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Functional Characterization of Human γ-Aminobutyric Acid$_A$ Receptors Containing the α4 Subunit

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SUMMARY

The α subunits are an important determinant of the pharmacology of γ-aminobutyric acid$_A$ (GABA$_A$) receptors with respect to agonists, antagonists, and modulatory compounds, particularly the benzodiazepines. The α4 subunit is the least abundant subunit in the brain and the most similar in deduced primary amino acid sequence to the α6 subunit. We demonstrate that the human α4 subunit forms a functional receptor when expressed with β γ2, demonstrating some properties similar to α6β γ2 and some properties more akin to α1β γ2. It also exhibited some properties that were unlike any other α subunit-containing receptor. GABA affinity seemed to be identical to that of the α1β1γ2 receptor; however, the partial agonists 4,5,6,7-tetrahydroisoxazolo-[5,4-c]pyridin-3-ol and piperidine-4-sulfonic acid showed lower efficacy than at either α1β1γ2 or α6β1γ2. Benzodiazepine pharmacology of α4-containing receptors was similar to that of α6-containing receptors with the exception of dimethoxy-4-ethyl-β-carboline-3-carboxylate, which behaved as a partial inverse agonist. Pentobarbital potentiated α4β1γ2 receptor GABA responses to a level comparable with α6β1γ2 (~700% of EC$_{20}$); however, unlike α6β1γ2 receptors, it did not elicit any direct activation of the receptor. Propofol also potentiated α4β1γ2 GABA responses but to a level more comparable to that of α1β1γ2, suggesting that these compounds act via different sites. Unlike other subunit combinations, propofol did not elicit a direct activation of the receptor. These results suggest that the mechanism for direct activation of the GABA$_A$ receptor by pentobarbital and propofol is absent on α4-containing receptors. Furosemide, which non-competitively inhibits the GABA$_A$ receptor, showed 700-fold selectivity for α6β3γ2 receptors over α1-, α2-, α3-, and α5-containing receptors and exhibited selectivity for α4β3γ2 receptors (>50-fold). These experiments reveal a unique pharmacology for α4-containing receptors with some similarities to both α6- and α1-containing receptors.

GABA is the primary inhibitory neurotransmitter in the vertebrate central nervous system and exerts the majority of its postsynaptic effects through GABA$_A$ receptors. These receptors are chloride-gated ion channels and are present on most of the neurons in the central nervous system. These receptors are known to be the site of action for a large number of clinically important drugs such as benzodiazepines and barbiturates, which are prescribed as anxiolytics, sedatives, antiepileptic agents, and hypnotics (1, 2). Molecular cloning techniques have revealed the presence of multiple gene families in the mammalian central nervous system (α1-6, β1-3, γ1-3, δ, ρ1-2), which encode GABA$_A$ receptor subunits that can coassemble into many different receptor subtypes, containing at least three different subunits (an αβ and a γ, and in cerebellar granule cells, an αβ and a γ, or a δ) (2, 3).

Pharmacological analysis of different subunit combinations expressed in recombinant systems have demonstrated that different subunits confer distinct properties to the GABA$_A$ receptors that are formed and that the properties of αβγ represent the minimum requirement to confer sensitivity to benzodiazepines (4, 5; for review, see Refs. 2, 3, and 6). The α subunit variant present has been shown to exhibit a major effect on the affinity and maximum degree of modulation or efficacy by benzodiazepine ligands (7–9). It has also been demonstrated that the β subunit has little or no effect on benzodiazepine pharmacology (10) but is a critical component of native GABA$_A$ receptors. The γ subunit is required for benzodiazepine affinity and influences the benzodiazepine pharmacology. The most abundant of these is the γ2 (11, 12), which confers to GABA$_A$ receptors a pharmacology comparable to that of most native brain receptors (4, 5, 8). The γ1 and γ3 subunit-containing receptors make up a much smaller proportion of GABA$_A$ receptors in the rat brain (10–15% each) (12). Both γ1- (13, 14) and γ3- (15–18) containing receptors have benzodiazepine binding sites with pharmacological profiles different from those of receptors that contain γ2.

ABBREVIATIONS: GABA, γ-aminobutyric acid; THIP, 4,5,6,7-tetrahydroisoxazolo-[5,4-c]pyridin-3-ol; P4S, piperidine-4-sulfonic acid; DMCM, dimethoxy-4-ethyl-β-carboline-3-carboxylate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; β-CCE, ethyl-β-carboline-3-carboxylate; β-CCP, propyl-β-carboline-3-carboxylate; YAC, yeast artificial chromosome; PCR, polymerase chain reaction; bp, base pair(s); TM, transmembrane domain.

A subset of $GABA_A$ receptors have been labeled diazepam insensitive. These have a very low affinity for classic benzodiazepines such as flunitrazepam but still form a benzodiazepine-like site that has a high affinity for the compounds Ro15-4513 and bretazenil (19, 20). These receptors are primarily localized in cerebellar granule cells (21) and correspond to receptors containing an α6 subunit. Another α subunit has been identified that confers diazepam insensitivity (22). The deduced amino acid sequence of the rat α4 subunit was found to be most homologous to that of the α6 subunit. When cotransfected with β2γ2, it formed a receptor with a high affinity binding site for [^{3}H]Ro15-4513 and [^{3}H]muscimol, similar to α6, and low affinity for diazepam, flunitrazepam, and CL218,872 (23). This subunit was of fairly low abundance in the rat brain and found mainly in thalamic and hippocampal brain regions (23–25).

When it was first cloned (22), the α4 subunit was shown to form functional receptors when coexpressed with a β1 subunit; however, since then, there has been little published on the functional pharmacology of receptors containing this subunit. We cloned and sequenced a human cDNA encoding the α4 subunit of the $GABA_A$ receptor. The α4 gene has been assigned to human chromosome 4p12-q12. Recombinant human α4β1γ2S and α4β3γ2 $GABA_A$ receptors were expressed in *Xenopus laevis* oocytes, and the functional pharmacology of these receptors was investigated using the two-electrode voltage-clamp method.

Materials and Methods

Isolation of a cDNA encoding the human $GABA_A$ receptor α4 subunit. A short human α4 cDNA sequence was first generated by PCR using oligonucleotide primers derived from the bovine α4 sequence (25): 5′-TTTCAGGAATTCAGTGCTGAGAGAAAAGCATCCTGAAAC-3′ (bp 1121–1160, incorporating an *Eco*RI site) and 5′-ATCCAGAAGCTTGTGGAGCAGAGGGAGTAGTAGTGGC-3′ (antisense, bp 1540–1577, incorporating a *Hin*dIII site). PCR was performed as described previously (26) using a human fetal brain cDNA library in λZAP (Stratagene, La Jolla, CA)) as template, and the PCR product was subcloned into pBluescript SK(−) (Stratagene).

A human fetal brain cDNA library in λ ZAP was screened using the ^{32}P-labeled α4 probe described above, and a single cDNA clone was isolated. DNA sequence analysis indicated that the clone contained 3′-untranslated sequence and 3′-coding region extending to bp 1162 equivalent of bovine α4 cDNA sequence. The missing 5′ sequence was obtained by anchored PCR using human brain 5′-RACE-Ready cDNA (Clontech, Palo Alto, CA). The antisense oligonucleotides used for the nested PCR were 5′-ATTGGCATTTGTATTCTGCAGAGG-3′ and 5′-GGAAGATTTGCTTGAATGGTTTGG-3′. A 1200-bp PCR product was obtained; the DNA sequencing of this product confirmed that it extended to 130 bp 5′ of the initiating ATG codon.

A cDNA containing the complete coding region of the α4 subunit was obtained using oligonucleotide primers generated from sequences of the 5′- and 3′-untranslated regions: 5′ sense primer, 5′-CCTGGATCCGTGAACAGGCTTGAAGTATG-3′ (incorporating a *Bam*HI site); and 3′ antisense primer, 5′-ACGAATTCACATTAGACTTTCTGATTTCTC-3′ (incorporating an *Eco*RI site). PCR was performed using human thalamus cDNA. The PCR product generated was subcloned into pCDNAI/Amp and sequenced on both strands using an Applied Biosystems (Norwalk, CT) 373A sequencer with dye terminator chemistry.

For expression of α4 in *X. laevis* oocytes, the cDNA was engineered to contain the 5′-untranslated region and sequences encoding the signal peptide of bovine α1 subunit. This construct was generated using the unique *Pme*I site at bp 128 (equivalent to the putative signal cleavage point in the translated protein) of the α4 cDNA clone. The α1 sequence was obtained by PCR using bovine α1 cDNA as a template, the sense primer 5′-CATGATGGATCCGCCCGCTCAGAC-3′ (bp 12–35 of α1, incorporating a *Bam*HI site), and the antisense primer 5′-TAATGAGTTTAAACCATAGCTTCTTCCAGT-3′ (bp 269–298, incorporating a *Pme*I site). The PCR product was digested with *Bam*HI and *Pme*I and subcloned into similarly cut α4 pCDNAI/Amp.

Mapping of human α4 gene. Chromosome assignment of the human α4 cDNA was achieved by PCR from NIGMS human × rodent somatic cell hybrid DNAs (Coriell Institute, Camden, NJ). Primers for PCR (5′-GATGAGTGAATGTGTTCTGGG-3′ and 5′-TGGAATACCTAGAAAGACAGCC-3′) were chosen from the 3′-untranslated region using the computer program PRIMER (The Whitehead Institute for Biomedical Research). PCR was performed as described previously (10), and products were analyzed on a 0.8% agarose gel. Fine mapping of the α4 gene was performed by isolating YAC clones containing the α4 PCR product from the CEPH megabase insert library (27) using a previously described strategy (28).

Oocyte expression. Adult female *X. laevis* 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. 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 medium 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 $NaHCO_3$, pH 7.5. Cells were impaled with two 1–3 MΩ electrodes containing 2 M KCl and voltage-clamped between −40 and −70 mV.

Experimental design. In all experiments, drugs were applied in the perfusate until the peak of the response was observed. Noncumulative concentration-response curves to agonists were constructed allowing ≥3 min between each agonist application to prevent desensitization. Curves were fitted using a nonlinear square-fitting program to the equation $f(x) = B_{max}/[1+(EC_{50}/x)^n]$, where x is the drug concentration, EC_{50} is the concentration of drug eliciting a half-maximal response, and n is the Hill coefficient. The effects of $GABA_A$ receptor modulators were examined on control GABA EC_{20} responses with a preapplication time of 30 sec.

All data are shown as mean ± standard error. Differences between mean values were evaluated by Student's t test and considered significant if $p < 0.05$.

Results and Discussion

The deduced primary amino acid sequence of the human $GABA_A$ receptor α4 subunit is shown in Fig. 1 aligned with sequences of the other human α subunits. The sequence has an open reading frame of 554 amino acids, 2 more than the rat subunit (23) and 2 less than the bovine subunit (22). Overall, the human deduced amino acid sequence is different from the rat sequence by 62 amino acids (i.e., 88% identity). All of the motifs found in the other members of this gene family are present [i.e., a putative signal peptide, four hydrophobic putative transmembrane domains (TM1–TM4), two cysteine residues separated by 13 amino acids in the putative large extracellular domain]. The sequence contains three putative *N*-glycosylation sites, four putative protein kinase A phosphorylation sites, and a number of

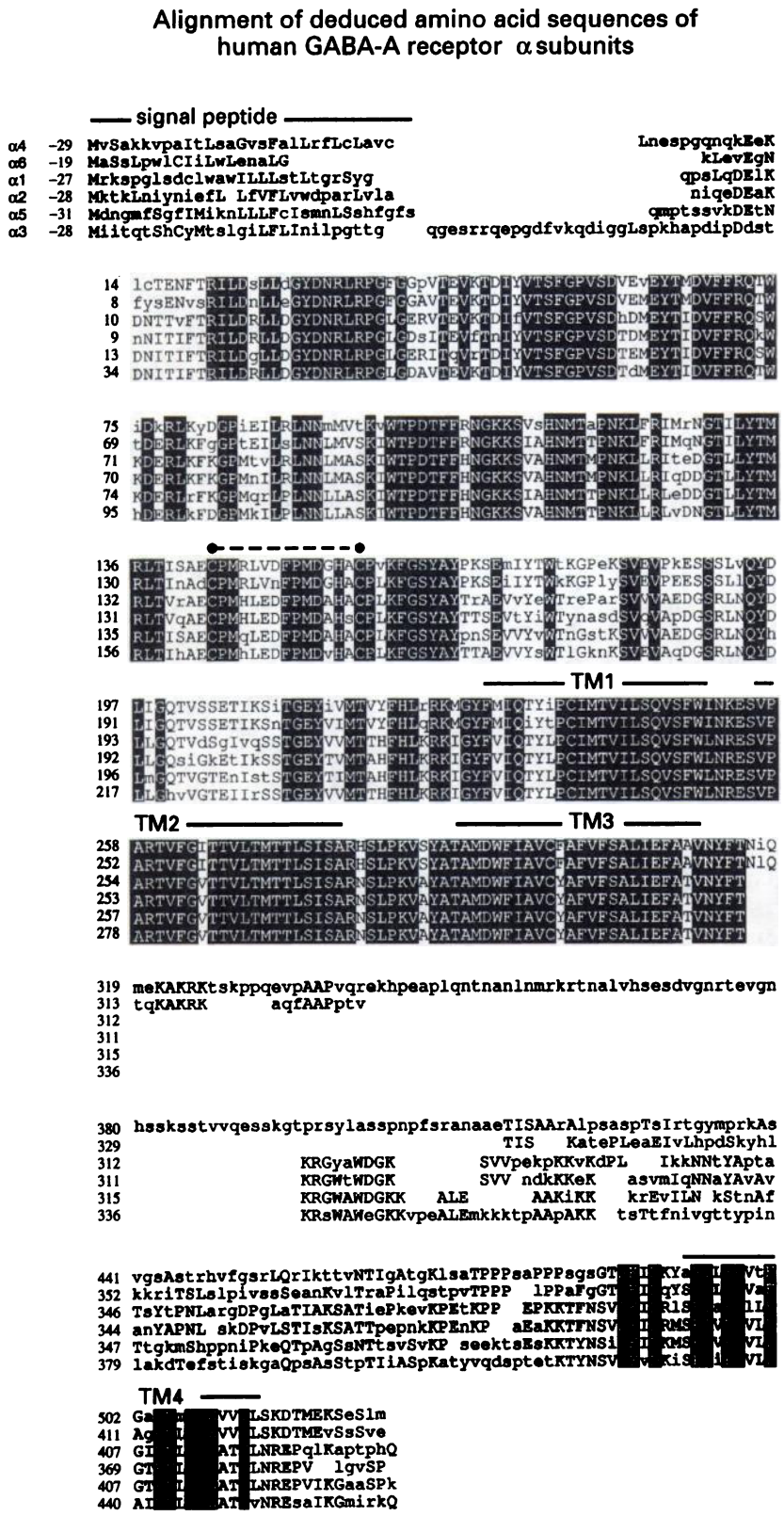

Fig. 1. Alignment of deduced amino acid sequences of human $GABA_A$ receptor α subunits. The human $\alpha 4$ subunit is the largest human $GABA_A$ subunit, being composed of 554 amino acids. Note that a large putative cytoplasmic domain is found between TM3 and TM4. The $\alpha 1$ sequence is from Ref. 41, the $\alpha 2$ and $\alpha 3$ sequences are from Ref. 8, the $\alpha 4$ sequence is from the current report, the $\alpha 5$ sequence is from Ref. 42, and the $\alpha 6$ sequence is from Ref. 20.

putative protein kinase C phosphorylation sites. As has been found for other $GABA_A$ receptor subunits, most of the interspecies amino acid differences are in the putative signal peptide and the putative cytoplasmic domain between TM3 and TM4.

From the alignment in Fig. 1, it can be seen that the $\alpha 4$ subunit is significantly larger than the other subunits, with the additional residues occurring in the putative cytoplasmic domain between TM3 and TM4. $\alpha 4$ is most homologous to the $\alpha 6$ subunit. Like the interspecies amino acid differences be-

tween the same α subunit, the amino acid differences between the different α subunits of the same species are localized primarily to the putative signal peptide and the putative large cytoplasmic domain. The TM1–TM3 region is highly conserved.

Chromosome assignment of the human α4 gene. A 250-bp product was amplified from human genomic DNA and from DNA from the human × rodent hybrids containing human chromosome 4, with a concordance frequency of 94%. The address of the YAC containing the sequence tagged site derived from the α4 gene was 920 f 5. This YAC was linked to two sequence tagged sites on chromosome 4 at the genetic position 0.57. Taking YAC sizes into consideration, this resulted in a regional chromosome location of 4p12-q12. It is of interest to note that the α2 and β1 genes have been assigned to 4p12-q13 using radioactive *in situ* hybridization (29). Similarly, the γ1 gene, which was originally mapped to 4p14-q21.1 by PCR amplification of radiation hybrids, has subsequently been fine mapped to a YAC that maps to 4p12-q12.[1] Thus, it is probable that these four genes form a cluster. Other $GABA_A$ receptor genes were also found clustered at discrete sites in the human genome. α1, α6, β2, and γ2 are located at 5q31.3-q33.2, whereas α5, β3, and γ3 are located at 15q11-q13 (for review, see Ref. 3).

Functional expression of α4-containing receptors. In this study, we compared the pharmacology of human α4βxγ2 with that of human α6-containing receptors (the typical diazepam-insensitive receptors) and human α1-containing receptors (the major population of diazepam-sensitive receptors). As described below, the pharmacology of α4βxγ2 is similar in some cases to that of α6-containing receptors but has some properties similar to α1-containing receptors and some unique properties.

Expression of human α4 with β1γ2 resulted in currents consistently smaller than with either α6β1γ2 or α1β1γ2. This was also observed with the use of β3γ2 subunits. As a consequence, subunits were injected in the ratio of 3:1:1 (α/β/γ) to maximize the expression of α4-containing receptors. When α4 was coinjected with β2γ2, the currents that were measured were too small to work with, suggesting that β2γ2 was not assembling efficiently with the α4 subunit.

GABA agonists and antagonists. Concentration-response curves to GABA were constructed and revealed an EC_{50} of 33.1 ± 3.1 μM (five experiments) with a Hill coefficient of 1.29 ± 0.05 (Fig. 2). This EC_{50} value is similar to that obtained for α1β1γ2S (25 ± 4 μM) but significantly higher than that obtained for α6β1γ2S (14.4 ± 1.4 μM) (Table 1). Although these differences were relatively small (2-fold) for GABA, other agonist compounds displayed greater variance among subunit combinations (Table 1).

Two $GABA_A$ receptor partial agonists were studied on α4β1γ2 and compared with α1β1γ2 and α6β1γ2. THIP and P4S have previously been shown to be partial agonists with varying efficacy on different recombinant receptors, with the efficacy varying according to the α subunit variant (α1, α3, or α5) that is present (30). As can be seen in Fig. 3 and Table 1, both THIP and P4S have lower efficacy on α1β1γ2 and even lower efficacy on α4β1γ2 compared with α6β1γ2s. These results confirm our previous report (30) that the degrees of efficacy and affinity for THIP and P4S are dependent on the

[1] J. Sikela, A. S. Wilcox, and P. J. Whiting, unpublished observations.

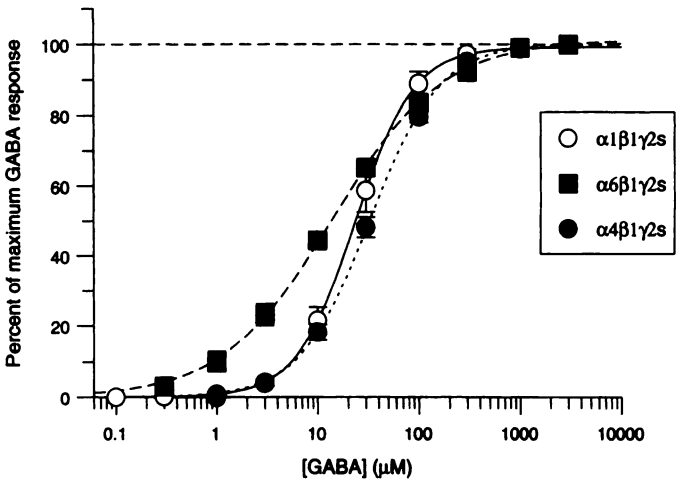

Fig. 2. Concentration-response curves for GABA in oocytes injected with α1β1γ2s, α6β1γ2s, and α4β1γ2s. Data represent the mean ± standard error for at least four oocytes.

TABLE 1

Affinity, maximum efficacy relative to GABA, and Hill coefficient of GABA, THIP, and P4S in oocytes injected with α1β1γ2s, α6β1γ2s, and α4β1γ2s

Values were calculated for each individual oocyte and represent the mean ± standard error of the data in Figs. 2 and 3.

	EC_{50}	Maximum efficacy	n_H
GABA			
α1β1γ2s	25 ± 4	100	1.5 ± 0.2
α6β1γ2s	14 ± 1	100	0.8 ± 0.1
α4β1γ2s	33 ± 3	100	1.3 ± 0.1
THIP			
α1β1γ2s	358 ± 60	78 ± 6	1.05
α6β1γ2s	66 ± 13	133 ± 6	0.9 ± 0.1
α4β1γ2s	835 ± 165	53 ± 9	1.1 ± 0.1
P4S			
α1β1γ2s	52 ± 5	32 ± 11	1.0
α6β1γ2s	51 ± 10	70 ± 10	0.6 ± 0.1
α4β1γ2s	nd	6 ± 1	nd

α subunit variant. THIP and P4S have the highest affinity and efficacy on α6β1γ2s and the lowest affinity and efficacy on α4β1γ2. The affinities and efficacies for the other α subunits when expressed with β1γ2 lie between these two extremes (30). These observations suggest that with regard to GABA agonist activation, α6 and α4 represent the two most diverse subunits. An interesting observation was that on α6β1γ2 receptors, the maximum response to THIP was consistently larger than that obtained with a maximum concentration of GABA. One possible explanation for this may be that GABA acts as a partial agonist at α6β1γ2 receptors, although differences in activation/desensitization rates for the two agonists also may account for this. Further experiments in which the kinetics of these responses are examined are necessary.

The efficacy of P4S on α4β1γ2 was only 6% of maximum GABA, being effectively an antagonist. As well as rightward shifts, however, depressed maximum responses were observed when GABA concentration-response curves were constructed in the presence of 100 μM P4S. One could speculate that P4S is behaving as a mixed competitive and noncompetitive antagonist at the $GABA_A$ receptor. For this reason, inhibition curves to P4S were constructed on α4β1γ2, reveal-

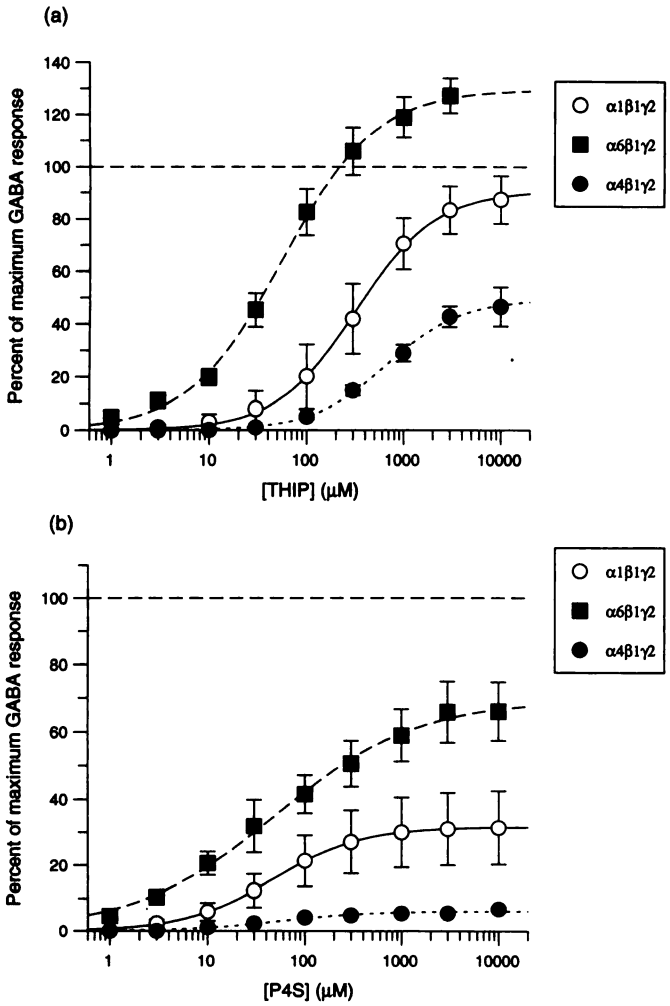

Fig. 3. Concentration-response curves for the partial agonists THIP (a) and P4S (b) on oocytes injected with $\alpha1\beta1\gamma2$s, $\alpha6\beta1\gamma2$s, and $\alpha4\beta1\gamma2$s. Data are normalized to the maximum response to GABA (3 mM) on each cell and represent the mean ± standard error for at least four oocytes.

ing an IC_{50} of 29 ± 3.1 μM, which is similar to the agonist affinities for this compound at $\alpha6\beta1\gamma2$ and $\alpha1\beta1\gamma2$ (51 and 52 μM, respectively).

Human $\alpha4\beta1\gamma2$ receptors were sensitive to the competitive antagonists bicuculline (100 μM) and SR95531 (1 μM) and the noncompetitive antagonist picrotoxin (100 μM) (Fig. 4). Bicuculline behaved as a competitive GABA antagonist with a pK_B of 5.86 ± 0.03 (data not shown). The pK_B for bicuculline on $\alpha4\beta1\gamma2$s is similar to the value of 5.96 obtained for $\alpha1\beta1\gamma2$s (31) and other $\alpha x\beta1\gamma2$s-containing receptors.[2] Bicuculline therefore shows no selectivity for any particular α subunit expressed with $\beta1\gamma2$s subunits.

Modulation at the benzodiazepine site. A number of compounds that act at the benzodiazepine site were examined for modulation of GABA currents at $\alpha4\beta1\gamma2$s receptors. These were compared directly with modulation on $\alpha1\beta1\gamma2$ and $\alpha6\beta1\gamma2$. Several benzodiazepines and β-carbolines and some "atypical" compounds, all of which act via the benzodiazepine site, were examined at a concentration of 1 μM (Fig. 5a). The "classic" benzodiazepine full agonist flunitrazepam had no effect on $\alpha4\beta1\gamma2$ receptors at 1 μM. Similarly, zolpidem and CL218,872, which are α1 selective, had no effect on $\alpha4\beta1\gamma2$. These results agree with the previous finding that rat α4-containing receptors have low affinity for classic benzodiazepines such as diazepam and flunitrazepam and the type I-selective zolpidem and CL218,872 (23, 32, 33). This is consistent with the α4 subunit's containing an arginine residue at position 106 (also present in α6), which is the position at which α1, α2, and α3 subunits have a histidine, conferring high affinity for these compounds (34).

Like α6-containing receptors, α4 also retains high affinity for Ro15-4513 (23, 33), corresponding to a diazepam-insensitive receptor. We previously demonstrated that this compound behaves as a partial agonist at $\alpha6\beta2\gamma2$ receptors (20), as does the close analogue Ro15-1788. Both of these compounds are also partial agonists at $\alpha4\beta1\gamma2$ receptors, with efficacies of 53% and 38%, respectively, potentiating GABA currents to a similar degree as $\alpha6\beta1\gamma2$ receptors (Fig. 5a). It is interesting to note how the efficacy of these two compounds is shifted from antagonist on $\alpha1\beta1\gamma2$ receptors to partial agonists on α6- and α4-containing receptors. Bretazenil has been demonstrated to have high affinity (12–50 nM) at $\alpha6\beta3\gamma2$ and at α4-containing receptors (20, 33). This compound acted as a partial agonist with the highest efficacy of all of the benzodiazepine-site compounds tested at $\alpha4\beta1\gamma2$ (78%). This was similar to that on $\alpha6\beta1\gamma2$ (87%). FG8205 is an imidazobenzodiazepine partial agonist, which has a relatively high affinity and efficacy on $\alpha6\beta2\gamma2$ (20). At 1 μM, this compound potentiated $\alpha4\beta1\gamma2$ (51%) to the same level as $\alpha1\beta1\gamma2$ and $\alpha6\beta1\gamma2$ (Fig. 5a).

The β-carbolines DMCM, β-CCE, and β-CCP bind to two sites on the $GABA_A$ receptor: with high affinity to the benzodiazepine site and low affinity to the loreclezole site, which is present only on β2- and β3-containing receptors (35). To investigate these compounds, we used $\alpha4\beta1\gamma2$, $\alpha1\beta1\gamma2$, and $\alpha6\beta1\gamma2$ combinations to eliminate the low affinity potentiat-

[2] B. Ebert, unpublished observations.

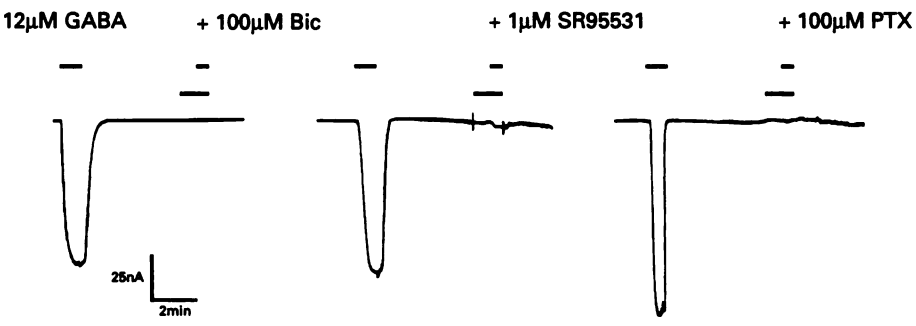

Fig. 4. Effects of $GABA_A$ antagonists on currents elicited by GABA in oocytes injected with $\alpha4\beta1\gamma2$s. The competitive $GABA_A$ antagonists bicuculline (*Bic*) (100 μM) and SR95531 (1 μM) and the noncompetitive antagonist picrotoxin (*PTX*) (100 μM) completely inhibited the response to 12 μM GABA in oocytes injected with $\alpha4\beta1\gamma2$s.

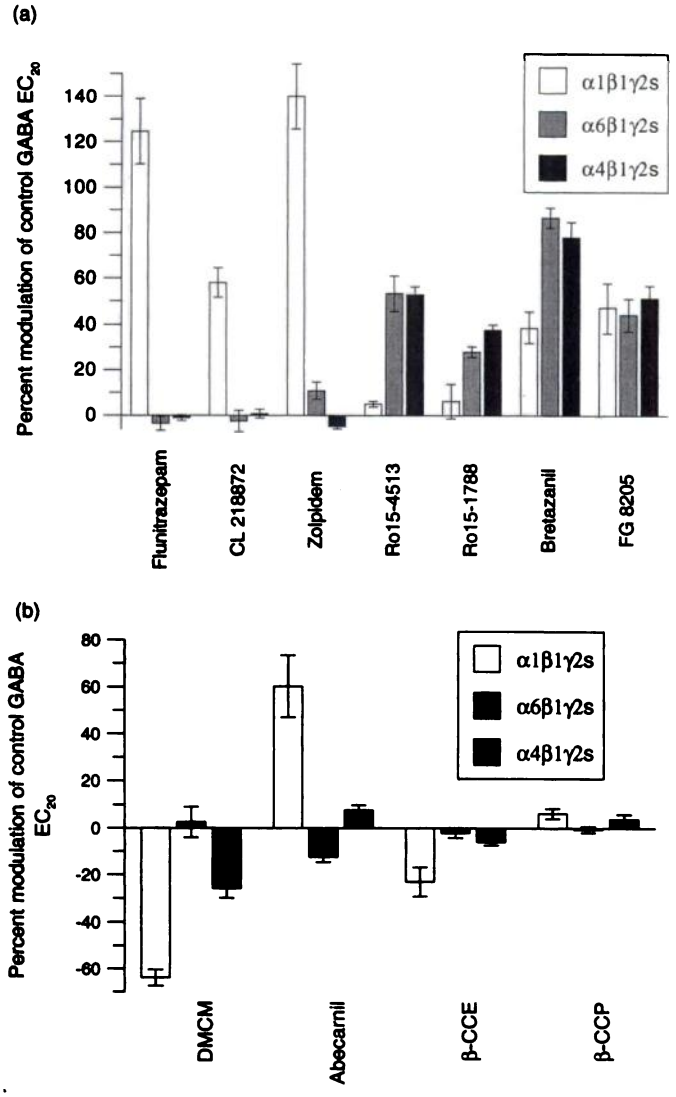

Fig. 5. Modulation of control GABA EC_{20} responses by benzodiazepine and β-carboline ligands. a, Modulation of control GABA EC_{20} responses by benzodiazepine ligands in oocytes injected with α1β1γ2s, α6β1γ2s, and α4β1γ2s. b, Modulation of control GABA EC_{20} responses by β-carboline ligands in oocytes injected with α1β1γ2s, α6β1γ2s, and α4β1γ2s. All compounds were examined at a concentration of 1 μM, and the data shown are the mean ± standard error at least four oocytes.

ing effects observed on α6β2γ2s and α6β3γ2. The effects of DMCM, abecarnil, β-CCE, and β-CCP were compared on α1β1γ2, α4β1γ2, and α6β1γ2 at a concentration of 1 μM. DMCM, which is an antagonist on α6β1γ2 but full inverse agonist on α1β1γ2, also showed inverse agonist activity on α4β1γ2, but this inhibition was not as great as at α1β1γ2 (Fig. 5b). Because the lowest affinity for DMCM on these receptors was 134 nM at α6β3γ2 (20) and was reported to be 14 nM at α4β2γ2 (33), the concentration of 1 μM should be sufficient to fully saturate the benzodiazepine binding site on all receptor combinations. Abecarnil showed potentiation on α1β1γ2 but had little effect on either α6β1γ2 or α4β1γ2 at concentrations of ≤10 μM. This may, however, be due to a low affinity rather than to a difference in efficacy. β-CCE has an affinity of 0.6 nM for α1β3γ2[3] and is an inverse agonist on α1β1γ2 but did not modulate α6β1γ2 or α4β1γ2 at 1 μM (Fig. 5b). Recent findings suggest a low affinity for this compound at α6- and α4-containing receptors (33). β-CCP, despite having 2 nM affinity for the benzodiazepine site,[4] did not modulate any of the combinations tested, suggesting that it is an antagonist at the benzodiazepine site.

Modulation by anesthetics. GABA responses (EC_{20} values) were potentiated by the steroid 5α-pregnan-3α-ol-20-one (367 ± 26%). This potentiation was not significantly different from that obtained on α1β2γ2S (298 ± 60%) or α6β2γ2S (312 ± 79%), suggesting little differences at the steroid modulatory site on α4-containing receptors.

Both pentobarbital and propofol have three effects on the GABA$_A$ receptor: potentiation of submaximal GABA responses, direct activation of the receptor, and, at high concentrations, block of the GABA current (36–38). Marked potentiation of the GABA response at α4β1γ2s receptors (706 ± 82%) was observed with pentobarbital (100 μM) (Fig. 6a) compared with 288 ± 32% on α1β1γ2S and 719 ± 52% on α6β1γ2S. The direct activation by pentobarbital on α4β1γ2S receptors was extremely small, being only 6% of maximum GABA at 1 mM, compared with 31% and 147% on α1β1γ2S and α6β1γ2S, respectively (Fig. 6b). Therefore, pentobarbital potentiates α4β1γ2 receptors to a level equivalent to the total effect (potentiation and direct effect) on α6β1γ2, without eliciting any direct activation of the receptor up to a concentration of 3 mM. Pentobarbital (100 μM; maximal effective concentration) potentiated α1β1γ2 receptors to a much lower extent, without eliciting a direct effect on this subunit combination. Although pentobarbital potentiated α6β1γ2 EC_{20} responses to a maximum of ~700%, this includes the direct activation component, which is significant at 100 μM on α6β1γ2 (38). In the absence of an antagonist-to-pentobarbital direct activation, it is impossible to resolve the potentiating and direct components on α6-containing receptors.

The anesthetic propofol did not elicit the same degree of potentiation as pentobarbital and, compared for these different subunit combinations, gave a different profile to pentobarbital. Propofol (10 μM; a concentration that elicited negligible direct activation on any subunit combination) was found to potentiate the GABA response (EC_{20}) of α1β1γ2 (214%) to a greater extent than α6β1γ2 receptors (120%) (Fig. 6c). This is consistent with other reports using Ltk$^-$ cells.[5] Propofol also potentiated the GABA EC_{20} at α4β1γ2 by 214%. Thus, in contrast to pentobarbital, the potentiating effect of propofol is greater at α1β1γ2s and α4β1γ2s than at α6β1γ2s. At high concentrations, propofol also directly activates GABA$_A$ receptors (37). Like pentobarbital, direct activation by propofol was greatest at α6β1γ2, lower at α1β1γ2, and negligible on α4β1γ2, up to concentrations of 1 mM (Fig. 6d).

Pentobarbital and propofol therefore show opposite profiles with regard to potentiation of the GABA response but similar profiles for direct activation of GABA$_A$ receptor subtypes. These data suggest that propofol and pentobarbital act at different sites to potentiate the response but may work in a similar manner to directly gate the channel, which is absent on α4β1γ2 receptors.

We have previously shown that direct activation of GABA$_A$

[3] R. McKernan, unpublished observations.

[4] R. McKernan, unpublished observations.

[5] N. Harrison, unpublished observations.

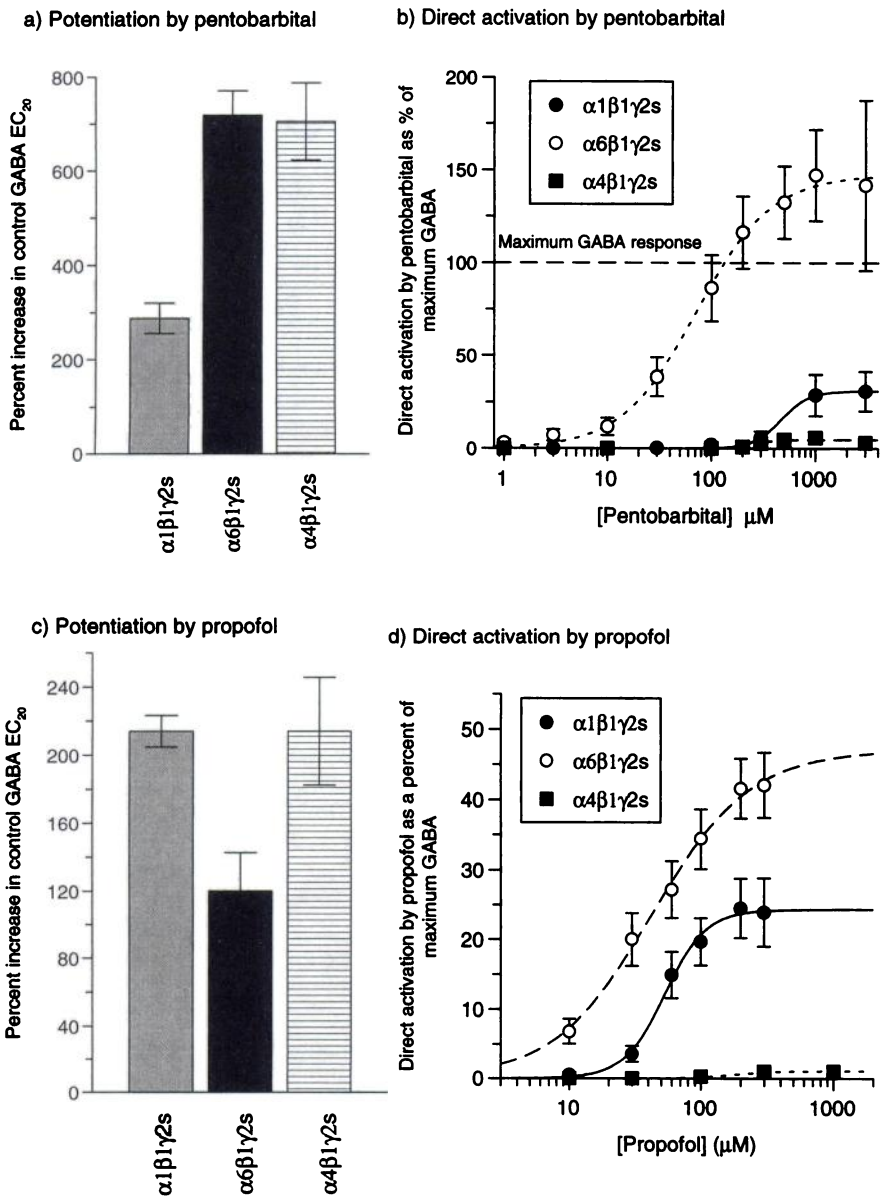

Fig. 6. Potentiation of control GABA EC_{20} responses and direct activation of $GABA_A$ receptors by pentobarbital and propofol in oocytes injected with $\alpha1\beta1\gamma2s$, $\alpha6\beta1\gamma2s$, and $\alpha4\beta1\gamma2s$. a, Potentiation of control GABA EC_{20} responses by pentobarbital (100 μM). b, Direct activation of $GABA_A$ receptors by pentobarbital. Data have been normalized to the maximum response to GABA (3 mM) on each cell. c, Potentiation of control EC_{20} responses by 10 μM propofol. d, Direct activation of $GABA_A$ receptors by propofol. Data have been normalized to the maximum response to GABA (3 mM) on each cell. The data shown are the mean ± standard error of at least four oocytes.

receptors by pentobarbital does not occur via the GABA recognition site because antagonism by bicuculline is not observed (39). In addition, mutations decreasing the GABA affinity have no effect on pentobarbital affinity (39). It is interesting to note that direct activation by pentobarbital and propofol is negligible on $\alpha4\beta1\gamma2$ receptors compared with other $\alpha x\beta1\gamma2s$ receptors, particularly α6-containing receptors, where pentobarbital will elicit a greater response than GABA (38). These effects may be related to the low efficacy obtained with the GABA recognition site partial agonists (P4S and THIP) at α4-containing receptors, even though the two sets of compounds act through different sites. One possibility is that the probability of channel opening is low for a4-containing receptors, regardless of the agonist recognition site. This hypothesis could be further resolved using single-channel analysis.

Modulation by furosemide. Furosemide has recently been shown to be a subtype-selective $GABA_A$ receptor antagonist, eliciting a greater affinity for $\alpha6\beta2\gamma2$ receptors than for $\alpha1\beta2\gamma2$ receptors (40). Because β1-containing receptors have low sensitivity to furosemide (40), the inhibition of GABA-induced currents was compared at human receptors containing the β3 subunit ($\alpha1\beta3\gamma2s$, $\alpha2\beta3\gamma2s$, $\alpha3\beta3\gamma2s$, $\alpha4\beta3\gamma2s$, $\alpha5\beta3\gamma2s$, and $\alpha6\beta3\gamma2s$) (Fig. 7). An IC_{50} of 162 μM was found for $\alpha4\beta3\gamma2$ compared with 6 μM for $\alpha6\beta3\gamma2$ and >5 mM for the other α subunits. Similar to the results of previous reports, furosemide was selective for the $\alpha6\beta3\gamma2$ receptor subtype (40); in addition, it showed some selectivity for $\alpha4\beta3\gamma2$ subtype. The $\alpha4\beta3\gamma2$ receptor was less sensitive to furosemide than the $\alpha6\beta3\gamma2$ receptor combination but >50-fold more sensitive than receptors containing other α subunits. It may be speculated that the intermediate affinity of furosemide for $\alpha4\beta3\gamma2$ receptors is because some, but not all, of the amino acid residues in the α6 subunit, which determine the high affinity for furosemide, are conserved in the α4 subunit.

The role of receptors containing the α4 subunit is unclear. It is the lowest abundant α subunit in the central nervous system, and it is located mostly in the thalamus and pronounced in certain lateral and ventral nuclei. It is also

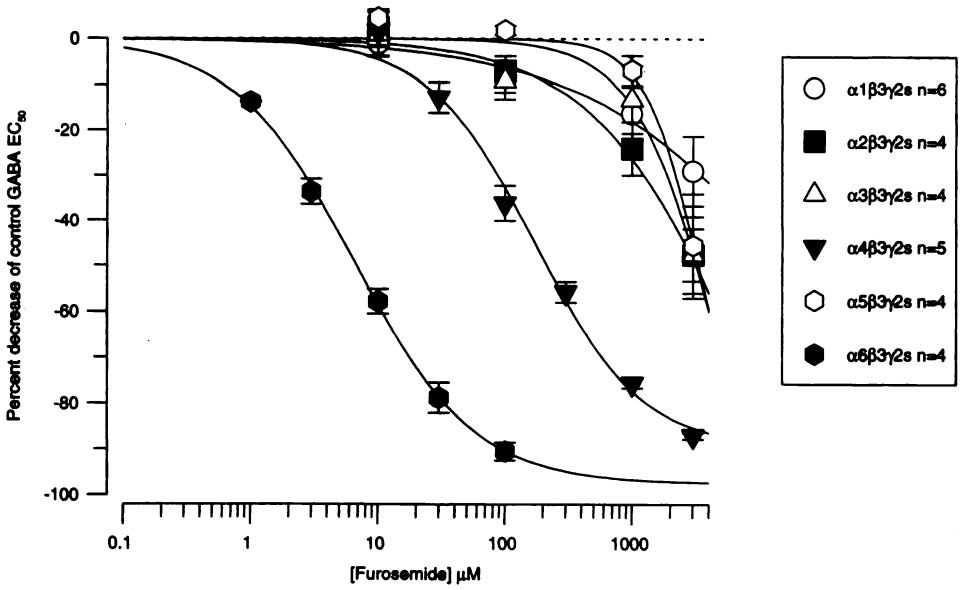

Fig. 7. Concentration-response curves for the inhibition of a GABA EC_{50} response by furosemide on GABA$_A$ receptors consisting of the different subunit combinations α1β3γ2, α2β3γ2, α3β3γ2, α4β3γ2, α5β3γ2, and α6β3γ2. Data represent mean values from the number of cells indicated in the *legend*.

present at low levels in the hippocampus, cortex, and basal ganglia (25). It is clear from the pharmacological profile reported here that these receptors are not involved in the behavioral responses evoked by classic benzodiazepines such as diazepam. Also, any effects mediated by direct activation of receptors by pentobarbital or propofol would not occur via this receptor subtype.

In conclusion, we demonstrated that the α4 subunit can confer a unique pharmacological profile to GABA$_A$ receptors. GABA partial agonists have relatively lower affinity and efficacy. Classic benzodiazepines show an identical profile to α6-containing receptors; however, atypical compounds and β-carbolines, which also act at the benzodiazepine site, behave differently. Modulatory sites for neurosteroids, pentobarbital, propofol, and furosemide are also present on α4-containing receptors. α4β1γ2s receptors are unique in not being directly activated by anesthetic compounds such as pentobarbital or propofol.

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