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Mapping the Agonist Binding Site of the $GABA_A$ Receptor: Evidence for a β-Strand

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$GABA_A$ receptors, along with the receptors for acetylcholine, glycine, and serotonin, are members of a ligand-gated ion channel superfamily (Ortells and Lunt, 1995). Because of the paucity of crystallographic information for these ligand-gated channels, little is known about the structure of their binding sites or how agonist binding is transduced into channel gating. We used the substituted cysteine accessibility method to obtain secondary structural information about the GABA binding site and to systematically identify residues that line its surface. Each residue from α_1 Y59 to K70 was mutated to cysteine and expressed with wild-type β_2 subunits in *Xenopus* oocytes or HEK 293 cells. The sulfhydryl-specific reagent *N*-biotinylaminoethyl methanethiosulfonate (MTSEA-Biotin) was used to covalently modify the cysteine-substituted residues. Receptors with cysteines substituted at positions α_1 T60, D62, F64, R66, and S68 reacted with MTSEA-Biotin, and α_1 F64C, R66C, and S68C were protected from reaction by agonist. We conclude that α_1 F64, R66, and S68 line part of the GABA binding site. The alternating pattern of accessibility of consecutive engineered cysteines to reaction with MTSEA-Biotin indicates that the region from α_1 Y59 to S68 is a β-strand.

Key words: GABA; $GABA_A$ receptor; binding site; substituted cysteine accessibility method; β-strand; cysteine mutagenesis; molecular model

GABA is the major inhibitory neurotransmitter in the mammalian brain, and $GABA_A$ receptors are the primary transducers of its action. $GABA_A$ receptors are likely to be heteropentameric proteins (Nayeem, 1994) assembled from distinct subunit classes with multiple subtypes, $\alpha(1–6)$, $\beta(1–4)$, $\gamma(1–3)$, $\delta(1)$, $\epsilon(1)$, and $\pi(1)$ (Rabow et al., 1995; Sieghart, 1995; Davies et al., 1997; Barnard et al., 1998). The binding of GABA to $GABA_A$ receptors promotes conformational changes leading to the opening of an integral anion-selective channel. Because the GABA binding sites reside on the extracellular surface of the protein and the channel gate is located close to the cytoplasmic end of the channel (Xu and Akabas, 1996), the local changes that occur at the binding site when GABA binds must be propagated to distant parts of the receptor. To understand the transduction of GABA binding to channel gating, one must identify the amino acid residues involved in GABA binding and then locate these residues in a three-dimensional structure of the receptor.

Photoaffinity labeling (Smith and Olsen, 1994) experiments have identified α_1F64 as forming part of the GABA binding site. In the β_2 subunit, mutations of Y157, T160, T202, and Y205 decrease the apparent affinity of GABA, and the results suggest that these residues are also part of the GABA binding site (Amin and Weiss, 1993). Although mutagenesis and photoaffinity labeling experiments are very useful, these methods cannot identify all the residues that form a ligand binding site or provide detailed structural information about the site.

To systematically identify residues that line the surface of the GABA binding site and to investigate the secondary structure of peptide segments involved in the formation of this site, we used the substituted cysteine accessibility method (Karlin and Akabas, 1998). This approach has been used to identify residues that line the ion-conducting pores of numerous channel proteins (Akabas et al., 1994; Cheung and Akabas, 1996; Kuner et al., 1996; Perez-Garcia et al., 1996; Sun et al., 1996; Xu and Akabas, 1996; Egan et al., 1998) as well as residues forming the surface of the binding site crevice of the dopamine D2 receptor (Javitch et al., 1995; Javitch, 1998).

In the current study, each $GABA_A$ receptor residue in the region from α_1Y59 to K70 was mutated to cysteine. This region of the receptor was selected for study because it contains α_1F64, a binding site residue identified by photoaffinity labeling. Mutant α_1 subunits were heterologously expressed with wild-type β_2 subunits. A sulfhydryl-specific reagent, *N*-biotinylaminoethyl methanethiosulfonate (MTSEA-Biotin; Toronto Research Chemicals), was used to covalently modify the substituted cysteines. We identify an engineered cysteine as being in the binding site by two criteria: (1) the reaction with MTSEA-Biotin covalently alters function, and (2) the sulfhydryl-specific reaction is impeded by the presence of binding site ligands.

Here, we show that five residues, α_1T60, D62, F64, R66, and S68, are accessible to MTSEA-Biotin. We confirm that α_1F64 is part of the GABA binding site, and identify two new binding site residues, α_1R66 and S68. By examining the pattern of accessibility of consecutive engineered cysteines, we infer that the region from α_1Y59 to S68 is a β-strand. On the basis of these results, a structural model of the GABA binding site is discussed. Because GABA binding is not diffusion-limited and binding most likely depends on receptor structure (Jones et al., 1998), this study

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provides insight into receptor mechanisms that define GABA affinity.

MATERIALS AND METHODS

Site-directed mutagenesis. The α_1 cysteine mutant constructs were made either by "Altered Sites II: *in vitro* Mutagenesis Systems" (Promega, Madison, WI) or by recombinant PCR. Cysteine substitutions were made in the α_1 subunit at positions Y59, T60, I61, D62, V63, F64, F65, R66, Q67, S68, W69, and K70 (see Fig. 1). The α_1 cysteine-substituted mutants were subcloned into pGH19 (Liman et al., 1992; Robertson et al., 1996) for expression in *Xenopus laevis* oocytes or into pCEP4 (Invitrogen, San Diego, CA) for transient expression in human embryonic kidney (HEK) 293 cells.

All α_1 cysteine mutants were verified by double-stranded DNA sequencing. The α_1 cysteine mutants have been named using the single letter code, as (wild-type residue) (residue number) (mutated residue).

Expression in oocytes. Oocytes from *Xenopus laevis* were prepared and injected with cRNA as described previously (Boileau et al., 1998). $GABA_A$ receptor rat α_1, β_2, and α_1 cysteine mutants in pGH19 were expressed by injection of cRNA into oocytes at molar ratios of 1:1, α/β. The oocytes were maintained in ND96 (in mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES, 1.8 $CaCl_2$, pH 7.4, supplemented with 100 μg/ml gentamicin and 100 μg/ml BSA for 2–14 d and used for electrophysiological recordings.

Voltage-clamp analysis. Oocytes under two-electrode voltage-clamp ($V_{hold} = -80$ mV) were perfused continuously with ND96 recording solution at a rate of 5 ml/min. Drugs and reagents were dissolved in ND96. To correct for slow drift in responsiveness, GABA dose–response plots were scaled to a low, nondesensitizing concentration of drug applied just before the drug concentration tested. Standard two-electrode voltage-clamp recording was performed using a GeneClamp 500 (Axon Instruments) interfaced to a computer with an IT-16 A/D device (Instrutech). Electrodes were filled with 3 M KCl and had a resistance of 0.5–1.5 MΩ.

All oocytes were tested for stability of responses to GABA before addition of MTSEA-Biotin by applying two to five pulses of GABA over a period of 10–30 min. The criterion for acceptable stability was that the peak currents varied by <3%. Routinely, GABA concentrations ranged between EC_{20} and EC_{60} and were chosen to obtain 0.5–8 μA of current. In general, we tested the covalent effects of MTSEA-Biotin by the following protocol: we determined the peak current evoked by several 5–10 sec applications of GABA, washed for 5 min, applied 2 mM MTSEA-Biotin for 2 min, washed for 5 min, and again determined the peak current evoked by GABA at the same concentration used before MTSEA-Biotin treatment. The covalent effect of MTSEA-Biotin was taken as $1 - (I_{GABA,\ after}/I_{GABA,\ before})$. To determine whether this response was reversible and repeatable, some cells were incubated for 2 min with ~20 mM DTT after MTSEA-Biotin exposure and current measurement. After a 15–20 min wash with ND96, current recovery was measured, and in some cases, inhibition by MTSEA-Biotin was tested by repeat exposure.

The protocol for agonist protection experiments was as follows. Various concentrations of MTSEA-Biotin were applied to mutant receptors to determine a low concentration that would yield near-maximal blockage with a 30 sec application. For α_1F64C and α_1R66C, 50 μM MTSEA-Biotin was chosen; for α_1S68C, 200 μM sulfhydryl reagent was required. The effect of those concentrations on mutant receptors served as controls for GABA protection experiments in separate cells. Cells were incubated for 30 sec with the appropriate concentration of MTSEA-Biotin plus a concentration of GABA approximately three times the concentration required for maximal current response (see Fig. 2). After determining the extent of protection from inhibition, the same cells were reexposed to the same concentration of MTSEA-Biotin alone to demonstrate that the full inhibitory effect, as compared with control cells, was obtainable.

Data acquisition and analysis were performed using AxoData, AxoGraph (Axon Instruments), and Prism software (Graphpad). Dose–response data were fit to the following four-parameter equation derived from the Hill equation: $Y = Min + (Max - Min)/(1 + 10^{(LogEC_{50}-X)\cdot(n_H)})$, where *Max* is the maximal response, *Min* is the response at the lowest drug concentration tested, *X* is the logarithm of agonist concentration, EC_{50} is the half-maximal response, and n_H is the Hill coefficient.

Transient expression in HEK 293 cells. Wild-type rat α_1, β_2, and cysteine mutant α_1 cDNAs in the mammalian expression vector pCEP4 were used for transient transfection of HEK 293 cells (ATCC CRL 1573). Cells were grown on 100 mm tissue culture dishes in Minimum Essential Medium with Earle's salts (Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) in a 37°C incubator under a 5% CO_2 atmosphere. Cells were cotransfected at 40–50% confluency with pCEP-α_1 or pCEP- α_1 cysteine mutant and pCEP-β_2. The vector pAdVAntage (Promega) was also added to enhance expression levels (6 μg of each subunit DNA/plate and 12 μg of pAdVAntage). Transient transfection of HEK 293 cells was performed using a standard $CaHPO_4$ precipitation method (Graham and vander Eb, 1973). Cells were harvested, and membrane homogenates were prepared 48 hr after transfection.

Binding assays. Cells were scraped from the dishes and pelleted by centrifugation (1000 × *g*, 10 min, 4°C). The cells were washed once and resuspended in a HEPES buffer containing (in mM): 124 NaCl, 2.9 KCl, 1.3 $MgSO_4$, 1.2 KH_2PO_4, 25.0 HEPES, 5.2 D-glucose, 2 EDTA, pH 7.4, and homogenized using a Brinkman polytron. The homogenates were centrifuged (30,000 × *g*, 20 min, 4°C), and the resulting pellets were resuspended in HEPES buffer. Protein concentrations were determined using a Bradford Assay (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard.

Saturation and competition binding experiments were performed as described previously (Boileau et al., 1998). In brief, membrane homogenates (100 μg) were incubated at room temperature with [^{3}H]muscimol (20 Ci/mmol; DuPont NEN, Wilmington, DE) in a final volume of 250 μl. Nonspecific binding was determined in the presence of 1 mM GABA or 100 μM muscimol, and specific binding was defined as the amount of tritiated drug bound in the absence of displacing ligand minus the amount bound in the presence of displacer. For saturation binding experiments, K_D and B_{max} were determined by fitting specific binding data to a single site using the equation $y = (B_{max} * x)/(K_D + x)$, where *y* is the specifically bound dpm and *x* is radiolabeled drug concentration (Prism software; Graphpad). Data from competition binding experiments were fit by using the equation $y = B_{max}/(1 + (x/IC_{50}))$, where *y* is the specifically bound dpm, B_{max} is maximal binding, and *x* is concentration of displacing drug (Prism software; Graphpad). K_I was calculated according to the Cheng–Prusoff/Chou equation (Cheng and Prusoff, 1973; Chou, 1974).

MTSEA-Biotin reaction and protection assay in HEK cells. HEK cells were harvested and washed by centrifugation as described above. After the second 1000 × *g* centrifugation, the cells were gently resuspended in a small volume of HEPES buffer and incubated for 10 min at room temperature with 5 mM MTSEA-Biotin (Toronto Research Chemicals) or buffer as a control. After the incubations, 50 ml of cold HEPES buffer was added, and the cell suspension was centrifuged (2000 × *g*, 10 min, 4°C). The cells were washed with an additional 50 ml of HEPES buffer, centrifuged (2000 × *g*, 10 min, 4°C), and then resuspended, and a membrane homogenate was prepared as described above. For protection experiments, cells were incubated for 15 min with 3 mM muscimol (~50 × K_D) before the incubation with MTSEA-Biotin, and the muscimol remained present during the subsequent incubation with MTSEA-Biotin.

Statistics. We analyzed the effects of MTSEA-Biotin by one-way ANOVA, applying the Dunnett post-test for significance of differences between the effects of MTSEA-Biotin on a mutant receptor and the effects on wild-type receptor ($p < 0.01$).

RESULTS

Expression of cysteine-substituted receptors in *Xenopus* oocytes

Twelve cysteine mutants were made in the α_1 subunit at positions Y59, T60, I61, D62, V63, F64, F65, R66, Q67, S68, W69, and K70 (Fig. 1). Because we test whether an engineered cysteine reacts with MTSEA-Biotin by whether MTSEA-Biotin covalently alters the GABA-induced current in oocytes expressing the mutant, we require that the cysteine substitution mutants be functional. Cysteine mutant α_1 subunits were individually expressed with wild-type β_2 subunits in *Xenopus laevis* oocytes, and current responses to GABA were measured. Because expression of single α_1 or β_2 subunits (Boileau et al., 1998) does not produce detectable GABA-mediated chloride currents, a robust current response confirms the expression of both subunits in a fully assembled functional receptor. Application of GABA to receptors contain-

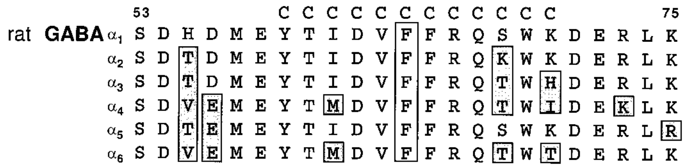

Figure 1. Aligned partial sequences of the rat $GABA_A$ receptor α subunit subtypes, numbered by alignment with the α_1 subunit. This region is highly conserved in all species from which α subunits have been cloned. Residues not identical to α_1 residues are *boxed* and highlighted in *gray*. α_1 F64, an identified GABA binding site residue, and aligned residues are *boxed*. Twelve individual cysteine-substituted α_1 subunits were made in the region from α_1 Y59 to K70 and are denoted by a *C* above the corresponding wild-type α_1 residues.

Table 1. Summary of GABA dose–response data from cysteine-substituted and wild-type $\alpha_1\beta_2$ $GABA_A$ receptors

	EC_{50} (μM)	Mutant/WT
αβ	8.2 ± 0.4	1.00
αT60Cβ	7.5 ± 0.6	0.92
αI61Cβ	3.4 ± 0.3	0.41
αD62Cβ	28 ± 2	3.46
αV63Cβ	3.0 ± 0.2	0.37
αF64Cβ	594 ± 11	72.8
αF65Cβ	19 ± 2	2.34
αR66Cβ	2610 ± 160	320
αQ67Cβ	n.d.	
αS68Cβ	8.0 ± 0.7	0.97
αW69Cβ	n.d.	
αK70Cβ	4.6 ± 0.3	0.56

Oocytes were treated with increasing concentrations of GABA, and current responses were recorded by two-electrode voltage clamp. Data were fit by nonlinear regression analysis as described in Materials and Methods. Mutant/WT, the ratio of the EC_{50} for GABA of cysteine mutant receptors to the EC_{50} for GABA of wild-type $\alpha_1\beta_2$ $GABA_A$ receptors; n.d., no detectable current.

ing α_1 T60C, I61C, D62C, V63C, F64C, F65C, R66C, S68C, and K70C gave robust current responses, whereas no significant GABA-mediated chloride current was detected after expression of α_1Q67Cβ_2 and α_1W69Cβ_2 receptors. Thus, cysteine was a functionally tolerated substitute for every residue except α_1 Q67 and W69, and it is likely that the positions occupied by the cysteine side chains in the functional mutant receptors are similar to the positions of the native amino acid side chains. Cysteine substitution had little effect (<3.5-fold) on the EC_{50} for GABA of α_1T60Cβ_2, α_1I61Cβ_2, α_1D62Cβ_2, α_1V63Cβ_2, α_1F65β_2, α_1S68Cβ_2, and α_1K70Cβ_2 receptors, whereas two mutants, α_1 F64Cβ_2 and R66Cβ_2, had 75-fold and 320-fold increases in EC_{50}, respectively (Table 1, Fig. 2).

Reactions of the cysteine-substituted receptors with MTSEA-Biotin in *Xenopus* oocytes

A 2 min application of 2 mM MTSEA-Biotin had no effect on the currents recorded from wild-type $\alpha_1\beta_2$ receptors or receptors containing α_1 I61C, V63C, F65C, and K70C (Fig. 3). The result that MTSEA-Biotin had no effect on wild-type receptors suggests either that the free sulfhydryls in wild-type $GABA_A$ receptors are inaccessible to MTSEA-Biotin or that reaction with wild-type cysteines has no effect on the function of the receptor. In either case, the absence of effects on wild-type $GABA_A$ receptors allows us to interpret the effects of MTSEA-Biotin on cysteine-substituted mutants as covalent modifications of the introduced cysteine.

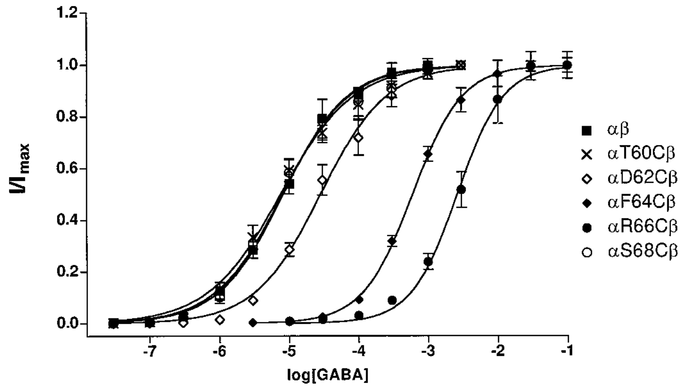

Figure 2. GABA dose–response curves of selected cysteine mutant and wild-type $\alpha_1\beta_2$ $GABA_A$ receptors. Oocytes were injected with wild-type or cysteine mutant α_1 cRNA and β_2 cRNA and were treated with increasing concentrations of GABA. Data were fit by nonlinear regression analysis as described in Materials and Methods. The apparent affinities for GABA of both α_1T60Cβ_2 and α_1S68Cβ_2 receptors are similar to wild-type $\alpha_1\beta_2$ receptors. Cysteine substitution shifts the apparent affinity for GABA of α_1D62Cβ_2, α_1F64Cβ_2, and α_1R66Cβ_2 receptors by ~3.5-, 75-, and 320-fold, respectively. Data points represent mean peak current from four or more cells from two or more batches of oocytes. Error bars are the SD. EC_{50} values determined from the curve fits are presented in Table 1.

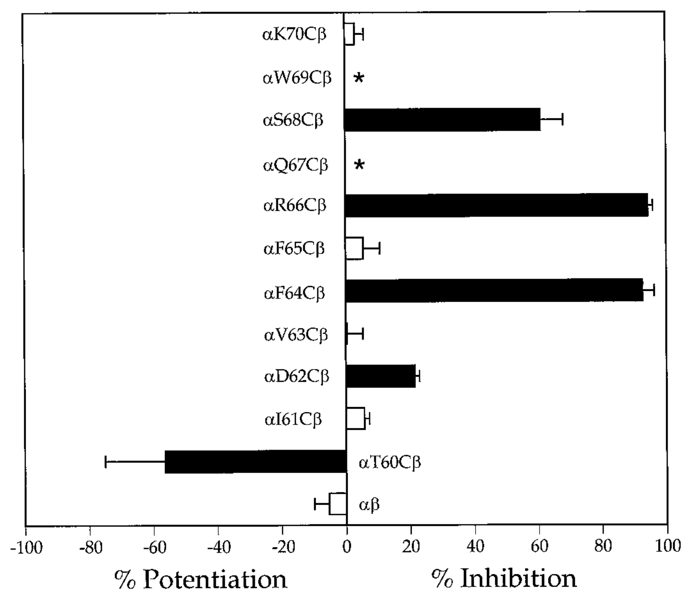

Figure 3. The alteration of GABA-activated current in wild-type and cysteine mutant $GABA_A$ receptors expressed in oocytes resulting from a 2 min application of 2 mM MTSEA-Biotin. The % change was calculated as $(1 - (I_{GABA,\ after}/I_{GABA,\ before})) \times 100$. Positive numbers indicate an inhibition of the current response, whereas negative numbers indicate a potentiation. Results are the means and SDs from three to six independent experiments. *Filled bars* indicate mutants for which the change in current was significantly different ($p < 0.01$) than wild-type receptor by one-way ANOVA. *Asterisks* indicates no detectable current.

MTSEA-Biotin had significant effects on the GABA-evoked currents recorded from receptors containing α_1 T60C, D62C, F64C, R66C, and S68C (Figs. 3, 4). In receptors containing α_1 D62C, F64C, R66C, and S68C, 2 mM MTSEA-Biotin inhibited the subsequent response to GABA by 21, 93, 95, and 61%, respectively. In receptors containing α_1 T60C, MTSEA-Biotin increased the GABA response by 56% (Fig. 3). The inhibition

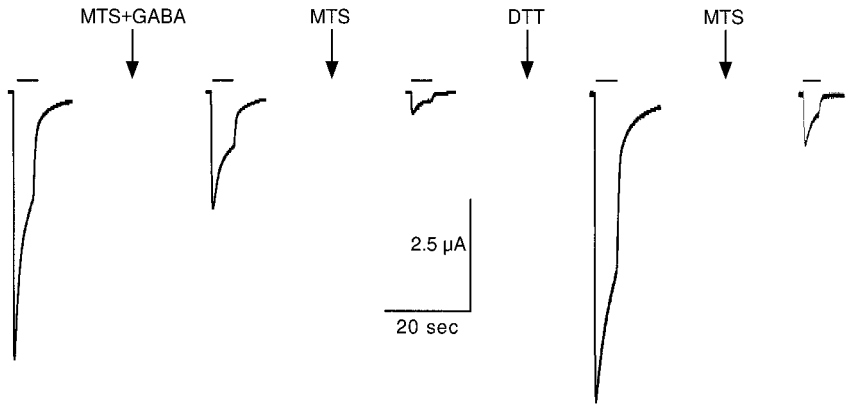

Figure 4. The effect of MTSEA-Biotin, applied in the presence and absence of GABA, on the subsequent GABA-activated currents of α_1F64Cβ_2 receptors. Current traces recorded by two-electrode voltage clamping of a single oocyte are shown. The current responses to applications of 10 mM GABA (*horizontal bars*) were recorded subsequent to the application of the following sequence of solutions (*arrows*): buffer, 50 μM MTSEA-Biotin + 300 mM GABA (*MTS+GABA*, 30 sec), 50 μM MTSEA-Biotin (*MTS*, 30 sec), 20 mM dithiothreitol (*DTT*, 2 min), and 50 μM MTSEA-Biotin (*MTS*, 30 sec). After all GABA applications, a 10 min wash in ND96 buffer occurred before any reagent application. The results demonstrate that the effects of MTSEA-Biotin on α_1F64Cβ_2 receptors are protectable by GABA, recoverable by DTT treatment, and repeatable.

and potentiation of the GABA current by disulfide linking of -SCH_2CH_2(NH)Biotin to the mutant receptors were reversed by treating the oocytes with the reducing agent dithiothreitol (DTT; 20 mM, 2 min) followed by a 15–20 min wash (Fig. 4). After the DTT treatment, MTSEA-Biotin produced the same effect on the GABA-evoked current as before the DTT treatment, demonstrating that the reversibility was caused by reduction of the disulfide bond rather than an artifact of the DTT treatment (Fig. 4).

To determine whether GABA could protect the cysteine mutant receptors from covalent modification by MTSEA-Biotin, a saturating concentration of GABA was added during the sulfhydryl reaction. In these experiments, the duration of the MTSEA-Biotin reaction and its concentration were adjusted so that the minimal amount of MTSEA-Biotin needed to produce a near-maximal effect was used (Figs. 4, 5). In the presence of GABA, the reaction of MTSEA-Biotin with receptors containing α_1 F64C, R66C, and S68C was significantly inhibited (Figs. 4, 5), whereas the reaction of MTSEA-Biotin with receptors containing α_1 T60C and D62C was not changed (data not shown). The presence of GABA caused a 60–70% protection of α_1F64Cβ_2, α_1R66Cβ_2, and α_1S68Cβ_2 receptors, where % protection = $(1 - (\text{Inhibition}_{\text{GABA +MTS}}/\text{Inhibition}_{\text{MTS}})) \times 100$. Because the reaction with MTSEA-Biotin is covalent and the binding of GABA is reversible, complete protection was not observed. Nevertheless, the results indicate that α_1 F64, R66, and S68 are near or part of the GABA binding site.

MTSEA-Biotin (2 mM, 2 min) had markedly different magnitudes of effect on some substituted cysteines than on others (Fig. 3). MTSEA-Biotin had the largest effects on cysteines substituted for α_1 F64 and R66. For cysteines substituted for α_1 T60 and S68, MTSEA-Biotin had intermediate effects, whereas MTSEA-Biotin had the smallest effect on α_1D62Cβ_2 receptors. Even brief exposure to micromolar concentrations of MTSEA-Biotin resulted in almost complete inhibition of current responses of α_1F64Cβ_2 and α_1R66Cβ_2 receptors (Figs. 4, 5).

Expression of cysteine mutant receptors in HEK 293 cells

To provide additional evidence that the effect of covalently adding -SCH_2CH_2(NH)Biotin to some of the cysteine-substituted receptors is caused by a direct effect at the binding site, we expressed some of the substituted cysteine α_1 subunits with wild-type β_2 subunits in HEK 293 cells and examined the ability of MTSEA-Biotin to alter the binding of [^{3}H]muscimol (a GABA agonist) and [^{3}H]SR95531 (a GABA antagonist). Although binding studies with agonists do not necessarily measure binding affinity because agonists induce conformational changes that lead

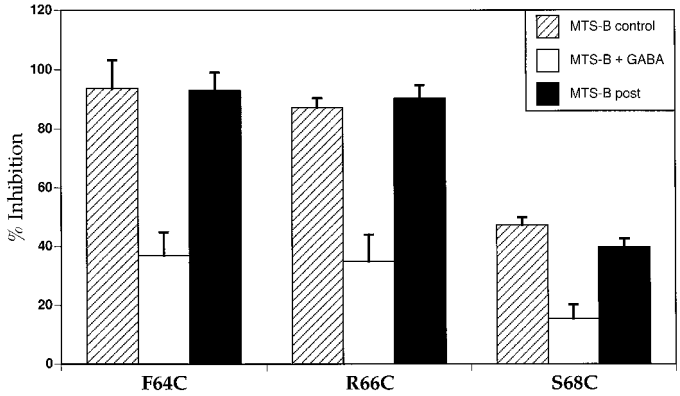

Figure 5. Protection of α_1F64Cβ_2, α_1R66Cβ_2, and α_1S68Cβ_2 receptors by GABA. Oocytes expressing mutant receptors were incubated for 30 sec with either MTSEA-Biotin alone or MTSEA-Biotin + GABA. Concentrations of MTSEA-Biotin were as follows: 50 μM for α_1F64Cβ_2 and α_1R66Cβ_2 receptors; 200 μM for α_1S68Cβ_2 receptors. Results are the means and SDs from three to five independent experiments. *MTS-B control* (*hatched bars*) is the inhibition of GABA-activated current resulting from MTSEA-Biotin treatment in control oocytes. *MTS-B + GABA* (*open bars*) is the inhibition of GABA-activated current resulting from incubation of oocytes with MTSEA-Biotin in the presence of a saturating concentration of GABA. Concentrations of GABA used for protection were ~3 × [GABA], which produced a maximal current: 300 mM for α_1F64Cβ_2, 600 mM for α_1R66Cβ_2, and 9 mM for α_1S68Cβ_2 receptors. *MTS-B post* (*filled bars*) is the inhibition of GABA-activated current resulting from MTSEA-Biotin treatment of oocytes that previously had been treated with MTSEA-Biotin + GABA. Protection by GABA was significant by one-way ANOVA ($p < 0.01$) as compared with MTS-B control. MTS-B post inhibitions were not different from MTS-B control inhibitions.

to receptor gating (Colquhoun, 1998), binding studies with antagonists avoid this complication and most likely measure binding directly.

Receptors containing α_1 Y59C T60C, I61C, D62C, V63C, F65C, R66C, and S68C specifically bound [^{3}H]muscimol (75–92 nM). At five positions, cysteine substitution had little effect on the affinity of [^{3}H]muscimol binding: α_1Y59Cβ_2, α_1T60Cβ_2, α_1I61Cβ_2, α_1V63Cβ_2, and α_1S68Cβ_2 receptors had equilibrium dissociation constants (K_D) for [^{3}H]muscimol not significantly different from wild-type $\alpha_1\beta_2$ receptors (Table 2). The largest change measured was for α_1Y59Cβ_2 receptors, which had a 2.9-fold decrease in muscimol affinity as compared with $\alpha_1\beta_2$ receptors. Although specific [^{3}H]muscimol binding was detectable in receptors containing α_1 D62C, F64C, F65C, and R66C, the amount of binding was low, and these mutant receptors were not

Table 2. Characteristics of [^{3}H]muscimol binding to wild-type and cysteine-substituted $GABA_A$ receptors

Receptor	K_D (nM)	K_{MUT}/K_{WT}	n
$\alpha\beta$	62 ± 5	1.0	22
αY59Cβ	177 ± 21	2.9	2
αT60Cβ	142 ± 30	2.3	5
αI61Cβ	129 ± 8	2.1	3
αD62Cβ	L.B.		3
αV63Cβ	89 ± 15	1.4	5
αF64Cβ	L.B.		3
αF65Cβ	L.B.		5
αR66Cβ	L.B.		3
αQ67Cβ	No significant binding		3
αS68Cβ	65 ± 8	1.1	3
αW69Cβ	No significant binding		3

The affinity of muscimol binding to wild-type and mutant receptors was determined by [^{3}H]muscimol saturation and competition binding assays as described in Materials and Methods. The means and SEM are shown for n independent experiments, each with triplicate determinations. Cysteine substitution of α_1 Y59, T60, I61, V63, or S68 had little effect on muscimol affinity. L.B., Low levels of specific binding made it difficult to accurately determine the affinity of muscimol binding to these mutant receptors.

analyzed further. No significant specific [^{3}H]muscimol binding was detected after expression of single α_1 or β_2 subunits or α_1Q67Cβ_2 and α_1W69Cβ_2 receptors.

Reactions of cysteine mutant receptors with MTSEA-Biotin in HEK 293 cells

Cysteine mutant receptors with near-normal binding affinity and expression were analyzed further by covalently reacting them with MTSEA-Biotin. Incubation with MTSEA-Biotin (2 mM, 15 min) caused a 42 ± 2.3% (n = 10) inhibition of [^{3}H]muscimol binding to α_1S68Cβ_2 receptors and a 40 ± 12% (n = 5) potentiation of binding to α_1T60Cβ_2 receptors (Fig. 6). The binding of [^{3}H]SR95531 (a GABA antagonist) to α_1 S68C-containing receptors was also decreased 40% after MTSEA-Biotin treatment (n = 2). MTSEA-Biotin did not have a significant effect on [^{3}H]muscimol binding to $\alpha_1\beta_2$, α_1Y59Cβ_2, α_1I61Cβ_2, or α_1V63Cβ_2 receptors (Fig. 6).

To determine whether muscimol could protect α_1T60Cβ_2 and α_1S68β_2 receptors from covalent modification by MTSEA-Biotin, nonradioactive muscimol (3 mM, ~50 × K_D) was added before and during the MTSEA-Biotin reaction. The inhibition caused by the reaction of MTSEA-Biotin with α_1S68Cβ_2 receptors was 10.3 ± 6% (n = 4) when 3 mM muscimol was added before and during the MTSEA-Biotin reaction (Fig. 7). Thus, the presence of 3 mM muscimol caused a 76% protection of α_1S68Cβ_2 receptors, where % protection = (1 − (10.3/42)) × 100. Addition of 3 mM muscimol to α_1T60Cβ_2 receptors before and during the MTSEA-Biotin reaction did not significantly decrease the potentiation observed (Fig. 7). The results obtained in HEK 293 cells confirm and supplement the data obtained electrophysiologically in *Xenopus* oocytes and show that α_1 S68 is near or part of the GABA binding site.

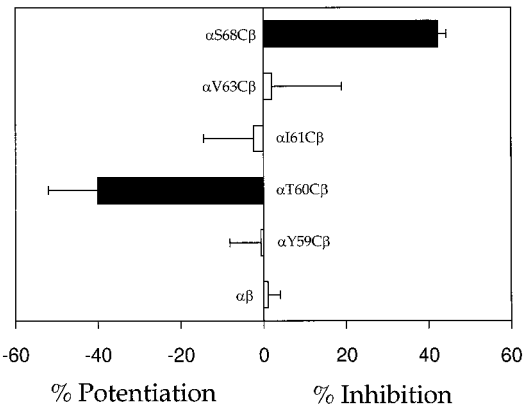

Figure 6. MTSEA-Biotin irreversibly alters [^{3}H]muscimol binding to α_1T60Cβ_2 and α_1S68Cβ_2 receptors expressed in HEK 293 cells. MTSEA-Biotin (2 mM, 15 min) was added extracellularly to intact cells expressing wild-type and mutant $GABA_A$ receptors and the specific binding of [^{3}H]muscimol (150–200 nM) was measured. % Change = (1 − (Specific $DPM_{[+MTSEA\text{-}Biotin]}$/Specific $DPM_{[control]}$)) × 100. Positive numbers indicate an inhibition of binding, whereas negative numbers indicate a potentiation of binding. Results are the means and SEM from four to five independent experiments, each with triplicate determinations for wild-type, α_1T60Cβ_2, and α_1S68Cβ_2 receptors, and from two independent experiments for α_1Y59Cβ_2, α_1I61Cβ_2, and α_1V63Cβ_2 receptors. *Filled bars* indicate mutants for which the change in binding was significantly different ($p < 0.05$) than wild-type receptors by one-way ANOVA.

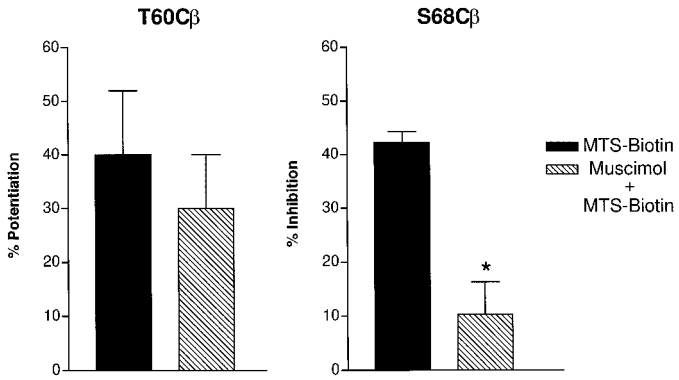

Figure 7. Muscimol protects α_1S68Cβ_2 receptors from covalent modification by MTS-Biotin. α_1T60Cβ_2 and α_1S68Cβ_2 receptors were incubated in the presence or absence of 3 μM muscimol before and during the application of 2 mM MTSEA-Biotin. The receptor preparations were washed thoroughly, and the binding of [^{3}H]muscimol (150–200 nM) was measured. The means and SEM of four independent experiments, each performed with triplicate determinations, are shown. *Filled bars*, % change in binding by MTSEA-Biotin alone; *hatched bars*, % change in binding in the presence of muscimol. In α_1T60Cβ_2 receptors, muscimol did not significantly slow the reaction of MTSEA-Biotin with the engineered cysteine. In α_1S68Cβ_2 receptors, muscimol significantly protected the reactive cysteine from covalent modification by MTSEA-Biotin (*$p < 0.05$).

DISCUSSION

Residues accessible to MTSEA-Biotin

We used the substituted cysteine accessibility method to investigate the secondary structure of a 12 amino acid segment of the α_1 polypeptide chain surrounding F64, a known GABA binding site residue (Sigel et al., 1992; Smith and Olsen, 1994). Furthermore, the approach was used to identify additional residues within this segment that are part of the GABA binding site. We made the following assumptions. (1) The GABA binding site is most likely at a water-accessible surface of the protein because under physiological conditions GABA is zwitterionic (Krogsgaard-Larsen et al., 1984); (2) MTSEA-Biotin is relatively impermeant and reacts preferentially at the water-accessible surface of a protein (Chen et al., 1998); and (3) if a cysteine-substituted residue is part of the

GABA binding site, the addition of -SCH_2CH_2(NH)Biotin will covalently alter binding, and site-selective ligands will protect the introduced cysteine from reaction with MTSEA-Biotin. On the basis of these assumptions, we show that five residues, α_1T60, D62, F64, R66, and S68, are solvent-exposed and accessible to MTSEA-Biotin, and three of them, α_1F64, R66, and S68, are part of or close to the GABA binding site because GABA slows their reaction with MTSEA-Biotin. Two residues, α_1Q67 and W69, do not tolerate cysteine substitution. These two residues are invariant in GABA α (Fig. 1), β, and γ subunits and are highly conserved in all superfamily subunits. We speculate that they play an essential structural role in this receptor superfamily.

The effects of covalently adding -SCH_2CH_2(NH)Biotin to a substituted cysteine could be attributed to a direct effect such as steric block and/or an indirect allosteric effect on the binding site. Regardless of the mechanism, the observation of a change in receptor function after MTSEA-Biotin treatment is proof that the reaction has occurred. For α_1T60Cβ_2 receptors, the effect of MTSEA-Biotin is not caused by steric overlap because modification of α_1T60C with MTSEA-Biotin leads to a potentiation of both the GABA current response and [^{3}H]muscimol binding (Figs. 4, 6). Furthermore, agonist does not protect α_1T60C from MTSEA-Biotin reaction (Fig. 7). For this residue, an indirect effect of the modification leading to an increase in GABA affinity and an enhancement of efficacy ("gating") are the most likely explanations. MTSEA-Biotin modification of α_1D62C decreases GABA-gated current (Fig. 3). This result is consistent with either a direct steric block or an indirect allosteric effect. The fact that GABA does not protect α_1D62C from MTSEA-Biotin modification supports an indirect action. However, it is also possible that -SCH_2CH_2(NH)Biotin, when attached to α_1D62C, is long enough to swing into the GABA binding site and sterically hinder GABA binding, although GABA is too small to protect α_1D62C from modification. Experiments using sulfhydryl reagents of different sizes will help distinguish between these possibilities. For α_1F64Cβ_2, α_1R66Cβ_2, and α_1S68Cβ_2 receptors, the inhibition measured after MTSEA-Biotin modification and the ability of agonist to protect these residues from modification (Figs. 4, 5, 7) strongly suggest that steric hindrance underlies the inhibition and is consistent with the idea that these residues are near or part of the GABA binding site.

Residues exposed in the GABA binding site

Although allosteric effects cannot be completely ruled out, several lines of evidence argue that α_1F64, R66, and S68 line part of the GABA binding site. Results from photoaffinity labeling (Smith and Olsen, 1994) and mutagenesis (Sigel et al., 1992) studies provide evidence that α_1F64 is a GABA binding site residue. Our results, showing that the reaction of MTSEA-Biotin with α_1F64Cβ_2 receptors irreversibly inhibits GABA-mediated chloride current (Fig. 4) and that GABA protects α_1F64C from the reaction (Fig. 5), provide independent evidence that α_1F64 is part of the binding site. These results demonstrate the validity of using the substituted cysteine accessibility method to identify binding site residues. Thus, on the basis of our criteria and the results reported in this paper, we reason that α_1R66 and S68 are also part of or near the GABA binding site.

Further proof that α_1F64 and R66 are both in the binding site is provided by the result that introducing cysteines at these positions causes 75- and 320-fold shifts in GABA EC_{50} values of α_1F64Cβ_2 and α_1R66Cβ_2 receptors, respectively (Table 1). The shifts in EC_{50} values are larger than one would predict if the mutations only affected gating (Amin and Weiss, 1993, their Fig. 3b). It is possible, however, that the mutations affect both binding and gating. Interestingly, treatment of purified $GABA_A$ receptors with an arginine-specific reagent, 2,3-butanedione, results in a time- and concentration-dependent loss of [^{3}H]muscimol binding (Widdows et al., 1987) and provides supplementary evidence that an arginine residue is important for GABA binding. The ability of MTSEA-Biotin to inhibit not only the GABA-activated chloride current but also the radioligand binding of both a GABA agonist and antagonist to α_1S68Cβ_2 receptors lends further support for the conclusion that α_1S68C is located near the GABA binding site. Finally, the identification of α_1R66 and S68 as binding site residues is concordant with their proximity to α_1F64 in a β-strand (Fig. 8).

Together, these observations are explained most simply by a model in which α_1F64, R66, and S68 line part of the GABA binding site (Fig. 8). However, not every one of these residues need to contact GABA. Some of these residues may be important for maintaining the local physico-chemical properties of the site or be involved in the local changes that occur at the binding site when agonist binds. GABA could protect noncontact residues in the binding pocket by blocking the passage of MTSEA-Biotin from the extracellular medium to that particular part of the binding site.

Secondary structure of the polypeptide chain flanking α_1F64

Our results, that alternating residues in the primary amino acid sequence from α_1Y59 to α_1S68 are accessible to MTSEA-Biotin, are consistent with this region forming a β-strand (Fig. 8). Because the accessibility of α_1Q67C and α_1W69C could not be tested (α_1Q67C and α_1W69C do not assemble into functional channels), the strictly alternating exposure surrounding α_1S68 is not absolutely established. The residues accessible to MTSEA-Biotin, with the exception of α_1F64, are hydrophilic amino acid residues. Because MTSEA-Biotin is relatively impermeant (Chen et al., 1998), the accessibility of these residues to reaction suggests that they are exposed at the protein, water-accessible surface. The inaccessible residues are mostly hydrophobic residues and are likely to be buried within the protein. We must be cautious, however, in our interpretation of apparently unreactive residues because we cannot rule out silent reactions that appear to have no functional consequences. Nevertheless, taken together, the results of this study strongly suggest that the polypeptide chain from α_1Y59 to S68 forms a β-strand and that a portion of this strand lines the GABA binding site. In agreement with our experimental results, a part of this region (α_1 M57-R66) is predicted by secondary structure modeling algorithms (Chou and Fasman, 1978; Smith and Olsen, 1995), to adopt a β-strand conformation.

Theoretical model of the GABA binding site

By analogy to the agonist binding site of the nicotinic acetylcholine receptor (Czajkowski et al., 1993), the GABA binding site of the $GABA_A$ receptor has been proposed to lie at the interface between the α and β subunits (Galzi and Changeux, 1994; Smith and Olsen, 1995). We propose that one domain of the GABA binding site on the α_1 subunit is formed in part by a β-strand and that α_1F64, R66, and S68 are facing into the GABA binding site (Fig. 8). Previous mutagenesis studies (Amin and Weiss, 1993) have suggested that two domains on the β_2 subunit, Y157 -T160 and T202-Y205, also form part of the GABA binding site. Although experimental evidence is lacking, we have tentatively

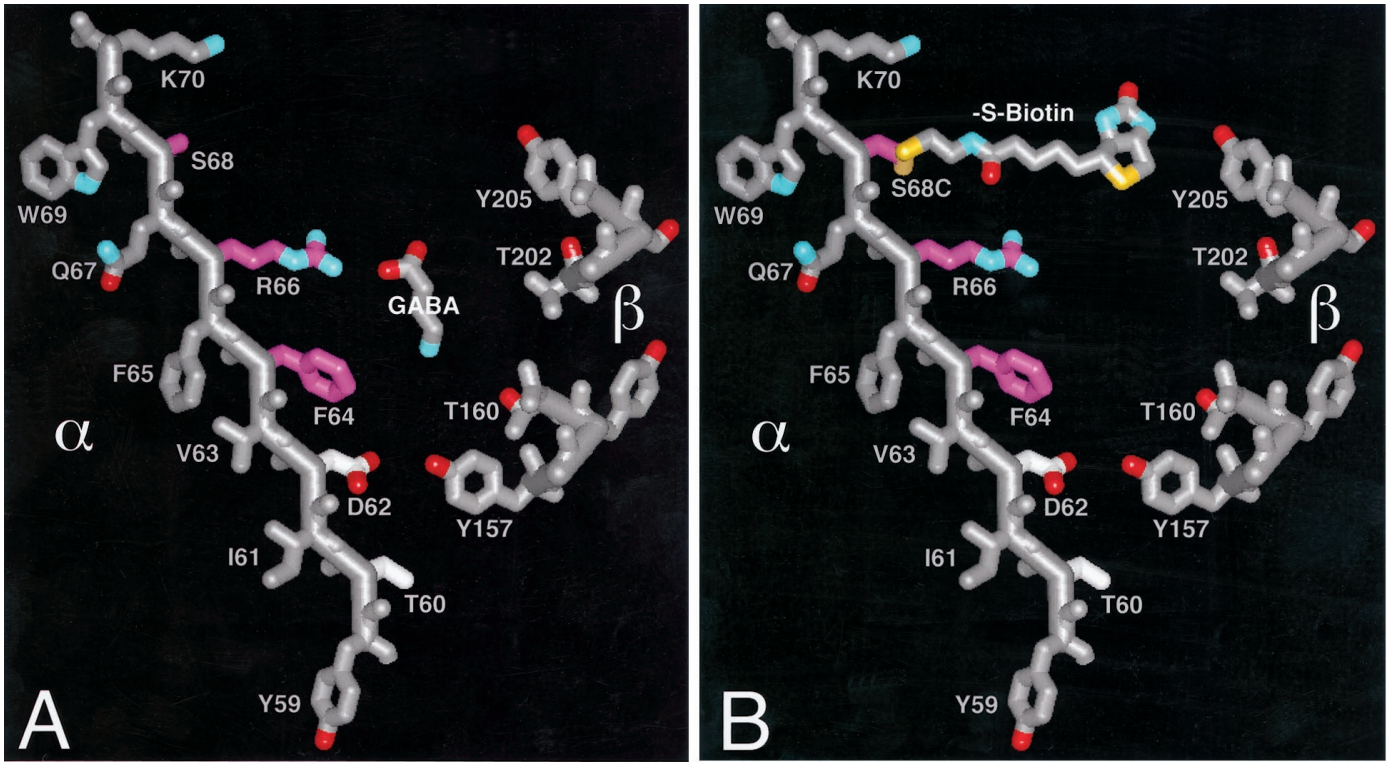

Figure 8. Theoretical structure of the agonist-binding site of the $GABA_A$ receptor. *A*, Molecular model of the GABA binding site pocket, with residues from the α_1 subunit (Y59-K70, *left*) and the β_2 subunit (Y157-T160 and T202-Y205, *right*) surrounding a GABA molecule. α_1 residues are arranged in an idealized β-strand conformation, with MTSEA-Biotin reactive side chains highlighted in *white* (reactive but not protected by agonist) or *magenta* (protected by agonist). β_2 subunit segments are shown in α-helical conformation. Selected oxygens (*red*) and nitrogens (*cyan*) are depicted for orientation and to show charged moieties. *B*, Model of a covalently modified α_1S68C mutant receptor binding site. After reaction with MTSEA-Biotin, the introduced cysteine forms a covalent disulfide bond with $-SCH_2CH_2$-Biotin (*-S-Biotin*). Orientation of the $-SCH_2CH_2$-Biotin is purely speculative and is shown in an extended conformation. Sulfur atoms are shown in *orange*. Peptide chains were created using Sybyl software (Tripos Associates) and rendered using WebLab (Molecular Simulations) and Adobe Photoshop (Adobe Systems) software. Cartoon depictions of other molecules were created using ISIS (MDL Information Systems) chemical modeling software.

modeled these segments as two α-helices because the identified residues in each segment are three residues apart. We are currently using the substituted cysteine accessibility method on these β_2 subunit domains to directly test this hypothesis.

The orientation of GABA relative to these identified binding site residues is not known. The stabilization of GABA binding will most likely involve electrostatic interactions and hydrogen bonding between GABA's charged groups and the side chains of binding site amino acid residues. We speculate that an electrostatic interaction between the positive guanidinium group of α_1R66 and the negative carboxyl group of GABA stabilizes GABA binding. α_1R66 is conserved in all $GABA_A$ receptor α, π, and ρ subunits. At the positive end of GABA, hydrogen bonding with β_2T160 and Y157 as well as interactions with the aromatic ring of α_1F64 may be important. Experiments using engineered GABA affinity reagents that can be "tethered" to cysteines substituted for α_1F64, R66, or S68 will be helpful in determining GABA's exact placement in the site.

These studies are a step toward constructing a detailed molecular model of the GABA binding site and ultimately will help explain how GABA binds and initiates the conformational changes that result in anion channel opening. Because the GABA binding site is most likely formed by residues from two adjacent subunits, we hypothesize that GABA and other agonists bridge the binding site. Agonist binding could promote a change in the distance between the α_1 and β_2 subunits that causes a shift of one subunit relative to the other, and this movement could then be propagated to the opening of the channel. With the methods described in this report and sulfhydryl-specific cross-linking reagents, we are now in a position to test this and alternative hypotheses.

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