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Mutagenesis of the Rat α1 Subunit of the γ-Aminobutyric Acid$_A$ Receptor Reveals the Importance of Residue 101 in Determining the Allosteric Effects of Benzodiazepine Site Ligands

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ABSTRACT

The γ-aminobutyric acid$_A$ (GABA$_A$) receptor contains a binding site (or sites) for benzodiazepines and related ligands. Previous studies have shown that the residue occupying position 101 (rat numbering) of the α subunit is particularly important in determining how some of these compounds interact with the receptor. We have made multiple substitutions (F, Y, K, Q, and E) of the histidine at this position of the rat α1 subunit and coexpressed the mutant subunits with β2 and γ2 subunits in *Xenopus* oocytes. The effects of flunitrazepam, Ro15-1788, and Ro15-4513 on GABA-gated currents were then examined using electrophysiological techniques. Three substitutions (F, Y, and Q) had little effect on the ability of flunitrazepam to potentiate GABA-induced currents and had relatively modest effects on the EC$_{50}$ value of the flunitrazepam response. Other mutations (K and E) resulted in drastic reduction of flunitrazepam recognition. All substitutions also affected the EC$_{50}$ values for Ro15-1788 and Ro15-4513, and some led to dramatic changes in their efficacy. For example, H101Y, H101K, and H101Q produced receptors at which Ro15-1788 acted as a partial agonist (maximum potentiation of 164, 159, and 130%, respectively), whereas Ro15-4513 acted as a partial agonist at H101F, H101K, and H101E (potentiation of 122, 138, and 110%, respectively) and an antagonist at H101Y and H101Q. These results indicate that the characteristics of the residue at position 101 of the α1 subunit play a crucial role in determining the efficacy of benzodiazepine-site ligands.

γ-Aminobutyric acid (GABA)$_A$ receptors are the most abundant inhibitory neurotransmitter receptors in the mammalian brain and are the site of action of many clinically important compounds, including benzodiazepines, barbiturates, and the general anesthetics propofol and etomidate (Dunn et al., 1994; Sieghart, 1995; Peters and Lambert, 1997). These receptors, which are members of the ligand-gated ion channel family, are composed of five subunits that are arranged in the membrane in a cylindrical fashion to form a central chloride ion channel (Nayeem et al., 1994). GABA$_A$ receptor subunits display a high degree of heterogeneity and are divided into a number of families based on sequence homology, with most families having several members. Thus far, the genes that encode mammalian GABA$_A$ receptor subunits include α1 to α6, β1 to β4, γ1 to γ3, ρ1 to ρ3, δ, π, and ϵ (Barnard et al., 1998). Although there is evidence for the existence of several different receptor types (McKernan and Whiting, 1996), it appears that the major subtype in mammalian brain contains the α1, β2, and γ2 subunits (Whiting et al., 1995). In studies of receptor binding sites, heterologous expression approaches have been used to demonstrate that the presence of both an α and a γ subunit is required to form a benzodiazepine binding site (Pritchett et al., 1989b; Zezula et al., 1996). Furthermore, the manner in which benzodiazepine-site ligands affect GABA-gated chloride ion flux is largely determined by which specific members of the α and γ subunit families are present within the receptor oligomer (Pritchett et al., 1989a; Herb et al., 1992; Puia et al., 1991).

Molecular biological studies have shown that specific regions in the N-terminal domain of both the α and γ subunits are important for recognition of benzodiazepines and related ligands. In a manner analogous to that described for ligand recognition by the nicotinic acetylcholine receptor (Changeux, 1995), residues important for benzodiazepine-site ligand binding to GABA$_A$ receptors appear to be clustered within discrete regions of the N termini of specific subunits (see Sigel and Buhr, 1997). Alterations of amino acids within

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ABBREVIATIONS: GABA, γ-aminobutyric acid; GABA$_A$, γ-aminobutyric acid type A, FNZ, flunitrazepam.

these domains can have significant effects on both the affinity and efficacy of ligands that interact with this site (Davies et al., 1998; Sigel et al., 1998). One particular residue that occurs at position 101 (rat numbering) of the α1 subunit appears to be absolutely essential for the recognition of classic agonist benzodiazepines such as diazepam (Wieland et al., 1992). In α subunits that confer agonist sensitivity (α1, α2, α3, and α5), the residue in this position is a histidine, whereas in the subunits that confer insensitivity (α4 and α6), an arginine is present. The importance of this residue in agonist recognition was further emphasized when it was shown that His102 of the bovine α1 subunit is the major site of photoaffinity labeling by [^{3}H]flunitrazepam (FNZ; Duncalfe et al., 1996).

Interestingly, although some ligands such as Ro15-4513 and Ro15-1788 recognize both diazepam-sensitive and -insensitive receptors, their efficacies differ between the two receptor types. Ro15-4513, for example, is a partial inverse agonist at α1-containing receptors but acts as a partial agonist in receptors containing the α6 subunit (Hadingham et al., 1996; Wafford et al., 1996). Similarly, Ro15-1788 is a partial agonist at α6-containing receptors (Hadingham et al., 1996; Wafford et al., 1996) but an antagonist at α1-containing receptors. Recently, we constructed a number of mutant rat α1 subunits that contained substitutions at the position normally occupied by His101 (Davies et al., 1998). It was found that when the mutant α subunits were incorporated into receptor oligomers, the substitutions had differential effects on the affinity of different benzodiazepine ligands. In addition, measurements of the effects of GABA on their binding provided preliminary evidence for changes in their pharmacological specificity. Thus, it appears that a single amino acid substitution at position 101 of the α subunit can change the manner in which benzodiazepine-site ligands allosterically modulate GABA-gated chloride flux.

In the present study, we examined in detail the functional changes resulting from amino acid substitutions of His101. Wild-type or mutated α1 subunits were coexpressed with the β2 and γ2 subunits in *Xenopus laevis* oocytes, and GABA-induced currents were investigated using the two-electrode voltage-clamp technique. We characterized the effects of three benzodiazepine-site ligands: FNZ, Ro15-1788, and Ro15-4513, which are generally considered to be an agonist, an antagonist, and a partial inverse agonist at this receptor type (Sieghart, 1995).

Materials and Methods

Preparation of Transcripts and Oocyte Injection. Site-directed mutagenesis of α1 subunits was performed as described previously (Davies et al., 1998) using the Altered Sites kit (Promega, Madison, WI). The α1 subunit was subcloned into the pAlter-1 vector for mutagenesis. Potential mutants were identified by the presence of a restriction site that was introduced as a silent mutation by the mutagenic oligonucleotide. The presence of the correct substitutions was verified by sequencing. The mutant α1 cDNAs were then subcloned into the pcDNA3 expression vector (InVitrogen, San Diego, CA).

cDNAs encoding the α1, β2, and γ2L subunits in the pcDNA3 vector were linearized, and cRNA transcripts were prepared by standard procedures as previously described (Hope et al., 1993). The cRNA transcripts were injected (50 nl of 1 mg/ml cRNA) into *X. laevis* oocytes (stage V to VI) that had been previously defolliculated by treatment with 2 mg/ml collagenase A (Boehringer-Mannheim, Indianapolis, IN) for 3 h at room temperature (Belelli et al., 1996). Injected oocytes were individually maintained at 19–20°C for up to 14 days in 96-well plates containing 200 μl of standard Barth's solution [15 mM HEPES, pH 7.6, 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.5 mM $Ca(NO_3)_2$, 0.5 mM $CaCl_2$, 1 mM $MgSO_4$] supplemented with 0.1 mg/ml gentamycin.

Electrophysiological Recordings. Oocytes were used for experimentation 2 to 14 days after cRNA injection. The methodology was essentially the same as described previously (Pistis et al., 1997). Briefly, electrical recordings were made from oocytes voltage-clamped at −60 mV using a GeneClamp 500 amplifier (Axon Instruments, Inc., Foster City, CA) in the two-electrode voltage-clamp mode. The oocytes were held in a chamber (0.5 ml) and continuously superfused (7–10 ml/min) with frog Ringer's solution (5 mM HEPES, pH 7.4, 120 mM NaCl, 2 mM KCl, and 1.8 mM $CaCl_2$). The voltage-sensing and current passing electrodes were filled with 3 M KCl and had resistances of 0.5 to 1.5 MΩ when measured in frog Ringer's solution. Agonist-induced responses were low pass filtered at 100 Hz, recorded onto videotape, and simultaneously displayed on a chart recorder. The peak amplitude of the agonist-evoked response was measured manually. All drugs were applied via the superfusion system.

For each oocyte, a maximal concentration of GABA (3 mM) was applied, and the resulting peak current amplitude was measured. This concentration of GABA was reapplied at approximately 20-min intervals until the current amplitude was consistent to within ±2% over three successive challenges.

To investigate the effects of the benzodiazepines, GABA concentrations eliciting a peak current approximately 10% (EC_{10}) or 50% (EC_{50}) of the maximum GABA response were used as indicated in the text. Pharmacological effects were first screened using a concentration of GABA approximating its EC_{50} value. Subsequently, the EC_{10} concentration of GABA was used to examine potentiating effects and, unless where noted in the text, the EC_{50} concentration of GABA was used to further characterize inverse agonist and antagonist effects. Benzodiazepines were dissolved in dimethyl sulfoxide to give stock concentrations of 30 mM, and these stocks were stored frozen at −20°C. In all experiments, the concentration of dimethyl sulfoxide was constant at 0.02%, a concentration that produced no overt vehicle effects. In each experiment, control currents using the indicated concentrations of GABA were first recorded. This was followed by 5-min washing with frog Ringer's solution and then by 3-min perfusion with the benzodiazepine before coapplication with the appropriate concentration of GABA. After recording of the evoked current, the oocyte was again washed with frog Ringer's for at least 5 min before further benzodiazepine perfusion. During each experiment, control currents were recorded periodically to verify that the response remained stable throughout and that any benzodiazepine effects were fully reversible.

Data Analysis. All data represent the mean (±S.E.) of observations made from at least three oocytes. Data were analyzed by nonlinear regression techniques using InPlot 4 (GraphPad Software, San Diego, CA). The GABA concentration dependence of the observed current was fit by the equation:

$$I = \frac{I_{max} \cdot [L]^n}{EC_{50}{}^n + [L]^n}$$

where I is the measured amplitude of the evoked current, [L] is the GABA concentration, EC_{50} is the concentration of GABA producing 50% of the maximal response (I_{max}), and n is the Hill coefficient. In each experiment, the observed current was normalized to the I_{max} (100%), and these normalized data were used to construct concentration-effect curves.

Unless otherwise indicated, the effects of the benzodiazepines were fit by the equation:

$$I = I_0 + \frac{(E_{max} - I_0)10^{X,n}}{10^{X,n} + 10^{C,n}}$$

where I is the amplitude of the observed current; [X] is the logarithm of the concentration of the benzodiazepine; I_0 and E_{max} are the currents observed in the absence of benzodiazepine and in the presence of the maximally effective concentration, respectively; C is the logarithm of the EC_{50} for the benzodiazepine response; and n is the Hill coefficient. As shown in the figures, the concentration dependencies of many of the benzodiazepine effects deviated from a simple process, giving Hill coefficients of greater than or less than 1. Unfortunately, without detailed knowledge of the mechanisms underlying these allosteric interactions, it is not presently feasible to develop a molecular framework within which to interpret these Hill coefficients.

Curves that were obviously biphasic were fit by the equation:

$$I = I_0 + \frac{E_1 \cdot 10^X}{10^{C_1} + 10^X} + \frac{E_2 \cdot 10^X}{10^{C_2} + 10^X}$$

where E_1 and E_2 are the amplitudes of the first and second phases, C_1 and C_2 are the logarithms of the corresponding EC_{50} values, and the other parameters are as described above.

In experiments to estimate antagonist affinities, the effects of Ro15-1788 or Ro15-4513 on the FNZ-induced potentiation were investigated. IC_{50} values were calculated from the equation:

$$I = I_0 + \frac{(I_{FNZ} - I_0)10^{X,n}}{10^{X,n} + 10^{C,n}}$$

where I is the observed current, I_{FNZ} is the current in the presence of FNZ, I_0 is the current in the presence of both FNZ and a saturating concentration of benzodiazepine antagonist, [X] is the logarithm of the antagonist concentration, C is the logarithm of the IC_{50} concentration, and n is the Hill coefficient. In these experiments, the data were normalized by first expressing the magnitude of the FNZ-induced potentiation of the GABA response (in the absence of antagonist) as 100% and then calculating the inhibition of this current in the presence of different concentrations of antagonist.

Drugs. GABA and FNZ were obtained from Sigma Chemical Co. (St. Louis, MO). Ro15-4513 and Ro15-1788 were generous gifts from Hoffmann-La Roche and Co. (Basel, Switzerland).

Results

At a holding potential of −60 mV, oocytes preinjected with cRNAs encoding rat α1, β2, and γ2 $GABA_A$ receptor subunits responded to bath-applied GABA with an inward current response. Similarly, receptors carrying the various α subunit mutations displayed robust current responses to GABA application. All receptors showed some modulation by benzodiazepine-site ligands as illustrated for the wild-type and H101Y mutant receptor in Fig. 1. Thus, all receptors examined displayed functional coupling between the GABA and benzodiazepine binding sites.

Analysis of the GABA concentration-response curve for the wild-type $\alpha1\beta2\gamma2$ receptor gave an EC_{50} value of 31.8 ± 3.4 μM (n = 4) and a Hill coefficient of 1.32 ± 0.16 (n = 4). None of the His101 mutations examined had a dramatic effect on GABA-induced currents (Table 1), and although the EC_{50} value was slightly increased in each case, this remained within a factor of about 2 of the wild-type receptor.

Mutations of the α1 subunit His101 to residues of altered chemical specificity did, however, have significant effects on both the potencies of the benzodiazepine-site ligands and on the manner in which they modulated GABA-evoked currents. For clarity, the qualitative changes in the pharmacological effects of FNZ, Ro15-1788, and Ro15-4513 are first summarized in Table 2.

The effects of FNZ on wild-type receptors were distinctly biphasic (Fig. 2A), suggesting a relatively potent potentiating effect (EC_{50} = 5.8 nM) and a lower affinity inhibitory response (EC_{50} = 178 nM). In the H101F, H101Y, and H101Q mutants, FNZ potentiated currents to a similar extent (E_{max} = 217–290% of control; Table 3). In contrast, FNZ had no effect on GABA-evoked currents in the H101K receptor (Fig. 2A), which is consistent with the lack of measurable FNZ binding to this receptor mutant when expressed in tsA201 cells (Davies et al., 1998). In the H101E mutant, which also displayed no measurable FNZ binding in mammalian cells, FNZ did have a potentiating effect but only at high ligand concentrations (>1 μM; Fig. 2B). Representative results for the effects of FNZ on GABA-induced currents are shown in Fig. 2, and the data are summarized in Table 3, where their measured EC_{50} values are compared with their apparent affinities, measured in binding assays of receptors expressed in tsA201 cells. Thus, FNZ is an agonist of all receptors that recognize this ligand.

The consequences of H101 mutations on the effects of Ro15-4513 on functional responses were more complex. In the wild-type receptor, Ro15-4513 was a very limited inverse agonist, decreasing GABA-evoked currents by only 6 to 10% with an EC_{50} of 1.8 nM (Fig. 3 and Table 4). However, in the H101K and H101E mutants (and to a small extent in the H101Y mutant), this ligand became a partial agonist (see Fig. 3). Ro15-4513 had no direct effect on currents elicited by the H101Q mutant, but it did inhibit the potentiation induced by FNZ, indicating that it is a potent antagonist of this receptor. As previously reported for these receptors expressed in tsA201 cells (Davies et al., 1998), none of the mutations caused a pronounced decrease in the apparent affinities for Ro15-4513, and in some cases, notably H101F and H101Q, the potency of this ligand was increased compared with the wild-type receptor (Fig. 3 and Table 4).

Figure 4 illustrates the effects of the mutations on the ability of Ro15-1788 to modulate GABA-gated currents. In the wild-type receptor, this ligand had no direct effect on the GABA-evoked currents (Fig. 4A) but inhibited the FNZ-induced potentiation with an IC_{50} value of 7.9 ± 1.9 nM (Fig. 4B). In the H101F mutant, Ro15-1788 also acted as an antagonist, but in the H101Y, H101K, and H101E receptors, it became a partial agonist (Fig. 4 and Table 5). Ro15-1788 had no direct effect on GABA-evoked currents in the H101E mutant receptor. Unfortunately, the lack of a robust agonist response of FNZ (Fig. 2B) or Ro15-4513 (Table 4) precluded further characterization of its possible antagonist properties on this receptor.

Discussion

A central issue concerning the benzodiazepine site of the $GABA_A$ receptor is how its occupation can result in a spectrum of pharmacological effects ranging from inverse agonism to full agonism. We examined the effects of mutations of His101 of the rat α1 subunit on the pharmacological specificity of FNZ, Ro15-1788, and Ro15-4513 (i.e., compounds that are commonly regarded as having agonist, antagonist, and partial inverse agonist properties, respectively). Although it has recently been suggested that His102 (human numbering) is not directly involved in determining the efficacies of benzodiazepine-site ligands (McKernan et al., 1998),

the above results demonstrate that the chemical nature of the residue at the homologous position of the rat α1 subunit has a significant effect on the actions of these compounds.

The mutations that were introduced had relatively minor effects on the concentration dependence of GABA-evoked currents (Table 1), suggesting that none of the substitutions had serious adverse effects on the overall structure of the receptor. Other groups have, however, reported larger shifts in the EC_{50} values for GABA activation when the corresponding residue in the α6 subunit was modified (Im et al., 1997).

The shifts in EC_{50} values obtained for the effects of FNZ, Ro15-1788, and Ro15-4513 on GABA-gated currents tended to parallel the shifts in binding affinities reported previously (Davies et al., 1998). In most cases, there was excellent agreement between the present functional data for receptors expressed in oocytes and previous binding data using receptors expressed in tsA201 cells. One exception is the effect of FNZ on the H101F mutant, where the apparent affinity obtained from binding experiments was 92.8 ± 9.1 nM compared with 8.6 ± 1.0 nM measured in the functional studies. In the absence of any other interpretation, it must be assumed that this is a cell type-specific phenomenon.

The binding of FNZ is particularly sensitive to the nature of the residue occupying position 101. Incorporation of the positively charged lysine resulted in receptors that were insensitive to FNZ. This is consistent with previous reports (Wieland et al., 1992; Benson et al., 1998) in which substitution of His101 in different α subunits by arginine produced receptors that were insensitive to diazepam. Similarly, the introduction of the negatively charged amino acid glutamate drastically affected FNZ binding, and in the H101E mutant, this ligand potentiated GABA-evoked currents only at high concentrations. In contrast, substitution by the uncharged glutamine restored sensitivity to FNZ (Table 3). The ability of glutamine to substitute for histidine in the recognition site has been discussed previously (Davies et al., 1998). In all mutant receptors that recognized FNZ, this drug acted as a strong agonist, potentiating GABA-induced currents to levels similar to those seen with wild-type receptors. This suggests that although the mutations reduce the affinity for FNZ, they do not produce qualitative changes in the protein conformational transitions that allosterically modulate GABA-gated chloride flux.

The efficacies of Ro15-1788 and Ro15-4513 were dramati-

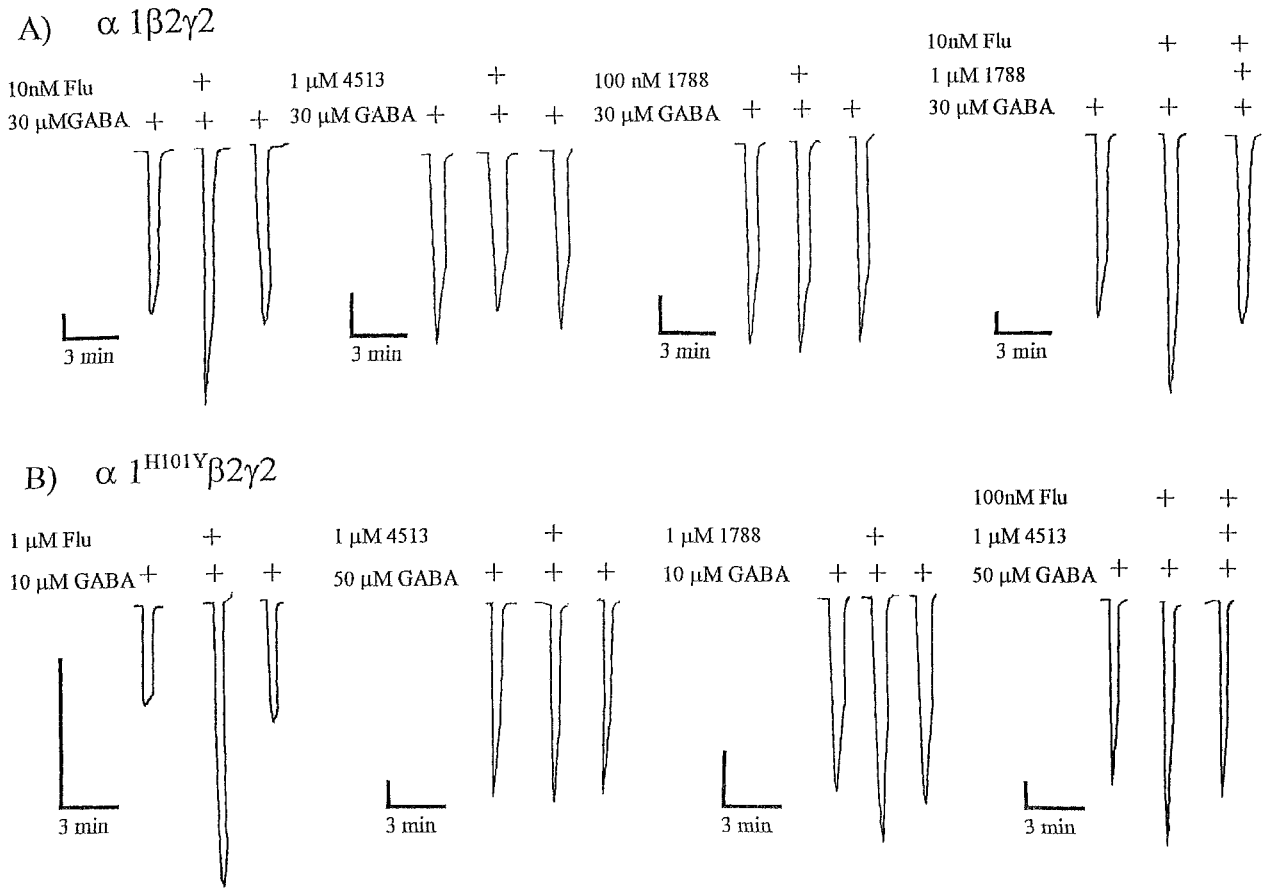

Fig. 1. Representative traces showing GABA-evoked currents and their modulation by benzodiazepine-site ligands for (A) the wild-type receptor and (B) the H101Y mutant. The oocytes were perfused with the benzodiazepine-site ligands (+) for 3 min before coapplication with the indicated concentrations of GABA to elicit the observed current (see the text for details). The maximum current (E_{max}) in each case lay in the range of 1 to 3 μA, and control currents were recorded using GABA concentrations approximating its EC_{50} or EC_{10} value for that receptor. Vertical bars represent 100 nA (A) and 50 nA (B). A, potentiation of the GABA current by FNZ, the rather limited inhibition by 1 μM Ro15-4513, and the lack of effect of Ro15-1788. In all cases, any observed effects were fully reversible as indicated by the control currents obtained after washing out of the modulator (third trace in each panel). Ro15-1788 (1 μM), however, inhibited the potentiation of the current by FNZ (10 nM), indicating that it acts as an antagonist on the wild-type receptor. The data obtained with H101Y (B) also show the reversible potentiation by FNZ (first three traces), but for this receptor, Ro15-4513 had no direct effect on GABA-induced currents, whereas Ro15-1788 potentiated the GABA response. Observed effects were again fully reversible on washout. B, the final three traces show that Ro15-4513 inhibited the FNZ-induced potentiation, suggesting that this ligand is an antagonist of the H101Y receptor complex.

cally altered by some of the mutations, but the pattern of the changes differed for the two ligands. In the wild-type receptor, Ro15-4513 was a poor inverse agonist, decreasing GABA-evoked currents by less than 10%. Decreases of a similar magnitude have been reported previously in studies using both the human recombinant $\alpha1\beta2\gamma2$ subtype (Hadingham et al., 1996) and acutely dissociated rat pyramidal neurons (Smith et al., 1998). As expected from previous studies (Sieghart, 1995), Ro15-1788 acted as an antagonist of the wild-type receptor. It has previously been shown that both Ro15-4513 and Ro15-1788 act as partial agonists in $\alpha6$ receptors in which arginine is present in this position (Hadingham et al., 1996; Wafford et al., 1996). Recently, the importance of this residue was further confirmed by showing that substitution of His101 by arginine in the $\alpha1$, $\alpha2$, $\alpha3$, and $\alpha5$ subunits resulted in Ro15-4513 becoming a positive allosteric modulator (Benson et al., 1998). Another mutation in the $\gamma2$ subunit has been shown to change the efficacy of Ro15-4513 (Mihic et al., 1994), suggesting that determinants in both the α and γ subunits affect the interaction of this compound with the receptor. A recent report by Im et al. (1997) suggests that residues C terminal to position 101 may be involved in determining the efficacy of Ro15-1788 at $\alpha6$-containing receptors. In the present study, we have shown that manipulation of this amino acid, at least in $\alpha1$ subunits, is sufficient to bring about changes in efficacy, not only for Ro15-1788 but also for Ro15-4513.

Relatively small differences in the structure of the residue at position 101 had differential effects on receptor modulation by Ro15-1788 and Ro15-4513. This is illustrated by comparing the effects of these compounds on the H101Y and H101F receptors. In the H101Y mutant, Ro15-1788 is an agonist, whereas Ro15-4513 acts as an antagonist. In the H101F receptor, the converse is true. Thus, the pharmacological specificity of these ligands is dictated by the presence or absence of a hydroxyl group in this position. The affinities for both agents were reduced in the tyrosine mutant, indicating that changes in efficacy do not parallel changes in apparent affinity.

Because structural information on the $GABA_A$ receptor is lacking, any rationalization of the differential effects of the mutations on the actions of Ro15-4513 and Ro15-1788 is speculative. Molecular modeling has suggested that agonists, inverse agonists, and antagonists differentially affect the receptor by interacting with different lipophilic pockets

TABLE 1

Effect of mutation of $\alpha1$ subunit His101 on GABA-induced currents

cRNAs encoding the wild-type or mutated $\alpha1$ subunits were coexpressed with the $\beta2$ and $\gamma2$ subunits in *X. laevis* oocytes, and currents in response to bath applied GABA were recorded.

α Subunit (no. of oocytes)	EC_{50}	n_H
	μM	
Wild type (5)	31.8 ± 3.4	1.32 ± 0.16
H101F (3)	60.3 ± 6.9	0.96 ± 0.11
H101Y (4)	42.5 ± 3.0	1.33 ± 0.12
H101K (7)	56.6 ± 8.8	1.61 ± 0.43
H101E (4)	66.9 ± 3.7	1.04 ± 0.06
H101Q (3)	51.4 ± 3.0	1.28 ± 0.10

TABLE 2

Qualitative description of the effects of mutation of $\alpha1$ subunit His101 on the pharmacology of benzodiazepine-site ligands

Mutated $\alpha1$ subunits were coexpressed with the wild-type $\beta2$ and $\gamma2$ subunits in *X. laevis* oocytes, and the effects of the modulators on GABA-induced currents were recorded.

α Subunit	FNZ	Ro15-4513	Ro15-1788
Wild type	Agonist	Poor inverse agonist	Antagonist
H101F	Agonist	Partial agonist	Antagonist
H101Y	Agonist	Poor partial agonist	Partial agonist
H101K	No effect	Partial agonist	Partial agonist
H101E	Agonist at high [FNZ]	Partial agonist	Antagonist
H101Q	Agonist	Antagonist	Partial agonist

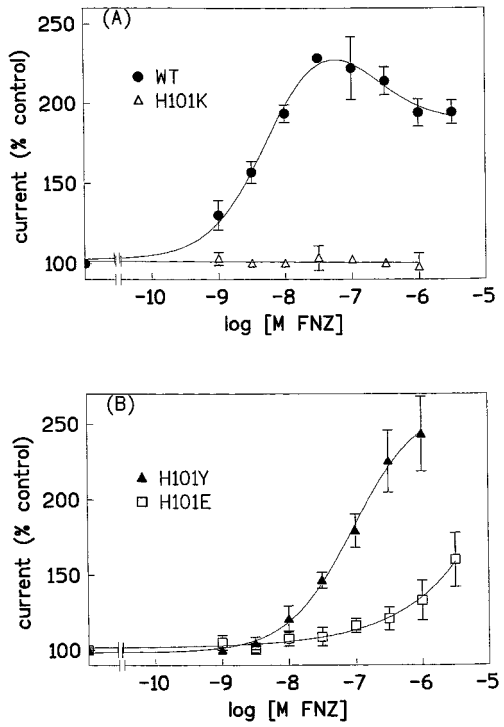

Fig. 2. Effects of FNZ on GABA-mediated currents in wild-type and mutant receptors. A, concentration dependence of FNZ effects on wild-type currents (●) induced by a concentration of GABA approximately equal to its EC_{10} value and its lack of effect on the receptor carrying the $\alpha1$ H101K mutation, again recorded using a GABA concentration approximating its EC_{10} concentration (△). B, the effects of FNZ on currents induced by GABA (at a concentration approximately equal to its EC_{10} value) in the $\alpha1$ H101Y (▲) and H101E (□) mutations are shown. Best-fit values obtained from these curves and for the H101F and H101Q mutants are listed in Table 3.

TABLE 3

Effects of mutation of $\alpha1$ subunit His101 on modulation of the $\alpha1\beta2\gamma2$ receptor by FNZ

$\alpha1$ Subunit (no. of oocytes)	K_i (from binding studies[a])	EC_{50}	Maximum Effect
	nM		*% of control*
Wild type[b] (5)	6.12 ± 0.36	(1) 5.8 ± 1.6 (2) 178 ± 132	(1) 256 ± 18 (2) −70 ± 17
H101F (4)	92.8 ± 9.1	8.6 ± 1.0	290 ± 8
H101Y (4)	142 ± 2	93 ± 19	266 ± 12
H101K (3)	No binding	No effect	
H101E (4)	No binding	>1 μM	>160%
H101Q (4)	103 ± 11	54 ± 49	217 ± 16

[a] Data from Davies et al. (1998), in which wild-type and mutant receptors were expressed in tsA201 cells. K_i values were obtained in experiments in which FNZ was used to displace [^{3}H]Ro15-4513, which was present at a concentration equivalent to its K_d value for each receptor type.

[b] As shown in Fig. 2, a plot of the effects of FNZ had a bell-shaped appearance. The parameters quoted were obtained from curve-fitting as described in the text in which (1) describes the potentiation of GABA-induced currents observed at low FNZ concentrations and (2) describes the inhibitory effect at higher concentrations.

within the binding site or sites. In the models proposed by Villar et al. (1989) and Zhang et al. (1995), the lipophilic pocket or pockets occupied by antagonists and inverse agonists are structurally related to each other, but the pocket that accommodates the 5-phenyl ring of benzodiazepine agonists lies in a much different position. Because of the similarities in the binding of antagonists and inverse agonists, small perturbations in binding site structure may be sufficient to change the particular lipophilic pocket with which these compounds interact, thus changing the efficacy of a ligand from inverse agonist to antagonist. To explain the partial agonism of Ro15-1788 and Ro15-4513 at some mutant receptors, two possibilities must be considered. First, both ligands may interact with the same regions of the binding cleft as in wild-type receptors but now act as agonists (i.e., the mutations affect events that are downstream from the initial binding interaction). Second, the structural changes induced by the mutations allow these compounds to interact with the lipophilic pocket that is normally "seen" only by agonists (i.e., the signal transduction mechanism remains the same as in wild-type receptors, but the initial receptor-ligand interaction is altered). We cannot presently distinguish between these possibilities, but it is hoped that further information will come from direct identification of binding domains for different ligands by peptide mapping of photolabeled mutant receptors.

It is generally assumed that agonists, antagonists, and inverse agonists interact with a common binding site because their binding is mutually exclusive at equilibrium. However, Buhr et al. (1997) recently showed that a single mutation in the γ2 subunit produced receptors that displayed biphasic displacement curves in which zolpidem, methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate, and Ro15-1788 were able to displace only about 50% of bound [^{3}H]FNZ. The authors suggested that this may indicate the presence of two

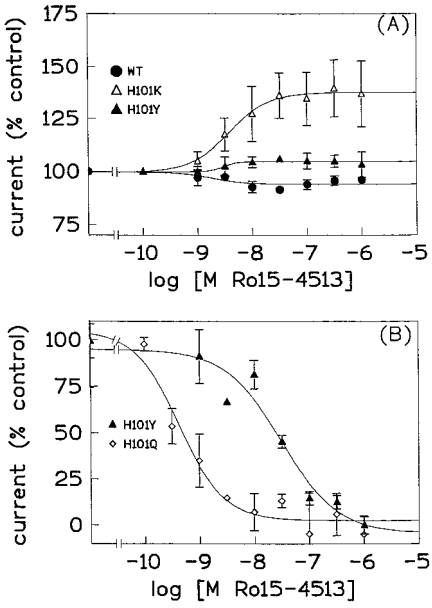

Fig. 3. Effects of Ro15-4513 on GABA-mediated currents. A, data for wild-type (●), H101K (△), and H101Y (▲) receptors, indicating that Ro15-4513 is a poor inverse agonist of the wild-type receptor but becomes a partial agonist after the H101K mutation. B, inhibition of the FNZ potentiation by Ro15-4513 in the H101Y (▲) and H101Q (◇) mutant receptors. In each case, both the GABA concentration (50 μM) and the FNZ concentration (100 nM) used were approximately equal to their EC_{50} values. Best-fit parameters from curve fitting are listed in Table 4.

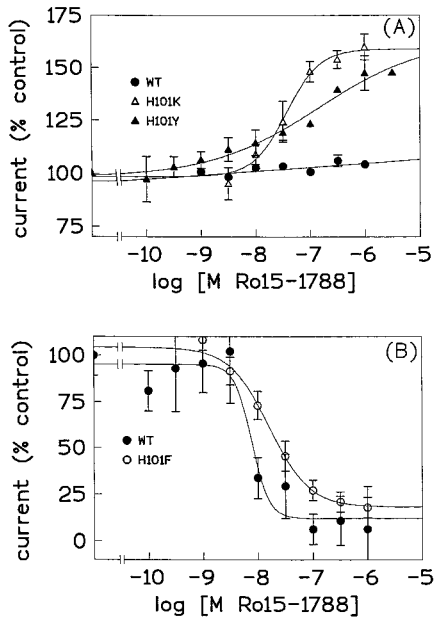

Fig. 4. Effects of Ro15-1788 on GABA-mediated currents. A, effects of Ro15-1788 on the wild-type (●), H101K (△), and H101Y (▲) receptors. B, inhibition of the FNZ-evoked potentiation by Ro15-1788 in the H101F (○) and wild-type (●) mutant receptors. The data for the wild type were obtained using GABA and FNZ concentrations of 30 μM and 10 nM (i.e., approximately equal to their EC_{50} values). In the case of H101F, the concentrations of GABA and FNZ were 10 μM and 10 nM, respectively. Best-fit parameters obtained from curve fitting are given in Table 5.

TABLE 4

Effect of mutation of α1 subunit His101 on modulation of the α1β2γ2 receptor by Ro15-4513

α1 Subunit (no. of oocytes)	K_d (from binding studies)[a]	EC_{50} or IC_{50}[b]	Maximum Effect
	nM		*% of control*
Wild type (5)	6.21 ± 0.54	1.8 ± 2.7	94.1 ± 1.2
H101F (4)	0.95 ± 0.07	0.19 ± 0.06	122 ± 1.0
H101Y (3)	7.01 ± 0.71	28 ± 21[b]	Antagonist
H101K (4)	1.54 ± 0.11	3.85 ± 0.72	137.6 ± 1.2
H101E (3)	2.13 ± 0.13	36 ± 23	110 ± 2.9
H101Q (3)	0.49 ± 0.07	0.64 ± 0.52[b]	Antagonist

[a] Data from Davies et al. (1998), in which wild-type and mutant receptors were expressed in tsA201 cells. K_d values were obtained in saturation binding assays using [^{3}H]Ro15-4513.

[b] IC_{50} values were obtained based on the ability of Ro15-4513 to inhibit the FNZ potentiation of GABA-induced currents in these receptors.

TABLE 5

Effect of mutation of α1 subunit His101 on modulation of the α1β2γ2 receptor by Ro15-1788

α1 Subunit (no. of oocytes)	K_i (from binding studies)[a]	EC_{50} or IC_{50}[b]	Maximum Effect
	nM		*% of control*
Wild type (4)	1.29 ± 0.46	7.9 ± 1.9[b]	Antagonist
H101F (4)	5.05 ± 0.48	16.3 ± 2.6[b]	Antagonist
H101Y (4)	90 ± 10	147 ± 30	164 ± 16
H101K (6)	26.6 ± 3.9	37 ± 6	159 ± 3
H101E (3)	79.1 ± 7.0		
H101Q (4)	8.45 ± 0.87	4.3 ± 1.6	130 ± 2

[a] Data from Davies et al. (1998), in which wild-type and mutant receptors were expressed in tsA201 cells. K_i values were obtained in experiments in which Ro15-1788 was used to displace [^{3}H]Ro15-4513, which was present at a concentration equivalent to its K_d value for each receptor type.

[b] IC_{50} values were obtained based on the ability of Ro15-1788 to inhibit the FNZ potentiation of GABA-induced currents in these receptors.

benzodiazepine binding sites within a single receptor molecule. In the present study, we identified one mutant (H101Q) that displays a shallow concentration dependence (Hill coefficient = 0.45 ± 0.11) for FNZ potentiation, suggesting that two benzodiazepine sites of different affinities may be present in these receptors. However, no evidence of binding site heterogeneity was obtained in direct binding studies in which FNZ was shown to completely displace [^{3}H]Ro15-4513 (Davies et al., 1998). Also in the current study, we found that high concentrations of FNZ (>1 μM) potentiated GABA-gated ion flux in the H101E mutant, yet previous competition studies using this receptor showed that FNZ at concentrations up to 10 μM was unable to displace bound [^{3}H]Ro15-4513. This was previously interpreted to mean that these receptors did not recognize FNZ. However, in light of the functional data for this mutant, it is possible that there is an additional low-affinity binding site for FNZ that is distinct from the high-affinity site for Ro15-4513.

The bell-shaped appearance of the curve for FNZ potentiation of the wild-type $\alpha1\beta2\gamma2$ receptor (Fig. 2A) suggests that the presence of more than one benzodiazepine site is not exclusive to the mutant receptors. Evidence for more than one diazepam-responsive component in this subtype has also been reported by others (Amin et al., 1997). Im et al. (1993) showed that an additional benzodiazepine site may lie at subunit interfaces other than α/γ, and it has been suggested by Sieghart (1995) that $GABA_A$ receptors may carry both a high-affinity and a low affinity site for benzodiazepine agonists. The significance of the lower-affinity benzodiazepine site or sites is not yet known.

In conclusion, the results described above show that His102 of the $\alpha1$ subunit is an important determinant of both the affinity and pharmacological efficacy of ligands that bind to the benzodiazepine site of the rat $\alpha1\beta2\gamma2$ $GABA_A$ receptor.

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