A β-Strand in the γ2 Subunit Lines the Benzodiazepine Binding Site of the GABA_A Receptor: Structural Rearrangements Detected during Channel Gating

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Benzodiazepines (BZDs) exert their effects in the CNS by binding to a modulatory site on GABA_A receptors. Individual amino acids have been implicated in BZD recognition and modulation of the GABA_A receptor, but the secondary structure of the amino acids contributing to the BZD binding site has not been elucidated. In this report we used the substituted cysteine accessibility method to understand the structural dynamics of a region of the GABA_A receptor implicated in BZD binding, γ2Y72–γ2Y83. Each residue within this region was mutated to cysteine and expressed with wild-type α1 and β2 subunits in Xenopus oocytes. Methanethiosulfonate (MTS) reagents were used to modify covalently the engineered cysteines, and the subsequent effects on BZD modulation of the receptor were monitored functionally by two-electrode voltage clamp. We identified an alternating pattern of accessibility to sulfhydryl modification, indicating that the region γ2T73–γ2T81 adopts a β-strand conformation. By monitoring the ability of BZD ligands to impede the covalent modification of accessible cysteines, we also identified two residues within this region, γ2A79 and γ2T81, that line the BZD binding site. Sulfhydryl modification of γ2A79C or γ2T81C allosterically shifts the GABA EC₅₀ of the receptor, suggesting that certain MTS compounds may act as tethered agonists at the BZD binding site. Last, we present structural evidence that a portion of the BZD binding site undergoes a conformational change in response to GABA binding and channel gating (opening and desensitization). These data represent an important step in understanding allosteric communication in ligand-gated ion channels.

Key words: benzodiazepine; binding site; allostery; ligand-gated ion channel; GABA; GABA_A receptor; substituted cysteine accessibility method; Xenopus oocytes; secondary structure.

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Benzodiazepines (BZDs) are among the most commonly prescribed therapeutics in the treatment of panic disorder, sleeplessness, and epilepsy (Dobie and Martin, 1996). BZDs exert their anxiolytic and hypnotic effects by binding to a unique site on the GABA_A receptor, the main inhibitory ligand-gated ion channel (LGIC) in the CNS (Hevers and Lüddens, 1998). BZD ligands encompass a full spectrum of efficacy and can potentiate, inhibit, or have no effect on GABA currents, depending on the ligand that is bound. BZD agonists increase GABA-gated Cl⁻ conductance by allosterically decreasing the GABA concentration needed to elicit half-maximal channel activity (EC₅₀; Hevers and Lüddens, 1998), thus making them powerful modulators of inhibitory tone in the brain. Although several studies have made progress toward identifying amino acids on the GABA_A receptor involved in BZD binding, a detailed structural map of the BZD binding pocket does not exist yet.

Both GABA_A receptor α- and γ-subunits play critical roles in BZD binding and modulation of GABA-activated current (I_GABA). It has been hypothesized that the BZD binding site is localized at the interface of these two subunits (for review, see Sigel and Buhr, 1997). To date, six residues in the γ2 subunit have been shown to affect ligand discrimination at the BZD site: γ2F77 (Buhr et al., 1997; Sigel et al., 1998), γ2A79 and γ2T81 (Kucken et al., 2000), γ2M130 (Buhr and Sigel, 1997; Wingrove et al., 1997), and γ2M57 and γ2Y58 (Buhr and Sigel, 1997; Kucken et al., 2000). Because these amino acids were identified by using chimeric and site-directed mutagenesis, none has been shown conclusively to line the BZD binding site itself.

The substituted cysteine accessibility method (SCAM) has been used previously to gain insight into the secondary structure of ion channels and ligand binding sites (for review, see Karlin and Akabas, 1998). In this study we used SCAM to examine the structure and dynamics of the γ2F77 region of the BZD binding site. We demonstrate that the polypeptide backbone surrounding γ2F77 is a β-strand, that γ2A79 and γ2T81 line the BZD binding pocket, and that the structure of the BZD binding site undergoes a conformational change during gating. Additionally, we provide evidence that modification of the BZD binding site by MTSEA-biotin or MTSEA-biotin-CAP, two sulfhydryl-specific reagents, allosterically shifts the sensitivity of the GABA_A receptor for GABA. Our data provide a detailed molecular model of a portion of the BZD binding site and potentially describe the allosteric transitions that underlie BZD modulation of the GABA_A receptor.

MATERIALS AND METHODS

Cysteine mutagenesis. Rat cDNAs encoding α1, β2, and γ2 GABA_A receptor subunits were used for all molecular cloning and functional studies. γ2 Cysteine mutants were made by a modified form of recombinant PCR described previously (Kucken et al., 2000). Wild-type and mutant subunits were subcloned into pGH19 (Liman et al., 1992; Robertson et al., 1996) for expression in Xenopus laevis oocytes. All γ2

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cysteine mutants were verified by double-stranded DNA sequencing and restriction enzyme analysis. The γγ cysteine mutants are named with single letter amino acid code as follows: wild-type residue, residue number of the mature protein, mutant residue (e.g., A79C).

RNA expression in Xenopus laevis oocytes. Capped cRNAs encoding individual γγ cysteine mutants were transcribed in vitro from Nheli- linearized cDNA template with the mMessage mMachine T7 kit (Ambion, Austin, TX). Oocytes were harvested from X. laevis and prepared for injection as described previously (Boileau et al., 1999). Briefly, oocytes were incubated in collagenase (0.25 mg/ml) in Ca2+-free ND96 [(in mm) 96 NaCl, 2 KCl, 1 MgCl2, and 5 HEPES, pH 7.2] for 20 min at room temperature and defolliculated in osmotic shock solution [130 mM K$_2$HPO$_4$ and 1 mM/ml bovine serum albumin (BSA), pH 6.5] for 30 min at room temperature. Single oocytes were injected within 24 hr with 27 nl of cRNA (10-100 ng/μl) per oocyte in the ratio 1:1:10 (αβγγ, Boileau et al., 1998; Boileau and Czajkowski, 1999). Oocytes were stored for 2-14 d at 16°C in ND96 (as above, with 1.8 mM CaCl$_2$) supplemented with 100 μg/ml gentamycin and 100 μg/ml BSA and were assayed functionally at least 2 d after RNA injection.

Two-electrode voltage clamp. Oocytes were perfused continuously with ND96 (5 ml/min) while being held under two-electrode voltage clamp at −80 mV. The bath solution was −200 μM Borosilicate electrodes used in recording (0.4-1.6 MΩ) were filled with 3 M KCl. Electrophysiological data were acquired with a GeneClamp 500 (Axon Instruments, Foster City, CA) interfaced to a computer with an IT16 analog-to-digital device (Instrutech, Great Neck, NY). Dr. Sepinwall (Hoffman-La Roche, Nutley, NJ) generously supplied the BZDs used in this study. Working concentrations of BZD ligands (FLZM and zolpidem) were made up in 10 mM CH$_3$SO$_2$SCH$_2$CH$_2$NH$_3$ hydroxide (CH$_3$SO$_2$SCH$_2$CH$_2$NH$_3$Cl) and stored at −20°C. The solutions were adjusted to pH 7.4 by the addition of 10 mM CH$_3$SO$_2$SCH$_2$CH$_2$NH$_3$OH. The bath volume was 1.5 ml.

Methanethiosulfonate (MTS) reagents. Three derivatives of methanethiosulfonate (CH$_3$SO$_2$SCH$_2$CH$_2$NH$_3$Cl) were used to modify covalently the introduced cysteines: MTS ethylammonium (X = NH$_3$+; MTSEA), N-biotinylaminomethyl MTS (X = NH$_2$; MTSEA-biotin), and N-biotinylaminomethyl CAP MTS (X = NHCO(CH$_2$)$_n$NH$_2$; MTSEA-biotin-CAP). MTSEA-biotin was used for initial accessibility studies. For rate determinations, MTSEA, MTSEA-biotin, and MTSEA-biotin-CAP were each used to modify accessible cysteines covalently. These reagents were chosen because they had the greatest effect on BZD potentiation of γγ receptors for receptors containing γγ2T75C, γγ2A79C, and γγ2T81C, respectively.

Concentration–response analysis. GABA concentration–response–reactions were scaled to a low, nondesensitizing concentration of GABA (EC$_{50}$; 3% variance of peak current) applied just before the test GABA concentration to correct for any slow drift in GABA responsiveness over the course of the experiment. All concentration–response–data were fit by the following equation:

$$ I = I_\text{max} \cdot \left[ \frac{[L]}{[L] + [EC_{50}]} \right]^{n} $$

where $I$ is the current response, $I_\text{max}$ is the maximal current response, $[L]$ is the drug concentration, $EC_{50}$ is the drug concentration that evokes half-maximal current response, and $n$ is the Hill coefficient. The FLZM potentiation of I$_\text{GABA}$ was defined as:

$$ P = (I_{\text{GABA}+\text{FLZM}} - I_{\text{GABA}}) - 1 $$

where I$_{\text{GABA}+\text{FLZM}}$ is the current response in the presence of GABA and FLZM, and I$_{\text{GABA}}$ is the current evoked solely by GABA. FLZM potentiation was measured at low concentrations of GABA (EC$_{50}$; 3% variance of peak current).

GABA concentration–response properties of γγ2A79C and γγ2T81C-containing receptors also were measured after MTSEA-biotin and MTSEA-biotin-CAP modification. In these experiments the responses of αβγγ2A79C or αβγγ2T81C receptors to different concentrations of GABA were measured in the same oocyte before and after the application of 2 mM MTS reagent for 2 min. We also examined the ability of FLZM to shift the GABA EC$_{50}$ of wild-type and γγ2A79C-containing receptors by measuring GABA concentration–response curves in the presence of 1 μM FLZM. For both GABA and FLZM concentration–response curves, individual curve fits were obtained from single oocytes. Log EC$_{50}$ values and their standard errors derived from the single curve fits were averaged and compared statistically by one-way ANOVA with Dunnnett’s post test for significance of differences. Data analysis and curve fitting were performed by using A xoGraph (Axon Instruments) and Prism software (GraphPad, San Diego, CA).

RESULTS

Expression and functional characterization of cysteine mutants

The 12 amino acids within the region γγ2Y72–γγ2Y83 were each mutated to cysteine (Fig. 1). This region of the γγ subunit includes γγF77, which has been shown previously to participate in BZD ligand discrimination and likely participates in the formation of the BZD binding site (Buhr et al., 1997; Sigel et al., 1998). To assess whether cysteine mutations affected GABA$_{A}$ receptor function and/or expression, we characterized the responsiveness of αβγγ mutant receptors to GABA and BZDs. Individual
Cysteine substitutions had no effect on the calculated Hill coefficient. Hill numbers of 2.9 ± 1.2 and 2.4 ± 0.7, respectively. An increased Hill coefficient may be an indication of mutational gain of cooperativity (Colquhoun, 1998). However, because Hill coefficients are based on a scale of whole numbers, these numbers may not be different from wild-type values.

FLZM did not potentiate I_{GABA} in γ2F77C- and γ2W82C-containing receptors. To determine whether these mutant subunits specifically disrupted FLZM potentiation or impaired receptor assembly, we assessed the Zn^{2+} sensitivities of αββγγ2F77C and αββγγ2W82C receptors. GABA receptors composed of αββγγ2 subunits are more sensitive to Zn^{2+} blockade than αββγγ2 receptor; thus Zn^{2+} sensitivity of I_{GABA} can be used to assess γ-subunit expression (Draguhn et al., 1990; Gingrich and Burkat, 1998). ZnCl2 (10 μM), when coapplied with 10 μM GABA, reduces I_{GABA} by 80% ± 7% in αββγγ2 receptors but only by 22% ± 4% in αββγγ2 receptors (n = 3; Fig. 3). For αββγγ2W82C and αββγγ2F77C receptors, ZnCl2 reduced I_{GABA} by 80% ± 14% and 30% ± 3%, respectively (n = 3; Fig. 3). Because the Zn^{2+} block of I_{GABA} in αββγγ2W82C receptors is indistinguishable from αββγγ2 receptors, it is likely that cysteine substitution at this residue is detrimental to assembly and/or cell surface expression of the γ2W82C subunit.

In contrast, the small amount of Zn^{2+} block observed for αββγγ2F77C receptors indicates that cysteine substitution at γ2F77 does not impair γ-subunit assembly and/or surface expression; thus the inability of FLZM to potentiate I_{GABA} is likely attributable to a direct effect of the mutation on BDZ binding. FLZM was unable to potentiate I_{GABA} in αββγγ2F77C receptors even at high concentrations (>10 μM), suggesting that this mutation severely disrupts the BDZ potentiation of I_{GABA}. Several structurally diverse BDZ agonists also were applied to oocytes expressing αββγγ2F77C receptors, including zolpidem and Cl218-872, to identify a BDZ for which this mutation did not disrupt recognition. None of the BDZs that were tested had an effect on I_{GABA}, suggesting that cysteine substitution at γ2F77 disrupts BDZ binding site architecture. In addition, αββγγ2F77C receptors were expressed in human embryonic kidney (HEK) 293 cells, and the specific binding of [3H]flunitrazepam and [3H]Ro 15-1788 was measured. No specific binding was detected (data not shown).

| Table 1. Summary of GABA and flurazepam concentration–response data from cysteine mutant and wild-type αββγγ2 GABA receptors |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Receptor       | EC_{50} (μM)    | n_{H}           | n   | EC_{50} mut/EC_{50} | EC_{50} (nm)    | n_{H}           | n   | EC_{50} mut/EC_{50} |
| αβγ             | 18 ± 4.7        | 1.7 ± 0.3       | 3   | 1.0                       | 250 ± 49        | 1.2 ± 0.1       | 3   | 1.0                        |
| αβγY72C         | 70 ± 12**       | 1.3 ± 0.2       | 3   | 3.9                       | 270 ± 64        | 1.3 ± 0.3       | 3   | 1.1                        |
| αβγI72C         | 20 ± 5.6        | 1.6 ± 0.3       | 3   | 1.1                       | 160 ± 15        | 1.8 ± 0.3       | 3   | 0.6                        |
| αβγI74C         | 30 ± 4.5        | 1.2 ± 0.1       | 3   | 1.7                       | 80 ± 12         | 2.9 ± 1.2       | 3   | 0.3                        |
| αβγD75C         | 39 ± 4.9**      | 1.5 ± 0.3       | 3   | 2.2                       | 4700 ± 1300**   | 1.6 ± 0.6       | 3   | 18.8                       |
| αβγI76C         | 30 ± 13         | 1.4 ± 0.5       | 3   | 1.7                       | 2600 ± 1200**   | 2.4 ± 0.7       | 3   | 10.4                       |
| αβγF77C         | 24 ± 11         | 1.0 ± 0.3       | 3   | 1.3                       | >10,000         | 3   | 3   |                            |
| αβγF78C         | 33 ± 5.8*       | 1.3 ± 0.2       | 4   | 1.8                       | 130 ± 35        | 1.8 ± 0.3       | 3   | 0.5                        |
| αβγA79C         | 30 ± 12         | 1.4 ± 0.3       | 9   | 1.7                       | 310 ± 45        | 1.4 ± 0.3       | 3   | 1.2                        |
| αβγO99C         | 9.0 ± 2.5       | 1.6 ± 0.4       | 3   | 0.5                       | 260 ± 28        | 1.0 ± 0.1       | 3   | 1.0                        |
| αβγT81C         | 15 ± 16.6       | 1.5 ± 0.1       | 4   | 0.8                       | 350 ± 170       | 1.5 ± 0.2       | 3   | 1.4                        |
| αβγW82C         | No expression   | 1.6 ± 0.2       | 3   | 0.6                       | 170 ± 17        | 1.1 ± 0.2       | 4   | 0.7                        |
| αβγY83C         | 10 ± 1.9        | 1.6 ± 0.2       | 3   | 0.6                       | No expression   | No expression   | 4   | 0.7                        |

Data represent mean ± SD values. n, Number of independent experiments; n_{H}, calculated Hill coefficient. ** Indicates values significantly different from wild-type receptors, with p < 0.05 and p < 0.01, respectively.
shown). Taken together, these results suggest that cysteine substitution at γ2F77 disrupts BZD binding and supports previous evidence that this residue is crucial for BZD recognition (Buhr et al., 1997; Sigel et al., 1998).

**Reaction of substituted cysteines with MTSEA-biotin**

SCAM has been used previously to generate novel information about the secondary structure and conformational dynamics of the GABA<sub>A</sub> receptor agonist binding site (Boileau et al., 1999; Wagner and Czajkowski, 2001) and constituent ion channel (Xu and Akabas, 1996; Williams and Akabas, 1999, 2000). In this method, consecutive amino acids are mutated one at a time to cysteine, expressed heterologously in *Xenopus* oocytes, and treated with MTSEA-biotin. Accessibility is defined by observing whether changes in receptor function occur after treatment. A major assumption of SCAM is that the mutation of a candidate amino acid to cysteine does not disrupt the orientation or accessibility of the native side chain radically. Given our evidence that the GABA EC<sub>50</sub> values for eight cysteine mutants have not been altered radically by mutation (see Table 1), it is likely that the positions of these introduced cysteine side chains reflect wild-type orientations. Although γ2D75C- and γ2I76C-containing receptors display decreased sensitivity to FLZM, GABA EC<sub>50</sub> values for these cysteine mutants are unchanged (see Table 1), suggesting that mutation at these positions does not disrupt the native structure of the receptor protein fundamentally.

We measured FLZM modulation of *I*<sub>GABA</sub> in *X. laevis* oocytes expressing γ2D75C- or γ2I76C-mutant GABA<sub>A</sub> receptors before and after treatment with 2 mm MTSEA-biotin for 2 min. Exposure of wild-type GABA<sub>A</sub>-receptor MTSEA-biotin had no significant effect on *I*<sub>GABA</sub> or on the FLZM potentiation of *I*<sub>GABA</sub> (Figs. 4B, 6C). Therefore, if effects on FLZM potentiation were observed in cysteine mutant receptors after treatment with MTSEA-biotin, we interpreted this result as evidence that covalent modification occurred at the introduced cysteine. MTSEA-biotin treatment of receptors containing γ2Y72C, γ2I74C, γ2I76C, γ2F78C, γ2Q80C, or γ2Y83C had no effects on the FLZM potentiation of *I*<sub>GABA</sub> (Fig. 4). Thus either these introduced cysteines were not accessible to MTSEA-biotin, or their modification by MTSEA-biotin had no observable effect on FLZM potentiation.

In contrast, MTSEA-biotin treatment of receptors containing γ2I73C, γ2D75C, γ2A79C, and γ2T81C significantly altered the FLZM modulation of *I*<sub>GABA</sub> (Fig. 4). After the application of MTSEA-biotin, the FLZM potentiation of *I*<sub>GABA</sub> was increased by 38 ± 25% for γ2I73C-containing receptors, whereas potentiation was decreased by 22 ± 8%, 95 ± 2%, and 23 ± 4% for γ2D75C-, γ2A79C-, and γ2T81C-containing receptors, respectively. The alternating pattern of accessibility within the region bounded by γ2T73 and γ2T81 suggests that this domain of the BZD binding site forms a β-strand.

**Identification of BZD binding site residues**

We examined the extent to which both FLZM and Ro 15-1788 could slow the rate of reaction of MTSEA reagents with accessible...
cysteines to identify residues within $\gamma_2Y72$–$\gamma_2Y83$ that line the BZD binding pocket. Although Ro 15-1788 is a BZD antagonist that competitively blocks the binding of FLZM, it does not enhance or inhibit $I_{\text{GABA}}$. Thus if the rate at which a MTS reagent reacts with an introduced cysteine is slowed by both FLZM and Ro 15-1788, then it is likely that both compounds are blocking the MTS reaction sterically and that the introduced cysteine is positioned in the BZD binding site.

MTS reaction rates were measured by examining the decrease in FLZM potentiation of $I_{\text{GABA}}$ after repeated exposure to FLZM potentiation of $I_{\text{GABA}}$ before and after a 2 min application of 2 mM MTS reaction rates were measured by examining the decrease in FLZM potentiation of $I_{\text{GABA}}$ after repeated exposure to FLZM. $I$-bars denote potentiation of $I_{\text{GABA}}$ measured during an application of 1 mM FLZM in the presence of 1 mM GABA. Note the decrease in FLZM potentiation and the increase in $I_{\text{GABA}}$ after MTS reaction after MTS modification (arrow). B, Changes in FLZM potentiation after MTS modification of $\alpha 2\beta 2\gamma 2$ (wild-type; wt) and mutant receptors. The percentage of change in FLZM potentiation after modification is defined as $\frac{((I_{\text{GABA}}\text{Potentiation}_{\text{After}})/I_{\text{GABA}}\text{Potentiation}_{\text{Before}}) - 1)\times 100}$. A negative value represents a decrease in FLZM potentiation after MTS modification, and a positive value represents an increase in FLZM potentiation after MTS modification. Black bars indicate mutants in which the change in potentiation was significantly different ($p < 0.01$) from wt receptor calculated by a one-way ANOVA with a Dunnett’s post test. Data represent mean $\pm$ SD from at least three independent experiments. *No detectable BZD potentiation of $I_{\text{GABA}}$; **no detectable $\gamma 2$ subunit expression.

MTSEA-biotin effects on the $\gamma 2Y72C$–$\gamma 2Y83C$ region. A, Representative current traces from $\alpha 1\beta 2\gamma 2A79C$ receptors showing FLZM modulation of $I_{\text{GABA}}$ before and after a 2 min application of 2 mM MTSEA-biotin. $I$-bars denote potentiation of $I_{\text{GABA}}$ measured during an application of 1 mM FLZM in the presence of 1 mM GABA. Note the decrease in FLZM potentiation and the increase in $I_{\text{GABA}}$ after MTS modification (arrow). B, Changes in FLZM potentiation after MTS modification of $\alpha 2\beta 2\gamma 2$ (wild-type; wt) and mutant receptors. The percentage of change in FLZM potentiation after modification is defined as $\frac{((I_{\text{GABA}}\text{Potentiation}_{\text{After}})/I_{\text{GABA}}\text{Potentiation}_{\text{Before}}) - 1)\times 100}$. A negative value represents a decrease in FLZM potentiation after MTS modification, and a positive value represents an increase in FLZM potentiation after MTS modification. Black bars indicate mutants in which the change in potentiation was significantly different ($p < 0.01$) from wt receptor calculated by a one-way ANOVA with a Dunnett’s post test. Data represent mean $\pm$ SD from at least three independent experiments. *No detectable BZD potentiation of $I_{\text{GABA}}$; **no detectable $\gamma 2$ subunit expression.

Figure 4. MTSEA-biotin effects on the $\gamma 2Y72C$–$\gamma 2Y83C$ region. A, Representative current traces from $\alpha 1\beta 2\gamma 2A79C$ receptors showing FLZM modulation of $I_{\text{GABA}}$ before and after a 2 min application of 2 mM MTSEA-biotin. $I$-bars denote potentiation of $I_{\text{GABA}}$ measured during an application of 1 mM FLZM in the presence of 1 mM GABA. Note the decrease in FLZM potentiation and the increase in $I_{\text{GABA}}$ after MTS modification (arrow). B, Changes in FLZM potentiation after MTS modification of $\alpha 2\beta 2\gamma 2$ (wild-type; wt) and mutant receptors. The percentage of change in FLZM potentiation after modification is defined as $\frac{((I_{\text{GABA}}\text{Potentiation}_{\text{After}})/I_{\text{GABA}}\text{Potentiation}_{\text{Before}}) - 1)\times 100}$. A negative value represents a decrease in FLZM potentiation after MTS modification, and a positive value represents an increase in FLZM potentiation after MTS modification. Black bars indicate mutants in which the change in potentiation was significantly different ($p < 0.01$) from wt receptor calculated by a one-way ANOVA with a Dunnett’s post test. Data represent mean $\pm$ SD from at least three independent experiments. *No detectable BZD potentiation of $I_{\text{GABA}}$; **no detectable $\gamma 2$ subunit expression.

Figure 5. Rate of sulfhydryl modification of $\alpha 1\beta 2\gamma 2A79C$ and $\alpha 1\beta 2\gamma 2T81C$ receptors in the presence and absence of FLZM and Ro 15-1788. A, B, Representative GABA (1 mM) and GABA plus FLZM (1 mM each) current traces recorded from $\alpha 1\beta 2\gamma 2A79C$ receptors. Arrows indicate 5 sec applications of 200 mM MTSEA-biotin alone (A) or 200 mM MTSEA-biotin plus 5 mM FLZM (B). FLZM potentiation of $I_{\text{GABA}}$ was measured before and after each MTS treatment. $I$-bars on traces show BZD-potentiated current. C, Observed decreases in FLZM potentiation of $I_{\text{GABA}}$ were plotted versus cumulative MTSEA-biotin exposure in $\alpha 1\beta 2\gamma 2A79C$ receptors. Data obtained from individual experiments were normalized to the potentiation measured at $t = 0$ and fit to single-exponential decay curves (■, MTS alone; ●, MTS + 5 mM FLZM). Data points are mean $\pm$ SD from at least three independent experiments. D, Rate experiments were performed similarly for receptors containing $\gamma 2T81C$, except that 5 sec applications of 20 mM MTSEA-biotin-CAP were used in place of MTSEA-biotin (■, MTS alone; ●, MTS + 5 mM FLZM). The calculated second-order rate constants for the MTS reaction are presented in Table 2.
were observed after MTSEA-biotin-CAP, but not MTSEA-biotin, modification of $\gamma_2T81C$-containing receptors (Fig. 6B).

We hypothesized that the increases in $I_{GABA}$ were attributable to changes in the GABA EC$_{50}$ values of $\alpha_1\beta_2\gamma_2A79C$ and $\alpha_1\beta_2\gamma_2T81C$ receptors after MTS modification. To test this hypothesis, we measured complete GABA concentration–response curves in single oocytes expressing $\alpha_1\beta_2\gamma_2A79C$ receptors before and after the application of 2 mM MTSEA-biotin (Fig. 7A) or 2 mM MTSEA-biotin-CAP (Fig. 7B). Covalent modification of $\gamma_2A79C$-containing receptors by MTSEA-biotin resulted in a significant $\sim$1.6-fold increase in GABA EC$_{50}$ ($p < 0.05$; Table 3). Likewise, MTSEA-biotin-CAP modification of $\gamma_2A79C$-containing receptors resulted in a $\sim$2.6-fold increase in GABA EC$_{50}$ ($p < 0.01$; Table 3). The GABA EC$_{50}$ shifts that were measured after the covalent modification of $\gamma_2A79C$ were similar to the shift in GABA EC$_{50}$ observed in the presence of FLZM.

To gain insight into the chemical specificity of this effect, we examined whether other MTS reagents, including MTSEA, MTS-ethyltrimethylammonium (MTSET), MTS-ethyarsulfonate (MTSES), and benzyl-MTS, also modulated $I_{GABA}$ when tethered to $\gamma_2A79C$. This result suggests that $\gamma_2T73$ is not within the BZD binding domain because it does not disrupt BZD recognition once it has been derivitized. It is possible that the increased BZD efficacy we have observed after modification of $\gamma_2T73$ is attributable to conformational changes in the BZD site that correspondingly increase the sensitivity to FLZM.

**Conformational changes detected within the BZD binding site**

According to allosteric theory, modulators bind to a site on the receptor protein that is distinct from the agonist binding site and exert their effects by initiating an allosteric transition in the protein that indirectly modifies the conformation of the agonist binding site (Changeux and Edelstein, 1998). Both radioligand binding and electrophysiological studies of the GABA$_A$ receptor have demonstrated functional interactions between the GABA and BZD binding sites (Skerritt and Johnston, 1983; Boileau and Czajkowski, 1999). Structural evidence, however, for GABA binding-site–BZD binding site communication is scarce. To detect directly whether structural changes of the BZD binding site occur during GABA binding and activation of the receptor, we examined whether GABA (100 $\mu$m; approximately EC$_{70}$/EC$_{50}$) altered the rates of reaction of MTS reagents with $\alpha_1\beta_2\gamma_2A79C$, $\alpha_1\beta_2\gamma_2T81C$, and $\alpha_1\beta_2\gamma_2A79C$ receptors. GABA significantly increased the rate of MTS modification of $\gamma_2A79C$-containing, but not $\gamma_2D75C$- or $\gamma_2T81C$-containing, receptors (Fig. 8; see Table 2). The ability of GABA to increase the accessibility of $\gamma_2A79C$ to sulfhydryl modification demonstrates that a domain of the BZD binding site undergoes an allosteric structural rearrangement during GABA binding and channel gating.
DISCUSSION

We used SCAM to examine the structure and dynamics of a region of the GABA<sub>A</sub> receptor implicated in BZD binding, γ<sub>2</sub>Y<sub>72</sub>–γ<sub>2</sub>Y<sub>83</sub> (Buhr et al., 1997; Sigel et al., 1998). Our data indicate that this region is a β-strand. We directly demonstrate that two residues that had been implicated previously in BZD binding, γ<sub>2</sub>A<sub>79</sub> and γ<sub>2</sub>T<sub>81</sub> (Kucken et al., 2000), line the BZD binding site. We show that MTSEA-biotin and MTSEA-biotin-CAP have the ability to act as covalent agonists of the BZD binding site. Last, we demonstrate that a portion of the BZD binding site undergoes structural rearrangements during GABA binding and/or gating.

Identification of amino acids in the BZD binding site

Four residues within γ<sub>2</sub>Y<sub>72</sub>–γ<sub>2</sub>Y<sub>83</sub> are accessible to MTSEA-biotin: γ<sub>2</sub>T<sub>73</sub>C, γ<sub>2</sub>D<sub>75</sub>C, γ<sub>2</sub>A<sub>79</sub>C, and γ<sub>2</sub>T<sub>81</sub>C. Of these four accessible residues, both γ<sub>2</sub>A<sub>79</sub>C and γ<sub>2</sub>T<sub>81</sub>C are protected from MTS modification by Ro 15-1788, whereas only γ<sub>2</sub>A<sub>79</sub> is protected from MTS modification by FLZM. Although antagonists may induce conformational changes in the BZD binding site, it is unlikely that these binding-associated structural movements are similar to those induced by an agonist. Thus protection observed when linked covalently to γ<sub>2</sub>A<sub>79</sub>C, A, B, GABA concentration–response curves obtained from single oocytes expressing α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>A<sub>79</sub>C receptors before (■) and after (▲) reaction with 2 mM MTSEA-biotin (A) or before (■) and after (▲) reaction with 2 mM MTSEA-biotin-CAP (B). The experiments were repeated two additional times with similar results. C, GABA concentration–response curves obtained from α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>A<sub>79</sub>C receptors in the absence (■) and presence (▲) of 1 μM FLZM. Data were fit by nonlinear regression, as described in Materials and Methods. Data represent mean ± SEM from three independent experiments. EC<sub>50</sub> values obtained from the curve fits are reported in Table 3.

Figure 6. MTSEA modification of α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>A<sub>79</sub>C and α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>T<sub>81</sub>C receptors increases I<sub>GABA</sub>. Traces represent the effect of 2 min applications (arrows) of 2 mM MTSEA-biotin or 2 mM MTSEA-biotin-CAP on current evoked by 3 μM GABA in oocytes expressing receptors containing either γ<sub>2</sub>A<sub>79</sub>C (A) or γ<sub>2</sub>T<sub>81</sub>C (B) subunits. The application of 2 mM MTSEA-biotin to oocytes expressing α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>C (C) or α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>T<sub>81</sub>C (B) receptors had no significant effect on I<sub>GABA</sub>.

Figure 7. MTSEA-biotin and MTSEA-biotin-CAP shift GABA EC<sub>50</sub> when linked covalently to γ<sub>2</sub>A<sub>79</sub>C. A, B, GABA concentration–response curves obtained from single oocytes expressing α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>A<sub>79</sub>C receptors before (■) and after (▲) reaction with 2 mM MTSEA-biotin (A) or before (■) and after (▲) reaction with 2 mM MTSEA-biotin-CAP (B). The experiments were repeated two additional times with similar results. C, GABA concentration–response curves obtained from α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>A<sub>79</sub>C receptors in the absence (■) and presence (▲) of 1 μM FLZM. Data were fit by nonlinear regression, as described in Materials and Methods. Data represent mean ± SEM from three independent experiments. EC<sub>50</sub> values obtained from the curve fits are reported in Table 3.
at an introduced cysteine in the presence of both an agonist and antagonist is good evidence that the cysteine lines the binding site. Therefore, we believe that \(\gamma_2\)T79 is facing into the BZD binding pocket. Because only Ro 15-1788 is able to protect \(\gamma_2\)T81C from sulphhydryl modification, we cannot conclude definitively that \(\gamma_2\)T81 lines the BZD binding site by our criteria. However, other evidence also suggests that \(\gamma_2\)T81 is facing into the binding site. In our study we demonstrate that MTSEA-biotin-CAP acts as a tethered agonist at this site. Moreover, we have shown previously via chimeric mutagenesis studies that both \(\gamma_2\)A79 and \(\gamma_2\)T81 are important determinants of BZD binding (Kucken et al., 2000). Although we could not evaluate the accessibility of \(\gamma_2\)F77C, it has been well established in previous studies that this residue is a critical determinant of BZD binding (Buhr et al., 1997; Sigel et al., 1998). Thus our data support a model in which \(\gamma_2\)F77, \(\gamma_2\)A79, and \(\gamma_2\)T81 line the BZD binding site.

### Secondary structure of the \(\gamma_2\)Y72–\(\gamma_2\)Y83 region of the BZD binding site

Alternating residues within the region \(\gamma_2\)T73–\(\gamma_2\)T81 are accessible to MTSEA-biotin. These data are consistent with a model in which this region forms a \(\beta\)-strand. Because the accessibility of \(\gamma_2\)F77C could not be tested, a strict pattern of alternating exposure has not been established absolutely. The residues accessible to MTSEA-biotin, with the exception of \(\gamma_2\)A79, are hydrophilic amino acid residues. Because MTSEA-biotin is relatively impermeant (Chen et al., 1998) and MTS reagents react from 10- to 100-fold faster with ionized sulphhydryl groups than protonated sulphhydryls (Roberts et al., 1986) and ionization of a sulphhydryl is much more probable in an aqueous environment, the accessible residues likely are exposed at the water-accessible surface of the protein. 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 reactions that appear to have no functional consequences. Nevertheless, it is unlikely that the addition of a large biotin moiety would have no effect on BZD modulation of \(I_{\text{GABA}}\) if \(\gamma_2\)Y72C, \(\gamma_2\)Y74C, \(\gamma_2\)Y76C, \(\gamma_2\)F78C, or \(\gamma_2\)Q80C actually face into the BZD binding pocket. We were unable to test the accessibility of \(\gamma_2\)W82C, because cysteine substitution at this residue impaired receptor assembly and/or expression. This tryptophan is highly conserved across many ligand-gated ion channel subunits and previously has been shown to regulate GABA<sub>\(\alpha\)1</sub> receptor \(\alpha_1\) subunit assembly (Srinivasan et al., 1999). Thus, it is reasonable to assume that \(\gamma_2\)W82 is not solvent-accessible, because it is hydrophobic and likely participates in intraprotein contacts that are associated with subunit assembly.

Taken together, the results of this study strongly suggest that the polypeptide chain from \(\gamma_2\)T73 to \(\gamma_2\)T81 forms a \(\beta\)-strand and that a portion of this strand lines the BZD binding site. In agreement with our experimental results, this region is predicted by secondary structure modeling algorithms (Chou and Fasman, 1978) to adopt a \(\beta\)-strand conformation. Interestingly, an aligned region of the \(\alpha_3\) subunit has been shown to form part of the GABA binding site and displays a similar secondary structure (Boileau et al., 1999).

### Structural rearrangements in the BZD binding site

A central question in GABA<sub>\(\alpha\)</sub> receptor pharmacology is how the binding of BZD ligands is transduced into allosteric modulation of the GABA<sub>\(\alpha\)</sub> receptor. It is likely that functional coupling between the BZD and GABA binding sites is accompanied by structural rearrangements in the receptor protein that change the apparent affinity of both sites (Changeux and Edelstein, 1998; Colquhoun, 1998). We demonstrate that a residue that faces into the BZD binding pocket \((\gamma_2\)A79) experiences an increase in accessibility to MTSEA-biotin modification during GABA binding and channel gating (see Fig. 8). In the time course of our experiments GABA induces both channel opening and desensitization; thus we cannot distinguish which gating transition is responsible for the increase in accessibility. Nevertheless, our results are consistent with a model in which \(\gamma_2\)A79 (or residues near \(\gamma_2\)A79) move(s) during GABA-associated gating transitions. We hypothesize that GABA gating causes movement within the BZD binding site that makes it easier for MTS reagents or BZDs to approach physically and interact with the site. Alternatively, an increase in accessibility could reflect a change in the ionization state of the introduced cysteine. Regardless of the mechanism, these data provide direct physical evidence that confirms allosteric theory; structural rearrangements occur within the BZD binding site in response to GABA binding to its own distinct site on the receptor. A recent study also has detected movements within the third transmembrane domain of the GABA<sub>\(\alpha\)</sub> receptor during allosteric modulation by BZDs (Williams and Akabas, 2000).

### Theoretical model of the BZD binding site

We demonstrate that \(\gamma_2\)T73, \(\gamma_2\)D75, \(\gamma_2\)A79, and \(\gamma_2\)T81 line the accessible surface of a \(\beta\)-strand in the \(\gamma_2\) subunit of the GABA<sub>\(\alpha\)</sub> receptor, with \(\gamma_2\)A79 and \(\gamma_2\)T81 in close proximity to the BZD ligand binding domain. We hypothesize that FLZM is topologically close to both \(\gamma_2\)F77 and \(\gamma_2\)A79 in the BZD binding site. Previous reports have speculated that the 5'-phenyl substituent of classical BZDs, such as FLZM, may participate in \(\pi\)-\(\pi\) stacking interactions with \(\gamma_2\)F77 (Buhr et al., 1997; Sigel et al., 1998), whereas others have suggested that these interactions also may

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**Table 3. GABA EC<sub>50</sub> values of \(\alpha_1\)\(\beta_2\)\(\gamma_2\) and \(\alpha_1\)\(\beta_2\)\(\gamma_2\)A79C receptors before and after treatment with 2 mM MTSEA-biotin, 2 mM MTSEA-biotin-CAP, or 1 \(\mu\)M FLZM**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>GABA EC&lt;sub&gt;50&lt;/sub&gt; ((\mu)M) before</th>
<th>(n)</th>
<th>Treatment</th>
<th>GABA EC&lt;sub&gt;50&lt;/sub&gt; ((\mu)M) after</th>
<th>(n)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; before/ EC&lt;sub&gt;50&lt;/sub&gt; after</th>
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<tbody>
<tr>
<td>(\alpha_1)(\beta_2)</td>
<td>18 ± 4.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>FLZM</td>
<td>5.5 ± 0.9&lt;sup&gt;**&lt;/sup&gt;</td>
<td>3</td>
<td>3.3</td>
</tr>
<tr>
<td>(\alpha_1)(\beta_2)A79C</td>
<td>30 ± 12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9</td>
<td>FLZM</td>
<td>7.8 ± 5.6&lt;sup&gt;**&lt;/sup&gt;</td>
<td>3</td>
<td>3.8</td>
</tr>
<tr>
<td>(\alpha_1)(\beta_2)A79C</td>
<td>43 ± 6.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3</td>
<td>MTSEA-biotin</td>
<td>28 ± 7.4&lt;sup&gt;**&lt;/sup&gt;</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>(\alpha_1)(\beta_2)A79C</td>
<td>29 ± 1.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3</td>
<td>MTSEA-biotin-CAP</td>
<td>11 ± 3.3&lt;sup&gt;**&lt;/sup&gt;</td>
<td>3</td>
<td>2.6</td>
</tr>
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</table>

Data represent mean ± SD values. *,** indicate values significantly different from GABA before (control), with \(p < 0.05\) and \(p < 0.01\), respectively.

<sup>a</sup>EC<sub>50</sub> values taken from Table 1.

<sup>b</sup>EC<sub>50</sub> values from single oocyte experiments before and after MTSEA-biotin application.

<sup>c</sup>EC<sub>50</sub> values from single oocyte experiments before and after MTSEA-biotin-CAP application.
Figure 8. A. Structures and lengths (in angstroms) of the different MTS reagents used in our experiments. Lengths were measured after energy minimization (<0.5 kcal/Å) and represent only the portion of the MTS reagent that covalently modifies an introduced cysteine. Cleavage points of each MTS reagent are indicated by an arrow. B. Summary of the second-order rate constants calculated for MTS derivitization of γ2D75C-, γ2A79C-, and γ2T81C-containing receptors. Oocytes expressing mutant receptors were incubated in the presence of MTS alone (control), MTS + FLZM, MTS + Ro 15-1788, or MTS + GABA. MTS reagents used were as follows: γ2D75C, MTSEA; γ2A79C, MTSEA-biotin; γ2T81C, MTSEA-biotin-CAP. Second-order rate constants were calculated for each MTS reaction and were normalized to the rate measured in the absence of ligand (control). Displayed values are mean ± SD from at least three independent experiments. **Indicate values significantly different from control MTS values, with p < 0.05 and p < 0.01, respectively.

include ααH101 (Davies et al., 1998; McKernan et al., 1998). We hypothesize that FLZM is oriented such that its S′-phenyl is in close contact with γ2F77 and that it occupies space within the binding site that is in close proximity to γ2A79. Although it is unlikely that FLZM chemically interacts with this alanine, the small size of the methyl group at this position may be important in maintaining the architecture of the BZD site because cysteine substitution at this position reduces the FLZM sensitivity of the receptor.

Our data demonstrate that MTSEA-biotin and MTSEA-biotin-CAP, after the modification of γ2A79, are oriented in a manner such that they are able to modulate allosterically the EC50 of the GABA binding site for GABA. Interestingly, although MTSEA-biotin shifts the GABA EC50 for γ2A79C-containing receptors within the range expected for a BZD partial agonist, this reagent has little-to-no effect on the GABA EC50 of γ2T81C-containing receptors. In contrast, MTSEA-biotin-CAP modification of γ2A79C-containing receptors shifts the GABA EC50 within the range of a full agonist and partially shifts the GABA EC50 of γ2T81C-containing receptors. Because MTSEA-biotin-CAP is 8 Å longer than MTSEA-biotin (see Fig. 8), these data suggest that γ2T81 lies farther than γ2A79 from a domain of the BZD binding site that drives allosteric interaction with the GABA binding site.

We speculate that MTSEA-biotin and MTSEA-biotin-CAP bridge the BZD binding site and are capable of exerting their allosteric effects on the GABA binding site by inducing shifts in the distance of α1 and γ2 subunits relative to each other. This mechanism may represent one set of conformational changes that may be required to transduce the binding of BZDs into allosteric modulation of the GABA binding site. Further studies that use the cross-linking of α1 and γ2 residues to span the BZD binding site will be necessary to test this hypothesis. Our results confirm the long-held belief that structural changes in the GABAγ receptor protein underlie allosteric communication between the GABA and BZD binding sites.

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