Two Tyrosine Residues on the α Subunit Are Crucial for Benzodiazepine Binding and Allosteric Modulation of γ-Aminobutyric Acid A Receptors

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SUMMARY

Benzodiazepines (BZs) exert their therapeutic effects in the mammalian central nervous system at least in part by modulating the activation of γ-aminobutyric acid (GABA)-activated chloride channels. To gain further insight into the mechanism of action of BZs on GABA receptors, we have been investigating structural determinants required for the actions of the BZ diazepam (dzp) on recombinant α1β2γ2 GABA A receptors. Site-directed mutagenesis was used to introduce point mutations into the α1 and γ2 GABA A receptor subunits. Wild-type and mutant GABA A receptors were then expressed in Xenopus laevis oocytes or human embryonic kidney 293 (HEK 293) cells and studied using two-electrode voltage-clamp and ligand-binding techniques. With this approach, we identified two tyrosine residues on the α1 subunit (Tyr159 and Tyr209) that when mutated to serine, dramatically impaired modulation by dzp. The Y209S substitution resulted in a >7-fold increase in the EC50 for dzp, and the Y159S substitution nearly abolished dzp-mediated potentiation. Both of these mutations abolished binding of the high affinity BZ receptor antagonist [3H]Ro 15–1788 to GABA A receptors expressed in HEK 293 cells. These tyrosine residues correspond to two tyrosines of the β2 subunit (Tyr157 and Tyr205) previously postulated to form part of the GABA-binding site. Mutation of the corresponding tyrosine residues on the γ2 subunit produced only a slight increase in the EC50 for dzp (~2-fold) with no significant effect on the binding affinity of [3H]Ro 15–1788. These data suggest that Tyr159 and Tyr209 of the α1 subunit may be components of the BZ-binding site on α1β2γ2 GABA A receptors.

BZs are frequently prescribed as anxiolytics, sedatives, anticonvulsants, and muscle relaxants (1–3). It is generally accepted that these compounds exert their therapeutic effects in the mammalian brain (2–8). Thus, a substantial effort has been directed at understanding the molecular mechanism by which BZs modulate GABA receptor function (9–12).

Molecular cloning studies (13–15) have revealed multiple subunits that contribute a vast number of pharmacologically distinct GABA A receptors. Site-directed mutagenesis studies have identified a threonine residue at position 101 (22) and a glycine residue at position 200 (21) have been implicated in BZ binding to the GABA receptor complex (Fig. 1).

Although the α subunit seems to form part of the BZ-binding site, the presence of a γ subunit is essential for the normal modulatory actions of BZs on GABA A receptors (19, 24, 25; although see Ref. 26). The γ subunit is photoaffinity labeled by [3H]flunitrazepam (27), suggesting that it may also contribute part of the BZ-binding site. Site-directed mutagenesis studies have identified a threonine residue at position 142 of the human γ2 subunit (Fig. 1) implicated in the efficacy of BZ ligands (28).

We previously identified two tyrosines at position 157 and 205 of the β2 subunit (Fig. 1) that when mutated, dramatically impaired GABA-mediated activation of the α1β2γ2 GABA A receptor/pore complex (29). These two tyrosine residues are conserved in all α, β, and γ subunit isoforms. Mutation of the homologous tyrosines in the α1 or γ2 subunits did not alter GABA-dependent activation of the α1β2γ2 GABA A receptor (29). Here, we demonstrate that mutagenec-
sis of these two tyrosines in the α1 (α1Y159S and α1Y209S) subunit, but not in the γ2 subunit (γ2Y172S and γ2Y220S), has profound effects on BZ binding and modulation of GABA-activated currents, suggesting that these amino acids may be components of the BZ-binding site.

Materials and Methods

Site-directed mutagenesis and in vitro transcription. Rat α1, β2, and γ2 cDNAs were cloned into the pSELECT vector (Promega, Madison, WI), and oligonucleotide-mediated site-directed mutagenesis was achieved with the Altered Sites Kit (Promega) as previously described (30). Successful mutagenesis was verified by sequencing.

cDNAs were linearized with SspI, which leaves a several-hundred-base pair tail that may increase cRNA stability in the oocyte. cRNA was transcribed from the linearized cDNAs through the use of standard in vitro transcription procedures or the Megascript Kit (Ambion, Austin TX). Integrity and yield of the cRNA were verified on a 1% formaldehyde-containing agarose gel. The test solution containing GABA (3 m M) or GABA plus dzp. The vehicles.

Data analysis. The fractional potentiation (FP) was calculated for each dzp concentration as follows:

\[
FP = \frac{I_{\text{dzp}} - I_{\text{control}}}{I_{\text{control}}}
\]  

where \(I_{\text{dzp}}\) is the amplitude of the GABA-activated current in the presence of dzp, and \(I_{\text{control}}\) is the amplitude of the GABA-activated current in the absence of dzp. Thus, a fractional potentiation of 1.0 represents a 2-fold increase over the control current amplitude. To quantify dzp sensitivity, the dzp dose-potentiation relationship was fit with the following Hill equation using a nonlinear least-squares method:

\[
FP = \frac{FP_{\text{max}}}{1 + (EC_{50}/[\text{dzp}])^n}
\]  

where FP is the fractional potentiation as defined by eq. 1, \(FP_{\text{max}}\) is the maximal fractional potentiation, \(EC_{50}\) is the concentration of dzp yielding a half-maximal enhancement of the GABA-activated current, and \(n_H\) is the Hill coefficient.

Transfection of mammalian cells. Cloned cDNAs encoding the rat wild-type or mutated subunits were subcloned into the polylinker site of appropriate expression vectors by standard recombinant DNA techniques (wild-type α1, β2, and γ2; pCDM8, pRK5, and pRc/CMV, respectively; mutant subunits: pRK7). Expression plasmid DNA was prepared by CsCl gradient centrifugation. HEK 293 cells were transfected using calcium phosphate precipitation with the combinations of plasmid DNAs (20 μg/10-cm plate) indicated in the text. After 48 hr, cells were harvested, pelleted by centrifugation at 4000 × g, and frozen at −70°C.

Binding assay. Cell membrane pellets were washed three times by homogenization in 20 volumes of ice-cold buffer (10 mM potassium phosphate, pH 7.2), centrifuged, and then homogenized in a mixture of 10 mM potassium phosphate and 100 mM potassium chloride, pH 7.2. Incubations contained 200-μl aliquots of membrane suspension; 25 μl of [3H]Ro 15–1788 (83.7 Ci/mmol; New England Nuclear Research Products, Boston, MA) or [3H]muscimol (16 Ci/mmol; Amer sham, Arlington Heights, IL). [3H]Ro 15–1788 was used at concen-
trations of 0.1–10 nm, and [3H]muscimol was used at concentrations of 1.5–50 nm. After incubation for 60 min at 4°, the membranes were collected by rapid filtration on Whatman GF/C filters and immediately washed two times with 5 ml of ice-cold buffer (10 mM potassium phosphate, 100 mM potassium chloride, pH 7.2). Radioactivity was measured by liquid scintillation spectroscopy. Specific binding was defined as the difference between total binding and nonspecific binding in the presence of clonazepam or GABA. Protein concentrations were determined using the BCA Protein Assay (Pierce Chemical, Rockford, IL).

Results

The dzp-mediated modulation of wild-type α1β2γ2 receptors. cRNA encoding rat wild-type α1, β2, and γ2 subunits were coinjected into X. laevis oocytes, and 1–2 days later, GABA-activated currents were examined using the two-electrode voltage-clamp technique. The traces in Fig. 2A are GABA-activated currents (3 μM GABA) from oocytes expressing wild-type α1β2γ2 GABA<sub>a</sub> receptors in the absence or presence of increasing concentrations of dzp. The dzp produced a concentration-dependent enhancement in the amplitude of the GABA-evoked currents. The fractional potentiation of the current is plotted as a function of dzp concentration in Fig. 2B. Note that the dose-response relationship for dzp-mediated modulation has three components (also obvious in the current traces of Fig. 2A); a potentiation that seems to plateau at around 1 μM, a depression apparent at 1-20 μM dzp, and a further potentiation at dzp concentrations of >20 μM.

The Hill equation was fit to the data points of ≤1 μM dzp, where the potentiation seemed to plateau. This fit (extrapolated as a dashed line) yielded an EC<sub>50</sub> value of 64.8 ± 3.7 nm, a Hill coefficient of 1.16 ± 0.04, and a fractional potentiation of 2.57 ± 0.02 (Table 1). Although this potentiation seemed to plateau around 1 μM dzp, this may be in part due to the depression that is evident in this concentration range. Thus, the EC<sub>50</sub> and fractional potentiation may be underestimated.

Fig. 2. The dzp-mediated potentiation of recombinant α1β2γ2 GABA<sub>a</sub> receptors. A, GABA-activated currents (3 μM GABA) in the absence and presence of increasing concentrations of dzp (indicated above the traces). B, Plot of the fractional potentiation as a function of dzp concentration. These data represent the mean ± standard error values for 24 oocytes. Note that there is a potentiation at ≤1 μM dzp, a depression obvious at 1-20 μM dzp, and a further potentiation at >20 μM dzp. Statistical comparison between the 1 and 5 μM data point demonstrated that the depression was statistically significant (p = 0.0017). Continuous line (extrapolated as a dashed line), from the best fit of the Hill equation for the means up to 1 μM dzp (see Discussion). This yielded an EC<sub>50</sub> value for dzp potentiation of 66 nm, a Hill coefficient of 1.03, and a maximal fractional potentiation of 2.6. The mean ± standard error values for the fits of the Hill equation to the data for each oocyte are presented in Table 1.

Mutations in conserved domains of the α1 subunit. The tyrosine at position 159 of the α1 subunit (Fig. 1) was mutated to serine (α1Y159S) and coexpressed with wild-type β2 and γ2 subunits. The traces in Fig. 3A are GABA-activated currents (3 μM GABA) in the absence or presence of increasing concentrations of dzp for the α1Y159Sβ2γ2 receptor. Note the dramatic decrease in potentiation at lower dzp concentrations compared with that of the wild-type receptor (Fig. 2A). This mutation did not impair activation by GABA (α1β2γ2: EC<sub>50</sub> = 45.8 ± 3.6 μM, Hill coefficient = 1.57 ± 0.09, I<sub>max</sub> = 381 ± 508 nA; α1Y159Sβ2γ2: EC<sub>50</sub> = 44.9 ± 4.5 μM, Hill coefficient = 1.62 ± 0.18, I<sub>max</sub> = 586 ± 405 nA; see Ref. 29). Fig. 3B plots the potentiation of GABA-activated currents for α1Y159Sβ2γ2 (open symbols) as a function of dzp concentration. For comparison, the potentiation of the wild-type α1β2γ2 receptor is also plotted (filled symbols). The α1Y159S substitution nearly abolished the dzp-mediated potentiation at <1 μM, and therefore the Hill equation could not be reliably fitted to these data points. In contrast, the potentiation at >20 μM greatly exceeded that of the wild-type receptor. One possible interpretation of the increased potentiation at high dzp concentrations is that the α1Y159S mutation impaired dzp sensitivity of the lower component, thereby shifting it to the right. Thus, this lower component might now overlap with the upper component, yielding the increased potentiation at high dzp concentrations (fractional potentiation of 7.5 compared with 4.4). Based on these data, however, we cannot rule out the possibility that the α1Y159S mutation enhanced the efficacy of the actions of dzp at these higher concentrations.

The second homologous tyrosine, at position 209 of the α1 subunit (Fig. 1), was mutated to serine, and the resulting α1Y209S was coexpressed with wild-type β2 and γ2 subunits. Similar to the tyrosine at position 159, mutation of the tyrosine at position 209 decreased the potentiation by dzp compared with that of the wild-type receptor. This mutation did not affect the EC<sub>50</sub> or I<sub>max</sub> values for GABA-mediated
activation (α1β2γ2: EC\textsubscript{50} = 45.8 ± 3.6 μM, \(I_{\text{max}}\) = 381 ± 508 nA; α1Y209Sβ2γ2: EC\textsubscript{50} = 38.2 ± 12.2 μM, \(I_{\text{max}}\) = 627 ± 379; see Ref. 29), although there was a slight but significant (\(p = 0.021\)) decrease in the Hill coefficient (α1β2γ2: Hill coefficient = 1.57 ± 0.09; α1Y209Sβ2γ2: Hill coefficient = 1.38 ± 0.10; see Ref. 29). Fig. 3C plots the potentiation of GABA-activated currents (3 μM GABA) for α1Y209Sβ2γ2 (open symbols) as a function of dzp concentration. Fitting a Hill equation to the data points at ≤10 μM diazepam yielded an EC\textsubscript{50} value of 463 ± 51.2 nM, a Hill coefficient of 1.03 ± 0.08, and a fractional potentiation of 1.54 ± 0.11 (Table 1). Thus, in comparison with the wild-type receptor, substitution of the tyrosine at position 209 imparted a 7.1-fold increase in the EC\textsubscript{50} value for dzp and a 1.7-fold reduction in the maximal potentiation.

Impaired dzp sensitivity is not due to the absence of the α subunit. Evidence suggests the α subunit contributes a major component of the BZ-binding site (21, 22, 31). Thus, we considered the possibility that the tyrosine substitutions impair the assembly of the mutant α subunit, resulting in a preponderance of β2γ2 GABA\textsubscript{γ} receptors that are less affected by dzp. It has previously been shown that β2γ2 GABA\textsubscript{α} receptors are dzp sensitive (32–34), and Fig. 4 demonstrates that the dzp sensitivity of β2γ2 GABA receptors is similar to that of α1β2γ2 GABA receptors (parameters provided in Table 1). These data suggest that the impairment of dzp-mediated modulation with the α1Y159S and α1Y209S substitutions (Figs. 2 and 3) cannot be accounted for by a mutation-induced impairment in the assembly of the α subunit.

More conservative substitutions at positions 159 and 209. To gain insight into the structural requirements at positions 159 and 209, more conservative substitutions with respect to the amino acid size and aromatic ring were introduced at these positions (i.e., α1Y159F and α1Y209F). Fitting the Hill equation to dose-response relationships (not shown) from the α1Y159Fβ2γ2 receptors (≤1 μM dzp) yielded an EC\textsubscript{50} value of 118.2 ± 39.4 nM, a Hill coefficient of 1.09 ± 0.06, and a fractional potentiation of 2.3 ± 0.03 (Table 1). Fitting the Hill equation to dose-response relationships (not shown) from the α1Y209Fβ2γ2 receptors (≤1 μM dzp) yielded an EC\textsubscript{50} value of 140.9 ± 3.2 nM, a Hill coefficient of 1.31 ± 0.02, and a fractional potentiation of 2.26 ± 0.24 (Table 1). Thus, in comparison with the serine substitution, the more conservative phenylalanine substitution at these two positions produced a moderate rightward shift in the dose-response relationship for dzp.

**Mutation of other tyrosines in the vicinity of α1Tyr159.** To test the relative importance of these two conservative tyrosines of the α1 subunit in dzp-mediated potentiation of the GABA-activated currents, we mutated other tyrosine residues in the vicinity of α1Tyr159 (positions 161 and 168). Substitution of the tyrosine at position 161 (α1Y161S) with serine (just two amino acids away from the crucial α1Tyr159) had no effect on the EC\textsubscript{50} value for dzp (Table 1), although there was a slight decrease in the maximal potentiation of the initial component. Similar to α1Y161S, substitution of the tyrosine at position 168 (α1Y168S) did not alter the EC\textsubscript{50} value for dzp (Table 1). The α1Y168S mutation also induced a slight depression in the maximal potentiation at low dzp concentrations.

**Mutation of a conserved threonine at position 162.** Previous studies have shown that the threonine at position 160 of the β2 subunit plays a crucial role in GABA-mediated activation (29). We mutated the homologous threonine in the α1 subunit (T162A) to investigate its potential role in dzp-mediated modulation of the GABA current. α1T162Aβ2γ2 mutant receptors demonstrated a similar sensitivity to dzp as that of the wild-type receptor (Table 1).

**Mutations in conserved domains of the γ2 subunit.** The α and β subunit tyrosines crucial for dzp-dependent potentiation (Figs. 2 and 3) and GABA-mediated activation (29) of the GABA\textsubscript{γ} receptor, respectively, are also conserved in the γ2 subunit (Fig. 1; γ2Tyr172 and γ2Tyr220). Because the γ2 subunit is essential for the modulatory effects of BZs (19), we examined the potential role of these two γ2 subunit tyrosines in the actions of dzp.

Fig. 5, A and B, shows plots of the dose-response relationships for dzp-mediated potentiation of α1β2γ2Y172S and
A. α1Y159Sβ2γ2

![Graph of α1Y159Sβ2γ2](image)

B. α1β2γ2 α1Y159Sβ2γ2

![Graph of α1β2γ2 and α1Y159Sβ2γ2](image)

C. α1β2γ2 α1Y209Sβ2γ2

![Graph of α1β2γ2 and α1Y209Sβ2γ2](image)

Fig. 3. The dzp-mediated potentiation of recombinant α1β2γ2, α1Y159Sβ2γ2, and α1Y209Sβ2γ2 GABAA receptors. A. GABA-activated currents (3 μM GABA) from oocytes expressing α1Y159Sβ2γ2 GABA receptors in the absence and presence of increasing concentrations of dzp (indicated above the traces). In comparison with the wild-type receptor, the potentiation at < 1 μM dzp was greatly diminished. The potentiation at > 20 μM, however, was enhanced compared with the wild-type receptor, possibly due to a rightward shift in the more dzp-sensitive component so that it now overlaps with the upper component. B. Plot of the fractional potentiation as a function of dzp concentration for α1Y159Sβ2γ2 GABAA receptors (○). These data represent the mean ± standard error values for 14 oocytes. The Hill equation could not be reliably fit to the initial component of the dzp dose-potentiation relationship. The wild-type data have been replotted for comparison (●). C. Plot of the fractional potentiation as a function of dzp concentration for α1Y209Sβ2γ2 GABAA receptors (○). These data represent the mean ± standard error values for 11 oocytes. Continuous line (extrapolated as a dashed line), from the best fit of the Hill equation to the open symbols (see Materials and Methods) for the mean values at ≤10 μM dzp. This yielded an EC50 value for dzp potentiation of 412 nM, a Hill coefficient of 1.03, and a maximal fractional potentiation of 1.4. The mean ± standard error values for the fits of the Hill equation to the data from each oocyte are presented in Table 1.

α1β2γ2Y220S (shaded circles) GABAA receptors, respectively. Both substitutions produced a ~2-fold increase in the EC50 value of the initial component for dzp (i.e., 118.5 ± 12.0 and 129.7 ± 5.3 nM for Y172S and Y220S, respectively). These are moderate shifts in comparison with those observed with mutation of the corresponding tyrosines of the α1 subunit. These two substitutions did not affect the sensitivity to activation by GABA (α1β2γ2: EC50 = 45.8 ± 3.6 μM, Hill coefficient = 1.57 ± 0.09, Imax = 381 ± 508 nA; α1β2γ2Y172S: EC50 = 40.4 ± 5.0 μM, Hill coefficient = 1.49 ± 0.14, Imax = 453 ± 492 nA; α1β2γ2Y220S: EC50 = 38.4 ± 6.2 μM, Hill coefficient = 1.43 ± 0.10, Imax = 495 ± 514 nA; see Ref. 29).

We considered the possibility that in the absence of the α1 subunit, the γ2 subunit can assume the role of the α1 subunit role in dzp sensitivity. Because the crucial tyrosines are conserved in the γ2 subunit, we coexpressed the β2 subunit along with these mutant γ2 subunits (γ2Y172S and γ2Y220S) to test their potential role in dzp-mediated potentiation of the β2γ2 receptor. Fig. 6 shows the wild-type β2γ2 dose-response relationship for dzp-mediated potentiation of the GABA-activated current (already presented in Fig. 4). The potentiation of GABA-activated currents from β2γ2Y172S (○) and β2γ2Y220S (□) by 10, 100, and 1000 nM dzp is also plotted. These nonconservative substitutions did not impair dzp sensitivity, suggesting that in the absence of the α1 subunit, these conserved γ2 subunit tyrosines do not assume the same role as their α1 subunit counterparts.

Effects of tyrosine mutations on BZ binding. The observed impairment of the sensitivity of the GABAA receptor to dzp imparted by the α1Y159S and α1Y209S mutations (Figs. 1 and 2) could be accounted for by two mechanisms: (a) impairment of dzp binding or (b) impairment of the coupling of dzp binding to receptor/channel modulation. In an effort to distinguish between these two possibilities, we compared the binding of the high affinity BZ antagonist Ro 15–1788 to wild-type and mutant receptors. Fig. 7 (●) is a representative Scatchard plot of [3H]Ro 15–1788 binding to a membrane preparation from HEK 293 cells expressing α1β2γ2 GABAA receptors. [3H]Ro 15–1788 bound to these receptors with a
dissociation constant ($K_d$) of $0.98 \pm 0.21 \text{ nM}$ (Table 2), which is in agreement with previously published reports (27). Substitution of either of the two crucial tyrosines in the $\alpha$ subunit with serine eliminated specific binding of $^{3\text{H}}$Ro 15–1788 to the receptor. Mismatch binding to these mutant receptors was similar to that of the wild-type receptor (Table 2). $^{3\text{H}}$Ro 15–1788 binding to transfected receptors containing the more conservative substitution, $\alpha 1Y209F$, was also examined. A representative Scatchard analysis is also presented in Fig. 7 (○). The $K_d$ value for $\alpha 1Y209F\beta 2\gamma 2$ was $4.07 \pm 0.38 \text{ nM}$ (Table 2), which represents a 4-fold decrease in affinity compared with the wild-type receptor ($p = 0.0002$). Receptors containing substitutions of the corresponding tyrosines in the $\gamma 2$ subunit ($\alpha 1\beta 2\gamma 2Y172S$, $\alpha 1\beta 2\gamma 2Y220F$) had $^{3\text{H}}$Ro 15–1788 binding that was not significantly different from the wild-type receptor (Fig. 8, Table 2).

**Discussion**

**Actions of dzp on wild-type $\alpha 1\beta 2\gamma 2$ and $\beta 2\gamma 2$ GABA_{A} receptors.** We examined the potentiation of GABA-activated currents in $\alpha 1\beta 2\gamma 2$ GABA_{A} receptors by dzp at concentrations ranging from 5 nM to 200 $\mu$M. Three apparent components were consistently observed in these dzp dose-potentiation relationships: (a) a fractional potentiation of 2.6 in the GABA-activated current that appeared to saturate around 1 $\mu$M and demonstrated an apparent EC_{50} value of 65 nM, (b) a slight depression evident at 1- 20 $\mu$M dzp, and (c) a further potentiation that > 20 $\mu$M dzp that imparted an additional 1.9-fold increase (with 200 $\mu$M diazepam) in the GABA-activated current over that seen at lower dzp concentrations. We examined the higher dzp concentration range based on the expectation that BZ-binding site mutants might induce rightward shifts in the dose-potentiation relationships for dzp.

The EC_{50} value and fractional potentiation that we ob-
activated currents in rat cortical neurons demonstrate an EC₅₀ range (300 nM to 10 μM) examined potentiation at a relatively high dzp concentration 4.5-fold increase in the amplitude (37), although that study presented in Table 2.

Scatchard analysis of the individual membrane preparations are presented in Table 2. Dissociation constants (Kₐ) were determined for the ligands [³H]Ro 15-1788 and [³H]muscimol on membrane preparations isolated from transfected HEK 293 cells. Bₘₐₓ values for Ro15-1788 and muscimol were not significantly different between wild-type and mutant receptors, when binding was seen. Values are mean ± standard error.

<table>
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<th>Combination</th>
<th>[³H]Ro 15-1788</th>
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<th>[³H]Muscimol</th>
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<tr>
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<td>Bₘₐₓ</td>
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N.D., not determined.

Fig. 8. Substitution of the tyrosines at position 172 or 220 of the γ2 subunit produces no significant change in BZ binding affinity. Representative Scatchard analysis showing binding affinity of the BZ antagonist [³H]Ro 15-1788 to membrane preparations from HEK 293 cells transfected with DNA encoding either α1β2γ2Y172S (■) or α1β2γ2Y220F (○) GABA receptors. The wild-type data are replotted for comparison (●). The mean Kₐ value is 0.80 ± 0.12 nM for the wild-type receptor, 0.73 ± 0.03 for α1β2γ2Y172S, and 0.96 ± 0.10 nM for α1β2γ2Y220F, as shown in Table 2. Parameters determined from a Scatchard analysis of the individual membrane preparations are presented in Table 2.

Binding site on the GABAₐ receptor complex. Micromolar-affinity BZ-binding sites have been reported in the mammalian central nervous system (38, 39).

The observation that β2γ2 GABA receptors show a similar dzp sensitivity as α1β2γ2 GABA receptors is intriguing given that a significant component of the BZ-binding site is presumed to be on the α subunit (16–23). One possibility is that the β2 or γ2 subunit could substitute for the absence of the α subunit in the actions of dzp. The α1 subunit tyrosine residues we identified in this study are conserved in both the β2 and γ2 subunits. The role of the β2 tyrosines (β2Tyr157 and β2Tyr205) would be difficult to assess because substitution of either of these tyrosines with serine nearly abolishes GABA-mediated activation (29). We tested the potential role of the γ2 tyrosines in the actions of dzp on β2γ2 GABA receptors. Mutation of either of these tyrosines to serine (γ2Y172S and γ2Y220S) did not impair dzp sensitivity, indicating homologous regions of the γ2 subunit do not substitute for the α subunit. Other possibilities are that the β2 subunit substitutes for the α1 subunit or other regions of the γ2 subunit (not γ2Tyr172 or γ2Tyr220) are involved in the actions of dzp. A third possibility is that a subunit endogenous to the oocyte is substituting for the α1 subunit and imparting dzp sensitivity on the expressed GABA receptors.

α1Tyr159 and α1Tyr209 may form part of the BZ-binding site. Structure-function studies of ligand-receptor interactions have typically revealed that binding sites are formed by contributions from several disparate regions of a subunit, as well as domains from neighboring subunits. Thus, the previously identified residues of the α1 subunit (21, 22) may contribute only part of the binding site. In this study, we identified two residues on the α1 subunit and γ2 subunits (positions 159 and 209) that seem to be crucial for the actions of dzp. The α1 subunit tyrosine residues we identified in this study are conserved in both the β2 and γ2 subunits. The role of the β2 tyrosines (β2Tyr157 and β2Tyr205) would be difficult to assess because substitution of either of these tyrosines with serine nearly abolishes GABA-mediated activation (29). We tested the potential role of the γ2 tyrosines in the actions of dzp on β2γ2 GABA receptors. Mutation of either of these tyrosines to serine (γ2Y172S and γ2Y220S) did not impair dzp sensitivity, indicating homologous regions of the γ2 subunit do not substitute for the α1 subunit. Other possibilities are that the β2 subunit substitutes for the α1 subunit or other regions of the γ2 subunit (not γ2Tyr172 or γ2Tyr220) are involved in the actions of dzp. A third possibility is that a subunit endogenous to the oocyte is substituting for the α1 subunit and imparting dzp sensitivity on the expressed GABA receptors.

α1Tyr159 and α1Tyr209 may form part of the BZ-binding site. Structure-function studies of ligand-receptor interactions have typically revealed that binding sites are formed by contributions from several disparate regions of a subunit, as well as domains from neighboring subunits. Thus, the previously identified residues of the α1 subunit (21, 22) may contribute only part of the binding site. In this study, we identified two residues on the α1 subunit and γ2 subunits (positions 159 and 209) that seem to be crucial for the actions of BZs on GABA receptors. The mutation Y159S nearly eliminated the potentiation seen at low dzp concentrations, whereas Y209S shifted the dzp EC₅₀ value and reduced the maximal potentiation. The more conservative substitution of these tyrosines with serine nearly abolishes GABA-mediated activation (29). We tested the potential role of the γ2 tyrosines in the actions of dzp on β2γ2 GABA receptors. Mutation of either of these tyrosines to serine (γ2Y172S and γ2Y220S) did not impair dzp sensitivity, indicating homologous regions of the γ2 subunit do not substitute for the α1 subunit. Other possibilities are that the β2 subunit substitutes for the α1 subunit or other regions of the γ2 subunit (not γ2Tyr172 or γ2Tyr220) are involved in the actions of dzp. A third possibility is that a subunit endogenous to the oocyte is substituting for the α1 subunit and imparting dzp sensitivity on the expressed GABA receptors.

binding site on the GABAₐ receptor complex. Micromolar-affinity BZ-binding sites have been reported in the mammalian central nervous system (38, 39).

The observation that β2γ2 GABA receptors show a similar dzp sensitivity as α1β2γ2 GABA receptors is intriguing given that a significant component of the BZ-binding site is presumed to be on the α subunit (16–23). One possibility is that the β2 or γ2 subunit could substitute for the absence of the α subunit in the actions of dzp. The α1 subunit tyrosine residues we identified in this study are conserved in both the β2 and γ2 subunits. The role of the β2 tyrosines (β2Tyr157 and β2Tyr205) would be difficult to assess because substitution of either of these tyrosines with serine nearly abolishes GABA-mediated activation (29). We tested the potential role of the γ2 tyrosines in the actions of dzp on β2γ2 GABA receptors. Mutation of either of these tyrosines to serine (γ2Y172S and γ2Y220S) did not impair dzp sensitivity, indicating homologous regions of the γ2 subunit do not substitute for the α1 subunit. Other possibilities are that the β2 subunit substitutes for the α1 subunit or other regions of the γ2 subunit (not γ2Tyr172 or γ2Tyr220) are involved in the actions of dzp. A third possibility is that a subunit endogenous to the oocyte is substituting for the α1 subunit and imparting dzp sensitivity on the expressed GABA receptors.

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15–1788. Although caution must be exercised in interpreting binding studies under these conditions (40), the simplest interpretation is that mutation of the tyrosine residues impaired binding of dzp to the BZ receptor. Thus, α1Tyr159 and α1Tyr209 may be components of the BZ-binding site/pocket itself.

The correlation of the two tyrosine residues of the γ2 subunit produced a ∼2-fold increase in the EC_{50} (Table 1) with no significant change in the binding affinity of [3H]Ro 15–1788 (Table 2). Thus, the γ2 subunit mutations may impair dzp-mediated potentiation at steps subsequent to BZ binding. Nevertheless, such slight shifts for nonconservative substitutions suggest that these two residues are not key determinants in the actions of BZs on GABA_{A} receptors.

Although there was a consistency in the effects of the mutations on the EC_{50} values (Table 1) and K_{d} values (Table 2), one cannot directly compare these parameters because different ligands were used in the binding and electrophysiological studies. Understanding the correlation between the K_{d} and the EC_{50}/fractional potentiation, however, must await further understanding of the relation between the observed affinity and efficacy of a ligand.

Other studies. A putative model of the BZ-binding site is beginning to emerge from structure-function studies of the GABA_{A} receptor. In this model, several disparate domains of the α subunit contribute components of the BZ-binding site (Fig. 1). The histidine at position 101 is photoaffinity labeled by BZ-site ligands (23) and, when mutated to arginine, eliminates dzp binding (22) and dzp-mediated potentiation (41). A separate region associated with the glycine residue at position 200 of the α subunit (21) seems to be important for BZ affinity. More recently, Buhr et al. (31) observed that substitution of an alanine at the threonine position at 202 or tyrosine at position 161 of the α1 subunit enhanced the maximal potentiation by dzp. In the current study, we replaced this tyrosine at position 161 and did not observe an effect on dzp-dependent potentiation of the GABA-induced currents (see Table 1), suggesting a more distal role for α1Tyr202 in comparison with αHis100, α1Tyr159, and α1Gly200 in dzp-dependent modulation of GABA-induced currents.

In summary, three domains of the α1 subunit in the vicinity of His101, Tyr159, and Gly200 seem to be associated with the BZ-binding site. In addition, mutation of the threonine at position 142 (28) or the phenylalanine at position 77 (31) of the γ2 subunit alters BZ efficacy (Fig. 1), suggesting the BZ-binding site may be at the α/γ subunit interface (23).

Of the domains implicated in BZ binding, in the vicinity of α1Tyr159 and α1Gly200, are homologous to domains of the β subunit implicated as components of the GABA binding site (Fig. 1). Furthermore, the two tyrosines at positions 159 and 209 that we have identified in the α1 subunit crucial for BZ binding are homologous to tyrosine residues in the β2 subunit that seem to play a key role in the binding of GABA.

It has been suggested that the homology observed between the two ligand-binding segments for GABA and Bzs may have arisen from gene duplication resulting in a modified agonist site that now functions as an allosteric modulatory site in the GABA_{A} receptor (23). Given the dramatic differences in the molecular structures of dzp and GABA, however, a significant correspondence in the structures of their respective binding sites would not necessarily be expected. Another intriguing possibility is that Bzs increase the sensitivity of the GABA receptor by uncovering an additional GABA-binding site(s). An increase in the number of binding sites with no change in the number of binding events required to open the pore could increase the GABA sensitivity without increasing the Hill coefficient (42). In this scenario, the Y159S and Y209S substitutions reported here impair dzp-mediated potentiation by impairing the binding of GABA to this site, and the observed elimination of [3H]Ro 15–1788 binding would be an indirect consequence of the strict coupling between the GABA and BZ binding domains. A comparison of the actions of dzp on the kinetics of single wild-type and mutant GABA_{A} receptors may help to distinguish these two possible mechanisms.

References


Benzodiazepine Binding Site on GABA<sub>A</sub> Receptors

Potentiation of γ-aminobutyric acid-induced chloride currents by various benzodiazepine site agonists with the α1γ2, β2γ2, α1β2γ2 subtypes of cloned γ-aminobutyric acid type A receptors.


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