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Channel Gating of the Glycine Receptor Changes Accessibility to Residues Implicated in Receptor Potentiation by Alcohols and Anesthetics*

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The glycine receptor is a target for both alcohols and anesthetics, and certain amino acids in the α1 subunit transmembrane segments (TM) are critical for drug effects. Introducing larger amino acids at these positions increases the potency of glycine, suggesting that introducing larger residues, or drug molecules, into the drug-binding cavity facilitates channel opening. A possible mechanism for these actions is that the volume of the cavity expands and contracts during channel opening and closing. To investigate this hypothesis, mutations for amino acids in TM1 (I229C) and TM2 (G256C, T259C, V260C, M263C, T264C, S267C, S270C) and TM3 (A288C) were individually expressed in *Xenopus laevis* oocytes. The ability of sulfhydryl-specific alkyl methanethiosulfonate (MTS) compounds of different lengths to covalently react with introduced cysteines in both the closed and open states of the receptor was determined. S267C was accessible to short chain (C3–C8) MTS in both open and closed states, but was only accessible to longer chain (C10–C16) MTS compounds in the open state. Reaction with S267C was faster in the open state. I229C and A288C showed state-dependent reaction with MTS only in the presence of agonist. M263C and S270C were also accessible to MTS labeling. Mutated residues more intracellular than M263C did not react, indicating a floor of the cavity. These data demonstrate that the conformational changes accompanying channel gating increase accessibility to amino acids critical for drug action in TM1, TM2, and TM3, which may provide a mechanism by which alcohols and anesthetics can act on glycine (and likely other) receptors.

The glycine receptor (GlyR)[1] is a target for both alcohols and anesthetics. Three amino acids were previously identified as critical for alcohol and/or volatile anesthetic action on glycine receptors (as well as the homologous residues on $GABA_A$ receptors): Ile^{229} (in TM1), Ser^{267} (in TM2), and Ala^{288} (in TM3) (1–8). To study and identify water accessible residues of ion channels, such as those in drug binding pockets, methanethiosulfonate (MTS) reagents may be used as structural probes using the substituted cysteine accessibility method (9). MTS reagents rapidly react to form disulfide bonds with cysteines in the presence of water, and an irreversible change in receptor function is taken as evidence of disulfide bond formation. By use of this method, residues accessible in the presence and/or absence of neurotransmitter to sulfhydryl-specific reagents have been determined for TM2 in $GABA_A$ and acetylcholine receptors and for TM3 and the TM2–TM3 loop for $GABA_A$ receptors (10–14). Lynch *et al.* (15) demonstrated conformational changes occurring in the TM2–TM3 loop in the glycine receptor with gating. Williams and Akabas (16–18) have shown that different $GABA_A$ receptor conformations are stabilized by the drugs diazepam and propofol. Additionally, Mascia *et al.* (19) found that covalent reaction of propyl methanethiosulfonate with a cysteine introduced in the putative alcohol/anesthetic binding site of the glycine receptor irreversibly enhanced receptor function and abolished further potentiation by alcohols and anesthetics (19).

Glycine receptors predominate in the spinal cord and brain stem and are present in the ventral tegmental area, a brain region of importance in the rewarding effects of alcohol (20–24). Clinically relevant concentrations of ethanol, longer chain alcohols, and volatile anesthetics enhance the function of the glycine receptor (and the homologous $GABA_A$ receptor) in heterologous expression systems (25–27). Numerous studies have shown ethanol potentiation of glycine activated currents in cultured cells, including neurons of the hippocampus and ventral tegmental area, brain synaptoneurosomes, and mouse and chick spinal cord neurons (21, 24, 28–31). As mediators of inhibition in the nervous system, glycine receptors may be involved in the sedative and anesthetic effects of alcohol, a hypothesis supported by a recent study showing decreased alcohol effects in transgenic mice expressing a mutant, alcohol-resistant, α1 subunit (32). The glycine receptor is one of the most credible candidates for mediating immobility caused by volatile anesthetics (33).

These studies raise the question of the mechanism by which occupation of this protein cavity by alcohols, anesthetics, or MTS reagents facilitates activation (or prevents inactivation) of the channel. It is established that channel gating causes tertiary structural rearrangements within receptor subunits (34), so we were interested in how channel gating causes changes in accessibility to the alcohol and anesthetic binding pocket. We propose that the volume of this cavity, bounded by amino acids in TM1, TM2, and TM3, is larger in the open state

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[1] The abbreviations used are: GlyR, glycine receptor; TM, transmembrane segment; MTS, methanethiosulfonate; $pCMBS^-$, *para*-chloromercuribenzene sulfonate; GABA, γ-aminobutyric acid; MBS, modified Barth's solution; Me_2SO, dimethyl sulfoxide; ANOVA, analysis of variance.

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of the channel than in the closed state. This would provide a mechanism by which occupation of the cavity by diverse small molecules can change receptor function. Our experiments expand on previous work (10–19) to use alkyl MTS compounds of different lengths as molecular instruments to estimate the volume of the drug binding pocket. To map the shape and organization of this binding cavity, we introduced cysteines at nine positions in transmembrane segments 1, 2, and 3. We studied the ability of MTS reagents of different lengths to covalently react with these nine positions in both the open and closed conformations of the glycine receptor.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression of Human GlyR α1 Subunit cDNA—Site-directed mutagenesis in the human GlyR α1 subunit was performed on cDNA subcloned into pBK-CMV N/B-200 or pCIS2 vectors using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Point mutations were verified by partial sequencing of the sense and antisense strands. As previously described (35), *Xenopus laevis* oocytes were isolated and injected with 1 ng of wild-type GlyR α1 cDNA or the following α1 subunit mutants: I229C, G256C, T259C, V260C, M263C, T264C, S267C, S270C, or A288C. Injected oocytes were singly stored in incubation media and incubated at 15 °C. Incubation media is composed of modified Barth's solution (MBS) containing in mM: 88 NaCl, 1 KCl, 10 HEPES, 0.82 $MgSO_4$, 2.4 $NaHCO_3$, 0.91 $CaCl_2$, and 0.33 $Ca(NO_3)_2$ (Sigma), adjusted to pH 7.5, and supplemented with 10 mg/liter streptomycin, 10,000 units/liter penicillin, 50 mg/liter gentamicin, 90 mg/liter theophylline, and 220 mg/liter pyruvate.

Electrophysiology—Electrophysiological measurements were made 1 to 10 days after injection with previously published methods (26). Oocytes were perfused with MBS at a rate of 2.0 ml/min and clamped at −70 mV using a Warner Instruments OC725C (Hamden, CT) oocyte clamp. Currents were continuously plotted using a Cole-Parmer chart recorder (Vernon Hills, IL).

Oocytes expressing wild-type and mutant receptors were perfused for 90 s with a 50 μM solution of propyl MTS, hexyl MTS, octyl MTS, decyl MTS, dodecyl MTS, hexadecyl MTS, benzyl MTS, or *para*-chloromercuribenzene sulfonate ($pCMBS^-$) (Toronto Research Chemicals, Toronto, ON) in either the absence of glycine or in the presence of 1 mM glycine (Bio-Rad). All MTS applications were for 90 s, unless otherwise specified as this gave a steady-state reaction with S267C. MTS compounds were dissolved in dimethyl sulfoxide (Me_2SO) and diluted in MBS to a final Me_2SO concentration not exceeding 0.05% (for hexadecyl MTS the final concentration was 0.1%). These concentrations of Me_2SO did not affect GlyR function. MTS solutions were prepared immediately before application to prevent degradation in all experiments (unless otherwise noted). For each oocyte tested, responses to the EC_{5-10} of glycine (*i.e.* concentration of glycine producing peak currents equal to 5 to 10% of the 1 mM glycine maximal current) were determined before and 10, 20, and 30 min after application of MTS. During co-applications of MTS reagents with 1 mM glycine, the voltage clamp circuit on the oocyte was inactivated to prevent cell damage and run down of the glycine current. For G256C, T259C, V260C, and A288C, 10 mM glycine was used as the maximum glycine concentration for labeling and to determine the EC_{5-10} because 10 mM glycine induced a maximal response in the concentration-response curves for these mutants (Table III).

To detect whether decyl MTS was reacting "silently" with S267C, but producing no observable change in current, the EC_{5-10} of glycine was determined, and an application 100 μM decyl MTS (in MBS) was followed by measurement of the glycine response. This was followed by an application of 50 μM propyl MTS (in MBS) and measurement of the glycine response. Responses to the initial EC_{5-10} of glycine were measured 10 min after each MTS application. We also tested the stability of propyl MTS in room temperature MBS. We observed no change in effectiveness of 1-h old 50 μM propyl MTS solutions compared with freshly prepared solutions on S267C.

The rate of reaction of propyl MTS (50 μM) with S267C was determined in four conditions: 1) no glycine; 2) no glycine plus 0.6 mM isoflurane; 3) 1 mM glycine; and 4) 1 mM glycine plus 0.6 mM isoflurane. For conditions 1 and 3, the EC_{5-10} of glycine was first determined for each oocyte. After 10 min, propyl MTS (50 μM) was applied for 15 s in the absence of glycine (10-s applications in the presence of glycine because the cumulative reaction time was shorter). Ten minutes following the propyl MTS application (or 15 min for MTS applications with glycine to allow time for receptor recovery from desensitization), the original EC_{5-10} of glycine was re-applied. This procedure was repeated until the glycine response reached a steady state. For conditions 2 and 4, the application procedure was identical and 0.6 mM isoflurane was co-applied with propyl MTS in the presence and absence of glycine. All MTS and isoflurane solutions were prepared immediately before each application to ensure that a uniform concentration of these compounds reached the oocytes. The normalized responses for each oocyte were fit to a one-phase exponential association curve to determine the time and rate constants of each curve. These time constants were then averaged and presented with their standard errors and the second-order rate constants were calculated by dividing the averaged rate constants by the concentration of MTS applied. The rates of reaction of hexyl and decyl MTS were determined in the same manner. Ten-second applications of 50 μM hexyl MTS were applied in the closed state, and 10-s applications of 5 μM hexyl MTS (or 1 μM decyl MTS) were applied in the presence of 1 mM glycine until a steady state response was reached.

To differentiate the receptor states in which MTS reacts, the potentiation by 0.8 mM isoflurane was measured following decyl MTS reaction with S267C receptors in the closed (as a control), desensitized, and open/desensitized states. The isoflurane potentiation of the EC_{5-10} glycine current was measured for each condition and compared with that of unlabeled receptors. As shown previously, receptors that could be labeled by MTS would have eliminated or reduced isoflurane potentiation (19). Receptors were labeled (90 s) in the closed state (50 μM decyl MTS) and the open/desensitized state (50 μM decyl MTS + 1 mM glycine). Desensitized receptors were labeled after a 10–14-min application of 1 mM glycine that left only 1–5% of the maximal current activable. Then the oocyte was washed in MBS (30 s), followed by application of 50 μM decyl MTS in MBS to label in either the desensitized or closed state. For all of the above conditions, the isoflurane potentiation was determined 15 min after MTS labeling. Potentiation was calculated by dividing the drug-induced current by the average EC_{5-10} glycine-induced currents applied 10 min before and after each drug application. For the control, unlabeled receptors, the isoflurane potentiation was determined as above. Isoflurane (Ohmeda Caribe Inc., Liberty Corner, NJ) was dissolved in MBS or glycine solutions immediately prior to each experiment. Samples of bath solutions of isoflurane reaching the oocyte were measured by gas chromatography to have a 50% loss from the prepared vial solutions. Thus, we prepared a vial solution of 1.6 mM to produce a bath concentration at the oocyte of 0.8 mM isoflurane (~2.4 times the anesthetic EC_{50}) (36).

Data Analysis—Data analysis was performed using GraphPad Prism version 3.02 (GraphPad Software Inc., San Diego CA). The software was used to fit concentration-response curves with non-linear regression curve fitting and to define significance of the glycine responses measured, either following MTS reagent applications or responses modulated by drugs, *versus* the control EC_{5-10} glycine responses using the paired Student's *t* test. For the rate of reaction experiments, the response of each oocyte was fit to a one-phase exponential association curve to determine the time and rate constants. These values were averaged and presented with their standard errors.

Molecular Volume Calculations and Modeling—The volumes of the MTS reagents, the volumes of the corresponding alkyl thiols that functionally react with the substituted cysteine residue, and the volumes of the amino acids cysteine and serine were calculated using Spartan 5.0 (Wavefunction, San Diego, CA).

Molecular modeling of the alcohol and anesthetic binding site was conducted as previously described (37). A model of the four transmembrane segments of a glycine receptor was built by threading the primary sequence of GlyR α1 over a template of a four-helix bundle found in the high-resolution structure of the cytochrome *c* oxidase (Protein Data Bank code 2OCC). An initial constraint on the model was that amino acid residues known to modulate anesthetic potency were in direct proximity to one another (Ile^{229} (TM1), Ser^{267} (TM2), and Ala^{288} (TM3)). A second set of constraints was that the pore-facing and lipid-facing residues identified in the literature should have appropriate positions. The entire structure was subjected to restrained molecular mechanics energy optimization with the CFF91 force field using Insight II (version 2000.1, Accelrys, San Diego, CA). The model revealed that other residues in TM2 might be in proximity to Ser^{267} and could be accessible to MTS reagents (Gly^{256}, Thr^{259}, Val^{260}, Met^{263}, Thr^{264}, and Ser^{270}). The positioning of hexyl MTS was based on forming the disulfide bond and then re-optimizing the GlyR model with harmonic restraints (100 kcal/Å^2) on all the backbone atoms of the subunit to illustrate a likely orientation and show the scale of the molecule relative to the subunit.

We used a homology model of the GlyR α1 to predict residues to mutate and to interpret the data (37). However, for these purposes we also evaluated a model for ligand-gated ion channels based on cryo-electron micrographs of the Torpedo nAChR (Protein Data Bank code

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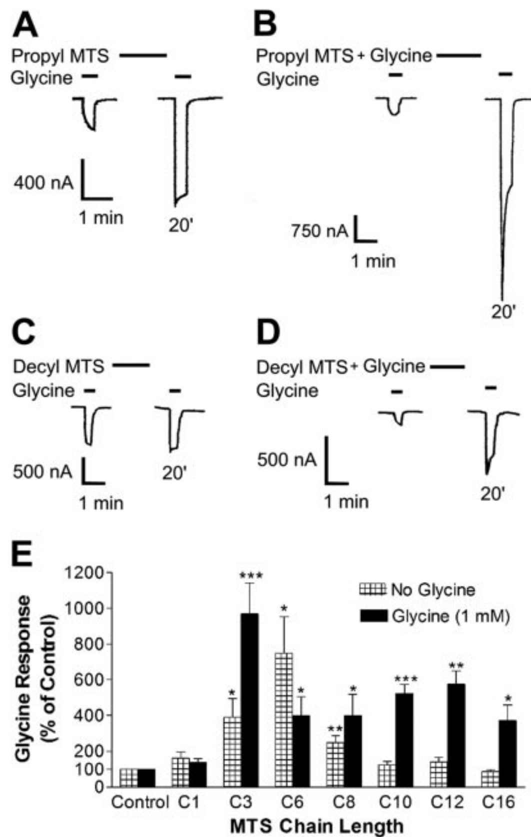

FIG. 1. **Effect of MTS reagents of different chain lengths on GlyR α1(S267C).** *A* and *B*, the glycine current resulting from an EC_{5-10} of glycine is enhanced 20 min after application of 50 μM propyl MTS in the absence and presence of glycine (1 mM). *C*, the glycine current is not enhanced significantly when 50 μM decyl MTS is applied in the absence of glycine. *D*, significant enhancement of receptor function occurs after 50 μM decyl MTS is co-applied with 1 mM glycine. *E*, MTS compounds (50 μM) with chain lengths ranging from propyl (C3) to hexadecyl (C16) were found to irreversibly enhance the receptor when co-applied in the presence of glycine (1 mM). However, when applied in the absence of glycine, propyl through octyl (C8) MTS could cause a significant enhancement and longer MTS compounds had no effect on receptor function. Data are expressed as mean ± S.E. of 6–13 oocytes. The paired Student's *t* test was used to determine significance of differences in the glycine EC_{5-10} responses before (control) and after treatment of MTS (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

1OED) (38). We have previously aligned GlyR α1 Ser[267] with nAChR L257 (Glu[266] in Protein Data Bank code 1OED) and GlyR α1 Ala[288] with nAChR Leu[279] (Glu[288] in Protein Data Bank code 1OED) (39). Recently, the corresponding residue in $GABA_A$ receptor α1 (A291) was aligned with nAChR Met[278] (Glu[287] in Protein Data Bank code 1OED) (40), so we included that residue in the evaluation. We measured the distance between pairs of Cα carbons on the backbone chain using the Viewer module of Insight II.

As a second independent evaluation of Protein Data Bank code 1OED, we highlighted two critical residues in a nAChR α subunit that were identified in a tryptophan scanning mutagenesis study (41). We rendered those residues with space filling surfaces to visualize their orientation with respect to the lipid bilayer and the center of the subunit.

RESULTS

We first tested the ability of MTS reagents of different lengths to covalently react with a cysteine introduced at amino acid residue 267 (S267C). Propyl MTS irreversibly potentiated the glycine response after being applied in both the absence of glycine (Fig. 1*A*) and in the presence of 1 mM glycine (Fig. 1*B*). In contrast, decyl MTS failed to irreversibly potentiate the S267C response following application in the absence of glycine (Fig. 1*C*), but could react and enhance S267C when applied in the presence of glycine (Fig. 1*D*).

We extended these observations by testing a series of MTS compounds of different sizes ranging from C1 (methyl) to C16 (hexadecyl) to determine their ability to react with S267C. These neutral MTS reagents have structural similarities to alcohols and anesthetics. Exposure to MTS reagents was carried out in the absence of glycine (closed state) and in the presence of a maximal concentration of glycine (1 mM, open and desensitized states). Methyl MTS did not cause receptor enhancement after application in either the closed or open state. We found that MTS compounds of shorter chain lengths (propyl to octyl MTS) reacted with S267C when applied in both the presence and absence of glycine, but the longer chain MTS compounds (decyl to hexadecyl MTS) were able to irreversibly react and alter the glycine response only when applied in the presence of glycine (Fig. 1*E*). In all cases, except hexyl MTS, the enhancement observed was greater after the MTS reagent was applied in the presence of glycine, but the enhancement values by hexyl MTS in the open and closed states were not significantly different. In addition to the *n*-alkyl MTS compounds, we observed that benzyl MTS and the negatively changed $pCMBS^-$ both caused enhancement of S267C after application in both conditions, but had no effect on the wild-type (Table I).

Wild-type receptors did not show an irreversible change in function following application of any of the MTS compounds at 50 μM (Table I). Also, the highest concentrations of propyl and decyl MTS used in our studies had no effect on wild-type glycine receptor function. Application of 1 mM propyl MTS (90 s) resulted in no significant change in current from control in either the absence (92 ± 9% of control, $n = 4$) or presence (86 ± 5% of control, $n = 4$) of 1 mM glycine. Likewise, decyl MTS (300 μM, 90 s) resulted in no significant change in either the absence (91 ± 3% of control, $n = 4$) or presence (88 ± 9% of control, $n = 5$) of 1 mM glycine.

Of consideration was the possibility that although longer chain MTS compounds did not cause enhancement of the glycine response, they could be silently reacting with the receptor without altering receptor function. In this way, the presence of MTS would go undetected. This possibility was tested by exposing the receptor to 100 μM decyl MTS (a compound having no effect following application in the closed state) and measuring the glycine response, and following this with a subsequent application of 50 μM propyl MTS (a compound that caused significant potentiation following application in the closed state) and measuring the glycine response in the same oocyte (Fig. 2). Decyl MTS produced no change in the glycine response, and the subsequent application of propyl MTS produced a percent enhancement of 530 ± 160, a value not statistically different from the 390 ± 100% enhancement viewed following a single application of propyl MTS to the S267C mutant in the absence of glycine (Table I).

We examined the state dependence of propyl, hexyl, and decyl MTS reaction with S267C by measuring the rate of reaction in the presence and absence of glycine (Table II). (For decyl MTS, no reaction occurred in the absence of glycine, and the rate of reaction was only measured with glycine.) The rate of reaction of propyl MTS in the presence of 1 mM glycine (τ = 29 ± 6.5 s, k = 1050 ± 190 s^{-1} M^{-1}) was significantly faster than in the absence (τ = 138 ± 20 s, k = 169 ± 29 s^{-1} M^{-1}). The rate of hexyl MTS reaction with S267C was also faster in the presence of glycine, and the reaction rates increased with increasing MTS chain lengths (Table II).

We also determined the rates of reaction of propyl MTS in both states in the presence of isoflurane, hypothesizing that the presence of an anesthetic could slow the reaction of MTS with S267C. The presence of isoflurane (0.6 mM) did not change the rate of propyl MTS reaction significantly in the absence (τ = 124 ± 41 s,

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TABLE I
Glycine responses, expressed as percent of control, of wild-type (WT) and GlyR α1(S267C) receptors following application of sulfhydryl-specific reagents of different sizes

Glycine responses of receptors 20 min following a 50 μM application of MTS compounds or $pCMBS^-$ in either the absence of glycine or presence of 1 mM glycine. Responses are expressed as percent of control initial EC_{5-10} responses before MTS application, and represent a mean ± S.E. of 4 to 13 oocytes.

MTS chain length	WT (no Gly)	WT (1 mM Gly)	S267C (no Gly)	S267C (1 mM Gly)
C1	100 ± 5	120 ± 9	160 ± 35	140 ± 22
C3	110 ± 11	92 ± 11	390 ± 100[a]	970 ± 170[b]
C6	87 ± 9	110 ± 13	750 ± 200[a]	400 ± 110[a]
C8	95 ± 5	88 ± 8	250 ± 39[c]	400 ± 120[a]
C10	86 ± 10	83 ± 6	120 ± 21	520 ± 53[b]
C12	110 ± 5	92 ± 7	140 ± 24	580 ± 74[c]
C16	88 ± 6	100 ± 7	86 ± 10	370 ± 85[a]
Ring-substituted				
Benzyl MTS	73 ± 13	85 ± 6	2200 ± 670[a]	1700 ± 340[c]
Charged				
$PCMBS^-$	82 ± 11	98 ± 12	500 ± 98[c]	510 ± 120[a]

[a] $p < 0.05$, significantly different from control before MTS by Student's paired t test.
[b] $p < 0.001$; significantly different from control before MTS by Student's paired t test.
[c] $p < 0.01$, significantly different from control before MTS by Student's paired t test.

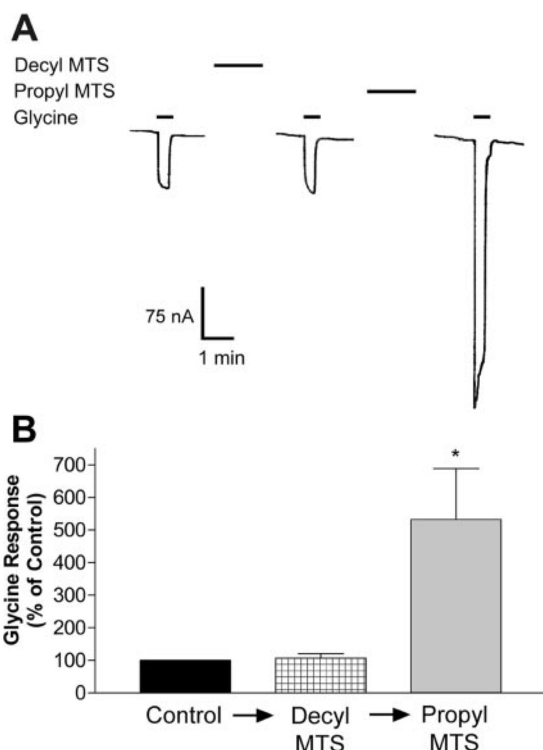

FIG. 2. **Decyl MTS does not block action of propyl MTS when applied in the closed state to GlyR α1(S267C).** To ensure that long chain MTS compounds were unable to label the cysteine in the closed state, labeling with 100 μM decyl MTS (no significant effect) was followed by a subsequent application of 50 μM propyl MTS (significant potentiation). *A*, this is an example tracing of the glycine responses; *B* shows the mean ± S.E. of the responses of 10 experiments. The EC_{5-10} was determined for each oocyte (average = 48 ± 8 μM). The paired Student's t test was used to determine significance of differences in the glycine EC_{5-10} responses before and after treatment of propyl and decyl MTS (* $p < 0.05$).

$k = 220 \pm 41\ s^{-1}\ M^{-1}$) or in the presence of 1 mM glycine ($\tau = 19 \pm 4.5$ s, $k = 1300 \pm 280\ s^{-1}\ M^{-1}$).

One possible explanation of the MTS labeling results obtained in the presence of glycine is that MTS is covalently reacting with and stabilizing both the open and desensitized states of the receptor. To differentiate between these two states, potentiation by isoflurane (0.8 mM) was tested following labeling S267C receptors (with 50 μM decyl MTS) in three states: closed (as a control), desensitized, and open (Fig. 3; see "Experimental Procedures"). The potentiation values for each

TABLE II
Rates of reactions of propyl, hexyl, and decyl MTS with S267C in the absence of glycine and presence of 1 mM glycine

The steady-state rates of reaction of both propyl MTS and hexyl MTS increased significantly in the presence of glycine. Additionally, reaction rates increased with increasing MTS chain length. Rates are expressed as a mean ± S.E. of 3 to 12 oocytes.

MTS chain length	No glycine	1 mM Glycine
	$s^{-1}\ M^{-1}$	
Propyl MTS	169 ± 29	1,050 ± 190[a]
Hexyl MTS	629 ± 91	10,700 ± 1,400[a]
Decyl MTS	No reaction	70,600 ± 7,400

[a] $p < 0.01$; significantly different from no glycine rate of reaction by Student's t test.

condition were compared with the isoflurane potentiation of unlabeled receptors with the hypothesis that receptors that reacted with MTS would have eliminated or reduced isoflurane potentiation, as previously demonstrated (19). As expected, isoflurane potentiation following labeling in the closed state (Fig. 3*C*) did not differ from that of unlabeled receptors (Fig. 3*B*), further supporting the conclusion that decyl MTS is unable to react in the closed state. MTS appears to react with and stabilize both the desensitized (Fig. 3*D*) and open states (Fig. 3*E*). Following reaction of decyl MTS, isoflurane produced a current independent of glycine, as shown in the tracings, likely indicating that some channels were open in the absence of glycine.

It has been shown that the MTS reaction at a position homologous to GlyR α1(S267C) in the 5-HT3 receptor (L293C) resulted in channels locked in the open state (42). We tested for this possibility by applying strychnine (10 μM) to S267C receptors following MTS labeling. There was no effect of strychnine after propyl MTS (50 μM) was applied in either the open or closed state ($n = 4-6$). Predictably, there was also no effect of strychnine after application of 50 μM decyl MTS to the oocytes in the closed state ($n = 5$). However, following decyl MTS (50 μM) reaction in the presence of 1 mM glycine, 10 μM strychnine blocked a small inward current of 110 ± 34 nA ($n = 7$), indicating that some channels were constitutively open following labeling. In some cases, MTS reagents produced a current when applied in the absence of glycine on mutant receptors as seen in studies at this position in the GABA receptor with $pCMBS^-$ (40). The currents observed here were blocked by 10 μM strychnine, indicating that MTS alone can open some channels. In all cases, the currents produced by 50 μM MTS alone were very small (never exceeding 1% of the maximal current), returned to baseline after the application, and were never

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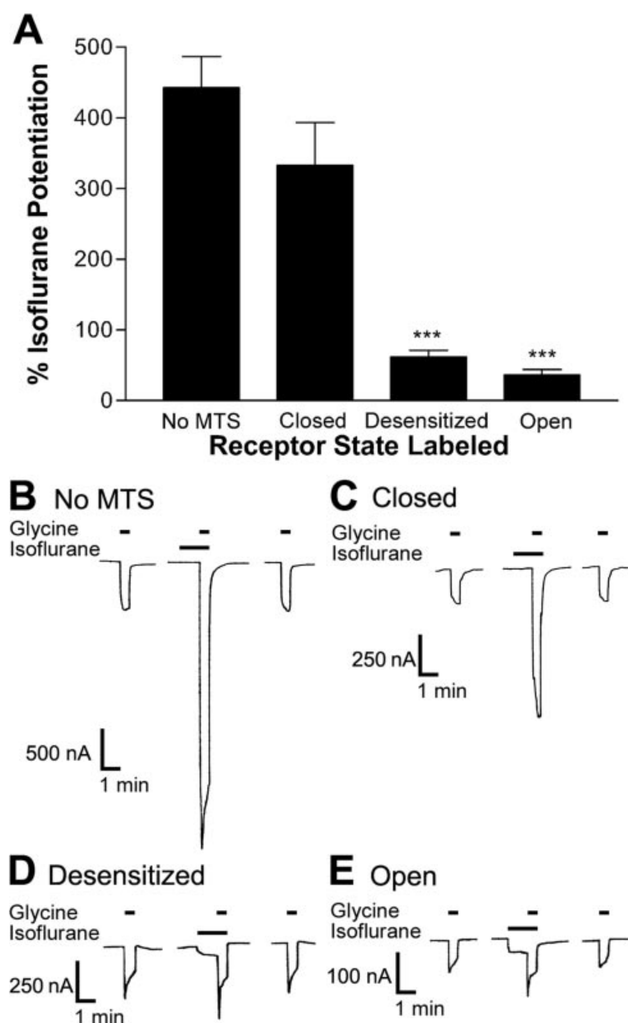

FIG. 3. **Isoflurane potentiation of the EC_{5-10} glycine response of S267C following labeling of the receptor in different states with 50 μM decyl MTS.** *A*, the potentiation of the EC_{5-10} glycine response by 0.8 mM isoflurane was measured on unlabeled receptors (*B*, *No MTS*) as the control. Following application of 50 μM decyl MTS in the absence of glycine (*closed*) or in the presence of 1 mM glycine (*open*), the potentiation by isoflurane was measured. Potentiation by isoflurane was also measured on receptors labeled in the "desensitized" state, where reaction with 50 μM decyl MTS followed application of 1 mM glycine for 11–14 min to desensitize receptors. *C*, the closed state was not labeled by decyl MTS because there was no significant elimination in the isoflurane potentiation. Both the desensitized and open states were labeled by decyl MTS, resulting in significant reduction of isoflurane potentiation. In addition, isoflurane alone potentiated receptors after reaction with decyl MTS in the desensitized and open states indicating that these receptors now had tonic activity (*D* and *E*). Data are expressed as a mean ± S.E. of 6 to 9 oocytes. The average current elicited by EC_{5-10} of glycine before and after each isoflurane application was used to calculate the percent potentiation for each condition. The mean average currents (nA) ± S.E. produced by an EC_{5-10} of glycine are as follows: no MTS = 550 ± 100, closed = 400 ± 70, desensitized = 330 ± 120, and open = 230 ± 50). One-way ANOVA with the Dunnett's post test was used to determine significance of differences in the isoflurane potentiated glycine EC_{5-10} of the labeled receptors *versus* the control, "No MTS" isoflurane response in unlabeled receptors (***, $p < 0.001$).

observed in the wild-type. Most often, we did not observe a current induced by MTS alone at all. Because the MTS alone current is not appreciable, this should not change our interpretation of data for reactions in the closed state.

The volumes of MTS reagents before and after reaction were calculated using Spartan 5.0 (Wavefunction, San Diego, CA). The relevant volume for diffusion into the binding site is the whole molecular volume of the MTS reagent, whereas the relevant volume for functional analysis is the portion of the MTS molecule that reacts covalently with the substituted cysteine residue and causes the observed effects. For each functional MTS volume that has covalently reacted given below, the sulfinic acid leaving group contributes ~65 Å^3 to the MTS reagent volumes. Propyl MTS (102 Å^3) through octyl MTS (204 Å^3) were able to covalently react with both open and closed states of S267C to produce enhancement of the glycine response. Benzyl MTS, with a functional volume of 150 Å^3, caused the largest responses after reaction in both states. Hexadecyl MTS (368 Å^3) was the largest compound tested that could react in the presence of glycine to cause enhancement. Substituting cysteine (123 Å^3) in place of serine (110 Å^3) resulted in a volume increase of 13 Å^3.

TABLE III
Amino acid positions (50), glycine EC_{50} and Hill coefficients for the wild-type (WT) receptor and the cysteine substitution mutants studied

The glycine EC_{50} and Hill coefficients were experimentally calculated from concentration response curves and are expressed as a mean ± S.E. of 4 to 10 oocytes.

Glycine receptor	TM2 position	EC_{50}	Hill coefficient
		μM	
WT	NA[a]	280 ± 47	2.3 ± 0.56
I229C	NA	110 ± 7	3.5 ± 0.48
S270C	18′	370 ± 90	1.3 ± 0.18
S267C	15′	330 ± 56	1.1 ± 0.11
T264C	12′	69 ± 32	3.5 ± 2.4
M263C	11′	270 ± 80	3.0 ± 1.3
V260C	8′	41 ± 24	2.8 ± 1.1
T259C	7′	770 ± 110[b]	1.8 ± 0.31
G256C	4′	670 ± 97	1.3 ± 0.11
A288C	NA	1800 ± 270[c]	2.0 ± 0.34

[a] NA, not applicable.
[b] $p < 0.05$, significantly different from wild-type receptors by one-way ANOVA with the Dunnett's post test.
[c] $p < 0.01$, significantly different from wild-type receptors by one-way ANOVA with the Dunnett's post test.

Two other amino acids, Ile^{229} in TM1 and Ala^{288} in TM3, have been implicated to be critical for alcohol and anesthetic action making them attractive targets for mutagenesis and probing with MTS reagents (1–3, 5, 8). Additionally six other neighboring residues in TM2 were mutated to cysteine and tested for accessibility to MTS reagents to determine the "ceiling" and "floor" of the binding cavity. A recent nuclear magnetic resonance study of the TM2 segment (43), a consensus of 10 secondary structure prediction algorithms for ligand gated-ion channels (39) and the cryo-electron micrograph structure of the acetylcholine receptor (38) all provide evidence that TM2 is an α helix. We selected the amino acids in TM2 that would be in close proximity to Ser^{267} in an α helix. The residues targeted by mutagenesis were Met^{263}, Thr^{264}, Val^{260}, Thr^{259}, Gly^{256} (helical turns toward the cytoplasm from Ser^{267}), and Ser^{270} (approximately a helical turn up toward the extracellular surface). The glycine EC_{50} values and the Hill slopes for these mutants were compared with the wild-type receptor (Table III). The EC_{50} values for T259C and A288C increased significantly from that of the wild-type receptor. We chose propyl and decyl MTS to characterize these eight mutant receptors because of the labeling distinction we observed on S267C (Fig. 1).

Both I229C and A288C showed state dependence of accessibility. For I229C, propyl MTS (500 μM) resulted in significant enhancement after application in the presence of glycine, but caused no change after application in the absence of glycine. Likewise, decyl MTS (50 μM) showed labeling in the presence, but not in the absence of glycine (Fig. 4*A*). For A288C, a 500 μM propyl MTS application resulted in significant labeling in the presence of glycine, but no change after application in the absence of glycine. Decyl MTS (50 μM) also showed labeling of

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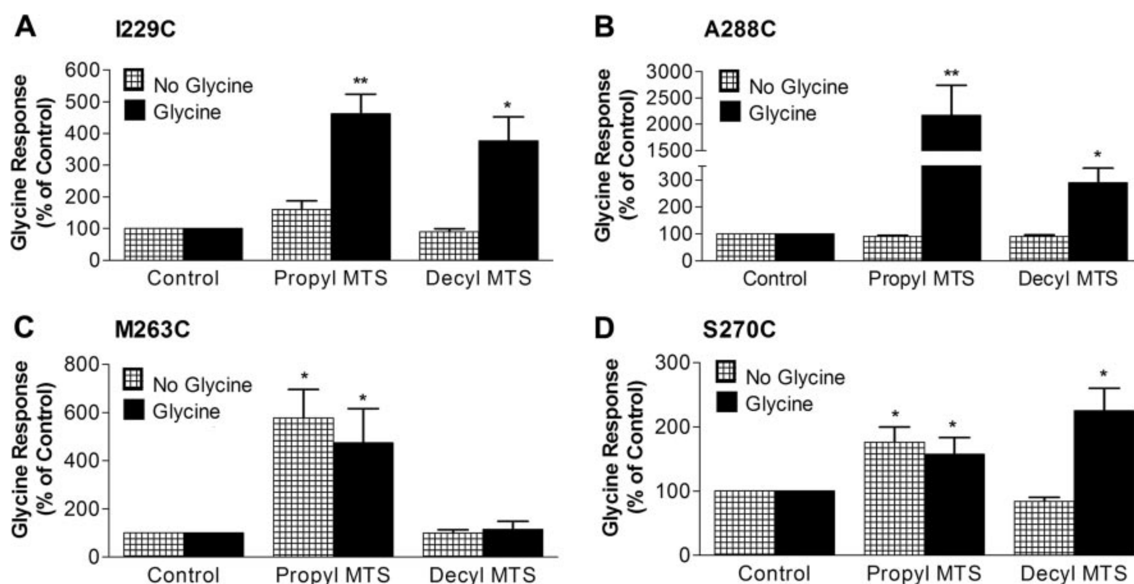

FIG. 4. **Labeling of GlyR α1 mutants I229C, A288C, M263C, and S270C with propyl and decyl MTS.** *A*, GlyR α1(I229C): propyl MTS (500 μM) and decyl MTS (50 μM) resulted in enhancement only after application in the presence of 1 mM glycine. *B*, GlyR α1(A288C): propyl MTS (500 μM) and decyl MTS (50 μM) caused receptor enhancement only when applied in the presence of 10 mM glycine (note that the scale of the *y* axis is broken). *C*, GlyR α1(M263C): propyl MTS (500 μM) resulted in significant enhancement when applied in both the absence and presence of glycine (1 mM). Decyl MTS (50 μM) produced no change in receptor function in either condition. *D*, GlyR α1(S270C): propyl MTS (500 μM) resulted in significant enhancement when the MTS was applied in both the absence and presence of glycine (1 mM). Decyl MTS (50 μM) only caused enhancement after being co-applied with 1 mM glycine. Data are expressed as a mean ± S.E. of 5 to 9 oocytes. The paired Student's *t* test was used to determine significance of differences in the glycine EC_{5-10} before and after treatment of MTS (*, $p < 0.05$ and **, $p < 0.01$).

A288C in the presence, but not in the absence of glycine (Fig. 4*B*). A lower concentration of propyl MTS (50 μM) had no significant effect on the A288C receptor when applied in either the open or closed state (data not shown).

For M263C, propyl MTS (500 μM) enhanced receptor function when applied in both the presence and absence of glycine. Decyl MTS (50 μM) caused no enhancement of M263C in either condition (Fig. 4*C*). For S270C, reaction with propyl MTS (500 μM) caused an irreversible change in receptor function when applied in both the presence and absence of glycine. Decyl MTS (50 μM) was able to enhance S270C current after application in only the presence of glycine (Fig. 4*D*). No labeling was observed for the other four mutants (G256C, T259C, V260C, and T264C) under any condition tested: 500 μM propyl MTS or 50 μM decyl MTS in either the presence or absence of glycine (Table IV).

All of the results presented above were obtained by using an EC_{5-10} concentration of glycine, determined individually for each oocyte. Mascia *et al.* (19) previously determined that application of propyl MTS resulted in a leftward shift in the glycine concentration-response curve with no change in the maximum glycine response. Consistent with this, we found that current induced by 1 mM glycine in the S267C, A288C, M263C, and S270C mutants was not significantly changed by exposure to propyl MTS.

In the homology model of GlyR α1 (6, 37), the reactive positions are rendered with space-filling surfaces, whereas the non-reactive positions tested are shown as ball and stick surfaces (Fig. 5, *A* and *B*). The disulfide bond to hexyl sulfide (formed after the reaction with hexyl MTS) was modeled for the S267C receptor. Positioning was based by forming the disulfide bond and then re-optimizing the GlyR model with harmonic restraints on all the backbone atoms of the subunit to illustrate a likely orientation and show the scale of the molecule relative to the subunit (Fig. 5, *C* and *D*).

Using the nAChR structure (38), the Cα to Cα distances between GlyR α1 Ser^{267} and either Glu^{287} or Glu^{288} in Protein Data Bank code 1OED were 13.8 and 14.5 Å, respectively (Fig. 6*A*). An important point is that Glu^{288} in Protein Data Bank code 1OED points toward the lipid bilayer and away from the center of the subunit. As a result, it is not in proximity to GlyR α1 Ser^{267}.

TABLE IV
Percent of control responses to EC_{5-10} glycine following application of propyl or decyl MTS on wild-type and TM2 mutant GlyRs in the presence or absence of glycine

TM2 mutants (T264C, V260C, T259C, G256C), in proximity to Ser^{267}, were tested for accessibility to MTS reagents. For the WT and T264C receptors, the maximal glycine concentration co-applied with propyl (500 μM) and decyl MTS (50 μM) was 1 mM, and for V260C, T259C, and G256C, the maximal glycine concentration used was 10 mM. Data are expressed as a mean ± S.E. of 4 to 7 oocytes. $p > 0.05$ for all receptor responses compared to the original EC_{5-10} response (control) before MTS by the Student's paired *t* test.

Glycine receptor	Propyl MTS		Decyl MTS	
	No Gly	Max Gly	No Gly	Max Gly
WT	100±6.4	110±4.5	86±10	83±6.0
T264C	130 ± 33	85 ± 12	93 ± 24	139 ± 41
V260C	98 ± 6.5	88 ± 11	110 ± 9.0	110 ± 19
T259C	110 ± 6.6	103 ± 8.6	91 ± 13	91 ± 5.3
G256C	90 ± 3.7	103 ± 6.8	86 ± 6.8	80 ± 6.2

The model of nAChR was built by aligning four polyalanine α helices onto the electron density of a cryo-electron micrograph and then threading residues of nAChR onto the polyalanine helices by matching protrusions in the 4-Å resolution electron density (38). In the notes section of the Protein Data Bank file, the authors of Protein Data Bank code 1OED noted the possibly of imperfect alignment. We evaluated the threading of residues by highlighting residues in TM3 (Fig. 6*B*) that were identified by Guzman *et al.* (41) using tryptophan scanning mutagenesis. Based on levels of expression and channel activation they suggested that Phe^{284} (Ala^{284} in Protein Data Bank code 1OED) should face the lipid and Met^{284} (Ala^{282} in Protein Data Bank code 1OED) should face the center of the α subunit. As shown in Fig. 6*B*, these positions are reversed, suggesting that the threading of TM3 in Protein Data Bank code 1OED is off by either two or five residues.

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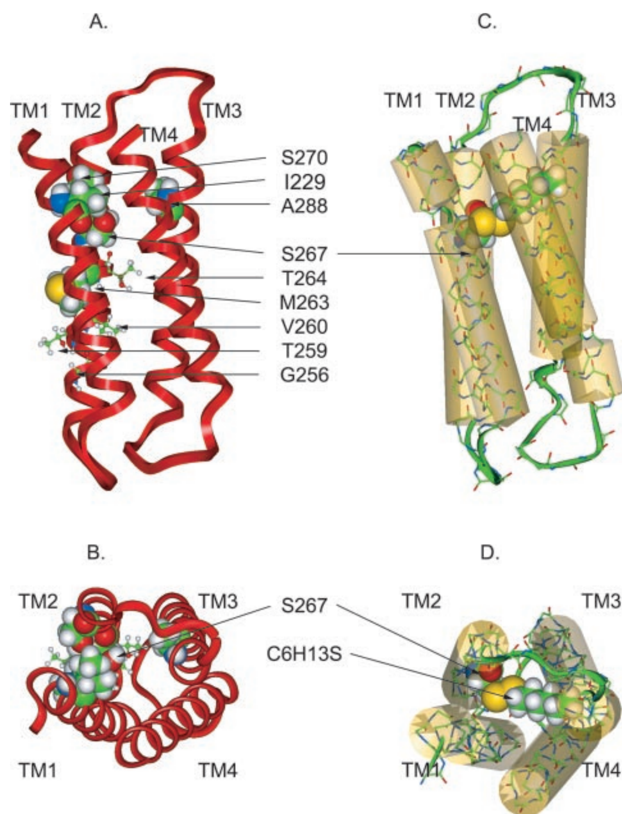

FIG. 5. **Molecular model of the transmembrane domain of one subunit of GlyR α1 that was built by threading the primary sequence of GlyR α1 onto a template of a four-helical bundle.** *A*, the homology model viewed from the side in the plane of the membrane shows a putative alcohol and anesthetic binding pocket; a cavity in the center of the receptor subunit. Residues that did react with MTS reagents are rendered with space-filling surfaces (Ile^{229}, Met^{263}, Ser^{267}, Ser^{270}, and Ala^{288}), and those that did not are rendered with ball and stick surfaces (Gly^{256}, Thr^{259}, Val^{260}, and Thr^{264}). The peptide backbone is shown as a *red ribbon*. *B*, the same model as *A*, viewed from the extracellular surface and looking into the center of the four-helical bundle. *C*, the model viewed from the side in the plane of the membrane with a disulfide bond to hexyl sulfide (formed after the reaction with hexyl MTS) from the sulfur atom of S267C in the mutated receptor. The four α helices are rendered as *transparent yellow cylinders*, the random coils as *green ribbons*. *D*, the same model as *C*, viewed from the extracellular surface and looking into the center of the four-helical bundle.

DISCUSSION

Our results indicate that conformational changes occur in transmembrane segments 1, 2, and 3 with channel gating. Experiments conducted on five mutants, I229C, M263C, S267C, S270C and A288C, provide evidence that accessibility to the region of the putative alcohol and anesthetic binding pocket changes with channel gating. Under different conditions, MTS compounds covalently reacted at these positions to result in enhancement of glycine receptor function.

Experiments on S267C demonstrated that MTS reagents of longer lengths are able to react in the open state. Importantly, there is a distinct length (octyl MTS) after which larger MTS compounds do not react with substituted cysteines in the closed state, but do react in the open state. Methyl MTS, the smallest compound tested, did not cause a change in receptor function after application in either the closed or open state, reinforcing the idea that a certain volume is needed to produce receptor enhancement. We noted the possibility that longer MTS compounds could silently react with the receptor without altering function, in which case we would not be able to detect the reaction. By showing that propyl MTS enhancement was not blocked by a previous application of decyl MTS, we determined that decyl MTS is unable to reach the cysteine in the closed state. Extrapolating, MTS compounds longer than decyl are not silently reacting with S267C and failing to enhance the receptor.

As in other studies using sulfhydryl reagents as probes, our experimental design leaves the native cysteines of the receptor intact (9, 19, 44). Observing no change in the wild-type response following any of the MTS reagents used, we assume that mutant receptors have a structure and properties similar to the wild-type receptors and that enhancement is because of specific reaction of MTS at the introduced cysteines. Many other studies (10–15) have identified differences in accessibility in receptor subunits when neurotransmitter was present or absent, and our results indicate that this is the case for residues in TM1, TM2, and TM3 of the glycine receptor as well.

In particular, our results on S267C coincide with the reactivity results in the $GABA_A$ receptor that show that this homologous position is accessible in the open and closed states (12). Accessibility studies of the site homologous to Ala^{288} in TM3 in the $GABA_A$ receptor found that this position was also reactive in both the closed and open states (12); however, our results for the glycine receptor showed distinct reactivity only in the presence of agonist. This may reflect a difference in the arrangement of the residues involved with alcohol/anesthetic between these two overall very similar inhibitory receptors. One piece of experimental evidence that may reflect this difference between these receptors is the finding that nonhalogenated, alkane anesthetics potentiate glycine receptors, but have little to no effect on $GABA_A$ receptors (45, 46).

The reaction rates of both propyl and hexyl MTS with S267C were faster in the presence of glycine than in the closed state, which provides further evidence of increased accessibility to Ser^{267} with channel opening. These rates of reaction with propyl MTS were not altered with the addition of isoflurane. It is interesting to note that propofol also did not protect the homologous TM2 positions from reaction with pCMBS$^-$ in a recent study in the GABA receptor α1 and β2 subunit, whereas protection was only seen for the β2 subunit TM3 position (40). This can be interpreted in at least two ways: 1) the on- and off-rates of anesthetic binding at Ser^{267} are too quick to provide observable competition with a compound that covalently reacts at its target, or 2) the drug binding site is elsewhere and isoflurane is not competing with MTS to bind at S267C. Present data do not allow us to definitively distinguish between these two possibilities.

We found that increasing the MTS chain length increased the rate of reaction. This may indicate that MTS compounds with longer chain lengths can reach the reactive cysteines more effectively through an amphipathic pathway or that the longer chain length MTS compounds are better stabilized near the reactive cysteine than shorter ones because of their lipophilic properties. It should be noted that the potency of *n*-alcohols for potentiation of glycine receptor function increases with chain length (8, 26).

When MTS reagents are coapplied with glycine, fractions of receptors exist in transitions between the closed, open, and desensitized states. By testing isoflurane potentiation of S267C following decyl MTS application in different receptor states, we found that both the desensitized and open states could be labeled, as indicated by the elimination of the isoflurane potentiation. Additionally, a fraction of receptors were constitutively open following reaction with decyl MTS, suggesting that the open state had reacted and was stabilized.

For both I229C and A288C, there is a distinct conformational change occurring with channel gating that allows MTS to react only in the presence of glycine. The state dependence of reac-

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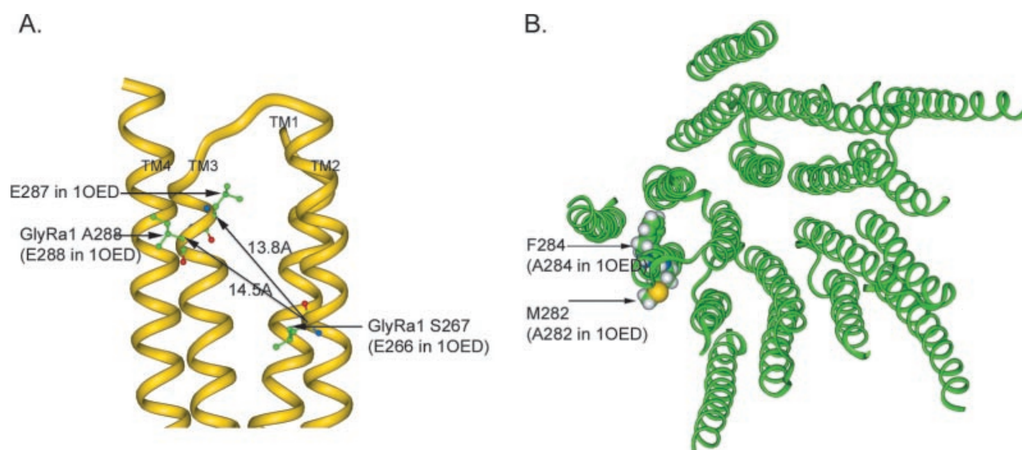

FIG. 6. **Positioning of amino acids in the nAChR α subunit Protein Data Bank code 1OED structure.** *A*, a view from the plane of the membrane of one nAChR α subunit in the Protein Data Bank code structure of 1OED. In TM2, the residue corresponding to GlyR α1 Ser^{267} (Glu^{266} in Protein Data Bank code 1OED) is rendered in *ball and stick*. In TM3, the residues corresponding to GlyR α1 Ala^{288} (Glu^{288} in Protein Data Bank code 1OED according to Ref. 39 or Glu^{287} according to Ref. 40) are rendered in *ball and stick*. The distance between pairs of Cα carbons on the backbone chain are indicated by *arrows*. The residue Glu^{288} faces toward the lipid bilayer and is on the opposite side of TM3 from TM2. *B*, a view of the TM domain in the nAChR model from Protein Data Bank code 1OED. In one nAChR α subunit, residues corresponding to Phe^{284} and Met^{282} (Ala^{284} and Ala^{282} in Protein Data Bank code 1OED) are rendered with space filling surfaces. The orientation of these two residues with respect to the interior of the α subunit is opposite to the assignment based on tryptophan scanning mutagenesis (41).

tion indicates there are specific conformational changes with channel gating occurring even in TM1 and TM3 of the GlyR, along with the necessary changes in TM2, completely altering the accessibility to these two positions. Previously, A288C was not observed to react with propyl MTS because of the lower concentration used (19), which may mean propyl MTS has less access to the position and a slower rate of reaction than what was measured for S267C. This may be because agonist is required for MTS reaction with A288C, but is not required for reaction with S267C. M263C reacted with propyl MTS, but not with decyl MTS, demonstrating that compounds of this length are too large to access this cysteine. For S270C, propyl MTS reacted in both states, but the larger decyl MTS reacted only in the presence of glycine, indicating that the water-filled space around this position increased in the open state.

For the five substituted amino acids that reacted (I229C, M263C, S267C, S270C and A288C), we observe distinctions in which compounds are able to access the site. Some positions in the putative alcohol/anesthetic pocket accommodate longer MTS reagents in the presence of glycine, and others require agonist for reaction. We attribute this change in accessibility to a change in the size and shape of the drug binding cavity. An alternate hypothesis is that the access pathway to the cysteine has changed, allowing larger MTS reagents access to this region; however, such a mechanism does not appear to be sufficient to fully explain our data. In particular, if glycine only increased the size of an access pathway to the drug binding region, we would expect M263C to react with decyl MTS in the open state in the same manner as I229C, S267C, A288C, and S270C. Since M263C reacts with propyl MTS, it must be in a water-filled cavity accessible to small MTS reagents. However, decyl MTS does not react with M263C, so access is not increased, and our data are explained more accurately by a change in the volume/size of the drug binding cavity with channel gating.

Mutations further toward the cytoplasm (G256C, T259C, V260C, and T264C) were inaccessible to labeling by propyl and decyl MTS in the presence and absence of glycine. Reaction with MTS is much faster when the cysteine is in a water-filled environment (9) and our results suggest that if the water-filled cavity does extend to this depth, it is not of sufficient size to admit MTS reagents. This concurs with other evidence in the $GABA_A$ receptor β1 subunit that the cytoplasmic end of TM2 is tightly packed against the rest of the protein, whereas the extracellular half of the helix is more loosely packed (47). Thus, the putative alcohol and anesthetic binding pocket of the glycine receptor extends three α helical turns into the transmembrane region from the extracellular surface as shown in our model of the four TM α helices (Fig. 5). Our model of the GlyR α1 subunit contains an internal cavity in the transmembrane domain that could be the site of alcohol and anesthetic action. The mutations are predicted to face toward this region, and the model is consistent with MTS reagents entering from the extracellular side, but being blocked from diffusing below Met^{263}. MTS reagents may enter via either a water-filled pathway or the lipid bilayer or a combination of the two, because both charged and neutral MTS compounds reacted. This drug-binding region may correspond to the space between the five TM2 α helices and the ring of 15 α helices surrounding them in the recent crystallographic structure of the acetylcholine receptor (38). Additionally, our model is supported by the NMR structure of glycine receptor TM2 segments (43). In comparing the NMR structure of wild-type and S267Y (anesthetic resistant) TM2 segments of the glycine receptor, Tang *et al.* (43) noted that the mutant caused only local conformation changes. They predicted Met^{263} could border the amphipathic drug binding cavity (43), which is supported by our MTS labeling data.

The structural model of GlyR α1 that we derived (37) shows Ser^{267} and Ala^{288} in proximity (Fig. 5). The proximity of these two residues and their orientation toward the center of the subunit are consistent with studies that showed additivity of the side chain volumes of these residues in changing the cutoff of long chain alcohols (8) and potentiation by anesthetics (2). In addition, the proximity of these residues is consistent with the ability of a double mutation (S267C/A288C) to form disulfide bonds (48). Although it is possible for side chains of distant residues to form disulfide bonds during thermal-motion induced excursions from their mean positions, the highest reaction rate is expected when the Cα to Cα distance is ~6 Å (48). In contrast, the corresponding residues in Protein Data Bank code 1OED (Fig. 6*A*) are not in proximity. In fact, the residue corresponding to Ala^{288} faces the lipid and is on the opposite side of TM3 from Ser^{267}. It is difficult to see how the effects of side chain volumes on Ser^{267} and Ala^{288} (2, 8) could be accommodated in this model. Moreover, formation of a disulfide bond between these residues in the Protein Data Bank code 1OED

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model would require considerable distortions while in the resting state.

The results of tryptophan scanning mutations in TM3 are also inconsistent with the present threading of TM3 in Protein Data Bank code 1OED. In fact, the positions of Phe[284] and Met[282] relative to the center of the subunit are the opposite of the orientations suggested by Guzman *et al.* (41). As a result, we have chosen to interpret our data in terms of the homology model in Fig. 5 rather than the model in Protein Data Bank code 1OED that is based on an intermediate resolution cryo-electron micrograph.

Previous work estimates the anesthetic binding site in the anesthetic-sensitive protein firefly luciferase to be 250 ml/mol (or 415 Å^3/molecule of protein) (49). Using a combination of mutagenesis and anesthetics of different sizes, Jenkins *et al.* (2) estimated the volume of the anesthetic binding site in $GABA_A$ receptors to be between 250 and 370 Å^3. In our experiments, octyl MTS (269 Å^3; 204 Å^3, after reaction with the cysteine) was the largest compound to produce glycine receptor enhancement in the S267C mutant in both states. The largest compound tested, hexadecyl MTS, which affected the receptor only after application to the open state, has a molecular volume of 433 Å^3 (368 Å^3, after reaction). This suggests that the volume of this cavity in the glycine receptor is similar to the anesthetic binding cavity in firefly luciferase and the $GABA_A$ receptor.

Our results lead us to a potential mechanism for alcohol and anesthetic action on this receptor and other related ligand-gated ion channels. Because we have evidence that the site of action of alcohols and volatile anesthetics experiences conformational changes during channel gating, this in turn suggests that drugs occupying this pocket may stabilize the open state of the channel to produce their effects.

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