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$GABA_A$ receptor β subunit heterogeneity: functional expression of cloned cDNAs

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Cloned cDNAs encoding two new β subunits of the rat and bovine $GABA_A$ receptor have been isolated using a degenerate oligonucleotide probe based on a highly conserved peptide sequence in the second transmembrane domain of $GABA_A$ receptor subunits. The $\beta 2$ and $\beta 3$ subunits share ~72% sequence identity with the previously characterized $\beta 1$ polypeptide. Northern analysis showed that both $\beta 2$ and $\beta 3$ mRNAs are more abundant in the brain than $\beta 1$ mRNA. All three β subunit encoding cDNAs were also identified in a library constructed from adrenal medulla RNA. Each β subunit, when co-expressed in *Xenopus* oocytes with an α subunit, forms functional $GABA_A$ receptors. These results, together with the known α subunit heterogeneity, suggest that a variety of related but functionally distinct $GABA_A$ receptor subtypes are generated by different subunit combinations.

Key words: $GABA_A$ receptor/β subunit/receptor subtypes/molecular cloning/oocyte expression

Introduction

GABA (γ-aminobutyric acid), the major inhibitory neurotransmitter in the vertebrate brain, mediates neuronal inhibition by opening a chloride channel integral to the $GABA_A$ receptor, which is also the target for a variety of therapeutically important drugs (reviewed in Olsen and Venter, 1986). Affinity-purified receptor is electrophoretically resolved into two major bands (α, 48-53 kd and β, 55-57 kd). The smaller band can be photoaffinity-labelled by benzodiazepine derivatives and the larger one by GABA agonists (reviewed by Stephenson, 1988). Molecular cloning of cDNAs encoding $GABA_A$ receptor subunits was facilitated by peptide sequences derived from purified receptor (Schofield *et al.*, 1987; Levitan *et al.*, 1988). Analysis of these cDNAs established that the 'α band' is heterogeneous, consisting of several variants of the α subunit (Levitan *et al.*, 1988) and of other subunits (Pritchett *et al.*, 1989). This confirmed the molecular heterogeneity of $GABA_A$ receptors, postulated on the basis of pharmacological (Squires *et al.*, 1979; Braestrup and Nielsen, 1981; Unnerstall *et al.*, 1981; Cooper *et al.*, 1987) and photoaffinity labelling (Möhler *et al.*, 1980; Sieghart *et al.*, 1983; Fuchs *et al.*, 1988) studies. However, the 'β band' was regarded as being homogeneous (Häring *et al.*, 1985; Mamalaki *et al.*, 1987). This band is so far molecularly characterized by only one cloned cDNA that encodes a subunit ($\beta 1$) with significant sequence similarity to the α subunits and, when co-expressed with these in *Xenopus* oocytes, produces functional $GABA_A$ receptors (Schofield *et al.*, 1987; Levitan *et al.*, 1988). Sequence homology also extends, in part, to other ligand-gated ion channels, reflecting the existence of a receptor superfamily (Schofield *et al.*, 1987; Grenningloh *et al.*, 1987a).

Comparison of the polypeptide sequences of the three $GABA_A$ receptor α subunits, the $\beta 1$ subunit as well as the 48 kd subunit of the glycine receptor revealed that the highest sequence identity resides in the four putative transmembrane segments (M1-M4). In particular, a contiguous sequence of eight amino acids rich in threonines is found in M2 of all these polypeptides (Grenningloh *et al.*, 1987b) which, by analogy to M2 of nicotinic acetylcholine receptor (Imoto *et al.*, 1988; Leonard *et al.*, 1988), is thought to form part of the channel lumen. We have used a highly degenerate oligonucleotide probe encoding this peptide sequence to screen for additional $GABA_A$ receptor subunits. We report the isolation of two new β subunit encoding cDNAs, $\beta 2$ and $\beta 3$, from both rat and bovine brain cDNA libraries and show that these new β subunits are more abundant in brain than the $\beta 1$ subunit. Functional expression in *Xenopus* oocytes demonstrates that these β subunits are capable of combining with an α subunit to form $GABA_A$ receptor chloride channels.

Results

Cloning of $\beta 2$ and $\beta 3$ subunit cDNAs

A cDNA library constructed from bovine brain RNA was screened with the degenerate oligonucleotide probe and numerous hybridizing signals were obtained. Among these were cDNAs encoding the known $GABA_A$ receptor subunits $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\beta 1$ (Schofield *et al.*, 1987; Levitan *et al.*, 1988), which were identified by subunit specific oligonucleotides. The remaining hybridizing clones were sequenced. Preliminary sequence data were obtained either directly from λ DNA or from recombinant M13 DNA, using the degenerate oligonucleotide as a primer. Clones encoding new subunits were identified by homology of their deduced amino acid sequence with the third transmembrane region of previously characterized $GABA_A$ receptor subunits.

Two cDNA clones were isolated that encoded different polypeptides showing high sequence identity to the $GABA_A$ receptor $\beta 1$ subunit. These polypeptides were designated as $\beta 2$ and $\beta 3$ subunits. By comparison with the $\beta 1$ polypeptide, the encoded $\beta 2$ subunit sequence lacked an initiation codon and the $\beta 3$ subunit sequence lacked the N-terminal 45 amino acids of the complete polypeptide. Full-length

cDNA clones were not identified upon rescreening, but a complete β3 subunit encoding clone was isolated from a bovine adrenal gland cDNA library (see below). We then screened a rat forebrain cDNA library with oligonucleotides based on the bovine β subunit coding sequences and isolated full-length cDNA clones for the rat β1, β2 and β3 subunits. The nucleotide and deduced amino acid sequences of the rat subunits, including part of the 5′ and 3′ non-coding regions, are shown in Figure 1. All three cDNAs encode polypeptides of ~450 amino acid residues (M_r 52 kd) with a 25-residue signal peptide (Von Heijne, 1986). The predicted mature polypeptide sequences contain four putative membrane-spanning regions, three potential N-linked glycosylation sites and a 15-residue-long, cysteine-flanked region, characteristic of all subunits of ligand-gated ion channels (Criado *et al.*, 1986; Schofield *et al.*, 1987). Notably, all three β subunits contain a cAMP-dependent phosphorylation site in a homologous position within their putative intracellular domain, suggesting involvement in the cellular control of receptor activity.

Comparison of the three β polypeptides predicted from either rat or bovine cDNAs (Figure 2) shows that 72% of the residues are invariant, reflecting an identity similar to that seen between different α subunits (Levitan *et al.*, 1988). As for the α variants, regions of highest homology include the membrane-spanning domains and large extracellular region. Low sequence similarity is seen in the signal peptides and in the intracellular domain located between M3 and M4.

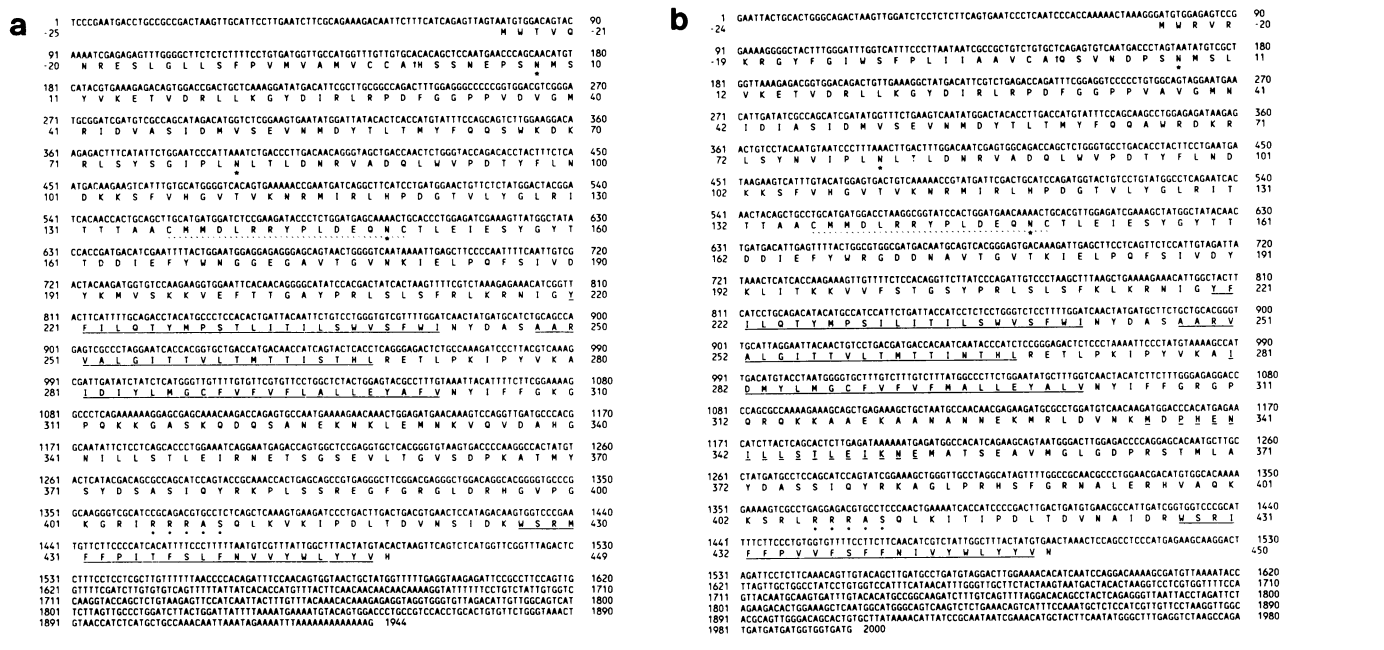

Fig. 1. Nucleotide and deduced amino acid sequences of rat $GABA_A$ receptor β1 (**a**), β2 (**b**) and β3 (**c**) subunits. Nucleotide and amino acid positions are indicated. Negative numbers refer to signal peptide residues. Potential N-linked glycosylation sites carry asterisks, the β-structural loop flanked by cysteines is indicated by a broken line and the four transmembrane regions are underlined. Putative regulatory sites in the intracellular region of the β subunits are denoted by small circles. Underlined residues in β2 (positions 336–352) correspond to a chemically determined peptide sequence of the homologous bovine subunit.

Importantly, in both the rat and bovine $\beta 2$ subunits this domain contains one of the peptide sequences (MXPHENILLSTLEIKNE) chemically determined from cyanogen bromide-cleaved affinity-purified bovine GABA$_A$ receptor (Schofield *et al.*, 1987). The $\beta 2$ subunit sequence is the only one in which a methionine residue (the site of cyanogen bromide cleavage) precedes this peptide sequence (Figures 1 and 2) and residues P_3, E_5 and K_{15} uniquely distinguish the $\beta 2$ from the $\beta 1$ and $\beta 3$ sequences. This demonstrates that the $\beta 2$ polypeptide is a component of the natural GABA$_A$ receptor complex. Interspecies homologies for β subunits are extremely high since on average only 10 residues are substituted between the rat and bovine homologues. A similarly high degree of sequence conservation has been observed for the bovine (Schofield *et al.*, 1987) and human (Schofield *et al.*, 1989) $\alpha 1$ and $\beta 1$ subunits.

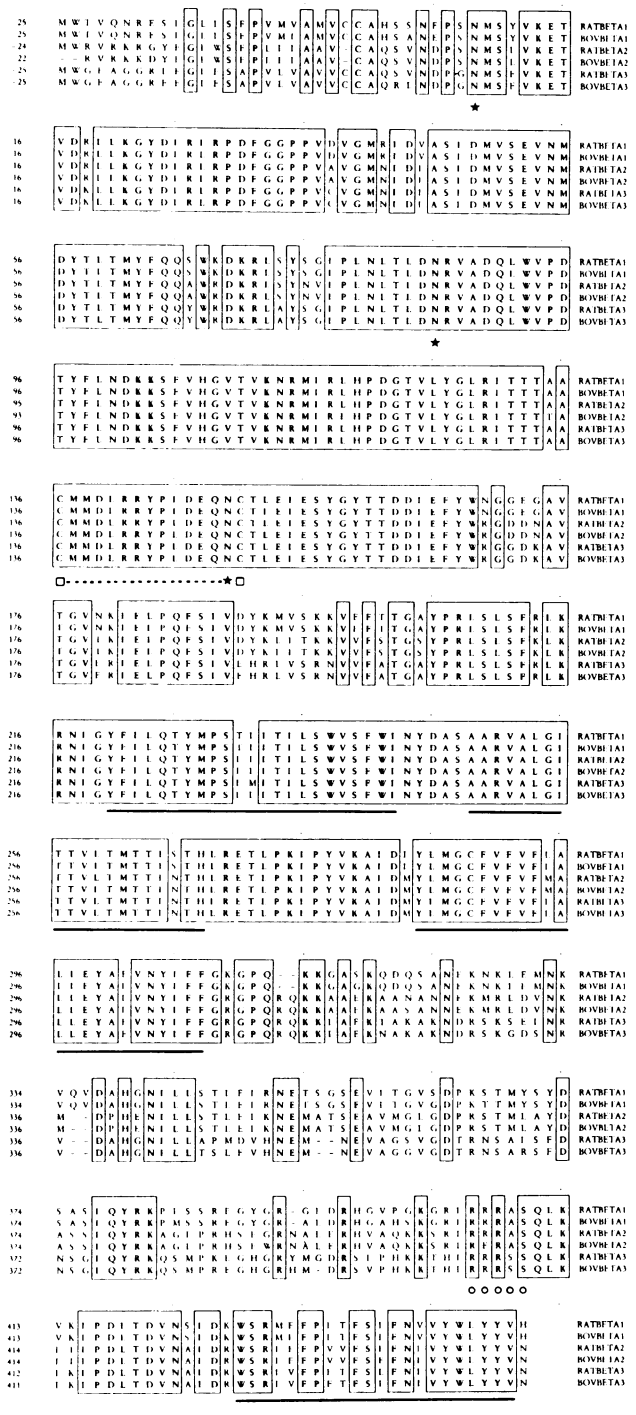

Fig. 2. Comparison of three rat and bovine GABA$_A$ receptor β subunits. Numbering and symbols are as in Figure 1.

β subunit localization

The extent of β subunit expression in the brain was investigated by Northern blot analysis. RNA samples were prepared from rat and calf total brain as well as from the cortex, hippocampus and cerebellum. Northern blots of these RNAs were hybridized with ^{32}P-labelled oligonucleotides complementary to DNA sequences encoding the divergent intracellular domain of the three β subunits. The autoradiographs (Figure 3) document that in the rat and bovine brain both the $\beta 2$ and $\beta 3$ subunits are considerably more abundant than the $\beta 1$ subunit. The relative abundance of the β subunit mRNAs parallels that of the respective cDNA clones in the brain libraries.

In rat brain, the hippocampus and cerebellum contain the highest levels of β subunit mRNAs while the cortex shows only small amounts of β-specific RNA. $\beta 2$ subunit mRNA is altogether more abundant than $\beta 3$ mRNA. The three β subunit encoding mRNAs differ in size, with $\beta 1$ mRNA being the largest ($\sim$12 kb) followed by $\beta 2$ mRNA ($\sim$8 kb) and $\beta 3$ mRNA, which is represented by two size forms

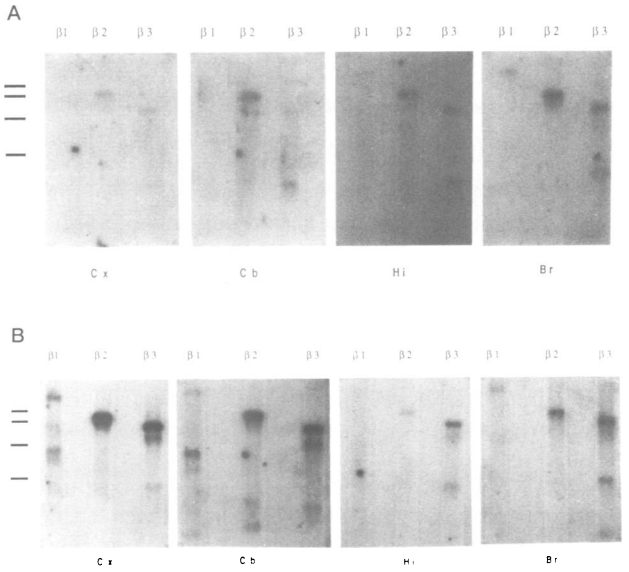

Fig. 3. Regional brain distribution of β subunit mRNAs. Northern blots of poly(A)$^+$ RNA from rat (A) and calf (B) cortex (Cx), hippocampus (Hi), cerebellum (Cb) and total brain (Br) were probed using $\beta 1$, $\beta 2$ and $\beta 3$ subunit-specific oligonucleotides. Size markers indicated on the left represent 9.5, 7.5, 4.4 and 2.4 kb.

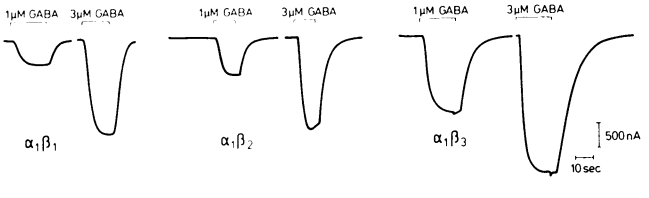

Fig. 4. Expression of GABA$_A$ receptor β subunits in *Xenopus* oocytes. Clamp potential was -70 mV. Downward deflections reflect inward currents (see calibration bar). GABA-application (1 and 3 μM for each subunit pair) is indicated by horizontal bars.

(~6 kb and ~2.5 kb), the smaller transcript being the major form in the rat cerebellum.

As observed for the α subunit mRNAs (M.Köhler and P.H.Seeburg, unpublished), the bovine brain contains substantially higher amounts of β subunit mRNA than the rat brain. In particular, the cortex and cerebellum display high levels of β2 and β3 mRNAs. Two β1 mRNAs (13 and 4 kb) are observed which are of equal abundance in calf cortex, whereas the smaller transcript appears to be the major form in cerebellum; β2 mRNA is represented by a single 8 kb transcript, while β3 mRNA exists as 8 kb (major form) and 7 kb transcripts in cortex and cerebellum. Several transcript sizes originating from one gene have been observed previously and usually reflect the use of different polyadenylation sites to generate different-sized 3′ flanking regions (Setzer *et al.*, 1980; Tosi *et al.*, 1981).

Three β subunits in the adrenal medulla

Determination of the *in vivo* subunit composition of a single type of $GABA_A$ receptor may help to resolve the role of multiple α and β subunit polypeptides. The adrenal medullary chromaffin cells express electrophysiologically (Bormann and Clapham, 1985) and pharmacologically (Kataoka *et al.*, 1984) well-characterized $GABA_A$ receptors that might constitute a single population of receptors. To analyse these receptors in molecular terms we constructed a bovine adrenal medulla cDNA library and screened it at low stringency using as probes ^{32}P-labelled α2 (Levitan *et al.*, 1988) and β1 (Schofield *et al.*, 1987) subunit cDNAs as well as the degenerate oligonucleotide based on the conserved octameric peptide sequence in M2 (see above). Clones encoding α1, β1, β2 and β3 subunits were obtained, but no α2 or α3 subunit cDNAs were found. While these results do not prove the absence of α variants, they suggest that these subunits represent at best very minor species in the adrenal medulla. This is substantiated by the absence of α2 and α3 subunit specific hybridization signals on Northern blots of adrenal medullary RNA (not shown). However, the presence of three β subunits may indicate that chromaffin $GABA_A$ receptors constitute a heterogeneous population.

All adrenal cDNA sequences were colinear with those of brain-derived cDNAs. Occasionally observed third-base substitutions in codons are probably a consequence of different RNA sources used in cDNA library construction. These results show that many of the genes encoding $GABA_A$ receptor subunits are expressed both centrally and peripherally without the use of alternative splicing. Hence, alternate exon usage is not a major mechanism for generating diversity of $GABA_A$ receptors.

Expression in Xenopus oocytes

We investigated whether the novel β subunits could contribute to the formation of functional $GABA_A$ receptors by expressing each rat β subunit in combination with the rat α1 subunit (unpublished) in *Xenopus* oocytes. Expression was achieved by nuclear injection (Voellmy and Rungger, 1982) of pairs of recombinant CDM8 vectors (Seed, 1987) in which the cloned cDNAs are under the transcriptional control of the cytomegalovirus promoter. This mode of expression is sensitive to small amounts of DNA and circumvents the need for the *in vitro* synthesis of RNA (Ballivet *et al.*, 1988). Electrophysiological recordings (Figure 4) showed that either the β2 or β3 polypeptide can be substituted for the β1 subunit to yield dose-dependent GABA-evoked currents. These were always inhibited by bicuculline, enhanced by the barbiturate pentobarbital and had reversal potentials (E_r) close to the chloride equilibrium potential (E_r −23 mV) of *Xenopus* oocytes (Dascal *et al.*, 1984), indicating that each receptor forms GABA-gated chloride channels (not shown). Thus, substitution of either the β2 or β3 subunit for the β1 subunit does not appear to change the qualitative properties of the expressed $GABA_A$ receptors.

Discussion

Molecular cloning has revealed heterogeneity of the $GABA_A$ receptor β subunit. The assignment of the two novel subunits β2 and β3 as true components of the $GABA_A$ receptor is based on the observations that (i) β1, β2 and β3 subunits share a high degree of sequence identity, (ii) the β2 subunit contains a peptide sequence obtained by chemical means from affinity-purified $GABA_A$ receptor complex and (iii) all three subunits form GABA-responsive chloride channels when co-expressed with the α1 subunit. The β subunits are highly sequence-conserved and contain a larger intracellular domain than other subunits (Levitan *et al.*, 1988; Pritchett *et al.*, 1989). This domain in the β subunits contains a consensus site for cAMP-dependent phosphorylation by protein kinase A (Feramisco *et al.*, 1980). The presence of this site strengthens our earlier hypothesis (Schofield *et al.*, 1987) that the β subunit provides a target for the cellular regulation of $GABA_A$ receptor activity.

While molecular heterogeneity of the α subunit was anticipated, the existence of β subunit variants is unexpected. Northern analysis indicates that the novel β subunits far exceed the β1 subunit in abundance. This result provides an explanation for the previously noted imbalance of *in situ* hybridization signals for α and β subunit mRNA in regions of rat and bovine brain (Séquier *et al.*, 1988; Siegel, 1988). In fact, evidence from this laboratory indicates that the different β subunits are indeed expressed in distinct, possibly overlapping neuronal populations (B.D.Shivers, unpublished).

The increasing number of variants of $GABA_A$ receptor α and β subunits poses the problem of determining the subunit composition of natural $GABA_A$ receptors. As the analysis of a homogeneous $GABA_A$ receptor population may provide one way of dissecting the subunit composition, we investigated the receptor in adrenal medullary chromaffin cells by molecular cloning. Besides finding only one α subunit we were surprised by the presence of all three β subunits. Thus, either all β variants are part of the same receptor or the adrenal chromaffin $GABA_A$ receptors are composed of different subtypes. Of these alternatives, the first is less likely considering the different expression levels of the three β subunit mRNAs and their distinct localization in the brain (unpublished). While $GABA_A$receptor diversity in chromaffin cells has yet to be substantiated by electrophysiology or pharmacology, the presence of several conductance states in the $GABA_A$ receptor of adrenal chromaffin cells (Bormann and Clapham, 1985) might indicate such a heterogeneity. It will be of interest to examine other peripheral $GABA_A$ receptor populations, such as those involved in the control of pituitary hormone release (Grandison and Guidotti, 1979; Racagni *et al.*, 1979).

The role of the β subunits in generating different $GABA_A$

receptor subtypes remains to be established. Combinations of different α and β subunits may define receptors that can be distinguished by pharmacology and channel properties, generating a greater diversity of GABA responses than would be achieved with fewer receptor subtypes. While the true extent of α and β subunit heterogeneity is unknown, recent cDNA cloning experiments in this laboratory have provided evidence for the existence of additional GABA$_A$ receptor α subunits and of novel subunits not of the α or β type (Pritchett *et al.*, 1989). However, no additional β subunit variants were identified.

Materials and methods

Isolation of cDNA clones

A λgt10 bovine brain cDNA library (Schofield *et al.*, 1987) was screened with a 96-fold degenerate ^{32}P-labelled 23mer oligonucleotide encoding a conserved octameric peptide sequence in M2 of GABA$_A$ receptor subunits, 5′ AC(A,C)AC(A,T)GT(G,T)CT(A,C,G)AC(A,C)ATGAC(A,C)AC 3′. Only indicated third position choices were included. Known subunits were identified using α1, α2, α3 and β subunit-specific oligonucleotides (Levitan *et al.*, 1988; and see below). cDNAs hybridizing to the 23mer but not to the subunit-specific oligonucleotides were sequenced in λg10 or after subcloning into M13 vectors (Vieira and Messing, 1987) by the chain termination method (Sanger *et al.*, 1977). Sequencing reactions using the 23mer oligonucleotide were performed with 0.5 μM primer and reactions were at 55°C when recombinant λ DNA was used as template. A rat forebrain cDNA library was screened with the following ^{32}P-labelled β subunit-specific oligonucleotides complementary to sequences encoding part of the large intracellular domain of the three subunits: β1; 75mer (5′ TCCCACGCCCGTGAGCACTTCAGAGCCGCTCGTCTCGTTCCTGATCTCCAGGGTACTGAGGAGAATGTTGCCGTG 3′); β2, 60mer (5′ TTTCCGATACTGGATGCTGGAGGCATCATAGGCCAGCATTGTGCTCCTTGGGTCTCCAAG 3′); β3, 60mer (5′ TCTTGCTGAATTCCGGGTATCACCAACGCCGCCGGCAACCTCGTTCATCTCATTGTGAAC 3′). The longest cDNA clones were completely sequenced. Furthermore, a bovine adrenal medulla cDNA library was constructed in λgt10 by standard methods (Huynh *et al.*, 1985) and screened using as probes both the degenerate 23mer oligonucleotide and two internally labelled *Eco*RI fragments of cloned bovine β1 and α2 subunit encoding cDNAs. The β1 cDNA fragment comprised nucleotides 1–726 (Schofield *et al.*, 1987) and the α2 cDNA fragment contained nucleotides 133–1755 (Levitan *et al.*, 1988). Sequence analysis was as described above.

Northern blot analysis

RNA was isolated by published methods (Chomczynski and Sacchi, 1987) from three brain regions of 8-month-old calf and young adult rats (200 g). Poly(A)$^+$ RNA was prepared using oligo(dT)–cellulose chromatography. For Northern analysis, RNA (3 μg) was electrophoresed in 1.2% formaldehyde-containing agarose gels and blotted onto nitrocellulose. These blots were hybridized to subunit-specific ^{32}P-labelled (sp. act. 10^6 c.p.m./pmol) oligonucleotides in 40% formamide at 42°C, washed in 2 × SSC, 0.1% SDS at 55°C and exposed to X-ray film, using an intensifying screen at −80°C for 5 days (bovine) or 14 days (rat). For the bovine Northerns the oligonucleotide sequences are listed above. For the rat experiment, the β1- and β2-specific oligonucleotides were 5′ GTAAGAGAGAAGCCCCAAACTCACTTAGTCTGTCTGCGATTTTGTACTGTC 3′ and 5′ AGAGAGGAGATCCACCCAGTGCAGTAATTC 3′, while the same β3 probe was used.

Expression in Xenopus oocytes

The rat α1 (unpublished), β1, β2 and β3 subunit-encoding cDNAs were cloned into the CDM8 vector (Invitrogen, San Diego, CA). Either *Xho*I or *Eco*RI fragments containing the entire coding sequences were converted to blunt ends and ligated with adaptor sequences to generate *Bst*XI cohesive termini. The adaptor sequences were 5′ CGAATTCAGAGAACA 3′ and 5′ CTCTGAATTCG 3′. The terminally modified cDNAs were then used to replace the stuffer fragment in CDM8 (Seed, 1987). Orientations of subcloned cDNAs relative to the vector-carried cytomegalovirus promoter were determined by restriction analysis. The nuclei of oocytes were injected (Voellmy and Rungger, 1982) with these constructs (10 nl, 350 pg of each expression plasmid). After incubation of injected oocytes at 19°C for 3–6 days, currents were recorded in a conventional two-electrode voltage clamp in normal frog Ringer solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM Hepes, pH 7.2) before and during superfusion with frog Ringer containing different concentrations of GABA. Bicuculline was used at 10 μM and pentobarbital at 5 μM.

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