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Key Amino Acids in the γ Subunit of the γ-Aminobutyric Acid$_A$ Receptor that Determine Ligand Binding and Modulation at the Benzodiazepine Site

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SUMMARY

Pharmacological analyses of γ-aminobutyric acid$_A$ (GABA$_A$) receptor subtypes have suggested that both the α and γ subunits, but not the β subunit, contribute to the benzodiazepine binding site. We took advantage of the different pharmacological properties conferred by the inclusion of different γ subunits in the receptor macromolecule to identify amino acids γ2Phe77 and γ2Met130 as key determinants of the benzodiazepine binding site. γ2Phe77 was required for high affinity binding of the benzodiazepine site ligands flumazenil, CL218,872, and methyl-β-carboline-3-carboxylate but not flunitrazepam. This amino acid was, however, required for allosteric modulation by flunitrazepam, as well as other benzodiazepine site ligands. In contrast, γ2Met130 was required for high affinity binding of flunitrazepam, clonazepam, and triazolam but not flumazenil, CL218,872, or methyl-β-carboline-3-carboxylate and did not affect benzodiazepine efficacy. Introduction of the phenylalanine and methionine into the appropriate positions of γ1 was not sufficient to confer high affinity for the benzodiazepine site ligand zolpidem. These data show that γ2Phe77 and γ2Met130 are necessary for high affinity binding of a number of benzodiazepine site ligands. Although most previous studies have focused on the contribution of the α subunit, we demonstrated a critical role for the γ subunit at the benzodiazepine binding site, indicating that this modulatory site is located at the interface of these two subunits. Furthermore, γ2Phe77 is homologous to α1Phe64, which has been previously shown to be a key determinant of the GABA binding site, suggesting a conservation of motifs between different ligand binding sites on the GABA$_A$ receptor.

The GABA$_A$ receptor, a member of the ligand-gated ion channel family, mediates synaptic inhibition through the gating of chloride ions, resulting in hyperpolarization of the cell membrane. It is the site of action of a number of pharmacological agents, including BZs, barbiturates, and anesthetics. The hetero-oligomeric receptor is formed from the coassembly of five different subunit classes [α, β, γ, δ (1, 2), and ε (3, 4)] in a presumed pentameric arrangement (5, 6) to yield a family of receptor subtypes. It is the heterogeneity within these subunits that provides the molecular basis for the differences in pharmacology of receptor subtypes (7).

Classic BZ pharmacology is exhibited by receptors containing a γ2 subunit in combination with an α and a β subunit (8). The affinity of BZ ligands for the receptor is dependent on the α subunit isoform, and hence compounds such as CL218,872 and zolpidem have higher affinity for α1βnγ2 (n = 1, 2, or 3) receptors than for other α subunit-containing receptors (9, 10), and flunitrazepam and diazepam (11, 12) have very low affinity (>10 μM) for α4βnγ2 and α6βnγ2. Mutagenesis studies have identified two amino acids on the α subunit as contributing to the BZ binding site (13, 14). Photoaffinity-labeling of the receptor by BZ ligands [^{3}H]flunitrazepam and [^{3}H]Ro15–4513 also highlights the proximity of the α subunit (15, 16); His102 has been shown to be the major site of incorporation of [^{3}H]flunitrazepam into the α1 subunit (17). It is clear, however, from the studies of both Stephenson *et al.* (15) and McKernan *et al.* (16) that the γ subunit also contributes significantly to the BZ binding site. In addition, pharmacological studies have demonstrated that the type of γ subunit (γ1, γ2, or γ3) coexpressed with an α and a β subunit profoundly influences the affinity and efficacy of BZs such as flumazenil and flunitrazepam (18–21). GABA$_A$ receptors containing a γ1 subunit have a >5000-fold lower affinity for the antagonist flumazenil than do those containing a γ2 or γ3 subunit, whereas γ1- and γ3-containing receptors have a 10–30-fold lower affinity for flunitrazepam than do receptors containing γ2 (18–22). In this study, we used these two observations as a starting point to identify the key amino acids of the γ2 subunit that contribute to the BZ site of the GABA$_A$ receptor.

ABBREVIATIONS: GABA, γ-aminobutyric acid; BZ, benzodiazepine; β-CCM, methyl-β-carboline-3-carboxylate; PCR, polymerase chain reaction; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Materials and Methods

Construction of Chimeric Subunits

Human α1, β1, γ1, γ2S, and γ3 cDNAs have been reported previously (10, 19, 21). The γ2S splice isoform is used throughout this study and is referred to simply as γ2. A PCR-based method, as described previously (23), was used in construction of the γ1/γ2 chimeric subunits.

γ1Δ2.1. A γ2 PCR product was generated with the pCDM8 vector-specific, sense oligonucleotide 5′-AGTCCGAAAGAATCTGCTCCCTGCTT-3′ and the γ2-specific, antisense primer 5′-GACAATGAGTATGCATGGGATATAGG-3′. This was digested with *Hin*dIII and *Nsi*I and inserted into similarly cut γ1 in pCDM8.

γ1Δ2.2. A γ2 fragment was obtained using the pCDM8 sense primer and the γ2-specific antisense primer 5′-GTGTTCATCCATGGGAAAATTGTGCA-3′. This was digested with *Hin*dIII and *Nco*I and inserted into similarly cut γ1 in pBS. The construct was subcloned into pcDNAIamp.

γ1Δ2.3. Two γ2-specific primers, 5′-TGCACAATTTTCCCATGGATGAACA C-3′ (sense) and 5′-GACAATGAGTATGCATGGGATATAGG-3′ (antisense), were used to amplify the γ2 portion, which was cut with *Nco*I and *Nsi*I and inserted into similarly cut γ1 in pBS. The construct was subcloned into pcDNAIamp.

γ1Δ2.4. A *Bgl*II site was introduced into γ1 in pcDNAIamp by site-directed mutagenesis using the primer 5′-GTGGCTGATCCTAGATCTTGGAGATTAT AT-3′ (γ1BglII). A γ2 fragment generated with the γ1Δ2.3 sense primer and 5′-AAGCCTCCAAGATCTTGTGTCGCC-3′ was digested with *Nco*I and *Bgl*II and inserted into similarly cut γ1BglII.

γ1Δ2.5. A γ2 fragment was obtained with the γ1Δ2.3 antisense primer and 5′-AAGCCTCCAAGATCTTGTGTCGCC-3′, cut with *Bgl*II and *Nsi*I, and inserted into similarly cut γ1BglII.

γ1Δ2.6. A γ2 fragment generated with the γ1Δ2.4 antisense primer and 5′-CACTGTCATCTTGAATTCCCTGCTGGAAG-3′ was digested with *Eco*RI and *Bgl*II and inserted into similarly cut γ1BglII.

γ1Δ2.7. A γ1 PCR fragment was generated using the sense primer 5′-ATAGATATATTTTTTGCGCAAACCT-3′ and antisense 5′-CTTAAAATAGGTACCATACTAGTCACATTTTA-3′ and then digested with *Fsp*I and *Spe*I. This was inserted into γ2 in pCDM8 digested with *Fsp*I and *Xba*I.

Site-Directed Mutagenesis

Oligonucleotide-directed mutagenesis was performed as described previously (23) using single-stranded γ1, γ2, or γ3 cDNAs in pcDNAIamp as template and sense-strand oligonucleotides. Mutations were verified by DNA sequencing.

Transient Expression and Radioligand Binding

The γ subunit constructs were cotransfected with α1 and β1 cDNAs and the vector pAdVAntage (Promega, Madison, WI) to enhance expression levels (2 μg of each subunit DNA/plate and 6 μg of pAdVAntage). Transient transfection in human embryonic kidney 293 cells (4×10^6 cells/10-cm plate) was performed through calcium phosphate precipitation (24). After 2 days, the cells were harvested by being scraped into phosphate-buffered saline and pelleted through centrifugation. The cell pellet was washed twice in 10 mM potassium phosphate, pH 7.4, with pelleting between washes before being resuspended in assay buffer (10 mM potassium phosphate, pH 7.4, 100 mM potassium chloride) and homogenization by passage through a 27-gauge needle.

Saturation binding curves were obtained by incubation of membranes with [^{3}H]flumazenil, [^{3}H]flunitrazepam, or [^{3}H]Ro15–4513 (all from New England Nuclear Research Products, Boston, MA) at 0.1–30 nM in a total volume of 0.5 ml. Nonspecific binding was determined in the presence of 10 μM flunitrazepam, except for γ1ΔI79Y, for which 10 μM Ro15–1788 was used. After 90 min at 4°, the assay was harvested by filtration onto GF/B filters (Brandel, Montreal, Quebec, Canada) using a TOMTEC (Orange, CT) cell harvester. Filters were washed three times with ice-cold assay buffer and dried before filter-retained radioactivity was detected by liquid scintillation counting. Dissociation constants, K_d values, were calculated by Scatchard analysis using GraFit. Displacement of [^{3}H]flumazenil or [^{3}H]flunitrazepam (at a concentration equivalent to the calculated K_d value) by β-CCM (Research Biochemicals, Natick, MA), CL218,872 (Lederle, Mont-St-Guibert, Belgium), clonazepam (Sigma Chemical, Poole, Dorset, UK), triazolam (Sigma), flunitrazepam (Sigma), and zolpidem (Synthelabo, Paris, France) was performed in a similar manner. The structures of the compounds are given in Fig. 1. Experimental data points were fitted to a single-site dose-response curve using GraFit, 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 and expressed as mean ± standard error.

Electrophysiology

Adult female *Xenopus laevis* specimens were anesthetized by immersion in a 0.4% solution of 3-aminobenzoic acid ethylester for 30–45 min (or until unresponsive). Ovary tissue was removed via a small abdominal incision, and stage V and VI oocytes were isolated with fine forceps. After mild collagenase treatment to remove follicle cells (Type IA; 0.5 mg/ml for 8 min), the oocyte nuclei were directly injected with 10–20 nl of injection buffer (88 mM NaCl, 1 mM KCl, 15 mM HEPES, pH 7, filtered through nitrocellulose) or sterile water containing different combinations of human $GABA_A$ subunit cDNAs (20 ng/μl) engineered into the expression vector pCDM8 or pcDNAI/Amp. α1, β1, γ1, and γ1 mutant subunit cDNAs were mixed in a 1:1:3 or 1:1:10 ratio to ensure preferential assembly of αβγ receptors. After incubation for 24–72 hr, oocytes were placed in a 50-μl bath and perfused at 4–6 ml/min with modified Barth's solution consisting of 88 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, and 2.4 mM $NaHCO_3$, at pH 7.5. Cells were impaled with two 1–3-MΩ electrodes containing 2 M KCl and voltage-clamped between −40 and −70 mV.

In all experiments, drugs were applied in the perfusate until the peak of the response was observed. The effects of $GABA_A$ receptor modulators were examined on control GABA responses using a concentration that elicited 20% of a maximum GABA response on each oocyte (EC_{20}) and a BZ preapplication time of 30 sec. Three minutes were allowed between each application to prevent desensitization. All values are shown as mean ± standard error.

Results

The initial search for determinants of the γ2 subunit that contribute to the BZ binding site was based on the observation that flumazenil has a ~5000-fold higher affinity for α1β1γ2 and α1β1γ3 receptors than α1β1γ1 (18) (Table 1). A simple, single-point assay was used to identify γ2 sequences

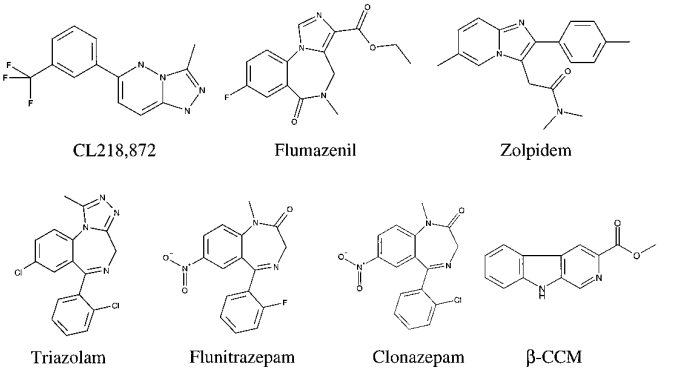

Fig. 1. Structures of BZ site ligands used in this study.

TABLE 1
Affinities for selected BZ site ligands at $GABA_A$ receptors containing wild-type, chimeric, or mutant γ subunits

	Flumazenil	Flunitrazepam	β-CCM	CL218-872	Zolpidem	Triazolam	Clonazepam
				μM			
α1β1γ1	>5000[c]	37.74 ± 5.07[a]	>5000[c]	>5000[c]	>5000[c]	25.65 ± 7.52[c]	150.0 ± 8.5[c]
α1β1γ2	0.91 ± 0.22[a]	3.90 ± 0.80[a]	0.45 ± 0.10[b]	46.75 ± 11.32[b]	40.23 ± 12.60[b]	0.45 ± 0.13[b]	1.03 ± 0.13[b]
α1β1γ3	2.80 ± 1.01[a]	150 ± 30[b]			5530 ± 1350[b]	6.04 ± 0.29[b]	25.95 ± 1.67[b]
α1β1γ1Δ2.2	3.07 ± 1.21[a]	9.75 ± 1.50[b]					
α1β1γ1Δ2.6	2.59 ± 1.85[a]	10.69 ± 2.45[b]			17.95 ± 2.52[b]		
α1β1γ1Δ2.7	1.62 ± 0.28[b]	38.94 ± 1.55[b]					
α1β1γ1ΔI79F	3.13 ± 0.40[a]	44.64 ± 3.69[b]	0.71 ± 0.07[b]	27.22 ± 2.94[b]	>5000[b]	3.44 ± 0.73[b]	14.58 ± 1.94[b]
α1β1γ1ΔL132M	>5000[c]	3.72 ± 0.57[a]	1390 ± 300[c]	>5000[c]	>5000[c]	1.58 ± 0.36[c]	2.68 ± 0.59[c]
α1β1γ1ΔI79F, L132M	10.56 ± 2.62[c]	6.98 ± 1.73[a]			1380 ± 270[c]		
α1β1γ1ΔI79Y	5.56 ± 2.19[a]	>5000[b]					
α1β1γ2ΔF77I	1420 ± 190[a]	7.62 ± 1.18[b]	264.7 ± 19.1[b]	>5000[b]	>5000[b]		
α1β1γ3ΔL133M	3.90 ± 0.98[a]	33.19 ± 1.21[b]			330 ± 80[b]	1.49 ± 0.25[b]	4.80 ± 0.97[b]

[a] K_d values.
[b] K_i values with [^{3}H]flumazenil as displaced radioligand.
[c] K_i values with [^{3}H]flunitrazepam as displaced radioligand.

contained within chimeric γ1/γ2 subunits that conferred high affinity binding. A series of six chimeric γ1/γ2 subunits were constructed (Fig. 2) that, when coexpressed with α1 and β1 cDNAs in human embryonic kidney 293 cells, allowed delineation of the determinants for high affinity binding to residues Asn33 to Pro159 of γ2 (numbering as for mature peptide). The affinities (K_d values) of [^{3}H]flumazenil for α1β1γ1Δ2.2 and α1β1γ1Δ2.6 receptors were 3.07 and 2.59 nM, which is very similar to the affinity at α1β1γ2 (0.91 nM). A comparison of the γ subunit amino acid sequences for this region (Fig. 3) reveals six positions at which the amino acid is conserved in γ2 and γ3 but not in γ1. These residues were targeted for site-directed mutagenesis, with the γ1 subunit sequence being changed to that of γ2. When coexpressed with α1 and β1 subunits, only one point mutant (γ1ΔI79F) conferred high affinity binding of [^{3}H]flumazenil (Fig. 4).

The affinity of [^{3}H]flumazenil for receptors containing γ1ΔI79F was 3.13 nM (Table 1), close to that of receptors containing wild-type γ2. Receptors containing γ2ΔF77I (as in γ1) had an affinity of 1.42 μM. These data confirm the critical role of γ2Phe77. To assess the nature of the interaction between the ligand and receptor, a series of subsequent point

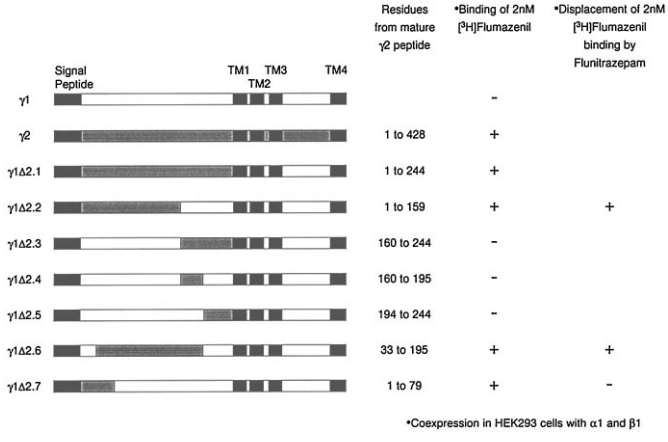

Fig. 2. Localization of the BZ binding site within the γ subunit. *Black areas*, chimeric γ1/γ2 subunits represented with the putative signal peptide and transmembrane spanning domains (TM1–4). *Shaded areas*, portions contributed by the γ2 subunit. *Column 1*, amino acid range. *Column 2*, subunits coexpressed with α1β1 and assayed for binding of 2 nM [^{3}H]flumazenil. *Column 3*, displacement of this binding by 1 μM flunitrazepam and 10 nM zolpidem.

mutations were made at Ile79 of the γ1 subunit. This residue was changed to aspartate, glutamate, histidine, tryptophan, and tyrosine. Only introduction of a tyrosine residue conferred high affinity binding of [^{3}H]flumazenil (Fig. 5, Table 1), demonstrating the requirement of a phenyl ring at this position. It was apparent, however, that the binding of [^{3}H]flumazenil was not displaced by flunitrazepam. Indeed, the affinity for flunitrazepam was significantly lower for receptors containing γ1ΔI79Y. K_i values for β-CCM and CL218,872 are similar for α1β1γ1ΔI79F and α1β1γ2 receptors, demonstrating the importance of this residue. The affinities for triazolam and clonazepam are also significantly increased at α1β1γ1ΔI79F, although not quite to the affinities at α1β1γ2, suggesting the requirement for additional determinant or determinants in the γ2 subunit. In contrast, the affinity for flunitrazepam and zolpidem at receptors containing γ1ΔI79F is not increased to the affinity of receptors containing a γ2 subunit (Table 1). This also suggested that additional amino acids within the γ2 subunit were required for the high affinity binding of these compounds. Two of the previously constructed chimeras (γ1Δ2.2 and γ1Δ2.6) were coexpressed with α1 and β1, and the ability of flunitrazepam and zolpidem to displace [^{3}H]flumazenil binding was determined (Fig. 2). An additional γ subunit chimera, γ1Δ2.7, was then constructed to further delineate the critical residue (Fig. 2). This last chimera has the phenylalanine residue necessary for [^{3}H]flumazenil binding, but the radioligand was not displaced by flunitrazepam or zolpidem; hence, a second residue delineated by Gln80 and Pro159 of the γ2 subunit was conferring higher affinity for flunitrazepam and zolpidem. Receptors containing the γ2 subunit (but not those containing γ1 or γ3) have a high affinity for both flunitrazepam and zolpidem (Table 1); therefore, point mutants were made in the γ1 subunit between Gln82 to Pro161 equivalent to positions at which γ2 has a different residue from either γ1 or γ3, regardless of whether these latter two subunits had an identical residue (Fig. 3). Six amino acid positions satisfied this criterion, and the γ1 point mutant γ1ΔI79F was also mutated at each of these positions so that they could be assayed for displacement of [^{3}H]flumazenil binding by flunitrazepam and zolpidem on coexpression with α1 and β1.

All of these double mutants were able to bind [^{3}H]flumazenil with high affinity (data not shown), but only one,

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             *                           *                                                         *           ●   ●               ●
γ1  35...s L i q G Y D N K L R P D I G V r P T V I e T D v Y Y V N S I G P V d p I N M E Y T I D I i F A Q T W f D S R L K F N S T M K V L
γ2  33...n L l e G Y D N K L R P D I G V k P T l I h T D m Y Y V N S I G P V n a I N M E Y T I D I f F A Q m W y D r R L K F N S T i K V L
γ3  36...k L l r e Y D k K L R P D I G i k P T V I d v D i Y Y V N S I G P V s s I N M E Y q I D I f F A Q T W t D S R L r F N S T M K i L

         ●                                       *   *                     ●                                   ●       *
γ1  99   m L N S N M V G K I W I P D T F F R N S r K s D A H W I T T P N R L L R I W N D G R V L Y T L R L T I N A E C y L Q L H N F P . . .
γ2  97   r L N S N M V G K I W I P D T F F R N S k K a D A H W I T T P N R m L R I W N D G R V L Y s L R L T I d A E C q L Q L H N F P . . .
γ3  100  t L N S N M V G l I W I P D T i F R N S k t a e A H W I T T P N q L L R I W N D G k i L Y T L R L T I N A E C q L Q L H N F P . . .
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Fig. 3. Alignment of human γ1, γ2, and γ3 subunit sequences. The partial amino acid sequences are shown aligned to the γ2 portion encompassed by residues Asn33 and Pro159 (numbering as mature peptide). *Residues in capital letters*, conserved between sequences. *, Residues common to only γ2 and γ3. ●, Residues between γ2Gln80 and γ2Pro159 where γ2 differs from γ1 and γ3, regardless of whether the latter two are the same (note that γ2Ser142 was not mutated in this study and thus is not indicated with ●). *Boxes*, positions of γ1 Ile79/γ2Phe77 and γ1Leu132/γ2Met130.

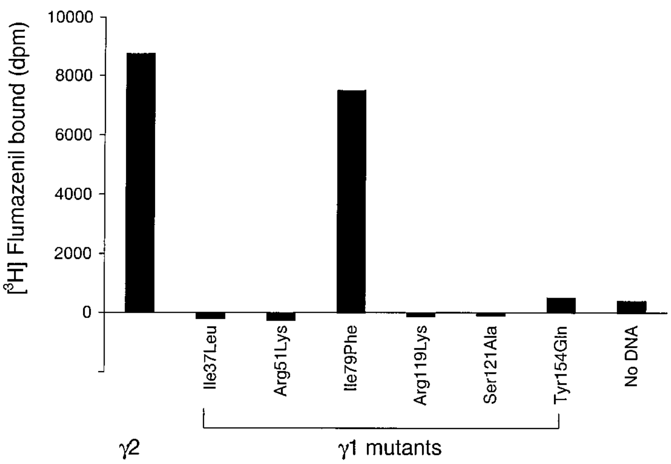

Fig. 4. Binding of [^{3}H]flumazenil to recombinant receptors containing mutant γ1 subunits. The γ1 mutants were coexpressed with α1 and β1 and assayed for binding of [^{3}H]flumazenil. Results are the mean values of two experiments.

γ1ΔI79F/L132M, showed displacement by flunitrazepam and zolpidem at the concentrations chosen for the assay. The single-point mutant, γ1ΔL132M, was subsequently constructed and on coexpression with α1 and β1 formed receptors with high affinity for [^{3}H]flunitrazepam (K_d = 3.72 nM; Table 1). K_i values for a number of compounds were obtained for α1β1γ1ΔL132M receptors by displacement of [^{3}H]flunitrazepam (Table 1). Both triazolam and clonazepam had significantly increased affinities at α1β1γ1ΔL132M (16- and 56-fold, respectively, compared with α1β1γ1), approaching their affinities at α1β1γ2. Flumazenil, β-CCM, CL218,872, and zolpidem have low affinity for α1β1γ1ΔL132M, suggesting γ2Met130 is not a critical residue for the binding of these compounds. The affinity of zolpidem was further investigated at receptors containing the γ1ΔI79F/L132M double mutant and found be to ≥10-fold higher than for receptors containing γ1 subunits with either single-point mutation but still 30-fold lower than for α1β1γ2, indicating an interaction with additional determinants.

The γ3 subunit is similar to γ2 in having a phenylalanine residue at position 80 and similar to γ1 in having a leucine residue at position 133 (Fig. 3), and receptors containing a γ3 subunit have a distinct BZ pharmacology (21). To confirm the importance of the residues identified above, the γ3 subunit was altered to give the mutants γ3ΔF80I and γ3ΔL133M. Binding of [^{3}H]flumazenil was abolished to receptors containing γ3F80I (n = 2; data not shown), confirming the importance of the phenylalanine residue at this position. Receptors containing γ3ΔL133M had a 4–5-fold increase in affinities for flunitrazepam, triazolam, and clonazepam (Table 1) and a 17-fold increase in affinity for zolpidem, confirming the importance of this residue at the BZ binding site. In contrast, the affinity for flumazenil was essentially unaffected by changes at this position of the γ3 subunit, further demonstrating that amino acids at this position of the γ subunit do not contribute to flumazenil binding.

In addition to affecting affinity, γ subunits can confer differences in BZ efficacy (19, 21). To determine the contributions to BZ efficacy of the individual amino acids identified in this study, mutated γ subunits were coexpressed with α1β1 in *X. laevis* oocytes, and modulation by BZs was compared with wild-type receptors (Table 2). We reported previously that α2β1γ1 receptors are modulated by BZs, although with generally lower efficacy than α2β1γ2 receptors (19). Here, we report that α1β1γ1 receptors were not modulated by flunitrazepam, CL218,872, β-CCM, or zolpidem (Table 2). The presence of the γ1 subunit in the α1β1γ1 receptor complex was confirmed by the higher GABA EC_{50} value compared with α1β1 and the relative insensitivity to zinc compared with α1β1 (Table 3). α1β1γ1ΔI79F receptors, however, were potentiated by flunitrazepam, but unlike α1β1γ2, 100 nM flunitrazepam did not elicit a maximum response (Fig. 6), suggesting a lower affinity for the former subunit combination, which in fact is the case (Table 1). A comparison of the data in Fig. 6 with the affinities for flunitrazepam given in Table 1 reveals that the EC_{50} value for flunitrazepam at α1β1γ2 and α1β1γ1ΔI79F is a little higher than the K_i value derived from radioligand binding. This is not unusual (25) and presumably reflects the fact that one is a direct measurement of the binding energy, whereas the other is a functional determination. α1β1γ1ΔI79F receptors were also potentiated by CL218,872 and inhibited by the inverse agonist β-CCM, although with lower efficacy than receptors containing γ2 (Table 2). They were not modulated by zolpidem, reflecting the low affinity of this compound for α1β1γ1ΔI79F receptors. Like α1β1γ1, receptors containing γ1ΔL132M were not modulated by any of the compounds tested, including flunitrazepam, which binds with an affinity of 3 nM. Coassembly of the γ1ΔL132M subunit into the receptor complex was again confirmed by higher GABA EC_{50} values and insensitivity to zinc (Table 3). Taken together, these data suggest a critical role for γ2Phe77 in conferring BZ efficacy to the receptor. To confirm this hypothesis, the mutant γ2ΔF77I was constructed and coexpressed with α1 and β1 subunits.

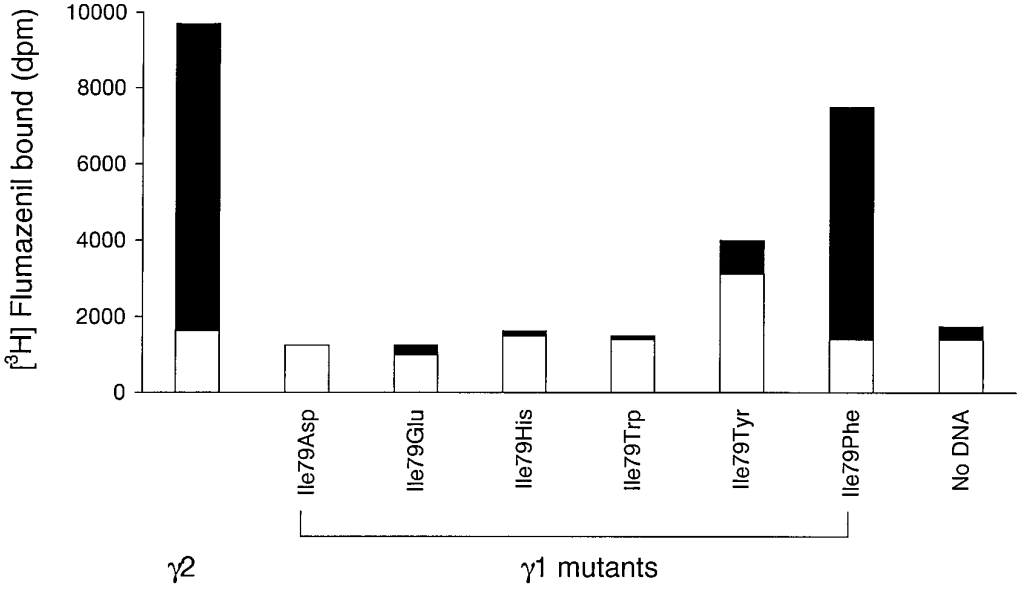

Fig. 5. Introduction of various amino acids at position 79 of the γ1 subunit. The isoleucine at position 79 of the γ1 subunit was mutated to aspartic acid, glutamic acid, histidine, tryptophan, and tyrosine. Recombinant receptors α1β1γ were assayed for binding of [^{3}H]flumazenil. *Height of bar*, total binding. *White area*, binding not displaced by 10 μM flunitrazepam. Values are mean of three experiments.

TABLE 2

Modulation by BZ ligands of GABA$_A$ receptors expressed in *X. laevis* oocytes

Data represent the percent modulation of a submaximal (EC_{20}) response to GABA by the BZ ligand; each value is the mean ± standard error from at least four individual oocytes. A positive value represents an agonist-like effect and a negative value represents an inverse-agonist effect. A value of zero represents no modulatory effect.

Subunit combination	Flunitrazepam		β-CCM 100 nM	CL 218 872 1 μM	Zolpidem 1 μM
	100 μM	1 μM			
α1β1γ2	94.6 ± 20.1	108.6 ± 8.9	−43.8 ± 9.6	58.0 ± 6.5	140 ± 14
α1β1γ1	−5.8 ± 3.5	−10.0 ± 5.5	−0.9 ± 1.9	1.9 ± 2.3	2.4 ± 2.3
α1β1γ1ΔI79F	25.0 ± 5.0	68.7 ± 14.8	−14.0 ± 3.8	28.4 ± 4.5	0.75 ± 1.4
α1β1γ1ΔL132M	−10.6 ± 2.2	−14.8 ± 1.7	0.1 ± 2.2	−1.3 ± 0.75	0.3 ± 1.3
α1β1γ2ΔF77I	9.0 ± 1.8	11.1 ± 2.3	−6.5 ± 3.4	0.1 ± 4.8	−1.5 ± 4.1

TABLE 3

GABA EC_{50}, Hill coefficients, and sensitivity to zinc of GABA$_A$ receptors expressed in *X. laevis* oocytes

EC_{50} values are geometric mean (− standard error, + standard error) and the slope arithmetic mean ± standard error. Inhibition by 3 μM zinc represents the percent inhibition of a GABA EC_{50} response for each receptor combination.

Subunit combination	GABA EC_{50}	Slope	*n*	Inhibition by 3 μM Zinc
	μM			%
α1β1	5.4 (4.4;6.7)	1.32 ± 0.06	7	−53.4 ± 3.8
α1β1γ2	16.2 (12.3;21.4)	1.36 ± 0.18	7	−4.5 ± 2.6
α1β1γ1	15.0 (11.9;19.1)	1.33 ± 0.12	4	−14.7 ± 3.1
α1β1γ1ΔI79F	25.0 (20.3;30.6)	1.8 ± 0.20	4	−10.0 ± 2.4
α1β1γ1ΔL132M	51.3 (46.0;57.2)	1.43 ± 0.07	4	−12.2 ± 3.7
α1β1γ2ΔF77I	23.7 (15.4;36.5)	1.5 ± 0.06	4	−0.5 ± 1.6

α1β1γ2ΔF77I receptors were not modulated by flunitrazepam (which binds with an affinity of 7.62 nM; Table 1) or any of the other BZ site ligands tested (Table 2), confirming the importance of this residue in conferring BZ efficacy to the receptor.

Discussion

Although some studies have been performed to identify residues responsible for the α subunit-selective binding profile of BZ site ligands (13–14), few studies have been made of the role of the γ subunit. The γ subunit is an essential component of the BZ binding site (8), and the BZ pharmacology is profoundly affected by the type of γ subunit present in the receptor complex (18–21). For example, flumazenil has a much greater affinity for γ2- and γ3-containing receptors than those containing γ1. This observation provided the criterion that initiated this study. The sequence homology between the γ subunits made it possible to construct chimeric γ subunits that would coassemble with α1 and β1 subunits. At this stage, and during the creation of subunit point mutants, the strategy was to introduce the determinants that conferred high affinity binding.

A single amino acid, γ2Phe77, was found to be necessary for high affinity binding of [^{3}H]flumazenil. The presence of

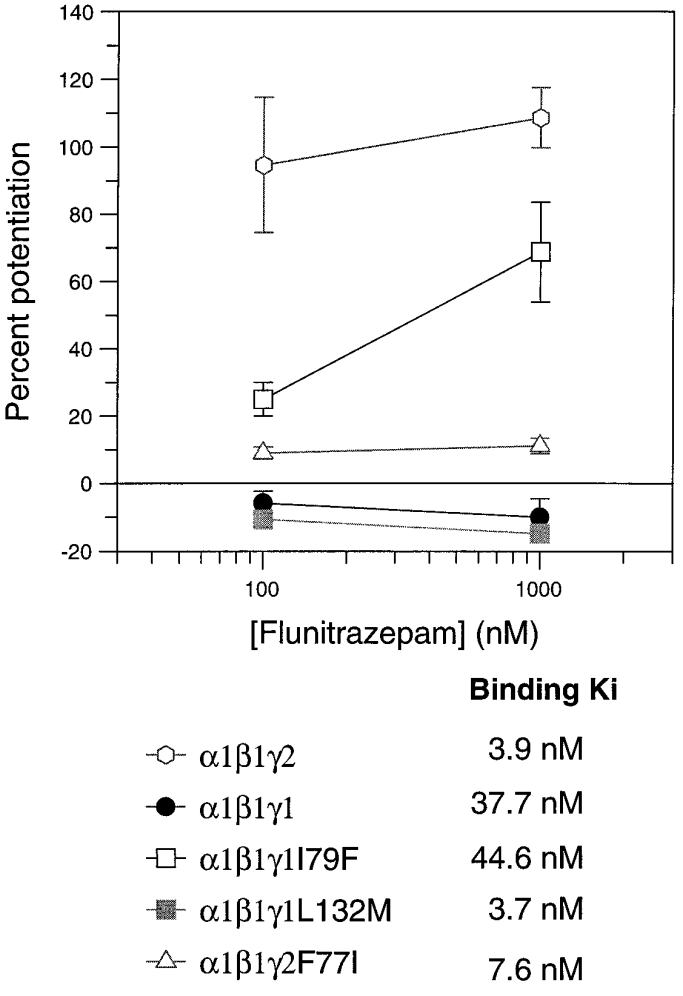

Fig. 6. Percent modulation of EC_{20}GABA responses at $\alpha1\beta1\gamma1$, $\alpha1\beta1\gamma2$, $\alpha1\beta1\gamma1\Delta I79F$, $\alpha1\beta1\gamma1\Delta L132M$, and $\alpha1\beta1\gamma2F77I$ $GABA_A$ receptors by 100 nM and 1 μM flunitrazepam. Modulation is expressed as the percent potentiation of an EC_{20} concentration of GABA for each oocyte. Values are mean ± standard error of at least four oocytes. For reference, the affinity (determined by radioligand binding; see Table 1) of flunitrazepam at the various subunit combinations is also shown.

this residue confers ≥5000-fold increase in affinity, indicating that it is one of the key constituents of the BZ binding site. It is a similarly important determinant for the binding of other structurally diverse BZ site ligands (i.e., CL218,872 and β-CCM). High affinity ("γ2-like") binding of triazolam and clonazepam is also dependent on the presence of this phenylalanine residue. It was hoped that more could be learned of the interaction between receptor and ligand by making further amino acid substitutions at γ1Ile79. Of the five substitutions made, only $\gamma1\Delta I79Y$ conferred a high affinity for [^{3}H]flumazenil (Table 1 and Fig. 5). Phenylalanine and tyrosine differ only by the addition of a *para*-hydroxyl group, suggesting the common benzene ring is interacting with flumazenil. Interestingly, receptors containing $\gamma1\Delta I79Y$ had a >100-fold reduction in affinity for flunitrazepam compared with $\gamma1\Delta I79F$- containing receptors, suggesting the presence of the *para*-hydroxy group disrupts binding.

A second residue in the γ subunit, γ2Met130, also contributes to the BZ binding site. This residue seems to have no significant effect on the binding affinity of flumazenil, β-CCM, or CL218,872; however, it is an important determinant for the binding of flunitrazepam, triazolam, and clonazepam, as demonstrated by the increased binding affinity when a methionine residue is introduced at the equivalent position in both γ1 and γ3. The latter three compounds have a pendant phenyl ring (Fig. 1), allowing speculation that the interaction with the methionine residue occurs through this moiety.

The affinity for zolpidem of receptors containing either of the γ1 point mutants is >5 μM. When both changes are introduced together, the affinity is increased but remains >30-fold lower than that at receptors containing γ2. Similarly, the presence of both the phenylalanine and methionine residues in $\gamma3\Delta L133M$ increases the affinity for zolpidem to 330 nM but is still 8-fold less than that at γ2-containing receptors. Additional amino acid determinants in the γ2 subunit may therefore be necessary to attain the 40 nM affinity achieved at $\alpha1\beta1\gamma2$ receptors. The location of the two (or possibly more) determinants required for high affinity binding of zolpidem on the γ subunit reveals the considerable contribution of this subunit to the binding site. However, zolpidem (a so-called BZ1-selective compound) has higher affinity for α1-containing receptors than for receptors containing other α subunits (9, 10). These data may be reconciled if the binding site for zolpidem is formed largely by determinants from the γ2 subunit; the lower affinity of zolpidem for $\alpha3\beta1\gamma2$ receptors compared with $\alpha1\beta1\gamma2$ receptors is due to increased steric hindrance by the large amino acid residue in α3, which is responsible for the selectivity (α3Glu225; Ref. 13) compared with the small glycine residue at the equivalent position in α1.

The functional properties observed when the various γ subunit mutants where coexpressed with α1 and β1 indicate that Phe77 is also required for allosteric modulation of the receptor by the BZ. When this position is occupied by an isoleucine (as in γ1, $\gamma1\Delta L132M$, and $\gamma2\Delta F77I$), the receptor is not modulated, despite affinities of 3–44 nM for flunitrazepam. Conversely, the receptors containing $\gamma1\Delta I79F$ were modulated by all the BZs tested, with the exception of zolpidem. The lack of modulation of $\alpha1\beta1\gamma1$ by BZs is in contrast to that observed for $\alpha2\beta1\gamma1$ (a combination likely to exist *in vivo*; Refs. 26 and 27), in which BZs are able to allosterically modulate the receptor (19). This suggests that a residue or residues in the α2 subunit can partially compensate for the effects of the phenylalanine residue and confer a degree of positive modulation to the receptor, albeit less than that conferred by Phe77; all BZ compounds tested had lower efficacy on $\alpha2\beta2\gamma1$ (19). One apparent contradiction is that although Phe77 is not required for the binding of flunitrazepam, it is a requirement for efficacy. For other BZ site ligands, such as β-CCM and CL218,872, Phe77 is required for both binding and, presumably, modulation. These data can be reconciled if Phe77 is an absolute requirement for allosteric modulation, not necessarily by direct interaction with the ligand, and is used as a binding determinant by some classes of BZ site ligands. An alternative hypothesis is that Phe77 could be a contact point for flunitrazepam but not necessary for the compound to bind (i.e., other contact points satisfy the energy requirements for high affinity binding); in the absence of Phe77, the compound could occupy the binding site in a conformation that is incapable of initiating the allosteric changes leading to modulation of the channel.

The data reported here, in conjunction with previous stud-

ies characterizing the BZ pharmacology of γ3-containing receptors, also suggest that Met132 does not influence the efficacy of BZs because despite differences in affinity, in a comparison of γ2- and γ3-containing receptors, flunitrazepam, dimethoxy-4-ethyl-β-carboline-3-carboxylate, bretazenil, zolpidem, and CL218,872 have similar degrees of efficacy (21).

An interesting insight from this study is that γ2Phe77 is at a position homologous to α1Phe64. The latter is a critical residue at the GABA binding site; mutations at this position affect the affinity of GABA (28), and this residue is the site of photoincorporation of the GABA site radiolabel [^{3}H]muscimol (29). The GABA site has contributions from both the α (28, 29) and β subunits (30), whereas as discussed, the BZ site has contributions from both the α and γ subunits. One interpretation is that the BZ site is a vestigial GABA binding site that over time has mutated and lost its ability to bind GABA, but by chance synthetic molecules (i.e., BZs) are able to bind to this site and thereby modulate receptor function. Indeed, the recent observation by Amin *et al.* (31) that α1Tyr159 and α1Tyr 209 (both conserved in all α subunits) are components of the BZ binding site supports this hypothesis; these two residues are homologous to β2Tyr157 and β2Tyr205, previously demonstrated to be part of the GABA binding site (30).

The observation that the aromatic residues tyrosine and phenylalanine are key components of both the GABA and BZ binding sites is a recurring theme in ligand-gated ion channels. The aromatic residues phenylalanine and tyrosine are thought to also contribute to the acetylcholine binding site on the nicotinic receptor α subunit (32), the glycine binding site of the strychnine-sensitive glycine receptor (33), and the glycine coagonist site of the *N*-methyl-D-aspartate-type glutamate receptor (34).

A recent report has also demonstrated that γ2Phe77 is an important determinant for the binding of BZ site ligands (35), which is in good agreement with the current data. However, this study also reported that diazepam was able to potentiate receptors containing γ2F77I; in contrast, we found that this phenylalanine is a key determinant for modulation of receptors by a number of BZ site ligands. The reason for this apparent discrepancy is unclear. Another amino acid in the γ2 subunit that has also been shown to directly affect the efficacy of BZ compounds is Thr142, which when mutated to serine increased the efficacy of BZ ligands, changing flumazenil and Ro15–4513 to agonists (36). However, this mutation did not affect BZ affinity.

In conclusion, we demonstrated that at least two residues in the γ subunit are key determinants of the BZ site of the $GABA_A$ receptor. γ2Phe77 is required for high affinity binding of some, but not all, BZ site ligands, but according to current results, it seems to be an absolute requirement for functional modulation by these compounds. γ2Met130 is also required for high affinity binding of some but not all BZ site ligands, but it does not seem to influence allosteric modulation.

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