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Cloning of cDNA Sequences Encoding Human α2 and α3 γ-Aminobutyric Acid$_A$ Receptor Subunits and Characterization of the Benzodiazepine Pharmacology of Recombinant α1-, α2-, α3-, and α5-Containing Human γ-Aminobutyric Acid$_A$ Receptors

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SUMMARY

cDNAs encoding human α2 and α3 γ-aminobutyric acid$_A$ receptor subunits have been cloned. Their deduced amino acid sequences show much sequence identity with the published bovine sequences (98.2% and 97.0% for α2 and α3, respectively). Human α1β1γ2, α2β1γ2, α3β1γ2, and α5β1γ2 subunit combinations were expressed in transiently transfected cells and their pharmacologies were characterized using a series of benzodiazepine (BZ) binding site ligands. Human α1-containing receptors exhibited a BZ1-type pharmacology, and α2-, α3-, and α5-containing receptors exhibited a broadly BZ2-type pharmacology. The partial inverse agonist Ro15-4513 showed an approximately 10–15-fold higher affinity for α5-containing than for α1-, α2-, or α3-containing receptors and is thus the first compound shown to have a significantly higher affinity for another receptor subtype than for α1β1γ2.

GABA is the major inhibitory neurotransmitter in the mammalian central nervous system. GABA acts by binding to its receptor, the GABA$_A$ receptor, resulting in the opening of an intrinsic chloride channel and hyperpolarization of the cell membrane (1). GABA$_A$ receptors are modulated by a number of pharmacological agents, including barbiturates, BZs, neurosteroids, and ethanol (2).

The application of molecular genetic approaches to the mammalian GABA$_A$ receptor demonstrated the existence of a gene family consisting of numerous subunits (α1–α6, β1–β3, γ1–γ3, and δ) (3–16). Additional diversity exists through alternative splicing of the γ2 subunit (13, 14). Expression of subunit combinations in either *Xenopus* oocytes or transfected mammalian cells has demonstrated that α, β, and γ subunits are required to form a GABA$_A$ receptor with the expected properties (9, 17). The BZ pharmacology of GABA$_A$ receptor subtypes appears to be determined by both the α and γ subunits (8, 11, 17). Receptors containing γ1 exhibit what appears to be an atypical BZ pharmacology (8). Receptors containing a γ2 subunit exhibit a classical BZ pharmacology, the nature of which appears to be determined by the type of α subunit present (9, 17). Receptors containing γ3 appear to bind BZs, but the precise pharmacology is as yet unclear (18, 19). Using the classical BZ1/BZ2 nomenclature (2, 20), α1βxγ2-containing receptors (with βx indicating β1, β2, or β3) are proposed to have a BZ1 pharmacology, whereas α2-, α3-, and α5 (βxγ2)-containing receptors have a broadly BZ2 pharmacology (17, 21).

Here we report the cloning and sequencing of human GABA$_A$ receptor α2 and α3 cDNAs. Additionally we report the characterization of the BZ pharmacology of human α1β1γ2, α2β1γ2, α3β1γ2, and α5β1γ2 subunit combinations expressed in transiently transfected cells.

Materials and Methods

Cloning and Sequencing of cDNAs

cDNA libraries. cDNAs were cloned from human fetal brain, and hippocampal cDNA libraries were constructed in the λZAP vector (Stratagene). For screening, cDNA libraries were seeded at 40,000 plaque-forming units/137-mm plate. Filter lifts were taken using Hybond N filters (Amersham). Probes were labeled with ^{32}P to high specific activity (>10^9 cpm/μg) by random priming. Positive clones were plaque purified and rescued according to manufacturer's instructions (Stratagene). Analysis of nucleotide and amino acid sequences was performed using Intelligenetics software (Intelligenetics, Inc., Mountain View, CA).

Cloning of human α2 cDNAs. A bovine α2 cDNA (4) was used as a probe to screen a human fetal brain cDNA library as described previously (22, 23). Several positive clones were isolated, one of which contained the full coding region. This was fully sequenced on both strands using Sequenase 2 (United States Biochemicals).

Cloning of human α3 cDNAs. A bovine α3 cDNA (4) was used as

ABBREVIATIONS: GABA, γ-aminobutyric acid; BZ, benzodiazepine; βCCM, methyl-β-carboline-3-carboxylate; bp, base pair(s); PCR, polymerase chain reaction.

a probe to screen a human fetal brain cDNA library as described previously (22, 23). Several positive clones were isolated, the longest of which was missing approximately 100 bp of the 5′ end of the α3 coding region. This was obtained by PCR, using as primers an oligonucleotide "anchor" derived from the T7 primer sequence of the Bluescript (Stratagene) vector (5′-AGCGCGCGTAATACGACTCACTATAGG GCGAA-3′) and an oligonucleotide derived from the sequence near the 5′ end of the truncated α3 cDNA that contained an internal *Hpa*I restriction site (5′-CAGCATGAATTGTTAACCTCATTGTA-3′). PCR was performed under standard conditions using Amplitaq enzyme and buffer (Perkin Elmer Cetus) with 200 μM deoxynucleoside triphosphates (50-μl final volume), cycling at 94° for 45 sec, at 55° for 2 min, and at 72° for 2 min for 30 cycles, and using 2 μl of human fetal brain cDNA library as template. PCR products were extracted with phenol/chloroform, precipitated with ethanol, digested with restriction enzymes, and engineered into the truncated α3 cDNA. The engineered cDNA was sequenced as described above.

α1, α5, β1, and γ2 cDNAs. The sequence of human α1 cDNA has been published previously by Schofield *et al.* (24). It differs from the bovine sequence (3) at a single amino acid (Trp-95 in bovine α1, Arg in human α1). To create a human α1 cDNA, the bovine sequence was converted to the human by site-directed mutagenesis of amino acid 95 with the oligonucleotide 5′-GCAATGAAAATCCGGACTCGGCAT-3′, using methods described elsewhere (25). Cloning and sequencing of human α5 cDNA have been reported elsewhere (26, 27). The sequence of human β1 has been published previously by Schofield *et al.* (24). A human β1 cDNA was isolated by screening a human fetal brain cDNA library with a bovine β1 cDNA probe as described previously and was sequenced as described above. The sequence of human γ2 has been published previously by Pritchett *et al.* (9). A human γ2 cDNA was isolated by PCR using conditions described above, human hippocampal cDNA library as template, and oligonucleotide primers derived from the 5′ and 3′ untranslated regions of the published γ2 sequence, incorporating a *Hind*III restriction site for subcloning (5′-GGGAGGGAAGCTTCTGCAACCAAGAGGC-3′ and 5′-ACCACATAGAAGCTTATTTAAGTGGAC-3′). Sequencing indicated that the form of γ2 used in this study is the short form, γ2S, lacking the 24-bp insert in the putative cytoplasmic loop region (13). Additionally, residue 81 of the published γ2 sequence is a methionine, whereas in the cDNA used in this study residue 81 is a threonine (a change at bp 585 of thymidine to cytidine).

Expression of GABA$_A$ Receptor Subunit Combinations in Transiently Transfected Cells

For transient expression studies, subunit cDNAs were subcloned into the pCDM8 vector (Invitrogen). The α2 cDNA was initially subcloned as a 1688-bp *Pst*I fragment that included 153 bp of 5′ untranslated region containing a $(CT)_{34}$ repeat sequence (Fig. 1). This was expressed poorly when cotransfected into HEK 293 cells with β1 and γ2, compared with the expression obtained with the α1, β1, and γ2 subunit combination (data not shown). In an attempt to increase expression the α2 cDNA was re-engineered; the sequence 5′ of the *Eco*RI site at bp 345 (Fig. 1) was removed and replaced with the equivalent bovine α1 sequence cloned as a PCR product derived from bovine α1 cDNA by using the oligonucleotide primers 5′-GCGGCGAAGCTTGGACGCCCCTCCGCTGCC-3′ (bp 47–76 of bovine α1, incorporating a *Hind*III restriction site) and 5′-TCGGTCCAGAATTCTGGTGAAGAC-3′(bp 326–349 of bovine α1, incorporating an *Eco*RI restriction site). This cDNA was expressed considerably more efficiently. The human β1 cDNA was also expressed poorly when cotransfected into HEK 293 cells with an α subunit and γ2 (data not shown). To overcome this the 5′ 847 bp (24) of the human β1 cDNA were replaced by the equivalent bovine β1 sequence using the conserved *Pst*I restriction site at this position. The resulting cDNA, which differed from the deduced human β1 amino acid sequence at only two positions (residue 3, threonine to alanine; residue 10, proline to serine), was expressed well.

DNA was prepared for transfection by double-banding on CsCl gradients, and transfections of HEK 293 cells (4×10^6 cells/10-cm plate) were performed according to the method of Chen and Okayama (28). For radioligand binding, 48 hr after transfection cells were washed with phosphate-buffered saline, scraped into phosphate-buffered saline, and pelleted by centrifugation (500 × *g*). The cell pellet was homogenized (three 5-sec bursts at setting 5, using a Semat Ultra-Turrax) in 10 mM potassium phosphate, pH 7.4, and then pelleted by centrifugation at 48,000 × *g* for 30 min at 4°. The pellets were washed twice in this manner before final resuspension in 10 mM potassium phosphate, pH 7.4, containing 100 mM potassium chloride (assay buffer).

Radioligand Binding

Saturation binding curves were obtained by incubating membranes (150 μg of protein) with various concentrations of [^{3}H]Ro15-1788 (83 Ci/mmol; NEN). Nonspecific binding was measured in the presence of 10 μM flunitrazepam (Sigma). Displacement of [^{3}H]Ro15-1788 (0.4 nM) by Ro15-4513 (Research Biochemicals, Natick, MA), βCCM (Research Biochemicals), FG8205 (29), CL218,872 (obtained from Lederle), flunitrazepam (Sigma), triazolam (Sigma), abecarnil (obtained from Schering AG, Berlin, Germany), bretazenil (obtained from Hoffmann La Roche, Basel, Switzerland), or zolpidem (obtained from Synthelabo, Paris, France) was performed in a similar manner. Each ligand concentration was assayed in triplicate in a 0.5-ml volume, incubated for 90 min at 4°, and harvested onto GF/B filters (Brandel) by filtration using a Tomtec cell harvester and washing with ice-cold assay buffer. After drying, filter-retained radioactivity was detected by liquid scintillation counting. Experimental data points were fitted to single-site dose-response curves using the least-squares iterative fitting routine of RS/1 analysis software (BBN Research Systems, Cambridge, MA). K_i values were calculated from three or more independent experiments using the equation $K_i = IC_{50} \times (1 + [[^3H]Ro15\text{-}1788]/K_d)$.

Results and Discussion

The nucleic acid and deduced primary amino acid sequences of the human GABA$_A$ receptor α2 and α3 subunits are shown in Fig. 1. The human α2 cDNA sequence contains an open reading frame of 451 amino acids. The 5′ untranslated region of this sequence contains the notable feature of a $(CT)_{34}$ dinucleotide repeat. This is also present in the rat α2 sequence (16). The deduced amino acid sequence has 98.2% identity with the bovine α2 subunit (4) and contains features found in other GABA$_A$ receptor subunits, i.e., a putative 28-residue signal peptide, three putative *N*-glycosylation sites, and four hydrophobic putative transmembrane domains. The human α3 cDNA sequence contains a deduced open reading frame of 492 amino acids that has 97.0% identity with the published bovine α3 deduced amino acid sequence (4) and again has the expected features of a GABA$_A$ receptor subunit, i.e., a putative 28-amino acid signal peptide, four putative *N*-glycosylation sites, and four putative transmembrane domains, with a large putative cytoplasmic loop between transmembrane domains 3 and 4.

Fig. 2 shows the alignment of human GABA$_A$ receptor α1, α2, α3, and α5 subunits, aligned so that the most homologous sequences are placed next to each other. Overall there is significant identity, particularly in the putative transmembrane domains. Only the putative signal peptide and putative large cytoplasmic loop domain show regions of significant diversity.

The BZ pharmacologies of human α1β1γ2, α2β1γ2, α3β1γ2, and α5β1γ2 GABA$_A$ receptor subunit combinations were determined by transient cotransfection into HEK 293 cells. Between 0.3 and 1.0 pmol/mg of membrane protein [^{3}H]Ro15-1788 binding sites could be detected in the transfected cell membranes. Scatchard analysis of saturation binding curves (data

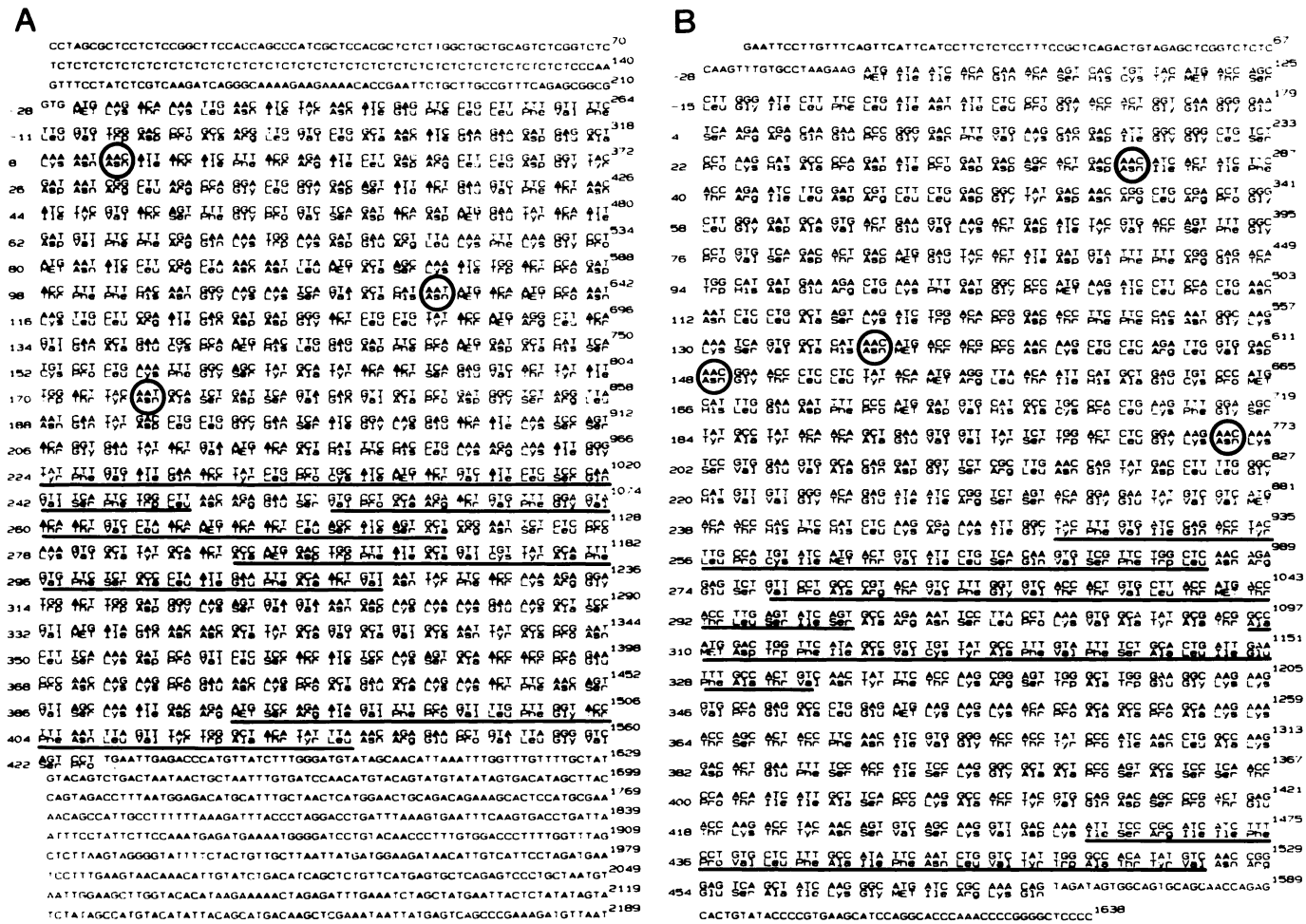

Fig. 1. Nucleotide and deduced amino acid sequences of human $GABA_A$ receptor $\alpha 2$ subunit (A) and $\alpha 3$ subunit (B). The amino acid numbers are indicated on the *left*; negative numbers refer to the putative signal peptide, with the first amino acid of the putative mature polypeptide being residue +1. Putative *N*-glycosylation sites are *circled*, and putative transmembrane domains are *underlined*.

not shown) for the binding of the antagonist [^{3}H]Ro15-1788 gave K_d values of 0.81 ± 0.07, 0.90 ± 0.09, 1.09 ± 0.04, and 0.56 ± 0.03 nM for $\alpha 1\beta 1\gamma 2$, $\alpha 2\beta 1\gamma 2$, $\alpha 3\beta 1\gamma 2$, and $\alpha 5\beta 1\gamma 2$ subunit combinations, respectively. To further characterize the BZ pharmacology of these subunit combinations, competition binding assays were performed using selected compounds, i.e., the full agonists flunitrazepam and triazolam, the BZ1-selective full agonist zolpidem, the partial agonists FG8205, abecarnil, and bretazenil, the BZ1-selective partial agonist CL218,872, the partial inverse agonist Ro15-4513, and the full inverse agonist β-CCM. The K_i values derived from these assays are shown in Table 1. Flunitrazepam, triazolam, abecarnil, bretazenil, and FG8205 bound with high affinity to all four $GABA_A$ receptors, with no significant selectivity between the various subunit combinations. β-CCM, in contrast, exhibited at least an 8–10-fold selectivity for $\alpha 1$-, $\alpha 2$-, and $\alpha 3$-containing receptors over $\alpha 5$-containing receptors. A similar result has been reported using rat $GABA_A$ receptor cDNAs (21). Of the compounds tested, CL218,882 exhibited a relatively lower affinity for all subunit combinations. It exhibited a 10-fold selectivity for $\alpha 1$-containing receptors, as reported previously (17, 21, 30), confirming that the $\alpha 1$ subunit combination corresponds to the BZ1 binding site of brain membrane. Similarly, another BZ1-selective compound, zolpidem, exhibited an approximately 10-fold selectivity for $\alpha 1$-containing receptors over $\alpha 2$- and $\alpha 3$-containing receptors and an almost 1000-fold selectivity for $\alpha 1$- over $\alpha 5$-containing receptors, as found previously using rat $GABA_A$ receptor cDNAs (21). In contrast, Ro15-4513 was unique in exhibiting a 10–15-fold higher affinity for $\alpha 5$-containing than for $\alpha 1$-, $\alpha 2$-, and $\alpha 3$-containing receptors.

The data confirm that the human $\alpha 1$-containing subunit combination corresponds to the BZ1 binding site, whereas the $\alpha 2$-, $\alpha 3$-, and $\alpha 5$-containing subunit combination corresponds to the BZ2 binding site. However, the lower affinity of zolpidem and β-CCM for $\alpha 5$-containing receptors indicates that there is considerable heterogeneity within the original BZ2 binding site. This is further emphasized by the binding of Ro15-4513, which showed higher affinity for $\alpha 5$- than for $\alpha 1$-, $\alpha 2$-, or $\alpha 3$-containing receptors. This compound is thus novel in being selective for another subunit combination over the $\alpha 1$-containing subunit combination. Ro15-4513 has previously been shown to bind to a class of BZ receptors in the cerebellum that do not bind diazepam, the so-called diazepam-insensitive sites (31). These binding sites are now known to be $\alpha 6$-containing $GABA_A$ receptors (11). $\alpha 6$-containing $GABA_A$ receptors have also been shown to have a higher affinity than $\alpha 1$-containing receptors for Ro15-4513, although the degree of selectivity was only 3-fold (11), compared with the 10–15-fold selectivity for $\alpha 5$- over

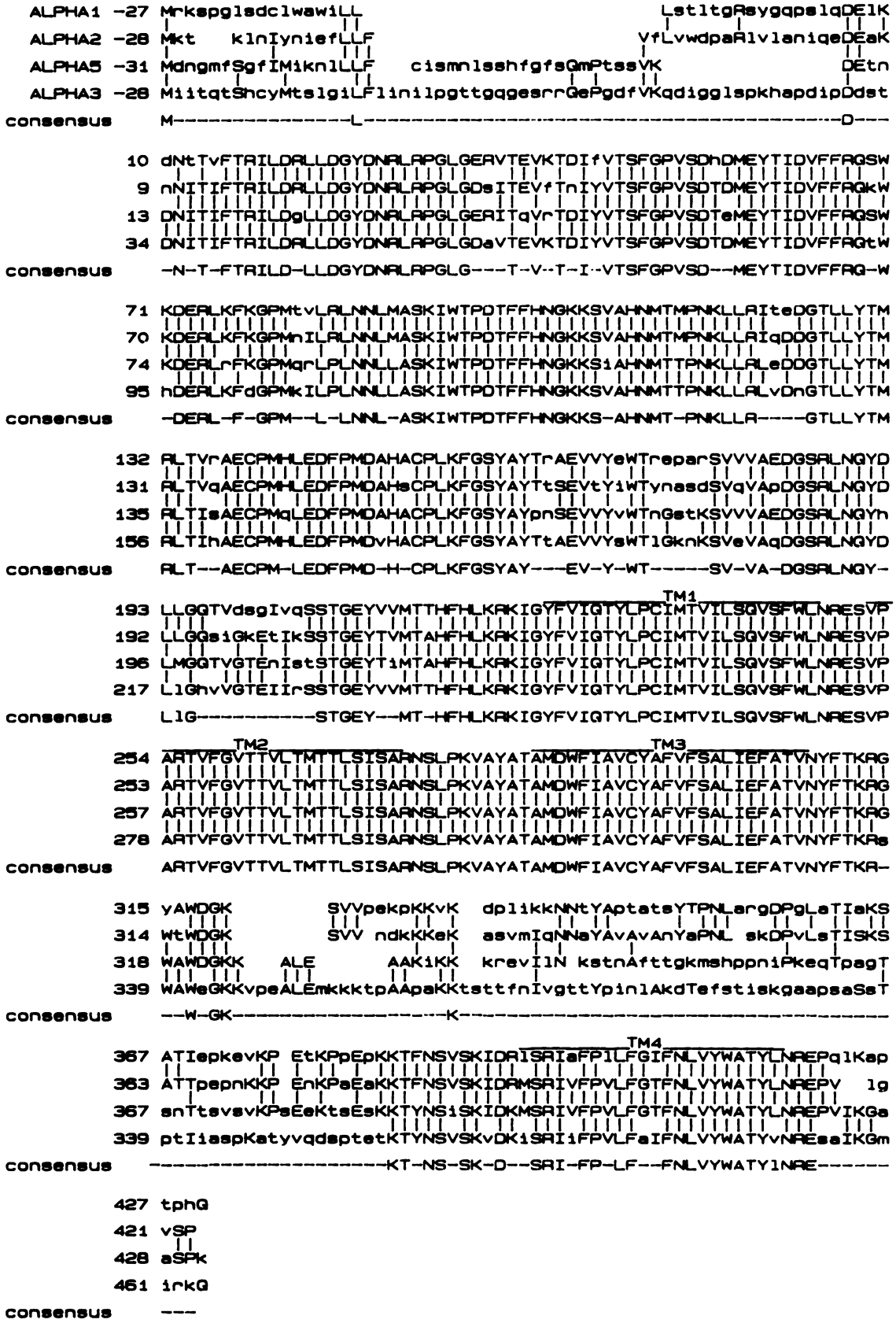

Fig. 2. Alignment of the deduced amino acid sequences of the human GABA$_A$ receptor α1 (24), α2, α3, and α5 (26, 27) subunits. Sequences were aligned using the Intelligenetics Genalign program so that the most homologous sequences are placed next to each other. The *consensus line* indicates where a residue is conserved in all sequences. The amino acid numbers are indicated on the *left*, with the putative mature polypeptide beginning at position +1. *TM1–4*, putative transmembrane domains.

TABLE 1

Affinity (K_i) of selected ligands for various human $GABA_A$ receptor subunit combinations transiently expressed in HEK 293 cells

The affinities were determined from displacement of 0.4 nM [^{3}H]Ro15-1788 by the various ligands, as described in detail in Materials and Methods. Values are given as mean ± standard error of at least three independent determinations.

	K_i			
	$\alpha1\beta1\gamma2S$	$\alpha2\beta1\gamma2S$	$\alpha3\beta1\gamma2S$	$\alpha5\beta1\gamma2S$
	nM			
Flunitrazepam	11.5 ± 1.6	5.2 ± 0.4	15.7 ± 3.6	5.5 ± 0.4
Triazolam	1.8 ± 0.4	1.2 ± 0.2	3.0 ± 0.7	1.2 ± 0.3
Bretazenil	1.2 ± 0.2	1.2 ± 0.2	1.3 ± 0.2	2.4 ± 0.5
Abecarnil	3.9 ± 1.0	4.4 ± 0.6	7.1 ± 0.6	8.4 ± 0.1
FG8205	2.3 ± 0.5	3.7 ± 0.2	6.4 ± 2.3	6.4 ± 0.1
Ro15-4513	10.0 ± 0.6	10.4 ± 1.1	7.8 ± 1.8	0.7 ± 0.1
β-CCM	2.2 ± 0.4	6.5 ± 1.2	9.2 ± 1.8	76.4 ± 7.8
Zolpidem	111.9 ± 17.0	760.6 ± 88.3	2,149.5 ± 492.3	25,846.1 ± 2,150.5
CL218,872	290.5 ± 31.9	2,903.2 ± 420.2	3,136.4 ± 597.6	1,154.1 ± 66.2

α1-, α2-, and α3-containing receptors reported here. It is interesting to note that Ro15-1788 and Ro15-4513 are quite similar in structure, yet the former is a nonselective BZ antagonist whereas the latter is an α5-selective partial inverse agonist. Thus, relatively small changes in the structures of these compounds can result in significant alterations in both their selectivity and efficacy.

We report in this investigation that abecarnil exhibits nanomolar affinity for all human $GABA_A$ receptor subunit combinations examined, with no significant selectivity. This is in disagreement with another report (32), which used recombinant rat $GABA_A$ receptor subunit combinations and found that α1-containing receptors had 30-fold and 10-fold higher affinity for abecarnil than did α3- and α5-containing receptors, respectively. The reason for this discrepancy is currently unclear.

A number of the BZ site agonists used in this study (flunitrazepam, triazolam, bretazenil, abecarnil, and FG8205) exhibited very similar profiles, binding with nanomolar affinity and showing no selectivity between the $GABA_A$ receptor subunit combinations. These compounds do, however, have different clinical/behavioral profiles; both flunitrazepam and triazolam are sedative agents and produce marked motor impairment, whereas bretazenil, abecarnil, and FG8205 show a much lower tendency to cause these effects (29, 33). It is possible to interpret this observation by postulating that flunitrazepam and triazolam cause sedation by binding with high affinity to other receptor subtypes to which bretazenil, abecarnil, and FG8205 do not bind. A more likely interpretation is that the different efficacies of these compounds lead to their different behavioral and clinical profiles. When tested electophysiologically on recombinant human $GABA_A$ receptors, flunitrazepam and triazolam are full agonists, whereas bretazenil, abecarnil, and FG8205 are partial agonists (30).[1] This supports previous observations that partial agonists have a lesser tendency to cause sedation (33). It remains to be demonstrated that the sedative properties of BZs can also be avoided by developing $GABA_A$ receptor subtype-selective BZ site compounds.

The BZ pharmacologies of the recombinant human $GABA_A$ receptors were in good agreement with the BZ pharmacology of receptor subtypes detergent solubilized from rat brain and immunoprecipitated with α subunit-specific antisera (34). The only compound that showed a significant difference was CL218,872, which had an approximately 6–7-fold lower affinity for recombinant human $\alpha2\beta1\gamma2$, $\alpha3\beta1\gamma2$, and $\alpha5\beta1\gamma2$ receptors, compared with the α2-, α3-, and α5-containing receptors immunoprecipitated from rat brain. However, the fact that the pharmacologies are overall very similar suggests that these recombinant receptors are a valid representation of native $GABA_A$ receptors. Additionally, because the native receptors immunoprecipitated from rat brain undoubtedly contained β subunits other than β1 (35, 36), this suggests that the type β subunit present does not significantly influence the BZ pharmacology of $GABA_A$ receptor subtypes. Indeed, a detailed study using different β subunits coexpressed with α and γ2 subunits in transfected cells confirms this observation.[2]

Previous studies investigating the BZ pharmacology of $GABA_A$ receptors have identified BZ1-selective compounds such as zolpidem and CL218872, which have higher affinity for α1-containing receptors. Here we have identified a compound, Ro15-4513, that has a higher affinity for α5-containing than for α1-, α2-, and α3-containing receptors. This suggests that in the future, using transfected cells transiently or stably (37) expressing $GABA_A$ receptor subunit combinations, it may be possible to identify receptor subtype-selective BZs that have improved profiles, lacking some of the undesirable effects such as tolerance and sedation that are features of the currently available drugs.

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