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# Two Tyrosine Residues on the $\alpha$ Subunit Are Crucial for Benzodiazepine Binding and Allosteric Modulation of $\gamma$-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 $\gamma$-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 $\alpha1\beta2\gamma2$ GABA$_A$ receptors. Site-directed mutagenesis was used to introduce point mutations into the $\alpha1$ and $\gamma2$ 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 $\alpha1$ 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 EC$_{50}$ for dzp, and the Y159S substitution nearly abolished dzp-mediated potentiation. Both of these mutations abolished binding of the high affinity BZ receptor antagonist [$^3$H]Ro 15–1788 to GABA$_A$ receptors expressed in HEK 293 cells. These tyrosine residues correspond to two tyrosines of the $\beta2$ subunit (Tyr157 and Tyr205) previously postulated to form part of the GABA-binding site. Mutation of the corresponding tyrosine residues on the $\gamma2$ subunit produced only a slight increase in the EC$_{50}$ for dzp (~2-fold) with no significant effect on the binding affinity of [$^3$H]Ro 15–1788. These data suggest that Tyr159 and Tyr209 of the $\alpha1$ subunit may be components of the BZ-binding site on $\alpha1\beta2\gamma2$ GABA$_A$ receptors.

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

Molecular cloning studies (13–15) have revealed multiple classes and isoforms of GABA$_A$ receptor subunits in the mammalian brain ($\alpha1$–6, $\beta1$–4, $\gamma1$–3, $\delta$). This diversity of $\alpha$, $\beta$, and $\gamma$ subunits allows the expression of a vast number of structurally unique GABA$_A$ receptor subtypes with distinct pharmacologies. Studies using exogenous expression, photoaffinity labeling, chimeric subunits, and site-directed mutagenesis have indicated that the $\alpha$ subunit contributes a major component of the BZ-binding site and, depending on the subtype, can confer either BZ1 or BZ2 pharmacology on the GABA$_A$ receptor (16–23). In particular, a histidine 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 $\alpha$ subunit seems to form part of the BZ-binding site, the presence of a $\gamma$ subunit is essential for the normal modulatory actions of BZs on GABA$_A$ receptors (19, 24, 25; although see Ref. 26). The $\gamma$ subunit is photoaffinity labeled by [$^3$H]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 $\gamma2$ subunit (Fig. 1) implicated in the efficacy of BZ ligands (28).

We previously identified two tyrosines at position 157 and 205 of the $\beta2$ subunit (Fig. 1) that when mutated, dramatically impaired GABA-mediated activation of the $\alpha1\beta2\gamma2$ GABA$_A$ receptor/pore complex (29). These two tyrosine residues are conserved in all $\alpha$, $\beta$, and $\gamma$ subunit isoforms. Mutation of the homologous tyrosines in the $\alpha1$ or $\gamma2$ subunits did not alter GABA-dependent activation of the $\alpha1\beta2\gamma2$ GABA$_A$ receptor (29). Here, we demonstrate that mutagene-

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**ABBREVIATIONS:** BZ, benzodiazepine; dzp, diazepam; GABA, $\gamma$-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEK, human embryonic kidney.

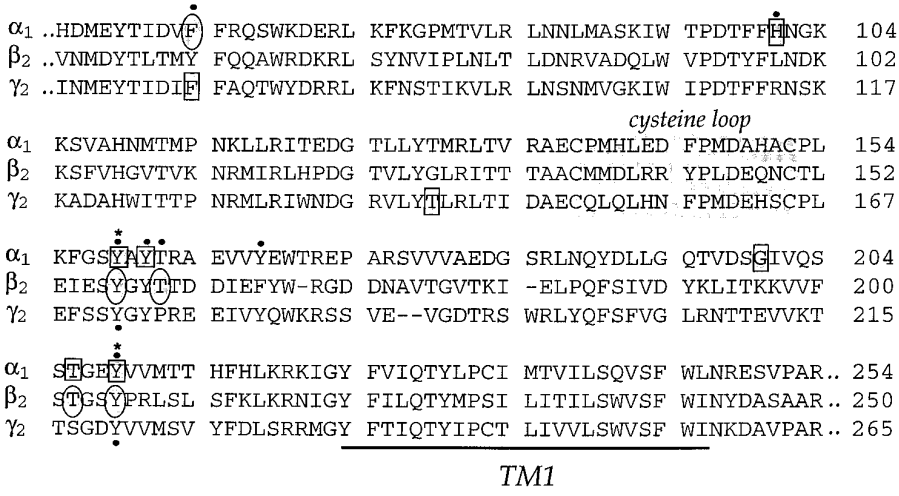


**Fig. 1.** Aligned amino acid sequences of the rat $GABA_A$ $\alpha$1, $\beta$2, and $\gamma$2 subunits. The sequences shown extend from residue 55 ($\alpha$ subunit numbering) to beyond the first putative membrane spanning domain (*TM1*). *Shaded 15-amino acid sequence*, highly conserved cysteine loop postulated to play a role in subunit assembly (30). *Boxed and shaded residues*, implicated in BZ-mediated modulation of the $GABA_A$ receptor. *Circled and shaded residues*, implicated in GABA-mediated activation. *Dot*, amino acids mutated. ∗, Crucial tyrosine residues.

sis of these two tyrosines in the $\alpha$1 ($\alpha$1Y159S and $\alpha$1Y209S) subunit, but not in the $\gamma$2 subunit ($\gamma$2Y172S and $\gamma$2Y220S), has profound effects on BZ binding and modulation of GABA-activated currents, suggesting these amino acids may be components of the BZ-binding site.

## Materials and Methods

**Site-directed mutagenesis and *in vitro* transcription.** Rat $\alpha$1, $\beta$2, and $\gamma$2 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 *Ssp*I, 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 agarose gel.

**Oocyte isolation and cRNA injection.** *Xenopus laevis* (Xenopus I, Ann Arbor, MI) were anesthetized by hypothermia, and oocytes were surgically removed from the frog and placed in a solution that consisted of 82.5 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM $Na_2HPO_4$, 50 units/ml penicillin, and 50 $\mu$g/ml streptomycin, pH 7.5. Oocytes were dispersed in this same solution minus $Ca^{2+}$ and plus 0.3% Collagenase A (Boehringer-Mannheim Biochemicals, Indianapolis IN). After isolation, the oocytes were thoroughly rinsed, and stage VI oocytes were separated and maintained overnight at 18°.

Micropipettes for injection of cRNA were pulled on a Sutter P87 horizontal puller, and the tips were cut off with microscissors. cRNAs for the desired subunit combinations were mixed (equimolar ratios), diluted 5–30-fold with diethylpyrocarbonate-treated water, and drawn up into the micropipette with negative pressure. The cRNA was injected into the oocytes by applying positive pressure using a Picospritzer II (General Valve Corporation, Fairfield, NJ).

To ensure that equal concentrations of cRNA per construct were injected, *in vitro* synthesized cRNA, at different set dilutions, were electrophoresed onto a 1% formaldehyde-containing agarose gel. The amount of cRNA was judged and matched by interpolation of lanes containing different dilutions of the corresponding cRNA.

**Recording.** At 1–3 days after injection, oocytes were placed onto a 300-$\mu$m nylon mesh suspended in a small volume chamber (<100 $\mu$l). The oocytes were continuously perfused and briefly switched to the test solution containing GABA (3 $\mu$M) or GABA plus dzp. The stock dzp (Sigma Chemical, St. Louis, MO) solution was made in PEG-300 or ethanol. No differences were observed between the two vehicles.

Recording microelectrodes were fabricated with a P87 Sutter horizontal puller and filled with 3 M KCl. The electrodes had resistances of 1–3 MΩ. Standard two-electrode voltage-clamp techniques were used to record currents in response to application of agonist. In all cases, the membrane potential was clamped to −70 mV. Data were played out on a Gould EasyGraf chart recorder during the experiment and recorded on tape for off-line analysis.

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

$$FP = \frac{I_{dzp} - I_{control}}{I_{control}} \quad (1)$$

where $I_{dzp}$ is the amplitude of the GABA-activated current in the presence of dzp, and $I_{control}$ 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:

$$FP = \frac{FP_{max}}{1 + (EC_{50}/[dzp])^{n_H}} \quad (2)$$

where FP is the fractional potentiation as defined by eq. 1, $FP_{max}$ 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 $\alpha$1, $\beta$2, and $\gamma$2: 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 $\mu$g/10-cm plate) indicated in the text. After 48 hr, cells were harvested, pelleted by centrifugation at 4000 × *g*, and frozen at −70°.

**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-$\mu$l aliquots of membrane suspension; 25 $\mu$l of [$^3$H]Ro 15–1788 (83.7 Ci/mmol; New England Nuclear Research Products, Boston, MA) or [$^3$H]muscimol (16 Ci/mmol; Amersham, Arlington Heights, IL). [$^3$H]Ro 15–1788 was used at concen-

trations of 0.1–10 nM, and [$^3$H]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 $\alpha1\beta2\gamma2$ receptors.** cRNA encoding rat wild-type $\alpha1$, $\beta2$, and $\gamma2$ 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 $\mu$M GABA) from oocytes expressing wild-type $\alpha1\beta2\gamma2$ $GABA_A$ receptors in the absence or presence of increasing concentrations of the BZ 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 around 1 $\mu$M, a depression apparent at 1- 20 $\mu$M dzp, and a further potentiation at dzp concentrations of $>$ 20 $\mu$M.

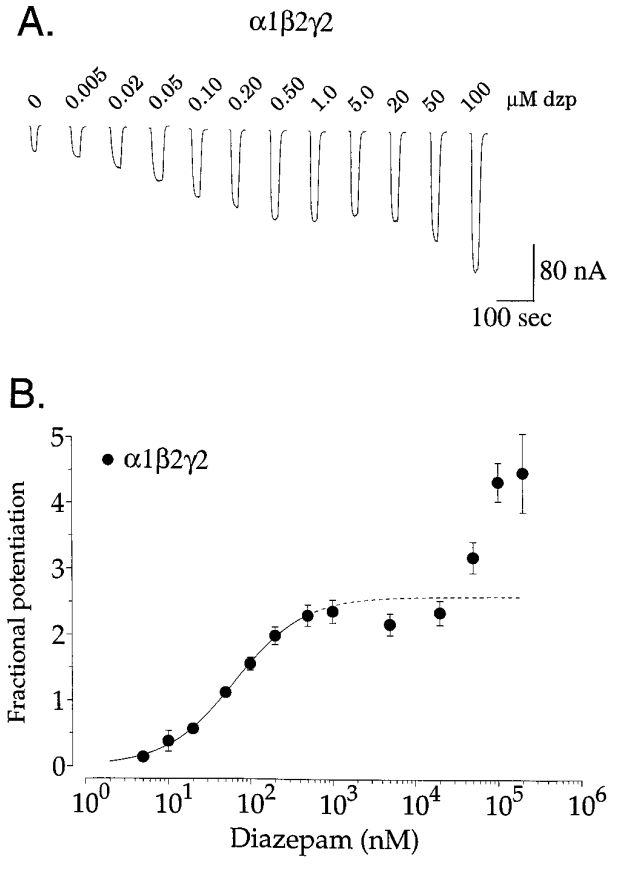


**Fig. 2.** The dzp-mediated potentiation of recombinant $\alpha1\beta2\gamma2$ $GABA_A$ receptors. A, GABA-activated currents (3 $\mu$M GABA) in the absence and presence of increasing concentrations of dzp (*indicated above the traces*). B, Plot of the fractional potentiation as a function of dzp concentration. These data represent the mean ± standard error values for 24 oocytes. Note that there is a potentiation at $\leq\sim1$ $\mu$M dzp, a depression obvious at 1- 20 $\mu$M dzp, and a further potentiation at $>$ 20 $\mu$M dzp. Statistical comparison between the 1 and 5 $\mu$M 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 $\mu$M dzp (see Discussion). This yielded an $EC_{50}$ 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.

The Hill equation was fit to the data points of $\leq$1 $\mu$M dzp, where the potentiation seemed to plateau. This fit (extrapolated as a *dashed line*) yielded an $EC_{50}$ 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 saturate around 1 $\mu$M dzp, this may be in part due to the depression that is evident in this concentration range. Thus, the $EC_{50}$ and fractional potentiation may be underestimated. The fractional potentiation at 200 $\mu$M dzp was 4.49 ± 0.60, which represents an additional 1.9-fold increase in the GABA-activated current above that seen at lower dzp concentrations. We examined these higher dzp concentrations of the wild-type receptor because mutations that impair the dzp sensitivity might shift the dose-response relationship to the right.

**Mutations in conserved domains of the $\alpha1$ subunit.** The tyrosine at position 159 of the $\alpha1$ subunit (Fig. 1) was mutated to serine ($\alpha1$Y159S) and coexpressed with wild-type $\beta2$ and $\gamma2$ subunits. The traces in Fig. 3A are GABA-activated currents (3 $\mu$M GABA) in the absence or presence of increasing concentrations of dzp for the $\alpha1$Y159S$\beta2\gamma2$ 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 ($\alpha1\beta2\gamma2$: $EC_{50}$ = 45.8 ± 3.6 $\mu$M, Hill coefficient = 1.57 ± 0.09, $I_{max}$ = 381 ± 508 nA; $\alpha1$Y159S$\beta2\gamma2$: $EC_{50}$ = 44.9 ± 4.5 $\mu$M, Hill coefficient = 1.62 ± 0.18, $I_{max}$ = 586 ± 405 nA; see Ref. 29). Fig. 3B plots the potentiation of GABA-activated currents for $\alpha1$Y159S$\beta2\gamma2$ (*open symbols*) as a function of dzp concentration. For comparison, the potentiation of the wild-type $\alpha1\beta2\gamma2$ receptor is also plotted (*filled symbols*). The $\alpha1$Y159S substitution nearly abolished the dzp-mediated potentiation at $<$ 1 $\mu$M, and therefore the Hill equation could not be reliably fitted to these data points. In contrast, the potentiation at $>$ 20 $\mu$M greatly exceeded that of the wild-type receptor. One possible interpretation of the increased potentiation at high dzp concentrations is that the $\alpha1$Y159S 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 $\alpha1$Y159S mutation enhanced the efficacy of the actions of dzp at these higher concentrations.

The second homologous tyrosine, at position 209 of the $\alpha1$ subunit (Fig. 1), was mutated to serine, and the resulting $\alpha_1$Y209S was coexpressed with wild-type $\beta2$ and $\gamma2$ 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 $EC_{50}$ or $I_{max}$ values for GABA-mediated

TABLE 1

**The dzp-mediated modulation of wild-type and mutant $GABA_A$ receptors**

Parameters were determined by fitting the Hill equation to the dose-response relationship for dzp at ≤1 μM except for the α1Y209S mutant, which was fit to ≤10 μM dzp. In all cases, the fractional potentiation was examined in the presence of 3 μM GABA. Values are mean ± standard error.

| Combination | Fractional potentiation | $EC_{50}$ | Hill coefficient | *n* |
|---|---|---|---|---|
| | | *nM* | | |
| Wild-type | | | | |
| α1β2γ2 | 2.57 ± 0.020 | 64.8 ± 3.7 | 1.16 ± 0.04 | 24 |
| β2γ2 | 2.60 ± 0.02 | 38.3 ± 12.4 | 1.32 ± 0.08 | 2 |
| α1 Mutants | | | | |
| F65Y | 1.98 ± 0.08 | 67.9 ± 0.8 | 1.22 ± 0.12 | 2 |
| H100R[a] | | Potentiation nearly abolished | | 4 |
| Y159F | 2.28 ± 0.03 | 118.2 ± 39.4 | 1.09 ± 0.06 | 3 |
| Y159S[a] | | Potentiation nearly abolished | | 3 |
| Y161S | 2.02 ± 0.18 | 58.6 ± 6.2 | 1.11 ± 0.07 | 6 |
| T162A | 3.17 ± 0.66 | 131.1 ± 37.8 | 1.29 ± 0.31 | 3 |
| Y169S | 1.55 ± 0.24 | 43.1 ± 7.0 | 1.18 ± 0.11 | 5 |
| Y209F | 2.26 ± 0.24 | 140.9 ± 3.2 | 1.31 ± 0.02 | 4 |
| Y209S | 1.54 ± 0.11 | 463.2 ± 51.2 | 1.03 ± 0.08 | 11 |
| γ2 Mutants | | | | |
| Y172S | 1.98 ± 0.10 | 118.5 ± 12.0 | 1.44 ± 0.06 | 3 |
| Y220S | 2.51 ± 0.36 | 129.7 ± 5.3 | 1.59 ± 0.30 | 2 |

[a] For these mutants, the potentiation was nearly abolished, preventing the fitting of the Hill equation to the dose-response relationships.

activation (α1β2γ2: $EC_{50}$ = 45.8 ± 3.6 μM, $I_{max}$ = 381 ± 508 nA; α1Y209Sβ2γ2: $EC_{50}$ = 38.2 ± 12.2 μM, $I_{max}$ = 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 $EC_{50}$ 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 $EC_{50}$ 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 $GABA_A$ receptors that are less affected by dzp. It has previously been shown that β2γ2 $GABA_A$ 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 $EC_{50}$ 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 $EC_{50}$ 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 conserved 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 $EC_{50}$ value for dzp (Table 1), although there was a slight decrease in the maximal potentiation of the initial component. Similar to αY161S, substitution of the tyrosine at position 168 with serine (α1Y168S) did not alter the $EC_{50}$ value for dzp-mediated potentiation (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 $GABA_A$ receptor, respectively, are also conserved in the γ2 subunit (Fig. 1; γ2Tyr172 and γ2Tyr220). Because the $\gamma_2$ 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

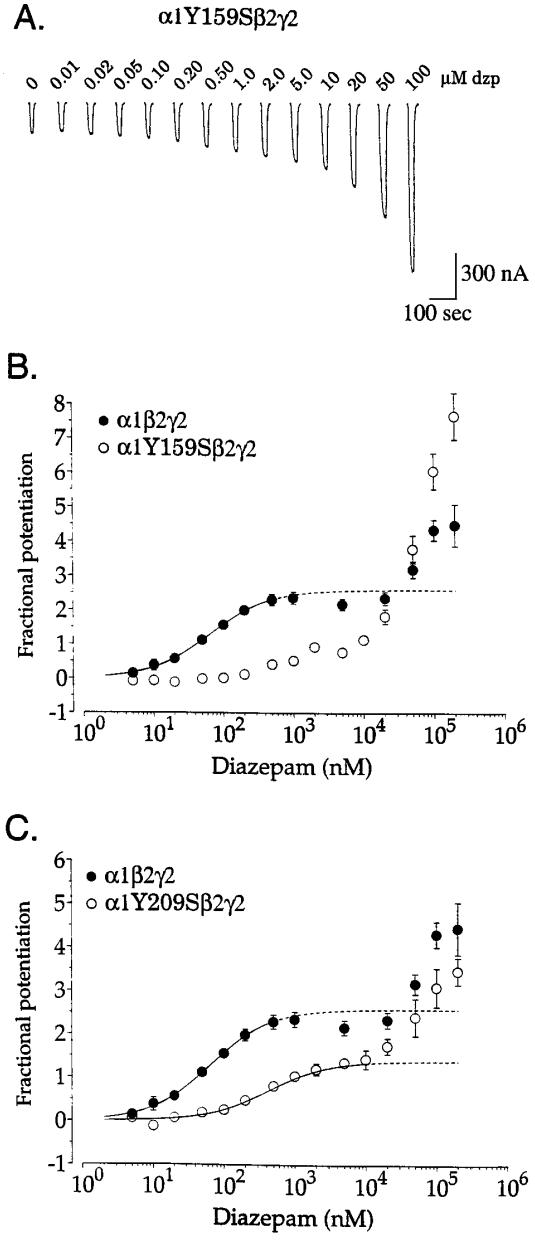


**Fig. 3.** The dzp-mediated potentiation of recombinant $\alpha1\beta2\gamma2$, $\alpha1Y159S\beta2\gamma2$, and $\alpha1Y209S\beta2\gamma2$ GABA$_A$ receptors. A, GABA-activated currents (3 $\mu$M GABA) from oocytes expressing $\alpha1Y159S\beta2\gamma2$ GABA receptors in the absence and presence of increasing concentrations of dzp (indicated *above the traces*). In comparison with the wild-type receptor, the potentiation at $< 1$ $\mu$M dzp was greatly diminished. The potentiation at $> 20$ $\mu$M, 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 $\alpha1Y159S\beta2\gamma2$ GABA$_A$ 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 $\alpha1Y209S\beta2\gamma2$ GABA 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 $\leq 10$ $\mu$M dzp. This yielded an EC$_{50}$ 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.

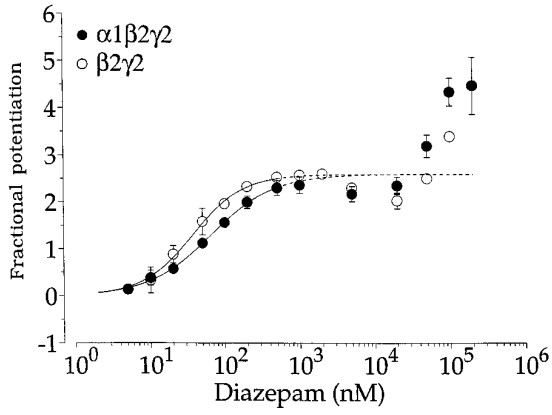


**Fig. 4.** Comparison of the dzp sensitivity of $\alpha1\beta2\gamma2$ and $\beta2\gamma2$ GABA$_A$ receptors. Plot of the fractional potentiation as a function of dzp concentration for $\alpha1\beta2\gamma2$ (●) and $\beta2\gamma2$ GABA receptors (○). *Continuous line through* ○ (extrapolated as a *dashed line*), from the best fit of the Hill equation at $\leq 1$ $\mu$M dzp. This yielded an EC$_{50}$ value for dzp potentiation of 37 nM, a Hill coefficient of 1.27, and a maximal fractional potentiation of 2.6. The mean ± standard error values for the fits of the Hill equation to the data from each oocyte are presented in Table 1.

$\alpha1\beta2\gamma2Y220S$ (*shaded circles*) GABA$_A$ receptors, respectively. Both substitutions produced a ~2-fold increase in the EC$_{50}$ 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 $\alpha1$ subunit. These two substitutions did not affect the sensitivity to activation by GABA ($\alpha1\beta2\gamma2$: EC$_{50}$ = 45.8 ± 3.6 $\mu$M, Hill coefficient = 1.57 ± 0.09, I$_{max}$ = 381 ± 508 nA; $\alpha1\beta2\gamma2Y172S$: EC$_{50}$ = 40.4 ± 5.0 $\mu$M, Hill coefficient = 1.49 ± 0.14, I$_{max}$ = 453 ± 492 nA; $\alpha1\beta2\gamma2Y220S$: EC$_{50}$ = 38.4 ± 6.2 $\mu$M, Hill coefficient = 1.43 ± 0.10, I$_{max}$ = 495 ± 514 nA; see Ref. 29).

We considered the possibility that in the absence of the $\alpha1$ subunit, the $\gamma2$ subunit can assume the role of the $\alpha1$ subunit role in dzp sensitivity. Because the crucial tyrosines are conserved in the $\gamma2$ subunit, we coexpressed the $\beta2$ subunit along with these mutant $\gamma2$ subunits ($\gamma2Y172S$ and $\gamma2Y220S$) to test their potential role in dzp-mediated potentiation of the $\beta2\gamma2$ receptor. Fig. 6 shows the wild-type $\beta2\gamma2$ dose-response relationship for dzp-mediated potentiation of the GABA-activated current (already presented in Fig. 4). The potentiation of GABA-activated currents from $\beta2\gamma2Y172S$ (○) and $\beta2\gamma2Y220S$ (□) 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 $\alpha1$ subunit, these conserved $\gamma2$ subunit tyrosines do not assume the same role as their $\alpha1$ subunit counterparts.

**Effects of tyrosine mutations on BZ binding.** The observed impairment of the sensitivity of the GABA$_A$ receptor to dzp imparted by the $\alpha1Y159S$ and $\alpha1Y209S$ 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 [$^3$H]Ro 15–1788 binding to a membrane preparation from HEK 293 cells expressing $\alpha1\beta2\gamma2$ GABA$_A$ receptors. [$^3$H]Ro 15–1788 bound to these receptors with a

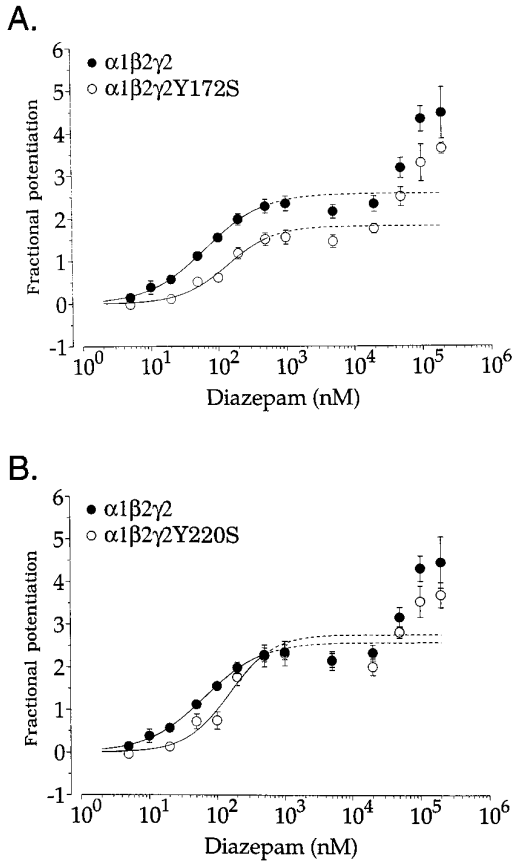


**Fig. 5.** The dzp sensitivity of oocytes expressing $\alpha1\beta2\gamma2$Y172S and $\alpha1\beta2\gamma$Y220S $GABA_A$ receptors. A, Plot of the fractional potentiation as a function of dzp concentration for $\alpha1\beta2\gamma2$Y172S $GABA_A$ receptors (○). ●, Wild-type data replotted for comparison. *Continuous line through* ○ (extrapolated as a *dashed line*), from the best fit of the Hill equation for the mean values at ≤1 μM dzp. This yielded an $EC_{50}$ value for dzp potentiation of 138 nM, a Hill coefficient of 1.27, and a maximal fractional potentiation of 1.8. B, Plot of the fractional potentiation as a function of dzp concentration for $\alpha1\beta2\gamma2$Y220S $GABA_A$ receptors (○). ●, Wild-type data replotted for comparison. *Continuous line through* ○ (extrapolated as a *dashed line*), from the best fit of the Hill equation for the mean value at ≤1 μM dzp, yielding an $EC_{50}$ value for dzp potentiation of 153 nM, a Hill coefficient of 1.34, and a maximal fractional potentiation of 2.8. The mean ± standard error values for the fits of the Hill equation to the data from each oocyte are presented in Table 1. The minimal effects of these mutations suggest these tyrosine residues do not play a key role in potentiation of the GABA receptor by dzp.

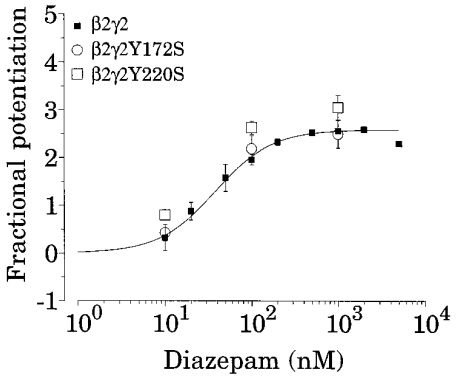


**Fig. 6.** The dzp-mediated potentiation of $\beta2\gamma2$Y172S and $\beta2\gamma2$Y220S receptors is not impaired. ■ and *continuous line*, from the fit of the Hill equation to the potentiation of the wild-type $\beta2\gamma2$ receptor. The parameters are provided in Table 1. ○ *and* □, dzp-mediated potentiation of the GABA-activated current (3 μM GABA) from $\beta2\gamma2$Y172S (○) and $\beta2\gamma2$Y220S (□) receptors by 10, 100, and 1000 nM dzp. Values are the mean ± standard error.

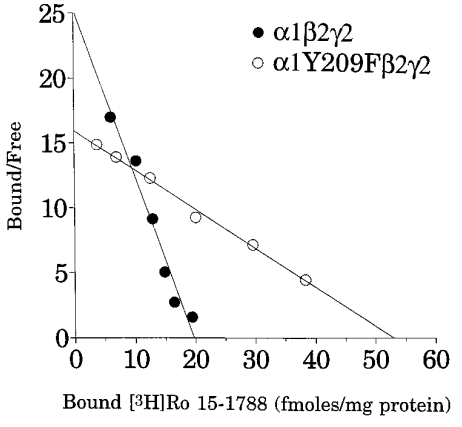


**Fig. 7.** Substitution of phenylalanine for tyrosine at position 209 of the α1 subunit results in a 5-fold decrease in BZ binding affinity. Representative Scatchard analysis showing binding affinity of the BZ antagonist [$^3$H]Ro 15–1788 to membrane preparations from HEK 293 cells transfected with DNA encoding either $\alpha1\beta2\gamma2$ (●) or $\alpha1$Y209F$\beta2\gamma2$ (○) $GABA_A$ receptors. The mean $K_d$ value is 0.80 ± 0.12 nM for the wild-type receptor and 4.07 ± 0.38 nM for the $\alpha1$Y209F$\beta2\gamma2$ receptor, as shown in Table 2. Parameters determined from a Scatchard analysis of the individual membrane preparations are presented in Table 2.

dissociation constant ($K_d$) of 0.98 ± 0.21 nM (Table 2), which is in agreement with previously published reports (27). Substitution of either of the two crucial tyrosines in the α subunit with serine eliminated specific binding of [$^3$H]Ro 15–1788 to the receptor. Muscimol binding to these mutant receptors was similar to that of the wild-type receptor (Table 2). [$^3$H]Ro 15–1788 binding to transfected receptors containing the more conservative substitution, α1Y209F was also examined. A representative Scatchard analysis is also presented in Fig. 7 (○). The $K_d$ value for $\alpha1$Y209F$\beta2\gamma2$ was 4.07 ± 0.38 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 γ2 subunit ($\alpha1\beta2\gamma2$Y172S, $\alpha1\beta2\gamma2$Y220F) had [$^3$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 $\alpha1\beta2\gamma2$ and $\beta2\gamma2$ $GABA_A$ receptors.** We examined the potentiation of GABA-activated currents in $\alpha1\beta2\gamma2$ $GABA_A$ receptors by dzp at concentrations ranging from 5 nM to 200 μM. 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 μM and demonstrated an apparent $EC_{50}$ value of 65 nM, (b) a slight depression evident at 1- 20 μM dzp, and (c) a further potentiation at > 20 μM dzp that imparted an additional 1.9-fold increase (with 200 μM 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-

TABLE 2

**BZ and muscimol binding to wild-type and mutant GABA$_A$ receptors**

Dissociation constants ($K_d$) were determined for the ligands [$^3$H]Ro 15-1788 and [$^3$H]muscimol on membrane preparations isolated from transfected HEK 293 cells. $B_{max}$ 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.

| Combination | [$^3$H]Ro 15-1788 | | $n$ | [$^3$H]Muscimol | |
|---|---|---|---|---|---|
| | $K_d$ | $B_{max}$ | | $K_d$ | $B_{max}$ |
| | nM | fmol/mg of protein | | nM | fmol/mg of protein |
| Wild-type | | | | | |
| $\alpha1\beta2\gamma2$ | 0.98 ± 0.21 | 84.9 ± 39.3 | 5 | 13.87 ± 0.85 | 244.2 ± 110.4 |
| $\alpha1$ Mutants | | | | | |
| Y159S | No binding observed | | 3 | 19.04 ± 3.22 | 250.7 ± 76.6 |
| Y209F | 4.07 ± 0.38 | 54.1 ± 5.1 | 3 | 14.10 ± 2.60 | 634.7 ± 315.9 |
| Y209S | No binding observed | | 3 | 16.57 ± 1.57 | 345.3 ± 145.9 |
| $\gamma2$ Mutants | | | | | |
| Y172S | 0.73 ± 0.03 | 22.3 ± 11.6 | 3 | N.D. | N.D. |
| Y220F | 0.96 ± 0.10 | 44.9 ± 35.1 | 3 | N.D. | N.D. |

N.D., not determined.

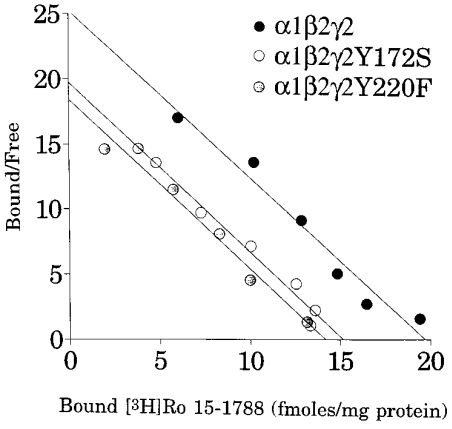


**Fig. 8.** Substitution of the tyrosines at position 172 or 220 of the $\gamma2$ subunit produces no significant change in BZ binding affinity. Representative Scatchard analysis showing binding affinity of the BZ antagonist [$^3$H]Ro 15–1788 to membrane preparations from HEK 293 cells transfected with DNA encoding either $\alpha1\beta2\gamma2$Y172S (○) or $\alpha1\beta2\gamma2$Y220F (◎) GABA$_A$ receptors. The wild-type data are replotted for comparison (●). The mean $K_d$ value is 0.80 ± 0.12 nM for the wild-type receptor, 0.73 ± 0.03 for $\alpha1\beta2\gamma2$Y172S, and 0.96 ± 0.10 nM for $\alpha1\beta2\gamma2$Y220F, as shown in Table 2. Parameters determined from a Scatchard analysis of the individual membrane preparations are presented in Table 2.

served for the more-sensitive dzp component is in good agreement with what others have reported for the actions of dzp on recombinant GABA$_A$ receptors (31–33, 35, 36). GABA-activated currents in rat cortical neurons demonstrate an EC$_{50}$ value for diazepam of 50 nM with a maximal fractional potentiation of 2.2-fold (35). GABA-activated currents in chick spinal neurons exhibit an EC$_{50}$ value for dzp of 570 nM and a 4.5-fold increase in the amplitude (37), although that study examined potentiation at a relatively high dzp concentration range (300 nM to 10 μM), suggesting they were likely examining the less-sensitive dzp component (Fig. 2). The depression seen at dzp concentrations of > 1 μM has also been observed in recombinant $\alpha5\beta2\gamma2$, $\alpha1\beta2\gamma2$, and $\beta2\gamma2$ GABA$_A$ receptors and in oocytes injected with mRNA isolated from chick brains (33, 35). This depression was not observed when the $\beta1$ subunit was substituted for the $\beta2$ subunit (32, 35), indicating this effect depends on the particular $\beta$ subunit isoform. The potentiation we observed at dzp concentrations of > 20 μM may represent an additional lower affinity BZ-binding site on the GABA$_A$ receptor complex. Micromolar-affinity BZ-binding sites have been reported in the mammalian central nervous system (38, 39).

The observation that $\beta2\gamma2$ GABA receptors show a similar dzp sensitivity as $\alpha1\beta2\gamma2$ GABA receptors is intriguing given that a significant component of the BZ-binding site is presumed to be on the $\alpha$ subunit (16–23). One possibility is that the $\beta2$ or $\gamma2$ subunit could substitute for the absence of the $\alpha$ subunit in the actions of dzp. The $\alpha1$ subunit tyrosine residues we identified in this study are conserved in both the $\beta2$ and $\gamma2$ subunits. The role of the $\beta2$ tyrosines ($\beta2$Tyr157 and $\beta2$Tyr205) 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 $\gamma2$ tyrosines in the actions of dzp on $\beta2\gamma2$ GABA receptors. Mutation of either of these tyrosines to serine ($\gamma2$Y172S and $\gamma2$Y220S) did not impair dzp sensitivity, indicating homologous regions of the $\gamma2$ subunit do not substitute for the $\alpha1$ subunit. Other possibilities are that the the $\beta2$ subunit substitutes for the $\alpha1$ subunit or other regions of the $\gamma2$ subunit (not $\gamma2$Tyr172 or $\gamma2$Tyr220) are involved in the actions of dzp. A third possibility is that a subunit endogenous to the oocyte is substituting for the $\alpha$ subunit and imparting dzp sensitivity on the expressed GABA receptors.

**$\alpha1$Tyr159 and $\alpha1$Tyr209 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 $\alpha$ and $\gamma$ subunits (21, 22) may contribute only part of the binding site. In this study, we identified two residues on the $\alpha1$ subunit (positions 159 and 209) that seem to be crucial for the actions of BZs on GABA$_A$ receptors. The mutation Y159S nearly eliminated the potentiation seen at low dzp concentrations, whereas Y209S shifted the dzp EC$_{50}$ value and reduced the maximal potentiation. The more conservative substitution of these tyrosines with phenylalanine produced moderate shifts in comparison with the serine substitutions.

Binding studies were carried out with the high affinity BZ antagonist [$^3$H]Ro 15–1788 on membrane preparations isolated from HEK 293 cells expressing either wild-type or mutant GABA receptors. The substitution of either $\alpha1$Tyr159 or $\alpha1$Tyr209 with serine abolished specific binding of [$^3$H]Ro

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, $\alpha$1Tyr159 and $\alpha$1Tyr209 may be components of the BZ-binding site/pocket itself.

Mutation of the corresponding tyrosine residues of the $\gamma$2 subunit produced a ~2-fold increase in the $EC_{50}$ (Table 1) with no significant change in the binding affinity of [$^3$H]Ro 15–1788 (Table 2). Thus, the $\gamma$2 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 $\alpha$ 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 $\alpha$ subunit (21) seems to be important for BZ affinity. More recently, Buhr *et al.* (31) observed that substitution of an alanine for the threonine at position 202 or tyrosine at position 161 of the $\alpha$1 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 $\alpha$1Tyr202 in comparison with $\alpha$1His100, $\alpha$1Tyr159, and $\alpha$1Gly200 in dzp-dependent modulation of GABA-induced currents.

In summary, three domains of the $\alpha$1 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 $\gamma$ subunit alters BZ efficacy (Fig. 1), suggesting the BZ-binding site may be at the $\alpha/\gamma$ subunit interface (23).

Two of the domains implicated in BZ binding, in the vicinity of $\alpha$Tyr159 and $\alpha$Gly200, are homologous to domains of the $\beta$ 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 $\alpha$1 subunit crucial for BZ binding are homologous to tyrosine residues in the $\beta$2 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 [$^3$H]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.

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