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Impact of β and γ Variants on Ligand-Binding Properties of γ-Aminobutyric Acid Type A Receptors

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SUMMARY

We expressed in cultured cells recombinant γ-aminobutyric acid type A ($GABA_A$) receptors of the subunit compositions $\alpha 1\beta j\gamma k$ and $\alpha 5\beta j\gamma k$ (j = 1, 2, or 3 and k = 2 or 3). A comparison of ligand-binding properties revealed a functional role for individual β variants, which depended on the α subunit in the $GABA_A$ receptor. Recombinant $\alpha 5\beta x\gamma 2/3$ receptors recognized the cage convulsant *t*-butylbicyclophosphoro[^{35}S]thionate, as well as the benzodiazepine (BZ) receptor inverse agonist [^{3}H]Ro 15–4513, only with the β3 variant. In contrast, the exchange of β variants in $\alpha 1\beta x\gamma 2$ receptors imparted differential modulation of *t*-butylbicyclophosphoro[^{35}S]thionate binding by BZ receptor ligands. The BZ site of γ3-containing receptors was partially independent of the accompanying α and β variants. $\alpha 1/5\beta 3\gamma 3$ receptors were zolpidem insensitive but distinguished from $\alpha 5\beta 3\gamma 2$ receptors by high affinity for the partial BZ receptor agonist CI 218,872. The distinct affinities of recombinant receptors for CI 218,872 suggested that the $\alpha 5\beta 3\gamma 2$ receptor is the dominant zolpidem-insensitive $GABA_A$ receptor in the brain. Hence, $\alpha 5\beta 3\gamma 3$ receptors are not a major fraction of the native zolpidem-insensitive receptors, even though their genes are colocalized on mouse chromosome 7 and on human chromosome 15.

$GABA_A$ receptors are heterooligomeric proteins, with the subunits being encoded by different genes (1). If naturally occurring mammalian $GABA_A$/BZ receptors assemble in subunit combinations of $\alpha i\beta j\gamma k$ (with i = 1–6, j = 1–3, and k = 1–3) and if a single stoichiometry is assumed for each assembly, then 54 different receptor types may be expressed in the brain (2). All natural $GABA_A$ receptors appear to recognize the GABA analog muscimol and the cage convulsant [^{35}S]TBPS (3, 4), the binding of which is regulated, in part, by BZ receptor ligands (5–7).

The role of the α variants in $\alpha i\beta 2\gamma 2$ receptors has been explored in some detail (for reviews, see Refs. 2, 8, and 9), and these variants are responsible for the differential BZ pharmacology of $GABA_A$/BZ receptors (10–14). For example, $\alpha 1\beta x\gamma 2$ receptors mimic the native BZ type I receptor, characterized by high sensitivity to diazepam and zolpidem (12, 13), and $\alpha 5\beta x\gamma 2$ receptors constitute a subtype of BZ type II receptors, characterized by high affinity for diazepam but insensitivity to zolpidem (12). The β variants were viewed as being necessary only for the functional characteristics of the ion channel (15). Their exchange does not alter the BZ ligand specificity in $\alpha 1\beta x\gamma 2$, $\alpha 2\beta x\gamma 2$, or $\alpha 3\beta x\gamma 2$ receptors (13). Furthermore, when coexpressed with α1, α3, or α5 in *Xenopus laevis* oocytes, all β variants give qualitatively similar responses to GABA and its analogs (15, 16). In contrast, the γ subunits are obligatory for the formation of the binding pocket for BZ ligands (17–19), but only γ2 has been explored in detail (see Refs. 2, 8, and 9), leaving obscure the functional role of γ1 and γ3.

We further characterized the β and γ3 variants in $\alpha 1\beta x\gamma 3$ and $\alpha 5\beta x\gamma 3$ receptors. α5 is predominantly expressed in adult rat hippocampus, where the mRNAs of all three β variants are detected at high levels, in addition to the γ3 mRNA (20). Furthermore, recent reports indicate that the genes coding for the α5, β3, and γ3 subunits are clustered on murine chromosome 7 and human chromosome 15 (21, 22). Such clustering is suggestive of a functional coexpression of the subunits. α1-containing BZ type I receptors constitute the majority of the $GABA_A$/BZ receptors in mammalian brain, but the distribution of α1 mRNA is widespread and it does not suggest a preference for assembly with any β or γ variant (20).

Experimental Procedures

Materials. All radioligands were purchased from DuPont-New England Nuclear. GABA, picrotoxinin, bicuculline, diazepam, and flunitra-

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ABBREVIATIONS: GABA, γ-aminobutyric acid; BZ, benzodiazepine; CI 218,872, 3-methyl-6-[(3-trifluoromethyl)phenyl]-1,2,4-triazolo[4,3-*b*]pyridazine; HEK, human embryonic kidney; Ro 15–1788, flumazenil; SR 95531, 2′-(3′-carboxy-2′,3′-propyl)-3-amino-6-*p*-methoxyphenylpyrazinium bromide; TBPS, *t*-butylbicyclophosphorothionate.

zepam were obtained from Sigma Chemical Co. (St. Louis, MO) and SR 95531 from Research Biochemicals Inc. (Natick, MA). Cl 218,872 was kindly donated by American Cyanamid Company (Pearl River, NY), 2-oxoquazepam by Schering-Plough (Kenilworth, NJ), zolpidem by Synthelabo Recherche (Bagneux, France), and Ro 15–4513, Ro 15–1788, and Ro 19–4603 by Dr. Hunkeler, Hofmann-LaRoche (Basle, Switzerland).

Transfection and membrane preparation. Expression vectors (12) for the α, β, and γ subunits were transfected in triple combination into HEK 293 cells (CRL 1573; American Type Culture Collection), as described (3). For optimal receptor expression, final concentrations were as follows: α1, 5; α5, 2; β1, 3; β2, 25; β3, 1; γ2, 0.5; and γ3, 0.2 μg of vector DNA/15-cm tissue culture plate. Forty hours after transfection, the cells were washed with phosphate-buffered saline, pH 7.4, at 37°, harvested in ice-cold phosphate-buffered saline, and centrifuged at 150 × g. The cell pellets were homogenized in an Ultraturrax homogenizer for 15 sec, pelleted at 23,000 × g, and used immediately or frozen at −80° and recentrifuged, with identical results. The membrane pellets were resuspended in 50 mM Tris/citrate buffer, pH 7.3.

Binding assays. Resuspended cell membranes (50–100 μg of protein/tube) were incubated in a final volume of 0.5 ml of 50 mM Tris/citrate buffer, pH 7.3, for [^{3}H]Ro 15–4513 and [^{3}H]muscimol or in 50 mM Tris/citrate buffer, pH 7.3, supplemented with 0.2 M NaCl for [^{35}S]TBPS. All radioligands were from DuPont-New England Nuclear. Nonspecific binding was determined with 10 μM Ro 15–1788, 100 μM GABA, and 20 μM picrotoxinin for the three radioligands, respectively. After 1 hr at 4° ([^{3}H]Ro 15–4513 and [^{3}H]muscimol) or 90 min at room temperature (24°) ([^{35}S]TBPS), the assay mixtures were rapidly diluted to 5 ml with 10 mM Tris·HCl, pH 7.5, and filtered through glass fiber filters (no. 52; Schleicher & Schuell). Filters were immersed in 4 ml of Packard Ultima Gold scintillation fluid, and the radioactivity was determined in a Beckman liquid scintillation counter using external standardization. Nonlinear regression was performed with the Inplot program (GraphPAD Software), to calculate the parameters of the saturation isotherms and displacement curves.

Results

We expressed in HEK 293 cells ternary GABA$_A$ receptors configured from the widely expressed α1 or the predominantly hippocampal α5 subunit with any of the three β subunits and either the γ2 or γ3 variant. All receptors were tested for their ability to bind [^{3}H]Ro 15–4513, an imidazo-BZ recognizing all known BZ receptors (4, 11), and [^{35}S]TBPS, the prototypic ligand for the GABA$_A$ receptor channel. We observed that all receptors bound [^{3}H]Ro 15–4513 (Table 1), indicating the presence of BZ sites. All α1 receptors recognized [^{35}S]TBPS, but only α5 receptors that contained the β3 subunit bound this ligand (Table 1). Native GABA$_A$ receptors bind [^{35}S]TBPS as well as [^{3}H]Ro 15–4513 (3, 4). Therefore, we determined the pharmacological profiles of α5β3γ2 and α5β3γ3 receptors for BZs, GABA analogs, and [^{35}S]TBPS and compared these profiles with those of α1 receptors. Furthermore, we studied in these receptors the allosteric modulation of [^{35}S]TBPS binding by BZs.

The BZ site pharmacology of α1β3γ2/3 and α5β3γ2/3 receptors was markedly affected by the α as well as the γ variant (Table 2). All γ3- or α5-containing receptors studied were zolpidem insensitive (Table 2). However, the zolpidem-insensitive receptors differed in binding specificities for other BZ site ligands. Remarkably, the affinity (K_i) for the prototypic BZ type I receptor compound Cl 218,872 was increased 10-fold in α1β3γ3 and α5β3γ3 receptors, compared with the homologous γ2-containing receptors (Table 2). The affinity for 2-oxoquazepam, another BZ type I receptor-preferring ligand, was decreased 10-fold in α1β3γ3 and α5β3γ3 receptors, compared with the homologous γ2-containing receptors (Table 2). Furthermore, α5β3γ2 and α5β3γ3 receptors displayed very high affinity for the inverse agonists [^{3}H]Ro 15–4513 and Ro 19–4603 (Table 2), whereas the affinity for the antagonist Ro 15–1788 was comparable in all receptors (Table 2). Finally, the affinity for the BZ site agonist flunitrazepam decreased from α1β3γ2 to α1β3γ3 receptors but varied only marginally between α5β3γ2 and α5β3γ3 receptors.

In contrast to the major differences in BZ site properties observed upon exchange of the α and γ variants, the neurotransmitter recognition sites of α1β3γ3, α5β3γ2, and α5β3γ3 receptors did not differ. This was indicated by the finding that the K_d for [^{3}H]muscimol and the K_i for GABA and SR 95531 displacement of [^{3}H]muscimol binding were not substantially altered by the subunit substitutions (Table 3). Moreover, the affinity for [^{35}S]TBPS was only marginally increased by substitution of γ2 for γ3 in α1- or α5-containing receptors (Table 3). This leaves the BZ site as the only major binding site on GABA$_A$/BZ receptors that is directly affected by a γ2 to γ3 exchange.

Although the affinity for [^{35}S]TBPS was similar in all α1- and α5-containing receptors, the modulation of [^{35}S]TBPS binding by BZ site ligands differed. In α1βxγ2 receptors, the [^{35}S]TBPS binding was affected by the BZ site ligands diazepam, Ro 15–4513, Cl 218,872, and zolpidem (Fig. 1A). The magnitude of the increase elicited by BZ site ligands in α1βxγ2 receptors differed markedly with the β variant and the particular ligand used. It ranged from a marginal effect of 109 ± 3% of control (four experiments; $p = 0.005$ versus control) for 10 μM Ro 15–4513 with α1β2γ2 receptors to a robust effect of 224 ± 7% (four experiments) for 10 μM zolpidem with α1β1γ2 receptors. The β substitutions did not change the rank order of potency of the BZ site compounds but the increase in [^{35}S]TBPS binding grew larger in the series β2 < β3 < β1 (Fig. 1A).

TABLE 1

BZ and TBPS binding to α1βxγ2/3 and α5βxγ2/3 receptors

α1βxγ2, α1βxγ3, α5βxγ2, and α5βxγ3 subunit combinations were expressed in HEK 293 cells and membranes were prepared as described. Binding was performed with 6 nM [^{3}H]Ro 15-4513 or [^{35}S]TBPS. The results are the means ± standard errors of three experiments.

	[^{3}H]Ro 15-4513 binding				[^{35}S]TBPS binding			
	α1		α5		α1		α5	
	γ2	γ3	γ2	γ3	γ2	γ3	γ2	γ3
	fmol/mg of protein				fmol/mg of protein			
β1	1120 ± 200	352 ± 24	218 ± 22	629 ± 4	50 ± 13	66 ± 4	5 ± 1	7 ± 1
β2	1220 ± 160	136 ± 7	42 ± 3	32 ± 8	136 ± 9	171 ± 6	1 ± 1	3 ± 4
β3	1570 ± 140	372 ± 47	287 ± 20	302 ± 8	129 ± 12	120 ±17	59 ± 3	90 ± 3

TABLE 2

K_d and K_i values of BZ ligands for some $GABA_A$ receptors

The indicated subunit combinations were expressed in HEK 293 cells and membranes were prepared as described. Shown are the K_i and K_d ([^{3}H]Ro 15-4513) values, as means ± standard errors, with the number of experiments given in parentheses.

Ligand	K_d or K_i				
	α1β1γ2	α1β3γ2	α1β3γ3	α5β3γ2	α5β3γ3
			nM		
[^{3}H]Ro 15-4513	4.1 ± 0.9 (3)	3.9 ± 0.8 (3)	2.8 ± 0.9 (3)	0.37 ± 0.03 (3)	0.54 ± 0.06 (6)
Cl 218,872	130 ± 40[a]	120 ± 18[b]	8 ± 1 (5)	280 ± 14 (3)	39 ± 5 (4)
Flunitrazepam	2.0 ± 0.3[a]	3.1 ± 0.4 (3)	67 ± 4 (5)	2.1 ± 0.2 (3)	9.3 ± 0.7 (3)
Diazepam	16 ± 1[a]		308 ± 116 (5)	17 ± 2 (3)	34 ± 2 (3)
Ro 15-1788	0.5 ± 0.2[a]		0.9 ± 0.3 (4)	0.5 ± 0.1[b]	0.23 ± 0.01 (3)
2-Oxoquazepam	20 ± 3[a]	16 ± 2[b]	210 ± 23 (5)	122 ± 24 (3)	1,040 ± 160 (5)
Ro 19-4603	4.0 ± 0.02 (3)	4.5 ± 0.7 (3)	3.3 ± 0.5 (4)	0.54 ± 0.02 (3)	0.7 ± 0.2 (4)
Zolpidem	30[a]	19 ± 3[b]	>10,000	>10,000	>10,000

[a] From Ref. 13.
[b] From Ref. 12.

TABLE 3

Kinetic parameters for [^{35}S]TBPS and [^{3}H]muscimol and K_i values for $GABA_A$ analogs with some $GABA_A$ receptors

The indicated subunit combinations were expressed in HEK 293 cells and membranes were prepared as described. Shown are the K_i and K_d ([^{3}H]muscimol and [^{35}S]TBPS) values and the B_{max}, as means ± standard errors, with the number of experiments given in parentheses.

Ligand	α1β3γ3	α5β3γ2	α5β3γ3
K_d (nM), [^{3}H]muscimol	5.9 ± 0.8 (3)	3.2 ± 0.6 (3)	4.1 ± 1.2 (3)
B_{max} (pmol/mg of protein), [^{3}H]muscimol	0.9 ± 0.2 (3)	0.69 ± 0.02 (3)	1.0 ± 0.2 (3)
K_i (nM)			
GABA	14 ± 2 (4)	16 ± 3 (3)	13 ± 2 (4)
SR 95531	118 ± 15 (4)	85 ± 9 (3)	105 ± 26 (4)
Bicuculline	9100 ± 1400 (4)		4400 ± 1300 (4)
K_d (nM), [^{35}S]TBPS	26 ± 3 (3)	57 ± 3 (3)	22 ± 2 (3)
B_{max} (pmol/mg of protein), [^{35}S]TBPS	1.3 ± 0.2 (3)	0.6 ± 0.1 (3)	3.1 ± 0.3 (3)

Diazepam increased [^{35}S]TBPS binding more effectively at 1 μM than at 10 μM in all α1βxγ2 receptor combinations. We observed an increase of [^{35}S]TBPS binding produced by 1 and 10 μM diazepam of 182 ± 3% and 164 ± 9% (α1β1γ2 receptors), 148 ± 4% and 130 ± 5% (α1β2γ2 receptors), and 169 ± 7% and 144 ± 4% of control (α1β3γ2 receptors), respectively. In contrast, 10 μM zolpidem was slightly more effective than 1 μM zolpidem in enhancing [^{35}S]TBPS binding to all α1βxγ2 receptors (data not shown). At 1 μM, the partial agonist Cl 218,872 was nearly as effective as diazepam with α1βxγ2 receptors (Fig. 1A). These results indicate that with recombinant receptors full and partial BZ site agonists may modulate [^{35}S]TBPS binding to similar extents. Diazepam and Ro 15–4513 did not modulate [^{35}S]TBPS binding to α1β3γ3, α5β3γ2, and α5β3γ3 receptors (Fig. 1B). However, Cl 218,872 (10 μM) decreased [^{35}S]TBPS binding to α1β3γ3 receptors (Fig. 1B). Addition of 50 μM bicuculline did not significantly alter the effect of the BZ site ligands on [^{35}S]TBPS binding to α1βxγ2, α1β3γ3, α5β3γ2, and α5β3γ3 receptors (data not shown). These data indicate that (i) the pharmacology of the BZ site is determined by single subunits and (ii) the modulation of [^{35}S]TBPS binding depends on the proper interaction of both the α and β subunits within a complex.

Discussion

We attempted to elucidate the roles of the β variants and the γ3 subunit in $GABA_A$ receptors by comparing α1βxγ2/3 and α5βxγ2/3 $GABA_A$ receptors with respect to the pharmacology of BZ and GABA site ligands. We also studied the [^{35}S]TBPS binding site and its allosteric interactions with the BZ receptor site.

Our results demonstrate the invariant affinity of [^{3}H]muscimol with α1- and α5-containing receptors and show that the affinity is independent of the β variant in α1βxγ2 receptors. This agrees with reports that $GABA_A$ receptors built from two (αxβ2) or three (αxβ2γ2) homologous subunits display identical

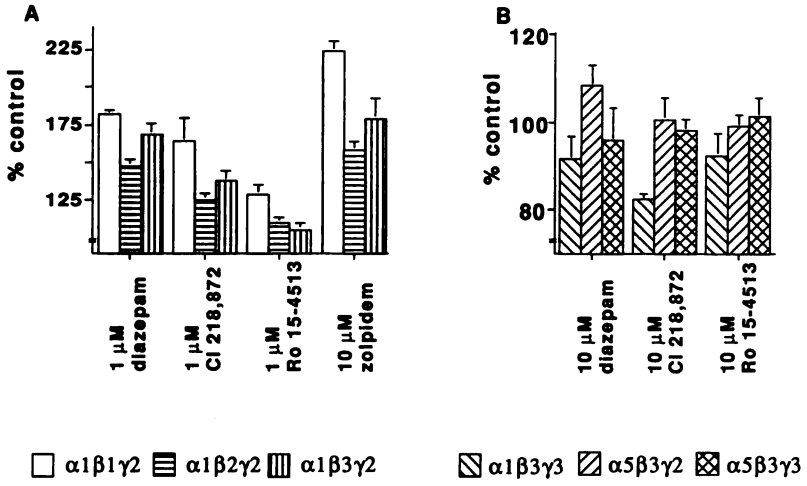

Fig. 1. Allosteric modulation of [^{35}S]TBPS binding to α1βxγ2 (A) and α1β3γ3 and α5β3γ2/3 (B) receptors by BZ receptor ligands. The indicated subunit combinations were expressed in HEK 293 cells and membranes were prepared as described. Given are the percentage of control values (means ± standard errors of four experiments) for [^{35}S]TBPS (5 nM) binding in the presence of the indicated substances.

[^{3}H]muscimol- and GABA-binding properties (3, 15, 23, 24). Receptors containing α1 recognized [^{3}H]Ro 15–4513 and [^{35}S] TBPS, regardless of the β variant, in α1βxγ2 or α1βxγ3 complexes. However, we found that the β3 variant is indispensable for [^{35}S]TBPS and [^{3}H]Ro 15–4513 binding to α5βxγ2 or α5βxγ3 receptors, which is surprising in view of the high level of sequence identity of the β subunits (16). This finding stresses the importance of subtle structural differences in the formation of GABA$_A$ receptor binding sites.

In spite of the apparent identity of the BZ binding site in α1βxγ2 receptors (13) (Table 2), the β variant affected the coupling of the BZ site to the [^{35}S]TBPS binding site. In agreement with recent electrophysiological results (25), [^{35}S] TBPS binding to α1β1γ2 receptors was more efficiently enhanced by BZ ligands than was that to the homologous β2 or β3 subunit-containing receptors. Surprisingly, 1 μM zolpidem was more effective than 1 μM diazepam in increasing [^{35}S] TBPS binding (Fig. 1A), possibly reflecting an interaction of diazepam, but not zolpidem, with the 4′-chlorodiazepam site on GABA$_A$/BZ receptors (26, 27). This notion agrees with a biphasic interaction of diazepam with [^{35}S]TBPS binding, because this binding is more effectively stimulated by 1 μM than 10 μM diazepam. This finding is also consistent with the more pronounced allosteric interaction between GABA and zolpidem, compared with that between GABA and flunitrazepam (28), and with the lower efficacy of diazepam, compared with zolpidem, in potentiating GABA-induced currents in recombinant GABA$_A$ receptors (29).

The α5β3γ2 receptor is a representative of BZ type II GABA$_A$ receptors and is additionally characterized by zolpidem insensitivity (12) (Table 2). We observed that α1β3γ3 and α5β3γ3 receptors also do not recognize zolpidem. Compared with α1βxγ2/3 receptors, the affinity of the α5β3γ2 and α5β3γ3 receptors for the inverse agonists [^{3}H]Ro 15–4513 and Ro 19–4603 was increased by nearly 1 order of magnitude, without a significant change in flumazenil affinity. A similarly high affinity for Ro 15–4513 has been found *in vitro* for human α5β1γ2 receptors (30), further emphasizing the negligible effect of different β variants on the conformation of the BZ site. As observed earlier for α1β2γ2/3 receptors (17), the exchange of γ2 to γ3 in α5β3γx receptors drastically modifies the affinity for agonists, while leaving the affinities for antagonists and inverse agonists largely unaffected. Substitution of the γ2 for the γ3 subunit seems to substantially alter the geometry of the BZ site agonist pharmacophore but appears not to affect the architecture of the BZ site binding pocket(s) for antagonists or inverse agonists. Importantly, the pharmacophores for agonists and inverse agonists are modulated by the α1 and α5 variants. These data indicate additional molecular heterogeneity of the classical BZ type II receptors (31, 32), rendering obsolete the current nomenclature of BZ type I and II receptors.

Given that all native GABA$_A$ receptors recognize [^{35}S]TBPS (3, 4), the lack of [^{35}S]TBPS binding sites in α5β1/2γ2 and α5β1/2γ3 receptors suggests that native zolpidem-insensitive α5 receptors exist either in the α5β3γ2 or the α5β3γ3 configuration. This notion is supported by the observation that the α5 subunit mRNA colocalizes with β3 mRNA in all α5-expressing brain regions (20, 33). GABA$_A$/BZ receptors affinity-purified from rat brain using α5 subunit-specific antibodies are zolpidem insensitive and show an affinity for Cl 218,872 in the high nanomolar range (34). Of the recombinant zolpidem-insensitive BZ receptors, only the α5β3γ2 receptor exhibits the same pharmacological pattern as the affinity-purified α5 receptor(s) (34) (Table 2). Therefore, the α5β3γ2 receptor seems to be the dominant zolpidem-insensitive GABA$_A$/BZ receptor in the hippocampus. A deletion of the chromosomal locus for the α5, β3, and γ3 subunits diminishes zolpidem-insensitive [^{3}H]BZ binding in an animal model of the Prader-Willi and Angelman syndromes (21) but this need not imply that the three subunits occur in the same receptor. Rather, our results suggest that receptors formed from the three subunits are not a major fraction of native α5-containing GABA$_A$ receptors. More selective ligands, or the availability of radiolabeled Cl 218,872, are required to prove the existence of native α1β3γ3 and α5β3γ3 receptors.

In conclusion, our data indicate that the α5 subunit requires β3 to create binding sites for BZs and convulsants in α5β3γ2 or α5β3γ3 receptors. Furthermore, the γ2 and γ3 variants differ in their effects on the BZ site agonist binding specificity, which in turn depends on the α subunit, indicating that the amino acid side chains of the α and γ subunits form the BZ pharmacophore(s). Finally, our data may provide a basis for the identification of new native GABA$_A$ receptor subtypes.

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