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A Single Amino Acid Residue on the α_5 Subunit (Ile215) Is Essential for Ligand Selectivity at $\alpha_5\beta_3\gamma_2$ γ-Aminobutyric Acid$_A$ Receptors

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ABSTRACT

Imidazobenzodiazepines such as RY-80 have been reported to exhibit both high affinity and selectivity for GABA$_A$ receptors containing an α_5 subunit. A single amino acid residue (α_5Ile215) has been identified that plays a critical role in the high-affinity, subtype-selective effects of RY-80 and structurally related ligands. Thus, substitution of α_5Ile215 with the cognate amino acid contained in the α_1 subunit (Val211) reduced the selectivity of RY-80 for $\alpha_5\beta_3\gamma_2$ receptors from ~135- to ~8-fold compared with $\alpha_1\beta_3\gamma_2$ receptors. This mutation produced a comparable reduction in the selectivity of RY-24 (a structural analog of RY-80) for $\alpha_5\beta_3\gamma_2$ receptors but did not markedly alter the affinities of ligands (e.g., flunitrazepam) that are not subtype-selective. Conversely, substitution of the α_1 subunit with the cognate amino acid contained in the α_5 subunit (i.e., α_1V211I) increased the affinities of α_5-selective ligands by a ~20-fold and reduced by 3-fold the affinity of an α_1-selective agonist (zolpidem). Increasing the lipophilicity (e.g., by substitution of Phe) of $\alpha_5$215 did not significantly affect the affinities (and selectivities) of RY-80 and RY-24 for α_5-containing GABA$_A$ receptors. However, the effect of introducing hydrophilic and or charged residues (e.g., Lys, Asp, Thr) at this position was no greater than that produced by the α_5I215V mutation. These data indicate that residue $\alpha_5$215 may not participate in formation of the lipophilic L$_2$ pocket that has been proposed to contribute to the unique pharmacological properties of α_5-containing GABA$_A$ receptors. RY-80 and RY-24 acted as inverse agonists in both wild-type $\alpha_5\beta_3\gamma_2$ and mutant α_5I215K$\beta_3\gamma_2$ receptors expressed in *Xenopus laevis* oocytes. However, both RY-24 and RY-80 acted as antagonists at mutant α_5I215V$\beta_3\gamma_2$ and α_5I215T$\beta_3\gamma_2$ receptors, whereas the efficacy of flunitrazepam was similar at all three receptor isoforms. The data demonstrate that amino acid residue $\alpha_5$215 is a determinant of both ligand affinity and efficacy at GABA$_A$ receptors containing an α_5 subunit.

The principal therapeutic actions of drugs such as the benzodiazepines (e.g., diazepam), imidazopyridines (e.g., zolpidem), and triazolopyridazines (e.g., zaleplon) are effected through the family of GABA$_A$ receptors (Lüddens et al., 1995; Korpi et al., 1997; Sigel and Buhr, 1997). Based on sequence homology, 17 distinct subunits belonging to six related families (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ϵ, ρ_{1-2}, θ) have been identified as members of this group of ligand-gated ion channels (for review, see McKernan and Whiting, 1996; Sigel and Buhr, 1997; Bonnert et al., 1999). Assuming a pentameric arrangement (Nayeem et al., 1994), there is a remarkable potential for GABA$_A$ receptor heterogeneity. Nonetheless, no more than 10 to 20 distinct GABA$_A$ receptor isoforms have been identified in the adult rat central nervous system (Fritschy and Mohler, 1995; De Blas, 1996; McKernan and Whiting, 1996), with the majority existing as heteromers composed of α-, β-, and γ-subunits (Fritschy and Mohler, 1995; De Blas, 1996). Although the stoichiometry of native GABA$_A$ receptors has not been definitively established, several studies have proposed a GABA$_A$ receptor configuration as consisting of 2α-, 2β-, and 1γ-subunit (Chang et al., 1996; Tretter et al., 1997).

Studies using recombinant GABA$_A$ receptors have demonstrated that subunit composition defines ligand pharmacology at these ligand-gated ion channels (Pritchett and Seeburg, 1990; Hadingham et al., 1993). This principle is amply illustrated by the impact of the α-subunit on the affinities of a chemically diverse group of substances often termed benzodiazepine site ligands (for review, see Lüddens et al., 1995; Korpi et al., 1997). For example, prototypic 1,4- benzodiazepines such as diazepam and flunitrazepam possess high (nM) affinities for GABA$_A$ receptors containing $\alpha_{1,2,3}$ and α_5

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ABBREVIATIONS: GABA, γ-aminobutyric acid; DS, diazepam sensitive.

subunits [comprising the "diazepam-sensitive" (DS) family of GABA$_A$ receptors], but are essentially inactive at receptors containing α_4 and α_6 subunits [the "diazepam-insensitive" family of GABA$_A$ receptors] (Korpi et al., 1992; Wong et al., 1992; Wieland and Lüddens, 1994; Fritschy and Mohler, 1995). This remarkable effect on ligand affinity is determined in a large part, by a single histidine residue in homologous positions $\alpha_1$101, $\alpha_2$101, $\alpha_3$126, and $\alpha_5$105 of the DS α-subunits and the cognate arginine in position 100 on the α_4 and α_6 receptors (Wieland et al., 1992; Benson et al., 1998).

The affinities of 1,4-benzodiazepines are very similar among both recombinant and native DS receptors (Mohler et al., 1978; Pritchett and Seeburg, 1990; Graham et al., 1996). Several nonbenzodiazepine molecules, including CL 218,872 and zolpidem, exhibit some selectivity for recombinant GABA$_A$ receptors bearing an α_1 subunit and possess higher affinities in brain regions (e.g., cerebellum) that are relatively enriched in this species (Squires et al., 1979; Pritchett and Seeburg, 1990; Hadingham et al., 1993). Only recently have very high-affinity, selective compounds been developed for less abundant GABA$_A$ receptor isoforms. Thus, based on the ~10-fold selectivity of Ro 15-4513 for GABA$_A$ receptors containing an α_5 subunits (Hadingham et al., 1993; Lüddens et al., 1994), compounds such as RY-80, RY-24, and L-655,708 have been developed (Liu et al., 1995, 1996; Quirk et al., 1996). These imidazodiazepine derivatives exhibit high affinity and selectivity for wild-type and recombinant GABA$_A$ receptors containing an α_5 subunit (Liu et al., 1995, 1996; Skolnick et al., 1997; Sur et al., 1998, 1999). Using these compounds as probes, we now identify a single amino acid residue on the α_5 subunit (Ile215) that is critical for ligand selectivity at recombinant $\alpha_5\beta_3\gamma_2$ receptors.

Materials and Methods

Transfection of Recombinant GABA$_A$ Receptors and Membrane Preparation. cDNAs encoding rat α_1 and α_5 subunits were subcloned into a pRc/CMV vector, as described elsewhere (Skolnick et al., 1997). The β_3 and γ_{2S} cDNAs were subcloned into pcDNA3 (Gunnersen et al., 1996). Site-directed mutagenesis was performed with QuickChange mutagenesis kit (Stratagene, La Jolla, CA). Presence of the desired mutations was verified by direct sequencing. To verify the absence of new, unwanted substitutions, the complete coding regions were sequenced for each mutant. In case of α_5I215F mutant, the plasmid resulting from the QuickChange mutagenesis reaction was digested with PflMI endonuclease, and the fragment containing the desired I215F substitution was gel-purified and ligated into the similarly digested wild-type pRc/CMV/a5 vector. Human embryonic kidney 293 cells (American Type Culture Collection, Manassas, VA) were maintained at 37°C in 5% CO_2 as previously described (Gunnersen et al., 1996). Cells were transfected with equal amounts (5 μg of each DNA/90-mm dish) by calcium phosphate precipitation as described previously (Gorman et al., 1990). The cells were harvested 48 h after transfection, by washing with ice-cold phosphate-buffered saline and centrifuged at 1000*g*. Cells were washed three times by homogenization in ice-cold 50 mM Tris-citrate buffer, pH 7.8, and centrifuged at 20000*g*. These membrane suspensions were stored at −70°C until needed.

Radioligand Binding. Incubations were performed in a final volume of 600 to 1000 μl and contained resuspended cell membranes (~0.02–0.1 mg of protein), 0.2 M NaCl, [^{3}H]Ro 15-1788, or [^{3}H]RY-80 (87 and 55.4 Ci/mmol, respectively; DuPont-New England Nuclear, Boston, MA), and 50 mM Tris-citrate buffer, pH 7.8, to volume. In competition experiments, 50 μl of buffer was replaced by drugs. [^{3}H]Ro 15-1788 was used at concentrations equal to its K_D values at the respective receptor subtype. Nonspecific binding was determined with Ro 15-1788 (10 μM). [^{3}H]Muscimol binding was determined using a membrane suspension (~0.02–0.1 mg of protein), [^{3}H]muscimol (20 Ci/mmol; DuPont-New England Nuclear), and 50 mM Tris-citrate buffer, pH 7.8, to volume. Nonspecific binding in this case was determined in presence of 1 mM GABA. Assays were incubated at 4°C for 2 h and terminated by rapid filtration (Brandel M-48R, Gaithersburg, MD) through GF/B filters followed by two 5-ml washes with ice-cold Tris-citrate buffer. The filter-retained radioactivity was determined by liquid scintillation counting. Data were analyzed with GraphPad Prism software (GraphPad Software Inc., San Diego, CA), and K_i values were calculated from the equation, $K_i = IC_{50}/(1 + [\text{radioligand}]/K_D)$. Both K_i and K_D values were calculated from at least three independent experiments performed in duplicate. Statistical significance was determined using a one-way ANOVA followed by a Dunnett's multiple comparison post hoc test. Protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). RY-24 and RY-80 were synthesized at the University of Wisconsin-Milwaukee, CL 218,872 was obtained from Lederle Laboratories (Mont-St-Guibert, Belgium), and zolpidem was obtained from Synthelabo (Laboratoire Experimental Recherche Synthelabo, Paris, France). Flunitrazepam was purchased from Research Biochemicals International (Natick, MA). The structures of Ro 15-1788 and related α_5-selective benzodiazepines are given in Fig. 1. All other reagents and chemicals were from standard commercial sources.

Expression in *Xenopus laevis* Oocytes. *X. laevis* frogs were purchased from Xenopus-1 (Dexter, MI). Collagenase B was from Boehringer Mannheim (Indianapolis, IN). All other compounds were from Sigma Chemical Co. Capped cRNA was synthesized from linearized template cDNA encoding the subunits using mMESSAGE mMACHINE kits (Ambion, Austin, TX). Oocytes were injected with cRNAs encoding the specified α_5 subunit variants along with the β_3 and γ_2 subunits in a ratio of 1:1:1 as determined by gel electrophoresis. Mature *X. laevis* frogs were anesthetized by submersion in 0.1% 3-aminobenzoic acid ethyl ester, and oocytes were surgically removed. Follicle cells were removed by treatment with collagenase B for 2 h. Each oocyte was injected with 5 to 25 ng of cRNA in 50 nl of water and incubated at 19°C in modified Barth's saline (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 100 μg/ml gentamicin, and 15 mM HEPES, pH 7.6). Recordings were performed 1 to 7 days post injection.

Electrophysiological Recordings. Oocytes were perfused at room temperature in a Warner Instruments oocyte recording chamber #RC-5/18 (Hamden, CT) with perfusion solution (115 mM NaCl, 1.8 mM $CaCl_2$, 2.5 mM KCl, 10 mM HEPES, pH 7.2). Perfusion solution was gravity fed continuously at a rate of 15 ml/min. GABA was dissolved in the perfusion solution. Drugs were added as a 10

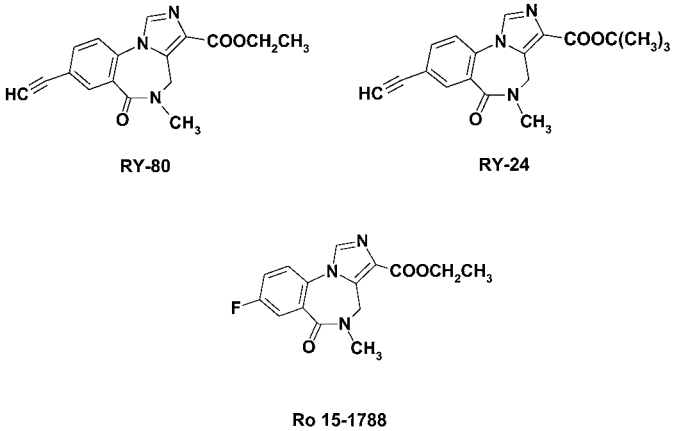

Fig. 1. Structures of imidazobenzodiazepines with selectivity for α_5-containing GABA$_A$ receptors: Comparison with Ro 15-1788.

mM solution in ethanol to the perfusion solution to achieve the appropriate concentration.

Unless otherwise indicated, current responses to GABA application were measured under two-electrode voltage clamp at a holding potential of −60 mV. Data were collected using a GeneClamp 500 amplifier and Axoscope software (Axon Instruments, Foster City, CA). GABA responses were measured at concentrations of GABA equal to its EC_{50} values for all receptors tested. GABA responses in the presence of saturating concentrations of drugs are reported as a percentage of the response to GABA alone ("percent control response", or "% control"). Data were fitted to a four-parameter logistic using GraphPad Prizm. Statistical significance was determined using a one-way ANOVA followed by a Bonferroni's multiple comparison post hoc test.

Results

Amino Acid Ile215 on the α_5 Subunit Contributes to Ligand Selectivity at $\alpha_5\beta_3\gamma_2$ Receptors. In an attempt to define amino acid residues on the α_5 subunit that are important for high affinity and selectivity to compounds such as RY-80, we considered amino acid residues that are conserved among all other DS α-subunits (Fig. 2). Based on this sequence comparison, four residues in the α_5 subunit N-terminal domain were selected for the initial analysis as the most likely to be involved in defining ligand selectivity. The corresponding amino acids on the other DS α-subunits were substituted on the α_5 subunit, yielding α_5G24R, α_5P166T, α_5H196D, and α_5I215V variants, respectively. Wild-type and mutant $\alpha_5\beta_3\gamma_2$ receptors were transiently expressed in the human embryonic kidney 293 cells. No additional mutations were found after sequencing the complete coding regions for each mutant. The screening strategy applied to identify amino acid(s) important for ligand selectivity at α_5 subunit was based on the premise that at the concentrations approximating the K_D value of each ligand the binding of [^{3}H]Ro 15-1788 and [^{3}H]RY-80 to the wild-type $\alpha_5\beta_3\gamma_2$ receptor will be similar, yielding a ratio of ~1. If a particular amino acid substitution introduced in the α_5 subunit altered [^{3}H]RY-80 binding, this ratio would change. Among the amino acid substitutions tested, only α_5I215V yielded a dramatically different ratio (17.8) than the value obtained (1.04) in wild-type $\alpha_5\beta_3\gamma_2$ receptors (data not shown). Based on this observation, the mutant receptor α_5I215V$\beta_3\gamma_2$ was chosen for fur-

```
                                                                  ↓
α1  ---------- ---------- -----QPSQD ELKDNTTVFT RILDRLLDGY 25
α2  ---------- ---------- -----NIQED EAKNNITIFT RILDRLLDGY 25
α3  QGESRRQEPG DFVKQDIGGL SPKHAPDIPD DSTDNITIFT RILDRLLDGY 50
α5  ---------- ---------- -QMPTSSVQD ETNDNITIFT RILDGLLDGY 29

α1  DNRLRPGLGE RVTEVKTDIF VTSFGPVSDH DMEYTIDVFF RQSWKDERLK 75
α2  DNRLRPGLGD SITEVFTNIY VTSFGPVSDT DMEYTIDVFF RQKWKDERLK 75
α3  DNRLRPGLGD AVTEVKTDIY VTSFGPVSDT DMEYTIDVFF RQTWHDERLK 100
α5  DNRLRPGLGE RITQVRTDIY VTSFGPVSDT EMEYTIDVFF RQSWKDERLR 79

α1  FKGPMTVLRL NNLMASKIWT PDTFFHNGKK SVAHNMTMPN KLLRITEDGT 125
α2  FKGPMNILRL NNSMASKIWT PDTFFHNGKK SVAHNMTMPN KLLRIQDDGT 125
α3  FDGPMKILPL NNLLASKIWT PDTFFHNGKK SVAHNMTTPN KLLRLVDNGT 150
α5  FKGPMQRLPL NNLLASKIWT PDTFFHNGKK SIAHNMTTPN KLLRLEDDGT 129

                                                  ↓
α1  LLYTMRLTVR AECPMHLEDF PMDAHACPLK FGSYAYTRAE VVYEWTREPA 175
α2  LLYTMRLTVQ AECPMHLEDF PMDAHSCPLK FGSYAYTTSE VTYIWTYNPS 175
α3  LLYTMRLTIH AECPMHLEDF PMDVHACPLK FGSYAYTKAE VIYSWTLGKN 200
α5  LLYTMRLTIS AECPMQLEDF PMDAHACPLK FGSYAYPNSE VVYVWTNGST 179

                  ↓                               ↓
α1  RSVVVAEDGS RLNQYDLLGQ TVDSGIVQSS TGEYVVMTTH FHLKRKIGYF 225
α2  DSVQVAPDGS RLNQYDLLGQ SIGKETIKSS TGEYTVMTAH FHLKRKIGYF 225
α3  KSVEVAQDGS RLNQYDLLGH VVGTEIIRSS TGEYVVMTTH FHLKRKIGYF 250
α5  KSVVVAEDGS RLNQYHLMGQ TVGTENISTS TGEYTIMTAH FHLKRKIGYF 229

                TM1
                ___________________
α1  VIQTYLPCIM TVILSQVSFW LNRESVPART VFGVTTVLTM TTLSISARNS 275
α2  VIQTYLPCIM TVILSQVSFW LNRESVPART VFGVTTVLTM TTLSISARNS 275
α3  VIQTYLPCIM TVILSQVSFW LNRESVPART VFGVTTVLTM TTLSISARNS 300
α5  VIQTYLPCIM TVILSQVSFW LNRESVPART VFGVTTVLTM TTLSISARNS 279
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Fig. 2. Alignment of the rat $GABA_A$ α_1, α_2, α_3, and α_5 subunits. The sequences shown represent N-terminal regions of the corresponding α-subunits up to the first putative transmembrane domain (TM1). Arrows: amino acids chosen for mutagenesis.

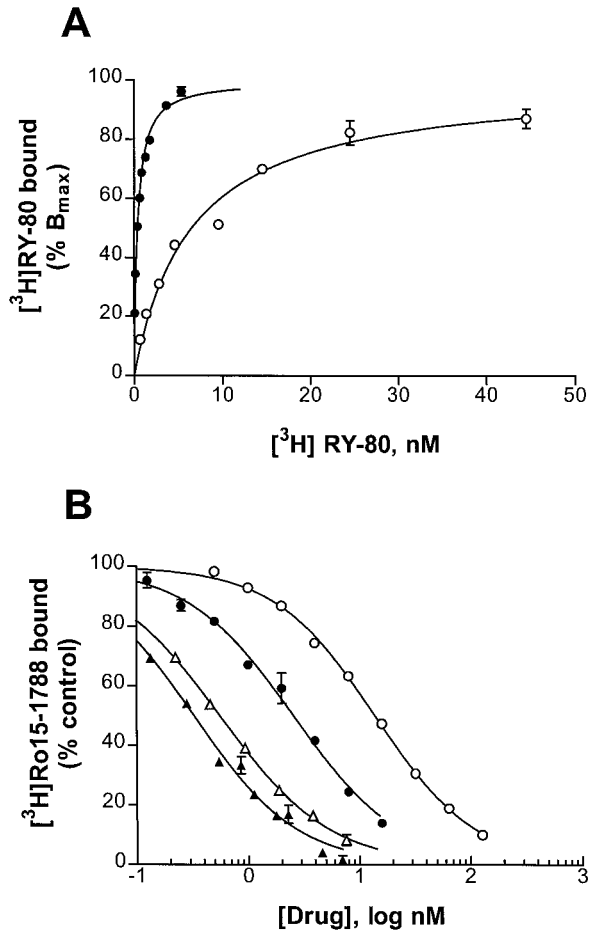

Fig. 3. Reduced affinities of α_5-selective ligands at α_5I215V$\beta_3\gamma_2$ receptor. A, binding of [^{3}H]RY-80 to wild-type $\alpha_5\beta_3\gamma_2$ (●) and α_5I215V$\beta_3\gamma_2$ (○) receptors. These are representative isotherms with K_D values for [^{3}H]RY-80 of 0.38 nM (B_{max} = 1008 fmol/mg of protein) and 6.5 nM (B_{max} = 2419 fmol/mg of protein) for the $\alpha_5\beta_3\gamma_2$ and α_5I215V$\beta_3\gamma_2$ receptors, respectively. Because of differences in receptor density and/or transfection efficiency, the quantity of bound [^{3}H]RY-80 was normalized to the estimated B_{max} values. K_D and B_{max} values were calculated by nonlinear least-squares fit of specifically bound [^{3}H]RY-80 (under *Materials and Methods*). B, displacement of [^{3}H]Ro 15-1788 binding with RY-24 from wild-type $\alpha_5\beta_3\gamma_2$ (●) and α_5I215V$\beta_3\gamma_2$ (○) receptors. [^{3}H]Ro 15-1788 was used at concentrations equal to its K_D value for each receptor. K_i values were calculated using the equation of Cheng and Prusoff (under *Materials and Methods*). K_i values for RY-24 were equal to 0.71 and 4.6 nM for $\alpha_5\beta_3\gamma_2$ and α_5I215V$\beta_3\gamma_2$ receptors, respectively. Representative curves are shown. Also shown are representative [^{3}H]Ro 15-1788 saturation isotherms for wild-type $\alpha_5\beta_3\gamma_2$ (▲) and α_5I215V$\beta_3\gamma_2$ (△) receptors. K_D values for [^{3}H]Ro 15-1788 were 0.34 and 0.57 nM in wild-type and mutant receptors, respectively. The data from [^{3}H]Ro 15-1788 saturation isotherms were transformed as $(1 - B/B_{max}) \times 100\%$ for ease of comparison.

ther analysis. Saturation analysis revealed a ~16-fold decrease in affinity for [^{3}H]RY-80 in α_5I215V$\beta_3\gamma_2$ receptor (5.6 ± 0.6 nM) compared with wild-type receptor (0.35 ± 0.02 nM) (Fig. 3A). Consistent with these data, the affinities of the α_5-selective compounds RY-80 and RY-24 in mutant receptors were reduced ~20- and ~10-fold, respectively, in competition studies using [^{3}H]Ro 15-1788 (Fig. 3B; Table 1). In contrast, the affinity of the nonselective ligand [^{3}H]Ro 15-1788 was not significantly affected by this mutation (Table 1; Fig. 3B).

Properties of α_1V211I$\beta_3\gamma_2$ Receptor. The dramatic effect produced by substitution of a valine in position $\alpha_5$215 on the affinities of the α_5-selective ligands prompted an examination of the effect of a cognate substitution (isoleucine for valine) on position $\alpha_1$211 (corresponding to $\alpha_5$215). Consistent with the previous reports (Liu et al., 1996) both RY-24 and RY-80 exhibited low affinities (56 ± 17 and 47 ± 8 nM, respectively) for wild-type $\alpha_1\beta_3\gamma_2$ receptors (Table 1). This exchange (α_1V211I$\beta_3\gamma_2$) increased the affinities of RY-24 and RY-80 by more than one order of magnitude (to 3.3 ± 0.3 and 3.7 ± 0.3 nM, respectively) (Table 1). Moreover, this mutation decreased the affinities of the α_1-selective ligands zolpidem and CL 218,872 by 3-fold without affecting the affinity of [^{3}H]Ro 15-1788 (Table 1; Fig. 3).

Properties of Mutant $\alpha_5\beta_3\gamma_2$ Receptors with Lipophilic Amino Acid Substitutions in Position $\alpha_5$215. Based on the hypothesis that interaction with a lipophilic pocket is required for ligand selectivity at the α_5-containing GABA$_A$ receptors (Liu et al., 1996), isoleucine in position 215 of the wild-type α_5 subunit was exchanged with alanine, leucine, or phenylalanine. The ligand binding properties of the α_5I215A$\beta_3\gamma_2$ receptor were similar to those of the α_5I215V$\beta\gamma$ receptor. The alanine substitution decreased (by >10-fold) the affinities of both RY-24 and RY-80 without altering the affinity of [^{3}H]Ro 15-1788. In contrast to the valine substitution, introduction of alanine in position $\alpha_5$215 increased the affinity of CL 218,872 by 10-fold (Table 1). Substitution of either leucine or the more lipophilic phenylalanine resulted in no significant change in the affinities of RY-24 and RY-80. Furthermore, substitution α_5I215F resulted in a slightly reduced affinity of Ro 15-1788 and a lower affinity of CL 218,872. The affinity of flunitrazepam was unchanged (compared with wild-type receptors) for both α_5I215A$\beta_3\gamma_2$ and α_5I215L$\beta_3\gamma_2$ receptors (K_i values of 0.6 ± 0.1 and 0.9 ± 0.2 nM, respectively), whereas the affinity for flunitrazepam at the α_5I215F$\beta_3\gamma_2$ receptors was decreased ~7-fold (K_i = 6.9 ± 1.8 nM). A decrease in the affinity of α_5-selective ligands at α_5I215A$\beta_3\gamma_2$ and α_5I215V$\beta_3\gamma_2$ receptors prompted us to further reduce the size of the side chain of the residue $\alpha_5$215. However, introduction of glycine resulted in levels of [^{3}H]Ro 15-1788, [^{3}H]RY-80, or [^{3}H]muscimol binding that were barely detectable.

Properties of Mutant $\alpha_5\beta_3\gamma_2$ Receptors with Charged or Polar Amino Acid Residues in Position $\alpha_5$215. Substitution of the negatively charged aspartate residue at position 215 produced a modest decrease in affinity of [^{3}H]Ro 15-1788 (1.7 ± 0.3 nM compared with 0.36 ± 0.04 nM for the wild-type receptor), and a similar, modest decrease in the affinities of RY-80 and RY-24 binding (Table 1). Substitution of a threonine (a more hydrophilic amino acid) for isoleucine yielded a receptor with properties similar to α_5I215V$\beta_3\gamma_2$ receptor. This receptor produced a 10-fold decrease in affinities of both RY-24 and RY-80 without significantly affecting [^{3}H]Ro 15-1788 binding. However, isoleucine-to-threonine substitution resulted in a small (3-fold) increase in the affinity of flunitrazepam (Table 1). Substitution of a basic lysine residue for isoleucine produced a 5- to 6-fold decrease in the affinities of both RY-24 and RY-80 without affecting the affinity of [^{3}H]Ro 15-1788. The affinity of flunitrazepam also was not substantially changed (Table 1). Additionally, α_5I215D$\beta_3\gamma_2$, α_5I215T$\beta_3\gamma_2$, and α_5I215K$\beta_3\gamma_2$ receptors displayed an increase in affinity of CL 218,872 (4-fold) compared with wild-type receptors; however, none of the amino acid substitutions in position $\alpha_5$215 yielded a receptor variant with any measurable affinity for zolpidem.

Efficacy of RY-24 and RY-80 at Wild-Type and Mutant $\alpha_5\beta_3\gamma_2$ Receptors. Mutation of a conserved histidine residue in the N-terminal domain of all DS α-subunits (α_1H101R, α_2H101R, α_3H126R, and α_5H105R) to arginine not only confers diazepam insensitivity to the respective $\alpha_x\beta_{2/3}\gamma_2$ receptors but also alters the efficacies of several ligands at these receptors (Benson et al., 1998). Based on these observations, the potential role of $\alpha_5$215 in modulating ligand efficacy was examined. Three mutant receptors, α_5I215V$\beta_3\gamma_2$, α_5I215K$\beta_3\gamma_2$, and α_5I215T$\beta_3\gamma_2$ were examined. Introduction of either valine, lysine, or threonine in position $\alpha_5$215 did not change the potency of GABA at these receptor

TABLE 1

Binding properties of the wild-type and mutant $\alpha_1\beta_3\gamma_2$ and $\alpha_5\beta_3\gamma_2$ receptors

K_D values for [^{3}H]Ro 15-1788 and [^{3}H]RY-80 were determined in saturation experiments. K_i values were derived from the displacement of [^{3}H]Ro 15-1788 at a concentration equivalent to its K_D value at each receptor subtype. Values are mean ± S.E.M. for at least three experiments performed in duplicate.

α-Subunit	K_D		K_i				
	[^{3}H]Ro15-1788	[^{3}H]RY-80	RY-24	RY-80	Flunitrazepam	CL 218,872	Zolpidem
	nM				*nM*		
Wild-type α_5	0.36 ± 0.04	0.35 ± 0.02	0.7 ± 0.1	0.4 ± 0.1	1.0 ± 0.1	411 ± 35	>5000
α_5I215G	ND	ND					
α_5I215A	0.43 ± 0.04	4.9 ± 0.2	5.7 ± 0.3	4.0 ± 0.4	0.6 ± 0.1	41 ± 5.0	>5000
α_5I215V	0.6 ± 0.1	5.6 ± 0.6	4.6 ± 0.2	6.1 ± 0.5	0.6 ± 0.1	244 ± 6.0	>5000
α_5I215L	0.36 ± 0.01	0.10 ± 0.01	0.4 ± 0.1	0.14 ± 0.02	0.9 ± 0.2	376 ± 70	>5000
α_5I215F	1.5 ± 0.1	0.30 ± 0.04	1.0 ± 0.3	0.23 ± 0.04	6.9 ± 1.8	642 ± 54	>5000
α_5I215T	0.53 ± 0.01	3.4 ± 0.2	6.1 ± 0.3	NA	0.4 ± 0.1	117 ± 26	>5000
α_5I215D	1.7 ± 0.3	0.66 ± 0.04	1.6 ± 0.2	NA	NA	97 ± 17	>5000
α_5I215K	0.31 ± 0.02	1.9 ± 0.3	4.5 ± 0.5	NA	1.9 ± 0.7	128 ± 27	>5000
Wild-type α_1	1.3 ± 0.3	47 ± 2	56 ± 17	47 ± 8	2.4 ± 0.6	245 ± 44	33 ± 7
α_1 V211I	1.1 ± 0.1	2.2 ± 0.3	3.3 ± 0.3	3.7 ± 0.3	9.0 ± 0.6	609 ± 99	99 ± 10

ND, no detectable binding; NA, not analyzed.

subtypes (EC_{50} = ~30 μM for all receptors tested; see Table 2, legend).

The benzodiazepine flunitrazepam potentiated GABA-mediated currents in wild-type $\alpha_5\beta_3\gamma_2$ receptors as well as the α_5I215V$\beta_3\gamma_2$, α_5I215K$\beta_3\gamma_2$, and α_5I215T$\beta_3\gamma_2$, mutants (Table 2). Consistent with previous results, RY-24 and RY-80 act as inverse agonists at $\alpha_5\beta_3\gamma_2$ receptors (Liu et al., 1995, 1996; Skolnick et al., 1997), producing a maximum reduction in GABA-evoked currents to 75 ± 2 and 70 ± 6% of the control response, respectively, when GABA was applied at its EC_{50} value (Table 2). A similar reduction of the GABA-evoked currents was produced by RY-24 and RY-80 at α_5I215K$\beta_3\gamma_2$ receptors (72 ± 4 and 62 ± 4% of control response, respectively). In contrast, neither RY-24 nor RY-80 affected GABA currents on either α_5I215V$\beta_3\gamma_2$ or α_5I215T$\beta_3\gamma_2$ receptors at concentrations of up to 1 μM, sufficient to saturate receptors.

Discussion

The objective of the present study was to localize the molecular features of the α_5 subunit responsible for the high affinity and selectivity of ligands such as RY-80. Because the N-terminal extracellular domain exhibits the greatest sequence divergence among α-subunits, it was hypothesized that this region was most likely to be involved in defining ligand selectivity. Four amino acid residues conserved in this region among the α_{1-3} subunits but different in the α_5 subunit (α_5G24, α_5P166, α_5H195, and α_5I215) were considered. Substitution of each of these four residues in the α_5 subunit with the corresponding amino acids conserved among the α_{1-3} subunits resulted in a significant reduction in [^{3}H]RY-80 binding only in the α_5I215V$\beta_3\gamma_2$ mutant (under *Results*). Saturation analysis confirmed that this reduction in [^{3}H]RY-80 binding reflects an ~16-fold increase in the K_D value of this radioligand (Table 1; Fig. 3) compared with wild-type α_5I215V$\beta_3\gamma_2$ receptors. This mutation concomitantly reduced the selectivity of RY-80 for $GABA_A$ receptors containing an α_5 subunit from ~134- to ~8.4-fold compared with cognate receptors containing an α_1 subunit. This mutation also increased the K_i of RY-24 by >6.0-fold and reduced its selectivity for α_5-containing $GABA_A$ receptors from ~80- to ~12-fold (Table 1; Fig. 3). Because all known α_5-selective ligands are structurally related (Fig. 1), it is not known whether the affinities of other, structurally unrelated compounds exhibiting α_5-subtype selectivity would be similarly affected. However, the observation that the affinity of Ro 15-1788 was not significantly altered in the α_5I215V$\beta_3\gamma_2$ mutant and that the affinity of CL 218,872 was slightly increased supports the hypothesis that this amino acid is essential for a selective interaction at $\alpha_5\beta_3\gamma_2$ receptors. We hypothesized that if Ile215 is essential for ligand selectivity at $\alpha_5\beta_3\gamma_2$ receptors, then substitution of this residue at this corresponding position in $\alpha_1\beta_3\gamma_2$ receptors (i.e., at Val211) should produce a significant *increase* in the affinity of compounds such as RY-80. Consistent with this hypothesis, the affinities of both RY-80 and RY-24 were increased ~20-fold in α_1V211I$\beta_3\gamma_2$, whereas the affinities of other ligands were either unchanged or slightly reduced (Fig. 4).

Based on the affinities of a structurally diverse group of

TABLE 2

Mutation of residue $\alpha_5$215 changes efficacy of benzodiazepine-site ligands

Wild-type or mutant $GABA_A$ receptors were expressed in X. laevis oocytes and electrophysiological recordings were performed as described under *Materials and Methods*. Responses to concentrations of GABA in presence of drugs are reported as a percentage of the response to GABA alone (% control). GABA was applied at concentration 30 μM, approximating its EC_{50} value at each receptor subtype tested (EC_{50} = 25.8 ± 7.8 μM). RY-80, RY-24, and flunitrazepam were used at a saturating concentration of 1 μM. Values are means ± S.D. for at least three oocytes.

Receptor Subtype	RY-80	RY-24	Flunitrazepam
$\alpha_5\beta_3\gamma_2$	70 ± 6	75 ± 2	168 ± 14
α_5I215V $\beta_3\gamma_2$	91 ± 7	94 ± 6	154 ± 5
α_5I215T $\beta_3\gamma_2$	97 ± 7	102 ± 7	167 ± 5
α_5I215K $\beta_3\gamma_2$	62 ± 4	73 ± 4	169 ± 18

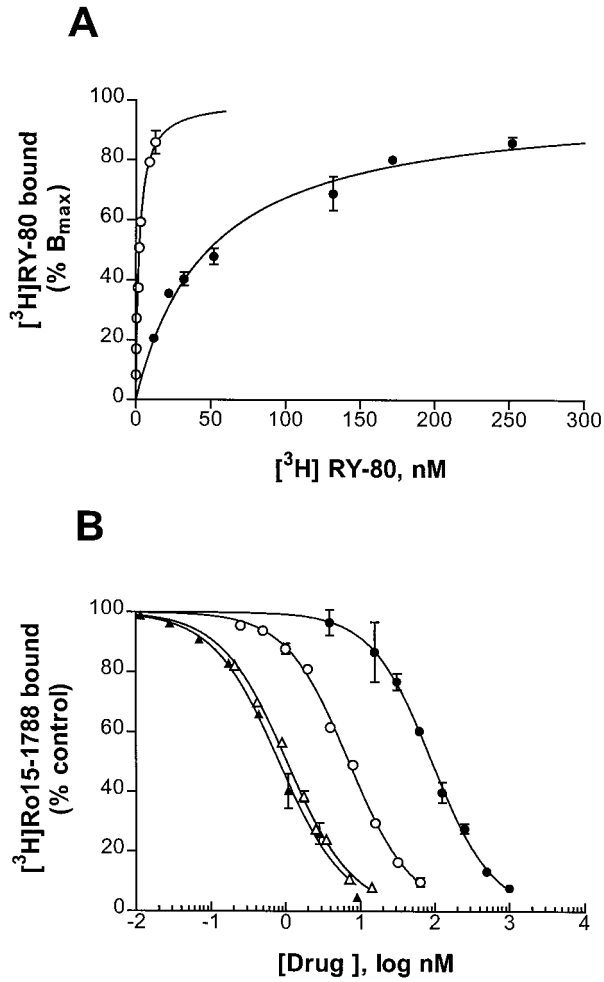

Fig. 4. Increased affinities of α_5-selective ligands at α_1V211I$\beta_3\gamma_2$ receptor. A, binding of [^{3}H] RY-80 to wild-type $\alpha_1\beta_3\gamma_2$ (●) and α_1V211I$\beta_3\gamma_2$ (○) receptors. These are representative isotherms with K_D values for [^{3}H] RY-80 of 48.4 nM (B_{max} = 353 fmol/mg of protein) and 2.3 nM (B_{max} = 1453 fmol/mg of protein) for the $\alpha_1\beta_3\gamma_2$ and α_1V211I$\beta_3\gamma_2$ receptors, respectively. Because of differences in receptor density and/or transfection efficiency the quantity of bound [^{3}H]RY-80 was normalized to the estimated B_{max} values. K_D and B_{max} values were calculated by nonlinear least-squares fit of specifically bound [^{3}H]RY-80 (under *Materials and Methods*). B, displacement of [^{3}H]Ro 15-1788 binding with RY-24 from wild-type $\alpha_1\beta_3\gamma_2$ (●) and α_1V211I$\beta_3\gamma_2$ (○) receptors. [^{3}H]Ro 15-1788 was used at concentrations equal to its K_D value at each receptor. K_i values were calculated using the equation of Cheng and Prusoff (under *Materials and Methods*). K_i values for RY-24 were equal to 55.9 and 3.3 nM for $\alpha_1\beta_3\gamma_2$ and α_1V211I$\beta_3\gamma_2$ receptors, respectively. Representative curves are shown. Also shown are representative [^{3}H]Ro 15-1788 saturation isotherms for wild-type $\alpha_1\beta_3\gamma_2$ (▲) and α_1V211I$\beta_3\gamma_2$ (△) receptors. K_D values for [^{3}H]Ro 15-1788 were 1.2 and 0.82 nM in wild-type and mutant receptors, respectively. The data from [^{3}H]Ro 15-1788 saturation isotherms were transformed as $(1 - B/B_{max}) \times 100\%$ for ease of comparison.

ligands, an inclusive pharmacophore of the $\alpha_5\beta_2\gamma_2$ receptor has been developed (Liu et al., 1996). A large lipophilic regionl (L_2) appears to contribute to the unique pharmacological properties of this $GABA_A$ receptor isoform (Liu et al., 1996). Thus, although the L_2 descriptor is common to the pharmacophore of other $GABA_A$ receptors (e.g., $\alpha_1\beta_2\gamma_2$ receptors), the larger volume of L_2 in $\alpha_5\beta_2\gamma_2$ receptors has been proposed to result in low affinities for ligands (e.g., zolipidem) that do not extend into this domain, and very high affinities for compounds (e.g., RY-80) capable of filling this area (Liu et al., 1996). Both the >10-fold reduction in the affinities of α_5-selective ligands produced by a subtle change in the lipophilicity of residue 215 (i.e., isoleucine-to-valine) and the corresponding increases in affinity produced by the cognate (i.e., "back") mutation in $\alpha_1\beta_3\gamma_2$ receptors prompted us to hypothesize that Ile215 constitutes a portion of this L_2 domain. If this hypothesis is correct, then increasing the lipophilicity of the residue at $\alpha_5$215 should increase the affinities of ligands such as RY-80 and RY-24 without remarkably affecting the affinities of nonselective ligands. Conversely, reducing lipophilicity, either by substituting a nonpolar amino acid with a smaller side chain or introduction of polar or charged residues at $\alpha_5$215 should produce a further reduction in the affinities of these compounds. Increasing the lipophilicity of this residue (e.g., leucine, phenylalanine) did not remarkably affect the affinities of RY-80 and RY-24. In contrast, 5- to 6-fold decreases in affinities of nonspecific ligands Ro 15-1788 and flunitrazepam were observed (Table 1). However, neither reducing lipophilicity by introducing an alanine residue nor substitution of polar and/or charged residues (e.g., threonine, lysine, and aspartic acid) decreased the affinities of the α_5-selective ligands beyond that produced by the original α_5I215V mutation (Table 1). These observations indicate that although contributing to the high affinity and selectivity of RY-80 and RY-24 at $\alpha_5\beta_3\gamma_2$ receptors, residue 215 may not directly participate in the formation of L_2. Thus, the original model, based on an L_2 lipophilic region exerting influence over ligand selectivity at α_5-containing $GABA_A$ receptors merits reconsideration. Furthermore, in the absence of crystallographic studies of ligand-bound receptor (Dingledine et al., 1999), it is possible that $\alpha_5$215 modulates the affinities of RY-24 and RY-80 through an allosteric mechanism rather than as an integral part of the binding pocket.

Although the present findings demonstrate that α_5Ile215 substantially contributes to ligand selectivity at $\alpha_5\beta_3\gamma_2$ receptors, it cannot be the sole determinant of the unique pharmacological profile of this receptor isoform. Thus, RY-80 and RY-24 retain modest selectivities (~8–14-fold) for α_5I215V, α_5I215A, and α_5I215T$\beta_3\gamma_2$ receptors compared with $\alpha_1\beta_3\gamma_2$ receptors. Moreover, despite a ~20-fold increase in the affinities of RY-80 and RY-24 for $\alpha_1\beta_3\gamma_2$ receptors containing a back mutation (i.e., α_1V211I$\beta_3\gamma_2$), these compounds remain significantly (~5-fold) more potent in wild-type $\alpha_5\beta_3\gamma_2$ receptors. Finally, the very low affinity of zolpidem at wild-type α_5-containing receptors is maintained through a range of mutations at this residue. Other likely candidates contributing to the pharmacological profile of $\alpha_5\beta_3\gamma_2$ receptors (i.e., selectivity for RY-80 and RY-24) are one or more of the other N terminus amino acid residues that differ between the α_5 and α_{1-3} subunits, as well as residues on the γ-subunit that may act in concert with α_5I215 to produce a unique pharmacology. This latter hypothesis is consistent with both the dramatic reduction in the affinity of zolpidem produced by substitution of a γ_3 for a γ_2 subunit in recombinant α_1-containing $GABA_A$ receptors (Lüddens et al., 1994), and the

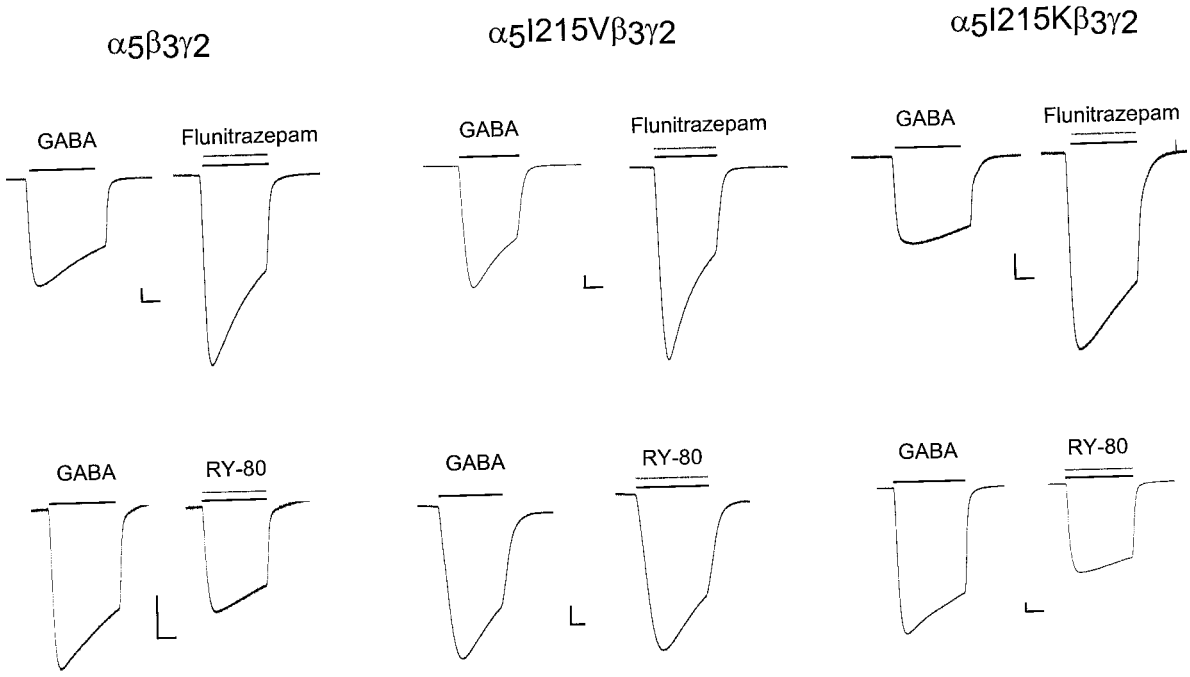

Fig. 5. Representative voltage-clamp trace recordings from *X. laevis* oocytes injected with the wild-type or mutant $\alpha_5\beta_3\gamma_2$ constructs $\alpha_5\beta_3\gamma_2$ (left), α_5I215V$\beta_3\gamma_2$ (center), and α_5I215K$\beta_3\gamma_2$ (right). GABA (30 μM) was perfused over oocyte for the duration indicated by the black bar. GABA plus saturating concentrations of compound (1 μM) were perfused over oocyte for the duration indicated by the gray bar. Oocytes were voltage-clamped at −60 mV. Scale bars, 50 nA/10 s. Note that flunitrazepam potentiates currents at all receptor subtypes tested, whereas RY-80 changes its mode of action from being an inverse agonist at the wild-type $\alpha_5\beta_3\gamma_2$ and mutant α_5I215K$\beta_3\gamma_2$ receptors to being an antagonist at the α_5I215V$\beta_3\gamma_2$ receptor.

absolute requirement for a γ-subunit for high-affinity binding of benzodiazepine-site ligands (Pritchett et al., 1989; Wong et al., 1992; Boileau et al., 1998).

The γ-subunit has been closely linked to the efficacy of benzodiazepine-site ligands (Knoflach et al., 1991; Puia et al., 1991). Nonetheless, a series of conservative mutations (α_1H101R, α_2H101R, α_3H126R, and α_5H105R) that imparts diazepam insensitivity to the corresponding $\alpha_x\beta_{2/3}\gamma_2$ receptors (Wieland et al., 1992; Benson et al., 1998) were recently shown to affect the efficacy of several benzodiazepine-site ligands (Benson et al., 1998). This finding prompted us to examine the role of $\alpha_5$215 in controlling ligand efficacy at recombinant $\alpha_5\beta_3\gamma_2$ receptors. Three mutations (α_5I215V, α_5I215T, and α_5I215K) were chosen for study based on their structural divergence from the residue present in wild-type receptors and the reduced affinities of α_5-selective agents. Introduction of valine, lysine, or threonine in position $\alpha_5$215 does not affect the potency of GABA compared with wild-type receptors. These mutations did not alter the ability of flunitrazepam to act as an agonist, increasing currents evoked by subsaturating concentrations of GABA (Fig. 5; Table 2). In contrast, the characteristic ability of RY-80 and RY-24 to reduce GABA-gated currents in wild-type $\alpha_5\beta_3\gamma_2$ receptors (Fig. 5; Table 2; Liu et al., 1996)) was abolished in α_5I215V$\beta_3\gamma_2$ and α_5I215T$\beta_3\gamma_2$ receptors, but retained in the α_5I215K$\beta_3\gamma_2$ mutants. The failure to observe a change in the efficacies of RY-24 and RY-80 in the α_5I215K mutants indicates this effect on ligand efficacy is independent of changes in ligand affinity because all three mutations reduced (albeit to different degrees) the affinities of these α_5-selective ligands. These data demonstrate that in addition to a well described role in defining the affinities of benzodiazepine-site ligands, the α-subunit can also impact ligand efficacy. The identification of amino acid residues contributing to ligand selectivity at $GABA_A$ receptor isoforms may provide insights resulting in compounds with a more limited spectrum of action than traditional 1,4-benzodiazepines.

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