

Cross-linking of glycine receptor transmembrane segments two and three alters coupling of ligand binding with channel opening

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Abstract

Contact points between transmembrane segments (TMs) two and three of the glycine receptor are undefined and may play an important role in channel gating. We tested whether two amino acids in TM2 (S267) and TM3 (A288), known to be critical for alcohol and volatile anesthetic action, could cross-link by mutating both to cysteines and expressing the receptors in *Xenopus laevis* oocytes. In contrast with the wild-type receptor and single cysteine mutants, the S267C/A288C double mutant displayed unusual responses, including a tonic leak activity that was closed by strychnine and a run-down of the response upon repeated applications of glycine. We hypothesized that these characteristics were due to cross-linking of the two cysteines on opposing faces of these

adjacent, alpha helical TMs. This would alter the movement of these two regions required for normal gating. To test this hypothesis, we used dithiothreitol to reduce the putative S267C–A288C disulfide bond. Reduction abolished the leak current and provided normal responses to glycine. Subsequent application of the cross-linking agent mercuric chloride caused the initial characteristics to return. These data demonstrate that S267 and A288 are near-neighbors and provide insight towards the location and role of the TM2–TM3 interface in ligand-gated ion channels.

Keywords: alcohols, anesthetics, cross-linking, glycine receptor, transmembrane segments, *Xenopus laevis* oocytes. *J. Neurochem.* (2004) **90**, 962–969.

Strychnine-sensitive glycine receptors (GlyRs) are a member of a family of ligand-gated ion channels. Receptors of this family are arranged as five subunits surrounding a central pore with each subunit composed of four alpha helical transmembrane segments (TM1–TM4; Rajendra *et al.* 1997; Miyazawa *et al.* 2003). Miyazawa *et al.* (2003) recently extended the resolution of the homologous *Torpedo* acetylcholine receptor to 0.4 nm, showing an inner ring of TM2 alpha helices line the channel pore with a second outer ring formed by the 15 alpha helices of TM1, 3, and 4.

Sites of alcohol and volatile anesthetic action on the glycine receptor (and the homologous GABA_A receptor) have been identified: in TM1 (I229), TM2 (S267), as well as a residue in TM3 (A288; Mihic *et al.* 1997; Wick *et al.* 1998; Ye *et al.* 1998; Greenblatt and Meng 1999; Ueno *et al.* 1999; Yamakura *et al.* 1999; Jenkins *et al.* 2001). These amino acids have been hypothesized to line a binding pocket for alcohols and volatile anesthetics between the four transmembrane segments of each subunit (Mascia *et al.* 2000; Jenkins *et al.* 2001; Yamakura *et al.* 2001). Though the pore lining residues of TM2 have been defined by

cysteine substitution and labeling in this family of proteins (Xu and Akabas 1996; Zhang and Karlin 1998; Horenstein *et al.* 2001), the contact points between the non-pore lining TM2 residues that may face TM1, 3, or 4 are undetermined. Considering that these amino acids are distant from one another in the primary amino acid sequence and that there is no complete crystal structure available for the glycine receptor, we were interested in determining how these amino acids were arranged.

Currently, there are two pieces of experimental evidence that suggest S267 and A288 may be near one another. First, Wick *et al.* (1998) exchanged amino acids in TM2 and TM3

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Abbreviations used: DTT, dithiothreitol; GABA, γ -aminobutyric acid; GlyR, glycine receptor; MBS, modified Barth solution; TM, transmembrane segment; WT, wild-type.

between the homomeric GABA ρ 1 and GlyRs to alter alcohol cut-offs of these two receptors. The alcohol cut-off is the point at which increasing the length of the alkyl chain of a primary alcohol no longer produces an increase in potency. The homomeric GlyR α 1 has an alcohol cut-off at decanol, while the GABA ρ 1 receptor cut-off is at heptanol, suggesting that the GABA ρ 1 alcohol binding pocket is smaller than that of the GlyR. When homologous positions of GABA ρ 1 (I307 and W328) were converted to their smaller glycine receptor counterparts (S267 and A288), the alcohol cut-off of the single mutant receptors increased from 7 to 9. Mutation of both of these amino acids to I307S/W328A increased the alcohol cut-off to be dodecanol or higher, suggesting that both residues were a part of a single alcohol-binding cavity (Wick *et al.* 1998). Secondly, Jenkins *et al.* (2001) studied homologous positions of the GABA ρ receptor, S270 and A291, and replaced them with the bulky amino acid tryptophan. The S270W single mutant was insensitive to the anesthetics isoflurane and halothane, but retained sensitivity to the smaller anesthetic chloroform. Meanwhile, when both were replaced to create a S270W/A291W double mutant, the receptor was insensitive to chloroform as well as isoflurane and halothane (Jenkins *et al.* 2001). Here, we tested for a direct association of the GlyR α 1 amino acid positions S267 and A288 to determine the orientation of TM2 and TM3.

Cross-linking has been used for decades to gather structural information about proteins. Existing and introduced cysteines have been cross-linked to determine near-neighbor relationships and associations of proteins, orientations of interactions as well as the activity, folding and three-dimensional structures of diverse proteins. In the GABA ρ receptor, cross-linking was used recently to identify extracellular domain residues that interact with the TM2–TM3 linker loop to couple agonist binding and gating (Kash *et al.* 2003), and to determine intersubunit TM2 segment contact points (Horenstein *et al.* 2001). Other studies have explored cross-linking between transmembrane helices in engineered and wild-type (WT) helical bundle proteins, such as keratin, cyclic nucleotide-gated channels and aspartate receptors (Fraser *et al.* 1988; Regan *et al.* 1994; Chervitz and Falke 1995; Matulef and Zagotta 2002). Disulfide cross-linking between adjacent alpha helices occurs when the residues are on opposing faces of the helices (Lee *et al.* 1995; Soskine *et al.* 2002) and have C α –C α distances less than 1 nm (Yang *et al.* 1996).

In the present study, we tested for cross-linking between cysteines introduced at two glycine receptor positions (S267 and A288) known to be involved with alcohol and inhaled anesthetic action. The S267C/A288C double mutant had different characteristics from the WT receptor. Its current decreased with repeated applications of glycine, and it displayed tonic activity in the absence of neurotransmitter. These characteristics were eliminated with the application of

the reducing agent dithiothreitol (DTT) and regained after application of mercuric chloride. Mercuric chloride (HgCl $_2$) is a cross-linking agent that reacts with vicinal pairs of cysteines to form an intermolecular mercury-linked dimer, even in transmembrane regions with a low dielectric environment (Soskine *et al.* 2002).

Our results indicate that a disulfide bond had formed between these two introduced cysteine residues to cross-link TM2 and TM3. This is strong evidence that S267 and A288 are near-neighbors in the tertiary glycine receptor structure and that these amino acids both could contribute to a binding pocket for alcohols and volatile anesthetics. Some of this work has been presented previously in abstract form (Lobo *et al.* 2003).

Materials and methods

Mutagenesis and expression of human GlyR α 1 subunit cDNA

Using the Quik-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), missense mutations were introduced in the human glycine receptor α 1 subunit (subcloned in the pBKCVMV N/B-200 or pCIS2 vectors). 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 GlyR α 1 WT, α 1(S267C), α 1(A288C), α 1(S267C) + α 1(A288C) in a 1 : 1 ratio or α 1(S267C/A288C) cDNA. When expressed in a heterologous system, such as *Xenopus laevis* oocytes, GlyR α 1 subunits can assemble homomericly to form functioning receptors with properties like those of native receptors (Taleb and Betz 1994). Colman's 'blind' method for nuclear injection of cDNAs was performed using a microdispenser (Drummond Scientific, Broomwall, PA, USA; Colman 1984). Injected oocytes were singly stored in incubation media [sterile modified Barth solution (MBS) containing 88 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.82 mM MgSO $_4$, 2.4 mM NaHCO $_3$, 0.91 mM CaCl $_2$, and 0.33 mM Ca(NO $_3$) $_2$, adjusted to pH 7.5] supplemented with 10 mg/L streptomycin, 10 000 U/L penicillin, 50 mg/L gentamicin, 90 mg/L theophylline, and 220 mg/L pyruvate and incubated at 15°C (Sigma Chemical Co., St Louis, MO, USA).

Electrophysiology

Electrophysiological measurements were made in oocytes 1–10 days after injection. Oocytes were placed in a rectangular chamber (volume approximately 100 μ L) and perfused with MBS at a rate of 2.0 mL/min with a peristaltic pump (Cole-Parmer Instruments Co., Chicago, IL, USA) through 18-gauge polyethylene tubing (Becton Dickinson, Sparks, MD, USA). Oocytes were impaled in the animal pole with two glass electrodes (0.5–10 M Ω) filled with 3 M KCl and clamped at –70 mV using a Warner Instruments OC725C (Hamden, CT, USA) oocyte clamp. Currents were continuously plotted using a chart recorder (Cole-Parmer Instrument).

Glycine 1 mM (Bio-Rad, Hercules, CA, USA) was dissolved in MBS and applied for 20 s (30 s for lower concentrations). Dithiothreitol (DTT; Sigma) was freshly prepared and dissolved in MBS at a concentration of 1 mM or 10 mM prior to each 3-minute

application. Mercuric chloride (HgCl_2 ; 10 μM ; Sigma) was prepared from a 1 mM stock in MBS and applied to cross-link (Soskine *et al.* 2002) for 1 min. Strychnine (10 μM ; Sigma) was prepared from a 1 mM stock in MBS and applied for 40 s.

Reduction experiments (Fig. 1) were performed as follows: 1 mM glycine was applied to the oocytes followed by a washout of 15 min. This was repeated twice. Then the oocyte was unclamped during the 1 mM DTT application because DTT affected the bath electrodes or impaling electrodes, causing the oocyte clamp to be unable to maintain the -70 mV potential in the mutant, WT, and uninjected oocytes. The oocyte was resealed, and was washed for 15 min. Glycine was then reapplied three times with 15-min washouts with the last response to glycine being measured 45 min after DTT. The reduction and cross-linking experiments (Fig. 3) were performed as follows: glycine (1 mM) was applied to the oocyte, followed by a 15-min washout. This was repeated once. This was followed by reduction with 10 mM DTT (oocyte unclamped) and a 15-min washout. Glycine was reapplied with a 15-min washout. Then the oocyte was unclamped again during a 1-min application of 10 μM HgCl_2 for cross-linking. Glycine was reapplied with a 15-min washout. This was followed by a second reduction with DTT and 15-min washout (as above) and a final application of glycine.

Ethanol (100 mM; AAPER Alcohol and Chemicals Co., Shelbyville, KY, USA), octanol (115 μM ; Sigma), isoflurane (0.77 mM; Marsam Pharmaceuticals, Inc., Cherry Hill, NJ, USA), and chloroform (2.0 mM; Sigma) were dissolved in MBS immediately prior to each experiment. Responses to these concentrations of alcohols and anesthetics were tested (following a 1-min pre-incubation with the

drug alone) on an EC_{5-10} of glycine (concentration of glycine eliciting 5–10% of the maximal glycine response), which was determined individually for each oocyte. S267C/A288C mutants were tested for their drug responses in the cross-linked state before DTT application. After reduction of S267C/A288C with 1 mM DTT, the EC_{5-10} concentration of glycine was re-determined for each oocyte, and the drugs were tested in the same manner. The drug responses were measured approximately 45 min after reduction to ensure that most receptors were uniformly in the reduced form.

Data analysis

Data analysis was performed using GraphPad Prism, Version 3.02 (GraphPad Software, Inc., San Diego, CA, USA). The software was used to define significance of glycine responses in the mutant receptors compared to WT controls, and the changes in responses after DTT application using one-way analysis of variance and the Student's paired *t*-test.

Molecular modeling

Molecular modeling of the glycine receptor transmembrane region was conducted as previously described (Trudell and Bertaccini 2004). A model of the four transmembrane segments of a glycine receptor was built by threading the primary sequence of GlyR α 1 over a template of a four-helix bundle found in the high-resolution structure of the cytochrome *c* oxidase (20CC). An initial constraint on the model was that amino acid residues known to modulate anesthetic potency were in direct proximity to one another [I229 (TM1), S267 (TM2), and A288 (TM3)]. A second set of constraints was that the pore-facing and lipid-facing residues identified in the

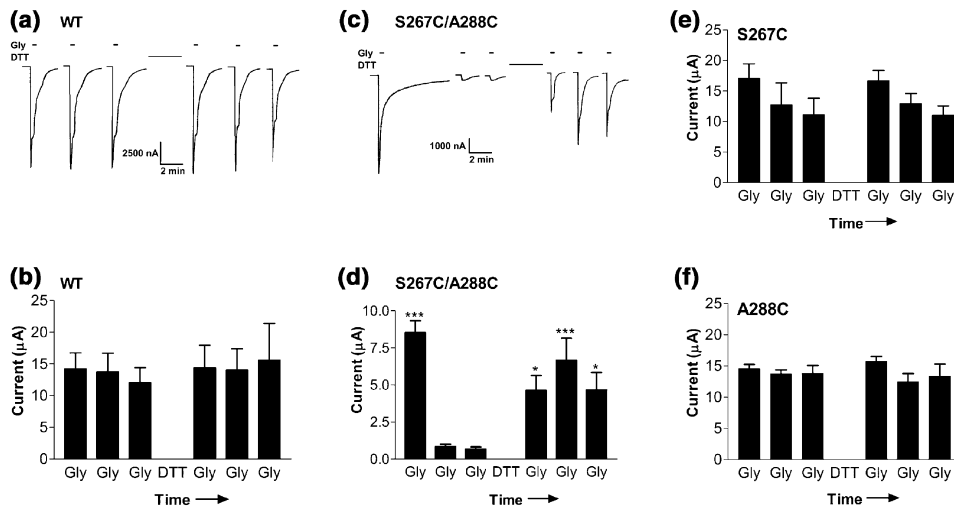


Fig. 1 Reduction with dithiothreitol has no effect on WT, S267C, and A288C glycine receptors, but increases the response of S267C/A288C receptors to subsequent applications of glycine. Glycine (1 mM, 20 s) was applied (at 15-min intervals) three times before and three times after a 3-min application of DTT (1 mM) to oocytes expressing WT or mutant glycine receptors. (a) A tracing of a single WT response shows no change in current after reduction. Oocytes were unclamped during DTT treatments, so this portion of the tracing is not shown. (b) Mean currents in the WT receptor show no change with repeated applications of glycine and no change after reduction. (c) A tracing of a single oocyte expressing S267C/A288C shows that exposure to 1 mM

glycine resulted in decrease in subsequent glycine responses, and that reduction causes an increase in the glycine response. (d) The mean currents in the S267C/A288C receptor show the decreasing current elicited by glycine before reduction, and recovery of current after reduction. (e and f) Mean currents in the S267C and A288C single mutants also show no change in current with repeated applications of glycine and no change after application of DTT. Mean values \pm SEM are shown for $n = 5$ –13 oocytes per condition from two to five batches of oocytes. * $p < 0.05$, *** $p < 0.001$ as compared to final glycine response pre-reduction by one-way analysis of variance followed by Dunnett's post-test.

literature should have appropriate positions. This molecular model positioned S267C and A288C in proximity to form a disulfide bond. Residues S267 and A288 were replaced with cysteines and an S–S bond was formed between them. All backbone atoms (C, Ca, N, O) were tethered to their initial positions with a force constant of 100 kcal/Å² and the structure was subjected to restrained molecular mechanics optimization with the CFF91 force field using Insight II (v 2000.1, Accelrys, San Diego, CA, USA).

Results

Effect of reduction on glycine currents in WT and mutant GlyRs

In WT glycine receptors, repeated exposures to 1 mM glycine elicited similar currents over time, and a 3-min application of 1 mM DTT produced no significant change in the receptor function (Figs 1a and b). Unlike the WT, exposure to glycine (1 mM) caused a run-down of the glycine response in the S267C/A288C mutant. This run-down was observed for 30 min after the first glycine application. Following application of 1 mM DTT, the response of S267C/A288C to glycine recovered significantly (Figs 1c and d). The single mutants, S267C and A288C, were tested as controls and displayed responses similar to the WT (Figs 1e and f). Oocytes co-injected with a 1 : 1 ratio of S267C + A288C did not show a current run-down and were similar to the WT, indicating that intersubunit cross-linking was not occurring ($n = 8$, data not shown).

Effect of reduction on GlyR α 1(S267C/A288C) leak current, tonic activity, and baseline current

Immediately after clamping, a large, inward leak current, sensitive to strychnine, was apparent in the majority of GlyR α 1(S267C/A288C)-expressing oocytes tested. This current, never seen in the WT receptor, declined immediately from the time of clamping to reach a stable baseline within 5–15 min. When a stable baseline was reached, all experiments were performed. WT glycine receptors do not respond to the channel antagonist strychnine in the absence of glycine. The S267C/A288C mutant; however, displayed a tonic current after reaching a stable baseline that was reduced by strychnine, which closed the open channels. This resulted in a decrease of the tonic inward current, suggesting that some channels were open in the absence of glycine (Fig. 2a). The strychnine effect on S267C/A288C receptors did not depend on prior activation with glycine. After reduction with 1 mM DTT, strychnine no longer had a significant effect on the mutant receptors (Figs 2a and b). After reduction, there was no leak current observed upon clamping. Also, the baseline shifted, indicating that the spontaneous inward leakage current was reduced in the mutant receptor and that the mutant channels had closed. This shift in baseline was significantly different from the WT receptors (Fig. 2c).

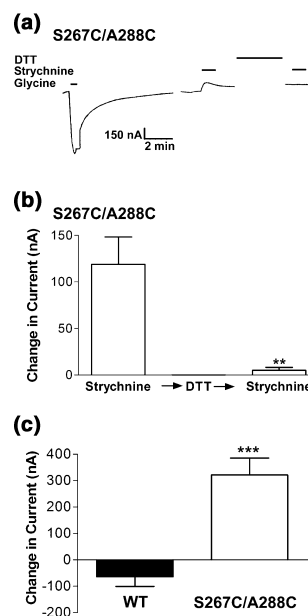


Fig. 2 Effects of reduction on S267C/A288C tonic activity and baseline shift following reduction. (a) Unlike WT receptors that do not respond to applications of strychnine, S267C/A288C receptors close with a 10 μ M strychnine application (40 s). Although shown after activation with glycine in the tracing, the strychnine response did not depend on prior activation with glycine. After reduction with DTT (1 mM, 3 min), strychnine no longer had an effect. (b) The mean decrease in the tonic inward current of S267C/A288C receptors by 10 μ M strychnine before and after reduction ($n = 9$ oocytes per condition from three batches of oocytes). $**p < 0.01$ as compared to the effect before reduction by the Student's t -test. (c) The baseline current shifted after reduction in the S267C/A288C mutant and appeared as a decrease in inward current. The change in baseline of the WT was compared to S267C/A288C. Mean values \pm SEM are shown for $n = 14$ WT oocytes from five batches of oocytes, and from $n = 34$ mutants from 16 batches of oocytes. $***p < 0.001$ as compared to the WT using the Student's t -test.

Effects of reduction and cross-linking on WT and S267C/A288C glycine receptors

We also asked if the mutant receptor could be cycled between its aberrant and WT characteristics by reduction with DTT, followed by re-cross-linking with HgCl₂ (Soskine *et al.* 2002) and then a second application of DTT. This experiment tested the reversibility of covalent bond formation between these two amino acids in the mutant receptor. In the WT receptors there was no significant change in current (elicited by 1 mM glycine) after applications of either DTT or HgCl₂ (Figs 3a and c). For either S267C or A288C, there was no significant change in mean current after applications of either DTT or HgCl₂, though both showed variable responses (some oocytes showed a decrease or an increase in current, while others showed no change) after the HgCl₂ applications (Fig. 3c). The current of the S267C/A288C mutant receptor

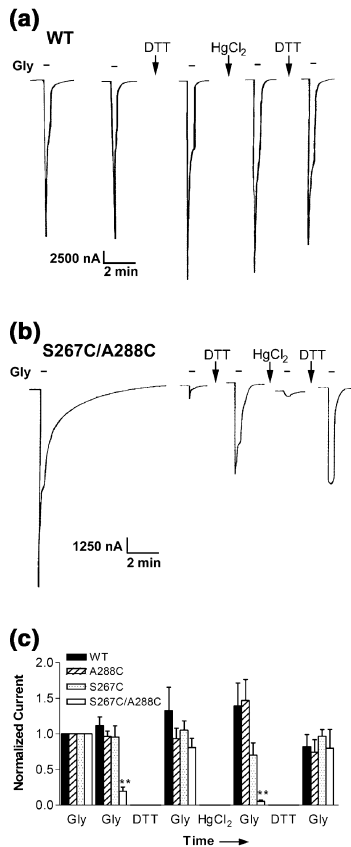


Fig. 3 Effects of reduction and cross-linking on WT, A288C, S267C, and S267C/A288C glycine receptors currents. Two applications of glycine (1 mM, 20 s) were applied at 15-min intervals, followed by reduction with DTT (10 mM, 3 min), glycine (1 mM), cross-linking with HgCl₂ (10 μ M, 1 min), glycine (1 mM), a second reduction step with DTT (10 mM, 3 min), and a final test with glycine (1 mM). (a) This tracing of the glycine response in the WT receptor shows that the current does not change significantly after reduction and cross-linking. (b) A tracing from the S267C/A288C mutant shows that reduction increases the glycine response, mercuric chloride nearly eliminates the current induced by 1 mM glycine, and that a subsequent reduction step again increases the glycine response. (c) The normalized mean currents \pm SEM are shown for the WT, A288C, S267C, and S267C/A288C receptors. Currents were normalized to 1.0 by dividing the currents of the WT and mutant receptors by the current induced by the initial glycine application. The data represent mean currents from $n = 5$ –6 oocytes per condition from two to four batches of oocytes. One-way analysis of variance followed by the Dunnett's post-test was used to determine differences in the mutant glycine responses in comparison to the respective WT response (** $p < 0.01$).

was altered significantly after applications of DTT or HgCl₂, with DTT causing a significant increase in the receptors' response and HgCl₂, causing a significant decrease in receptor function. These functional changes were measured 15 min after the DTT or HgCl₂ applications (Figs 3b and c).

Effects of alcohols and volatile anesthetics on WT and mutant glycine receptors

The effects of two alcohols (ethanol and octanol) and two volatile anesthetics (isoflurane and chloroform) were tested on the WT, S267C, A288C, and S267C/A288C receptors. The EC_{5–10} of glycine was determined for each oocyte. Responses were tested after a 1-min pre-incubation of the drug, using the EC_{5–10} glycine. The single S267C mutation reduced or eliminated all drug responses tested except that of isoflurane. Meanwhile, the A288C mutation reduced or eliminated all drug responses tested except chloroform. The double mutant, S267C/A288C, reduced or eliminated the responses of all four drugs in comparison to the WT receptors. The mean responses of the WT and mutant receptors to these four drugs and the average EC_{5–10} glycine values of the receptors are presented in Table 1.

Effects of volatile anesthetics on S267C/A288C glycine receptors before and after reduction

To determine whether reduction of the S267C/A288C receptors altered the drug responses, the drugs were tested approximately 45 min after application of 1 mM DTT while the receptors were in a uniformly reduced state. After reduction, the EC_{5–10} glycine was re-determined for each oocyte (average = 40 \pm 15 μ M), and the drugs were tested. The two alcohols still had no effect (data not shown), but the two anesthetics now potentiated the glycine response. The response of the reduced mutant to isoflurane was significantly greater than the response of the cross-linked S267C/A288C receptors, but did not recover to the level seen in the WT. Additionally, the response of the reduced S267C/A288C receptors to chloroform was significantly larger than that of the cross-linked receptors and recovered to a response similar to that of the WT receptors (Fig. 4).

Discussion

These results suggest that an intrasubunit disulfide bond forms between S267C and A288C in GlyR α 1(S267C/A288C) receptors. These two amino acids can covalently react with one another to link TM2 and TM3. Disulfide bond formation can occur spontaneously, in the absence of additional oxidizing or cross-linking agents, to change the receptor's characteristics. Reduction of the disulfide bond with dithiothreitol largely restores receptor function to that of WT. Also, these cysteines can be cross-linked again with an application of mercuric chloride, which adds a 0.4 nm bridge between the two cysteines.

One possibility is that disulfide bonds may form between S267C and A288C during protein folding and processing in the oxidizing environment of the endoplasmic reticulum. However, considering that one of the characteristics of this double mutant is a decrease in the current induced after an application of glycine, it seems likely that some disulfide

Table 1 Glycine EC₅₋₁₀ and alcohol and volatile anesthetic responses of the WT glycine receptors and the cysteine substitution mutants

Glycine receptor	EC ₅₋₁₀ (μM)	Percentage potentiation			
		Ethanol	Octanol	Isoflurane	Chloroform
WT	63 ± 4	80 ± 10	170 ± 20	450 ± 50	200 ± 30
S267C	43 ± 6	0 ± 6†	90 ± 30*	520 ± 150	10 ± 10†
A288C	230 ± 10†	- 10 ± 10†	30 ± 20†	150 ± 30†	190 ± 30
S267C/A288C	95 ± 10	10 ± 4†	20 ± 7†	110 ± 20†	60 ± 10†

Percentage potentiation of EC₅₋₁₀ glycine responses to ethanol (100 mM), octanol (115 μM), isoflurane (0.77 mM), and chloroform (2.0 mM) are expressed as a mean ± SEM of 4–28 oocytes per condition from two to 17 batches of oocytes. S267C/A288C receptors were tested for their responses here after application of maximal glycine and EC₅₋₁₀ glycine determination in the uniform cross-linked state. **p* < 0.05, †*p* < 0.01; significantly different from WT receptors by one-way ANOVA with Dunnett's post-test.

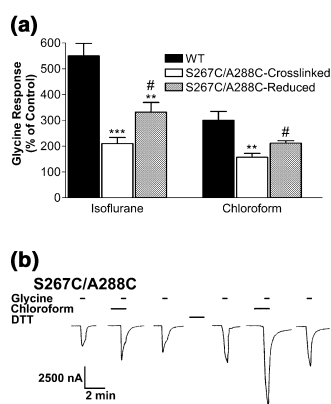


Fig. 4 Effects of volatile anesthetics on the WT glycine receptor and drug effects on S267C/A288C before and after reduction. (a) Isoflurane (0.77 mM) and chloroform (2.0 mM) both potentiate the WT (■) response to EC₅₋₁₀ glycine. These effects are smaller in the cross-linked S267C/A288C mutant (□). After reduction with 1 mM DTT (3 min), the max glycine response and EC₅₋₁₀ glycine was re-determined to test the drugs a second time. Post-reduction, both isoflurane and chloroform potentiated the glycine response to a greater extent than before (▒). Mean values ± SEM are shown for *n* = 4–7 oocytes per condition from at least two batches of oocytes. ***p* < 0.01, ****p* < 0.001, as compared to WT potentiation and #*p* < 0.05 as compared to cross-linked receptor potentiation, Student's *t*-test. (b) This example tracing of the S267C/A288C mutant shows the effect of chloroform on the EC₅₋₁₀ glycine response in the cross-linked and reduced states in a single oocyte.

bonds are forming during the process of channel gating. Support for this hypothesis includes evidence that in the GABA_A receptor the presence of GABA increases accessibility to amino acids in TM3, indicating movement of TM3 with channel gating (Williams and Akabas 1999). Also, in the glycine receptor, sulfhydryl-specific reagents react with the glycine receptor single cysteine substitution mutant A288C in the open state of the receptor, but not in the closed state (Harris *et al.* 2003). These results indicate that a conformational change occurs with channel gating to enlarge a water-filled, intrasubunit cavity and place residue 288 in the

putative drug-binding cavity facing S267. This movement could place S267C and A288C in proximity to form a disulfide bond. A recent abstract suggests a very low level of cross-linking using oxidizing agents between cysteines introduced into proximate positions (S270C/V292C) of the GABA_A receptor (Bali and Akabas 2003), which may generalize our findings in the glycine receptor to the rest of the ligand-gated ion channel family.

Intersubunit cross-linking has been demonstrated between TM2 segments in the GABA receptor (Horenstein *et al.* 2001). To further test our hypothesis that S267C and A288C faced a common pocket within each subunit, rather than facing the interface and forming intersubunit cross-links, we compared the behavior of S267C/A288C double mutants with oocytes co-injected with a 1 : 1 ratio of S267C + A288C cDNAs. The co-injected single mutant subunits behaved like the WT rather than the S267C/A288C double mutant, indicating that intersubunit cross-linking was not occurring. This supports our model that S267C and A288C face a common intrasubunit pocket.

Conformational analysis of disulfide bridges in high resolution crystal structures indicate the distance between the alpha carbons nearest to the sulfur atoms of covalently bound cysteines range from 0.46 to 0.74 nm (Thornton 1981). Analysis of 351 disulfide bridges showed the most common distance is approximately 0.56 nm with a Cα-Cβ-S (C-C-S) bond angle of 114 degrees (Petersen *et al.* 1999). From our molecular model of the glycine receptor transmembrane regions, S267C and A288C, are positioned at the interface between TM2 and TM3. The residues are in close enough proximity to form a right-handed disulfide bond with a distance and angle comparable to those above. In this model, the distance between the alpha carbons of S267 and A288 is 0.7 nm and the C-C-S bond angle is 112 degrees (Fig. 5). The two residues are nearer to one another than the ~1.0 nm separation previously reported for the GABA_A receptor (Bali and Akabas 2004).

Endogenous disulfide bonds necessary for glycine receptor function are located in the extracellular domain of the

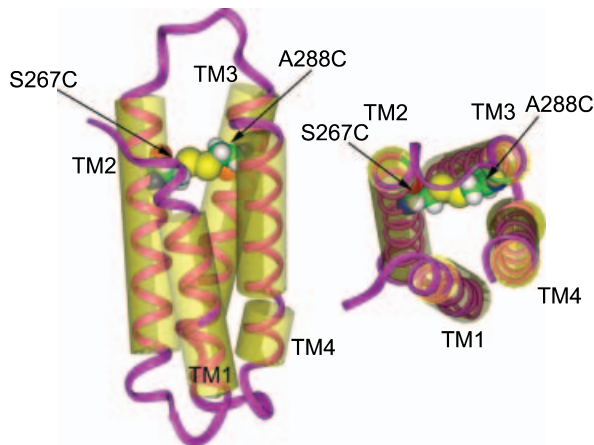


Fig. 5 Model of the disulfide bond formed between S267C and A288C of the GlyR α 1(S267C/A288C) receptor. Our data indicate disulfide bond formation in the absence of oxidizing agents between introduced cysteines at positions S267 and A288. This molecular model of the glycine receptor transmembrane regions illustrates this disulfide bond. The distance between the alpha carbons of S267 and A288 is 0.7 nm and the C-C-S bond angle is approximately 112 degrees.

receptor (Rajendra *et al.* 1995, 1997). These disulfide bonds must be well protected because the concentrations of DTT used in our experiments did not alter the WT glycine receptor function. Others have also noted that exposure of WT GlyRs and GABA $_A$ receptors to DTT had no significant effect on current magnitude following washout (Horenstein *et al.* 2001; Lynch *et al.* 2001; Kash *et al.* 2003). Also, there is an endogenous cysteine in TM3 (C290) of the WT and mutant receptor subunits. There may be a very low level of cross-linking between S267C and C290C because the S267C single mutant showed a non-significant trend in responses similar to S267C/A288C. Overall, because the currents of S267C were statistically similar to WT, C290 does not seem to be involved in cross-linking with the cysteine introduced in TM2 and other free cysteines in the WT to any significant extent.

Interestingly, all four of the alcohols and anesthetics tested had eliminated or reduced effects in the S267C/A288C mutant. The simplest explanation for the alcohol data is that alcohol potentiation for the glycine receptor is abolished by the cysteine substitutions independent of formation of disulfide bonds. It is not surprising that reduction cannot restore the WT effects of alcohols in the double mutant, as the single mutant S267C does not respond to ethanol, and A288C responds to neither ethanol nor octanol. In contrast, after reduction with dithiothreitol, the two volatile anesthetics tested were able to potentiate the S267C/A288C receptors. Isoflurane and chloroform both cause smaller potentiations than the WT receptors when the S267C/A288C receptors are cross-linked. After reduction, the chloroform potentiation of S267C/A288C recovered to be similar to WT and the potentiation by isoflurane increased. From these data, it is clear that the

disulfide bond is interfering with allosteric modulation by anesthetic drugs on this region of the receptor. One possibility is that reduction of the disulfide bond removes this obstruction from the binding site and increases the volume of the drug-binding cavity to allow the anesthetics to stably bind and cause receptor potentiation.

Natural disulfides serve to stabilize protein structure by decreasing the degrees of freedom of movement (or entropy) of the unfolded state, forcing the equilibrium to the folded state (Wetzel 1987). Likewise, introduced disulfide bridges have been shown to result in increased stability of mutant proteins (Matsumura *et al.* 1989). In the case of the S267C/A288C mutant, cross-linking the transmembrane segments to one another results in a functioning protein with an unnaturally limited flexibility. In particular, the region of TM2 where S267 is located has been demonstrated to have high flexibility in nuclear magnetic resonance experiments with TM2 peptides (Yushmanov *et al.* 2003). Because this region plays a role in conducting the signal of agonist binding to the TM2–3 linker and into TM2 (Kash *et al.* 2003), it makes sense that our introduced disulfide bond produces changes in channel function.

Restriction of the dynamic movement of TM2 by covalently linking it to TM3 changes channel properties. WT glycine receptors exist in multiple receptor states: the unliganded closed state, and many liganded open and desensitized states. The normal dynamics between these receptor states are disturbed by the double bond between S267C and A288C, and this is noticeable at the whole-cell level. As the population of S267C/A288C mutant channels open with the first application of glycine, they take much longer to close than the WT receptors, indicating the difficulty in moving from one channel conformation to another. Additionally, further applications of glycine produce minimal glycine responses in the mutant receptors, indicating that they may be preferentially stabilized in the desensitized state or ‘frozen’ in the resting state, where they are not responsive to glycine. These characteristics are eliminated with reduction, which shows that once the restrictive disulfide bonds are broken, the channels can behave in a more similar manner to the WT channels.

These data demonstrate the orientation and near-neighbor proximity of S267 and A288 because of the ability of these two amino acids to form a disulfide bond. These data locate the vertical position of TM2 with respect to TM3 and shows that they face one another. Most importantly, disulfide bonding between these two introduced cysteines provides insight regarding the location and role of the TM2–TM3 interface.

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