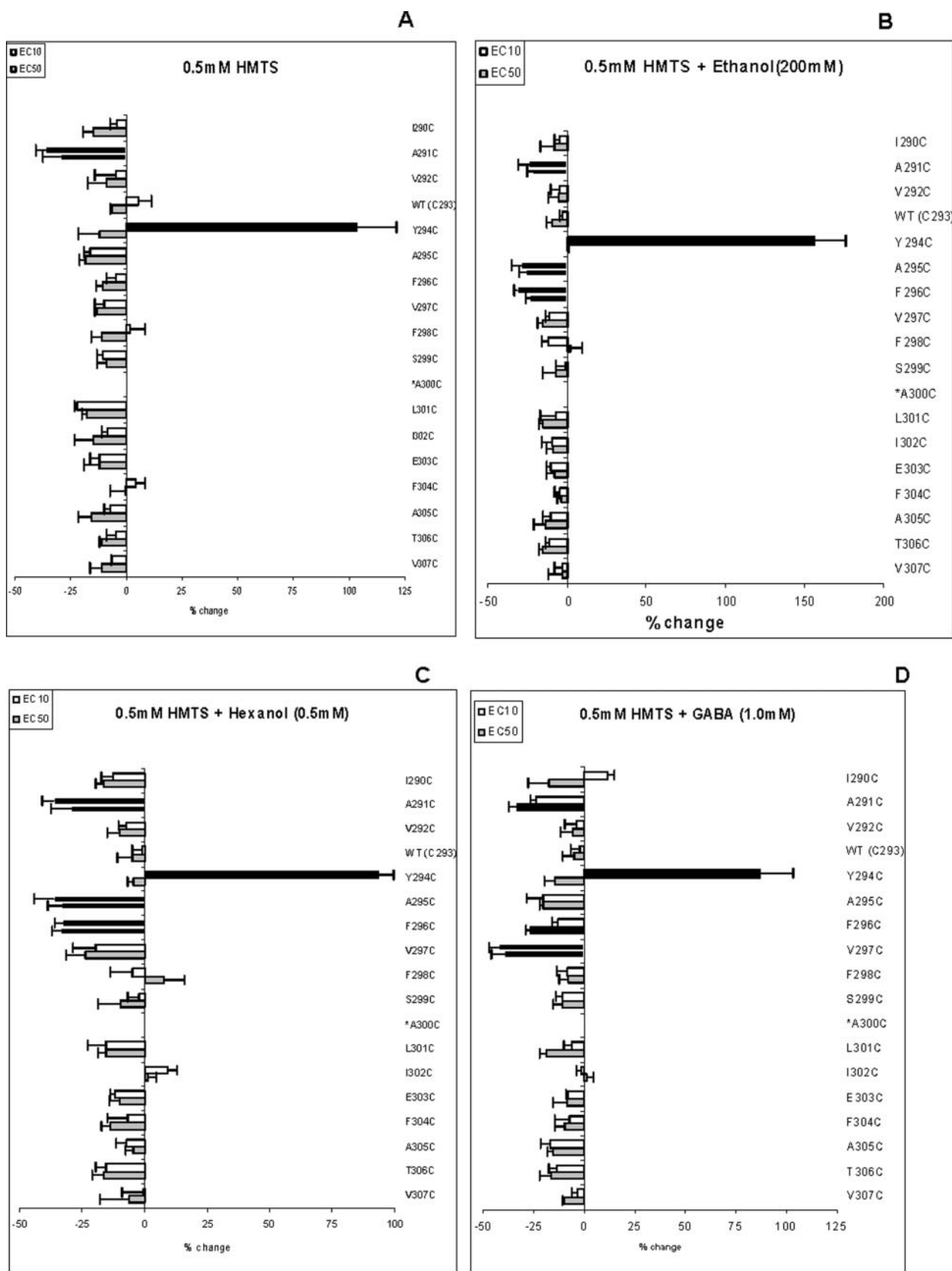


FIG. 2. Effects of HMTS on GABA-induced currents of wild type and mutant GABA_A receptors in the resting state (A), ethanol-bound state (B), hexanol-bound state (C), or GABA-bound state (D). For GABA-induced currents, the GABA EC₅₋₁₀ (top bar, white) and EC₅₀ (lower bar, gray) were applied on wild type (WT) and mutant GABA_A receptors. Black bars indicate effects that are significantly different statistically from the effect on wild type in each condition by a one-way analysis of variance, using the Dunnett's post hoc test. The percentage of change was calculated as $\{(I_{\text{after}}/I_{\text{before}}) - 1\} \times 100$, where I_{before} and I_{after} indicate the values of the two GABA-induced currents before and after the application of the sulphydryl reagent (0.5 mM applied for 90 s). All of the values are presented as the means \pm S.E. from three to eight oocytes. *, A300C receptor is not included because of little current response to GABA.

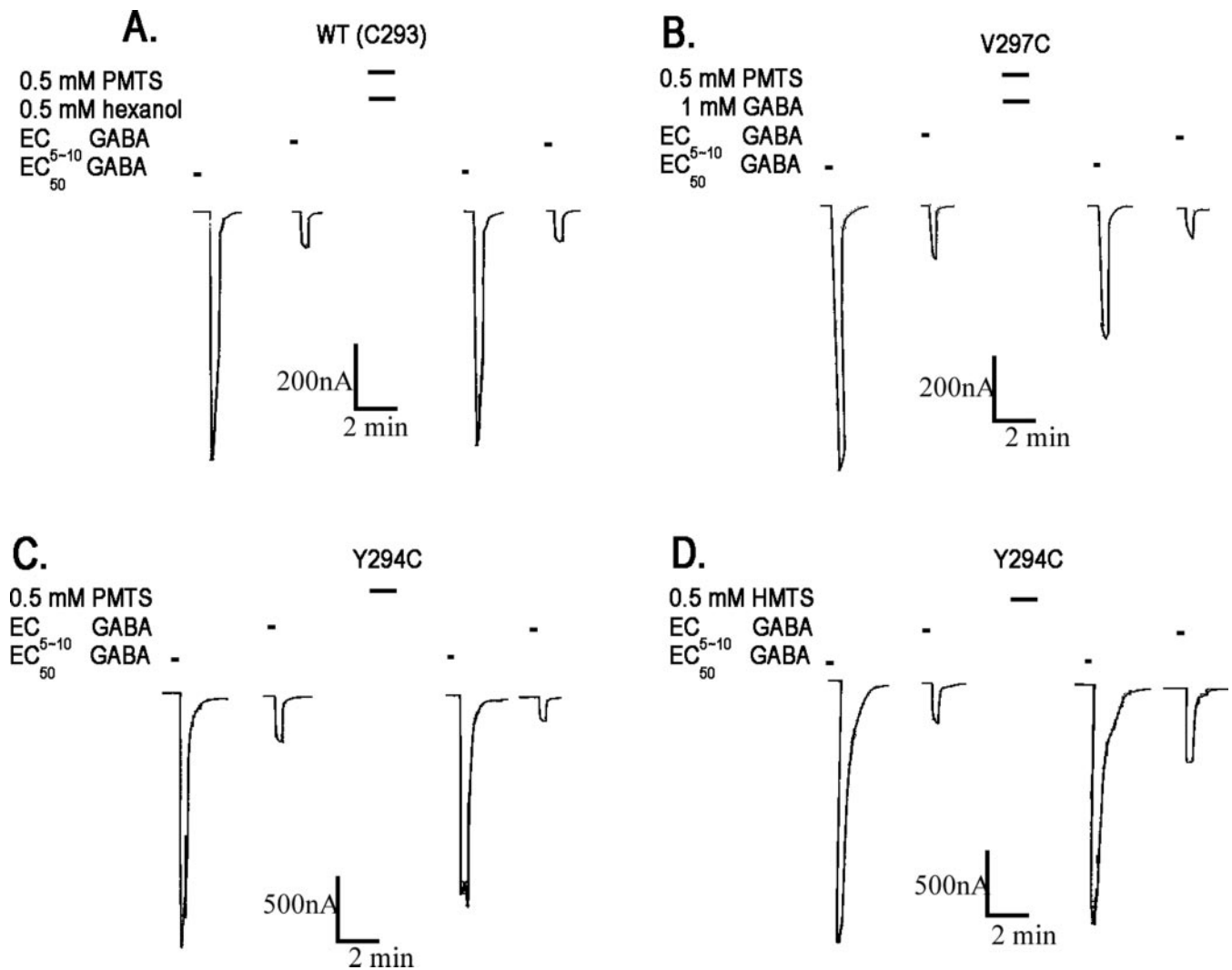


FIG. 3. Effects of MTS reagents on wild type and mutant GABA_A receptors. Sample tracings obtained from oocytes expressing wild type (WT (C293); A), V297C (B), and Y294C (C and D) GABA_A receptors demonstrating the effects of MTS solutions (0.5 mM) of various conditions on currents induced by GABA (EC₅₋₁₀ or EC₅₀). A, no effect of PMTS applied in the hexanol-bound state; B, inhibitory effect of PMTS applied in the GABA-bound state; C, inhibitory effect of PMTS applied in the resting state; D, potentiating effect of HMTS applied in the resting state.

(-S⁻) 10⁹ times faster than with un-ionized thiols (-SH) (18, 29). Previous results have indicated that the subset of cysteine substitution mutants that react with sulfhydryl-specific reagents are markers for the specific conformational states induced by different ligands (15–17, 19, 21, 25, 26, 30–32). In particular, using SCAM it was shown that TM3 of the GABA_A receptor α_1 subunit undergoes conformational changes during channel gating and allosteric benzodiazepine or propofol modulation (16, 17). Alcohols are also assumed to produce allosteric modulation of GABA_A receptors, but nothing is known about conformational changes produced by alcohols and possible overlap with changes produced by GABA, benzodiazepines, and propofol. In this study, we used SCAM to investigate whether there are common conformational changes in TM3 of the GABA_A receptor α_1 subunit during alcohol binding and GABA binding. In this study, we assume that the mutant is accessible to the MTS reagent if either of GABA EC₅₋₁₀ or EC₅₀-induced currents are affected after treatment by an MTS reagent. We found that the A291C and Y294C mutants in the resting state were significantly accessible to MTS reagents, consistent with these amino acids at the extracellular end of TM3 being on the water-accessible protein surface or facing a water-filled cavity as was proposed earlier (15, 23). It has been suggested that this water-filled cavity is connected to the extracellular solution, at

least transiently allowing water and MTS reagents to enter and that residues lining the cavity can therefore react with applied MTS reagents in the resting state (17). MTS reagents also reacted with the A291C, Y294C A295C, and F296C mutants in the presence of alcohols. The results imply that an alcohol-induced conformational change may induce the water-filled cavity around A291C and Y294C to extend deeper, causing the A295C and F296C residues to be accessible to MTS reagents (Fig. 5). Another possibility is that alcohol binding could induce the formation of a separate water-filled cavity around A295C or F296C that is transiently connected to the extracellular solution to allow PMTS or HMTS to reach these residues. In this case the cavity around A291C/Y294C may not be connected to the one that forms in the alcohol-bound state around A295C/F296C. Given the proximity of these residues, however, this seems unlikely. Furthermore, exposure of the A291C, Y294C, F296C, and V297C mutants to MTS reagents in the presence of GABA had significant effects on their GABA-induced currents, indicating that the water-accessible surface around A291C and Y294C residues widened to include F296C and V297C because of a structural movement induced by GABA-binding (Fig. 5). It is also possible that residue Val²⁹⁷ simply becomes accessible by distortion of this local environment in the presence of GABA. However, Val²⁹⁷ is located

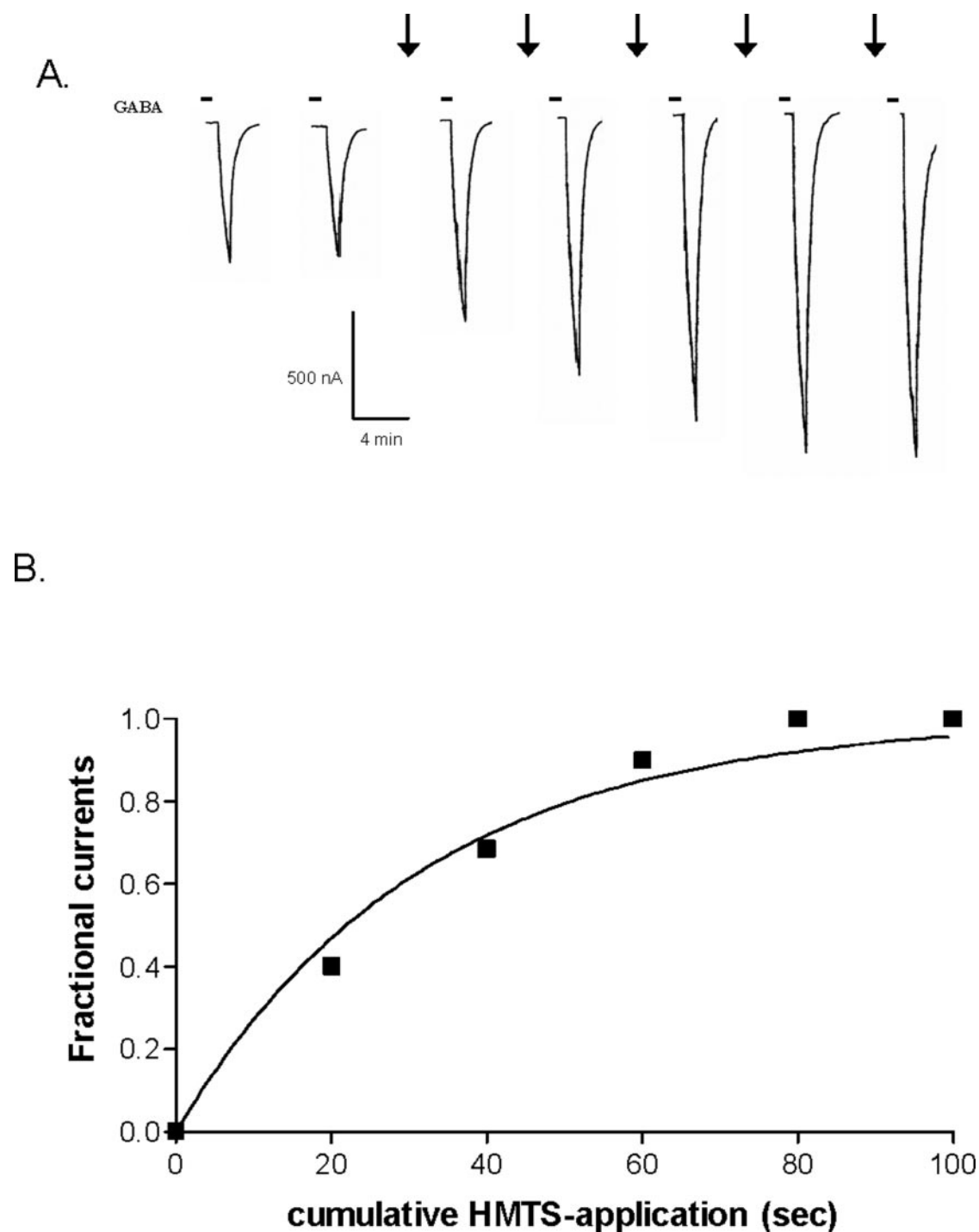
GABA_A Receptor Structure

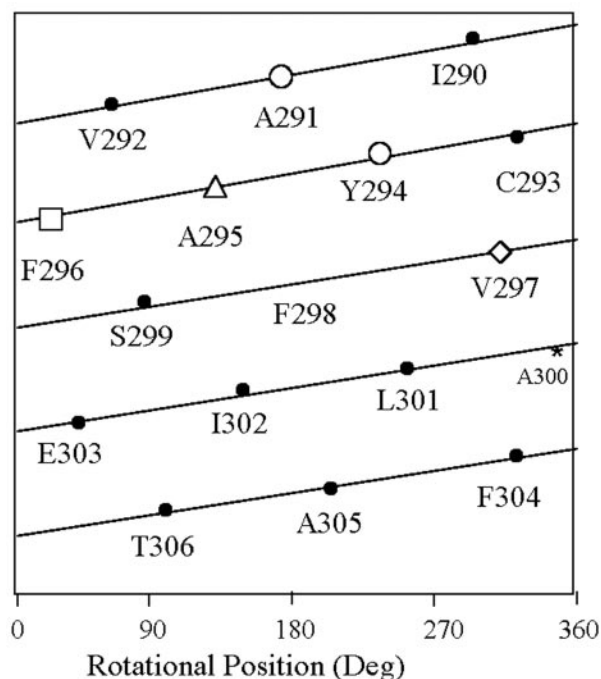
FIG. 4. **Measurement of MTS reaction rates with Y294C mutant.** *A*, representative current tracing was obtained from an oocyte expressing Y294C mutant. The EC₅₋₁₀ GABA-induced currents are shown. Each arrow indicates an application of 0.5 mM HMTS in the resting state for 20 s. After 10 min of washing, the GABA-induced current was redetermined. *B*, normalized currents to initial GABA-induced current are plotted against cumulative application time to HMTS and fitted with one phase exponential function. The calculated second order rate constants are shown in Table III.

TABLE III

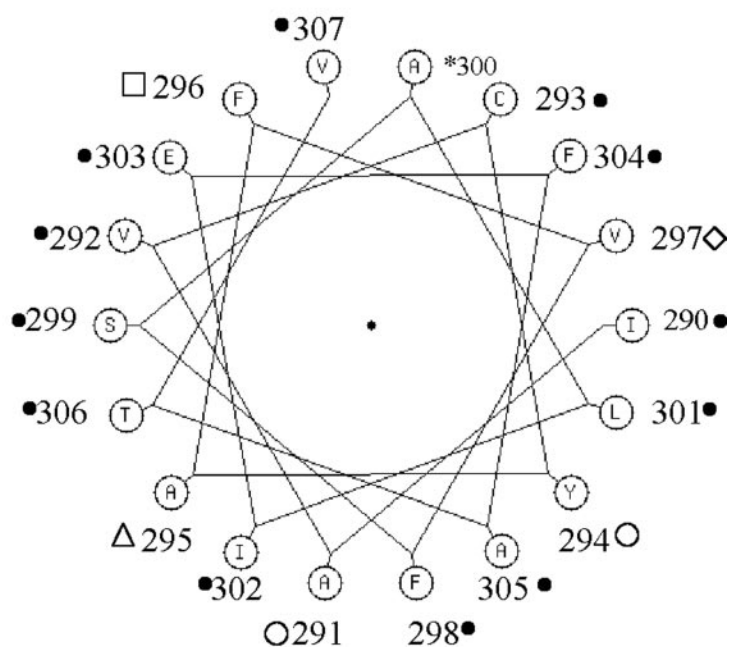
Second order rate constants of reactions of HMTS with accessible cysteine mutants in the resting, alcohol-bound, and GABA-bound states —, no reaction; GABA EC₅₋₁₀ was used for Y294C mutant, and GABA EC₅₀ was used for other mutants; the values are presented as the means \pm S.E. from four to seven oocytes.

Mutants	Resting state	GABA (1 mM)-bound state	Hexanol (0.5 mM)-bound state	Ethanol (200 mM)-bound state
A291C	69 \pm 8.0	60 \pm 4.4	74 \pm 3.0	66 \pm 5.7
Y294C	56 \pm 5.6	122 \pm 38	70 \pm 8.6	52 \pm 6.8
A295C	—	—	71 \pm 25	98 \pm 25
F296C	—	62 \pm 9.0	50 \pm 15.6	82 \pm 15.4
V297C	—	50 \pm 3.6	—	—

A. Helical net projection



B. Helical wheel projection



- , reactive with MTS in the resting, alcohol- and GABA-bound states △, reactive with MTS in the alcohol-bound states
 □, reactive with MTS in the alcohol- and GABA-bound states ◇, reactive with MTS in the GABA-bound states
 ●, no effect

*A300, not tested because of little current response to GABA

FIG. 5. α -Helical representations showing the change of GABA-induced currents before and after treatment of MTS reagents on each Cys substitution mutant of TM3 in the absence and presence of GABA or alcohols. A, helical net projection. The top side shows extracellular end, and bottom side shows the intracellular end. B, helical wheel projection. Each symbol in A and B shows reactivity with MTS in indicated functional states. *, A300C receptor is not included because of little current response to GABA.

below Tyr²⁹⁴ in our model (Fig. 5); therefore, it is likely that V297C becomes accessible to MTS reagents by expansion of the cavity containing water and MTS reagents in the GABA-bound state. These data also support the idea that an extracellular structural change produced by binding of GABA to the N-terminal region must be transferred to this TM3 region possibly through the TM2–3 linker (19, 33). The A291C and Y294C mutants are accessible to MTS reagents in the resting state and also in the alcohol-bound and in the GABA-bound states. Introduction of cysteines deeper than Val²⁹⁷ within TM3 did not reveal any effect of MTS reagents in the absence or presence of GABA, ethanol, or hexanol in our studies. This suggests that MTS reagents are not accessible to these amino acids, although we cannot rule out the alternative possibility that the modification of the amino acids by these alkyl MTS derivatives is functionally silent. It should be noted that some sites (F298C, S299C, L301C, I302C, or V307C in the different states) appeared to be reactive to MTS reagents, but statistical comparison of wild type and these mutants did not show significant effects (Figs. 1 and 2). Taken together, this suggests that the extracellular side of TM3 might be more flexible or dynamic compared with the intracellular side in the presence of agonist or modulator. Recently, it was shown that the protein packing is loose around the extracellular half of the GABA_A receptor β_1 subunit TM2 in the presence of GABA (34), consistent with this study that the extracellular side of TM3 might be more flexible. This region composed of A291C, Y294C, A295C, F296C, and V297C of TM3 may be involved in structural movements that transduce the allosteric modulation of GABA_A receptor.

It is of interest to compare our data with those from previous studies that used the same mutants but different thiol reagents (15, 16). The mutants A291C, Y294C, F296C, F298C, A300C, L301C, and E303C were found to be accessible to *para*-chloromercuribenzenesulfonate in the presence of GABA. Our data showed that MTS reagents were reactive with A291C, Y294C, F296C, and V297C in GABA-bound state. It should be noted that structurally and functionally different thiol reactive agents could show different accessibilities or reactivity with the same cysteine mutation or different reagents may have very different functional effects following modification (15, 26, 35, 36). Furthermore, to investigate the accessibility GABA EC_{5–10} or EC₅₀ was used in this study and GABA EC₅₀ or nearly saturating GABA were used in the study from Williams and Akabas (15), but the more important finding is that both studies show evidence for conformational changes in the TM3 of GABA_A receptor α_1 subunit induced by binding of agonist or modulators.

Reaction Rates of HMTS with the Reactive Mutants in the Different Functional States—The microenvironment and access pathway to the introduced cysteines can be characterized by determining the rate constants of reaction with MTS reagents (18, 24, 25). The rate at which MTS reagents react with a cysteine side chain depends on the collision frequency between the MTS reagent and the ionized sulfhydryl group. The collision frequency depends on the local concentration of the MTS reagent in the vicinity of the cysteine. This is influenced by steric factors in the access pathway from bulk solution and at the site of the cysteine. The extent to which a cysteine ionizes

depends on fractional time that the residue is in contact with water and bulk solution pH and on the local electrostatic potential. To investigate the local physical environment of the accessible introduced cysteines of TM3 of the GABA_A receptor α_1 subunit, we determined the rate constant of reaction of HMTS to each of the reactive cysteine mutants. The rate constants of the A291C mutant did not vary with treatments, suggesting that the local environment around the A291C residue in the alcohol-bound states did not change significantly from environment in the resting- or GABA-bound states. The rate constant of the Y294C mutant in the GABA-bound state is significantly faster than the rate constants in the resting or alcohol-bound states. This indicates that the local structure around Y294C and/or the access pathway stabilized by GABA is different from the resting and alcohol-bound states. This is consistent with previous results with Y294C (16). F296C is accessible to MTS reagents in the alcohol- and GABA-bound states. The reaction rate of the F296C mutant in the GABA-bound state is not significantly different from reaction rate in the alcohol-bound states. Therefore, the microenvironment of the F296C residue induced by alcohol binding seems to be similar to local environment induced by GABA binding.

Conclusions—Our data suggest that conformational changes induced by GABA or alcohol cause the water-accessible surface of the TM3 segment to increase from the region around A291C and Y294C to the deeper region surrounding A295C, F296C, or V297C. Based on results of functional accessibility and reaction rate of MTS reagents, our data also suggest that the extracellular side of TM3 is more flexible or dynamic during channel gating or alcohol modulation and the cytosolic side of TM3 might adopt a more tightly packed or rigid conformation.

There are several possible implications of these findings. First, if we assume that alcohols are binding in the existing water-filled cavity formed in part by TM3, then it appears that this binding promotes the expansion of this cavity or stabilizes a receptor conformational state in which the cavity is larger. This may allow binding of additional molecules of alcohol and may also mimic some of the actions of GABA, thereby promoting channel opening. In a more general extension, it is of interest to note that the alcohol-binding site in the *Drosophila* protein LUSH has some similarity to the putative alcohol-binding site TM2/3 region of GABA_A receptors (37). This binding region in LUSH is a water-filled cavity that adopts multiple conformations when occupied by water but is stabilized with limited movement when alcohols displace water (37, 38). Thus, the high flexibility observed for this region of TM3 in the present study may also allow for multiple conformations of the water-filled cavities, and perhaps, occupation of the cavity by alcohol selectively stabilizes substrates that in turn stabilize the open state of the channel.

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