Structural Requirements for Imidazobenzodiazepine Binding to GABA<sub>A</sub> Receptors

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ABSTRACT

Several structural subclasses of ligands bind to the benzodiazepine (BZD) binding site of the GABA<sub>A</sub> 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<sub>2</sub>A79 and γ<sub>2</sub>T81. Both classic BZDs and i-BZDs protect cysteines substituted at γ<sub>2</sub>A79 and γ<sub>2</sub>T81 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<sub>2</sub>A79 than classic BZDs or BZDs that lack a 3′-imidazo substituent (e.g., midazolam). The effect that γ<sub>2</sub>A79 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<sub>2</sub>A79 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<sub>2</sub>A79 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<sub>2</sub>A79.

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<sub>A</sub> receptor, the main effector of neuronal inhibition within the central nervous system (Hevner and Lüddens, 1998). The BZD binding site is on the extracellular surface of the GABA<sub>A</sub> 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; Kucen 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).

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<sub>2</sub> 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<sub>2</sub>A79 had a larger effect on the binding affinities of Ro 15-4513 and Ro 15-1788 than on the classic BZD ligand flunitrazepam (Kucen et al., 2000). Additionally, Ro15-1788 as well as the classic BZD flurazepam impeded the covalent modification of a cysteine substituted at γ<sub>2</sub>A79, whereas modification of γ<sub>2</sub>T81C was significantly impeded by Ro15-1788 but not by flurazepam.
(Teissère and Czajkowski, 2001). Based on these data, we hypothesized that γ2A79 and γ2T81 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 γ2A79. In addition, we examined the ability of several BZD ligands with different structures and functional efficacies to slow the rate of covalent modification of γ2A79 and γ2T81C. Our studies indicate that γ2A79 and γ2T81 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 GABAA receptor and describe in part the three-dimensional relationship between i-BZD ligands and the γ2 subunit of the GABAA receptor.

Materials and Methods

Mutagenesis. Rat cDNAs encoding α1, β2, and γ2S subunits were used for all molecular cloning, radioligand binding, and functional studies. Site-directed mutagenesis of γ2A79 and γ2T81 was carried out using recombinant oligonucleotides and the polymerase chain reaction. For radioligand binding, amino acid mutations were made in 9E10 epitope-tagged γ2 subunit as the template. The presence of the epitope had no detectable effect on ligand recognition or on expression of the γ2 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 pGHI9 (Liman et al., 1992; Robertson et al., 1996) for expression in Xenopus laevis oocytes. All γ2 mutants were verified by restriction enzyme analysis and double-strand DNA sequencing.

Transient Transfection and Radioligand Binding. HEK 293 cells were transiently transfected with α1, β2, and γ2myc or γ2myn mutant subunits using a standard CaHPO4 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 3H]flunitrazepam at sub-KD 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: $Y = B_{\text{max}}/(1 + XIC_{50})$, where $Y$ is the specifically bound disintegrations per minute, $B_{\text{max}}$ 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. [3H]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 α1, β2, γ2, γ2A79C, or γ2T81C subunits in pGHI9 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/μl/subunit) in the ratio 1:1:10 (α1/β2/γ2, Boileau et al., 2002). Expressed receptors were functionally assayed using two-electrode voltage clamp (Vhold = −80 mV, room temp) as described previously (Teissère 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 MgCl2, 1.8 mM CaCl2, and 5 mM HEPES, pH 7.2). In all electrophysiological experiments, both GABA-activated current (IGABA) and flurazepam-mediated potentiation of GABA-activated current (I_{GABA} + flurazepam) were measured. Flurazepam-mediated potentiation of IGABA was defined as [(IGABA + flurazepam/IGABA) − 1] × 100. Rates of sulfhydryl-specific covalent modification of α1β2,γ2A79C or α1β2γ2T81C receptors by methanethiosulfonate (MTS) reagents were determined using the following protocol: 1) flurazepam potentiation of IGABA 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_{50} concentration of flurazepam); 2) the oocyte was washed for 3 min in ND96 buffer; and 3) flurazepam potentiation of IGABA was measured again. This protocol was repeated until flurazepam potentiation of IGABA 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 IGABA and IGABA + flurazepam. Applications of the MTS reagent were repeated until flurazepam potentiation of IGABA no longer decreased. γ2A79-containing receptors were reacted with 200 μM N-biotinylaminooethyl MTS (MTSEA-Biotin; Biotium, Hayward, CA) and γ2T81C-containing receptors were reacted with 20 μM N-biotinylcaproylaminooethyl CAP MTS.
The affinity of different BZDs to slow the rate of MTS modification of \( \gamma_2 \)A79C and \( \gamma_2 \)T81C was assayed by coapplying a BZD with the MTS-reagent during the rate determinations. The following BZDs were tested (all applied at \( -EC_{50} \) concentrations): flurazepam, Ro 15-1788, Ro 15-4513, midazolam, Ro 40-6129, and Ro 41-3380. In these experiments, flurazepam potentiation of \( I_{\text{GABA}} \) was stabilized before measuring the rate of MTS modification as follows: 1) 1 \( \mu \)M GABA and 1 \( \mu \)M GABA + 1 \( \mu \)M flurazepam were applied to an oocyte; 2) the oocyte was treated with an \( EC_{50} \) concentration of BZD and then washed for 3 min in ND96; and 3) flurazepam potentiation of \( I_{\text{GABA}} \) was measured again using 1 \( \mu \)M GABA and 1 \( \mu \)M GABA + 1 \( \mu \)M flurazepam. This protocol was repeated until \( I_{\text{GABA}} \) and \( I_{\text{GABA}} + \text{flurazepam} \) changed by less than 10% and demonstrated that the wash time was sufficient to wash out the test \( EC_{50} \) 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 \( I_{\text{GABA}} \) could still be obtained.

### Homology Modeling of the BZD Binding Site

The mature protein sequences of the rat \( \alpha_1 \) and \( \gamma_2 \) 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 119B) and loaded into Swiss Protein Databank Viewer (http://ca.expasy.org/spdbv). The mature \( \alpha_1 \) protein sequence from T12 to T122 and the mature \( \gamma_2 \) 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 thread subunits were imported into SYBYL (Tripos, Inc., St. Louis, MO) and energy minimized (< 0.5 kcal/Å). The first 10 iterations were carried out using Simplex minimization (Press et al., 1988) followed by 1000 iterations using the Powell conjugate gradient method (Powell, 1977). An \( \alpha_1/\gamma_2 \) 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.

### Results

The Effect of Systematic \( \gamma_2 \)A79 Mutagenesis on i-BZD Binding Affinity

Ten point mutations were made at \( \gamma_2 \)A79 to evaluate the contribution of this residue to BZD ligand affinity (\( \gamma_2 \)A79—Gly, Ser, Cys, 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 (\( \alpha_1 \beta_2 \gamma_2 \)) and 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 \(^3\)Hflunitrazepam. 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 \( \gamma_2 \)A79 significantly altered flunitrazepam affinity. The \( \gamma_2 \)A79R, -C, and -Q mutations reduced flunitrazepam affinity 3-, 5-, and 9-fold, respectively. In contrast, 9 of the 10 mutations at \( \gamma_2 \)A79 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 \( \gamma_2 \)A79, in general, had larger effects on the binding affinities of i-BZDs than on flunitrazepam affinity, we hypothesized that \( \gamma_2 \)A79 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 \( \gamma_2 \)A79.

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

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Flunitrazepam</th>
<th>Midazolam</th>
<th>Ro 15-4513</th>
<th>Ro 15-1788</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_i )</td>
<td>( K_i )</td>
<td>( K_i )</td>
<td>( K_i )</td>
<td>( K_i )</td>
</tr>
<tr>
<td>( nM )</td>
<td>( nM )</td>
<td>( nM )</td>
<td>( nM )</td>
<td>( nM )</td>
</tr>
<tr>
<td>( a\beta \gamma )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \alpha_2 \gamma_9 )</td>
<td>8.9 ± 0.9</td>
<td>3</td>
<td>1.0</td>
<td>5.8 ± 1.1</td>
</tr>
<tr>
<td>( \alpha_2 \gamma_9 )</td>
<td>13 ± 3</td>
<td>4</td>
<td>1.5</td>
<td>15 ± 2*</td>
</tr>
<tr>
<td>( \alpha_2 \gamma_9 )</td>
<td>15 ± 2</td>
<td>5</td>
<td>1.7</td>
<td>N.D.</td>
</tr>
<tr>
<td>( \alpha_2 \gamma_9 )</td>
<td>46 ± 5**</td>
<td>3</td>
<td>5.4</td>
<td>40 ± 2**</td>
</tr>
<tr>
<td>( \alpha_2 \gamma_9 )</td>
<td>4.4 ± 1.6</td>
<td>3</td>
<td>0.5</td>
<td>5.7 ± 1.9</td>
</tr>
<tr>
<td>( \alpha_2 \gamma_9 )</td>
<td>44 ± 2**</td>
<td>3</td>
<td>2.2</td>
<td>6 ± 16**</td>
</tr>
<tr>
<td>( \alpha_2 \gamma_9 )</td>
<td>0 ± 1</td>
<td>3</td>
<td>1.1</td>
<td>N.D.</td>
</tr>
<tr>
<td>( \alpha_2 \gamma_9 )</td>
<td>6.1 ± 2.2</td>
<td>3</td>
<td>0.7</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>( \alpha_2 \gamma_9 )</td>
<td>24 ± 5*</td>
<td>3</td>
<td>2.7</td>
<td>47 ± 16**</td>
</tr>
<tr>
<td>( \alpha_2 \gamma_9 )</td>
<td>20 ± 4</td>
<td>3</td>
<td>2.2</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* 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 \( \gamma_2 \)A79 mutation, midazolam binding affinity was examined. Midazolam contains an imidazopyridine 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 \( \gamma_2 \)A79 mutant receptors (\( \gamma_2 \)A79C, -C, -E, -L, -R, and -Y). In general, the effects \( \gamma_2 \)A79 mutation had on midazolam affinity mirrored those observed for flunitrazepam rather than Ro 15-4513 or Ro 15-1788 (Table 1). For example, the \( \gamma_2 \)A79C, -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 \( \gamma_2 \)A79R 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 \( \gamma_2 \)A79 mutation is probably caused by the 3’-imidazo substituent of these ligands.

To examine the potential spatial relationship between \( \gamma_2 \)A79 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 Å³, 60.7 Å³, and 109.6 Å³, respectively), these compounds were ideal for testing the effect of 3’ substituent size on i-BZD binding affinity after mutation of \( \gamma_2 \)A79.

The ability of Ro 40-6129, Ro 41-0639, and Ro 41-3380 to displace the binding of \(^{3}H\)flunitrazepam in wild type, \( \gamma_2 \)A79C-, \( \gamma_2 \)A79E-, \( \gamma_2 \)A79L-, \( \gamma_2 \)A79Y-, and \( \gamma_2 \)A79R-containing receptors was measured (Fig. 2, Table 2). All three ligands bound to wild-type GABA\(_{\text{A}}\) 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 binding affinity decreased with increasing bulkiness of the 3’ position 79 (Fig. 3). For all the mutant receptors tested, BZD chain of the introduced residue and the wild-type alanine at the difference in accessible surface area between the side.

Previously, we reported that sulfhydryl-modification of \( \gamma_2 \)A79C was slowed by the presence of both flurazepam and Ro 15-1788, whereas modification of \( \gamma_2 \)T81C seemed to be slowed only by Ro 15-1788 (Teiss\`ere and Czajkowski, 2001). Although the presence of flurazepam showed a clear trend in slowing the rate of MTSEA-Biotin-CAP modification of
Imidazobenzodiazepine Binding to the GABA<sub>a</sub> Receptor 293

TABLE 2
Affinities of Ro 40-6129, Ro 41-0639, and Ro 41-3380 for wild-type (α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>) and mutant receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Accessible Surface Area</th>
<th>Ro 40-6129</th>
<th>Ro 41-0639</th>
<th>Ro 41-3380</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
<td>n</td>
<td>K&lt;sub&gt;i&lt;/sub&gt;/mut/</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>K&lt;sub&gt;i&lt;/sub&gt;/βγ</td>
</tr>
<tr>
<td>αβγ</td>
<td>115,140&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.6 ± 0.7</td>
<td>3</td>
<td>1.0</td>
</tr>
<tr>
<td>αβγ A79C</td>
<td>135</td>
<td>8.6 ± 1.1**</td>
<td>3</td>
<td>3.3</td>
</tr>
<tr>
<td>αβγ A79L</td>
<td>170</td>
<td>7.3 ± 1.5**</td>
<td>3</td>
<td>2.8</td>
</tr>
<tr>
<td>αβγ A79E</td>
<td>190</td>
<td>3.5 ± 0.6</td>
<td>3</td>
<td>1.3</td>
</tr>
<tr>
<td>αβγ A79R</td>
<td>225</td>
<td>4.3 ± 1</td>
<td>3</td>
<td>1.7</td>
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<tr>
<td>αβγ A79Y</td>
<td>230</td>
<td>27 ± 2**</td>
<td>3</td>
<td>7.3</td>
</tr>
<tr>
<td>αβγ T81C</td>
<td>135</td>
<td>19 ± 4**</td>
<td>3</td>
<td>3.0</td>
</tr>
</tbody>
</table>

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

The side-chains of γ<sub>2</sub>F77, γ<sub>2</sub>A79 and γ<sub>2</sub>T81 are located adjacent to each other on a β-strand within the BZD binding site (Teissèere and Czajkowski, 2001). Mutagenesis of γ<sub>2</sub>F77 affects the binding affinity of both classic BZDs and i-BZDs (Buhr et al., 1997a; Sigel et al., 1998), whereas mutagenesis of γ<sub>2</sub>A79 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<sub>2</sub>A79 and γ<sub>2</sub>T81 (Fig. 4, Table 3). Thus, although classic BZDs do not require γ<sub>2</sub>A79 for high affinity binding, when bound they are located close enough to γ<sub>2</sub>A79C and γ<sub>2</sub>T81C 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<sub>2</sub>A79 and γ<sub>2</sub>T81 line a region in which the 3′-imidazolo substituent of i-BZDs is positioned. Several lines of evidence support this model of i-BZD orientation. Overall, γ<sub>2</sub>A79 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<sub>2</sub>A79 within the BZD recognition site. The effect of γ<sub>2</sub>A79 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<sup>3</sup>, 60.7 Å<sup>3</sup> and 45.4 Å<sup>3</sup>, respectively; Fig. 3). Furthermore, larger amino acid side chains introduced at γ<sub>2</sub>A79 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<sub>2</sub>A79R 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<sup>3</sup>). 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...
Summary of second-order rate constants for reaction of MTSEA-Biotin \((\gamma_2\gamma_9C)\) or MTSEA-Biotin-CAP \((\gamma_2\gamma_8C)\) 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 MTSEA reagent used (see Materials and Methods). Data represent mean ± S.D.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(k_2) (\gamma_7YC) (M^{-1})s(^{-1})</th>
<th>(n)</th>
<th>(k_2) (\gamma_8YC) (M^{-1})s(^{-1})</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.250 ± 240</td>
<td>5</td>
<td>18.670 ± 5.430</td>
<td>5</td>
</tr>
<tr>
<td>Flurazepam</td>
<td>640 ± 330*</td>
<td>3</td>
<td>11,540 ± 1,520*</td>
<td>4</td>
</tr>
<tr>
<td>Midazolam</td>
<td>570 ± 190*</td>
<td>3</td>
<td>11,500 ± 4,160*</td>
<td>3</td>
</tr>
<tr>
<td>Ro 40-6129</td>
<td>350 ± 220**</td>
<td>3</td>
<td>5,700 ± 1,900**</td>
<td>3</td>
</tr>
<tr>
<td>Ro 41-3380</td>
<td>540 ± 310*</td>
<td>3</td>
<td>8,200 ± 1,700**</td>
<td>3</td>
</tr>
<tr>
<td>Ro 15-1788</td>
<td>590 ± 100*</td>
<td>3</td>
<td>3,630 ± 860**</td>
<td>3</td>
</tr>
<tr>
<td>Ro 15-4513</td>
<td>500 ± 100**</td>
<td>3</td>
<td>5,850 ± 400**</td>
<td>3</td>
</tr>
</tbody>
</table>

\(*, P < 0.05; **, P < 0.01; significantly different from control (MTS alone)."

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\(CH_3\)_2\), binding affinity is reduced 100-fold (Wong et al., 1993). Mutations at \(\gamma_2\gamma_7YC\) 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-azido 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., \(CO_2CH_2\)) 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 \(\gamma_2\) 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 \(\gamma_2\gamma_7YC\) 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 $\gamma_2$ subunit from T73 to T81 forms a $\beta$ strand (Teissère 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 $\beta$ strand (AChBP, $\beta_2$). Many of the residues previously identified by mutagenesis, the substituted cysteine accessibility method ($\alpha_1$Y159, $\alpha_2$T206, $\alpha_1$Y209, $\gamma_2$Y58, $\gamma_3$F77, $\gamma_2$A79, and $\gamma_2$T81), and photoaffinity labeling ($\alpha_1$Y101) as contributing to the BZD binding site (Duncan et al., 1996; Amin et al., 1997; Buhr et al., 1997a,b; Davies et al., 1998; Kucken et al., 2000; Teissère and Czajkowski, 2001) are positioned at the $\alpha_1/\gamma_2$ 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 AChBP 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 $\alpha$ subunit in a region that lies between G103 and the C terminus (Davies et al., 1996; Duncan 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 $\alpha_1$Y209 (Sawyer et al., 2002). Combining our data with this finding, we envision that Ro 15-4513 spans the binding site between $\alpha_1$Y209 and $\gamma_2$A79, with the azide substituent facing the $\alpha_1$ subunit and the 3'-imidazo substituent facing the $\gamma_2$ 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 $\gamma_2$A79 (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 (82.8 Å$^3$) 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 $\alpha_1$ and $\gamma_2$ 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 $\alpha_1/\gamma_2$ interface of the GABA$_A$ receptor. The $\alpha_1$ subunit is shown in red and the $\gamma_2$ subunit is yellow. Several residues that contribute to the BZD-binding site are highlighted, $\alpha_1$H101, $\alpha_1$Y209, $\gamma_3$F77, and $\gamma_2$A79. 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. $\alpha_1$ subunit residues are colored red; $\gamma_2$ 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 BDZ 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 BDZ binding site that can be detected at A79 (Teissèdre and Czajkowski, 2001). We speculate that BDZ binding also promotes structural rearrangements within the BDZ binding site and that these local changes determine how a particular BDZ will modulate the GABA response. Because the therapeutic value of BDZs depends on their efficacy, identification of the residues mediating these local movements is an important goal for future studies.

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