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Structural and functional characterization of the γ_1 subunit of $GABA_A$/benzodiazepine receptors

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The $GABA_A$ receptor γ_1 subunit of human, rat and bovine origin was molecularly cloned and compared with the γ_2 subunit in structure and function. Both γ subunit variants share 74% sequence similarity and are prominently synthesized in often distinct areas of the central nervous system as documented by *in situ* hybridization. When co-expressed with α and β subunits in *Xenopus* oocytes and mammalian cells, the γ variants mediate the potentiation of GABA evoked currents by benzodiazepines and help generate high-affinity binding sites for these drugs. However, these sites show disparate pharmacological properties which, for receptors assembled from α_1, β_1 and γ_1 subunits, are characterized by the conspicuous loss in affinity for neutral antagonists (e.g. flumazenil) and negative modulators (e.g. DMCM). These findings reveal a pronounced effect of γ subunit variants on $GABA_A$/benzodiazepine receptor pharmacology.

Key words: ion channels / γ subunit variants / receptor subtypes / benzodiazepine

Introduction

$GABA_A$ (γ-aminobutyric acid type A) receptors belong to the superfamily of ligand-gated ion channels (Unwin, 1989) and assemble from several homologous subunits to form a GABA-gated chloride conducting pore. Channel activity mediates synaptic inhibition in the central nervous system (CNS) and can be modulated by a number of psychoactive drugs, notably barbiturates and benzodiazepines (Olsen and Venter, 1986). $GABA_A$ receptors occur in virtually every neuron in the brain, are found on glia (Bormann and Kettenmann, 1988) and also exist in the periphery (Bormann and Clapham, 1985; Rorsman *et al.*, 1989).

Molecular biological studies have uncovered the existence of an array of subunits, indicating a considerable structural heterogeneity of $GABA_A$ receptors. Subunits of related structure and belonging to four different sequence classes (α, β, γ and δ) have been characterized with the α class comprising the largest number of variants (Schofield *et al.*, 1987; Levitan *et al.*, 1988; Ymer *et al.*, 1989a,b; Lolait *et al.*, 1989; Pritchett *et al.*, 1989a; Shivers *et al.*, 1989; Pritchett and Seeburg, 1990). This molecular diversity confirms and extends the observed pharmacological heterogeneity of $GABA_A$ receptors (Squires *et al.*, 1979; Braestrup and Nielsen, 1981; Unnerstall *et al.*, 1981; Cooper *et al.*, 1987).

Recombinant expression of diverse subunit combinations has reproduced many properties of natural $GABA_A$ receptors. Importantly, receptors having a high affinity benzodiazepine recognition site can be built from three different subunits, members of both the α and β class and a γ_2 subunit. In these receptors, the particular α subunit variant determines the affinity of benzodiazepines (Pritchett *et al.*, 1989b; Pritchett and Seeburg, 1990) while the γ_2 subunit is necessary for both the manifestation of the benzodiazepine binding site and the robust potentiation of GABA evoked chloride currents by these drugs (Pritchett *et al.*, 1989a). Since, in the receptor complex, the α subunit can be photoaffinity labelled by tritiated benzodiazepines (Sieghart *et al.*, 1987; Fuchs *et al.*, 1990), the γ_2 subunit seems to induce a conformation in the α subunit necessary for benzodiazepine recognition or may indeed form part of this site.

Most of our work on the properties of recombinantly expressed $GABA_A$/benzodiazepine receptors was performed using the γ_2 variant (Pritchett *et al.*, 1989a,b; Pritchett and Seeburg, 1990) although cDNAs encoding two variants of the γ subunit class had been isolated by us (Pritchett *et al.*, 1989a). We now present the primary structure of the γ_1 subunit from rat, bovine and man. We show that this γ variant is widely synthesized in rat brain and, when assembled with α_1 and β_1 subunits, participates in the formation of $GABA_A$/benzodiazepine receptors whose pharmacological properties differ in important aspects from receptors containing the γ_2 subunit.

Results

Isolation and structure of γ_1 subunit cDNAs

The γ_1 subunit cDNA was isolated as one of several cDNAs encoding new $GABA_A$ receptor subunits by screening human, bovine and rat brain libraries with a degenerate oligonucleotide probe. This oligonucleotide encoded the octapeptide TTVLTMTT present within the second transmembrane region of all $GABA_A$ receptor and ligand binding glycine receptor subunits (Grenningloh *et al.*, 1987; Schofield *et al.*, 1987; Shivers *et al.*, 1989). Novel subunit cDNAs were classified as γ_1, γ_2 and δ subunit cDNAs on the basis of their encoded polypeptide sequences (Pritchett *et al.*, 1989a; Shivers *et al.*, 1989). Figure 1 presents the nucleotide and deduced amino acid sequence of the rat γ_1 subunit cDNA. The human, bovine and rat γ_1

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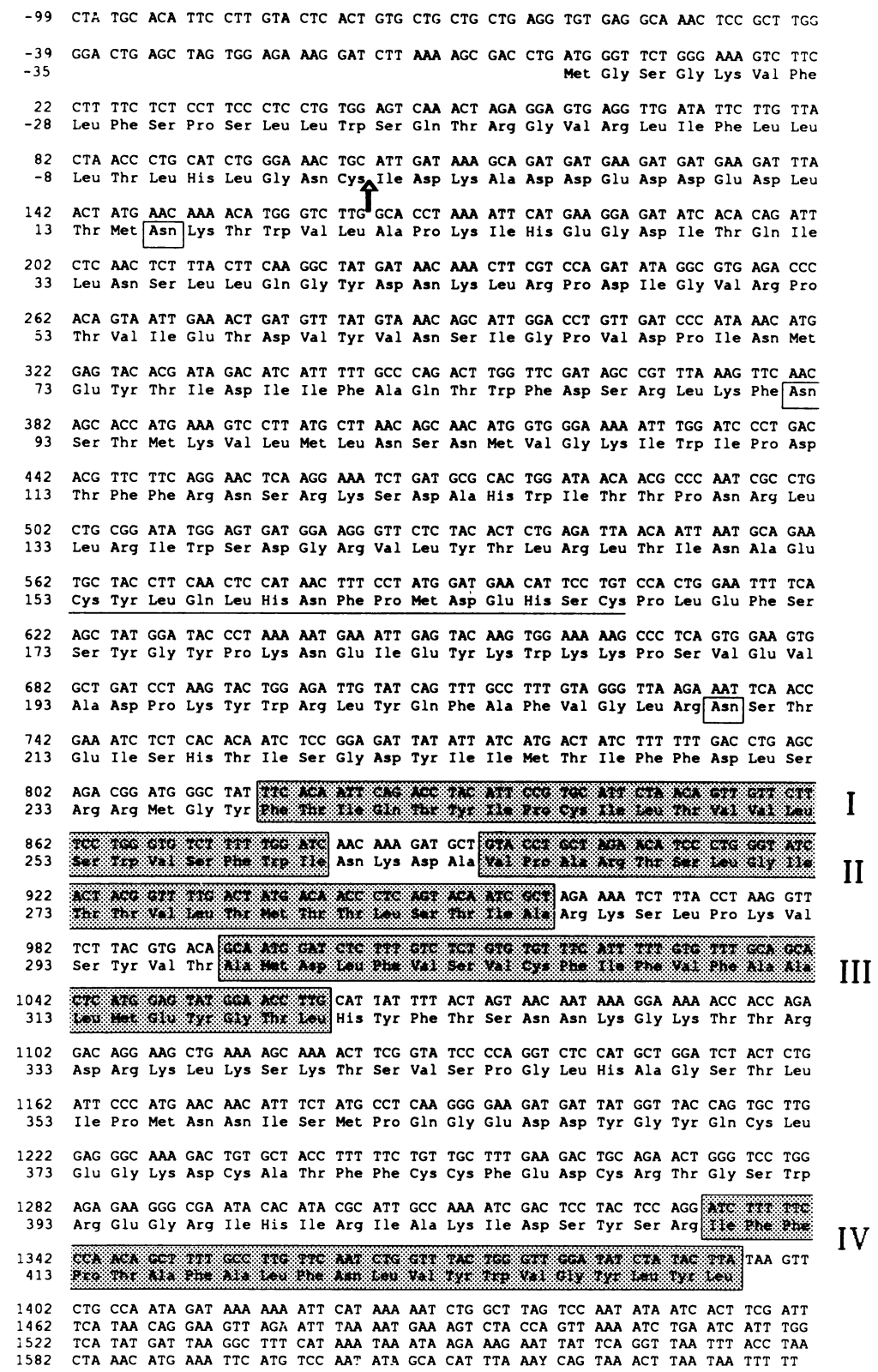

Fig. 1. Nucleotide and predicted amino acid sequence of rat $GABA_A$ receptor γ_1 subunit. The amino acid sequence is shown below the nucleotide sequence. Numbers on the left denote nucleotides and amino acid residues. Negative numbers specify residues in the signal sequence and nucleotides in the 5′ untranslated sequence. The proposed end of the signal sequence (von Heijne, 1986) is indicated by an arrow. Putative N-linked glycosylation sites are boxed, the invariant extracellular cysteine-flanked loop structure is underlined and the four transmembrane regions are stippled and numbered on the side.

polypeptides are compared with each other and with the bovine γ_2 subunit in Figure 2. The γ_1 cDNAs of all three species encode polypeptides of 465 amino acids including a putative 35 residue signal peptide (von Heijne, 1986) and share ~93% sequence identity. A similarly high degree of sequence conservation is seen for other $GABA_A$ receptor

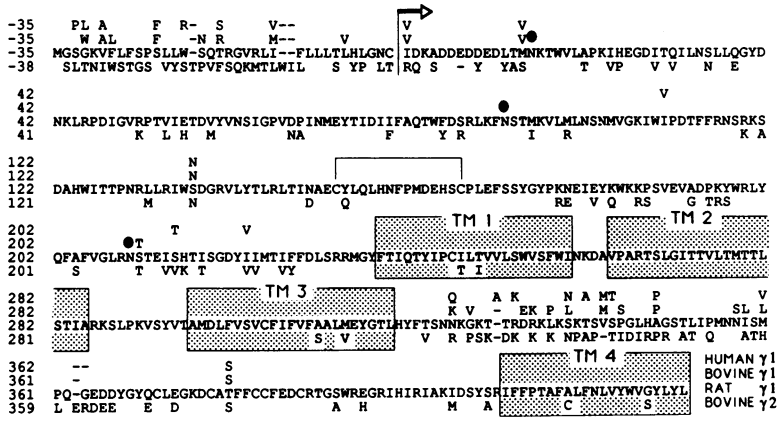

Fig. 2. Comparison of primary structures of human, bovine and rat γ_1 subunits and of the bovine γ_2 subunit. Amino acid sequences are given in the single letter code and are numbered for each polypeptide on the left. Negative numbers indicate signal peptides. The continuous sequence is that of the rat γ_1 subunit. Only substitutions in the other subunits are shown. For best alignment, dashes were introduced in several positions. The arrow marks the predicted mature N-termini. Filled circles denote N-linked glycosylation sites, the disulphide-bonded 15 residue β-structural loop is bracketed by a thin line and the four transmembrane regions TM1–TM4 are stippled and boxed.

subunits of different species (Schofield *et al.*, 1989; Ymer *et al.*, 1989b). The predicted mature γ_1 polypeptides ($M_r \approx 54$ kd) contain four putative membrane spanning regions and, in the extracellular domain, a 15 residue, cysteine-flanked β-structural loop, characteristic of subunits of ligand-gated ion channels (Criado *et al.*, 1986; Schofield *et al.*, 1987) as well as three N-linked glycosylation sites.

There is a 74% sequence identity between the two γ variants, γ_1 and γ_2, reflecting a similar level of identity to that seen in variants of the α and β subunit classes (Levitan *et al.*, 1988; Ymer *et al.*, 1989b). Regions best conserved are the membrane spanning domains and the large extracellular part including the positions of the three putative glycosylation sites. Low sequence similarity is observed in the signal peptide and the N-terminal half of the domain located between transmembrane regions three and four. Curiously, the C-terminal half of this domain is well conserved in the γ_1 and γ_2 variants, suggesting that the underlying sequence may serve an important receptor function. This segment begins with acidic residues and a tyrosine (residues RDEEY in γ_2) which may constitute the target of tyrosine-specific protein glycosylation and, hence, cellular control of channel expression by tyrosine kinases (Hopfield *et al.*, 1988).

Distribution of γ_1 mRNA in rat brain

Oligonucleotide-mediated *in situ* hybridization (Young *et al.*, 1986; Wisden *et al.*, 1988) was performed on sections of rat brain to evaluate the expression of γ_1 mRNA and to compare this expression with that of γ_2 mRNA. A survey of these mRNAs in whole brain horizontal and coronal sections is presented in Figures 3 and 4. Importantly, none of the probes employed labelled white matter tracts, indicating their specificity at even low signal strength. Hence, the pictures document that both γ_1 and γ_2 subunits are widely and often prominently expressed. They indicate that the γ_1 subunit should be regarded as a major player in modulating the structural and functional aspects of GABA$_A$ receptors. Furthermore the pictures reveal distinct differences in expression patterns of the two γ mRNAs. The γ_1 mRNA (Figure 3A) is expressed uniformly throughout the cortex caudate putamen, colliculi and in the hippocampal complex. In contrast to γ_2 mRNA expression (Figure 3B), γ_1 mRNA

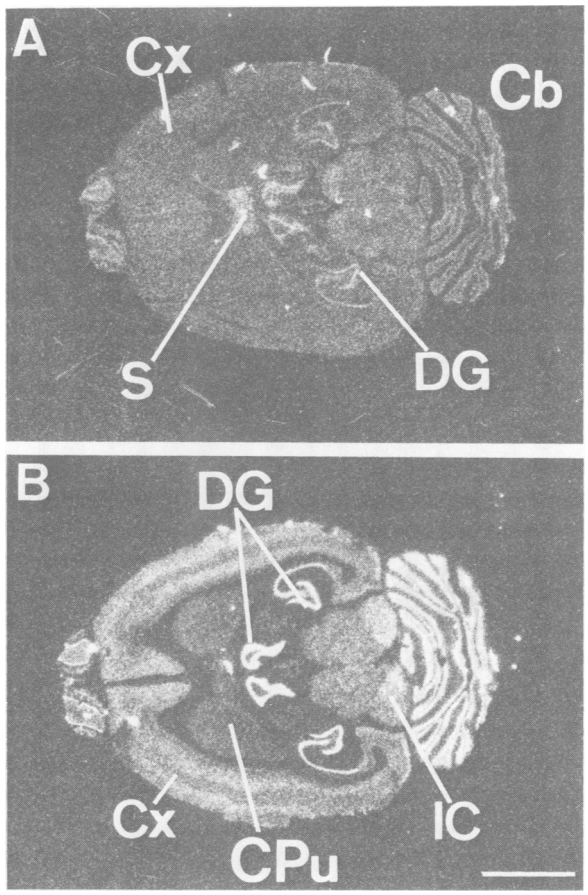

Fig. 3. Distribution of mRNAs encoding γ subunit variants γ_1 (**A**) and γ_2 (**B**) in horizontal sections of rat brain as visualized by *in situ* hybridization. Cb, cerebellum; CPu, caudate putamen; Cx, cortex; DG, dentate gyrus; IC, inferior colliculus; S, septal nuclei. Bar, 4 mm.

expression in cortex appears non-laminated. In addition, γ_1 mRNA is expressed prominently in the septal-forebrain nuclei, a region where γ_2 mRNA expression is relatively low (Figure 3A and B).

The most striking expression of the γ_1 mRNA occurs in certain amygdaloid and hypothalamic nuclei (Figure 4). In

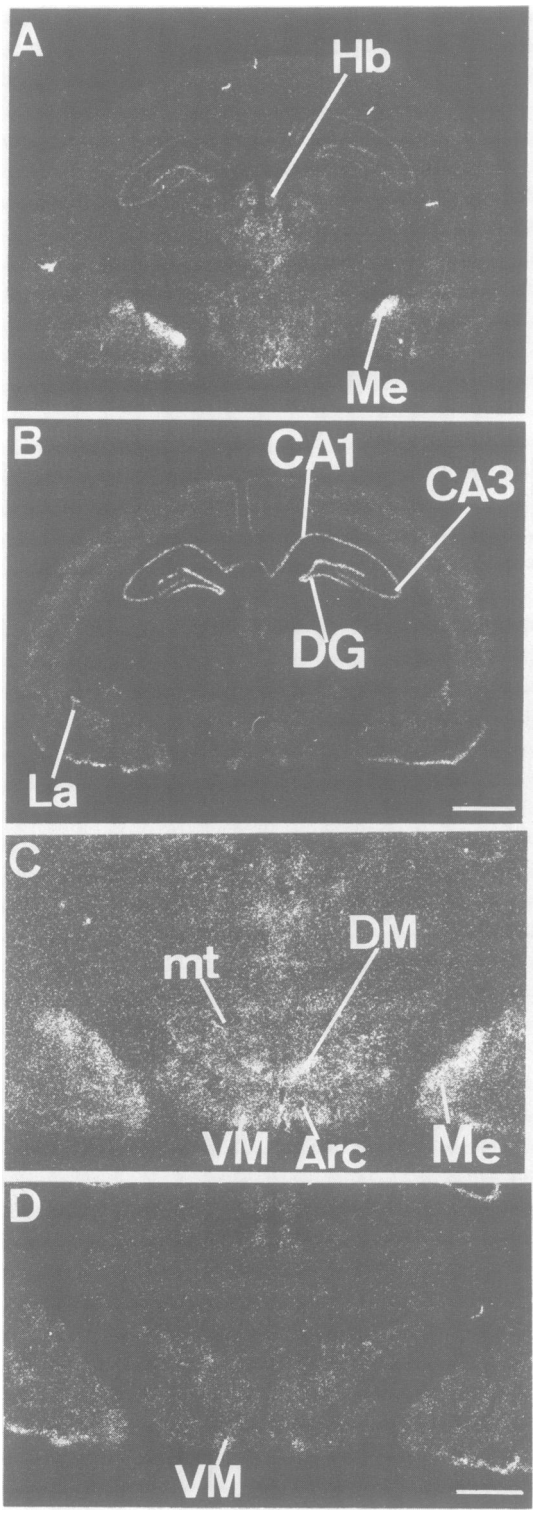

Fig. 4. Differential distribution of mRNAs encoding γ subunit variants γ_1 (**A** and **C**) and γ_2 (**B** and **D**) in the amygdala and hypothalamic region of the rat brain as observed in coronal sections. Arc, arcuate hypothalamic nucleus; DG, dentate gyrus; Hb, habenular nucleus; La, lateral amygdaloid nucleus; Me, medial amygdaloid nucleus; mt, mammillothalamic tract; Vm, ventromedial thalamic nucleus. Bar: A and B, 2 mm; C and D, 1.4 mm.

areas such as the medial amygdaloid nuclei, and in hypothalamic nuclei such as the dorsomedial, ventromedial and arcuate nuclei, γ_1 mRNA appears to be more abundant than γ_2 mRNA (Figure 4A–D). This suggests a prominent role of the γ_1 subunit in the functional make-up of $GABA_A$ receptors involved in amygdaloid/hypothalamic circuitry. In fact, the disparities in the relative amounts of γ_1 and γ_2 mRNA in hypothalamic and amygdaloid nuclei (Figure 4) highlight the receptor heterogeneity in this region.

Expression of α_1, β_1 and γ_1 subunits in Xenopus oocytes

To determine the functional properties of $GABA_A$ receptors containing the γ_1 subunit, *in vitro* synthesized RNA encoding either bovine α_1, β_1 or α_1, β_1, γ_1 subunits was expressed in *Xenopus* oocytes (Barnard *et al.*, 1982; Schofield *et al.*, 1987). Dose-dependent inward currents were observed in response to GABA and the reversal potential was close to the Cl^- equilibrium potential ($E_r \approx -20$ mV) (36), indicating that both subunit combinations form GABA-gated Cl^- channels. At 10 μM GABA, the $\alpha_1\beta_1$ receptors displayed 60–70% of their maximal current response while the $\alpha_1\beta_1\gamma_1$ combination operated at only 20% of maximal response, in keeping with the higher cooperativity of channel activation seen for $\alpha\beta\gamma$ receptors (Verdoorn *et al.*, 1990). The specificity of the GABA-induced current response was shown by blockage using the GABA antagonist bicuculline and the channel blocker picrotoxin (not shown). For both subunit combinations the lowest effective dose of GABA was between 0.01 and 1 μM, probably due to variability in receptor expression. Desensitization became apparent at GABA concentrations >20 μM, suggesting that the current amplitudes were underestimated at higher GABA concentrations since both channel activation and desensitization are contributing factors.

Potentiation of activation by barbiturates and benzodiazepines is a key feature of native $GABA_A$ receptors (Olsen and Venter, 1986). Oocyte responses were effectively potentiated by pentobarbital (Figure 5). At 10 μM GABA, the potentiation by 25 μM pentobarbital was 148 ± 95% (α_1, β_1; n = 10) and 430 ± 320% (α_1, β_1, γ_1; n = 10). In ten independent experiments, no BZ potentiation was observed with the α_1, β_1 subunit combination, using 10 μM midazolam (n =3; Figure 5), 10 μM diazepam or 20 μM flurazepam at 0.5 μM GABA (n = 8) and 10–20 μM diazepam or 20 μM clorazepam at 10 μM GABA (n = 33). In one experiment, 5–40% increase in current amplitude was observed in four of seven oocytes tested. However, channels reconstituted from α_1, β_1 and γ_1 subunits routinely showed potentiation by these agonists (Figure 5). At 10 μM GABA, 10 μM midazolam potentiated by 57 ± 9% (n = 5). At 0.5 μM GABA, 20 μM flurazepam increased the current amplitude by 36 ± 12% (n = 3, not shown) and 20 μM clorazepam by 85 ± 11% (not shown). Potentiation was also dose dependent. At 0.5 μM GABA the current was potentiated in the presence of 1 μM diazepam by 4 ± 3% (n = 4), at 5 μM by 19 ± 10% (n = 4), at 10 μM by 25 ± 10%, (n = 5) and at 20 μM by 33 ± 18% (n = 5). At 2 μM GABA, 5 μM diazepam increased the current response by 25 ± 9% (n = 14), at 10 μM GABA, 5 μM diazepam potentiated by 40 ± 12% (n = 6).

The BZ antagonist flumazenil (Ro 15-1788) was also tested (Figure 5). With the α_1, β_1 combination, 10 μM flumazenil showed marked potentiation of 71% ± 8% (n = 5) at 0.5 μM GABA. A reduced potentiation of 30 ± 19.5% (n = 5) was observed with receptors composed of α_1, β_1 and γ_1 subunits. With both subunit combinations, a potenti-

ation was sometimes observed with 10 μM DMCM (not shown). This unusual profile contrasts with that seen in natural $GABA_A$ receptors and in receptors containing the γ_2 subunit in place of γ_1 (Pritchett *et al.*, 1989a) although a weak agonistic activity of flumazenil has been reported (Hunkeler *et al.*, 1981; Haefely, 1988).

Benzodiazepine pharmacology

The pharmacological properties of human recombinant $GABA_A$ receptors containing α_1, β_1 and either γ_1 or γ_2 subunits were analysed. Membranes of cultured mammalian cells transiently expressing the appropriate subunit combinations (Pritchett *et al.*, 1988) were used for ligand binding and ligand competition studies of several benzodiazepines including flunitrazepam, flumazenil and of the β-carboline, DMCM (Table I). For both γ_1 and γ_2 subunit containing receptors, binding sites for [^{3}H]flunitrazepam were observed, not seen with receptors lacking a γ subunit (Pritchett *et al.*, 1989a). Although B_{max} values varied among transfections, the ratio of these values for γ_2 to γ_1 subunit containing receptors was ~5, indicating a higher efficiency of assembly for receptors formed from α_1, β_1 and γ_2 subunits.

Distinct differences were observed in the pharmacological properties of receptors based on which γ variant was co-expressed. Most ligands displayed >10-fold reduced affinities when the γ_1 subunit was co-expressed. Most notably, the affinities for flumazenil and DMCM dropped by more than four orders of magnitude, indicating that γ_1 and γ_2 subunits participate in pharmacologically distinct $GABA_A$ receptors. The finding that the α_1, β_1, γ_1 receptors possess an allosteric site for agonists but not for antagonists and inverse agonists suggests that agonist and antagonist sites in $GABA_A$ receptors may not be isosteric.

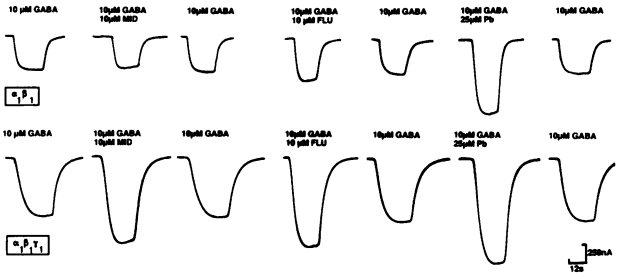

Fig. 5. Expression of cloned $GABA_A$ receptors in oocytes injected with α_1, β_1 **(top row)** and α_1, β_1 and γ_1 **(bottom row)** subunit RNAs. Oocytes were voltage clamped at −70 mV. Downward deflections indicate inward currents. Agonists and antagonists were MID, midazolam; FLU, flumazenil (Ro 15-1788); Pb, pentobarbital. GABA applications in the absence and presence of these drugs were for ~20 s until a current plateau was reached. The current traces in each row were from the same oocyte with the washout not shown.

Discussion

The present study describes a second member of the γ class of $GABA_A$ receptor subunits and, hence, provides a basis for better understanding the structural and functional features of these receptor constituents. The distinctive sequence features of the γ variants include an extended signal sequence, an acidic residue rich mature N-terminus and a well-conserved 40 amino acid region preceding the fourth putative transmembrane segment and possibly containing phosphorylation sites. As judged from a comparison with other $GABA_A$ receptor subunit classes, the γ subunits show highest sequence similarity (~40%) to α subunits and seem to cooperate with these in modelling the benzodiazepine recognition site of $GABA_A$ receptors.

Several fuctional properties of natural $GABA_A$ receptors can be traced to the signature of γ variants by our previous and present analyses of recombinantly expressed subunits. Thus, receptors containing a γ subunit show larger channel conductances than those found in receptors assembled from α and β subunits alone (Verdoorn *et al.*, 1990). Perhaps related to this property, the potentiation of GABA evoked currents by benzodiazepines is a consistent characteristic of γ subunit containing receptors and is rarely seen in receptors consisting only of α and β subunits (Levitan *et al.*, 1988; Pritchett *et al.*, 1989a; Malherbe *et al.*, 1990).

The most striking trait imparted by γ variants is the manifestation of a high-affinity binding site for benzodiazepines. Notably, this site is formed by co-expression of members of three subunit classes, indicating subunit interactions in its formation. The influence of different α variants on the pharmacology of the benzodiazepine binding site is well established (Pritchett *et al.*, 1989b, 1990). However, the pronounced alteration in this site generated by substituting the two γ variants highlights the effect of the γ subunits on the modelling of this clinically important allosteric effector site.

Using α_1 and β_1 subunits as partners for the γ variants we observed a striking loss in affinity for the benzodiazepine antagonist Ro 15-1788 and the inverse agonist DMCM when the γ_1 subunit was substituted for γ_2. The loss in affinity for these compounds may be functionally related to the increase in GABA evoked current produced by these drugs in *Xenopus* oocytes expressing α_1, β_1 and γ_1 subunits. Although potentiating effects of Ro 15-1788 have been reported (Haefely, 1988), the unusual pharmacological profile of receptors assembled from α_1, β_1, γ_1 subunits may indicate that such receptors are not formed *in vivo*.

Table I. Pharmacological properties of recombinant and cerebellar $GABA_A$ receptors

	$\alpha_1\beta_1\gamma_1$	$\alpha_1\beta_1\gamma_2$	Cerebellar membrane[a]
Flunitrazepam	20 ± 5$^+$	2 ± 0.4$^+$	1.5
Flumazenil	>10,000	1 ± 0.2$^+$	0.5
Clonazepam	320 ± 60	1 ± 0.1	0.7
2-Oxoquazepam	380 ± 52	19 ± 2.5	7
DMCM	>10,000	5.3 ± 1.5	2.5

Data represent K_i or $+K_d$ values ± SEM. Two columns list values obtained from ligand binding to crude membranes of 293 cells, transiently expressing $GABA_A$ receptor subunits α_1, β_1 and γ_1 or α_1, β_1 and γ_2.
[a]The third column lists values converted to K_i from IC_{50} values of rat cerebellum reported in Sieghart and Schuster (1984).

Due to the large number of $GABA_A$ receptor subunits many experiments with both natural and recombinant receptors are needed to determine the subunit composition of the diverse $GABA_A$ receptor subtypes in the brain. However, the abundant expression of both γ variants in different brain areas, the *in vitro* requirement for these subunits in the formation of benzodiazepine binding sites and the pronounced effect of the γ variants on the pharmacology of these sites serve as clear indicators that members of the γ subunit class participate in natural $GABA_A$/benzodiazepine receptors. It may be of considerable interest that particular hypothalamic nuclei seem rich in $GABA_A$ receptors containing the γ_1 variant. A GABA-ergic component in hypothalamic hypophysiotropic physiology is well known (Gudelsky *et al.*, 1983; Lamberts *et al.*, 1984; Adler and Crowley, 1986). Recombinant modelling of the receptors involved in this regulation may lead to the development of tailored drugs to better target their activity.

Materials and methods

Isolation and sequencing of cDNA clones

A bovine brain λgt10 cDNA library (Schofield *et al.*, 1987) (2×10^6 recombinant phage) was screened with a 96-fold degenerate ^{32}P end-labeled 23mer oligonucleotide (Shivers *et al.*, 1989) (specific activity 10^6 c.p.m./pmol) encoding the conserved octameric peptide sequence in TM2 of $GABA_A$ and glycine receptor subunits (Grenningloh *et al.*, 1987). Hybridization was at room temperature overnight in 20% formamide/0.9 M NaCl/0.09 M sodium citrate/5 × Denhardt's solution/100 μg per ml yeast RNA/0.025% sodium pyrophosphate. Washing was at 40°C in 0.15 M NaCl/0.015 M sodium citrate (1 × SSC). Known subunits were identified using α and β subunit specific oligonucleotides (Levitan *et al.*, 1988; Ymer *et al.*, 1989b). Clones only hybridizing to the 23mer were sequenced either in λgt10 (at 55°C) or after subcloning into M13 vectors (Vieira and Messing, 1987) by the chain termination method (Sanger *et al.*, 1977), using 0.5 μM of the degenerate 23mer as primer. To obtain full length γ_1 cDNAs, the library was rescreened with two specific probes: an oligonucleotide homologous to the most 5′ known sequence (5′-TTATATCTCCTTCGTGAATC-3′) and an *Eco*RI–*Eco*RV fragment covering the first 479 known base pairs (bp). A 60mer (5′-CTGCAGTCTTCAAAGCAACAGAAGAAGCTGGCACAATCTTTGCCCTCCAAACACTGGTAG-3′) encoding a portion of the intracellular loop domain was used to screen cDNA libraries constructed from rat forebrain and human fetal brain mRNAs for γ_1 homologs. A bovine γ_2 cDNA clone was isolated after screening an adrenal medulla cDNA library (Ymer *et al.*, 1989b) with the degenerate 23mer. The initial clone did not encode the entire subunit and the bovine brain cDNA library was subsequently screened with an oligonucleotide (5′-GTTCCGGAGGGTGATGTCAC-3′) specific for the missing 5′ sequences.

In situ hybridization

In situ hybridization was performed as described (Wisden *et al.*, 1988) using as probes oligonucleotides extended at their 3′ termini by [^{35}S]dAMP. Hybridization of probe (1 pg/μl; 1000 d.p.m./μl) was overnight at 42°C in a buffer containing 50% formamide/4 × SSC/10% dextran sulphate. Sections were washed at 60°C in 1 × SSC. Exposure time was 1 week on Kodak XAR-5 film. Brain structures were identified using the atlas of Paxinos and Watson (1986). Parallel sections hybridized with 20-fold excess of unlabelled oligonucleotides in addition to the labelled probe resulted in the absence of signal (not shown). The γ_1 signal was reproduced independently using two unique oligonucleotides constructed to different parts of the γ_1 cDNA sequence. The oligonucleotide sequences were 5′-GGGAATCAGAGTAGATCCAGCATGGAGACCTGGGGATACCG-3′ (complementary to γ_1 subunit residues 341–354), 5′-CCTTGAGGCATAGAAATGTTGTTCATGGGAATCAGAGTAGATCC-3′ (complementary to rat γ_1 subunit residues 357–371), and 5′-CATTTGGATCGTTGCTGATCTGGGACGGATATCAATGGTAGGGGC-3′ (complementary to rat γ_2 subunit residues 338–352 and kindly supplied by British Bio-Technology, Oxford, UK).

Expression in Xenopus oocytes

A 4.0 kb *Eco*RI fragment encoding the entire bovine γ_1 subunit sequence was subcloned into pBluescript SK$^-$ (Stratagene, La Jolla, CA) and the resulting construct linearized with *Hin*dIII. Similar vectors (Bluescript, SP65) were constructed carrying α_1 and β_1 subunit cDNAs and capped bovine α_1, β_1 (Schofield *et al.*, 1987) and γ_1 RNA transcripts were synthesized using Sp6 or T7 RNA polymerase in the presence of m^{7}G(5′)PPPc5′G according to Promega, Madison, WI. The *in vitro* synthesized RNAs were mixed in equimolar ratios prior to injection to yield the co-expression of bovine α_1 and β_1 or α_1, β_1 and γ_1 subunits. About 50 nl RNA (1 μg/μl in H_2O) were injected into *Xenopus* oocytes and the oocytes were incubated for 24 h at 19°C in modified Barth's solution [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 7.5 mM Tris–HCl pH 7.4] containing 100 U/ml each of streptomycin and penicillin. The oocytes were then treated with 1 mg/ml collagenase (Type I Sigma) for 1 h and the follicle cell layer was mechanically removed. Electrophysiological recordings were performed 2–6 days after injection, using a conventional two microelectrode voltage clamp amplifier (Methfessel *et al.*, 1986). The oocytes were clamped at −70 mV and superfused (5–7 ml/min) with normal frog Ringer solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES pH 7.4). The amplitude of inward currents following superfusion with frog Ringer containing various pharmacological compounds was measured.

Ligand binding studies

Supercoiled recombinant vector DNAs constructed for the expression of the human α_1, β_1 and γ_1 encoding cDNAs, subcloned individually into the expression vector pCIS2 (Gorman *et al.*, 1990), were used to transform human embryonic kidney 293 cells (10 plates; 4×10^6 cells and 20 μg DNA per plate). Cells washed twice with phosphate buffered saline (PBS) and scraped into 10 ml PBS. The cell pellet (500 mg) was homogenized in a Polytron tissue homogenizer (Brinkmann) in 10 ml of 10 mM potassium phosphate, pH 7.4, and centrifuged (50 000 *g*, 20 min). This procedure was repeated three times and the final pellet was resuspended in potassium phosphate buffer, pH 7.4, containing 100 mM KCl. For each concentration of displacing ligand, duplicate samples, each equivalent to 10^6 cells (100 μg protein), were incubated (4°C, 60 min) in 1 ml containing 4 pmol of [^{3}H]flunitrazepam (75 Ci/mmol). Non-specific binding was determined by competition in the presence of 1 μM clonazepam. Filtered samples were washed twice with 5 ml homogenization buffer and filter-retained radioactivity was determined by liquid scintillation counting.

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