

GABA-Induced Intersubunit Conformational Movement in the GABA_A Receptor α 1M1- β 2M3 Transmembrane Subunit Interface: Experimental Basis for Homology Modeling of an Intravenous Anesthetic Binding Site

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The molecular basis of general anesthetic interactions with GABA_A receptors is uncertain. An accurate homology model would facilitate studies of anesthetic action. Construction of a GABA_A model based on the 4 Å resolution acetylcholine receptor structure is complicated by alignment uncertainty between the acetylcholine and GABA_A receptor M3 and M4 transmembrane segments. Using disulfide crosslinking we previously established the orientation of M2 and M3 within a single GABA_A subunit. The resultant model predicts that the β M3 residue β 2M286, implicated in anesthetic binding, faces the adjacent α 1-M1 segment and not into the β 2 subunit interior as some models have suggested. To assess the proximity of β 2M286 to the α 1-M1 segment we expressed β 2M286C and γ 2 with 10 consecutive α 1-M1 cysteine (Cys) mutants, α 1I223C to α 1L232C, in and flanking the extracellular end of α 1-M1. In activated states, β 2M286C formed disulfide bonds with α 1Y225C and α 1Q229C based on electrophysiological assays and dimers on Western blots, but not with other α 1-M1 mutants. β 2F289, one helical turn below β 2M286, formed disulfide bonds with α 1I228C, α 1Q229C and α 1L232C in activated states. The intervening residues, β 2G287C and β 2C288, did not form disulfide bonds with α 1-M1 Cys mutants. We conclude that the β 2-M3 residues β 2M286 and β 2F289 face the intersubunit interface in close proximity to α 1-M1 and that channel gating induces a structural rearrangement in the transmembrane subunit interface that reduces the β M3 to α M1 separation by \sim 7 Å. This supports the hypothesis that some intravenous anesthetics bind in the β M3- α M1 subunit interface consistent with azi-etomidate photoaffinity labeling.

Introduction

Since the first successful use of general anesthesia in surgery in 1846 scientists have sought to understand the molecular and cellular basis of general anesthetic action. During the past decade, investigators recognized the importance of general anesthetic interactions with GABA_A receptors (Franks, 2008). To elucidate the molecular basis of these interactions, research has sought to determine the GABA_A receptor structure and the location of anesthetic binding sites.

GABA_A receptors belong to the Cys-loop receptor neurotransmitter-gated ion channel superfamily (Akabas, 2004). Subunits have an \sim 200 residue extracellular domain and four transmembrane segments (M1, M2, M3, M4). Medium to high resolution structures exist for the *Torpedo* nicotinic acetylcholine (ACh) receptor (4 Å resolution) (Unwin, 2005), a prokaryotic

homolog (3.3 Å resolution) (Hilf and Dutzler, 2008), and the snail ACh binding proteins (Celie et al., 2004). Difficulties obtaining sufficient quantities of eukaryotic membrane proteins make a GABA_A receptor crystal structure unlikely in the near future. Homology models could provide useful GABA_A molecular models for the interpretation of experimental results.

Sequence alignment is a critical step in homology modeling. Absolutely conserved residues between the pre-M1 region and the extracellular end of M2 provide confidence in the GABA_A-ACh sequence alignment in this region. The alignment of the M2-M3 loop and M3 segment are uncertain because of the lack of absolutely conserved residues in this region and to differences in the M2-M3 loop length of up to two amino acids. Previously, we used disulfide crosslinking between pairs of engineered cysteines (Cys) within the α 1-M2 and M3 segments to demonstrate that GABA_A M3 residues α 1L301 and α 1F304 were in close proximity to α 1T267 (M2-12') (Jansen and Akabas, 2006). In the ACh receptor structure, α I289 and α T292 in M3 are closest to M2-12'. This provides an experimental basis for constructing a GABA_A receptor model to the end of the M3 segment.

Residues implicated in general anesthetic action include the M2-15' position, aligned with β 2N265, and a position near the M3 extracellular end, aligned with β 2M286 (Belelli et al., 1997; Mihic et al., 1997; Hemmings et al., 2005). β 2M286 mutations

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altered the cutoff size for long chain alcohol effects (Krasowski et al., 2001). Propofol, an intravenous general anesthetic, protected β 2M286C from covalent modification by sulfhydryl-reactive reagents (Bali and Akabas, 2004). Azi-etomidate, a photo-labile anesthetic, labeled both β 2M286 and α 2M236 suggesting that the etomidate binding site is in the subunit interface (Li et al., 2006). In earlier GABA_A models, β N265 and β M286 were positioned facing the interior of a single subunit (Trudell and Bertaccini, 2004; Hosie et al., 2006; Campagna-Slater and Weaver, 2007). Our GABA_A receptor homology model (Jansen and Akabas, 2006) predicts that β 2M286 faces the subunit interface in close proximity to the α 1-M1 segment. We tested the hypothesis that β 2M286 is in close proximity to residues in or flanking the extracellular end of α 1-M1. The results validate our homology model and suggest that channel activation decreases the intersubunit separation between β 2-M3 and α 1-M1. The results are consistent with the idea that some intravenous anesthetics bind in the transmembrane subunit interface.

Materials and Methods

Constructs used, mRNA preparation, and oocyte expression. Because the rat α 1 and β 2 subunits have endogenous Cys in the M1 and M3 segments we used Cys-light constructs for these subunits to ensure that the endogenous Cys did not complicate the interpretation of our results. In the Cys-light constructs the endogenous α 1C234 (M1) and α 1C293 (M3) were mutated to serine, and β 2C288 (M3) was mutated to alanine. In this study we refer to the α 1C234S-C293S β 2C288A γ 2s as the wild-type Cys-light construct. All engineered Cys mutants were made and expressed in this background. All mutants were made using the QuikChange method (Stratagene) and sequenced completely to confirm the mutations. The α 1 Cys mutants studied in this work cover the range 223-IGYFVIQTYL-232.

Preparation and injection of mRNA in oocytes was performed as described previously (Bali and Akabas, 2007). Briefly, capped mRNA was synthesized by *in vitro* transcription using T7 RNA polymerase mMessage mMachine kit (Ambion) from *Nhe*I-linearized (New England Biolabs) pGEMHE vectors containing rat GABA_A α 1, β 2, γ 2s or the various mutants used. *Xenopus laevis* oocytes were harvested and defolliculated with collagenase for 1 h at 16°C in SOS medium (in mM): 100 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, and 5 HEPES, pH 7.5 with 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 250 ng/ml amphotericin B (Invitrogen) supplemented with 5% horse serum (Sigma-Aldrich). Oocytes were injected with 50 nl of 0.2 ng/ml mRNA 24–48 h after isolation and maintained at 16°C. The ratio of mRNA α 1: β 2: γ 2 injected was 1:1:2. We previously showed based on Zn²⁺ and diazepam sensitivity that this ratio gave us uniform incorporation of γ 2 into the surface expressed, functional receptors (Horenstein et al., 2005).

Electrophysiology. Two-electrode voltage-clamp experiments were conducted at room temperature 3–5 d after mRNA injection as described previously (Bali and Akabas, 2007). Oocytes, in an \sim 200 μ l chamber, were voltage clamped at -60 mV and perfused at 5–6 ml/min with calcium-free frog ringer (CFRR) solution (in mM) 115 NaCl, 2.5 KCl, 1.8 MgCl₂, 10 HEPES, pH 7.5 with NaOH. Currents were recorded and analyzed using a TEV-200 amplifier (Dagan Instruments), Digidata 1322A analog-digital converter and pClamp 8.2 software (Molecular Devices). The ground electrode was connected to the bath via a 3 M KCl/Agar bridge. Glass microelectrodes were filled with 3 M KCl and had a resistance <2 M Ω . All experiments were performed on at least three oocytes from at least two different batches of oocytes.

Stock solutions of GABA, DTT, CuSO₄ in water were prepared at concentrations of 0.1, 1.0, and 0.1 M, respectively. 1,10-phenanthroline (phen) was prepared as a 1.0 M stock solution in DMSO. Reagents were diluted into CFRR just before each experiment. DMSO concentration never exceeded 0.1%, a concentration at which no effect is observed on GABA-induced currents (data not shown). Copper phenanthroline (Cu:phen) solutions contained 100 μ M CuSO₄ and 200 μ M phenanthroline.

Reagent applications were separated by 4–5 min wash periods to allow

Table 1. Characterization of the GABA-activated currents of the single Cys mutants

	EC ₅₀ (μ M)	n_H	I_{max} (nA)	<i>n</i>
WT Cys-light	4.0 \pm 0.9	1.5 \pm 0.1	4350 \pm 240	4
α 1I223C	20 \pm 4	1.2 \pm 0.2	560 \pm 320	3
α 1G224C	15 \pm 5	0.8 \pm 0.2	2100 \pm 570	3
α 1Y225C	2.0 \pm 0.5	1.7 \pm 0.3	4500 \pm 1030	3
α 1F226C	2.7 \pm 0.4	1.7 \pm 0.1	1700 \pm 190	5
α 1V227C	2.2 \pm 0.4	1.2 \pm 0.05	1800 \pm 450	3
α 1I228C	1.6 \pm 0.3	0.7 \pm 0.2	5780 \pm 220	6
α 1Q229C	2.0 \pm 1.0	1.1 \pm 0.4	3860 \pm 290	5
α 1T230C	16 \pm 3.5	1.1 \pm 0.1	2430 \pm 250	3
α 1Y231C	^a	^a	20 nA at 200 μ M	5
α 1L232C	1.5 \pm 0.5	0.9 \pm 0.2	2670 \pm 260	3
β 2M286C	15	1.2		
β 2F289C	3.5 \pm 0.75	1.5 \pm 0.11	1700 \pm 260	3

GABA concentration–response relationships were determined by measuring the currents induced by sequential application of increasing concentrations of GABA to oocytes expressing the single Cys mutants in α 1 β 2 γ 2 receptors. α 1 and β 2 subunits were the Cys-light versions. Currents were normalized to the maximal current and fitted by nonlinear regression to the Hill equation. EC₅₀ is the GABA concentration inducing half-maximal responses, n_H is the Hill coefficient, and I_{max} is the maximal GABA-induced current. Means \pm SEM are given. *n*, Number of oocytes tested. Data for β 2M286C are from Bali and Akabas (2004).

^aCurrents too small to determine EC₅₀.

complete washout and recovery from desensitization. *p*-Chloromercuribenzenesulfonate (pCMBS[−]) was obtained from Toronto Research Chemicals.

Western blotting. Four days after mRNA injection, oocytes were washed once with CFRR. Cu:phen/GABA (100:200 μ M/1 μ M) was then applied for 2 min on intact oocytes. Surface proteins were biotinylated with 0.5 mg/ml sulfo-NHS-LC-biotin (Pierce) for 30 min at room temperature (RT) in CFRR, pH 8. Oocytes were then washed with CFRR and Tris-NaCl buffer (100 mM NaCl, 20 mM Tris, pH 7.5), tritirated at 4°C in 20 μ l/oocyte lysis buffer (Tris-NaCl buffer plus 1% Triton X-100, 0.5% deoxycholate, 10 mM *N*-ethyl maleimide, and HALT protease inhibitor cocktail (Pierce), solubilized by rotating at 4°C for 2 h, and spun twice at 16,000 \times g, 4°C, 20 min to remove debris and yolk. Biotinylated proteins were bound to streptavidin beads (Pierce) by rotating for 4 to 16 h at 4°C. Beads were washed with lysis buffer and bound proteins eluted with 4 \times SDS-sample buffer for 10 min, 37°C. Proteins were separated by SDS-PAGE (4–15% PAGE gel) (Bio-Rad), transferred to PVDF membranes (Bio-Rad) and processed according to standard procedures using a primary rabbit anti-GABA_A α 1 polyclonal (AB9820, Millipore Bioscience Research Reagents) and secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce) antibodies and ECL (Pierce).

Homology model. The construction of the homology model was described previously (Jansen and Akabas, 2006). All distance measurements are based on α carbon separation and were made using Deepview Swiss PDB Viewer version 3.7 (<http://spdbv.vital-it.ch/>). Figures were generated either in Deepview and rendered using PovRay version 3.6 (Persistence of Vision Pty. Ltd.) or using Pymol (DeLano Scientific LLC).

Statistical/data analysis. Results are presented as mean \pm SEM. GABA concentration–response relationships were determined by nonlinear regression fits to the Hill equation using Prism 4 software (GraphPad Software) as we have done previously (Bali and Akabas, 2004).

Results

Expression and functional characterization of the α 1-M1 Cys mutants

Coinjection of mRNA encoding each of the 10 individual α 1-M1 Cys mutants (223-IGYFVIQTYL-232) with wild-type Cys-light β 2 and wild-type γ 2 resulted in functional GABA-activated currents for all of the single α 1 Cys mutants. The GABA EC₅₀ for wild-type Cys-light was 4.0 \pm 0.9 μ M and for the mutants ranged from 1.5 \pm 0.5 μ M for α 1L232C to 20 \pm 4 μ M for α 1I223C (Table 1). The average currents induced by saturating concentrations of GABA were 4300 \pm 240 nA for wild-type Cys-light and for the mutants ranged from 20 nA for α 1Y231C to 5800 \pm 220 nA for

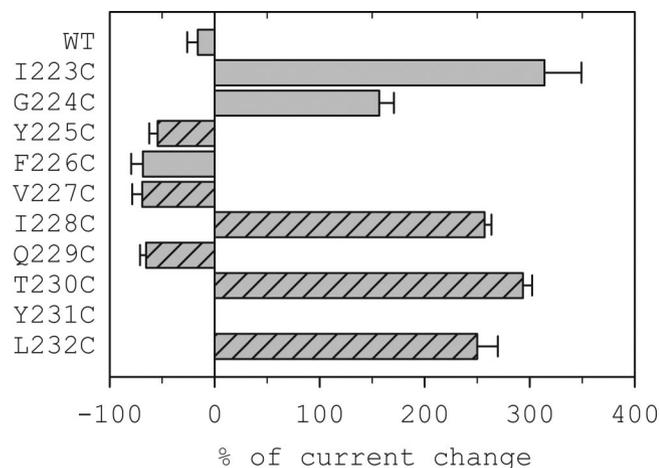


Figure 1. Effect of pCMBS[−] on GABA-induced current in α_1 Cys mutants expressed in *Xenopus* oocytes. GABA_A receptors with mutated α_1 subunits were treated with 200 μ M pCMBS[−] for 2 min and the resulting GABA-induced current was normalized relative to the current before treatment. To maximize the effect of pCMBS[−] modification to avoid missing reactive Cys mutants the GABA test concentration was adjusted based on the effect of pCMBS[−] modification. For mutants in which the subsequent currents were potentiated the GABA test concentration was EC₂₀–EC₃₀. For mutants in which pCMBS[−] application inhibited subsequent GABA currents the GABA test concentration was \sim EC₆₀–₈₀. The bars represent the percentage change for at least 3 cells per mutant. One way ANOVA followed by Holm–Sidak *post hoc* test shows a statistically significant difference for all nine α_1 M1 Cys mutants relative to WT Cys-light receptors. Hatched bars indicate Cys mutants that after modification by pCMBS[−] showed an increased holding current in the absence of GABA most likely due to an increase in spontaneous opening. The initial holding currents for oocytes expressing all of the mutants were similar to those in oocytes expressing wild-type receptors indicating that there was no significant increase in spontaneous channel opening in the unmodified Cys mutants.

α 1I228C (Table 1). A 90 s application of 5 mM DTT to α 1Y231C β 2 γ 2 receptors did not affect subsequent GABA-induced current amplitude (data not shown). The currents for α 1Y231C were too small and this mutant was not studied in additional experiments. The initial leak current before application of pCMBS[−] was not significantly different for any of the single Cys mutants compared with wild type. We infer that the Cys mutations did not significantly increase the spontaneous open probability for the mutant receptors.

Accessibility of α 1-M1 Cys mutants to pCMBS[−]

We tested the ability of the sulfhydryl-reactive reagent pCMBS[−] to react with each of the α 1-M1 Cys mutants. Application of 200 μ M pCMBS[−] for 2 min to oocytes expressing the wild-type Cys-light α 1 β 2 γ 2 had no effect on the subsequent GABA-induced currents (data not shown). In contrast, a 2 min application of 200 μ M pCMBS[−] in the absence of GABA to the α 1-M1Cys β 2 γ 2 single Cys mutant receptors irreversibly modified the subsequent GABA-induced currents in all nine of the α 1-M1 Cys mutants (Fig. 1). pCMBS[−] application inhibited the subsequent GABA-induced currents for four of the mutants (α 1Y225C, α 1F226C, α 1V227C, α 1Q229C) but potentiated subsequent GABA currents in the other five mutants (Fig. 1). For the β 2-M3 Cys mutant, we previously showed that application of pCMBS[−] to α 1 β 2M286C γ 2 receptors increased the subsequent GABA-induced currents by 75% (Bali and Akabas, 2004). For the β 2F289C mutant, pCMBS[−] application both increased the holding current and diminished subsequent GABA currents. However, the total current, holding + GABA-activated, was similar to the initial currents before pCMBS[−] application. We infer that pCMBS[−] covalently modified the engineered Cys residue in each

of these mutant receptors and that to react with pCMBS[−], the engineered Cys residues were at least transiently on the water-accessible surface of the protein.

The extensive accessibility of the M1 Cys mutants to reaction with pCMBS[−] suggests that this region of the protein is not tightly packed with other protein regions because tight protein packing would preclude pCMBS[−] from entering the region. pCMBS[−] would fit into a right cylinder that is \sim 6 Å in diameter and 10 Å in length. Thus, crevices or cavities at least this size must exist to allow pCMBS[−] to access these Cys mutants. Alternatively, this region may be relatively mobile, transiently exposing residues to the water accessible surface of the protein to allow reaction. Previous substituted cysteine accessibility method (SCAM) studies of the aligned region in the ACh receptor α subunit showed extensive accessibility to reaction with methanethiosulfonate reagents although a more limited pattern of accessibility was observed in the ACh β subunit M1 segment (Akabas and Karlin, 1995; Zhang and Karlin, 1997). The basis of these differences is uncertain, however, the ACh β subunit corresponds to the GABA_A γ 2 subunit, because neither forms part of the agonist binding sites.

Disulfide bond formation of α 1-M1 Cys mutants with β 2M286C under ambient oxidizing conditions

Coexpression of the nine individual α 1-M1 Cys mutants with β 2M286C and γ 2 also resulted in GABA-induced currents for all nine double Cys mutant pairs. We noted that for the α 1Q229C β 2M286C γ 2 receptors, with repeated GABA applications, the amplitude of the GABA-induced current diminished with each subsequent GABA application until the currents plateaued at a level significantly smaller than the magnitude of the first GABA application (Fig. 2A). To determine whether channel activation was necessary for this reduction in current amplitude with repetitive GABA applications we examined the effect of the time interval between GABA applications on the decrease in GABA-induced current amplitude. Regardless of whether the GABA applications were separated by five or 10 min intervals, the extent of current decline was independent of the duration between GABA applications and was directly related to the cumulative duration of GABA application (Fig. 2B). We infer that channel activation was essential for this process. In the presence of GABA the channels fluctuate between open and desensitized states. We cannot distinguish whether the reaction occurred in one or both of these states, thus, we will refer to them collectively as the activated states to distinguish them from the closed, resting state that the channels occupy in the absence of GABA.

To determine whether the decreased current was due to disulfide bond formation or heavy metal binding we assayed the effects of EGTA and DTT application. EGTA binds polyvalent heavy metals with high affinity whereas DTT can both chelate heavy metals and reduce disulfide bonds. After reaching the plateau level, a 60 s application of 2 mM EGTA had no effect on the subsequent GABA-induced current amplitude (Fig. 2C). The lack of effect of EGTA implies that the decline in currents with GABA application was not due to heavy metal binding to the engineered Cys residues. In contrast, a 90 s application of 5 mM DTT largely reversed the decrease in GABA-induced currents and restored the currents to 85% of their initial level. Because EGTA had no effect and DTT restored the GABA-induced currents, we infer that the decrease was due to formation of a disulfide bond between α 1Q229C and β 2M286C. This implies that under ambient oxidizing conditions the rate of disulfide bond formation was significantly faster in the activated channel states, open and/or desen-

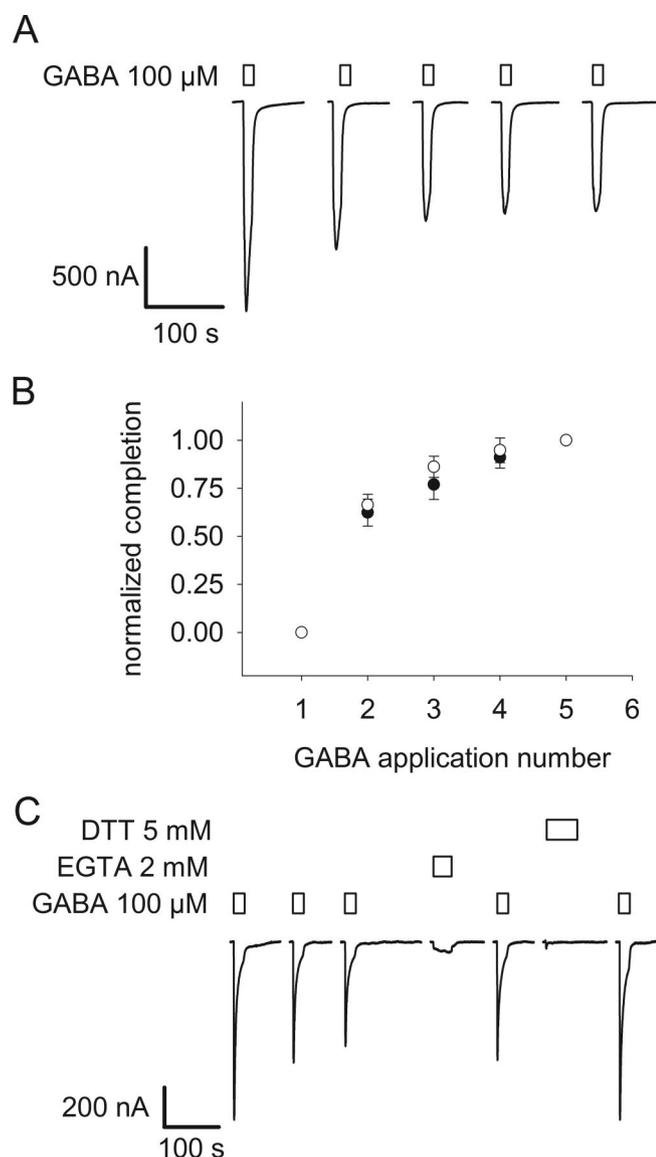


Figure 2. GABA-induced decrease of current in the double mutant α 1Q229C β 2M286C γ 2. **A**, GABA-induced currents in α 1Q229C β 2M286C γ 2 receptors decrease as a function of the application of GABA, here using a saturating concentration (100 μ M). **B**, Mean GABA-induced current normalized for the maximum effect (normalized completion = $(I_i - I_t)/(I_i - I_f)$, where I_i is the initial GABA current, I_t is the current at a time, t , and I_f is the final current) \pm SEM ($n = 3$) plotted as a function of the number of 10 s applications of an EC₅₀ GABA concentration (0.5 μ M) on α 1Q229C β 2M286C γ 2. Plain circles are the mean current \pm SEM for three oocytes plotted against the number of GABA applications, pulse frequency is one per 5 min; filled circles, pulse frequency is one per 10 min. **C**, The decrease in GABA-induced current is only reversed by 2 mM DTT in this representative experiment. Bars over traces indicate the period of application of the reagents shown to the left of each set of traces. Holding potential is -60 mV.

sitized, compared with the resting state in which the rate was negligible.

For the other eight α 1 Cys mutants coexpressed with β 2M286C γ 2, there were no changes in GABA-induced currents with repetitive applications of GABA. Furthermore, a 90 s application of 5 mM DTT did not alter the subsequent GABA currents indicating that disulfide bonds had not formed before the initial GABA application (data not shown). We infer that β 2M286C did not form disulfide bonds with the other eight α 1-M1 Cys mutants either spontaneously at rest or during channel activation under ambient oxidizing conditions.

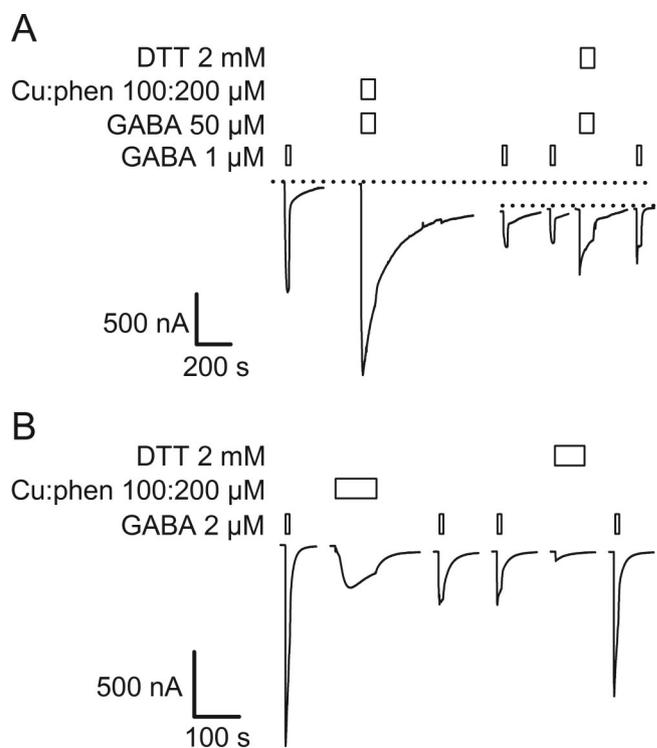


Figure 3. Oxidation alters GABA-induced currents in α 1Y225C β 2M286C γ 2 and α 1Q229C β 2M286C γ 2 receptors. **A**, Experiment illustrating the effect of application of 100:200 μ M Cu:phen in the presence of a saturating GABA concentration (50 μ M) on α 1Y225C β 2M286C γ 2 receptors. Subsequent GABA test currents are significantly diminished and the holding current in the absence of GABA after Cu:phen-induced oxidation is increased. The increased holding currents were blocked by picrotoxin (data not shown) and presumably result from an increased spontaneous open probability after disulfide bond formation. 2 mM DTT applied in the presence of GABA did not reverse the observed effect; a similar result was obtained with DTT applied in the absence of GABA (data not shown). **B**, Experiment showing a decrease in GABA-induced current after Cu:phen treatment of an oocyte expressing α 1Q229C β 2M286C γ 2 receptors. Application of 2 mM DTT significantly restored the currents to close to their initial level. The increase in current during Cu:phen application is reversible and was also observed in WT Cys-light receptors. This is due to an agonist effect of phenanthroline. Bars over traces indicate the period of application of the reagents shown to the left of each set of traces.

Copper phenanthroline induced oxidation at β 2M286C

Cu:phen creates a more oxidizing environment than exists under ambient conditions by catalyzing the formation of superoxide and hydroxyl radicals (Kobashi, 1968). We assayed the ability of Cu:phen to induce disulfide bond formation between the engineered Cys in the nine double mutants. A 60–90 s application of 100:200 μ M Cu:phen irreversibly diminished the subsequent GABA-induced currents for two of the Cys mutant pairs, α 1Y225C β 2M286C γ 2 and α 1Q229C β 2M286C γ 2 by $51 \pm 6\%$ ($n = 8$) and $62 \pm 5\%$ ($n = 8$), respectively (Fig. 3A,B). In the α 1Y225C β 2M286C γ 2 mutant, Cu:phen application also caused an increase in the holding current after Cu:phen without suggesting that it increased the spontaneous open probability of the receptors (Fig. 3A). For this double mutant a subsequent 90 s application of 5 mM DTT only restored a small percentage of the initial GABA-induced current. EGTA application (2 mM, 2 min) had no effect (data not shown). For the α 1Q229C β 2M286C γ 2 mutant, after Cu:phen application, a 60–90 s application of 2 mM EGTA, whether applied in the presence or absence of GABA, had no effect on the GABA-induced currents. In contrast, a 60–90 s application of 2 mM DTT restored the GABA currents to $86 \pm 5\%$ ($n = 8$) of their initial value. We also tested another reducing

agent, tris(2-carboxyethyl)phosphine (TCEP) and the results were similar (data not shown). Thus, 86% of the Cu:phen induced current decrease was restored by reduction with DTT. We infer that at least this fraction of the current decrease in response to Cu:phen application was due to disulfide bond formation. The inability to completely restore the GABA-induced currents suggests either that the disulfide bond was formed in a restricted space that limited access of the reducing agents DTT or TCEP to the disulfide, or some of the Cys residues may have been oxidized to higher order oxidation states (Cline et al., 2004). Neither DTT nor TCEP can reduce higher order sulfur oxidation states. Application of 100 μ M CuSO₄ had no irreversible effects on either of these double mutants.

Both Cu:phen and phenanthroline by itself appeared to act as GABA_A receptor activators. Application of 200 μ M phenanthroline induced a current in wild type and in some of the double Cys mutants that was as large as 20% of maximal GABA currents. This was completely reversible after phenanthroline washout. Thus, during the Cu:phen application the channels were in a mixture of closed and activated (open/desensitized) states.

In each α 1 β 2 γ 2 GABA_A receptor there are two β 2M3- α 1M1 interfaces. We tested whether any of the α 1Q229C β 2M286C γ 2 engineered Cys residues were accessible to react with pCMBS⁻ after Cu:phen oxidation. pCMBS⁻ can only react with free thiols and not with disulfide-bonded Cys or with higher order Cys oxidation states. As a control, we assayed for the effect of pCMBS⁻ application without previous Cu:phen oxidation. In the absence of Cu:phen oxidation, application of 200 μ M pCMBS⁻ inhibited the subsequent GABA currents and increased the holding current (Fig. 4A). Furthermore, the increase in holding current during and after pCMBS⁻ application occurred even if the pCMBS⁻ was applied in the presence of bicuculline, a GABA competitive antagonist (Fig. 4A). This suggests that the current was not due to an agonist effect of pCMBS⁻ at the GABA binding site but rather to its irreversible modification of the free cysteine(s). The increased holding current induced by the reaction of pCMBS⁻ was inhibited by picrotoxin indicating that it was mediated via spontaneously opened GABA_A receptor channels (Fig. 4A, final trace). This demonstrates that pCMBS⁻ modification causes a functional effect for this double mutant.

In contrast, in oocytes expressing α 1Q229C β 2M286C γ 2 receptors, after Cu:phen application, a 60 s application of 200 μ M pCMBS⁻ had little effect on the subsequent GABA-induced currents (Fig. 4B, compare traces 3 and 5). There are several possible interpretations of the result that after Cu:phen oxidation, pCMBS⁻ application had no effect: (1) both pairs of Cys residues were involved in disulfide bonds and thus unavailable to react with pCMBS⁻, (2) one member of a pair was oxidized to a higher order oxidation state and either precluded pCMBS⁻ reaction with the other Cys or it precluded a functional effect of pCMBS⁻ reaction with the other Cys thiol, (3) both Cys in a pair could be oxidized to higher order oxidation states, or (4) if a disulfide bond formed only at one β - α subunit interface and not the other then pCMBS⁻ modification of Cys at the nonmodified β - α interface had no effect. To determine whether Cu:phen-induced oxidation caused the formation of disulfide bonds we first oxidized α 1Q229C β 2M286C γ 2 receptors with Cu:phen, then reduced with DTT, and finally applied pCMBS⁻ which caused an increase in the holding current and a 70% reduction in subsequent currents elicited by a submaximal (1 μ M) GABA application (Fig. 4B, compare traces 7 and 10). Because DTT cannot reduce Cys oxidized to higher oxidation states, such as sulfenic acid, this result implies that a significant portion of the receptors

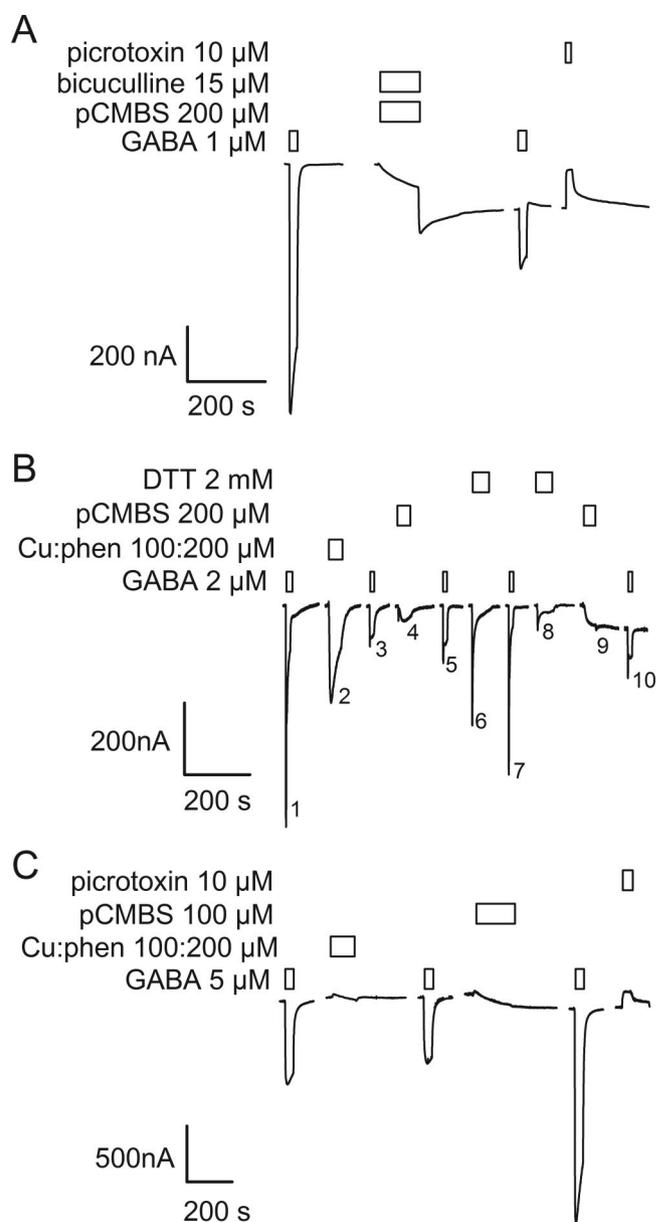


Figure 4. Application of the Cys-specific reagent pCMBS⁻ probes the absence or presence of disulfide bonds in double Cys mutant receptors. **A**, In the absence of previous oxidation, application of 200 μ M pCMBS⁻ in the presence of 15 μ M bicuculline to α 1Q229C β 2M286C γ 2 receptors induced an increased holding current in the absence of GABA. The holding current increased further after washout of pCMBS⁻ and bicuculline. The current elicited by a subsequent GABA application was significantly decreased. Picrotoxin inhibited the increased holding current suggesting that it was due to the spontaneous opening of pCMBS⁻ modified GABA_A receptors. **B**, Treatment of α 1Q229C β 2M286C γ 2 receptors with the oxidizing reagent Cu:phen (100:200 μ M) induced a decrease in subsequent GABA-induced current, as in Figure 3B. Subsequent treatment with 200 μ M pCMBS⁻ had no statistically significant effect on GABA-induced currents (compare traces 3 and 5). The lack of effect of pCMBS⁻ indicates that the engineered Cys were inaccessible for reaction because they were part of disulfide bonds. Reduction with DTT restored the GABA-induced currents (compare traces 5 and 7). Application of pCMBS⁻ after reduction (trace 9) increased the holding current and decreased the subsequent GABA-induced current (compare traces 7 and 10). **C**, Application of Cu:phen to α 1I228C β 2M286C γ 2 receptors either in the absence of GABA, or in the presence of GABA (data not shown) had no effect on subsequent GABA-induced currents. Subsequent application of pCMBS⁻ caused an irreversible increase in the subsequent GABA-induced current, as well as an increase in the holding current. The increased holding current was inhibited by picrotoxin consistent with it being mediated by spontaneously opened GABA_A receptors. Bars over traces indicate the period of application of the reagents shown to the left of each set of traces.

contained at least one pair of disulfide bonded engineered Cys after Cu:phen application. Similar results were obtained with α 1Y225C β 2M286C γ 2 receptors. Thus, oxidation by Cu:phen induced reversible disulfide bond formation indicating that α 1Y225C and α 1Q229C are in close proximity to β 2M286C in the activated state.

For the other seven Cys mutant pairs, Cu:phen application had no irreversible effects on the subsequent GABA-induced currents. When applied after Cu:phen, 200 μ M pCMBS⁻ (2 min) irreversibly altered the subsequent GABA currents for all seven Cys mutant pairs (Fig. 4C). Thus, the Cys must have been free to react with pCMBS⁻ implying that Cu:phen oxidation did not induce disulfide bond formation or higher order oxidation states for these other pairs of Cys residues.

Disulfide crosslinking between β 2F289C and α 1-M1 Cys mutants

In an α helix, β 2F289 would lie on the same face of the helix as β 2M286, one helical turn closer to the cytoplasmic end. In the homology model it is also predicted to face into the subunit interface toward α 1-M1. In the model, the α carbon separation distance between β 2F289 and the closest α 1-M1 residues, α 1I228, α 1Q229 and α 1L232 is <11 Å. We tested whether disulfide bonds would form between β 2F289C and Cys substituted for these residues and α 1T230, predicted to lie on the side of the α 1-M1 helix facing away from the subunit interface. α 1Y231C was not tested due to its low expression level (Table 1).

In oocytes expressing α 1L232C β 2F289C γ 2, repeated GABA applications resulted in a progressive reduction in the peak current to a plateau level \sim 31 \pm 5% (n = 3) of the initial peak current (Fig. 5A). A 60 s application of 2 mM EGTA did not have a significant effect on the subsequent GABA current amplitude but a 60–90 s application of 5 mM DTT restored 75% of the initial current (Fig. 5B). A 60 s application of 100:200 μ M Cu:phen caused an 80 \pm 3% (n = 3) decrease in the subsequent GABA current. We conclude that channel activation induced disulfide bond formation between these two engineered Cys residues under ambient oxidizing conditions. Disulfide bonds did not form spontaneously in the resting state between this Cys pair.

For the mutant α 1I228C β 2F289C γ 2 repeated GABA applications caused a 50% reduction in the GABA-induced currents. Once a plateau was reached, a 90 s application of 5 mM DTT restored the GABA-induced currents to \sim 80% of their initial value. A 60 s application of 100:200 μ M Cu:phen caused a 72 \pm 2% (n = 3) decrease in the subsequent GABA-induced test currents. The effect of Cu:phen was partially reversed by 2 mM DTT (2 min) but 2 mM EGTA had no effect (data not shown). We infer that a disulfide bond formed in the activated state between these two engineered Cys residues.

In contrast to the two double mutants above, oocytes expressing α 1Q229C β 2F289C γ 2 showed no progressive reduction or change in desensitization rate for the GABA currents with repeated GABA applications (Fig. 5C, control traces 1 and 2). EGTA application had little effect on the desensitization rate of a subsequent GABA test pulse (Fig. 5C, trace 3). However, the rate of desensitization of a GABA test pulse 5 min after a 60 s application of 5 mM DTT was significantly slower than the initial rate of desensitization (Fig. 5C, trace 4). This suggests that a disulfide bond had formed spontaneously under ambient oxidizing conditions in the resting state. DTT application had no effects on the desensitization rate in the single mutant α 1Q229C β 2 γ 2. A 60 s application of 100:200 μ M Cu:phen accelerated the desensitization rate and reduced the peak current induced by subsequent

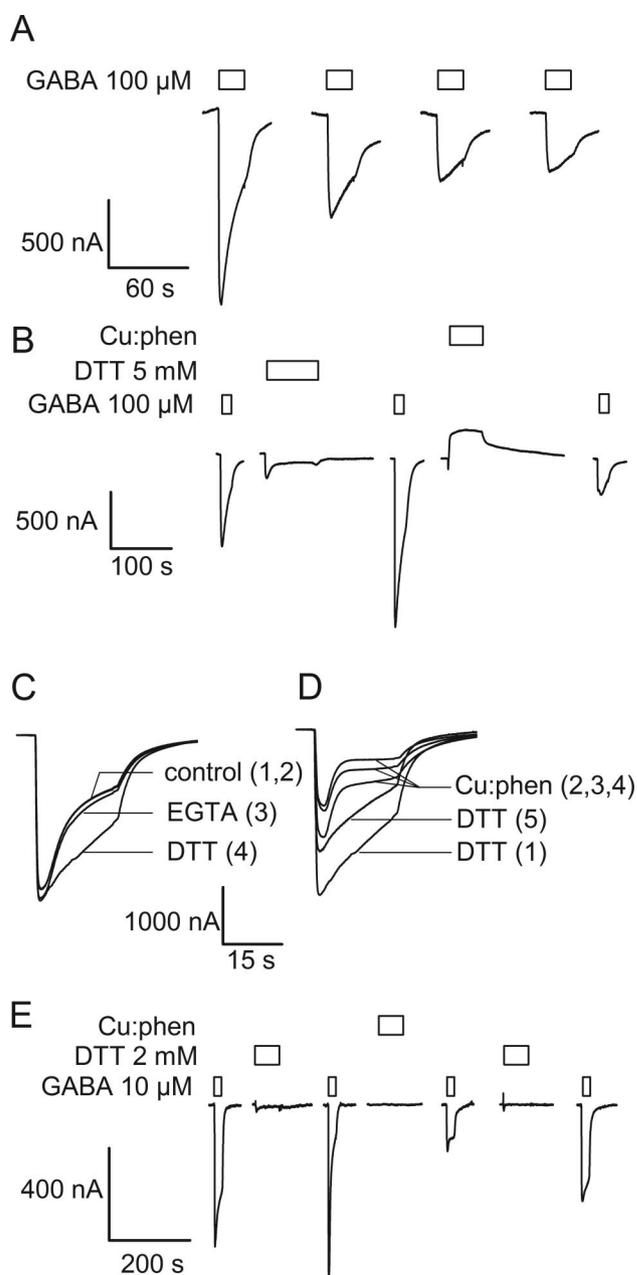


Figure 5. Electrophysiological effects of disulfide bond formation between β 2F289C and α 1L232C, α 1Q229C and α 1I228C. Currents recorded from oocytes expressing the double Cys mutants. **A**, Sequential GABA applications caused a decrease in the currents elicited from an oocyte expressing the α 1L232C β 2F289C γ 2 mutant. **B**, Same cell as in **A**. After the initial GABA-induced decrease observed in **A**, application of 2 mM DTT for 90 s induced a recovery of the initial current. A subsequent application of 100:200 μ M Cu:phen inhibited the subsequent GABA currents to a greater extent than sequential GABA applications in ambient oxygen. **C**, Superposition of four consecutive GABA pulses on α 1Q229C β 2F289C γ 2. Each trace is numbered sequentially. A reagent was applied to the oocyte (data not shown) and the subsequent GABA-induced current is shown and labeled with the reagent that was applied before the GABA pulse. The two control traces (1,2) are indistinguishable. A 60 s application of 2 mM EGTA did not significantly alter the desensitization rate (3), whereas a 60 s application of 5 mM DTT induced a significant decrease in the desensitization rate (4). **D**, Same cell as in **C**; trace 4 of **C** is trace 1 of **D**. Three successive 60 s Cu:phen pulses (data not shown) induced a gradual increase in the desensitization rate and a decrease in the peak amplitude of the GABA-induced current (traces 2, 3, 4). A 60 s application of 5 mM DTT completely reversed the increase in desensitization rate and partially reversed the decrease in current amplitude. **E**, Illustrative experiment showing that a 100:200 μ M Cu:phen application caused a 75% inhibition of a near-saturating GABA-induced current in α 1I228C β 2F289C γ 2 receptors. Prior treatment with 2 mM DTT caused a small increase in the GABA-induced currents. Application of 2 mM DTT after Cu:phen application caused a partial recovery of the initial current amplitude. Boxes over traces indicate the period of application of the reagents shown to the left of each set of traces.

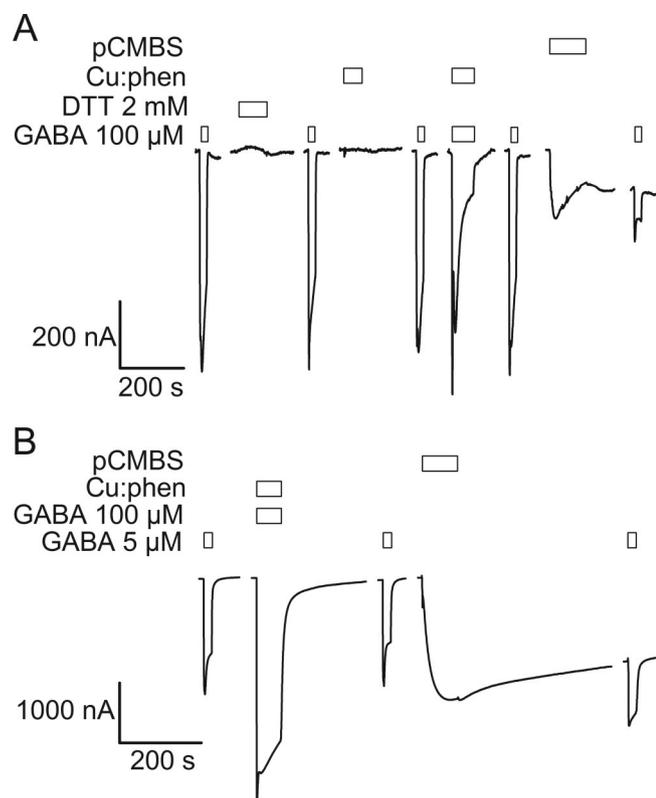


Figure 6. Absence of disulfide bond formation for α 1T230C β 2G287C γ 2 and α 1Q229C β 2C288(WT) γ 2 receptors. **A**, α 1T230C β 2G287C γ 2 receptors were insensitive to a 2 mM DTT application, and to 100:200 μ M Cu:phen applied in the absence (first pulse) or in the presence (second pulse) of saturating GABA. Furthermore, these oxidizing treatments did not prevent a large 200 μ M pCMBS[−]-induced decrease in the current measured at saturating GABA concentration ($80 \pm 12\%$, $n = 3$). Similar results were observed using submaximal GABA concentration (data not shown). **B**, The intensity of a submaximal GABA-induced current in α 1Q229C β 2C288C γ 2 receptors was not modified after treatment with 100:200 μ M Cu:phen in the presence of a saturating GABA concentration. Furthermore, subsequent pCMBS[−] reaction still induced a $50 \pm 10\%$ ($n = 3$) reduction of the current intensity, indicating that the cysteines were not involved in a disulfide bond. Similar results were obtained when the receptors were treated by Cu:phen in the absence of GABA (data not shown).

GABA applications (Fig. 5D). These changes were reversed by a 60 s DTT application (Fig. 5D, trace 5) but not by EGTA (data not shown). We conclude that at least one disulfide bond had formed spontaneously in the resting state of the receptor. The major functional effect of this bond was to increase the desensitization rate after GABA application. Application of Cu:phen in the presence of GABA seemed to accelerate the decrease in subsequent GABA currents suggesting that the disulfide bond formation rate was faster in the activated than in the resting state. We conclude that a disulfide bond formed spontaneously in the resting state of the α 1Q229C β 2F289C γ 2 receptors. Disulfide bonds also formed in the more oxidizing environment created by Cu:phen in which the rate of disulfide bond formation was faster in the activated state of the receptor.

There was no effect of Cu:phen or DTT application either in the absence or presence of GABA on the double Cys mutant α 1T230C β 2F289C γ 2. We infer that no disulfide bonds were formed by this pair of engineered Cys consistent with α 1T230 being on the opposite side of the α 1-M1 helix from the subunit interface.

β 2G287C and β 2C288 do not form disulfide bonds with α 1-M1 Cys mutants

We tested the ability of β 2G287C and β 2C288, the two amino acids between β 2M286 and β 2F289, to form disulfide bonds with α 1-M1 Cys mutants from α 1I228 to α 1L232 (excluding α 1Y231C). A 1 min application of 100:200 μ M Cu:phen had no effect on β 2G287C or β 2C288 when coexpressed with the α 1-Cys mutants and wild-type γ 2. In these experiments, Cu:phen was applied in the absence and then in the presence of saturating GABA concentration. Furthermore, the oxidizing treatment of the receptors did not prevent the effect of a subsequent pCMBS[−] application (Fig. 6), strongly suggesting that the cysteines were not engaged in a disulfide bond. Figure 6A shows an example of absence of cross link for α 1T230C β 2G287C γ 2, and Figure 6B an example for α 1Q229C β 2C288(WT) γ 2. Consistent with our model, β 2G287 and β 2C288, which are predicted to face into the β 2 subunit interior, do not form disulfide bonds with α 1-M1 Cys mutants.

Detection of disulfide crosslinked α 1- β 2 subunits by immunoassay

To confirm the presence of disulfide bonds after Cu:phen induced oxidation, we assayed for the presence of disulfide-linked dimers using Western blots. We tested the α 1Y225C β 2M286C γ 2 and α 1Q229C β 2M286C γ 2 double Cys mutants and the corresponding single Cys mutants. Intact oocytes expressing the single and double Cys mutants were incubated with 1 μ M GABA + 100:200 μ M Cu:phen for 2 min, washed and surface biotinylated with sulfo-NHS-LC-biotin. The oocytes were lysed and the membranes solubilized. Biotinylated proteins were purified using streptavidin-coated beads, separated by SDS-PAGE electrophoresis, and transferred to PVDF for Western blotting. Using an anti-GABA_A α 1 subunit primary antibody, bands consistent with dimer formation were present in the lanes from oocytes expressing the double Cys mutants α 1Y225C β 2M286C γ 2 and α 1Q229C β 2M286C γ 2 but not the corresponding single mutants (Fig. 7A). Treatment of the samples with 40 mM DTT before PAGE analysis diminished the dimer band intensity, consistent with dimer formation being due to the presence of disulfide bonds (Fig. 7B).

For the β 2F289C double Cys mutants, α 1L232C β 2F289C γ 2, α 1Q229C β 2F289C γ 2 and α 1I228C β 2F289C γ 2, where electrophysiological experiments were consistent with disulfide bond formation, dimers were observed on Western blots from oocytes expressing the double Cys mutants after treatment with Cu:phen + GABA but not with the corresponding single Cys mutants (Fig. 7C). Controls with the corresponding single Cys mutants showed little or no evidence of dimer formation (Fig. 7C).

Discussion

In the absence of a high resolution GABA_A receptor x-ray crystal structure, a homology model based on the 4 Å resolution model of the *Torpedo* ACh receptor closed state would be the best alternative (Unwin, 2005). A significant barrier to building an accurate homology model of the GABA_A transmembrane domain is the uncertainty in the GABA_A and ACh sequence alignment for the M2-M3 loop and M3 segment due to the lack of absolutely conserved residues in this region. We have determined experimentally validated proximity constraints using disulfide crosslinking that provide the foundation for constructing a realistic model of the transmembrane domain. Here we tested the ability of four consecutive β 2-M3 Cys-substitution mutants (β 2M286C, β 2G287C, β 2C288, β 2F289C) to form disulfide

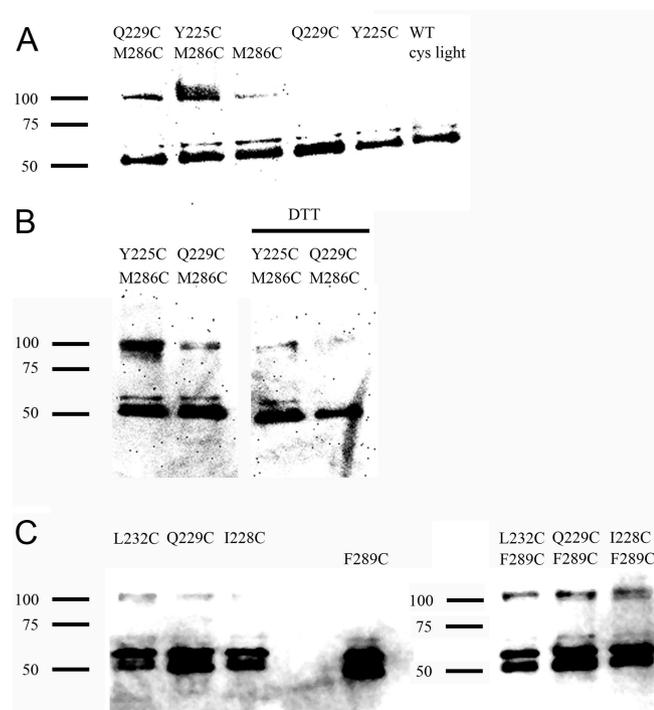


Figure 7. Western blots demonstrate disulfide linked dimers in double Cys mutant receptors inferred to form disulfide bonds in the electrophysiological assays. **A**, Representative Western-blot of the double Cys mutants containing β 2M286 (α 1Q229C β 2M286C γ 2 and α 1Y225C β 2M286C γ 2), the single Cys mutants (α 1 β 2M286C γ 2, α 1Q229C β 2 γ 2, and α 1Y225C β 2 γ 2), and the Cys-light WT after a 2 min treatment in intact oocytes with 100:200 μ M Cu:phen in the presence of 1 μ M GABA. Cys-light receptors (right) and the single mutants show a minimal level of dimers after the 2 min Cu:phen application. The two double mutants showed a significant amount of dimer on similar treatment. The positions of molecular size markers are indicated on the left. **B**, Effect of reduction with DTT after Cu:phen oxidation in the α 1Q229C β 2M286C γ 2 and α 1Y225C β 2M286C γ 2 double Cys mutants. Intact oocytes were treated with a 2 min application of Cu:phen/GABA 100 μ M:200 μ M:1 μ M. Left two lanes were treated only with Cu:phen + GABA, oocytes for the right two lanes were treated with Cu:phen + GABA and then with 40 mM DTT for 2 min. Note the decrease in dimers after reduction with DTT. **C**, Representative Western-blot of the double Cys mutants containing β 2F289C (α 1L232C β 2F289C γ 2, α 1Q229C β 2F289C γ 2 and α 1I228C β 2F289C γ 2), and the individual single mutants (α 1L232C β 2 γ 2, α 1Q229C β 2 γ 2, α 1I228C β 2 γ 2 and α 1 β 2F289C γ 2). The experimental conditions were the same as in **A**. Bands consistent with a dimer were observed in lanes containing the double Cys mutants (right panel), but at significantly lower levels or not at all, in the lanes loaded with membrane preparations from oocytes expressing the single mutant constructs (left panel).

bonds with α 1-M1 Cys-substitution mutants. In activated states β 2M286C formed disulfide bonds with α 1Y225C and α 1Q229C but not with neighboring α 1-M1 Cys-substitution mutants. β 2G287C and β 2C288 did not form disulfide bonds with any α 1-M1 Cys mutants. However, β 2F289C, predicted to be on the same helical face as β 2M286, formed disulfide bonds with α 1I228C, α 1Q229C and α 1L232C but not with α 1T230C that is predicted to face into the α subunit interior (Fig. 8). Thus, we conclude that β 2M286 and β 2F289 face into the subunit interface in close proximity to the α 1-M1 residues α 1Y225, α 1I228, α 1Q229 and α 1L232. These results are consistent with these regions being α helical and provide strong experimental support for our GABA_A model, which is based on the ACh receptor structure with the sequence alignment constrained by our previous M2-M3 crosslinking results (Unwin, 2005; Jansen and Akabas, 2006).

Other GABA_A receptor models have positioned β 2M286 facing into the interior of the β 2 subunit four-helix bundle (Trudell

and Bertaccini, 2004; Ernst et al., 2005; Hosie et al., 2007; Lobo et al., 2008). These models are incompatible with our current and previous crosslinking data (Jansen and Akabas, 2006) and therefore we conclude that the orientations of the M3 segments in those models are incorrect. This has important implications for interpreting experiments aimed at identifying the residues forming the binding sites for general anesthetics on the GABA_A receptor.

As noted above, work by several investigators suggests a role for residues aligned with β 2M286 in anesthetic action (Mihic et al., 1997; Krasowski et al., 2001; Bali and Akabas, 2004; Li et al., 2006). The fact that this residue points into the subunit interface rather than into the subunit interior suggests that a general anesthetic binding site may lie in the transmembrane domain subunit-subunit interfaces rather than in the interior of individual subunits. This would be consistent with azi-etomidate photoaffinity labeling of both α M236 and β M286, because both of these residues face a common subunit interface in our model and thus could be labeled as parts of a single binding site (Li et al., 2006). α 1M236 is \sim 17 Å from β 2M286 in our model (Fig. 8D). Mutation of α 1L232 was previously reported to alter anesthetic potentiation (Jenkins et al., 2001). In our model, this residue faces into the β - α subunit interface, one helical turn above α 1M236. It is \sim 13 Å from β 2M286 and Cys substituted at this position formed a disulfide bond with β 2F289C. Other studies have implicated the M2-15' position, aligned with β 2N265, in the actions of etomidate and other anesthetics (Belelli et al., 1997; Mihic et al., 1997; Jurd et al., 2003). These studies, including the *in vivo* knock-in mouse data, suggest that the amino acid at this position has important effects on the efficacy of various anesthetics. In our model β 2N265 is \sim 15 Å from β 2M286 and although it largely faces toward the β 2-M3 segment, it may have some exposure to the subunit interface, particularly if channel opening involves a coupled movement of β M2 and β M3 away from the channel axis toward α M1 in the adjacent subunit, which would be consistent with our current results.

Disulfide trapping has been used to study the proximity and mobility of pairs of residues in membrane proteins (Careaga and Falke, 1992; Yu et al., 1995; Wu and Kaback, 1996; Hastrup et al., 2001; Horenstein et al., 2001). In proteins of known crystal structure, the maximum α carbon separation for disulfide bonded Cys is 5.6 Å (Careaga and Falke, 1992). To form a disulfide bond two Cys must approach to this separation distance with sufficient frequency to allow covalent bond formation. This is not necessarily their average separation. The formation rate depends on the collision frequency, the orientation of the sulfur atoms and the presence of an oxidizing environment (Kobashi, 1968; Careaga and Falke, 1992). If the collision frequency and energetics are adequate, then ambient oxygen is sufficient to promote a measurable disulfide bond formation rate. In less favorable situations, a more oxidizing environment may be necessary to induce a measurable rate of disulfide bond formation. In the resting state, β 2M286C did not form disulfide bonds with any of the α 1-Cys mutants. We cannot distinguish whether in the resting state this is due to insufficient proximity, constrained mobility or an unfavorable orientation of β 2M286C relative to α 1-M1. In our model based on the ACh receptor closed state structure, the α carbon separation from β 2M286 to α 1Y225 and α 1Q229 is \sim 12 Å and the separation of β 2F289 to α 1Q229 and α 1L232 is \sim 11 Å (Fig. 8). These distances are too large to form a disulfide bond and imply that a conformational change during GABA-induced activation must bring these residues into closer proximity.

In the GABA-activated state a disulfide bond formed between

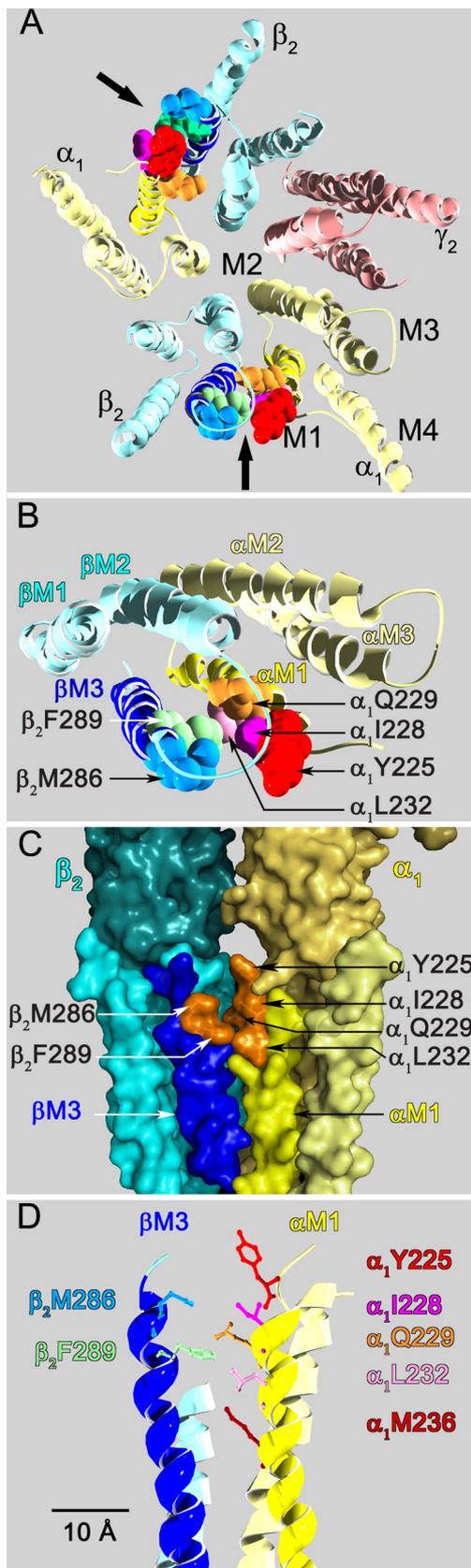


Figure 8. Views based on our GABA_A receptor homology model of the interface between the β 2-M3 and α 1-M1 transmembrane segments illustrating the positions of the crosslinked residues. **A**, View from the extracellular space of the entire transmembrane domain. The extracellular domain has been removed. The subunits are color coded α 1 (pale yellow), β 2 (light blue), γ 2 (pink). The transmembrane segments (M1, M2, M3, M4) are indicated on the α 1 subunit in the lower right. The black arrows indicate the β 2M3- α 1M1 subunit interfaces. The crosslinked

β 2M286C and α 1Q229C under ambient oxidizing conditions and with α 1Y225C in the presence of Cu:phen, which creates a more oxidizing environment. Because phenanthroline acts as an agonist on some of the receptors studied, we could not test whether a more oxidizing environment would promote disulfide bond formation with either of these α 1-M1 Cys mutants in the absence of activation. Because the α 1Q229C- β 2M286C disulfide bond formed under ambient conditions, whereas the α 1Y225C- β 2M286C disulfide only formed with Cu:phen, we infer that in the activated state the collision frequency and/or orientation are more favorable between β 2M286C and α 1Q229C than with α 1Y225C. For β 2F289C, disulfide bonds formed with both α 1Q229C and α 1L232C under ambient oxidizing conditions during channel activation.

This suggests that if the closed state model is correct, these residues must move at least 6–7 Å during channel activation to reduce the α carbon separation sufficiently to allow disulfide bond formation. Whether channel activation causes these residues to move into closer proximity or causes an increase in the mobility of one or both of the regions containing these Cys to allow increased collision frequency is uncertain. In a previous SCAM study of α 1-M3, we found that GABA activation significantly increased the number of pCMBS[−]-reactive α 1-M3 substituted Cys (Williams and Akabas, 1999). We inferred that in the activated state there was looser protein packing around M3 allowing water-filled crevices to extend, at least transiently, from the extracellular solution into the transmembrane protein interior. Our previous disulfide crosslinking between α 1-M2 and α 1-M3 indicates that M3 does not undergo significant rotational motion or translation perpendicular to the membrane plane. Thus, the motion is more likely to be a rigid translation of β 2-M3 toward α 1-M1. Consistent with this, we previously suggested that during activation M2 translates away from the central channel axis toward M1 in the adjacent subunit (Jansen and Akabas, 2006). We conclude that channel activation may involve a conformational movement in the β 2M3- α 1M1 subunit interface in which the β 2M2-M3 segments move as a relatively rigid body toward α 1M1 thereby narrowing the separation between the two subunits. The 6–7 Å movement needed for disulfide bond formation in our experiments would be sufficient to separate the M2 segments thereby opening the channel gate and allowing ion translocation through the pore. Similar conformational changes probably occur in other Cys-loop receptors during the process of channel opening.

Thus, our GABA_A receptor model, based on both intrasubunit and intersubunit constraints, shows that residues implicated in intravenous anesthetic action lie along the transmembrane do-

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residues in the β 2M3- α 1M1 subunit interface are shown in space filling format. The residues are colored as follows in this and all other panels in this figure: α 1Y225 (red), α 1I228 (dark pink), α 1Q229 (orange), α 1L232 (light pink), α 1M236 (maroon), β 2M286 (gray blue), β 2F289 (green). **B**, Closer top view of the β 2M3- α 1M1 subunit interface. Only two subunits are shown, the extracellular domain and the M4 segments in each of these subunits have been removed for clarity. **C**, Side view of the β 2M3- α 1M1 subunit interface from the lipid bilayer looking toward the channel lumen in the background, rendered as a molecular surface. The β 2M3 segment is in dark blue, the α 1M1 segment is in bright yellow. Part of the extracellular domain is visible above the transmembrane segments. The molecular surfaces of the disulfide crosslinked residues identified in this study are represented in orange. **D**, Side view of the residues in the β 2M3- α 1M1 subunit interface. Residues are shown in wireframe representation. Separation distances between α carbons are as follows: β 2M286 to: α 1Y225 (12 Å), α 1Q229 (11 Å), α 1L232 (13 Å), and α 1M236 (17 Å). From β 2F289 to: α 1Q229 (9 Å), α 1L232 (11 Å), α 1M236 (13 Å).

main β - α subunit interface. These transmembrane interfaces may form the binding sites for many volatile and intravenous general anesthetics in a manner similar to the agonist binding sites that are formed at the β - α subunit interface in the extracellular domain. The presence of two β - α interfaces suggests that anesthetic binding to one site may be responsible for the potentiation of GABA-induced currents observed at clinically relevant anesthetic concentrations. Anesthetic binding at both sites may be responsible for the direct activation observed at higher anesthetic concentrations (Rüsch et al., 2004). The length of the interface may in part account for differences in the efficacy of different anesthetics which may bind at slightly different positions along the interface. The fact that disulfide bond formation occurred in the GABA-activated but not in the closed state suggests that channel activation involves a rigid body movement of β M3 toward α M1. Thus, just as GABA binding promotes contraction of the agonist site in the extracellular domain β - α interface (Wagner and Czajkowski, 2001), anesthetic binding in the transmembrane β - α interface may promote the concerted movement of β M3 toward α M1.

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