0026-895X/03/6302-289–296$7.00
MOLECULAR PHARMACOLOGY
Vol. 63, No. 2
2057/1036686
Mol Pharmacol 63:289–296, 2003
Printed in U.S.A.

Structural Requirements for Imidazobenzodiazepine Binding to $GABA_A$ Receptors

AMY M. KUCKEN, JEREMY A. TEISSÉRE, JAMES SEFFINGA-CLARK, DAVID A. WAGNER, and CYNTHIA CZAJKOWSKI

Department of Physiology, University of Wisconsin-Madison, Madison, Wisconsin (A.M.K., J.S.-C., D.A.W., C.C.); Department of Pharmacology, Emory University, Atlanta, Georgia (J.A.T.)

Received August 7, 2002; accepted October 22, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Several structural subclasses of ligands bind to the benzodiazepine (BZD) binding site of the $GABA_A$ receptor. Previous studies from this laboratory have suggested that imidazobenzodiazepines (*i*-BZDs, e.g., Ro 15-1788) require domains in the BZD binding site for high-affinity binding that are distinct from the requirements of classic BZDs (e.g., flunitrazepam). Here, we used systematic mutagenesis and the substituted cysteine accessibility method to map the recognition domain of *i*-BZDs near two residues implicated in BZD binding, γ_2A79 and γ_2T81. Both classic BZDs and *i*-BZDs protect cysteines substituted at γ_2A79 and γ_2T81 from covalent modification, suggesting that these ligands may occupy common volumetric spaces during binding. However, the binding of *i*-BZDs is more sensitive to mutations at γ_2A79 than classic BZDs or BZDs that lack a 3′-imidazo substituent (e.g., midazolam). The effect that γ_2A79 mutagenesis has on the binding affinities of a series of structurally rigid *i*-BZDs is related to the volume of the 3′-imidazo substituents. Furthermore, larger amino acid side chains introduced at γ_2A79 cause correspondingly larger decreases in the binding affinities of *i*-BZDs with bulky 3′ substituents. These data are consistent with a model in which γ_2A79 lines a subsite within the BZD binding pocket that accommodates the 3′ substituent of *i*-BZDs. In agreement with our experimental data, computer-assisted docking of Ro 15-4513 into a molecular model of the BZD binding site positions the 3′-imidazo substituent of Ro 15-4513 near γ_2A79.

Benzodiazepines (BZDs) are therapeutic agents commonly used in the treatment of anxiety, sleeplessness, and epilepsy (Doble and Martin, 1996). BZDs exert their anxiolytic, hypnotic, and anticonvulsant effects by interacting with a unique modulatory site on the $GABA_A$ receptor, the main effector of neuronal inhibition within the central nervous system (Hevers and Lüddens, 1998). The BZD binding site is on the extracellular surface of the $GABA_A$ receptor at an interface formed by the α and γ subunits (Smith and Olsen, 1995; Sigel and Buhr, 1997). Several studies have identified residues on both the α subunit (Duncalfe et al., 1996; Amin et al., 1997; Buhr et al., 1997b; Davies et al., 1998; Schaerer et al., 1998; Renard et al., 1999; Davies et al., 2001) and the γ subunit (Buhr and Sigel, 1997; Buhr et al., 1997a; Wingrove et al., 1997; Kucken et al., 2000) that mediate high-affinity BZD binding; however, the specific interactions between individual amino acids and BZD ligands and the orientation of BZDs within the recognition site remain unclear (for review, see He et al., 2001).

This work was supported in part by National Institutes of Mental Health/National Research Service Award grant MH12966 (to J.A.T.) and National Institutes of Neurological Disorders and Stroke Grant NS34727 (to C.C.).

A.M.K. and J.A.T. contributed equally to this work.

The structures of BZD binding site ligands are quite diverse. Classic BZDs, such as flunitrazepam and flurazepam, possess a common 1,4-benzodiazepine nucleus with a 5-phenyl substituent (Fig. 1; Sternbach, 1979). A different class of BZD ligands possesses both a 5-phenyl substituent and an imidazo ring substituted at positions 1 and 2 of the diazepine nucleus (e.g., midazolam; Fig. 1). In contrast, BZDs such as Ro 15-4513 and Ro 15-1788 possess the imidazo ring but lack the 5-phenyl substituent (Fig. 1). Our research has sought to identify specific domains of the γ_2 subunit that are important for binding different structural classes of BZDs and to establish how each class of ligand is oriented within the BZD binding site.

Imidazobenzodiazepines (*i*-BZDs), such as Ro 15-4513 and Ro 15-1788, seem to possess structural requirements for binding that are distinct from classic BZDs. Previously, we demonstrated that mutation of γ_2A79 had a larger effect on the binding affinities of Ro 15-4513 and Ro 15-1788 than on the classic BZD ligand flunitrazepam (Kucken et al., 2000). Additionally, Ro15-1788 as well as the classic BZD flurazepam impeded the covalent modification of a cysteine substituted at γ_2A79, whereas modification of γ_2T81C was significantly impeded by Ro15-1788 but not by flurazepam

ABBREVIATIONS: BZD, benzodiazepine; *i*-BZD, imidazobenzodiazepine; AChBP, acetylcholine binding protein; HEK, human embryonic kidney; MTS, methanethiosulfonate; MTSEA-Biotin, *N*-biotinaminoethyl methanethiosulfonate; MTSEA-Biotin-CAP, *N*-biotincaproylaminoethyl methanethiosulfonate; I_{GABA}, GABA-gated Cl^- current.

(Teissére and Czajkowski, 2001). Based on these data, we hypothesized that γ_2A79 and γ_2T81 line part of an *i*-BZD subsite of the BZD binding site.

In this article, we extend these studies and further test our hypothesis. The binding affinities of seven different BZD ligands were measured after systematic mutation of γ_2A79. In addition, we examined the ability of several BZD ligands with different structures and functional efficacies to slow the rate of covalent modification of γ_2A79C and γ_2T81C. Our studies indicate that γ_2A79 and γ_2T81 contribute to a subsite of the BZD binding pocket that accommodates the 3′ substituent of the *i*-BZD imidazo ring (see Fig. 1). Using the recently crystallized molluscan acetylcholine binding protein (AChBP) (Brejc et al., 2001) as a structural template, we modeled the BZD binding site of the $GABA_A$ receptor and describe in part the three-dimensional relationship between *i*-BZD ligands and the γ_2 subunit of the $GABA_A$ receptor.

Materials and Methods

Mutagenesis. Rat cDNAs encoding α_1, β_2, and γ_{2S} subunits were used for all molecular cloning, radioligand binding, and functional studies. Site-directed mutagenesis of γ_2A79 and γ_2T81 was carried out using recombinant oligonucleotides and the polymerase chain reaction. For radioligand binding, amino acid mutations were made using a *myc* 9E10 epitope-tagged γ_2 subunit as the template. The presence of the epitope had no detectable effect on ligand recognition or on expression of the γ_2 subunit (Kucken et al., 2000). Wild-type and mutant subunits were subcloned into pCEP4 (Boileau et al., 1998) for transient expression in human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Manassas, VA) or into pGH19 (Liman et al., 1992; Robertson et al., 1996) for expression in *Xenopus laevis* oocytes. All γ_2 mutants were verified by restriction enzyme analysis and double-strand DNA sequencing.

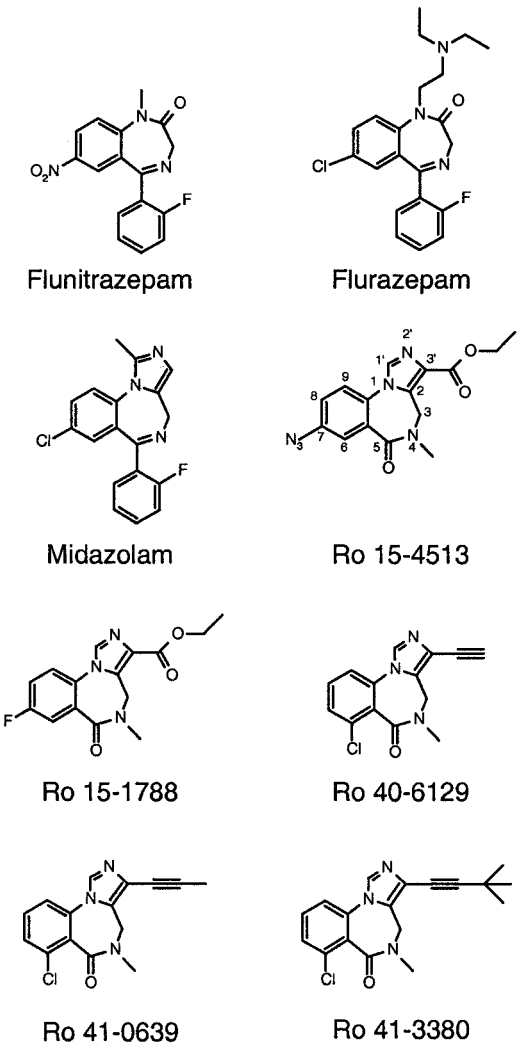

Fig. 1. Structures of BZD ligands used in this study. The numbering scheme for carbon atoms comprising the diazepine nucleus and 1,2-imidazo ring is illustrated for Ro 15-4513. Ligand structures were created using ISIS/Draw (MDL Information Systems, San Leandro, CA).

Transient Transfection and Radioligand Binding. HEK 293 cells were transiently transfected with α_1, β_2, and γ_{2myc} or γ_{2myc} mutant subunits using a standard $CaHPO_4$ precipitation method (Kucken et al., 2000). Cells were harvested 48 h after transfection, and membrane homogenates were prepared as described previously (Kucken et al., 2000). Membrane homogenates (100 μg) were incubated at room temperature with [^{3}H]flunitrazepam at sub-K_D concentrations for wild-type or mutant receptors and 8 to 11 concentrations of unlabeled ligand in a final volume of 250 μl. Data were fit using the equation: $Y = B_{max}/[1 + (X/IC_{50})]$, where Y is the specifically bound disintegrations per minute, B_{max} is the maximal binding, X is the concentration of unlabeled ligand, and IC_{50} is the concentration of unlabeled ligand that reduces the maximal specific binding by 50% (GraphPad Software, San Diego, CA). K_I values were calculated using the equation: $K_I = IC_{50}/(1 + [\text{radioligand}]/K_D)$ (Cheng and Prusoff, 1973; Chou, 1974), where K_D refers to the equilibrium dissociation constant of the radioligand. The use of this equation assumes that ligand binding follows the law of mass action, is competitive, and that the data reflect one-site binding with no cooperativity. K_I values were obtained from at least three independent experiments, each with triplicate determinations. [^{3}H]Flunitrazepam (85 Ci/mmol) was obtained from PerkinElmer Life Sciences (Boston, MA). Nonradioactive BZDs were obtained from Hoffman-La Roche (Nutley, NJ) or RBI/Sigma (Natick, MA).

Expression in Oocytes and Electrophysiology. Capped cRNAs encoding the α_1, β_2, γ_2, γ_2A79C, or γ_2T81C subunits in pGH19 were transcribed in vitro using the mMessage mMachine T7 kit (Ambion, Austin, TX). Oocytes were harvested from *X. laevis* and injected within 24 h with 27 nl of cRNA (10–200 pg/nl/subunit) in the ratio 1:1:10 ($\alpha/\beta/\gamma$; Boileau et al., 2002). Expressed receptors were functionally assayed using two-electrode voltage clamp ($V_{hold} = -80$ mV, room temp) as described previously (Teissére and Czajkowski, 2001). Working concentrations of GABA and BZD ligands were made up in ND96 oocyte perfusion solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.2). In all electrophysiological experiments, both GABA-activated current (I_{GABA}) and flurazepam-mediated potentiation of GABA-activated current ($I_{GABA + flurazepam}$) were measured. Flurazepam-mediated potentiation of I_{GABA} was defined as $[(I_{GABA + flurazepam}/I_{GABA}) - 1] \times 100$. Rates of sulfhydryl-specific covalent modification of $\alpha_1\beta_2\gamma_2$A79C or $\alpha_1\beta_2\gamma_2$T81C receptors by methanethiosulfonate (MTS) reagents were determined using the following protocol: 1) flurazepam potentiation of I_{GABA} was measured by applying 1 μM GABA and then applying 1 μM GABA + 1 μM flurazepam (corresponding to ~EC_{50} concentration of GABA and ~EC_{80} concentration of flurazepam); 2) the oocyte was washed for 3 min in ND96 buffer; and 3) flurazepam potentiation of I_{GABA} was measured again. This protocol was repeated until flurazepam potentiation of I_{GABA} changed by less than 5%. After potentiation stabilized, the rate of MTS reaction was measured by applying a subsaturating 5 s application of an MTS reagent 30 s after determination of I_{GABA} and $I_{GABA + flurazepam}$. Applications of the MTS reagent were repeated until flurazepam potentiation of I_{GABA} no longer decreased. γ_2A79C-containing receptors were reacted with 200 μM *N*-biotinylaminoethyl MTS (MTSEA-Biotin; Biotium, Hayward, CA) and γ_2T81C-containing receptors were reacted with 20 μM *N*-biotinylcaproylaminoethyl CAP MTS

(MTSEA-Biotin-CAP; Biotium) as described previously (Teissére and Czajkowski, 2001).

The decrease in flurazepam potentiation of I_{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 potentiation, 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, $M^{-1}s^{-1}$) by dividing by the concentration of MTS reagent used to correct for the concentration dependence of this effect (Pascual and Karlin, 1998). To verify the accuracy of the protocol, some of the rate determinations were conducted at two different concentrations of MTS-reagent.

The ability of different BZDs to slow the rate of MTS modification of γ_2A79C and γ_2T81C was assayed by coapplying a BZD with the MTS-reagent during the rate determinations. The following BZDs were tested (all applied at ~EC_{95} concentrations): flurazepam, Ro 15-1788, Ro 15-4513, midazolam, Ro 40-6129, and Ro 41-3380. In these experiments, flurazepam potentiation of I_{GABA} was stabilized before measuring the rate of MTS modification as follows: 1) 1 μM GABA and 1 μM GABA + 1 μM flurazepam were applied to an oocyte; 2) the oocyte was treated with an EC_{95} concentration of BZD and then washed for 3 min in ND96; and 3) flurazepam potentiation of I_{GABA} was measured again using 1 μM GABA and 1 μM GABA + 1 μM flurazepam. This protocol was repeated until I_{GABA} and $I_{GABA\ +\ flurazepam}$ changed by less than 10% and demonstrated that the wash time was sufficient to wash out the test EC_{95} concentration of BZD. In some cases, after treating the oocytes with MTS-reagent in the presence of a BZD, receptors were re-exposed to the same concentration of MTS-reagent alone to demonstrate that a maximal decrease in flurazepam potentiation of I_{GABA} could still be obtained.

Homology Modeling of the BZD Binding Site. The mature protein sequences of the rat α_1 and γ_2 subunits were modeled by comparison with the deduced three-dimensional structure of a subunit of the AChBP (Brejc et al., 2001). The crystal structure of the AChBP was downloaded from the Protein Data Bank (PDB code 1I9B) and loaded into Swiss Protein Databank Viewer (http://ca.expasy.org/spdbv). The mature α_1 protein sequence from T12 to I222 and the mature γ_2 protein sequence from D26 to M233 were aligned with the AChBP primary amino acid sequence (Cromer et al., 2002) and threaded onto the AChBP tertiary structure using the "Interactive Magic Fit" function of Swiss Protein Databank Viewer. The threaded subunits were imported into SYBYL (Tripos, Inc., St. Louis, MO) and energy minimized (< 0.5 kcal/Å). The first 100 iterations were carried out using Simplex minimization (Press et al., 1988) followed by 1000 iterations using the Powell conjugate gradient method (Powell, 1977). An α_1/γ_2 BZD binding site interface was assembled by overlaying the monomeric subunits on the AChBP scaffold and the resulting structure was imported into SYBYL and energy minimized. Docking of Ro 15-4513 was performed using AutoDock 3.0 (Morris et al., 1998). The ligand started out in an arbitrary conformation, orientation, and position and the docking simulation was carried out using a Lamarckian genetic algorithm (Morris et al., 1998). AutoDock 3.0, like other docking programs, treats the receptor protein as a fixed target; thus, in the final docked structure, the binding site residue side-chains have not moved.

Statistics. Data were analyzed by one-way analysis of variance, applying the Dunnett post-test for significance of differences between treatments (GraphPad). Comparisons used $\log(K_I)$ or $\log(k_2)$ values for the analysis.

Results

The Effect of Systematic γ_2A79 Mutagenesis on *i*-BZD Binding Affinity. Ten point mutations were made at γ_2A79 to evaluate the contribution of this residue to BZD ligand affinity (γ_2A79→Gly, Ser, Cys, Glu, Gln, Leu, Phe, Tyr, Arg, Trp). These residues were chosen to represent a range of amino acid properties (i.e., size, charge, hydrophobicity). Wild-type ($\alpha_1\beta_2\gamma_2$) or mutant ($\alpha_1\beta_2\gamma_2$-mutant) receptors were expressed in HEK 293 cells and the binding affinities (K_I) of flunitrazepam, Ro 15-4513 and Ro 15-1788 were measured by displacement of [^{3}H]flunitrazepam. Wild-type receptors bound flunitrazepam, Ro 15-4513, and Ro 15-1788 with K_I values of 8.9, 3.9, and 3.5 nM, respectively (Table 1). Only 3 of the 10 mutations at γ_2A79 significantly altered flunitrazepam affinity. The γ_2A79R, -C, and -Q mutations reduced flunitrazepam affinity 3-, 5-, and 9-fold, respectively. In contrast, 9 of the 10 mutations at γ_2A79 significantly reduced the binding affinities of Ro 15-4513 and Ro 15-1788 (Table 1). For Ro 15-4513, the decreases in affinity ranged from 6-(A79S) to 93-fold (A79F). For Ro 15-1788, the decreases ranged from 3-(A79S) to 21-fold (A79Y). Because mutations at γ_2A79, in general, had larger affects on the binding affinities of *i*-BZDs than on flunitrazepam affinity, we hypothesized that γ_2A79 lines a subsite of the BZD binding pocket important for *i*-BZD binding. Furthermore, the results implied that the chemical elements that are unique to *i*-BZDs (i.e., the imidazo ring and/or the 3′ substituent) are probably near γ_2A79.

To determine whether the 3′substituent of *i*-BZDs was the

TABLE 1

Affinities of flunitrazepam, midazolam, Ro 15-4513, and Ro 15-1788 for wild-type ($\alpha_1\beta_2\gamma_2$) and mutant receptors

K_I values were determined by displacement of [^{3}H]flunitrazepam binding. Data represent mean ± S.E.M. values.

Receptor	Flunitrazepam			Midazolam			Ro 15-4513			Ro 15-1788		
	K_I	n	K_I-mut/K_I-$\alpha\beta\gamma$	K_I	n	K_I-mut/K_I-$\alpha\beta\gamma$	K_I	n	K_I-mut/K_I-$\alpha\beta\gamma$	K_I	n	K_I-mut/K_I-$\alpha\beta\gamma$
	nM			*nM*			*nM*			*nM*		
$\alpha\beta\gamma$	8.9 ± 0.9	3	1.0	5.8 ± 1.1	5	1.0	3.9 ± 0.9	6	1.0	3.5 ± 0.2	3	1.0
$\alpha\beta\gamma$A79G	13 ± 3	4	1.5	15 ± 2*	7	2.6	100 ± 3**	3	26	48 ± 2**	3	14
$\alpha\beta\gamma$A79S	15 ± 2	5	1.7	N.D.			23 ± 3**	3	5.9	11 ± 2**	3	3.1
$\alpha\beta\gamma$A79C[a]	48 ± 5**	3	5.4	40 ± 2**	3	6.9	62 ± 15**	3	16	20 ± 1**	3	5.7
$\alpha\beta\gamma$A79E	4.4 ± 1.6	3	0.5	5.7 ± 1.9	4	1.0	45 ± 5**	4	12	22 ± 3**	3	6.3
$\alpha\beta\gamma$A79Q[a]	79 ± 13**	3	8.9	N.D.			112 ± 32**	3	29	65 ± 12**	3	19
$\alpha\beta\gamma$A79L	20 ± 3	3	2.2	26 ± 6**	3	4.5	71 ± 8**	3	18	25 ± 3**	3	7.1
$\alpha\beta\gamma$A79F	10 ± 1	3	1.1	N.D.			362 ± 66**	3	93	71 ± 1**	3	20
$\alpha\beta\gamma$A79Y[a]	6.1 ± 2.2	3	0.7	11 ± 3	7	1.9	202 ± 26**	3	52	74 ± 11**	3	21
$\alpha\beta\gamma$A79R[a]	24 ± 5*	3	2.7	47 ± 16**	3	8.1	5.6 ± 1.6	3	1.4	5.6 ± 0.8	3	1.6
$\alpha\beta\gamma$A79W	20 ± 4	3	2.2	N.D.			64 ± 18**	3	16	27 ± 6**	3	7.7

[a] Data for flunitrazepam, Ro 15-4513, and Ro 15-1788 binding reported in Kucken et al. (2000).

n, number of independent experiments; N.D., data not determined.

*, $P < 0.05$; **, $P < 0.01$; significantly different from wild-type receptors.

structural element responsible for the sensitivity of *i*-BZDs to γ_2A79 mutation, midazolam binding affinity was examined. Midazolam contains an imidazo ring that is similar to Ro 15-4513 and Ro 15-1788, but it does not possess a 3′ substituent (see Fig. 1). The K_I of midazolam was determined for several γ_2A79 mutant receptors (γ_2A79G, -C, -E, -L, -R, and -Y). In general, the effects γ_2A79 mutation had on midazolam affinity mirrored those observed for flunitrazepam rather than Ro 15-4513 or Ro 15-1788 (Table 1). For example, the γ_2A79G, -E, -L, and -Y mutations altered the affinities of midazolam and flunitrazepam less than 4.5-fold yet decreased Ro 15-4513 affinity between 12- and 52-fold. The γ_2A79R mutation did not significantly alter Ro 15-4513 or Ro 15-1788 affinities, yet flunitrazepam and midazolam affinities were significantly decreased 3- and 8-fold, respectively. These results suggest that the sensitivity of Ro 15-4513 and Ro 15-1788 binding to γ_2A79 mutation is probably caused by the 3′-imidazo substituent of these ligands.

To examine the potential spatial relationship between γ_2A79 and the 3′ substituent of the *i*-BZD imidazo ring, the binding affinities of three additional *i*-BZDs (Ro 40-6129, Ro 41-0639, Ro 41-3380) were measured. Like Ro 15-4513 and Ro 15-1788, these compounds each possess a 3′-imidazo substituent. In contrast to the 3′ substituents of Ro 15-4513 and Ro 15-1788, which are relatively flexible polar esters, the 3′ substituents of Ro 40-6129, Ro 41-0639, and Ro 41-3380 are rigid, hydrophobic alkynes. Because of their rigid nature, and the observation that Ro 40-6129, Ro 41-0639, Ro 41-3380 only differ from each other in the volume of their 3′ substituents (45.4 Å^3, 60.7 Å^3, and 109.6 Å^3, respectively), these compounds were ideal for testing the effect of 3′ substituent size on *i*-BZD binding affinity after mutation of γ_2A79.

The ability of Ro 40-6129, Ro 41-0639, and Ro 41-3380 to displace the binding of [^{3}H]flunitrazepam in wild type, γ_2A79C-, γ_2A79E-, γ_2A79L-, γ_2A79Y-, and γ_2A79R-containing receptors was measured (Fig. 2, Table 2). All three ligands bound to wild-type $GABA_A$ receptors with high affinity. All of the mutations significantly reduced, by 10-fold or more, the binding affinity of Ro 41-3380, which possesses the largest 3′ imidazo substituent. Three of the five mutations significantly reduced the affinities of Ro 41-0639 and Ro 40-6129, which possess progressively smaller 3′ substituents (Table 2).

The decreases observed in BZD binding affinity were examined as a function of 1) the 3′ substituent volume and 2) the difference in accessible surface area between the side chain of the introduced residue and the wild-type alanine at position 79 (Fig. 3). For all the mutant receptors tested, BZD binding affinity decreased with increasing bulkiness of the 3′ substituent. In addition, as the surface area of the amino acid introduced at γ_2A79 was increased, we observed correspondingly larger decreases in Ro 41-3380 binding affinity. Taken together, these data are consistent with a model in which γ_2A79 lines a subsite within the BZD binding pocket that accommodates the 3′ substituent of *i*-BZD ligands.

Ability of *i*-BZDs to Slow Sulfhydryl Modification of γ_2A79C and γ_2T81C. We compared the ability of flurazepam (positive modulator), midazolam (positive modulator), Ro 40-6129 (zero modulator), Ro 41-3380 (positive modulator), Ro 15-1788 (zero modulator) and Ro 15-4513 (negative modulator) to impede covalent modification of γ_2A79C and γ_2T81C. BZD ligands with different structures and functional efficacies were used to determine whether either of these properties influenced the ability of the ligand to slow the rate of covalent modification of cysteines substituted at γ_2A79 and γ_2T81. The rate of methanethiolsulfonate (MTS) modification of an engineered cysteine depends on several factors: 1) the permeability of the pathway to the substituted cysteine; 2) the electrostatic potential in the binding site and along the pathway; 3) the ionization of the sulfhydryl group; and 4) local steric restrictions. All of the ligands significantly slowed the rates at which MTSEA-Biotin and MTSEA-Biotin-CAP reacted with γ_2A79C and γ_2T81C, respectively (Fig. 4, Table 3).

Previously, we reported that sulfhydryl-modification of γ_2A79C was slowed by the presence of both flurazepam and Ro 15-1788, whereas modification of γ_2T81C seemed to be slowed only by Ro 15-1788 (Teissére and Czajkowski, 2001). Although the presence of flurazepam showed a clear trend in slowing the rate of MTSEA-Biotin-CAP modification of

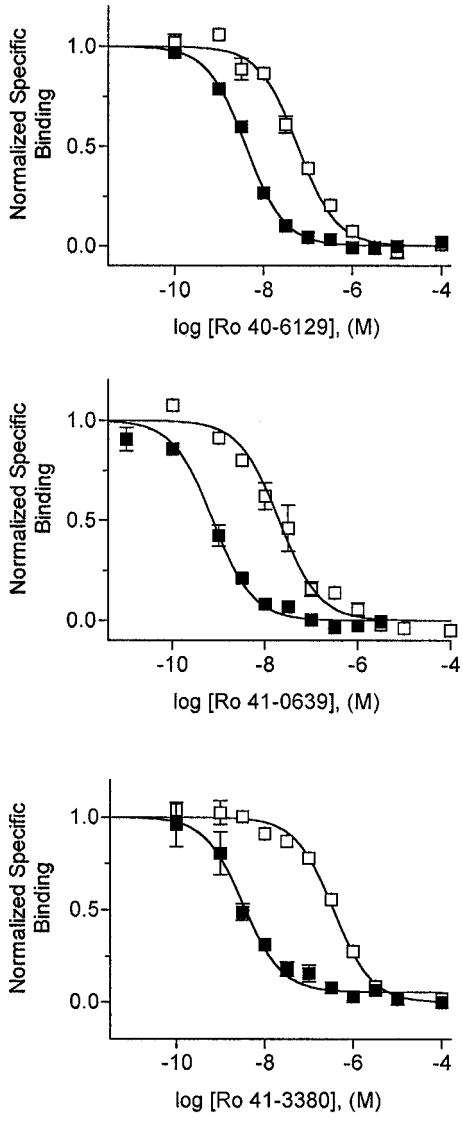

Fig. 2. Mutation of γ_2A79 to tyrosine differentially affects the binding of structurally similar *i*-BZDs to the BZD binding site. Displacement of [^{3}H]flunitrazepam binding by Ro 40-6129 (top), Ro 41-0639 (middle), and Ro 41-3380 (bottom) in $\alpha_1\beta_2\gamma_2$ (■) and $\alpha_1\beta_2\gamma_2$A79Y (□) receptors is shown. The displacing ligands differ only in the size of their respective 3′-imidazo substituents. Values are mean ± S.E.M. of triplicate determinations and were fit by nonlinear regression analysis. K_I values are reported in Table 2.

TABLE 2

Affinities of Ro 40-6129, Ro 41-0639, and Ro 41-3380 for wild-type ($\alpha_1\beta_2\gamma_2$) and mutant receptors

K_I values were determined by displacement of [^{3}H]flunitrazepam binding. Mutations at γ_2A79 are arranged in ascending order of accessible side-chain surface area (Å^2; Chothia, 1976). Data represent mean ± S.E.M.

Receptor	Accessible Surface Area	Ro 40-6129			Ro 41-0639			Ro 41-3380		
		K_I	n	K_I-mut/K_I-$\alpha\ \beta\ \gamma$	K_I	n	K_I-mut/K_I-$\alpha\ \beta\ \gamma$	K_I	n	K_I-mut/K_I-$\alpha\ \beta\ \gamma$
		nM			*nM*			*nM*		
$\alpha\beta\gamma$	115,140[a]	2.6 ± 0.7	3	1.0	0.4 ± 0.2	3	1.0	1.9 ± 0.2	4	1.0
$\alpha\beta\gamma$ A79C	135	8.6 ± 1.1**	3	3.3	2.5 ± 0.4**	4	6.3	18 ± 6**	5	9.5
$\alpha\beta\gamma$ A79L	170	7.3 ± 1.5**	3	2.8	1.1 ± 0.4	3	2.8	328 ± 87**	3	173
$\alpha\beta\gamma$ A79E	190	3.5 ± 0.6	3	1.3	1.9 ± 0.3**	3	4.8	119 ± 20**	3	63
$\alpha\beta\gamma$ A79R	225	4.3 ± 1	3	1.7	1.2 ± 0.2	3	3.0	501 ± 203**	5	264
$\alpha\beta\gamma$ A79Y	230	27 ± 2**	3	10	7.6 ± 3.4**	3	19	192 ± 2**	3	101
$\alpha\beta\gamma$ T81C	135	19 ± 4**	3	7.3	1.2 ± 0.3*	4	3.0	16 ± 3**	5	8.4

[a] Accessible surface areas for both alanine and threonine are reported, respectively.
n, number of independent experiments.
**, $P < 0.01$, significantly different from wild-type receptors.

γ_2T81C (29%), the rate was not statistically significant. Here, the inclusion of additional data demonstrated that flurazepam significantly slowed (39%) the rate of covalent modification of γ_2T81C (Fig. 4, Table 3). Because differences in BZD functional efficacy did not alter the ability of the ligands to protect γ_2A79C or γ_2T81C from sulfhydryl modification, it is likely the protection is caused by a direct steric block of the substituted cysteines and not caused by allosteric conformational changes in the protein that accompany BZD binding. Differences in ligand structure also did not affect the ability of the ligands to protect either γ_2A79C or γ_2T81C from sulfhydryl modification. The results indicate that both classic BZDs and *i*-BZDs, when bound, lie topologically close to γ_2A79 and γ_2T81.

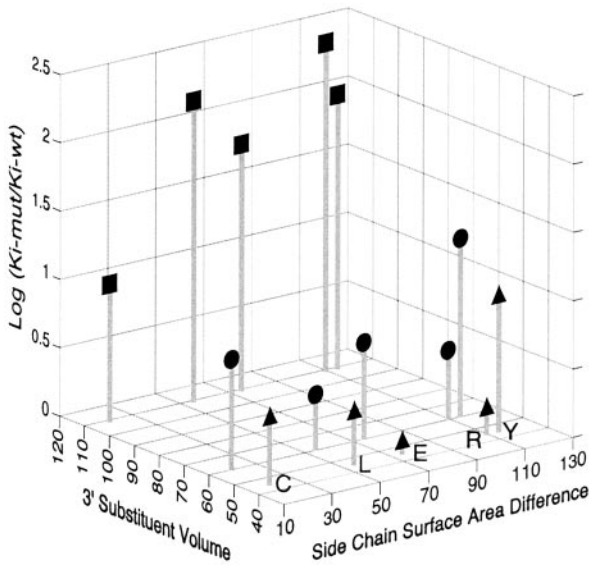

Fig. 3. The decreases observed in Ro 40-6129, Ro 41-0639, and Ro 41-3380 binding affinities after mutation of γ_2A79 is correlated to the volume of the 3′-imidazo substituent. The log of the ratio K_I-mutant/K_I-wild-type is plotted as a function of 1) the volume (Å^3) of the 3′-imidazo substituent of Ro 40-6129 (45.4, ▲), Ro 41-0639 (60.7, ●), and Ro 41-3380 (109.6, ■), and 2) the difference in accessible surface-area between the side chain of the introduced residue and the wild-type alanine. The accessible surface areas of the side chains were taken from Chothia (1976) and are listed in Table 2. The K_I values for mutant and wild-type receptors are reported in Table 2.

Discussion

The side-chains of γ_2F77, γ_2A79 and γ_2T81 are located adjacent to each other on a β-strand within the BZD binding site (Teissére and Czajkowski, 2001). Mutagenesis of γ_2F77 affects the binding affinity of both classic BZDs and *i*-BZDs (Buhr et al., 1997a; Sigel et al., 1998), whereas mutagenesis of γ_2A79 decreases the binding affinity of *i*-BZDs more than classic BZDs (Table 1) indicating that different residues within the binding pocket are important for stabilizing classic BZD and *i*-BZD binding. Both classic BZDs and *i*-BZDs, however, slow covalent modification of γ_2A79 and γ_2T81 (Fig. 4, Table 3). Thus, although classic BZDs do not require γ_2A79 for high affinity binding, when bound they are located close enough to γ_2A79C and γ_2T81C to sterically interfere with the covalent addition of a sulfhydryl reagent at these positions.

Orientation of *i*-BZDs in the BZD Binding Site. Based on our experimental data, we propose that γ_2A79 and γ_2T81 line a region in which the 3′-imidazo substituent of *i*-BZDs is positioned. Several lines of evidence support this model of i-BZD orientation. Overall, γ_2A79 mutations disrupt *i*-BZD binding to a greater extent than classic BZD binding which suggests that structural elements unique to *i*-BZDs (and not common elements, such as the fused diazepine nucleus) are positioned near γ_2A79 within the BZD recognition site. The effect of γ_2A79 mutagenesis on the binding affinities of structurally rigid *i*-BZDs (Ro 41-3380 > Ro 40-0639 > Ro 41-6129) is related to the volume of each 3′ substituent (109.6 Å^3, 60.7 Å^3 and 45.4 Å^3, respectively; Fig. 3). Furthermore, larger amino acid side chains introduced at γ_2A79 cause correspondingly larger decreases in the binding affinities of *i*-BZDs with bulky 3′ substituents. These data can be explained by a model in which the addition of bulky side chains at position 79 decreases the volume of the binding site pocket and hinders occupation of the site by ligands bearing large 3′ substituents, such as Ro 41-3380. A possible contradiction to this model is that the γ_2A79R mutation did not disrupt the binding affinities of Ro 15-4513 or Ro 15-1788, which also possess fairly large 3′ substituents (82.8 Å^3). However, a favorable interaction between the arginine side chain and the ester group of these compounds could overcome potential steric interference. It is also possible that the arginine side chain could interact with neighboring residues or with the

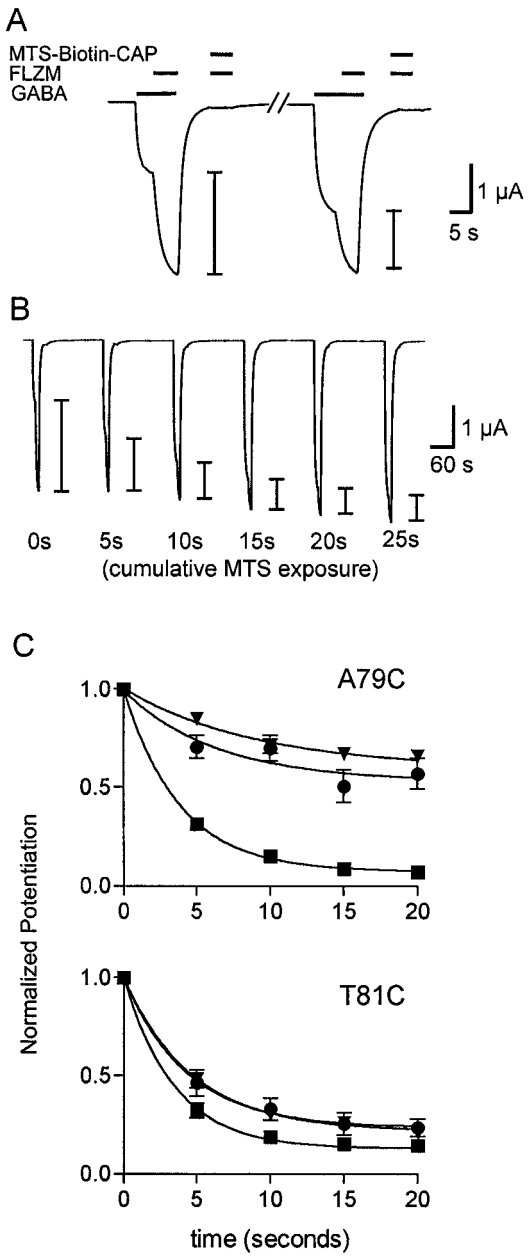

Fig. 4. Rate of sulfhydryl modification of $\alpha_1\beta_2\gamma_2$A79C and $\alpha_1\beta_2\gamma_2$T81C receptors is altered in the presence of BZDs. A, representative GABA (1 μM) and GABA + flurazepam (1 μM each) current traces from $\alpha_1\beta_2\gamma_2$T81C receptors before and after a single 5-s coapplication of 20 μM MTSEA-Biotin-CAP and 5 μM flurazepam (FLZM). I-bars denote BZD potentiation of GABA-activated current. Break in current trace represents a 3-min wash period. Traces shown are expanded from B (0- and 5-s exposure) to illustrate experimental protocol. B, current traces recorded from $\alpha_1\beta_2\gamma_2$T81C receptors after successive applications of MTSEA-Biotin-CAP + FLZM. FLZM potentiation of GABA current was measured before and after each MTSEA-Biotin-CAP treatment. Note that after MTSEA-Biotin-CAP + FLZM exposure, GABA-activated current increased in concert with a decrease in BZD potentiation as described previously in Teissére and Czajkowski (2001). C, rate of sulfhydryl modification of $\alpha_1\beta_2\gamma_2$A79C (top) and $\alpha_1\beta_2\gamma_2$T81C (bottom) receptors in the absence and presence of midazolam and Ro 41-3380. Observed decreases in the potentiation of I_{GABA} by FLZM were plotted versus cumulative MTSEA-Biotin (A79C) or MTSEA-Biotin-CAP (T81C) exposure as described under *Materials and Methods*. Data from individual experiments were normalized to the flurazepam potentiation measured at $t = 0$ and fit to single exponential decay curves (■, MTS alone; ●, MTS + 1 μM midazolam; ▼, MTS + 1 μM Ro 41-3380). Data points are mean ± S.D. from at least three independent experiments. The calculated second-order rate constants for the MTS reaction are presented in Table 3.

TABLE 3

Summary of second-order rate constants for reaction of MTSEA-Biotin (γ_2A79C) or MTSEA-Biotin-CAP (γ_2T81C) in the absence (control) or presence of BZD ligands

Second-order rate constants (k_2) were derived by dividing the calculated pseudo–first-order rate constants by the concentration of MTS reagent used (see *Materials and Methods*). Data represent mean ± S.D.

Treatment	$\alpha\beta\gamma$ A79C	n	$\alpha\beta\gamma$ T81C	n
	$M^{-1}s^{-1}$		$M^{-1}s^{-1}$	
Control	1,250 ± 240	5	18,670 ± 5,430	5
Flurazepam	640 ± 330*	3	11,540 ± 1,520*	4
Midazolam	570 ± 190*	3	11,500 ± 4,160*	3
Ro 40-6129	350 ± 220**	3	5,700 ± 1,900**	3
Ro 41-3380	540 ± 310*	3	8,200 ± 1,700**	3
Ro 15-1788	590 ± 100*	3	3,630 ± 860**	3
Ro 15-4513	500 ± 100**	3	5,850 ± 400**	3

n, number of independent experiments.
*, $P < 0.05$; **, $P < 0.01$; significantly different from control (MTS alone).

peptide backbone to minimize its impact on the binding of these ligands.

Previously, we reported that conservative amino acid substitutions of γ_2T81 resulted in small but significant decreases in *i*-BZD binding affinities (Kucken et al., 2000). In this study, mutation of γ_2T81 to cysteine significantly decreased the binding of the *i*-BZDs, Ro 40-6129, Ro 41-0639 and Ro 41-3380 between 3 and 8 fold (Table 2). Mutation of γ_2F77 to tyrosine has no affect on Ro 40-6129, Ro 41-0639 and Ro 41-3380 binding (Sigel et al., 1998). Taken together, these data, in addition to the result that *i*-BZDs protect γ_2A79C and γ_2T81C from covalent modification, support the conclusion that γ_2A79 and g_2T81 line a region of the BZD binding pocket in which the 3′-imidazo substituent of *i*-BZDs is positioned.

Our data also suggest that there is a size limit to what can be accommodated within the binding cavity. Consistent with this idea, large substitutions at the 3′ position of *i*-BZDs are not tolerated. For example, as the size of the ester group increases from $CO_2CH_2CH_3$ (Ro 15-1788) to $CO_2CH_2C(CH_3)_3$, binding affinity is reduced 100-fold (Wong et al., 1993). Mutations at γ_2A79 affect Ro 15-4513 binding to a greater extent than Ro 15-1788 (Table 1). One explanation of these results is that even though the 3′ substituents of Ro 15-4513 and Ro 15-1788 are the same size, the overall length of Ro 15-4513, from the 7-azide group to the end of the 3′ester substituent, is longer than Ro 15-1788 (Fig. 1).

Interestingly, *i*-BZDs with small 3′ substituents (e.g., CO_2CH_3) also have decreased binding affinities (Wong et al., 1993), suggesting that there may be an optimal size relationship between the 3′ substituent and the binding site. Mutation of γ_2A79 to a smaller residue (e.g., glycine) as well as to larger residues (e.g., phenylalanine, tyrosine) significantly decreases Ro 15-4513 and Ro 15-1788 binding affinities (Table 1) and suggests that size of the side chain at this position influences *i*-BZD binding affinity. Other amino acid properties, such as hydrophobicity, aromaticity, charge, and H-bonding capability, did not correlate with the decreases in *i*-BZD binding affinity measured after γ_2A79 mutagenesis.

Homology Model of the BZD Binding Site. Much of our experimental data were completed before the publication of the molluscan AChBP crystal structure (Brejc et al., 2001). To help facilitate discussion of our results and to provide additional support for our model of *i*-BZD orientation, we homology-modeled the benzodiazepine binding site using the

structure of the AChBP as a template (Fig. 5). It is important to recognize that the model is only a static picture of the BZD binding site captured in an indeterminate state. The homology model is based on the structure of the AChBP crystallized in the presence of the putative agonist HEPES. Because AChBP binds acetylcholine with high affinity, it has been hypothesized that this structure may represent receptor in either an open or desensitized state (Brejc et al., 2001).

Prior data from our laboratory demonstrated that the region of the γ_2 subunit from T73 to T81 forms a β strand (Teissére and Czajkowski, 2001). Our secondary structure prediction agrees with the crystal structure of the AChBP, where residues aligned with this region of the $GABA_A$ receptor form part of a β strand (AChBP, β2). Many of the residues previously identified by mutagenesis, the substituted cysteine accessibility method (α_1Y159, α_1T206, α_1Y209, γ_2Y58, γ_2F77, γ_2A79, and γ_2T81), and photoaffinity labeling (α_1H101) as contributing to the BZD binding site (Duncalfe et al., 1996; Amin et al., 1997; Buhr et al., 1997a,b; Davies et al., 1998; Kucken et al., 2000; Teissére and Czajkowski, 2001) are positioned at the α_1/γ_2 subunit interface and define a cavity that probably forms the BZD-binding site (Fig. 5A).

In the absence of a crystal structure of the $GABA_A$ receptor bound with a BZD ligand, it is difficult to identify which residues directly contact a ligand and to predict how the ligands are oriented in the binding pocket. Ro 15-4513 can be used as a photoaffinity label for the BZD binding site of the $GABA_A$ receptor (Mohler et al., 1984; Sieghart et al., 1987). The azide group at the 7 position of the fused diazepine ring (Fig. 1) is the photoreactive moiety and, when exposed to UV light, the aryl azide undergoes ring-expansion and subsequent bond formation with nearby nucleophilic groups (Hermanson, 1996). Studies using both bovine brain and recombinant $GABA_A$ receptors have established that Ro 15-4513 photoincorporates into the α subunit in a region that lies between G103 and the C terminus (Davies et al., 1996; Duncalfe and Dunn, 1996). Davies and Dunn (1998) mapped a region of incorporation to the extracellular loop between transmembrane regions two and three. Recently, however, a site of Ro 15-4513 incorporation was mapped to a tyrosine residue equivalent to rat α_1Y209 (Sawyer et al., 2002). Combining our data with this finding, we envision that Ro 15-4513 spans the binding site between α_1Y209 and γ_2A79, with the azide substituent facing the α_1 subunit and the 3′-imidazo substituent facing the γ_2 subunit. In agreement with our experimental data, computational docking of Ro 15-4513 into the BZD binding site positions the 3′-imidazo substituent of Ro 15-4513 near γ_2A79 (Fig. 5, B and C). The docking of Ro 15-1788 resulted in the same positioning of the 3′-imidazo substituent.

Although our data clearly define the orientation of *i*-BZDs within the binding site, how the binding of *i*-BZDs promote local movements within the binding site that are coupled to changes in GABA binding and/or GABA activation of the channel remains unknown. The size of the 3′ substituent alone does not seem to predict *i*-BZD efficacy. Ro 15-4513, which possesses a fairly large 3′ substituent (82.8Å^3) is a BZD inverse-agonist, Ro 15-1788 (82.8 Å^3) is a BZD antagonist, Ro 40-6129 (45.4 Å^3) is a BZD antagonist, Ro 41-0639 (60.7 Å^3) is a weak BZD partial agonist, and Ro 41-3380 (109.6 Å^3) is a BZD agonist. According to allosteric theory, modulators that bind to a receptor protein exert their effects

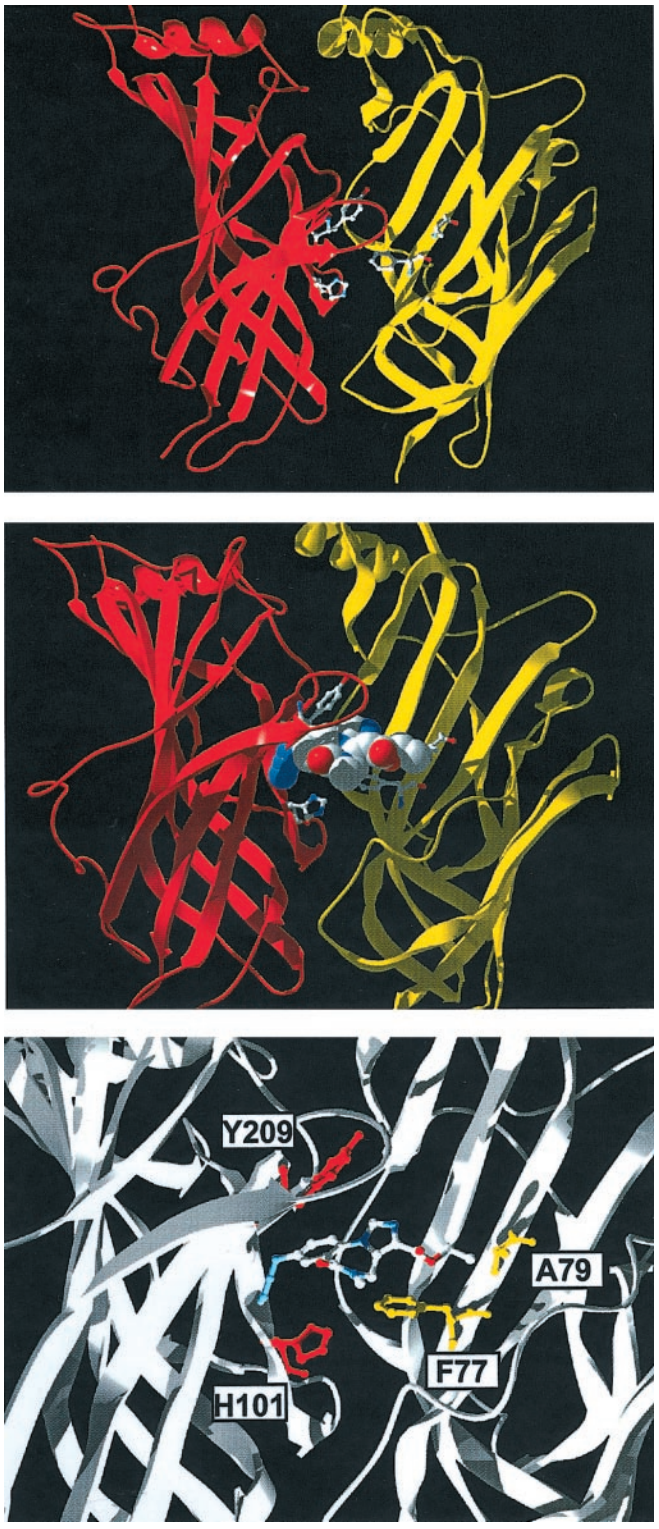

Fig. 5. Structural model of the BZD-binding site. The extracellular N-terminal regions of the $GABA_A$ receptor α_1 and γ_2 subunits were threaded onto the crystal structure of the AChBP (Brejc et al., 2001) and energy-minimized as described under *Materials and Methods*. Top, model of the α_1/γ_2 interface of the $GABA_A$ receptor. The α_1 subunit is shown in red and the γ_2 subunit is yellow. Several residues that contribute to the BZD-binding site are highlighted, α_1H101, α_1Y209, γ_2F77, and γ_2A79. Middle, Ro 15-4513 (space-filled) computationally docked into the BZD-binding site (see *Materials and Methods*). Bottom, magnified view of Ro 15-4513 (ball and stick representation) docked into the BZD-binding site. α_1 subunit residues are colored red; γ_2 subunit residues are colored yellow. The docked ligand had a binding energy of approximately −16 kcal/mol.

by initiating an allosteric transition in the protein that indirectly modifies the conformation of the agonist binding site (Changeux and Edelstein, 1998). 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. Previously, we demonstrated that GABA binding and/or GABA-mediated receptor activation causes structural rearrangements in the BZD binding site that can be detected at A79 (Teissére and Czajkowski, 2001). We speculate that BZD binding also promotes structural rearrangements within the BZD binding site and that these local changes determine how a particular BZD will modulate the GABA response. Because the therapeutic value of BZDs depends on their efficacy, identification of the residues mediating these local movements is an important goal for future studies.

Acknowledgments

We thank Lisa M. Sharkey for assistance in rate of modification experiments, Lisa A. Wheeler for assistance in binding assays, Eric Wise for aid in the design of loop structures, and Dr. J. Glen Newell for invaluable discussion and critical reading of this manuscript.

References

Amin J, Brooks-Kayal A, and Weiss DS (1997) Two tyrosine residues on the α subunit are crucial for benzodiazepine binding and allosteric modulation of γ-aminobutyric acid$_A$ receptors. *Mol Pharmacol* **51:**833–841.

Boileau AJ, Baur R, Sharkey LM, Sigel E, and Czajkowski C (2002) The relative amount of cRNA coding for gamma2 subunits affects stimulation by benzodiazepines in GABA$_A$ receptors expressed in *Xenopus* oocytes. *Neuropharmacology* **43:**695–700.

Brejc K, van Dijk WJ, Klaassen RV, Schuurmans M, van der Oost J, Smit AB, and Sixma TK (2001) Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature (Lond)* **411:**269–276.

Buhr A, Baur R, and Sigel E (1997a) Subtle changes in residue 77 of the γ subunit of $\alpha_{1\beta2\gamma2}$ GABA$_A$ receptors drastically alter the affinity for ligands of the benzodiazepine binding site. *J Biol Chem* **272:**11799–11804.

Buhr A, Schaerer MT, Baur R, and Sigel E (1997b) Residues at positions 206 and 209 of the α_1 subunit of γ-aminobutyric acid$_A$ receptors influence affinities for benzodiazepine binding site ligands. *Mol Pharmacol* **52:**676–682.

Buhr A and Sigel E (1997) A point mutation in the γ_2 subunit of the γ-aminobutyric acid type A receptors results in altered benzodiazepine binding specificity. *Proc Natl Acad Sci USA* **94:**8824–8829.

Changeux J and Edelstein SJ (1998) Allosteric receptors after 30 years. *Neuron* **21:**959–980.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.

Chothia C (1976) The nature of the accessible and buried surfaces in proteins. *J Mol Biol* **105:**1–12.

Chou T (1974) Relationships between inhibition constants and fractional inhibition in enzyme-catalyzed reactions with different numbers of reactants, different reaction mechanisms and different types and mechanisms of inhibition. *Mol Pharmacol* **10:**235–247.

Cromer BA, Morton CJ, and Parker MW (2002) Anxiety over GABA$_A$ receptor structure relieved by AChBP. *Trends Biochem Sci* **27:**280–287.

Davies M, Bateson AN, and Dunn SMJ (1998) Structural requirements for ligand interactions at the benzodiazepine recognition site of the GABA$_A$ receptor. *J Neurochem* **70:**2188–2194.

Davies M and Dunn SMJ (1998) Identification of a unique domain in bovine brain GABA$_A$ receptors that is photoaffinity labelled by [^{3}H]Ro15-4513. *Biochem and Biophys Res Comm* **246:**650–653.

Davies M, Martin IL, Bateson AN, Hadingham KL, Whiting PJ, and Dunn SMJ (1996) Identification of domains in human recombinant GABA$_A$ receptors that are photoaffinity labelled by [^{3}H]Ro15-4513. *Neuropharmacol* **35:**1199–1208.

Davies M, Newell JG, and Dunn SMJ (2001) Mutagenesis of the GABA$_A$ receptor α_1 subunit reveals a domain that affects sensitivity to GABA and benzodiazepine site ligands. *J Neurochem* **79:**55–62.

Doble A and Martin IL (1996) *The GABA$_A$/Benzodiazepine Receptor As a Target for Psychoactive Drugs.* Landes, Austin, TX.

Duncalfe LL, Carpenter MR, Smillie LB, Martin IL, and Dunn SMJ (1996) The major site of photoaffinity labeling of the γ-aminobutyric acid type A receptor by [^{3}H]flunitrazepam is histidine 102 of the α subunit. *J Biol Chem* **271:**9209–9214.

Duncalfe LL and Dunn SMJ (1996) Mapping of GABA$_A$ receptor sites that are photoaffinity labelled by [^{3}H]flunitrazepam and [^{3}H]Ro15-4513. *Eur J Pharmacol* **298:**313–319.

He X, Zhang C, and Cook JM (2001) Model of the BzR binding site: Correlation of data from site-directed mutagenesis and the pharmacophore/receptor model. *Med Chem Res* **10:**269–308.

Hermanson GT (1996) *Bioconjugate Techniques.* Academic Press, San Diego.

Hevers W and Lüddens H (1998) The diversity of GABA$_A$ receptors: pharmacological and electrophysiological properties of GABA$_A$ channel subtypes. *Mol Neurobiol* **18:**35–86.

Kucken AM, Wagner DA, Ward PR, Teissére JA, Boileau AJ, and Czajkowski C (2000) Identification of benzodiazepine binding site residues in the γ_2 subunit of the γ-aminobutyric acidA receptor. *Mol Pharmacol* **57:**932–939.

Liman ER, Tytgat J, and Hess P (1992) Subunit stoichiometry of a mammalian K^+ channel determined by construction of multimeric cDNAs. *Neuron* **9:**861–871.

Mohler H, Sieghart W, Richards JG, and Junkeler W (1984) Photoaffinity labeling of benzodiazepine receptors with a partial inverse agonist. *Eur J Pharmacol* **102:**191–192.

Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK, and Olson AJ (1998) Automated docking using a Lamarckian genetic algorithm and empirical binding free energy function. *J Comput Chem* **19:**1639–1662.

Pascual JM and Karlin A (1998) State-dependent accessibility and electrostatic potential in the channel of the acetylcholine receptor: inferences from rates of reaction of thiosulfonates with substituted cysteines in the M2 segment of the α subunit. *J Gen Physiol* **111:**717–739.

Powell MJD (1977) Restart procedures for the conjugate gradient method. *Math Prog* **12:**241–254.

Press WH, Flannery BP, Teukolsky SA, and Vetterling WT (1988) Conjugate gradients, simplex and BFGS, in *Numerical Recipes in C: the Art of Scientific Computing*, pp 301–327, Cambridge University Press, Cambridge, UK.

Renard S, Olivier A, Granger P, Avenet P, Graham D, Sevrin M, George P, and Besnard F (1999) Structural elements of the γ-aminobutyric acid type A receptor conferring subtype selectivity for benzodiazepine site ligands. *J Biol Chem* **274:**13370–13374.

Robertson GA, Warmke JM, and Ganetzky B (1996) Potassium currents expressed from *Drosophila* and mouse eag cDNAs in *Xenopus* oocytes. *Neuropharmacol* **35:**841–850.

Sawyer GW, Chiara DC, Cohen JB, and Olsen RW (2001) Characterization of the site of photoincorporation of [3H]Ro15-4513 into affinity purified GABA$_A$ receptor α subunit. *Soc Neurosci Abstr* **27:**591.15.

Sawyer GW, Chiara DC, Olsen RW, and Cohen JB (2002) Identification of the bovine γ-aminobutyric acid type A receptor α subunit residues photolabeled by the imidazobenzodiazepine [^{3}H]Ro15-4513. *J Biol Chem* **277:**50036–50045.

Schaerer MT, Buhr A, Baur R, and Sigel E (1998) Amino acid 200 on the α_1 subunit of GABA$_A$ receptors affects the interaction with selected benzodiazepine binding site ligands. *Eur J Pharmacol* **354:**283–287.

Sieghart W, Eichinger A, Richards JG, and Mohler H (1987) Photoaffinity labelling of benzodiazepine receptor proteins with a partial inverse agonist [^{3}H]Ro15-4513: a biochemical and autoradiographic study. *J Neurochem* **48:**46–52.

Sigel E and Buhr A (1997) The benzodiazepine binding site of GABA$_A$ receptors. *Trends Pharmacol Sci* **18:**425–429.

Sigel E, Schaerer MT, Buhr A, and Baur R (1998) The benzodiazepine binding pocket of recombinant $\alpha_{1\beta2\gamma2}$ γ-aminobutyric acid$_A$ receptors: relative orientation of ligands and amino acid side chains. *Mol Pharmacol* **54:**1097–1105.

Smith GB and Olsen RW (1995) Functional domains of GABA$_A$ receptors. *Trends Pharmacol Sci* **16:**162–168.

Sternbach LH (1979) The benzodiazepine story. *J Med Chem* **22:**1–7.

Teissére JA and Czajkowski C (2001) A β-strand in the γ_2 subunit lines the benzodiazepine binding site of the GABA$_A$ receptor: structural rearrangements detected during channel gating. *J Neurosci* **21:**4977–4986.

Wingrove PB, Thompson SA, Wafford KA, and Whiting PJ (1997) Key amino acids in the γ subunit of the γ-aminobutyric acid$_A$ receptor that determine ligand binding and modulation at the benzodiazepine binding site. *Mol Pharmacol* **52:**874–881.

Wong G, Koehler KF, Skolnick P, Gu ZQ, Ananthan S, Schonholzer P, Hunkeler W, Zhang W and Cook JM (1993) Synthetic and computer-assisted analysis of the structural requirements for selective, high-affinity ligand binding to diazepam-insensitive benzodiazepine receptors. *J Med Chem* **36:**1820–1830.

Address correspondence to: Cynthia Czajkowski, Ph.D., Dept. of Physiology, University of Wisconsin-Madison, 1300 University Ave., 197 MSC, Madison, WI 53706. E-mail: czajkowski@physiology.wisc.edu