

Accessibility to residues in transmembrane segment four of the glycine receptor

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Abstract

Glycine receptors (GlyRs) are members of the ligand-gated ion channel superfamily. Each subunit has four transmembrane segments (TM1–TM4). Several studies suggest that amino acids in all four TMs face into a water-filled, alcohol and anesthetic binding cavity in the extracellular portion of the transmembrane domain. TM4 should contribute a “wall” to this cavity, but the residues involved are unknown. Here, we determined the ability of an alcohol analog, propyl methanethiosulfonate (propyl MTS), to covalently react with twelve GlyR TM4 positions (I401–I412) after mutating the original amino acids to cysteines. Reactivity of a cysteine with propyl MTS implies that the cysteine is exposed to water. W407C, I409C, Y410C, and K411C showed altered receptor function following reaction with propyl MTS in the presence or absence of glycine. The cysteine mutations alone eliminated the effects of ethanol for I409C, Y410C, and K411C, and reduced the effects of octanol for I409C and isoflurane for K411C. The ability of propyl MTS to reduce isoflurane and chloroform potentiation was examined in the reactive mutants. Potentiation by isoflurane was significantly reduced for I409C after reaction. These data demonstrate water-accessibility of specific TM4 positions in the GlyR and suggest involvement of these residues with alcohol and anesthetic action.

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1. Introduction

Glycine receptors (GlyRs) are members of the cys-loop superfamily of ligand-gated ion channels. Each subunit of the pentameric receptor has four transmembrane segments (TM1–TM4). GlyRs are targets of alcohols and volatile anesthetics, and their function is potentiated by these drugs. Three amino acids were identified as critical for alcohol and/or volatile anesthetic action on GlyRs (and the homologous GABA_A

receptor): I229 (in TM1), S267 (in TM2) and A288 (in TM3) (Greenblatt and Meng, 1999; Jenkins et al., 2001; Mihic et al., 1997; Ueno et al., 2000; Wick et al., 1998; Yamakura et al., 1999; Ye et al., 1998). All three sites are located in the extracellular portion of the transmembrane domain. These three amino acids are believed to face into a water-filled, drug-binding cavity at the center of the transmembrane domain of each receptor subunit. This model is supported by mutagenesis, substituted cysteine accessibility method (SCAM), and electrophysiological studies, as well as biochemical crosslinking between S267C and A288C, and molecular modeling data (Jenkins et al., 2001; Jung et al., 2005; Lobo et al., 2004a, b; Mascia et al., 2000; Mihic et al., 1997; Wick et al., 1998; Williams and Akabas, 1999; Yamakura et al., 2001). We hypothesize that the drug-binding cavity is bound by amino acids contributed by all four TMs, including TM4.

Abbreviations: GlyR, glycine receptor; WT, wild type; TM, transmembrane segment; MTS, methanethiosulfonate; GABA, γ -aminobutyric acid; EC, effective concentration; SCAM, substituted cysteine accessibility method; nAChR, nicotinic acetylcholine receptor; MBS, modified Barth's solution; DMSO, dimethyl sulfoxide.

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While amino acids on TM1, TM2, and TM3 have been the subject of study in the GlyR, the orientation of amino acids involved in alcohol and anesthetic action suggest that residues in the extracellular portion of TM4 also contribute to form a wall of the drug-binding cavity. In the recently published 4 Å nicotinic acetylcholine receptor structure, the mostly lipid-facing TM4 forms a left-handed, alpha helical bundle with the other three transmembrane segments, and has probable contacts with TM1 and TM3 (Miyazawa et al., 2003; Unwin, 2005). Experimental evidence from mutagenesis in the GABA_A receptor suggest that positions in TM4 are important for anesthetic action (Jenkins et al., 2002). When 12 positions in the extracellular portion of TM4 were mutated to the bulky amino acid tryptophan in the $\alpha 1$ subunit of the GABA_A receptor, a number of positions were found to either increase or decrease the effects of the volatile anesthetics: isoflurane, halothane, and chloroform (Jenkins et al., 2002). Presently, it is unknown which amino acids in TM4 of the glycine receptor are contributing to the drug-binding pocket. Homology modeling of the GlyR implicated that the amino acids in the extracellular portion of TM4, Y406 to Y410, are the most likely participants in drug binding (Bertaccini et al., 2005).

Here, we tested 12 single cysteine mutants (I401C-I412C) in TM4 of the GlyR $\alpha 1$ subunit for their accessibility to the sulfhydryl-specific reagent propyl methanethiosulfonate (MTS) using the substituted cysteine accessibility method (SCAM; Karlin and Akabas, 1998). These amino acids were selected for mutation because they are on the extracellular side of the transmembrane domain, and we hypothesize that amino acids in this region could be facing into the water-filled, drug-binding pocket. Reaction with MTS reagents requires that the sulfhydryl side chain of the substituted cysteine is ionized, and ionization occurs predominantly in the presence of water (Karlin and Akabas, 1998). Because alcohols and anesthetics are proposed to bind in a water-filled cavity at the center of the transmembrane domain, SCAM results would provide information about the structure of TM4 and indicate possible candidate positions for drug action. As in our previous studies, we used an alkyl MTS reagent (rather than more conventionally used sulfhydryl-specific reagents), because when bound to a cysteine an alkyl thiol is structurally similar to an alcohol or anesthetic molecule bound covalently to the protein (Lobo et al., 2004a; Mascia et al., 2000). Here, we tested for reactivity under conditions where the channel was either closed or open (in the absence or presence of glycine) using two-electrode voltage clamping in *Xenopus* oocytes.

Covalent reaction with propyl MTS results in altered channel function, and we found that four positions covalently reacted with propyl MTS (W407C, I409C, Y410C, and K411C), indicating that these positions are water-accessible. To our knowledge, this study is the first to determine water-accessibility to residues in TM4 for any ligand-gated ion channel. We tested whether these four mutants were responsive to alcohols (ethanol and octanol) and tested two anesthetics (isoflurane and chloroform) both before and after reaction with propyl MTS. These experiments tested the effect of the

single cysteine substitutions on drug modulation and tested whether reaction with propyl MTS could occlude potentiation by anesthetic molecules, one criterion previously proposed for an alcohol/anesthetic binding site (Mascia et al., 2000). Additionally, following reaction of I409C with propyl MTS, potentiation of the glycine response by isoflurane was significantly reduced. These data suggest that there are water-accessible sites in TM4 of the glycine receptor and that these positions are important for alcohol and anesthetic action.

2. Methods

2.1. Mutagenesis and expression of human GlyR $\alpha 1$ subunit cDNA

Site-directed mutagenesis in the human glycine receptor $\alpha 1$ subunit was performed on cDNA subcloned into the pBK-CMV vector using the Quik-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). Point mutations were verified by partial sequencing of the sense and antisense strands. *Xenopus laevis* oocytes were isolated and injected (1 ng per 30 nl) with either human glycine receptor $\alpha 1$ wild type cDNA or with the following $\alpha 1$ mutants: I401C, F402C, N403C, M404C, F405C, Y406C, W407C, I408C, I409C, Y410C, K411C, or I412C. The cDNAs were injected into the nucleus using a microdispenser (Drummond Scientific, Broomwall, PA) (Colman, 1984). Injected oocytes were singly stored in incubation media and stored at 15 °C. Incubation media is composed of sterile modified Barth's solution [MBS; 88 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.82 mM MgSO₄, 2.4 mM NaHCO₃, 0.91 mM CaCl₂, and 0.33 mM Ca(NO₃)₂, adjusted to pH 7.5] and supplemented with 10 mg/l streptomycin, 10,000 units/l penicillin, 50 mg/l gentamicin, 90 mg/l theophylline, and 220 mg/l pyruvate. Electrophysiological reagent chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Extraction of oocytes from *Xenopus laevis* frogs was in accordance with the National Institutes of Health guide for the care and use of laboratory animals, as previously described (Beckstead et al., 2000). When expressed in *Xenopus laevis* oocytes, GlyR $\alpha 1$ subunits assemble homomerically, forming functioning receptors with properties similar to those of native receptors (Taleb and Betz, 1994).

2.2. Electrophysiology

Electrophysiological measurements were made in oocytes 1–10 days after injection. Oocytes were placed in a rectangular chamber (approximately 100 μ l volume) and perfused (2.0 ml/min) with MBS, in the presence or in the absence of drugs, via a roller pump (Cole-Parmer Instruments Co., Chicago, IL) through 18-gauge polyethylene tubing (Becton Dickinson, Sparks, MD). Oocytes were impaled in the animal pole with two glass electrodes filled with 3 M KCl and clamped at -70 mV using a Warner Instruments OC725C (Hamden, CT) oocyte clamp. Currents were continuously plotted using a chart recorder (Cole-Parmer Instrument Co, Chicago, IL).

Glycine (Biorad, Hercules, CA) was dissolved in MBS. Concentrations of 100 μ M glycine and higher were applied for 20 s, and lower concentrations were applied to oocytes for 30 s to reach a peak response. Isoflurane was purchased from Ohmeda Caribe Inc. (Liberty Corner, NJ), ethanol was purchased from AAPER Alcohol and Chemicals Co. (Shelbyville, KY), and octanol and chloroform were purchased from Sigma Chemical Co. (St. Louis, MO).

Glycine concentration response curves were determined for the WT and each of the 12 TM4 mutants. The maximal glycine response was elicited at 1 mM glycine for all mutants except Y410C, which required 5 mM glycine for the concentration response curve to plateau. Concentrations of glycine ranging from 10 μ M to 5 mM were applied to each oocyte to measure the response curves. For each mutant, the data were fitted for individual oocytes with nonlinear regression curve fitting, and the mean values of the EC₅₀ and Hill coefficients were calculated. These values, as well as the average maximal response to glycine, were compared to those obtained for the WT receptors by one-way ANOVA.

For the propyl MTS accessibility experiments, first the EC₅–EC₁₀ of glycine (5–10% of the maximal response to 1 mM or 5 mM glycine) was determined for each expressing oocyte. After a 10 min washout, propyl MTS (500 μM) was applied in either the absence of glycine (closed state) or in the presence of maximal glycine (1 mM or 5 mM, open/desensitized state) for 90 s. Then the response to the initial EC₅–10 of glycine was determined 10, 20, and 30 min after application of propyl MTS. The percent potentiation of the recorded glycine currents over the initial current (before the propyl MTS application) was calculated for each oocyte. Our previous experiments on glycine receptor mutants have indicated that application of 500 μM propyl MTS for 90 s is sufficient for a complete reaction (Lobo et al., 2004a). Because the three glycine responses after application of propyl MTS did not differ, we used the 20 min time point response for our analyses.

To test alcohol and volatile anesthetic responses, isoflurane (0.6 mM), ethanol (100 mM), octanol (114 μM), and chloroform (2.0 mM) were dissolved in solution immediately prior to each experiment. First the EC₅–10 of glycine was determined for each expressing oocyte. After 10 min, the alcohol or anesthetic solutions were applied as a 1-min preincubation in MBS alone followed by a 30-s application of the drug in an EC₅–10 solution of glycine. After a 10 min washout, a second glycine EC₅–10 test pulse was applied. Potentiation by drugs was calculated by dividing the drug-induced current by the average EC₅–10 glycine-induced currents applied 10 min before and after each drug application.

To test whether reaction with propyl MTS could reduce anesthetic responses, the responses of anesthetics were tested before and after application of propyl MTS (500 μM). The responses of isoflurane (0.6 mM) and chloroform (2.0 mM) were tested on the glycine EC₅–10 response, as described above. Potentiation by isoflurane and chloroform was calculated by dividing the drug-induced current by the average EC₅–10 glycine-induced currents (control) applied 10 min before and after each drug application, and expressing the data as a percent of the control.

After a 10 min washout, propyl MTS (500 μM) was applied for 90 s. Propyl MTS was applied in the absence of glycine for the WT, W407C, Y410C, and K411C mutants, and in the presence of maximal glycine (1 mM) for I409C, which reacted only in the open state. After a 10 min washout, the maximal glycine response was re-determined, and a new EC₅–10 of glycine was determined. The responses of isoflurane (0.6 mM) and chloroform (2.0 mM) on the new glycine EC₅–10 response were then tested, and potentiations were calculated, as above. The anesthetic potentiation before and after reaction with propyl MTS were compared using the paired Student's *t*-test for each oocyte. Re-determining the maximal glycine current and EC₅–10 after reaction with propyl MTS ensured that if there was any change in the maximal current after reaction with propyl MTS, anesthetic responses were being measured and compared at the same EC.

2.3. Data analysis

Data analysis was performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA). The software was used to fit concentration response curves with non-linear regression curve fitting. Glycine EC₅₀ and Hill coefficients were compared to the WT receptors by one-way ANOVA with the Dunnett's post-test. Mutant glycine responses measured following propyl MTS application were compared with the WT control using one-way ANOVA with the Dunnett's post-test. Responses of mutant receptors to alcohols and anesthetics were compared to the WT response using one-way ANOVA with the Dunnett's post-test. Differences in drug-modulated glycine responses before and after propyl MTS application for individual oocytes were statistically defined using the paired Student's *t*-test.

3. Results

Analysis of glycine concentration response curves showed that of the 12 mutants tested, the N403C, W407C, Y410C, and K411C mutants had significantly greater EC₅₀ values than the WT receptor (one-way ANOVA). All of the mutants tended to have a lower sensitivity to glycine than the WT receptor, but

the Hill coefficients of the mutants did not show significant changes from the WT receptors. The maximal currents from the Y406C and Y410C receptors significantly decreased (Table 1). Glycine concentration response curves are shown for the WT, W407C, I409C, Y410C, and K411C mutants in Fig. 1.

3.1. MTS reactivity summary

The 12 mutants and WT glycine receptor were tested for irreversible changes in receptor function after application of propyl MTS to test if the substituted cysteines were water-accessible. Glycine (EC₅–10) was applied 10 min before and 10, 20, and 30 min after application of 500 μM propyl MTS, as shown in Fig. 2. The initial response and the EC₅–10 glycine response at the 20 min time point were compared because the responses at the three time points after propyl MTS application did not differ. Responses indicated either no reaction or irreversible, covalent reaction. Reaction with propyl MTS was tested in both the presence and absence of a maximal glycine concentration to detect whether reaction was occurring in the closed or in the open/desensitized state.

The WT and the six most intracellular mutants (I401C to Y406C) were not affected after reaction with propyl MTS in either the presence or absence of glycine. W407C reacted with propyl MTS, resulting in irreversible potentiation, when applied in both the presence (245 ± 100%) and absence of glycine (180 ± 42%). I408C was inaccessible under both conditions. I409C only reacted in the presence of glycine, resulting in irreversible inhibition (−43 ± 11%). Y410C reacted with propyl MTS in both the presence (510 ± 73%) and absence (260 ± 65%) of glycine, resulting in irreversible potentiation. K411C reacted in both the presence (−72 ± 4.3%) and absence (−65 ± 6.5%) of glycine, resulting in irreversible inhibition of the glycine current. The final mutant, I412C, was not affected by application of propyl MTS in both conditions. Representative tracings of the glycine responses before and after application of propyl MTS are shown for the WT, W407C, Y410C, and K411C glycine receptors in Fig. 2, and the mean reactivity data for all of the mutants and the WT receptor are shown in Fig. 3.

3.2. WT, W407C, I409C, Y410C, and K411C responses to alcohols and volatile anesthetics

The responses of the four accessible mutants to alcohols and anesthetics were determined and compared to the WT receptor. Percent of control responses to ethanol (100 mM), octanol (114 μM), isoflurane (0.6 mM), and chloroform (2.0 mM) were measured with an EC₅–10 concentration of glycine, which was determined for each oocyte. The responses of I409C to ethanol and octanol were significantly reduced. The Y410C mutant showed no potentiation by ethanol. K411C also showed no potentiation by ethanol and had a reduced isoflurane effect (Table 2).

Table 1
Glycine EC₅₀, Hill coefficients and maximal glycine currents for the wild type (WT) receptor and the cysteine substitution mutants studied in TM4

Receptor	EC ₅₀ (μM)	Hill coefficient	Max current (μA)
WT	104 ± 5.8	3.2 ± 0.8	16.9 ± 1.2
I401C	198 ± 24	2.3 ± 0.2	11.9 ± 1.2
F402C	147 ± 24	3.5 ± 1.2	13.4 ± 1.9
N403C	283 ± 75**	2.9 ± 0.2	15.1 ± 1.7
M404C	112 ± 11	2.1 ± 0.2	16.6 ± 1.4
F405C	190 ± 16	2.2 ± 0.2	21.3 ± 2.0
Y406C	162 ± 22	2.9 ± 0.6	7.4 ± 1.4**
W407C	236 ± 28*	2.3 ± 0.1	13.7 ± 1.1
I408C	203 ± 17	2.8 ± 0.4	15.5 ± 1.5
I409C	197 ± 47	3.5 ± 0.5	13.6 ± 1.1
Y410C	550 ± 61**	2.6 ± 0.2	8.1 ± 1.0**
K411C	260 ± 33*	2.6 ± 0.3	14.9 ± 1.9
I412C	206 ± 15	2.5 ± 0.5	13.9 ± 1.1

The mean glycine EC₅₀, Hill coefficients and maximal (Max) currents were calculated from concentration response curves of single oocytes. Glycine EC₅₀ and Hill coefficient data are expressed as a mean ± SE of 4–10 oocytes, and maximal glycine currents are the mean ± SE of 15–48 oocytes. **p* < 0.05, ***p* < 0.01; significantly different from wild type receptors by one-way ANOVA with the Dunnett's post-test.

3.3. Effects of isoflurane and chloroform on TM4 mutants before and after reaction with propyl MTS

Because W407C, I409C, Y410C, and K411C reacted with propyl MTS and were water-accessible, we asked if the reaction of propyl MTS at these positions could block modulation of the glycine receptor response by anesthetics, as shown previously for the S267C mutant receptor (Mascia et al., 2000). If propyl MTS reaction blocked drug effects at these mutant receptors, then this would support the hypothesis that propyl MTS is permanently occupying the drug-binding cavity and

thereby preventing another drug molecule (isoflurane or chloroform) from binding to and affecting the receptor. This would provide strong evidence that an amino acid position lines the drug-binding cavity.

The effects of isoflurane (0.6 mM) and chloroform (2.0 mM) were tested on EC_{5–10} glycine responses of the WT and each mutant. Potentiation by drugs was calculated by dividing the drug-induced current by the average EC_{5–10} glycine-induced currents (control) applied 10 min before and after each drug application. Propyl MTS was applied for (90 s) in the absence of glycine to all receptor types except I409C, which required glycine for reaction. Then the maximal glycine response and EC_{5–10} glycine concentration was re-determined to ensure comparisons were made at the same EC, and the responses of these two volatile anesthetics were determined again. An example tracing showing the procedure is shown in Fig. 4A. The average maximal glycine current magnitudes did not change significantly following propyl MTS application for the WT or any of the mutants tested, though from oocyte to oocyte we observed both increases and decreases. The average maximal currents (in μA ± SEM) pre- and post-propyl MTS application were as follows: WT pre 13.8 ± 2.4, post 17.2 ± 5.6 (*n* = 5), W407C pre 7.7 ± 2.3, post 7.4 ± 1.7 (*n* = 9), I409C pre 10.7 ± 1.2, post 12.8 ± 2.6 (*n* = 5), Y410C pre 11.9 ± 1.9, post 12.7 ± 3.2 (*n* = 7), and K411C pre 17.5 ± 3.6, post 13.4 ± 2.9 (*n* = 10). Application of propyl MTS reduced the potentiation by isoflurane of the I409C mutant, but had no effect on the potentiation by chloroform (Fig. 4A and D). Reaction with propyl MTS did not alter the potentiation by isoflurane or chloroform on the WT, W407C, Y410C, and K411C mutants (Fig. 4B, C, E, and F).

4. Discussion

Mutagenesis of 12 positions in TM4 to cysteine and reaction with propyl MTS allowed us to determine water accessible positions in this region of the glycine receptor. Four positions in the extracellular portion of TM4 reacted with propyl MTS to produce irreversible changes in glycine receptor function. Altered drug responses due to the cysteine mutations suggest that these amino acids may be involved with alcohol and volatile anesthetic action. The four reactive positions (W407, I409, Y410, and K411) were all located in the most extracellular portion of TM4. Previously identified positions involved with alcohol and volatile anesthetic action in the other three transmembrane segments (I229, S267, and A288) are also located in the extracellular portion of the transmembrane domain, suggesting that the TM4 sites could play a role in drug binding along with the other known residues.

Both W407C and Y410C reacted with propyl MTS in the absence and presence of glycine, causing an increased response to glycine. The apparent differences in potentiation after reaction in the absence and presence of glycine may reflect a higher reaction rate when propyl MTS is applied in the presence of glycine. This may be due to increased ionization of the SH group, increased access to the propyl MTS reagent (Karlin

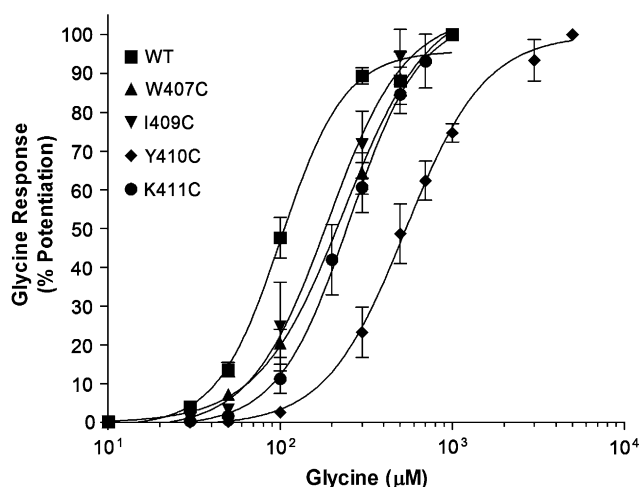


Fig. 1. Concentration response curves for the WT glycine receptor and the W407C, I409C, Y410C, and K411C mutants. Currents elicited by at least seven different concentrations of glycine are expressed as a fraction of the maximal glycine response in the WT and mutant receptors. The four mutant response curves were all right-shifted from the WT response, with significant right-shifts in the W407C, Y410C, and K411C mutants. The data presented are the mean ± SE from concentrations response curves of 4–10 oocytes, fitted with nonlinear regression curve fitting. In some cases, the error bars are smaller than the symbols.

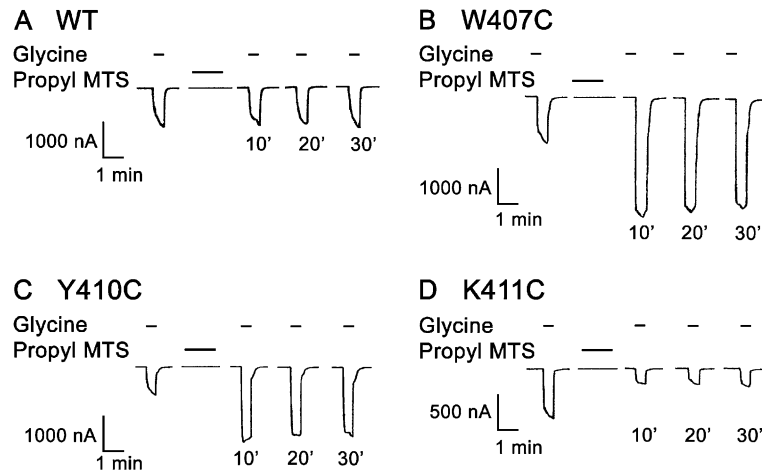


Fig. 2. Effect of propyl MTS on WT, W407C, Y410C, and K411C glycine receptors. The current resulting from an EC_{5–10} of glycine was tested before and 10, 20, and 30 min after application of 500 μ M propyl MTS in the absence of glycine. Covalent reaction of the cysteine and propyl MTS is indicated by a change in the glycine-induced current. Representative tracings are shown for the WT and three reactive mutants. (A) For the WT, the current elicited by glycine was not altered following application of propyl MTS. (B) and (C) Both W407C and Y410C currents were irreversibly enhanced after application of propyl MTS. (D) K411C showed irreversible inhibition following reaction of propyl MTS.

and Akabas, 1998), or tipping or rotation of TM4 during transition to the open state, as observed in other GlyR TMs (Lobo et al., 2004a). For I409C and K411C, reaction with propyl MTS resulted in an irreversible decrease in receptor current. Since I409C only reacts in the presence of glycine, this indicates that the position is lipid exposed in the resting state and moves or rotates during channel gating to position the side chain in a water-filled cavity where it reacts with propyl MTS. The increases and decreases in glycine receptor current

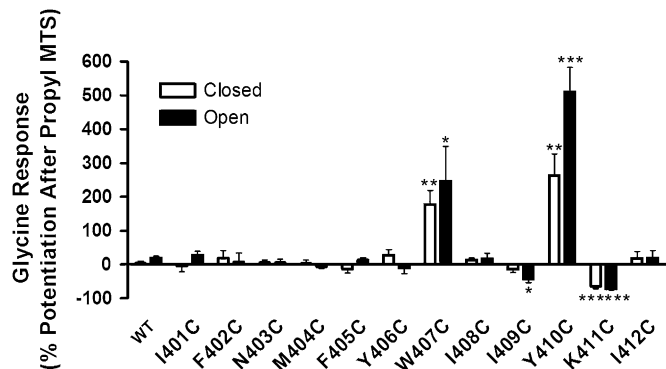


Fig. 3. Accessibility of propyl MTS to GlyR α 1 TM4 mutants. Propyl MTS (500 μ M) was applied to WT and mutant receptors in the absence (closed) and presence (open) of a maximal concentration of glycine. Shown, are the glycine responses 20 min following application of 500 μ M propyl MTS, expressed as a percent potentiation of the initial EC_{5–10} glycine response, for the WT and I401C–I412C mutants. A change in the glycine EC_{5–10} response after application of propyl MTS indicates that a covalent reaction has occurred. Four mutants reacted with propyl MTS to produce a change in the glycine EC_{5–10} response, indicating that they are water-accessible. W407C and Y410C reacted in both the presence and absence of glycine and had a potentiated response to glycine. I409C and K411C reacted with propyl MTS and showed inhibition of the initial glycine response. I409C only reacted with propyl MTS in the presence of glycine, while K411C reacted in both states. Data are expressed as a mean \pm SE of 5–11 oocytes. All mutant responses were compared to the respective WT control by one-way ANOVA and the Dunnett's post-test (* p < 0.05, ** p < 0.01 and *** p < 0.001).

caused by reaction of propyl MTS at these four sites suggest that the positions are important for stabilizing the open and closed states of the channel.

Because TM4 is believed to be partially surrounded by lipids (Miyazawa et al., 2003), it is somewhat surprising that three sequential amino acids (I409, Y410, and K411) are all in a water-filled environment. One aspect that complicates analysis of these data is the relative size of the native amino acids in TM4. Overall, the 12 native amino acids targeted for mutation were larger than cysteine. With each introduced cysteine, the mutation itself may enlarge existing water cavities or introduce new pockets of water where propyl MTS may react.

The N403, W407, Y410, and K411 residues should lie along the same helical face since TM4 is an alpha helix (Miyazawa et al., 2003). Interestingly, the cysteine mutants at only these positions had glycine EC₅₀ values that were significantly greater than the WT. Along with having increased glycine EC₅₀ values, both the W407C and Y410C mutants showed potentiation of the glycine response after reaction with propyl MTS. One possible explanation of this is that

Table 2

Responses of WT, W407C, I409C, Y410C, and K411C receptors to ethanol (100 mM), octanol (114 μ M), isoflurane (0.6 mM), and chloroform (2.0 mM)

Glycine receptor	Percent of control			
	Ethanol	Octanol	Isoflurane	Chloroform
WT	180 \pm 11	270 \pm 17	880 \pm 84	500 \pm 24
W407C	150 \pm 20	340 \pm 83	620 \pm 39	320 \pm 51
I409C	120 \pm 16*	110 \pm 7.3*	730 \pm 110	400 \pm 65
Y410C	94 \pm 22**	150 \pm 15	870 \pm 110	510 \pm 52
K411C	85 \pm 5.1**	200 \pm 74	420 \pm 49**	450 \pm 66

Drug responses were measured on the EC_{5–10} glycine responses (determined for each oocyte). Data are expressed as a percent of control of the mean \pm SE of 5–16 oocytes. Mutant responses were compared to the WT using one-way ANOVA and Dunnett's post-test (* p < 0.05, ** p < 0.01).

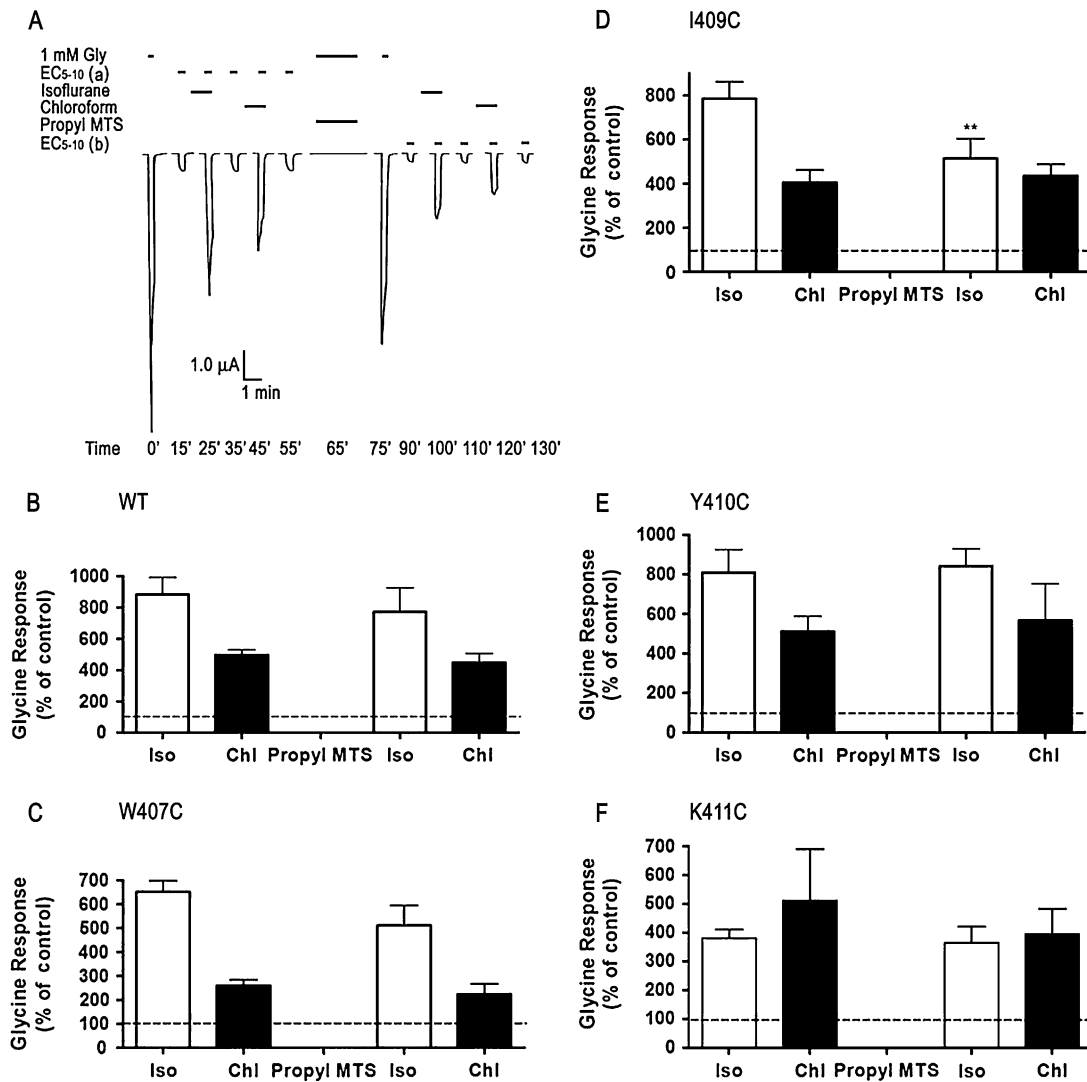


Fig. 4. Effects of volatile anesthetics on WT, W407C, I409C, Y410C, and K411C receptors before and after application of propyl MTS. To determine if reaction with propyl MTS could block anesthetic action, we tested the response of isoflurane and chloroform before and after application of propyl MTS. For each glycine receptor, the maximal glycine current and the glycine EC_{5-10} (a) were determined prior to measuring the potentiation by isoflurane (Iso; 0.6 mM) and chloroform (Chl; 2.0 mM). Following application of 500 μ M propyl MTS, the maximal glycine current and the glycine EC_{5-10} (b) were re-determined and the potentiation by isoflurane and chloroform were measured again using the new glycine EC_{5-10} (b). The control EC_{5-10} glycine response was determined for each drug response by averaging the EC_{5-10} glycine response from directly before and after the drug application. (A) A tracing of the full procedure is shown for the I409C mutant. Propyl MTS was applied in the absence of glycine to all receptor types except I409C, where glycine was required for reaction. During co-application of maximal glycine and propyl MTS, the oocyte was unclamped. Time elapsed between applications is indicated below the tracing. The bar graphs (B)–(F) illustrate the potentiation of the EC_{5-10} glycine response by isoflurane and chloroform, and are presented as a percent of the control. Application of propyl MTS to the (B) WT, (C) W407C, (E) Y410C, and (F) K411C receptors produced no change in the response of the receptor to isoflurane and chloroform. (D) There was a significant reduction of the isoflurane potentiation for the I409C receptor. The Student's paired *t*-test was used to determine differences in the mean drug potentiation from before and after the propyl MTS application (***p* < 0.01, *n* = 3–7 oocytes).

the reaction of propyl MTS at W407C and Y410C increased the volume occupied at these positions to mimic the native amino acids. Reaction of propyl MTS could cause the mutant receptors to mimic the WT receptor and result in a leftward shift of the glycine concentration response curve. W407 and Y410 may lie on a helical face of TM4 that either interacts with another TM region or faces into the drug-binding cavity to regulate both channel gating and drug action.

We compared our accessibility data in TM4 of the GlyR to published data on tryptophan mutants in TM4 of the homologous GABA_A receptor α 1 subunit (Jenkins et al., 2002)

(Table 3). Mutation of a number of residues to tryptophan in the GABA_A receptor were shown to alter receptor sensitivity to three volatile anesthetics: isoflurane, halothane and chloroform (Jenkins et al., 2002) (Table 3). While tryptophan and cysteine have different properties, looking for overlap between these positions could indicate that an amino acid is important for drug action because it is both water-accessible and because mutation at the residue alters anesthetic responses. The GlyR and GABA_A receptor sequences were aligned using the consensus sequences of the ligand-gated ion channels (Bertaccini and Trudell, 2002). While there was no reactivity with propyl

MTS observed for positions I401, F402, Y406, I408, and I412 in the glycine receptor, mutation to tryptophan of homologous sites in the GABA_A receptor altered anesthetic effects. The glycine receptor mutant W407C was reactive, but could not be compared to the GABA_A receptor results, where the position was already a tryptophan. Meanwhile, overlap between the data sets occurred with the three other water-accessible positions. Positions I409, Y410, and K411 were all water-accessible in the glycine receptor, and caused changes in anesthetic action when the homologous amino acids were mutated to tryptophan in the GABA_A receptor (Table 3), suggesting that these amino acids could participate in drug binding.

The comparison of our results to other data sets in Table 3 favors positioning W407 and Y410 inward toward a water-filled cavity that is involved with drug binding. In the GABA_A receptor, mutation of Y415 (homologous to GlyR Y410) to tryptophan resulted in an increased drug response, while for the other tryptophan mutants caused decreased drug responses (Jenkins et al., 2002). In the GlyR, increasing the volume at W407C and Y410C with propyl MTS leads to potentiation, which is consistent with other MTS reactivity studies at positions involved with alcohol and volatile anesthetic action in the GlyR: TM1 (I229C), TM2 (S267C) and TM3 (A288C) (Lobo et al., 2004a; Mascia et al., 2000). Additionally, increasing the volume in this cavity by mutations at TM2 (S270W) and TM3 (A291W, and double mutant S270W/A291W) in the GABA_A receptor, resulted in potentiation and spontaneous activity (Findlay et al., 2001; Jenkins et al., 2001). Overall, these data favor placement of W407 and Y410 in the putative drug-binding cavity, since increasing the volume at these positions either by mutation or by propyl MTS reaction leads to receptor potentiation.

The four accessible positions were tested to see if reaction with propyl MTS could block potentiation by isoflurane and chloroform. In contrast to S267C (Mascia et al., 2000) and A288C (Lobo, 2004), propyl MTS reaction did not block potentiation by drugs at W407C, Y410C, and K411C. Like the WT receptor, these mutants were potentiated by isoflurane and

chloroform both before and after reaction with propyl MTS. Only the potentiation of I409C by isoflurane was reduced following covalent reaction with propyl MTS, which indicates that this position may play a role in anesthetic binding. One possible reason that propyl MTS treatment cannot block isoflurane action is that propyl MTS is not large enough or is not the correct shape to mimic a drug molecule in the cavity, and therefore prevent the drug response. It is also possible that these positions are not in the drug-binding cavity, or that they are located in the binding cavity but are not playing a direct role in binding these drugs. Still, the cysteine mutations alone affected responses of some alcohols and anesthetics tested. I409C, Y410C, and K411C reduced or eliminated the response to ethanol, I409C had a reduced octanol potentiation, and K411C had a reduced isoflurane effect. Although propyl MTS did not prevent volatile anesthetic potentiation, it is not possible to exclude these residues as candidates for participation in a drug binding cavity.

Additionally, we compared our data to results from tryptophan substitution studies in TM4 of the *Torpedo californica* acetylcholine receptor α subunit (Tamamizu et al., 2000) and published data on the positions that reacted with hydrophobic probes in TM4 of the α , β , γ , and δ *Torpedo californica* acetylcholine receptor subunits (Blanton and Cohen, 1992; Blanton and Cohen, 1994; Blanton et al., 1998). Our data was consistent with these studies. As expected, the amino acids postulated to be lipid-facing in the acetylcholine receptor were not water-accessible in the glycine receptor, with the exception of I409C. Since I409C is reactive in only the presence of glycine, we interpret this to mean channel gating rotates or moves the I409C side chain from an unexposed, lipid-facing position into a water-filled cavity.

In conclusion, four cysteine mutants were accessible to propyl MTS: W407C, I409C, Y410C, and K411C. Overall, comparison of our data with published results favor placing W407 and Y410 in the putative alcohol and anesthetic binding cavity, since increased volume at these positions also leads to potentiation. Therefore, W407 and Y410 would face amino acids in the

Table 3

The glycine receptor TM4 reactivity data compared with previously published data on tryptophan mutants in TM4 of the $\alpha 1$ subunit of the GABA_A receptor (Jenkins et al., 2002) to look for overlap between water-accessible positions that reacted with propyl MTS and mutations which affected anesthetic responses

GlyR $\alpha 1$			GABAAR $\alpha 1$			
Position	Cys mutants		Position	Trp mutants, tested in GABAA $\alpha 1\beta 2\gamma 2\delta$		
	No glycine	Glycine		Isoflurane	Halothane	Chloroform
I401	NR	NR	I406	NE	NE	↓
F402	NR	NR	F407	NE	NE	↑
N403	NR	NR	N408			
M404	NR	NR	L409	NE	NE	NE
F405	NR	NR	V410	NE	NE	NE
Y406	NR	NR	Y411	↓	↓	NE
W407	↑	↑	W412			
I408	NR	NR	A413	↓	NE	↑
I409	NR	↓	T414	↓	NE	NE
Y410	↑	↑	Y415	NE	↑	↑
K411	↓	↓	L416	NE	↓	↓
I412	NR	NR	N417	NE	NE	↑

NR, no reaction; NE, no effect; ↑, increased response; ↓, decreased response.

three other transmembrane segments (I299, S267, and A288) that are involved with alcohol and anesthetic drug binding.

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