

# A $\beta$ -Strand in the $\gamma_2$ Subunit Lines the Benzodiazepine Binding Site of the GABA<sub>A</sub> 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<sub>A</sub> receptors. Individual amino acids have been implicated in BZD recognition and modulation of the GABA<sub>A</sub> 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<sub>A</sub> receptor implicated in BZD binding,  $\gamma_2$ Y72– $\gamma_2$ Y83. Each residue within this region was mutated to cysteine and expressed with wild-type  $\alpha_1$  and  $\beta_2$  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  $\gamma_2$ T73– $\gamma_2$ T81 adopts a

$\beta$ -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,  $\gamma_2$ A79 and  $\gamma_2$ T81, that line the BZD binding site. Sulfhydryl modification of  $\gamma_2$ A79C or  $\gamma_2$ T81C allosterically shifts the GABA EC<sub>50</sub> 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<sub>A</sub> receptor; substituted cysteine accessibility method; *Xenopus* oocytes; secondary structure

Benzodiazepines (BZDs) are among the most commonly prescribed therapeutics in the treatment of panic disorder, sleeplessness, and epilepsy (Doble and Martin, 1996). BZDs exert their anxiolytic and hypnotic effects by binding to a unique site on the GABA<sub>A</sub> 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<sup>−</sup> conductance by allosterically decreasing the GABA concentration needed to elicit half-maximal channel activity (EC<sub>50</sub>; 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<sub>A</sub> receptor involved in BZD binding, a detailed structural map of the BZD binding pocket does not exist yet.

Both GABA<sub>A</sub> receptor  $\alpha$ - and  $\gamma$ -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  $\gamma_2$  subunit have been shown to affect ligand discrimination at the BZD site:  $\gamma_2$ F77 (Buhr et al., 1997; Sigel et al., 1998),  $\gamma_2$ A79 and  $\gamma_2$ T81 (Kucken et al., 2000),  $\gamma_2$ M130 (Buhr and Sigel, 1997; Wingrove et al., 1997), and  $\gamma_2$ M57 and  $\gamma_2$ Y58 (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  $\gamma_2$ F77 region of the BZD binding site. We demonstrate that the polypeptide backbone surrounding  $\gamma_2$ F77 is a  $\beta$ -strand, that  $\gamma_2$ A79 and  $\gamma_2$ T81 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<sub>A</sub> 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<sub>A</sub> receptor.

## MATERIALS AND METHODS

**Cysteine mutagenesis.** Rat cDNAs encoding  $\alpha_1$ ,  $\beta_2$ , and  $\gamma_{2S}$  GABA<sub>A</sub> receptor subunits were used for all molecular cloning and functional studies.  $\gamma_2$  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  $\gamma_2$

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cysteine mutants were verified by double-stranded DNA sequencing and restriction enzyme analysis. The  $\gamma_2$  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).

**cRNA expression in *Xenopus laevis* oocytes.** Capped cRNAs encoding individual  $\gamma_2$  cysteine mutants were transcribed *in vitro* from *NheI*-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  $\text{Ca}^{2+}$ -free ND96 [(in mM) 96 NaCl, 2 KCl, 1  $\text{MgCl}_2$ , and 5 HEPES, pH 7.2] for 20 min at room temperature and defolliculated in osmotic shock solution [130 mM  $\text{K}_2\text{HPO}_4$  and 1 mg/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 (10–200 pg/nl per subunit) in the ratio 1:1:10 ( $\alpha$ : $\beta$ : $\gamma$ ; 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  $\text{CaCl}_2$ ) supplemented with 100  $\mu\text{g/ml}$  gentamycin and 100  $\mu\text{g/ml}$  BSA and were assayed functionally at least 2 d after cRNA 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 volume was  $\sim 200$   $\mu\text{l}$ . Borosilicate electrodes used in recording (0.4–1.6 M $\Omega$ ) 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 flurazepam (FLZM) were made up in ND96 by diluting from a 10 mM stock made in water. Concentrations of Ro 15-1788 were made up in ND96 by diluting from a 10 mM stock made in DMSO. The final concentration of DMSO in solution was always  $<1\%$  and did not affect GABA<sub>A</sub> receptor properties.

**Methanethiosulfonate (MTS) reagents.** Three derivatives of methanethiosulfonate ( $\text{CH}_3\text{SO}_2\text{SCH}_2\text{CH}_2\text{X}$ ; MTS) were used to modify covalently the introduced cysteines: MTS ethylammonium ( $\text{X} = \text{NH}_3^+$ ; MTSEA), *N*-biotinylaminoethyl MTS ( $\text{X} = \text{NH}$ -biotin; MTSEA-biotin), and *N*-biotinylaminoethyl CAP MTS ( $\text{X} = \text{NHCO}(\text{CH}_2)_5\text{NH}$ -biotin; 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  $I_{\text{GABA}}$  for receptors containing  $\gamma_2\text{D75C}$ ,  $\gamma_2\text{A79C}$ , and  $\gamma_2\text{T81C}$ , respectively.

**Concentration–response analysis.** GABA concentration–responses were scaled to a low, nondesensitizing concentration of GABA ( $\text{EC}_2$ – $\text{EC}_{10}$ ) applied just before the test GABA concentration to correct for any slow drift in  $I_{\text{GABA}}$  responsiveness over the course of the experiment. All concentration–response data were fit by the following equation:

$$I = I_{\text{max}} * [L]^n / [L]^n + [\text{EC}_{50}]^n,$$

where  $I$  is the current response,  $I_{\text{max}}$  is the maximal current response,  $[L]$  is the drug concentration,  $\text{EC}_{50}$  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:

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

where  $I_{\text{GABA+FLZM}}$  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 ( $\text{EC}_2$ – $\text{EC}_{10}$ ).

GABA concentration–response properties of  $\gamma_2\text{A79C}$ - and  $\gamma_2\text{T81C}$ -containing receptors also were measured after MTSEA-biotin and MTSEA-biotin-CAP modification. In these experiments the responses of  $\alpha_1\beta_2\gamma_2\text{A79C}$  or  $\alpha_1\beta_2\gamma_2\text{T81C}$  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  $\text{EC}_{50}$  of wild-type and  $\gamma_2\text{A79C}$ -containing receptors by measuring GABA concentration–response curves in the presence of 1  $\mu\text{M}$  FLZM. For both GABA and FLZM concentration–response curves, individual curve fits were obtained from single oocytes. Log  $\text{EC}_{50}$  values and  $n_H$  values derived from the single curve fits were averaged and compared statistically by one-way ANOVA with Dunnett's post test for significance of differences. Data analysis and curve fitting were performed by using AxoGraph (Axon Instruments) and Prism software (GraphPad, San Diego, CA).

**MTSEA-biotin modification.** GABA and BZD current responses of oocytes expressing  $\alpha_1\beta_2\gamma_2$  or  $\alpha_1\beta_2\gamma_2$ -mutant receptors were stabilized before exposure to MTS reagents (Toronto Research Biochemicals, Downsview, Ontario) by applying two to four pulses of each ligand over a 20 min period. Stability was defined as  $<3\%$  variance of peak current responses to both GABA and FLZM. For all experiments, FLZM was used to measure the BZD potentiation of  $I_{\text{GABA}}$  before and after the MTS treatment. GABA concentrations ranged from  $\text{EC}_2$  to  $\text{EC}_{10}$ , and FLZM concentrations were approximately  $\text{EC}_{80}$ . Because  $\gamma_2\text{D75C}$ - and  $\gamma_2\text{T81C}$ -containing receptors exhibited a rightward shift in responsiveness to FLZM, these mutants were tested with 5  $\mu\text{M}$  FLZM. The effects of covalent modification by MTSEA-biotin were tested as follows: after achieving current stability,  $I_{\text{GABA}}$  and  $I_{\text{GABA+FLZM}}$  were measured, followed by a 3 min wash; 2 mM MTSEA-biotin was bath-applied for 2 min, followed by a 5 min wash; then  $I_{\text{GABA}}$  and  $I_{\text{GABA+FLZM}}$  were redetermined at the same concentrations that were used before MTSEA-biotin treatment. The covalent effect of MTSEA-biotin was taken as:

$$[(\text{FLZM Potentiation}_{\text{After MTS}} / \text{FLZM Potentiation}_{\text{Before MTS}}) - 1] * 100.$$

**MTS rates of reaction.** Rates of sulfhydryl-specific covalent modification of  $\alpha_1\beta_2\gamma_2\text{D75C}$ ,  $\alpha_1\beta_2\gamma_2\text{A79C}$ , and  $\alpha_1\beta_2\gamma_2\text{T81C}$  receptors were determined by monitoring the effect of sequential subsaturating applications of MTS reagents on the potentiation of  $I_{\text{GABA}}$  by FLZM. Rates were determined as follows: after achieving current stability,  $I_{\text{GABA}}$  and  $I_{\text{GABA+FLZM}}$  were measured by applying 1  $\mu\text{M}$  GABA and 1  $\mu\text{M}$  GABA plus 1  $\mu\text{M}$  FLZM, respectively (except in the case of receptors containing  $\gamma_2\text{D75C}$ , when 5  $\mu\text{M}$  FLZM was used); the oocyte was washed for 30 sec in ND96; the MTS reagent was applied by using a concentration and duration of application for which a robust effect could be observed but that did not result in a complete block of BZD potentiation; the oocyte was washed for 3 min in ND96;  $I_{\text{GABA}}$  and  $I_{\text{GABA+FLZM}}$  were redetermined, and the entire sequence was repeated. This protocol was continued until the reaction was complete ( $I_{\text{GABA+FLZM}}$  no longer changed). Concentrations and durations of MTS application were as follows:  $\gamma_2\text{D75C}$ , 200  $\mu\text{M}$  MTS-EA, 10 sec;  $\gamma_2\text{A79C}$ , 200  $\mu\text{M}$  MTSEA-biotin, 5 sec; and  $\gamma_2\text{T81C}$ , 20  $\mu\text{M}$  MTSEA-biotin-CAP, 5 sec. The decrease in FLZM potentiation of  $I_{\text{GABA}}$  was plotted versus cumulative time of MTS exposure and fit to the single-exponential decay equation:

$$Y = AE^{-kt},$$

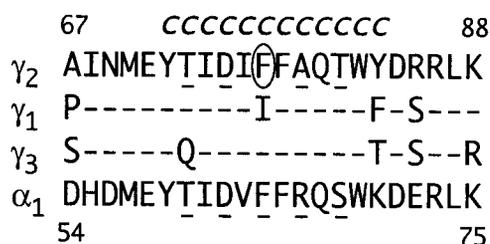
where  $A$  is the initial response,  $k$  is the pseudo-first-order rate constant of the reaction, and  $t$  is the time in seconds (GraphPad). The derived pseudo-first-order rate constant was converted into a second-order rate constant ( $k_2$ , M/sec) by dividing by the concentration of MTS reagent that was used (Pascual and Karlin, 1998). The effects of different drugs on the MTS reaction rates were assayed by the coapplication of GABA, FLZM, or Ro 15-1788 with the MTS reagent. Concentrations of drugs used in these experiments were as follows:  $\gamma_2\text{D75C}$ , 5  $\mu\text{M}$  FLZM, 1  $\mu\text{M}$  Ro 15-1788, 100  $\mu\text{M}$  GABA;  $\gamma_2\text{A79C}$ , 5  $\mu\text{M}$  FLZM, 5  $\mu\text{M}$  Ro 15-1788, 100  $\mu\text{M}$  GABA;  $\gamma_2\text{T81C}$ , 5  $\mu\text{M}$  FLZM, 1  $\mu\text{M}$  Ro 15-1788, 100  $\mu\text{M}$  GABA. With the exception of FLZM in experiments with  $\gamma_2\text{D75C}$ -containing receptors, the concentrations of ligands reflect approximate  $\text{EC}_{05}$  concentrations. In some cases, after treating the oocytes with MTS reagent in the presence of a BZD, we reexposed receptors to the same concentration of MTS reagent alone to demonstrate that a maximal decrease in FLZM potentiation of  $I_{\text{GABA}}$  was still obtainable.

**Statistics.** In all experiments the data were analyzed by one-way ANOVA, applying the Dunnett's post test for significance of differences between treatments ( $p < 0.05$ ; GraphPad).

## RESULTS

### Expression and functional characterization of cysteine mutants

The 12 amino acids within the region  $\gamma_2\text{Y72}$ – $\gamma_2\text{Y83}$  were each mutated to cysteine (Fig. 1). This region of the  $\gamma_2$  subunit includes  $\gamma_2\text{F77}$ , 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<sub>A</sub> receptor function and/or expression, we characterized the responsiveness of  $\alpha_1\beta_2\gamma_2$  mutant receptors to GABA and BZDs. Individual



**Figure 1.** Aligned partial sequences of the rat GABA<sub>A</sub> receptor  $\gamma_{1-3}$  and  $\alpha_1$  subunit isoforms. Numbering reflects alignment with the mature  $\gamma_2$  subunit. Residues in  $\gamma_1$  and  $\gamma_3$  that are identical to  $\gamma_2$  residues are represented by a dash.  $\gamma_2$ F77, the circled residue, has been implicated previously in BZD binding and modulation (Buhr et al., 1997; Sigel et al., 1998). Each amino acid in the region  $\gamma_2$ Y72– $\gamma_2$ Y83 was mutated individually to cysteine and is denoted by a C above the corresponding wild-type  $\gamma_2$  residue. Underlined residues in  $\alpha_1$  (Boileau et al., 1999) and  $\gamma_2$  represent amino acids that are accessible to sulfhydryl-specific modification after mutation to cysteine.

cysteine mutant  $\gamma_2$  subunits were coexpressed with wild-type  $\alpha_1$  and  $\beta_2$  subunits in *X. laevis* oocytes, and GABA-elicited currents ( $I_{GABA}$ ) as well as FLZM potentiation of  $I_{GABA}$  were measured with two-electrode voltage clamp.

Cysteine substitution was well tolerated within the region  $\gamma_2$ Y72– $\gamma_2$ Y83. The GABA EC<sub>50</sub> values for eight cysteine mutants were not significantly different from wild-type values. For  $\gamma_2$ Y72C-,  $\gamma_2$ D75C-, and  $\gamma_2$ F78C-containing receptors the GABA EC<sub>50</sub> values were shifted less than fourfold (Table 1; Fig. 2A). Cysteine substitutions had no effect on the calculated Hill coefficients for GABA-mediated activation of the receptor.

Because the presence of a  $\gamma_2$  subunit confers BZD sensitivity to GABA<sub>A</sub> receptors (Hevers and Lüddens, 1998), detectable potentiation of  $I_{GABA}$  in the presence of FLZM was taken to indicate functional expression of a mutant  $\gamma_2$  subunit. Cysteine substitution of eight mutants in the  $\gamma_2$  subunit did not disrupt sensitivity to FLZM. For  $\gamma_2$ D75C- and  $\gamma_2$ I76C-containing receptors, FLZM EC<sub>50</sub> values were increased 19- and 10-fold, respectively (Table 1; Fig. 2B). FLZM-associated Hill coefficients were not noticeably different from wild-type values, except for  $\gamma_2$ I74C- and  $\gamma_2$ I76C-containing receptors, which displayed Hill numbers

of  $2.9 \pm 1.2$  and  $2.4 \pm 0.7$ , 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  $\gamma_2$ F77C- and  $\gamma_2$ W82C-containing receptors. To determine whether these mutant subunits specifically disrupted FLZM potentiation or impaired receptor assembly, we assessed the Zn<sup>2+</sup> sensitivities of  $\alpha_1\beta_2\gamma_2$ F77C and  $\alpha_1\beta_2\gamma_2$ W82C receptors. GABA receptors composed of  $\alpha_1\beta_2$  subunits are more sensitive to Zn<sup>2+</sup> blockade than  $\alpha_1\beta_2\gamma_2$  receptors; thus Zn<sup>2+</sup> sensitivity of  $I_{GABA}$  can be used to assess  $\gamma$ -subunit expression (Draguhn et al., 1990; Gingrich and Burkat, 1998). ZnCl<sub>2</sub> (10  $\mu$ M), when coapplied with 10  $\mu$ M GABA, reduces  $I_{GABA}$  by  $80 \pm 7\%$  in  $\alpha_1\beta_2$  receptors but only by  $22 \pm 4\%$  in  $\alpha_1\beta_2\gamma_2$  receptors ( $n = 3$ ; Fig. 3). For  $\alpha_1\beta_2\gamma_2$ W82C and  $\alpha_1\beta_2\gamma_2$ F77C receptors, ZnCl<sub>2</sub> reduced  $I_{GABA}$  by  $80 \pm 14\%$  and  $30 \pm 3\%$ , respectively ( $n = 3$ ; Fig. 3). Because the Zn<sup>2+</sup> block of  $I_{GABA}$  in  $\alpha_1\beta_2\gamma_2$ W82C receptors is indistinguishable from  $\alpha_1\beta_2$  receptors, it is likely that cysteine substitution at this residue is detrimental to assembly and/or cell surface expression of the  $\gamma_2$ W82C subunit.

In contrast, the small amount of Zn<sup>2+</sup> block observed for  $\alpha_1\beta_2\gamma_2$ F77C receptors indicates that cysteine substitution at  $\gamma_2$ F77 does not impair  $\gamma$ -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 BZD binding. FLZM was unable to potentiate  $I_{GABA}$  in  $\alpha_1\beta_2\gamma_2$ F77C receptors even at high concentrations (>10  $\mu$ M), suggesting that this mutation severely disrupts the BZD potentiation of  $I_{GABA}$ . Several structurally diverse BZD agonists also were applied to oocytes expressing  $\alpha_1\beta_2\gamma_2$ F77C receptors, including zolpidem and CI 218–872, to identify a BZD for which this mutation did not disrupt recognition. None of the BZDs that were tested had an effect on  $I_{GABA}$ , suggesting that cysteine substitution at  $\gamma_2$ F77 disrupts BZD binding site architecture. In addition,  $\alpha_1\beta_2\gamma_2$ F77C receptors were expressed in human embryonic kidney (HEK) 293 cells, and the specific binding of [<sup>3</sup>H]flunitrazepam and [<sup>3</sup>H]Ro 15-1788 was measured. No specific binding was detected (data not

**Table 1.** Summary of GABA and flurazepam concentration–response data from cysteine mutant and wild-type  $\alpha_1\beta_2\gamma_2$  GABA<sub>A</sub> receptors

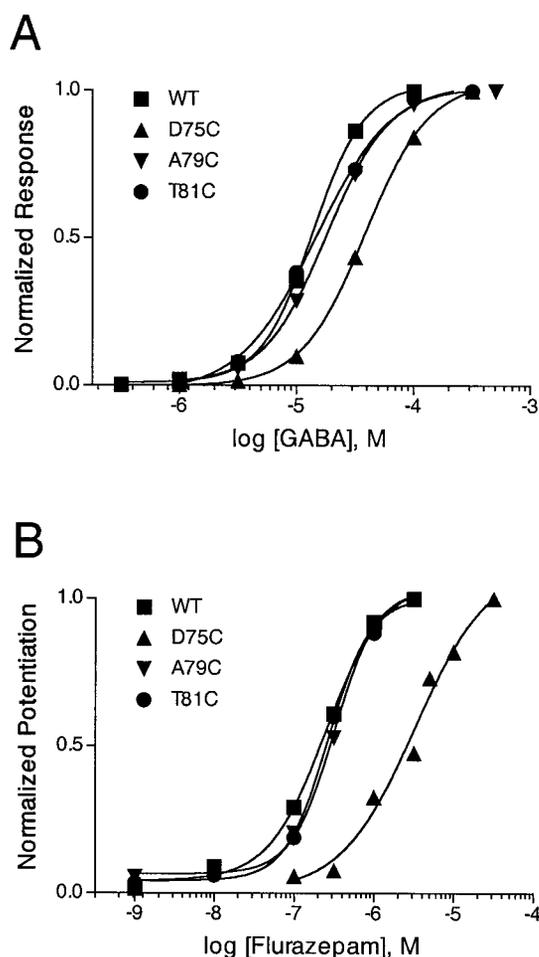
Receptor	GABA				Flurazepam			
	EC <sub>50</sub> ( $\mu$ M)	$n_H$	$n$	EC <sub>50</sub> mut/EC <sub>50</sub> $\alpha\beta\gamma$	EC <sub>50</sub> (nM)	$n_H$	$n$	EC <sub>50</sub> mut/EC <sub>50</sub> $\alpha\beta\gamma$
$\alpha\beta\gamma$	18 $\pm$ 4.7	1.7 $\pm$ 0.3	3	1.0	250 $\pm$ 49	1.2 $\pm$ 0.1	3	1.0
$\alpha\beta\gamma$ Y72C	70 $\pm$ 12**	1.3 $\pm$ 0.2	3	3.9	270 $\pm$ 64	1.3 $\pm$ 0.3	3	1.1
$\alpha\beta\gamma$ T73C	20 $\pm$ 5.6	1.6 $\pm$ 0.3	3	1.1	160 $\pm$ 15	1.8 $\pm$ 0.3	3	0.6
$\alpha\beta\gamma$ I74C	30 $\pm$ 4.5	1.2 $\pm$ 0.1	3	1.7	80 $\pm$ 12	2.9 $\pm$ 1.2	3	0.3
$\alpha\beta\gamma$ D75C	39 $\pm$ 4.9**	1.5 $\pm$ 0.3	3	2.2	4700 $\pm$ 1300**	1.6 $\pm$ 0.6	3	18.8
$\alpha\beta\gamma$ I76C	30 $\pm$ 13	1.4 $\pm$ 0.5	3	1.7	2600 $\pm$ 1200**	2.4 $\pm$ 0.7	3	10.4
$\alpha\beta\gamma$ F77C	24 $\pm$ 11	1.0 $\pm$ 0.3	3	1.3	>10,000		3	
$\alpha\beta\gamma$ F78C	33 $\pm$ 5.8*	1.3 $\pm$ 0.2	4	1.8	130 $\pm$ 35	1.8 $\pm$ 0.3	3	0.5
$\alpha\beta\gamma$ A79C	30 $\pm$ 12	1.4 $\pm$ 0.3	9	1.7	310 $\pm$ 45	1.4 $\pm$ 0.3	3	1.2
$\alpha\beta\gamma$ Q80C	9.0 $\pm$ 2.5	1.6 $\pm$ 0.4	3	0.5	260 $\pm$ 28	1.0 $\pm$ 0.1	3	1.0
$\alpha\beta\gamma$ T81C	15 $\pm$ 1.6	1.5 $\pm$ 0.1	4	0.8	350 $\pm$ 170	1.5 $\pm$ 0.2	3	1.4
$\alpha\beta\gamma$ W82C		No expression				No expression		
$\alpha\beta\gamma$ Y83C	10 $\pm$ 1.9	1.6 $\pm$ 0.2	3	0.6	170 $\pm$ 17	1.1 $\pm$ 0.2	4	0.7

Data represent mean  $\pm$  SD values.  $n$ , Number of independent experiments;  $n_H$ , calculated Hill coefficient. \*\*\*Indicate values significantly different from wild-type receptors, with  $p < 0.05$  and  $p < 0.01$ , respectively.

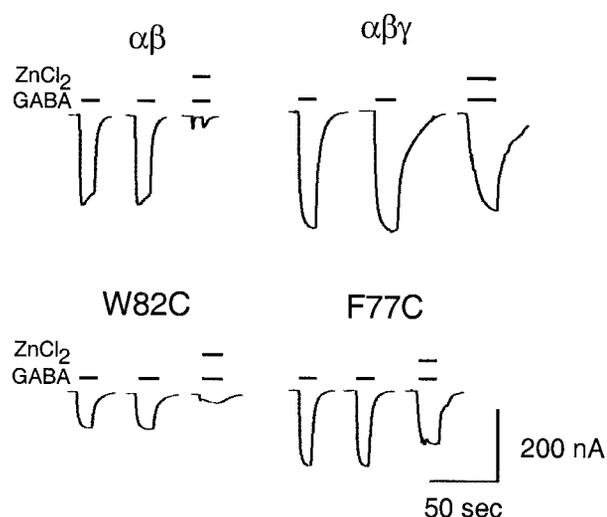
shown). Taken together, these results suggest that cysteine substitution at  $\gamma_2$ F77 disrupts BZD binding and supports previous evidence that this residue is crucial for BZD recognition (Buhr et al., 1997; Sigel et al., 1998).

#### 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 vitro*, and treated with sulfhydryl-specific reagents. 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



**Figure 2.** GABA (*A*) and BZD (*B*) concentration–response curves of wild-type  $\alpha_1\beta_2\gamma_2$  GABA<sub>A</sub> receptors and three representative mutant receptors:  $\alpha_1\beta_2\gamma_2$ D75C,  $\alpha_1\beta_2\gamma_2$ A79C, and  $\alpha_1\beta_2\gamma_2$ T81C. Oocytes expressing  $\alpha_1$ ,  $\beta_2$ , and  $\gamma_2$  or  $\gamma_2$ -mutant subunits were treated with increasing concentrations of GABA or flurazepam (FLZM) while current responses were recorded by using two-electrode voltage clamp. *A*, Responses to GABA are normalized to  $I_{GABA \text{ Max}}$ . *B*, FLZM potentiation of  $I_{GABA}$  was measured with 1  $\mu$ M GABA. For each mutant, FLZM potentiation is normalized to maximal potentiation. Data were fit by nonlinear regression, as described in Materials and Methods. Experiments were performed at least three times with similar results. EC<sub>50</sub> values obtained from the curve fits are reported in Table 1.



**Figure 3.** Zn<sup>2+</sup> sensitivity of  $\alpha_1\beta_2$ ,  $\alpha_1\beta_2\gamma_2$ , and  $\alpha_1\beta_2\gamma_2$  mutant GABA<sub>A</sub> receptors. GABA-activated current traces were recorded from oocytes expressing  $\alpha_1\beta_2$ ,  $\alpha_1\beta_2\gamma_2$ ,  $\alpha_1\beta_2\gamma_2$ F77C, or  $\alpha_1\beta_2\gamma_2$ W82C receptors. Bars represent 10–20 sec applications of 10  $\mu$ M GABA in the presence or absence of 10  $\mu$ M ZnCl<sub>2</sub>. Data reflect triplicate determinations.

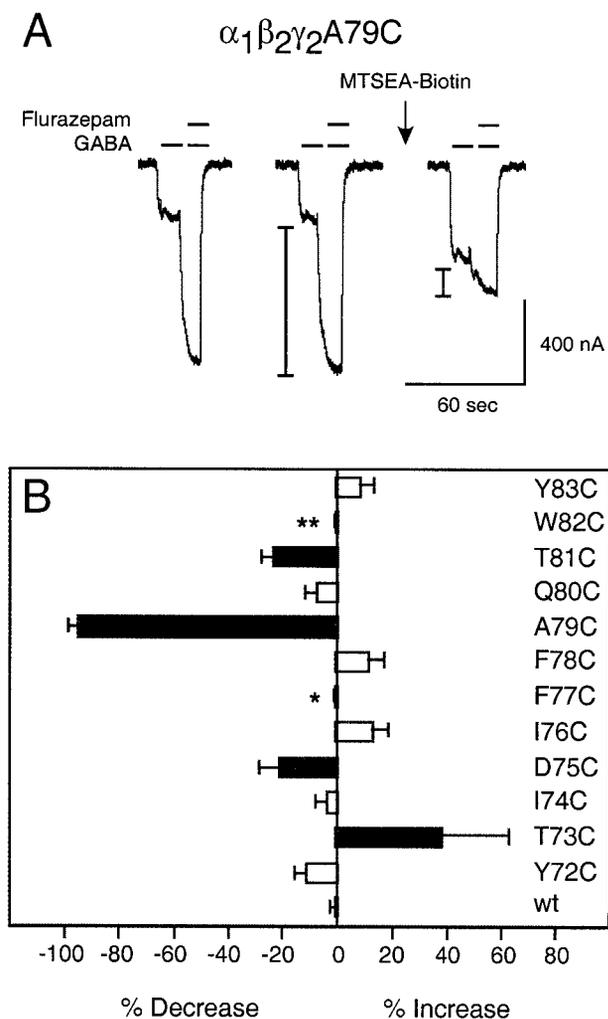
GABA and FLZM EC<sub>50</sub> values for eight cysteine mutants have not been altered radically by mutation (see Table 1), it is likely that the positions of these introduced cysteine side chains reflect wild-type orientations. Although  $\gamma_2$ D75C- and  $\gamma_2$ I76C-containing receptors display 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  $I_{GABA}$  in *X. laevis* oocytes expressing wild-type  $\alpha_1\beta_2\gamma_2$  or  $\alpha_1\beta_2\gamma_2$ -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> receptors to MTSEA-biotin had no significant effect on  $I_{GABA}$  or on the FLZM potentiation of  $I_{GABA}$  (Figs. 4*B*, 6*C*). 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  $\gamma_2$ Y72C,  $\gamma_2$ I74C,  $\gamma_2$ I76C,  $\gamma_2$ F78C,  $\gamma_2$ Q80C, or  $\gamma_2$ Y83C had no effects on the FLZM potentiation of  $I_{GABA}$  (Fig. 4). Thus either these introduced cysteines were not accessible to MTSEA-biotin modification, or their modification by MTSEA-biotin had no observable effect on FLZM potentiation.

In contrast, MTSEA-biotin treatment of receptors containing  $\gamma_2$ T73C,  $\gamma_2$ D75C,  $\gamma_2$ A79C, and  $\gamma_2$ T81C significantly altered the FLZM modulation of  $I_{GABA}$  (Fig. 4). After the application of MTSEA-biotin, the FLZM potentiation of  $I_{GABA}$  was increased by 38 ± 25% for  $\gamma_2$ T73C-containing receptors, whereas potentiation was decreased by 22 ± 8%, 95 ± 2%, and 23 ± 4% for  $\gamma_2$ D75C-,  $\gamma_2$ A79C-, and  $\gamma_2$ T81C-containing receptors, respectively. The alternating pattern of accessibility within the region bounded by  $\gamma_2$ T73 and  $\gamma_2$ T81 suggests that this domain of the BZD binding site forms a  $\beta$ -strand.

#### Identification of BZD binding site residues

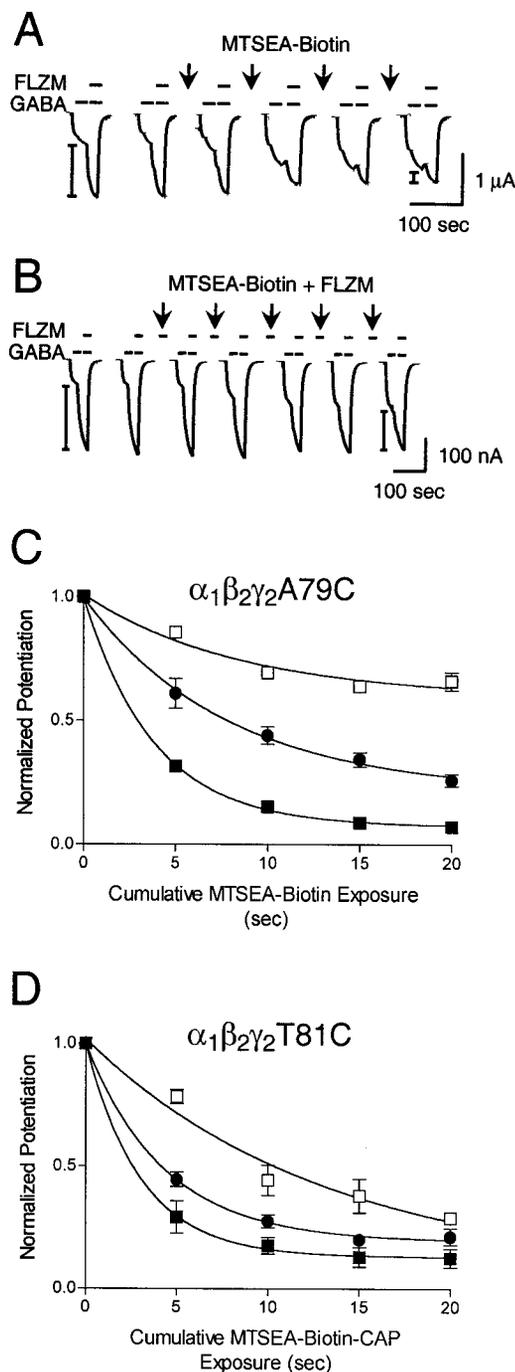
We examined the extent to which both FLZM and Ro 15-1788 could slow the rate of reaction of MTS reagents with accessible



**Figure 4.** MTSEA-biotin effects on the  $\gamma_2$ Y72C– $\gamma_2$ Y83C region. *A*, Representative current traces from  $\alpha_1\beta_2\gamma_2$ A79C receptors showing FLZM modulation of  $I_{GABA}$  before and after a 2 min application of 2 mM MTSEA-biotin. *I*-bars denote potentiation of  $I_{GABA}$  measured during an application of 1  $\mu$ M FLZM in the presence of 1  $\mu$ M GABA. Note the decrease in FLZM potentiation and the increase in  $I_{GABA}$  after MTSEA-biotin modification (arrow). *B*, Changes in FLZM potentiation after MTSEA-biotin modification of  $\alpha\beta\gamma$  (wild-type; *wt*) and mutant receptors. The percentage of change in FLZM potentiation after modification is defined as [(FLZM Potentiation<sub>After</sub>/FLZM Potentiation<sub>Before</sub>) – 1]·100]. A negative value represents a decrease in FLZM potentiation after MTSEA-biotin reaction, and a positive value represents an increase in FLZM potentiation after MTSEA-biotin reaction. 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  $I_{GABA}$ ; \*\*no detectable  $\gamma_2$  subunit expression.

cysteines to identify residues within  $\gamma_2$ Y72– $\gamma_2$ Y83 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  $I_{GABA}$ . 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  $I_{GABA}$  after repeated exposure to



**Figure 5.** Rate of sulfhydryl modification of  $\alpha_1\beta_2\gamma_2$ A79C and  $\alpha_1\beta_2\gamma_2$ T81C receptors in the presence and absence of FLZM and Ro 15-1788. *A*, *B*, Representative GABA (1  $\mu$ M) and GABA plus FLZM (1  $\mu$ M each) current traces recorded from  $\alpha_1\beta_2\gamma_2$ A79C receptors. Arrows indicate 5 sec applications of 200  $\mu$ M MTSEA-biotin alone (*A*) or 200  $\mu$ M MTSEA-biotin plus 5  $\mu$ M FLZM (*B*). FLZM potentiation of  $I_{GABA}$  was measured before and after each MTS treatment. *I*-bars on traces show BZD-potentiated current. *C*, Observed decreases in FLZM potentiation of  $I_{GABA}$  were plotted versus cumulative MTSEA-biotin exposure in  $\alpha_1\beta_2\gamma_2$ A79C 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  $\mu$ M FLZM; □, MTS + 5  $\mu$ M Ro 15-1788). Data points are mean  $\pm$  SD from at least three independent experiments. *D*, Rate experiments were performed similarly for receptors containing  $\gamma_2$ T81C, except that 5 sec applications of 20  $\mu$ M MTSEA-biotin-CAP were used in place of MTSEA-biotin (■, MTS alone; ●, MTS + 5  $\mu$ M FLZM; □, MTS + 5  $\mu$ M Ro 15-1788). The calculated second-order rate constants for the MTS reaction are presented in Table 2.

**Table 2. Summary of second-order rate constants for reaction of MTS compounds with receptors containing  $\gamma_2$ D75C,  $\gamma_2$ A79C, or  $\gamma_2$ T81C in the absence (control) or presence of BZD ligands**

Receptor	Reagent	Control		FLZM		Ro 15–1788		GABA	
		$k_2$ ( $M^{-1}s^{-1}$ )	$n$						
$\alpha\beta\gamma$ D75C	MTSEA	420 $\pm$ 200	3	530 $\pm$ 210	3	510 $\pm$ 250	3	580 $\pm$ 70	3
$\alpha\beta\gamma$ A79C	MTSEA-biotin	1300 $\pm$ 200	5	670 $\pm$ 250*	3	600 $\pm$ 100**	3	3700 $\pm$ 300**	3
$\alpha\beta\gamma$ T81C	MTSEA-biotin-CAP	17,000 $\pm$ 4400	3	12,000 $\pm$ 1700	3	3700 $\pm$ 800**	3	22,000 $\pm$ 2300	3

Second-order rate constants ( $k_2$ ) were derived by dividing the fit pseudo-first-order rate constants by the concentration of MTS reagent used (see Materials and Methods). The concentration of MTS compounds used was as follows (in  $\mu M$ ): MTSEA, 200; MTSEA-biotin, 200; MTSEA-biotin-CAP, 20. Data represent mean  $\pm$  SD values. \*\*Indicate values significantly different from control (MTS alone), with  $p < 0.05$  and  $p < 0.01$ , respectively.

MTSEA ( $\alpha_1\beta_2\gamma_2$ D75C), MTSEA-biotin ( $\alpha_1\beta_2\gamma_2$ A79C), or MTSEA-biotin-CAP ( $\alpha_1\beta_2\gamma_2$ T81C). The decrease in FLZM potentiation of the receptor was plotted against the cumulative time of MTS exposure, and the data were fit with a single-exponential decay curve. Second-order rate constants ( $k_2$ ) for the MTS reaction with the introduced cysteines were calculated from curve fits (Fig. 5; see Materials and Methods).

Introduction of a cysteine at position  $\gamma_2$ A79 created a free sulfhydryl that reacted with MTSEA-biotin. Both FLZM ( $\sim EC_{94}$ ) and Ro 15-1788 ( $\sim EC_{98}$ ) significantly slowed the rate of sulfhydryl modification of  $\gamma_2$ A79C by MTSEA-biotin ( $p < 0.05$ ; Table 2; Fig. 5).  $\alpha_1\beta_2\gamma_2$ T81C receptors reacted robustly only with MTSEA-biotin-CAP. Modification of  $\gamma_2$ T81C-containing receptors by MTSEA-biotin-CAP was slowed significantly by Ro 15-1788 ( $\sim EC_{98}$ ;  $p < 0.05$ ), but not by FLZM ( $\sim EC_{93}$ ; Table 2; Fig. 5). The rate of modification of  $\alpha_1\beta_2\gamma_2$ D75C receptors by MTSEA was unchanged in the presence of FLZM ( $\sim EC_{50}$ ) and Ro 15-1788 ( $\sim EC_{98}$ ; Table 2). Taken together, these data indicate that  $\gamma_2$ A79 and  $\gamma_2$ T81, but not  $\gamma_2$ D75, lie within the BZD binding site. In addition, Ro 15-1788, but not FLZM, protects  $\gamma_2$ T81C from covalent modification, suggesting that  $\gamma_2$ T81C may participate in forming an overlapping binding site subdomain for Ro 15-1788. We did not evaluate  $\gamma_2$ T73 as a potential binding site candidate because sulfhydryl-specific derivitization of this residue resulted in an increase in BZD potentiation of  $I_{GABA}$ . This result suggests that  $\gamma_2$ T73 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  $\gamma_2$ T73 is attributable to conformational changes in the BZD site that correspondingly increase the sensitivity to FLZM.

#### Tethered MTSEA-biotin and MTSEA-biotin-CAP allosterically modulate GABA apparent affinity

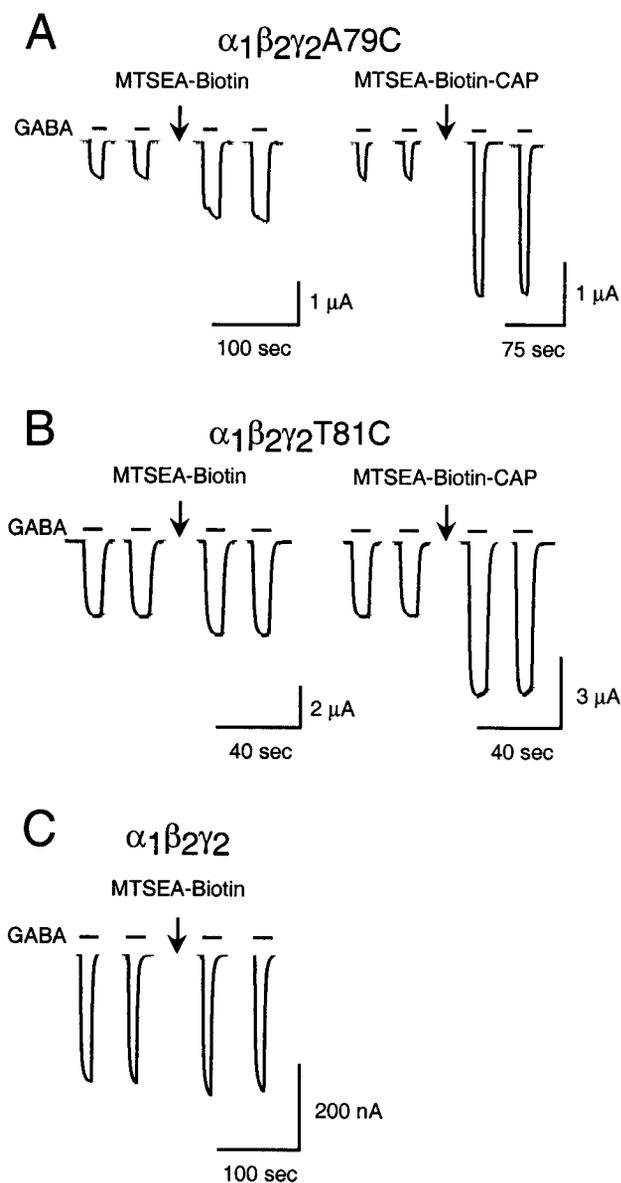
After the reaction of MTSEA-biotin or MTSEA-biotin-CAP with  $\alpha_1\beta_2\gamma_2$ A79C receptors, we observed that  $I_{GABA}$  was increased substantially (see Figs. 4A, 6A). To gain insight into the chemical specificity of this effect, we examined whether other MTS reagents, including MTSEA, MTS-ethyltrimethylammonium (MTSET), MTS-ethylsulfonate (MTSES), and benzyl-MTS, also could modulate  $I_{GABA}$  when tethered to  $\gamma_2$ A79C. Although all of the MTS reagents that were tested reacted with  $\gamma_2$ A79C, as evidenced by a decreased FLZM potentiation of  $I_{GABA}$ , only MTSET, MTSEA-biotin, and MTSEA-biotin-CAP increased  $I_{GABA}$  (data not shown). MTSEA-biotin and MTSEA-biotin-CAP are the largest reagents that were tested, and MTSET is positively charged. The data suggest that large and/or positively charged compounds may be better suited to initiate allosteric changes in the receptor protein once they are attached covalently to the BZD binding site. Interestingly, robust increases in  $I_{GABA}$

were observed after MTSEA-biotin-CAP, but not MTSEA-biotin, modification of  $\gamma_2$ T81C-containing receptors (Fig. 6B).

We hypothesized that the increases in  $I_{GABA}$  were attributable to changes in the GABA  $EC_{50}$  values of  $\alpha_1\beta_2\gamma_2$ A79C and  $\alpha_1\beta_2\gamma_2$ T81C receptors after MTS modification. To test this hypothesis, we measured complete GABA concentration–response curves in single oocytes expressing  $\alpha_1\beta_2\gamma_2$ A79C receptors before and after the application of 2 mM MTSEA-biotin (Fig. 7A) or 2 mM MTSEA-biotin-CAP (Fig. 7B). Covalent modification of  $\gamma_2$ A79C-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  $\gamma_2$ A79C-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  $\gamma_2$ A79C were similar to the shift in GABA  $EC_{50}$  observed in the presence of FLZM. Coapplications of 1  $\mu M$  FLZM during a GABA concentration–response protocol resulted in a significant  $\sim 3.8$ -fold increase in the  $EC_{50}$  of  $\alpha_1\beta_2\gamma_2$ A79C receptors for GABA ( $p < 0.01$ ; Fig. 7C) and a  $\sim 3.2$ -fold shift in wild-type receptors (Table 3). Taken together, our data suggest that the addition of a MTS reagent to the BZD binding site may initiate structural changes in the receptor that mimic perturbation by an agonist. This effect can be explained most simply by a model in which MTSEA-biotin and MTSEA-biotin-CAP mechanistically act like BZD partial agonists when tethered to  $\gamma_2$ A79C.

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

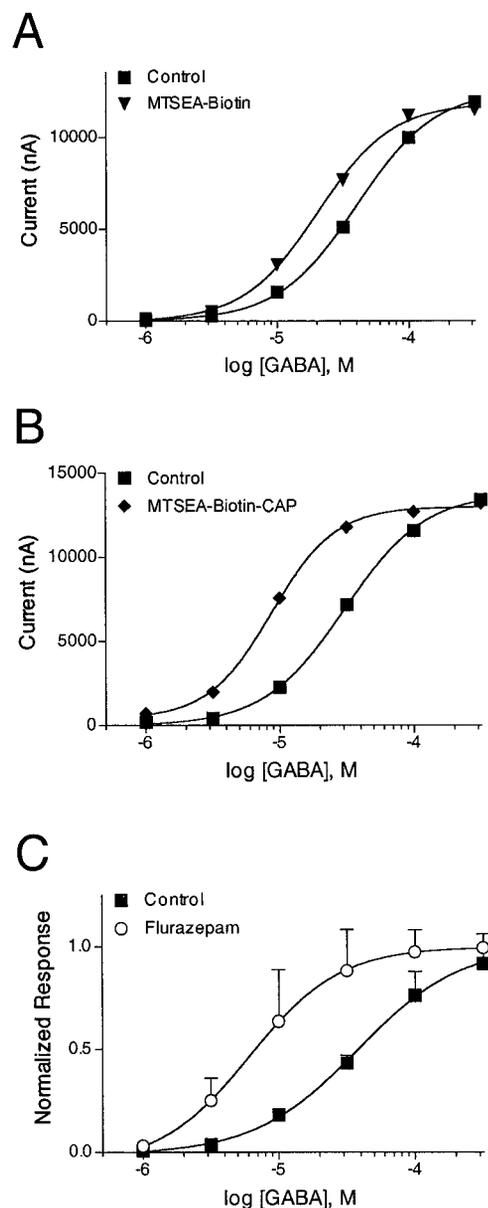
According to allosteric theory, modulators bind to a site on the receptor protein that is distinct from the agonist binding site and exert their effects by initiating an allosteric transition in the protein that indirectly modifies the conformation of the agonist binding site (Changeux and Edelstein, 1998). Both radioligand binding and electrophysiological studies of the GABA<sub>A</sub> 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  $EC_{70}$ – $EC_{86}$ ) altered the rates of reaction of MTS reagents with  $\alpha_1\beta_2\gamma_2$ D75C,  $\alpha_1\beta_2\gamma_2$ A79C, and  $\alpha_1\beta_2\gamma_2$ T81C receptors. GABA significantly increased the rate of MTS modification of  $\gamma_2$ A79C-containing, but not  $\gamma_2$ D75C- or  $\gamma_2$ T81C-containing, receptors (Fig. 8; see Table 2). The ability of GABA to increase the accessibility of  $\gamma_2$ A79C to sulfhydryl modification demonstrates that a domain of the BZD binding site undergoes an allosteric structural rearrangement during GABA binding and channel gating.



**Figure 6.** MTS modification of  $\alpha_1\beta_2\gamma_2A79C$  and  $\alpha_1\beta_2\gamma_2T81C$  receptors increases  $I_{GABA}$ . 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  $\mu$ M GABA in oocytes expressing receptors containing either  $\gamma_2A79C$  (A) or  $\gamma_2T81C$  (B) subunits. The application of 2 mM MTSEA-biotin to oocytes expressing  $\alpha_1\beta_2\gamma_2$  (C) or  $\alpha_1\beta_2\gamma_2T81C$  (B) receptors had no significant effect on  $I_{GABA}$ .

## DISCUSSION

We used SCAM to examine the structure and dynamics of a region of the GABA<sub>A</sub> receptor implicated in BZD binding,  $\gamma_2Y72$ – $\gamma_2Y83$  (Buhr et al., 1997; Sigel et al., 1998). Our data indicate that this region is a  $\beta$ -strand. We directly demonstrate that two residues that had been implicated previously in BZD binding,  $\gamma_2A79$  and  $\gamma_2T81$  (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.



**Figure 7.** MTSEA-biotin and MTSEA-biotin-CAP shift GABA EC<sub>50</sub> when linked covalently to  $\gamma_2A79C$ . A, B, GABA concentration–response curves obtained from single oocytes expressing  $\alpha_1\beta_2\gamma_2A79C$  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  $\alpha_1\beta_2\gamma_2A79C$  receptors in the absence (■) and presence (○) of 1  $\mu$ M FLZM. Data were fit by nonlinear regression, as described in Materials and Methods. Data represent mean  $\pm$  SEM from three independent experiments. EC<sub>50</sub> values obtained from the curve fits are reported in Table 3.

## Identification of amino acids in the BZD binding site

Four residues within  $\gamma_2Y72$ – $\gamma_2Y83$  are accessible to MTSEA-biotin:  $\gamma_2T73C$ ,  $\gamma_2D75C$ ,  $\gamma_2A79C$ , and  $\gamma_2T81C$ . Of these four accessible residues, both  $\gamma_2A79C$  and  $\gamma_2T81C$  are protected from MTS modification by Ro 15-1788, whereas only  $\gamma_2A79$  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

**Table 3.** GABA EC<sub>50</sub> values of  $\alpha_1\beta_2\gamma_2$  and  $\alpha_1\beta_2\gamma_2A79C$  receptors before and after treatment with 2 mM MTSEA-biotin, 2 mM MTSEA-biotin-CAP, or 1  $\mu$ M FLZM

Receptor	GABA EC <sub>50</sub> ( $\mu$ M) before	<i>n</i>	Treatment	GABA EC <sub>50</sub> ( $\mu$ M) after	<i>n</i>	EC <sub>50</sub> before/EC <sub>50</sub> after
$\alpha\beta\gamma$	18 ± 4.7 <sup>a</sup>	3	FLZM	5.5 ± 0.9**	3	3.3
$\alpha\beta\gamma A79C$	30 ± 12 <sup>a</sup>	9	FLZM	7.8 ± 5.6**	3	3.8
$\alpha\beta\gamma A79C$	43 ± 6.7 <sup>b</sup>	3	MTSEA-biotin	28 ± 7.4 <sup>b</sup> *	3	1.5
$\alpha\beta\gamma A79C$	29 ± 1.7 <sup>c</sup>	3	MTSEA-biotin-CAP	11 ± 3.3 <sup>c</sup> **	3	2.6

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.

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  $\gamma_2A79$  is facing into the BZD binding pocket. Because only Ro 15-1788 is able to protect  $\gamma_2T81C$  from sulfhydryl modification, we cannot conclude definitively that  $\gamma_2T81$  lines the BZD binding site by our criteria. However, other evidence also suggests that  $\gamma_2T81$  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  $\gamma_2A79$  and  $\gamma_2T81$  are important determinants of BZD binding (Kucken et al., 2000). Although we could not evaluate the accessibility of  $\gamma_2F77C$ , 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  $\gamma_2F77$ ,  $\gamma_2A79$ , and  $\gamma_2T81$  line the BZD binding site.

### Secondary structure of the $\gamma_2Y72$ – $\gamma_2Y83$ region of the BZD binding site

Alternating residues within the region  $\gamma_2T73$ – $\gamma_2T81$  are accessible to MTSEA-biotin. These data are consistent with a model in which this region forms a  $\beta$ -strand. Because the accessibility of  $\gamma_2F77C$  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  $\gamma_2A79$ , are hydrophilic amino acid residues. Because MTSEA-biotin is relatively impermeant (Chen et al., 1998) and MTS reagents react from 10<sup>9</sup> to 10<sup>10</sup> times faster with ionized sulfhydryl groups than protonated sulfhydryls (Roberts et al., 1986) and ionization of a sulfhydryl 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  $I_{GABA}$  if  $\gamma_2Y72C$ ,  $\gamma_2I74C$ ,  $\gamma_2I76C$ ,  $\gamma_2F78C$ , or  $\gamma_2Q80C$  actually face into the BZD binding pocket. We were unable to test the accessibility of  $\gamma_2W82C$ , 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>A</sub> receptor  $\alpha_1$  subunit assembly (Srinivasan et al., 1999). Thus, it is reasonable to assume that  $\gamma_2W82$  is not solvent-accessible, because it is hydrophobic and likely participates in intraprotein contacts that are associated with subunit assembly.

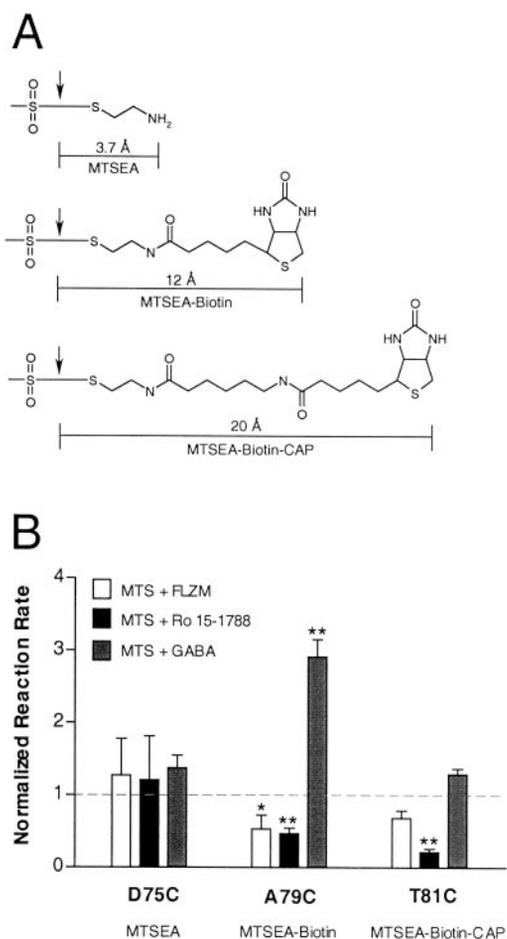
Taken together, the results of this study strongly suggest that the polypeptide chain from  $\gamma_2T73$  to  $\gamma_2T81$  forms a  $\beta$ -strand and that a portion of this strand lines the BZD binding site. In agreement with our experimental results, this region is predicted by secondary structure modeling algorithms (Chou and Fasman, 1978) to adopt a  $\beta$ -strand conformation. Interestingly, an aligned region of the  $\alpha_1$  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>A</sub> receptor pharmacology is how the binding of BZD ligands is transduced into allosteric modulation of the GABA<sub>A</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 ( $\gamma_2A79$ ) 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  $\gamma_2A79$  (or residues near  $\gamma_2A79$ ) 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>A</sub> receptor during allosteric modulation by BZDs (Williams and Akabas, 2000).

### Theoretical model of the BZD binding site

We demonstrate that  $\gamma_2T73$ ,  $\gamma_2D75$ ,  $\gamma_2A79$ , and  $\gamma_2T81$  line the accessible surface of a  $\beta$ -strand in the  $\gamma_2$  subunit of the GABA<sub>A</sub> receptor, with  $\gamma_2A79$  and  $\gamma_2T81$  in close proximity to the BZD ligand binding domain. We hypothesize that FLZM is topologically close to both  $\gamma_2F77$  and  $\gamma_2A79$  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  $\gamma_2F77$  (Buhr et al., 1997; Sigel et al., 1998), whereas others have suggested that these interactions also may



**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 derivatization of  $\gamma_2$ D75C-,  $\gamma_2$ A79C-, and  $\gamma_2$ T81C-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:  $\gamma_2$ D75C, MTSEA;  $\gamma_2$ A79C, MTSEA-biotin;  $\gamma_2$ T81C, 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  $\pm$  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  $\alpha_1$ H101 (Davies et al., 1998; McKernan et al., 1998). We hypothesize that FLZM is oriented such that its 5'-phenyl is in close contact with  $\gamma_2$ F77 and that it occupies space within the binding site that is in close proximity to  $\gamma_2$ A79. 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 creating an open volumetric space to accommodate BZD ligands of different sizes. Our data suggest that Ro 15-1788 binds near  $\gamma_2$ A79, but with the additional contribution of  $\gamma_2$ T81 to its binding site. Experiments that use a variety of chemically diverse MTS reagents that can modify  $\gamma_2$ A79C and  $\gamma_2$ T81C will be helpful in characterizing these structurally distinct binding subdomains further. Neither FLZM nor Ro 15-1788 appears to bind near  $\gamma_2$ D75 or  $\gamma_2$ T73. However, we propose that  $\gamma_2$ D75 may be important in maintaining the architecture of the BZD site be-

cause 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  $\gamma_2$ A79, are oriented in a manner such that they are able to modulate allosterically the  $EC_{50}$  of the GABA binding site for GABA. Interestingly, although MTSEA-biotin shifts the GABA  $EC_{50}$  for  $\gamma_2$ A79C-containing receptors within the range expected for a BZD partial agonist, this reagent has little-to-no effect on the GABA  $EC_{50}$  of  $\gamma_2$ T81C-containing receptors. In contrast, MTSEA-biotin-CAP modification of  $\gamma_2$ A79C-containing receptors shifts the GABA  $EC_{50}$  within the range of a full agonist and partially shifts the GABA  $EC_{50}$  of  $\gamma_2$ T81C-containing receptors. Because MTSEA-biotin-CAP is 8 Å longer than MTSEA-biotin (see Fig. 8), these data suggest that  $\gamma_2$ T81 lies farther than  $\gamma_2$ A79 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  $\alpha_1$  and  $\gamma_2$  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  $\alpha_1$  and  $\gamma_2$  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 GABA<sub>A</sub> receptor protein underlie allosteric communication between the GABA and BZD binding sites.

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