γ-Aminobutyric Acid Increases the Water Accessibility of M3 Membrane-Spanning Segment Residues in γ-Aminobutyric Acid Type A Receptors

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ABSTRACT γ-Aminobutyric acid type A ($GABA_A$) receptors are members of the ligand-gated ion channel gene superfamily. Using the substituted cysteine accessibility method, we investigated whether residues in the α_1M3 membrane-spanning segment are water-accessible. Cysteine was substituted, one at a time, for each M3 residue from α_1Ala^{291} to α_1Val^{307}. The ability of these mutants to react with the water-soluble, sulfhydryl-specific reagent $pCMBS^-$ was assayed electrophysiologically. Cysteines substituted for α_1Ala^{291} and α_1Tyr^{294} reacted with $pCMBS^-$ applied both in the presence and in the absence of GABA. Cysteines substituted for α_1Phe^{298}, α_1Ala^{300}, α_1Leu^{301}, and α_1Glu^{303} only reacted with $pCMBS^-$ applied in the presence of GABA. We infer that the $pCMBS^-$ reactive residues are on the water-accessible surface of the protein and that GABA induces a conformational change that increases the water accessibility of the four M3 residues, possibly by inducing the formation of water-filled crevices that extend into the interior of the protein. Others have shown that mutations of α_1Ala^{291}, a water-accessible residue, alter volatile anesthetic and ethanol potentiation of GABA-induced currents. Water-filled crevices penetrating into the interior of the membrane-spanning domain may allow anesthetics and alcohol to reach their binding sites and thus may have implications for the mechanisms of action of these agents.

INTRODUCTION

The binding of GABA to the $GABA_A$ receptors stimulates the opening and subsequent desensitization of an anion-selective channel that is an integral part of the receptor (Amin and Weiss, 1993; Macdonald and Olsen, 1994; Maconochie et al., 1994; Rabow et al., 1995; Jones et al., 1998). The nature of the conformational changes that underlie these functional state changes is unknown. Numerous $GABA_A$ receptor subunits have been cloned (Wisden and Seeburg, 1992; Macdonald and Olsen, 1994; Rabow et al., 1995). The subunits are members of the neurotransmitter-gated ion channel gene superfamily that includes receptors for glycine, acetylcholine (ACh), and serotonin (Macdonald and Olsen, 1994; Jackson and Yakel, 1995; Karlin and Akabas, 1995; Rabow et al., 1995). Each subunit has a similar transmembrane topology, with a ~200 amino acid extracellular N-terminal domain, four membrane-spanning segments (M1, M2, M3, and M4), a large intracellular loop between M3 and M4, and a short extracellular C-terminus (Schofield et al., 1987; Macdonald and Olsen, 1994). The $GABA_A$ receptor subunit stoichiometry varies in different brain regions, but a common subunit stoichiometry is 2 α, 2 β, and 1 γ subunits (Backus et al., 1993; Chang et al., 1996; McKernan and Whiting, 1996; Tretter et al., 1997; Farrar et al., 1999). The five subunits are arranged pseudosymmetrically around a central pore (Nayeem et al., 1994). Thus each subunit contributes to the channel lining. Using the substituted cysteine accessibility method (SCAM) (Akabas et al., 1992, 1994; Xu and Akabas, 1993), we previously identified nine putative, channel-lining residues in the α_1M2 membrane-spanning segment (Xu and Akabas, 1993, 1996). Based on the pattern formed by these residues, we proposed that the M2 segment secondary structure was largely α-helical. Furthermore, we inferred that the gate, the charge-selectivity filter, and the picrotoxin binding site were located near the cytoplasmic end of the channel (Xu et al., 1995; Xu and Akabas, 1996).

Little is known about the role of the M3 membrane-spanning segment in the structure and function of the $GABA_A$ receptor. Residues aligned with α_1Ala^{291}, near the extracellular end of M3, were implicated in the ability of volatile anesthetics and alcohols to potentiate GABA-induced currents (Mihic et al., 1997; Krasowski et al., 1998a,b; Wick et al., 1998). In the homologous ACh receptor mutations of M3 residues altered gating kinetics (Campos-Caro et al., 1997; Wang et al., 1999). The secondary structure of the ACh receptor M3 segment was inferred to be largely α-helical (Blanton and Cohen, 1994; Gorne-Tschelnokow et al., 1994; Corbin et al., 1998; Lugovskoy et al., 1998; Methot and Baenziger, 1998), although others inferred that M3 was not helical (Unwin, 1993).

We have used the substituted cysteine accessibility method to identify residues in the M3 membrane-spanning segment that are on the water-accessible surface of the $GABA_A$ receptor. Cysteines substituted for six M3 residues reacted with charged, hydrophilic, sulfhydryl-specific reagents in a GABA-dependent manner. We propose that conformational changes occurring during gating induce the formation of water-filled crevices that extend into the inte-

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rior of the membrane-spanning domain of the protein. The mechanism of action of general anesthetics and alcohols that potentiate GABA-induced currents may involve interactions with residues lining these water-filled crevices.

MATERIALS AND METHODS

Substituted cysteine accessibility method

SCAM provides a systematic approach to the identification of residues on the water-accessible surface of a protein. In this method individual residues in largely hydrophobic membrane-spanning segments are mutated, one at a time, to cysteine. The cysteine substitution mutants are expressed heterologously. If the functional properties of the mutant channels are similar to wild type, we assay the ability of charged, sulfhydryl-specific reagents to react with the engineered cysteines. In the $GABA_A$ receptor we assay modification electrophysiologically by a change in the peak current induced by test pulses of GABA applied before and after application of the sulfhydryl reagent. If the peak current after application of the sulfhydryl reagent is significantly different from the initial peak current, we infer that the reagent reacted with the engineered cysteine.

The reagents that we used include the mercurial derivative *p*-chloromercuribenzenesulfonate ($pCMBS^-$) and the methanethiosulfonate (MTS) derivatives MTS-ethylammonium ($MTSEA^+$) and MTS-ethyltrimethylammonium ($MTSET^+$). $pCMBS^-$ reacts with cysteine to covalently add $HgC_6H_4SO_3^-$ to the sulfhydryl. The MTS derivatives add $-SCH_2CH_2X$ to the cysteine, where X is NH_3^+ for $MTSEA^+$ and $N(CH_3)_3^+$ for $MTSET^+$. $pCMBS^-$ reacts 10^3 times faster with ionized thiolates ($-S^-$) than with un-ionized thiols (-SH), and the MTS reagents react 5×10^9 times faster with ionized thiolates than with thiols (Hasinoff et al., 1971; Roberts et al., 1986). We assume that only cysteines on the water-accessible surface of a protein ionize to any significant extent; thus we infer that reactive cysteines are on the water-accessible surface of the protein. In addition, $pCMBS^-$ and $MTSET^+$ are membrane impermeant (VanSteveninck et al., 1965; Holmgren et al., 1996; Olami et al., 1997), and thus when they are applied extracellularly they only have access to residues that are on the extracellular, water-accessible surface of the protein.

In solution $pCMBS^-$ becomes *p*-hydroxy-mercuribenzenesulfonate (Boyer, 1954). Although we were unable to find a measurement of the ionization constant for the hydroxyl from *p*-hydroxy-mercuribenzenesulfonate, the ionization constant for phenylmercuric hydroxide was reported to be 1×10^{-10} (Waugh et al., 1955); similar values have been reported for other organic mercurials (Waugh et al., 1955; Simpson, 1961). Assuming this ionization constant, at our bath pH, 7.5, less than 0.03% of the *p*-hydroxy-mercuribenzenesulfonate would be ionized and therefore zwitterionic. Thus we assume that $pCMBS^-$ is negatively charged in solution at pH 7.5.

Mutagenesis and expression

Residues α_1Ala^{291} to α_1Val^{307} in the rat $GABA_A$ α_1 subunit were mutated, one at a time, to cysteine, using the Altered Sites method (Promega, Madison, WI) (except for F304C, which was done by polymerase chain reaction). The mutants were subcloned into the α_1pGEMHE vector (Horenstein and Akabas, 1998) and confirmed by DNA sequencing. mRNA was synthesized from the α_1, β_1, and γ_2 pGEMHE plasmids with T7 RNA polymerase and injected into *Xenopus* oocytes at a ratio of 1 α_1:1 β_1:1 γ_2 (200 pg/nl) in 50 nl of diethylpyrocarbonate-treated water as described (Xu and Akabas, 1993).

Electrophysiology

GABA-induced currents were recorded from individual oocytes 1–5 days after mRNA injection, using a two-electrode voltage-clamp setup. Electrodes were filled with 3 M KCl and had a resistance of <2 MΩ. The ground electrode was connected to the bath with a 3 M KCl/agar bridge. Data were acquired and analyzed on a 486/33 MHz PC computer with a TEV-200 amplifier (Dagan, Minneapolis MN), a TL-1 DMA data interface (Axon Instruments, Foster City, CA), and programs written in Axobasic (Axon Instruments). The oocyte was continuously perfused at 5 ml/min with calcium-free frog Ringer's (CFFR) (115 mM NaCl, 2.5 mM KCl, 1.8 mM $MgCl_2$, 10 mM HEPES, pH 7.5) at room temperature. All solutions were made and diluted in CFFR. The holding potential was -80 mV.

Experimental protocol

Effects of sulfhydryl reagents on GABA-induced currents were assayed using the following sequence of reagents: 100 μM GABA, 20 s; 100 μM GABA, 20 s; EC_{50} GABA, 20 s; EC_{50} GABA, 20 s; sulfhydryl reagent $\pm$ 100 μM GABA, 1 min; 100 μM GABA, 20 s; 100 μM GABA, 20 s; EC_{50} GABA, 20 s; EC_{50} GABA, 20 s (see Fig. 1 for examples). The applications of GABA and reagents were separated by 3- to 5-min washes with CFFR to allow recovery from desensitization. The fractional effect of the sulfhydryl reagent was taken as $\{I_{GABA,\ after}/I_{GABA,\ before}) - 1\} * 100$, where $I_{GABA,\ after}$ is the average peak current of the two GABA test pulses after the application of the sulfhydryl reagent and $I_{GABA,\ before}$ is the average of the peak current of the initial two GABA applications. Test pulses of GABA were applied at two concentrations, EC_{50} and more than five times EC_{50}, i.e., near saturating GABA. Changes in the peak current induced by the EC_{50} GABA test pulses are more sensitive to effects of modification on gating kinetics, whereas changes in the peak current induced by the saturating GABA test pulses are more sensitive to effects of modification on conductance (Zhang and Karlin, 1998). The following concentrations of sulfhydryl reagents were used: 0.5 mM $pCMBS^-$, 2.5 mM $MTSEA^+$, and 1 mM $MTSET^+$. $pCMBS^-$ was obtained from Sigma (St. Louis, MO), MTS reagents were obtained from Toronto Research Chemicals (North York, ON, Canada).

For $pCMBS^-$, the primary reagent that we used to screen $GABA_A$ receptor cysteine-substitution mutants, we applied the reagent for 1 min at 0.5 mM. This combination of time and concentration was chosen because they were the maximum concentration and duration that caused no significant increase in the leak conductance of uninjected *Xenopus* oocytes. This limits our ability to detect reactive residues. As discussed below, for a given mutant, given the variability of responses, application of a reagent must cause a net change in current greater than $\sim$30% to be significantly different statistically from wild type by a one-way analysis of variance (for *n* between 4 and 6). Given this threshold and the reaction conditions that we used for $pCMBS^-$ (0.5 mM applied for 1 min), if complete reaction caused 100% inhibition of the GABA-induced current, we would detect as reactive positions with a second-order reaction rate greater than 12 l/mol-s.

Measurement of reaction rates

We measured the rates of reaction of $pCMBS^-$ with the engineered cysteines by determining the effect of sequential brief applications of $pCMBS^-$. A test pulse of GABA was applied, and the GABA-induced current was measured. GABA plus a lower concentration of $pCMBS^-$ (0.1 or 0.2 mM) was applied for 15–20 s. After washout of $pCMBS^-$, a test pulse of GABA was applied, and the GABA-induced current was measured. The effect of four or five brief, sequential applications of $pCMBS^-$ was determined. The magnitudes of the GABA test currents were normalized relative to the initial test pulse. The normalized current was plotted as a function of the cumulative duration of $pCMBS^-$ treatment and fitted with a single exponential decay function, using Prism2 software (GraphPad, San Diego, CA). The pseudo-first-order rate constant obtained from the exponential fit was divided by the $pCMBS^-$ concentration to yield the second-order rate constant.

Curve fitting and statistics

Data are expressed as the percentage change in current after modification for the appropriate concentration of GABA. The significance of the dif-

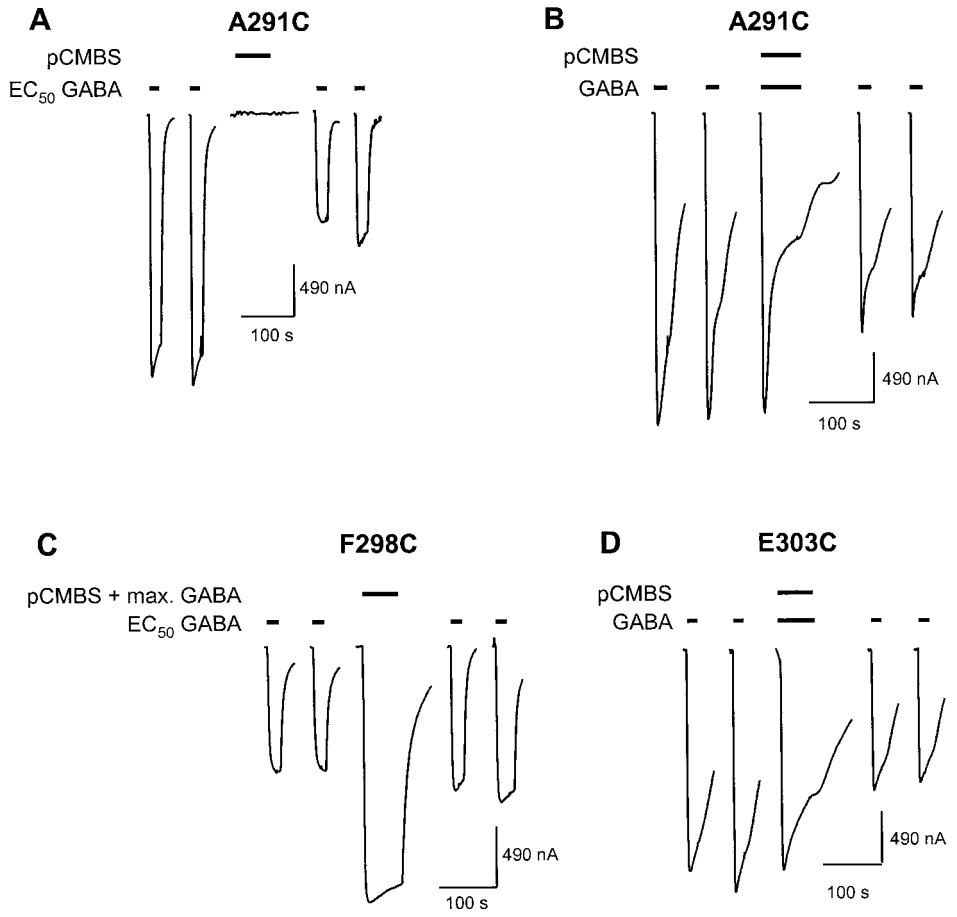

FIGURE 1 Effect of 0.5 mM pCMBS$^-$ on GABA-induced currents from individual oocyte expressing several M3 segment cysteine-substitution mutants. (*A*) pCMBS$^-$ applied in the absence of GABA inhibits subsequent EC_{50} GABA-induced currents in an oocyte expressing α_1A291C. (*B*) pCMBS$^-$ applied in the presence of GABA inhibits subsequent GABA-induced currents in an oocyte expressing α_1A291C. (*C*) pCMBS$^-$ potentiates subsequent EC_{50} GABA-induced currents for the mutant α_1F298C. (*D*) pCMBS$^-$ applied in the presence of GABA inhibits subsequent GABA-induced currents in an oocyte expressing α_1E303C. For all records, holding potential is -80 mV. Bars above traces indicate the period of application of reagents.

ference between each mutant and wild type was determined by one-way ANOVA, using the Student-Newman-Kuels post hoc test (SPSS for Windows; SPSS, Chicago, IL). Dose-response curves were fit using Prism software (GraphPad). A paired *t*-test was used to determine the significance of differences in GABA EC_{50} before and after treatment with pCMBS$^-$.

It is important to recognize that we assay reactive residues based on the functional effect of modification. We identify functional effects based on the statistical significance of the effect on a mutant relative to the effect on wild type. For mutants where the average effect after application of pCMBS$^-$ is small, whether the effect is judged to be significant depends, in part, on the stringency of the one-way ANOVA post hoc test used. For example, if we were to use the less stringent Duncan post hoc test, then an additional residue F296C would be judged to be reactive with pCMBS$^-$ in the presence of GABA (Fig. 2 *B*). The choice of post hoc test is, unfortunately, somewhat arbitrary.

RESULTS

Expression of the cysteine mutants

We substituted cysteines, one at a time, for 17 consecutive residues, from α_1Ala^{291} to α_1Val^{307}, in the M3 membrane-spanning segment of the rat α_1 subunit. The mutant α_1 subunits were expressed with wild-type β_1 and γ_2 subunits in *Xenopus* oocytes. One to two days after mRNA injection GABA-induced currents were observed for all of the mutants. For oocytes expressing wild type the average peak current induced by 100 μM GABA ($V = -80$ mV) was 1.9 ± 0.5 μA, and for the mutants it ranged between 0.2 ± 0.1 μA for A300C and 3.7 ± 0.2 μA for A305C (Table 1). We measured the EC_{50} for GABA activation of wild-type receptor and each of the cysteine substitution mutants. For wild-type receptor the GABA EC_{50} was 2.1 ± 1.3 μM ($n = 6$), and for the mutants the GABA EC_{50} ranged between 0.5 ± 0.1 μM for A291C and Y294C and 48 ± 15 μM for L301C (Table 1). Thus all of the mutants assembled and displayed GABA-induced currents similar to wild type. Therefore, we assumed that the structures of the mutants were similar to the structure of wild type. Cysteine substitutions in the ACh receptor αM1 and αM2 segments caused a similar range of changes in the ACh EC_{50} (Akabas et al., 1994; Akabas and Karlin, 1995). We infer that the changes in EC_{50} were due to changes in the gating kinetics and not to structural perturbations of the distant ligand-binding site.

Reactions with pCMBS$^-$ applied in the absence of GABA

In the absence of GABA the channels are largely in the closed state. We showed previously that when assayed with near-saturating GABA test pulses a 1-min application of 0.5 mM pCMBS$^-$ had no effect on the subsequent GABA-induced currents in wild-type receptors (Xu and Akabas, 1996). For wild type we have now obtained the same result when EC_{50} GABA test pulses were used (Fig. 2 *A*). We infer

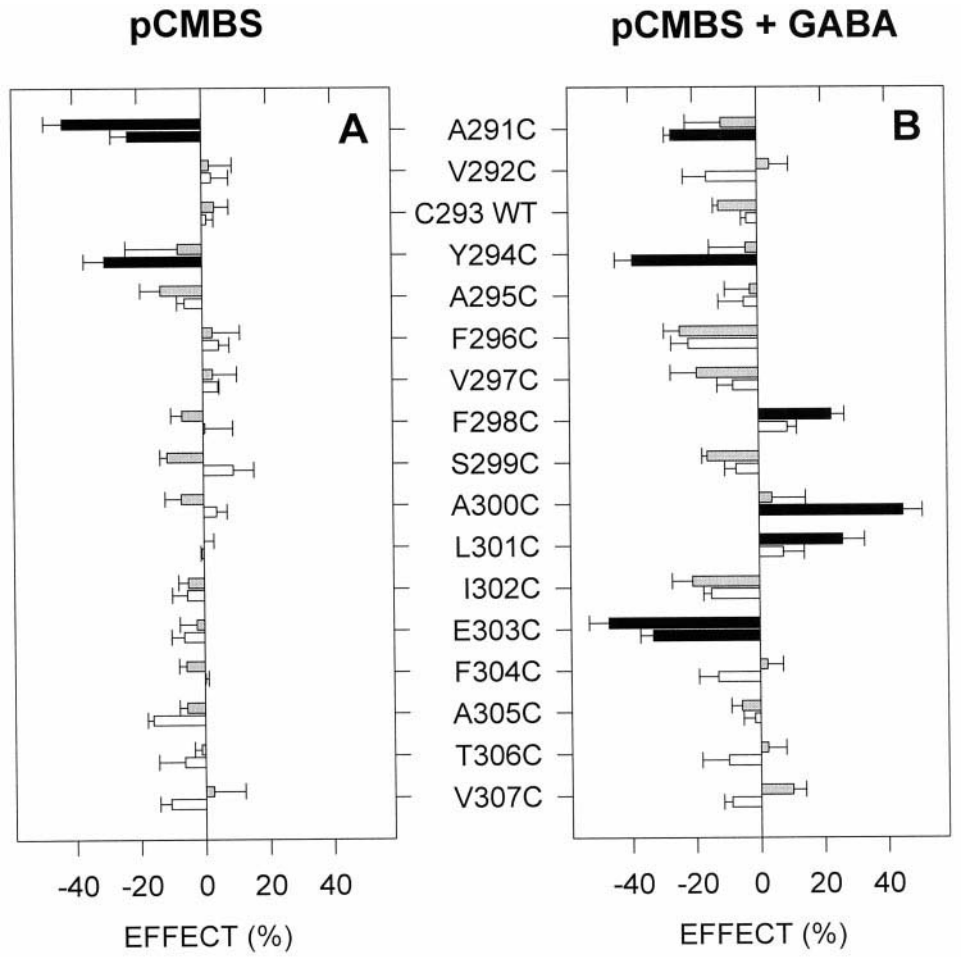

FIGURE 2 The irreversible effect of a 1-min application of 0.5 mM $pCMBS^-$, applied in the absence (*A*) or in the presence (*B*) of 100 μM GABA on subsequent GABA-induced currents of wild-type and mutant $GABA_A$ receptors. For each mutant the results are shown for test pulses at the GABA EC_{50} (*top bar, gray*) and at a saturating concentration of GABA (*lower bar, white*). Solid bars indicate effects that are significantly different statistically from the effect on wild type by a one-way ANOVA, using the Student-Newman-Kuels post hoc test. A negative effect indicates inhibition of subsequent GABA-induced currents; a positive effect indicates potentiation of subsequent GABA-induced currents. The means and SEMs are shown. For each condition the results of 3–20 oocytes are averaged.

that the endogenous cysteine residues were inaccessible for reaction with $pCMBS^-$, or reaction with $pCMBS^-$ had no functional effects.

TABLE 1 GABA EC_{50} and maximum currents for the M3 cysteine substitution mutants

Mutant	*n*	EC_{50} (μM)	I_{max} (nA)	Hill coeff.
A291C	3	0.5 ± 0.1	2420 ± 422	1.13 ± 0.40
V292C	3	4.9 ± 1.6	1516 ± 105	0.82 ± 0.12
WT, C293*	6	2.1 ± 1.3	1877 ± 467	1.29 ± 0.19
Y294C	3	0.5 ± 0.1	970 ± 235	1.72 ± 0.56
A295C	3	2.8 ± 0.7	1729 ± 410	0.59 ± 0.06
F296C	4	1.4 ± 0.4	1482 ± 78	0.80 ± 0.02
V297C	3	2.4 ± 0.6	2274 ± 63	1.00 ± 0.08
F298C	4	28.4 ± 4.0	1876 ± 526	1.06 ± 0.30
S299C	3	4.5 ± 0.2	1884 ± 439	0.91 ± 0.18
A300C	4	3.5 ± 0.5	224 ± 53	0.66 ± 0.01
L301C	4	48.0 ± 15.0	907 ± 225	1.01 ± 0.03
I302C	3	1.9 ± 0.8	921 ± 165	0.96 ± 0.18
E303C	4	1.4 ± 0.3	1033 ± 273	0.93 ± 0.11
F304C	3	1.7 ± 0.3	1564 ± 283	1.01 ± 0.12
A305C	3	1.2 ± 0.3	3743 ± 202	1.14 ± 0.21
T306C	4	0.9 ± 0.3	1076 ± 145	1.28 ± 0.24
V307C	4	9.0 ± 3.8	910 ± 234	0.82 ± 0.18

*Note that the results for wild type (WT) are given at the position of the endogenous M3 segment cysteine, α_1Cys^{293}.

When assayed using EC_{50} GABA test pulses, a 1-min application of 0.5 mM $pCMBS^-$ applied in the absence of GABA irreversibly inhibited the subsequent GABA-induced currents for the A291C mutant (Figs. 1 *A* and 2 *A*, *top bar in each pair of bars*). When assayed with near-saturating GABA test pulses, the subsequent GABA-induced currents of both A291C and Y294C were inhibited (Fig. 2 *A*). In the absence of GABA the receptor was in the closed state, although brief spontaneous openings may have occurred. The rate of spontaneous openings in $\alpha_1\beta_1\gamma_2$ $GABA_A$ receptors has not been measured; however, in the ACh receptor spontaneous openings occur with a probability of less than 1 in 10^5 (Jackson, 1984). Thus we feel that reactions of $pCMBS^-$ applied in the absence of GABA with A291C and Y294C were occurring in the closed state and not during brief spontaneous openings.

Reactions with $pCMBS^-$ applied in the presence of GABA

In the presence of GABA the receptor undergoes transitions mainly between the open and desensitized states. We showed previously that when assayed with near-saturating GABA test pulses a 1-min application of 0.5 mM $pCMBS^-$

applied in the presence of 100 μM GABA had no effect on the subsequent GABA-induced currents of wild type (Xu and Akabas, 1996). For wild type we have now obtained the same result when EC_{50} GABA test pulses were used (Fig. 2 *B*). We infer that in the presence of GABA the endogenous cysteine residues were inaccessible for reaction with $pCMBS^-$ or reaction with $pCMBS^-$ had no functional effects.

For the cysteine-substitution mutants, a 1-min application of 0.5 mM $pCMBS^-$ + 100 μM GABA irreversibly altered the subsequent GABA-induced currents of the mutants A291C and Y294C, which also reacted with $pCMBS^-$ applied in the absence of GABA. In addition, when $pCMBS^-$ was applied in the presence of GABA the currents for the mutants F298C, A300C, L301C, and E303C were also irreversibly altered (Figs. 1 and 2 *B*). For the mutants A291C, Y294C, and E303C $pCMBS^-$ modification resulted in inhibition of the subsequent currents induced by near-saturating concentrations of GABA. The E303C mutant also showed inhibition of currents induced by test pulses of GABA at the EC_{50} concentration (Fig. 2 *B*).

For the other mutants, F298C, A300C, and L301C, $pCMBS^-$ modification potentiated the subsequent GABA-induced currents. For F298C and L301C the potentiation was observed for currents induced by EC_{50} GABA concentrations. This potentiation was due to a decrease in the GABA EC_{50} after covalent modification. For the F298C mutant the GABA EC_{50} was 28 $\pm$ 4 μM before modification by $pCMBS^-$ and decreased to 15 $\pm$ 2 μM ($n = 4$; $p = 0.03$) after modification by $pCMBS^-$ (Fig. 3). Similarly, for the L301C mutant, the GABA EC_{50} was 48 $\pm$ 15 μM before modification by $pCMBS^-$ and decreased to 24 $\pm$ 8 μM ($n = 4$; $p = 0.05$) after modification by $pCMBS^-$. For both mutants $pCMBS^-$ modification did not alter the maximum current induced by saturating concentrations of GABA. Similar covalent modification induced changes in EC_{50} were seen for some cysteine substitution mutants in the M2 segment of the ACh receptor α and β subunits (Akabas et al., 1994; Zhang and Karlin, 1998). Because these residues are far from the agonist binding site, we assume that these changes in EC_{50} arose because of changes in gating kinetics and not because of changes in the affinity of the binding site for agonist (Akabas et al., 1994; Colquhoun, 1998). In the case of the A300C mutant, the post-$pCMBS^-$ test current induced by a near-saturating concentration of GABA was potentiated, but the post-$pCMBS^-$ test current induced by the EC_{50} GABA concentration was not altered (Fig. 2 *A*). For A300C, $pCMBS^-$ modification did not alter the GABA EC_{50} (data not shown). The increase in current for A300C may result from an increase in single-channel conductance.

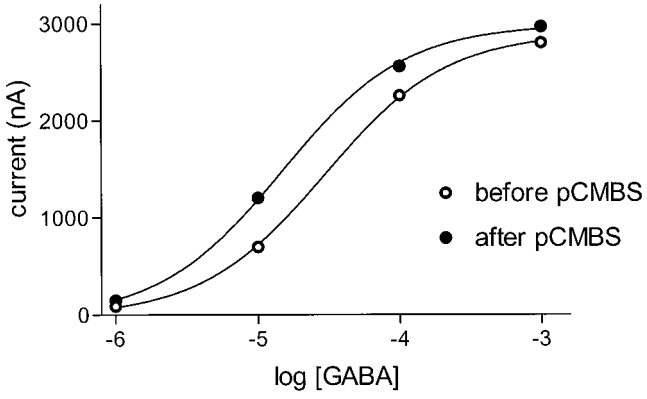

FIGURE 3 $pCMBS^-$ modification reduces the GABA EC_{50} of the F298C mutant. Results are shown for an individual oocyte. The GABA dose-response curve was determined (○). 0.5 mM $pCMBS^-$ + 100 μM GABA was applied for 1 min, and then the GABA dose-response curve was determined again (●). The data were fit with a sigmoid dose-response curve shown by the solid line. For this oocyte the EC_{50} was 29 μM before $pCMBS^-$ and 15 μM afterward. Similar results were obtained in three other oocytes.

We attempted to measure the voltage dependence of the rates of reaction of $pCMBS^-$ with E303C, the most cytoplasmic of the accessible residues. The second-order reaction rate constant for $pCMBS^-$ with E303C in the presence of GABA at a holding potential of -80 mV was 273 $\pm$ 62 l/mol-s ($n = 3$). This rate was about 5- to 10-fold slower than the rates of reaction of $pCMBS^-$ with M2 segment residues (Horenstein and Akabas, unpublished results). The reaction rate with E303C was similar at -80 and -10 mV. Thus there was no evidence that the reaction rate of $pCMBS^-$ with E303C was voltage dependent.

In the presence of GABA the receptor undergoes transitions between the open and desensitized states. The reaction with $pCMBS^-$ appeared to occur in a nonconducting state of the channel because for the F298C, A300C, L301C, and E303C mutants the current during a 1-min application of 0.5 mM $pCMBS^-$ + 100 μM GABA could be superimposed on the current induced by a 1-min application of 100 μM GABA alone (Fig. 4); however, after the reagents were washed out, subsequent applications of GABA test pulses showed that the peak currents were markedly altered after $pCMBS^-$ + GABA but not after GABA alone. If reaction were occurring in the open state we would have expected the time dependence of the current during the application of $pCMBS^-$ + GABA to be different from the current observed with GABA alone. Alternatively (but, we feel, less likely), modification in the open state may not affect conductance but may affect subsequent reopening from the closed state. Therefore, we infer that in the presence of GABA $pCMBS^-$ reacted during a nonconducting, desensitized state, a state that does not readily return to the open state during continuous application of GABA.

There are several potential interpretations for those cysteine substitution mutants where the effect of application of the sulfhydryl reagent was not significantly different from that of wild type. Some or all of these residues may be inaccessible to $pCMBS^-$, and thus there was no reaction at these positions. Similarly, they may have been accessible, but the rate of reaction of $pCMBS^-$ may have been lower than our detection threshold. Alternatively, reaction may have occurred at some or all of these positions, but covalent

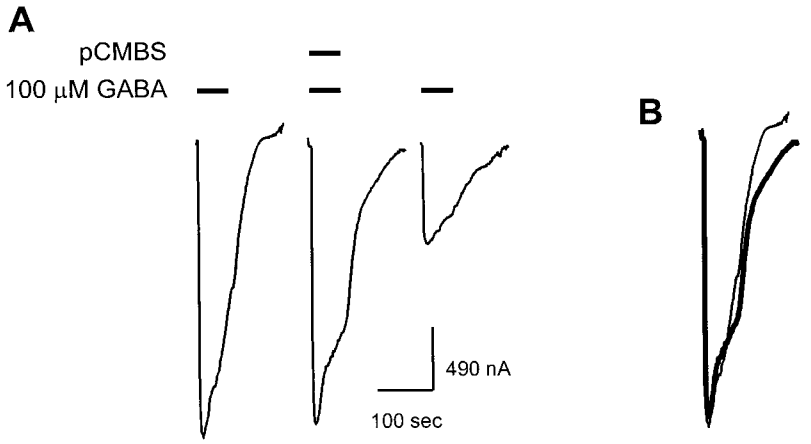

FIGURE 4 pCMBS$^-$ appears to react with α_1E303C in a desensitized state of the receptor. (*A*) Currents recorded from a single oocyte during 1-min applications of 100 μM GABA in the absence (*first and third traces*) and in the presence (*second trace*) of 0.5 mM pCMBS$^-$. Note that currents of the first and second traces are very similar (these traces are superimposed in *B*). This indicates that pCMBS$^-$ does not significantly alter the current during application of pCMBS$^-$, but the subsequent GABA-induced currents (*third trace*) are markedly reduced. (*B*) The first two traces in *A* are superimposed. *Thin line*, Trace of GABA alone; *thick line*, pCMBS$^-$ + GABA. Bars above traces indicate the period of application of reagents. Holding potential, −80 mV.

modification may have no functional effect on the subsequent GABA-induced currents. We cannot distinguish between these possibilities, and thus the negatives must be interpreted with caution. Finally, we do not believe that the negatives result from a failure of the α_1 subunit cysteine substitution mutants to express because expression of only the β_1 and γ_2 subunits gives currents that are less than 20 nA (Sigel et al., 1990) (data not shown), and all of the cysteine substitution negatives had peak GABA-induced currents greater than 750 nA (Table 1).

Reactions with cationic MTS reagents

A 1-min application of 2.5 mM MTSEA$^+$ in the absence of GABA had no effect on wild type or on any of the cysteine-substitution mutants (Fig. 5 *A*).

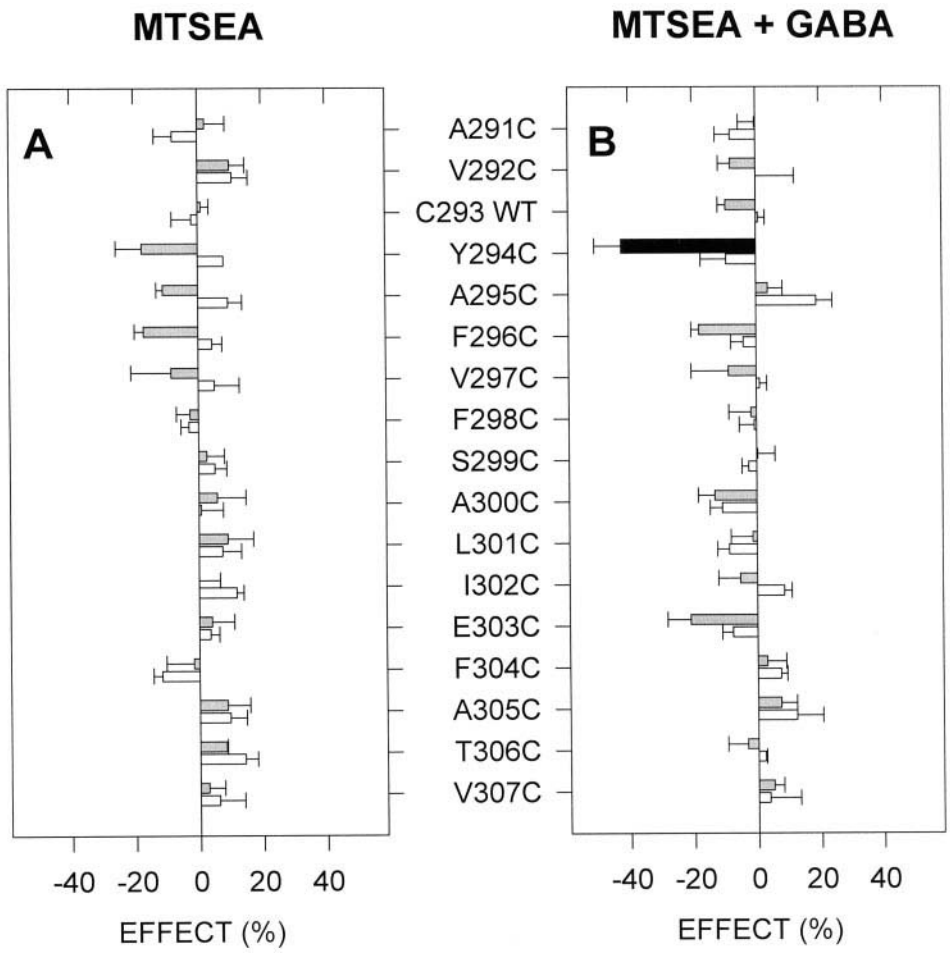

FIGURE 5 The irreversible effect of a 1-min application of 2.5 mM MTSEA$^+$, applied in the absence (*A*) or in the presence (*B*) of 100 μM GABA, on subsequent GABA-induced currents of wild-type and mutant $GABA_A$ receptors. For each mutant the results are shown for test pulses at the GABA EC_{50} (*top bar, gray*) and at a saturating concentration of GABA (*lower bar, white*). Solid bars indicate effects that are significantly different statistically from the effect on wild type by a one-way ANOVA, using the Student-Newman-Kuels post hoc test. A negative effect indicates inhibition of subsequent GABA-induced currents; a positive effect indicates potentiation of subsequent GABA-induced currents. The means and SEMs are shown. For each condition the results of three to eight oocytes are averaged.

A 1-min application of 2.5 mM $MTSEA^+$ + 100 μM GABA irreversibly inhibited the subsequent GABA-induced current of the mutant Y294C but had no effect on subsequent GABA-induced currents for the other cysteine substitution mutants (Fig. 5). For those mutants that reacted with $pCMBS^-$ + GABA we assayed to see if $MTSEA^+$ was reacting but having no effect on GABA-induced currents. If $MTSEA^+$ were reacting it would prevent a subsequent application of $pCMBS^-$ + GABA from having an effect. For the mutants A300C and E303C preapplication of $MTSEA^+$ prevented $pCMBS^-$ from reacting subsequently (Fig. 6 *B*). Similar effects were observed with a 1-min application of 1 mM $MTSET^+$, which is permanently positively charged (data not shown). Thus we inferred that the cationic MTS reagents reacted with Y294C, A300C, and E303C. $MTSEA^+$ and $MTSET^+$ did not react with the other M3 segment $pCMBS^-$ reactive residues. For example, as shown in Fig. 6 *A*, preapplication of $MTSEA^+$ did not prevent subsequent reaction by $pCMBS^-$ with α_1F298C.

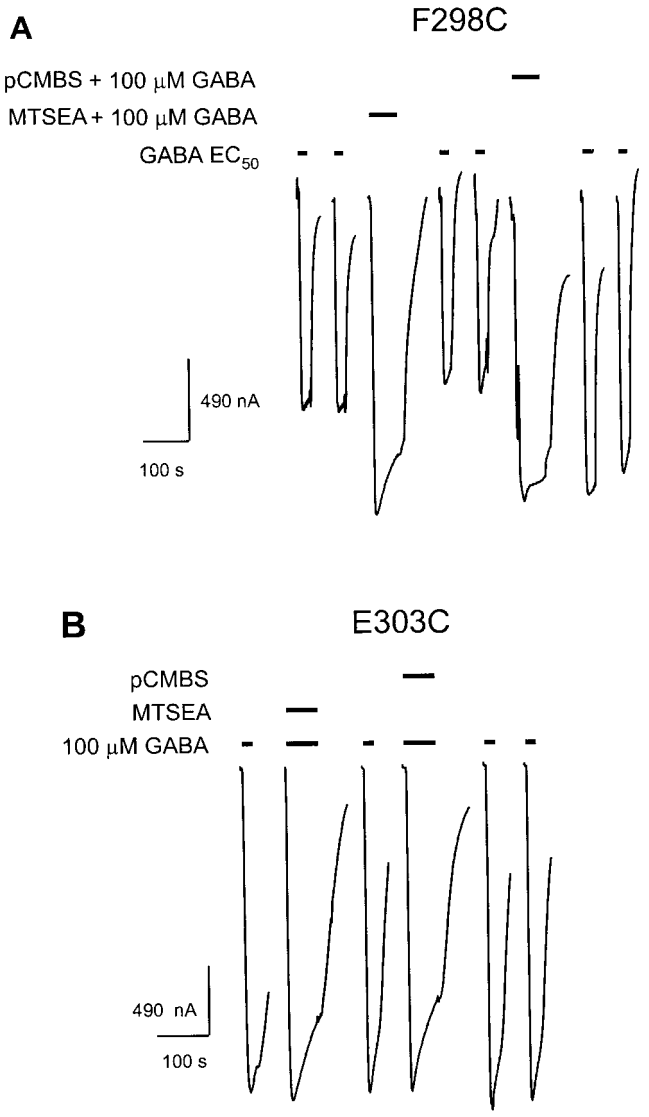

FIGURE 6 Effect of pretreatment with 2.5 mM $MTSEA^+$ on the subsequent response to 0.5 mM $pCMBS^-$ application in the $pCMBS^-$ reactive mutants. (*A*) For the F298C mutant, application of $MTSEA^+$ has little effect, but subsequent application of $pCMBS^-$ causes marked potentiation. This indicates that the $MTSEA^+$ had not reacted with this mutant. (*B*) For the E303C mutant, application of $MTSEA^+$ has no effect on the subsequent current; however, the subsequent application of 0.5 mM $pCMBS^-$ also has no effect. This indicates that the $MTSEA^+$ must have reacted with the cysteine, thereby preventing reaction with pCMBS, which would have inhibited this mutant. Bars above traces indicate the period of application of reagents. Holding potential, −80 mV.

Accessibility of the M2 segment residue α_1Ser^{270}

Mutations of α_1Ser^{270} and/or the aligned residues in the β subunits alter the ability of loreclezole and general anesthetics to potentiate GABA-induced currents (Wingrove et al., 1994; Belelli et al., 1997; Mihic et al., 1997). We had previously investigated the accessibility of α_1S270C to covalent modification by $pCMBS^-$. We showed that when a near-saturating concentration of GABA was used for the test responses, $pCMBS^-$ had no effect on the post-$pCMBS^-$ GABA-induced currents (Xu and Akabas, 1996). We reinvestigated the accessibility of α_1S270C, using GABA test concentrations at the GABA EC_{50}, which are more sensitive to modifications that effect only gating kinetics (Zhang and Karlin, 1998) because modification that alters gating kinetics but not conductance may not alter currents induced by near-saturating GABA concentrations. When the reactivity of α_1S270C$\beta_1\gamma_2$ was tested using EC_{50} GABA concentrations, a 1-min application of 0.5 mM $pCMBS^-$ applied in the absence of GABA inhibited subsequent GABA-induced currents by 43 ± 9% (n = 3). A 1-min application of 0.5 mM $pCMBS^-$ in the presence of 100 μM GABA inhibited the subsequent GABA-induced currents by 46 ± 4% (n = 5) (Fig. 7). Thus we infer that cysteine substituted for α_1Ser^{270} reacted with $pCMBS^-$ both in the presence and in the absence of GABA.

DISCUSSION

We have shown that $pCMBS^-$ added extracellularly reacted with cysteines substituted for six of 17 M3 membrane-spanning segment residues and with the M2 residue α_1S270C. We infer that these sulfhydryl-reactive residues were exposed, at least transiently, on the water-accessible surface of the protein for several reasons. First, the sulfhydryl reagents react faster with the ionized thiolate form of cysteine ($-S^-$) than with the uncharged thiol form (-SH); $pCMBS^-$ reacts 10^3 times faster and the MTS reagents react 5×10^9 times faster (Hasinoff et al., 1971; Roberts et al., 1986). Only cysteines on the water-accessible surface will ionize to a significant extent. Second, at pH 7.5, $pCMBS^-$ and $MTSET^+$ are permanently charged and membrane impermeant (VanSteveninck et al., 1965; Holmgren et al., 1996; Olami et al., 1997). Therefore, they are unlikely to enter hydrophobic regions in the lipid bilayer or in the interior of proteins. This implies that these reagents traverse

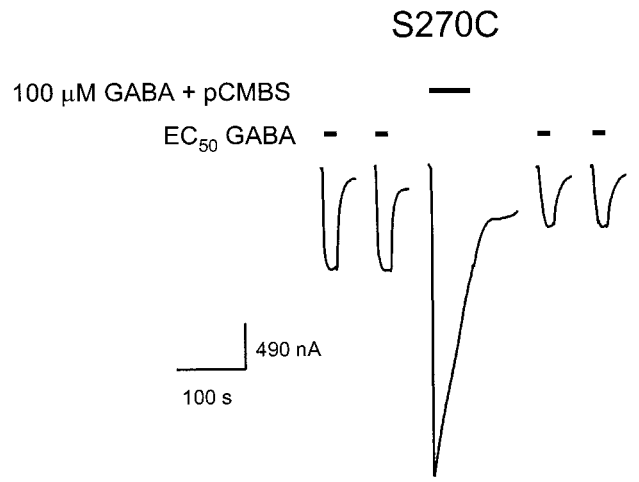

FIGURE 7 $pCMBS^-$ (0.5 mM) applied in the presence of GABA inhibits its subsequent GABA-induced currents in an oocyte expressing the M2 segment α_1S270C mutant when assayed with EC_{50} GABA test pulses. Bars above traces indicate the period of application of reagents. Holding potential, −80 mV.

an aqueous pathway to the reactive cysteines. Third, Falke and colleagues showed that cysteine reactivity was well correlated with solvent accessibility measured from the x-ray crystal structure in the extracellular domain of the aspartate chemotaxis receptor (Danielson et al., 1997). Thus we infer that the substituted cysteines and their corresponding wild-type residues, α_1Ala^{291}, α_1Tyr^{294}, α_1Phe^{298}, α_1Ala^{300}, α_1Leu^{301}, α_1Glu^{303}, and α_1Ser^{270} are exposed, at least transiently, on the water-accessible surface.

The reactivity of four of the six M3 segment residues was dependent on the functional state of the receptor. The two most extracellular reactive residues, A291C and Y294C, and the M2 residue S270C reacted both in the presence and in the absence of GABA. These were accessible both in the closed and in an activated state. In contrast, the reactions with the four residues closer to the cytoplasmic end of M3, F298C, A300C, L301C, and E303C, were state dependent. These four mutants only reacted with $pCMBS^-$ applied in the presence of GABA. In the presence of GABA the channels fluctuate mainly between the open and desensitized states. Our observation that the GABA-induced current during the 1-min application of $pCMBS^-$ could be superimposed on the current induced by GABA in the absence of $pCMBS^-$, yet the subsequent GABA-induced currents following $pCMBS^-$ were irreversibly altered (Fig. 4) suggests that reaction occurred in a desensitized state. Alternatively, reaction might have occurred in the open state if it did not alter conduction but rather the kinetics of subsequent reopening of the channels. This, we think, is unlikely because even during a 1-min application of GABA the channels pass through the closed state, and this would result in an alteration of the current profile in the presence of $pCMBS^-$. We conclude that $pCMBS^-$ most likely reacted with the four residues, F298C, A300C, L301C, and E303C, in a nonconducting state, a state from which channels rarely return to the open state in the continued presence of GABA, i.e., a desensitized state.

Our discovery that M3 segment residues are on the water-accessible surface of the protein is surprising. There was no previous evidence that M3 might form part of the channel lining or that water-filled crevices might extend into the interior of the protein. Thus a critical issue raised by our findings is the water-accessible surface with which these residues are in contact—the water-filled ion channel or a water-filled crevice that extends, perhaps transiently, into the interior of the membrane-spanning domain of the protein. The secondary structure of the M2 segment appears to be α-helical, and residues on one side of the helix line the channel (Xu and Akabas, 1996). The channel-lining residues in the GABA M2 segment, however, did not change markedly in the presence and absence of GABA (Xu and Akabas, 1996). Thus we think that it is unlikely that a major conformational change occurs in the structure of the channel lining, such as the M3 membrane-spanning segment moving into the channel lining in the presence of GABA, although we cannot exclude this possibility. Furthermore, in the ACh receptor the channel is largely lined by residues from the M2 segments (reviewed in Karlin and Akabas, 1995; Lester, 1995; Changeux and Edelstein, 1998). In the 9-Å resolution structure of the ACh receptor channel the M2 segments were closest to the central channel axis at the cytoplasmic end and angled away from the channel axis toward the extracellular end (Unwin, 1993). Thus other membrane-spanning segments must fill the gaps between the M2 segments near the extracellular end, although they were not seen in the 9-Å electron density map (Unwin, 1993). There were water-accessible residues near the extracellular end of the ACh receptor αM1 segment, and we suggested that these M1 residues might fill the spaces between the extracellular ends of the M2 segment (Akabas and Karlin, 1995). Residues from the M3 segment may also fill those gaps, but a conformational change would be required to increase the accessibility of the residues in the middle of the M3 segment, and as we noted above GABA did not induce major changes in the M2 segment residues that line the channel (Xu and Akabas, 1996). Another possibility is that the M3 segment faces the nonchannel lining side of the M2 segment, and in the absence of GABA a water-filled crevice extends between the M2 and M3 segments from the extracellular surface to the level of α_1Tyr^{294}. The conformational changes that follow GABA binding result in the crevice extending deeper into the interior of the membrane-spanning domain, thereby increasing the accessibility of the other M3 residues. There are several precedents for the existence of water-filled crevices extending into the interior of membrane-spanning domains of proteins. For example, in G-protein-coupled receptors the ligand-binding site is formed by residues in the membrane-spanning segments, and access to the binding site is via a water-filled crevice (Javitch et al., 1994, 1995a,b; Fu et al., 1996). In voltage-dependent Na^+ and K^+ channels, it appears that two water-filled crevices, one extending from the extracellular surface

and one from the cytoplasmic surface, contact the S4 voltage-sensing, membrane-spanning segment (Yang et al., 1996; Baker et al., 1998; Cha and Bezanilla, 1998). Furthermore, water-filled crevices and cavities have been identified in the interior of water-soluble proteins (Englander and Kallenbach, 1983; Yu et al., 1999). Thus we conclude that the sulfhydryl reactive M3 residues probably do not line the ion channel. Rather, we speculate that a water-filled crevice is present in the closed state. It is lined in part by the M3 segment residues α_1Ala^{291}, α_1Tyr^{294}, and the M2 segment residue α_1Ser^{270}. We do not know whether this crevice is continuously present or arises because of fluctuations in the protein structure. In the presence of GABA the crevice extends deeper into the membrane-spanning domain, thereby exposing α_1Phe^{298} and α_1Leu^{301}. Whether the water-filled crevice extends around the side of the helix to expose α_1Ala^{300} and α_1Glu^{303} or a second water-filled crevice extends from the surface to these residues is uncertain.

The lack of voltage dependence in the reaction of pCMBS$^-$ with E303C is consistent with reaction occurring in a nonconducting state where the potential falls across the gate rather than along the length of the channel or with the residue being in a dead-end crevice where the electrical potential does not fall in the crevice; these two possibilities are not mutually exclusive. In the ACh receptor the reaction rates of charged, sulfhydryl reagents with M2 segment channel-lining, substituted cysteines were voltage dependent in the open state but were not voltage dependent in the closed state (Pascual and Karlin, 1998). There are also other possible explanations for the lack of voltage dependence, but our results do not allow us to distinguish among the possibilities.

In contrast to our results in the M2 segment (Xu and Akabas, 1996), the pattern formed by the M3 water-accessible residues do not provide a clear indication of the M3 segment secondary structure. Furthermore, we do not know whether they are accessible in the same state of the receptor. The homologous ACh receptor evidence, including the pattern of labeling by hydrophobic compounds and spectroscopic studies of peptides and fragments of ACh receptor membrane-spanning domains, suggests that the M3 segment secondary structure is largely α-helical (Blanton and Cohen, 1994; Gorne-Tschelnokow et al., 1994; Methot et al., 1994; Baenziger and Methot, 1995; Blanton et al., 1998; Lugovskoy et al., 1998). Unwin, however, inferred that only M2 was α-helical, based on an electron density map (Unwin, 1993). Our results are inconsistent with a β-strand secondary structure for the M3 segment.

We conducted a BLAST search, using the sequence in and flanking the rat GABA$_A$ α_1 M3 segment, that identified 179 sequences from GABA$_A$, glycine, and GABA$_C$ receptor subunits and invertebrate glutamate-gated chloride channels (data not shown) (Altschul et al., 1990). Comparison of the aligned sequences showed that the α_1 subunit amino acid was conserved in less than 60% of the sequences at the positions aligned with α_1Ala^{291}, α_1Val^{292}, α_1Ala^{295}, α_1Ile^{302}, α_1Ser^{299}, and α_1Thr^{306}. When plotted on an α-helical wheel these positions lie on one face of the helix (Fig. 8). At one other position α_1Tyr^{294} tyrosine was conserved in less than 60% of the sequences; however, as we showed, this residue is on the water-accessible surface in both the absence and presence of GABA. The other M3 residues are conserved in more than 75% of the sequences, although, at the positions aligned with α_1Phe^{298} and α_1Phe^{304}, aromatic

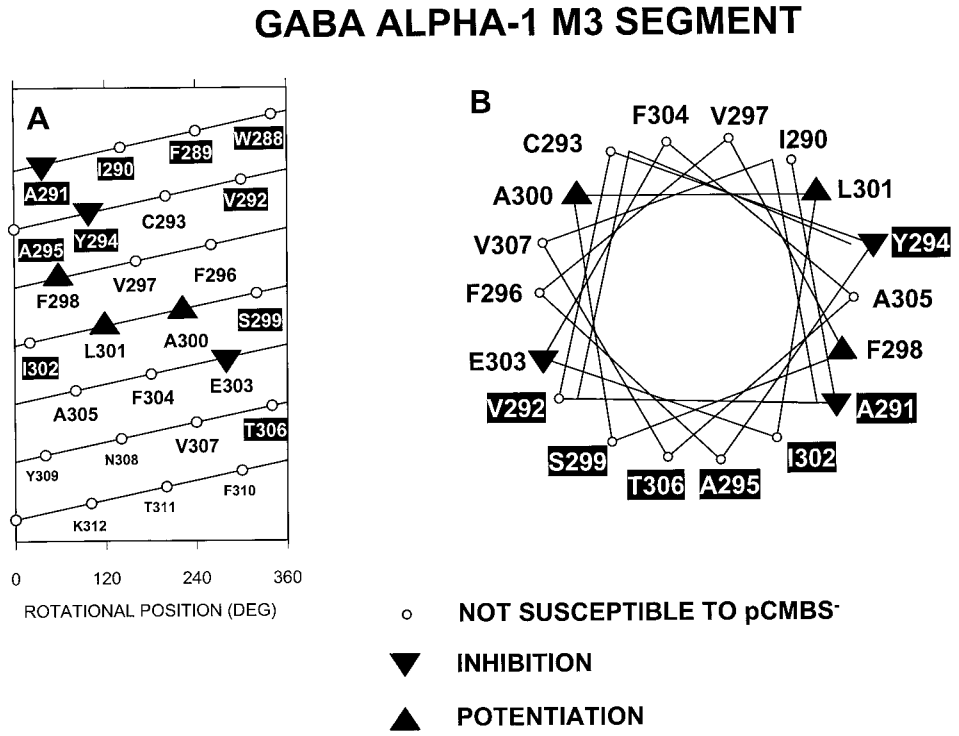

FIGURE 8 α-Helical representations of the residues in the rat α_1 M3 membrane-spanning segment showing the pCMBS$^-$ reactive residues. The pCMBS$^-$ reactive residues are indicated by solid triangles: upright triangles if modification caused potentiation of subsequent GABA-induced currents and downward triangles if modification caused inhibition of subsequent GABA-induced currents. (*A*) α-Helical net projection. The *x* axis represents the circumference of the helix. The *y* axis represents the distance along the helix; extracellular is at the top, intracellular at the bottom. (*B*) α-Helical wheel projection. In both *A* and *B* the residue names in black boxes indicate positions where the α_1 amino acid was conserved at the aligned position in less than 60% of 179 homologous sequences identified in a BLAST search. At the other positions the α_1 subunit amino acid was conserved in more than 75% of the aligned positions.

residues are conserved in more than 98% of the sequences, but a specific aromatic residue is not. Sequence variability is a common feature of solvent-exposed residues and of residues exposed to the lipid bilayer, where the substitutions are confined to hydrophobic amino acids (Baldwin, 1993; Donnelly et al., 1993). We hypothesize that the variable side of the M3 helix faces the lipid bilayer because the amino acid substitutions are almost entirely hydrophobic and the only polar substitutions are serine or threonine, which, in α-helices in hydrophobic environments, can satisfy their side-chain hydrogen bonding requirements through interactions with backbone carbonyls (Gray and Matthews, 1984). The exception is α_1Ala^{291}, which flanks the variable face of the helix at the extracellular end of M3 and is on the water-accessible surface of the protein. Aside from α_1Ala^{291}, all of the water-accessible residues lie on the conserved face of the helix (Fig. 8). In the ACh receptor, one side of the M3 segment appears to face the lipid bilayer (Blanton and Cohen, 1994; Blanton et al., 1998; Methot and Baenziger, 1998); however, the position of the other side relative to the channel and the interior of the protein is unknown.

It is important to note that one of the original assumptions of SCAM was that the only water-accessible residues in membrane-spanning segments would be channel-lining residues (Akabas et al., 1992; Xu and Akabas, 1993); this appears to be incorrect. It is likely that the M2 segment residues (Xu and Akabas, 1996) line the channel because the rates of reaction of $MTSES^-$ with the most cytoplasmic M2 residue, α_1V257C, were voltage dependent (Horenstein and Akabas, unpublished results). Our current results raise the possibility that water-filled crevices may extend into the interior of other integral membrane proteins with important functional consequences. The water-filled crevice that we have inferred to be present in the $GABA_A$ receptor may have a role in the molecular basis for the activity of several general anesthetics, barbiturates, antiepileptic drugs (loreclezole), alcohol, and neurosteroids. These drugs and hormones potentiate GABA-induced currents. Mutations of residues in various subunits aligned with α_1Ser^{270} in M2 and α_1Ala^{291} in M3 alter the effects of these compounds (Wingrove et al., 1994; Koltchine et al., 1996; Belelli et al., 1997; Mihic et al., 1997; Wick et al., 1998; Amin, 1999). α_1Ser^{270} is located on the opposite side of the M2 segment helix from the channel-lining side, and we inferred that it faced into the interior of the protein (Xu and Akabas, 1996). We have now shown that α_1Ser^{270} and α_1Ala^{291} are both on the water-accessible surface. These two residues may face each other across a water-filled crevice, although this remains to be determined. Perhaps the drugs intercalate into the water-filled crevice and stabilize the open state of the receptor. Consistent with this hypothesis, single-channel kinetic analysis indicates that barbiturates increase the mean open time, which is consistent with stabilization of an open state (Study and Barker, 1981; MacDonald et al., 1989). Further studies are in progress to investigate the effects of these cysteine substitutions on the action of general anesthetics and other GABA modulatory drugs and to determine whether activation of $GABA_A$ receptors by anesthetics and barbiturates induces changes in accessibility of these M3-substituted cysteines that are similar to the changes induced by GABA.

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