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Sequence and expression of a novel $GABA_A$ receptor α subunit

Sanie Ymer*, Andreas Draguhn+, Martin Köhler, Peter R. Schofield** and Peter H. Seeburg

Laboratory of Molecular Neuroendocrinology, Center for Molecular Biology (ZMBH), Im Neuenheimer Feld 282, D-6900 Heidelberg, and *+Dept. of Cell Physiology, Max Planck Institut für Medizinische Forschung, Jahnstr. 29 D-6900 Heidelberg, FRG*

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Cloned cDNA encoding the bovine α_4 subunit of the $GABA_A$ receptor has been isolated. The predicted 521 amino acid long mature protein contains an exceptionally long intracellular domain and shares 53–56% sequence similarity to the previously characterized α_1, α_2 and α_3 subunits. Co-expression of α_4 and β_1 in *Xenopus* oocytes resulted in the formation of GABA-gated chloride channels with expected pharmacology, although no benzodiazepine potentiation was observed. Northern analysis indicates that a 4 kb α_4 mRNA is expressed in the calf cerebellum, cortex and hippocampus but is barely detectable in the rat brain.

γ-Aminobutyric acid A receptor; Ligand-gated ion channel; Receptor subtype; Voltage clamp recording; cDNA cloning

1. INTRODUCTION

Fast synaptic inhibition in the central nervous system is primarily mediated by GABA (γ-aminobutyric acid). This neurotransmitter opens the intrinsic chloride channel of the $GABA_A$ receptor, the activity of which can be modulated by a variety of drugs, notably benzodiazepines (BZs) and barbiturates [1,2]. Peptide sequences derived from affinity-purified material have facilitated the isolation of cDNAs encoding $GABA_A$ receptor α and β subunits [3]. As a consequence, several structural features have emerged common to other ligand-gated receptor subunits [3,4]. These features include 4 transmembrane domains (M1–M4) and a disulphide loop formed by two cysteine residues located extracellularly. Another feature is the highly divergent intracellular loop domain between M3 and M4. Within the M2 of $GABA_A$ and glycine receptors there is a conserved 8 amino acid sequence [5] thought to form part of the channel lumen [6,7]. Degenerate oligonucleotide probes encoding these amino acids have been used to isolate further $GABA_A$ receptor subunit encoding cDNA clones. Currently 4 classes of subunits have been identified and designated α [8], β [9,10], γ [11] and δ [12]. All classes share 35–45% sequence similarity and within each class (with the exception of δ) variants exist which display in excess of 70% identity. These studies have confirmed and extended the functional heterogeneity of the $GABA_A$ receptor suggested by pharmacology [13–16] and photoaffinity labelling [17–19]. Using the same approach in this study, cloned cDNA encoding a novel α subunit (α_4) was isolated from a bovine brain cDNA library. Functional expression in *Xenopus* oocytes demonstrates that this α subunit is capable of combining with a β subunit [3,9] to form GABA-gated chloride channels.

Correspondence address: P.H. Seeburg, Laboratory of Molecular Neuroendocrinology, University of Heidelberg, ZMBH, INF 282, D-6900 Heidelberg, FRG

**Present address:* Division of Biochemistry and Molecular Biology, John Curtin School of Medical Research, The Australian University, GPO Box 334, Canberra City, 2601 Australia

***Present address:* Pacific Biotechnology Ldt, 72–76 McLachlan Avenue, Rushcutters Bay, 2011 Australia

The nucleotide sequence(s) presented here has (have) been submitted to the EMBL/Gen Bank database under the accession number no. Y07515

2. MATERIALS AND METHODS

A bovine brain cDNA library in λgt10 [3] was screened using a 96-fold degenerate ^{32}P-labelled 23-mer oligonucleotide pool [9,12] encoding the conserved 8 amino acids in M2 of $GABA_A$ and glycine receptor subunits. Known subunits were identified using a pool of α_1, α_2, α_3 and β_1 subunit-specific oligonucleotides [9]. cDNAs hybridizing only to the 23-mer were sequenced [21] in λgt10 [9,12] or after subcloning into M13 vectors [20]. The longest α_4 cDNAs were completely sequenced with the aid of internal primers (5′ GTG TCC TAT GCA ACT GCC 3′, 5′ CAC AAT GAG ACT CAC CAT 3′ and 5′ ATT TCA GCT GCT CCA GTG CTG 3′).

A 2.0 kb *Eco*RI fragment encoding the entire bovine α_4 subunit was subcloned into pGEM-2 (Promega, Madison, WI) and the resulting construct linearized with *Hin*dIII. Capped bovine α_4 and β_1 [3] RNA transcripts were synthesized using Sp6 RNA polymerase and $m^7G(5')ppp(5')G$ according to Promega. Approximately 50 nl of subunit-specific RNAs (1 μg/μl in H_2O) were co-injected into defolliculated *Xenopus* oocytes and after incubation at 19°C for 2–6 days, induced currents were recorded using a conventional two-electrode voltage clamp [22].

RNA was isolated [23] from whole bovine brain, from brains of young, sexually mature rats, and from the cerebellum, cortex and hippocampus of an 8-month-old calf. RNA was enriched for poly(A)$^+$ RNA by oligo(dT)-cellulose chromatography. Northern analysis was carried out [9] using an α_4 subunit-specific ^{32}P-end labelled 60-mer

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oligonucleotide (5′ AGC AGA GGG AGT AGT AGT GGC TGA TAA CTT CCC CGA AGT CCC TAT GCT ATT AAC TGT GGT 3′) as the probe. Other probes used were oligonucleotides specific for α_1, α_2 and α_3 subunits [8].

3. RESULTS AND DISCUSSION

Numerous hybridization signals resulted from screening a bovine brain λgt10 cDNA library with the degenerate M2 oligonucleotide probe. Those clones which did not also hybridize to the known subunit-specific oligonucleotide pool (α_1, α_2, α_3 and β_1) were initially characterized by sequencing using the degenerate M2-encoding oligonucleotide as a primer. Cloned cDNAs encoding new subunits were identified by homology of their deduced amino acid sequence with previously characterized $GABA_A$ receptor subunits. As a result, a cDNA clone was identified that contained an open reading frame of 1701 bp displaying significant similarity to the previously characterized $GABA_A$ receptor α subunit sequences [8]. This cDNA encodes a polypeptide (designated α_4) of 556 amino acids, including a 35-residue signal peptide [24]. The cDNA sequence was not generated by alternative splicing of any other α subunit transcript.

A comparison of the predicted α_4 polypeptide sequence with that of the α_1, α_2 and α_3 subunits [8] shows that, as for other $GABA_A$ receptor subunits, regions of highest similarity include the putative transmembrane domains, the most conserved being M2 (fig.1). Here, α_1, α_2 and α_3 are identical and share 96% identity with α_4 in which a valine residue substitutes for an isoleucine (position 258). The extracellular domain is conserved to about 73% between α_1, α_2, α_3 while α_4 shares 60% with each. This domain contains putative *N*-glycosylation sites (fig.1). Overall, α_4 shares only 56% invariant amino acid residues with α_1, α_2 and 53% with α_3. The relationship of α_4 to subunits of other classes is as follows: β_1, 30%; γ_2, 40%; δ, 29%; glycine 48 kDa, 33%.

The intracellular loop domain of the α_4 polypeptide is extremely long, making α_4 the largest $GABA_A$ receptor subunit to date with a predicted molecular mass of 64 kDa for the unglycosylated mature protein. This domain displays the greatest amino acid sequence diversity between different subunits and may contain sites for intracellular regulation of channel activity [3]. In fact, both β [9] and γ [11] subunits have consensus sequences for phosphorylation located here. No such sites have been found in α_4 (or any α) but other unidentified regulatory features may be present.

To study functional expression the novel α_4 subunit was co-expressed with the bovine β_1 subunit [3,9] in *Xenopus* oocytes (fig.2). Following injection of in vitro synthesized RNA, 89% of oocytes ($n = 131$) expressed GABA-induced inward currents that were dose-dependent between 0.01 and 100 μM. When measured, the slope of the log dose versus log response curve

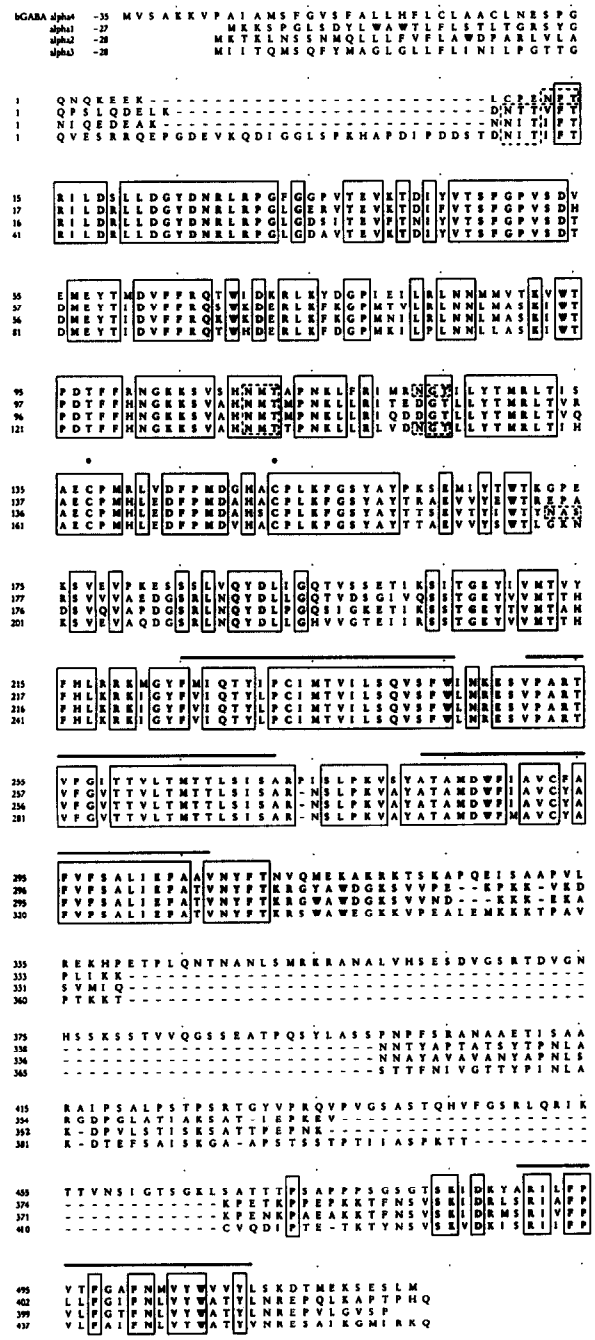

Fig.1. Comparison of the predicted amino acid sequence of the bovine $GABA_A$ receptor α_4 subunit with bovine α_1, α_2 and α_3 subunits [8]. The sequence alignment contains the following corrections to the previously published α_1, α_2 and α_3 sequences [8]: deletions of T at position 368 (α_1), T at position 365 (α_2), and KGA at positions 393–395 (α_3). These residues had been included in [8] as a result of a faulty alignment program and had escaped proof-reading. Amino acid sequence numbering starts at the proposed mature N-terminal residue [24], the presumptive signal sequences being negatively numbered. Invariant residues are enclosed in solid boxes and the putative *N*-linked glycosylation sites in dotted boxes. Postulated membrane-spanning hydrophobic sequences M1–M4 are marked by solid lines and the cysteine residues forming the disulphide loop by dots [3]. Dashes have been introduced to improve sequence alignment. The cDNA sequence of the α_4 subunit has been deposited in the EMBL database under accession no. Y07515.

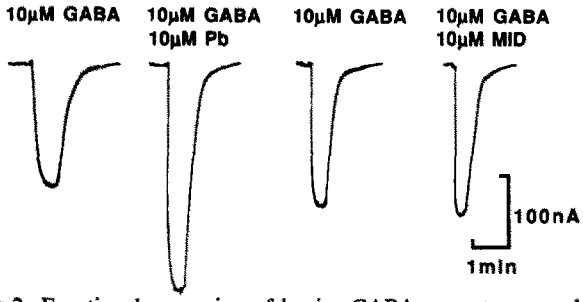

Fig.2. Functional expression of bovine $GABA_A$ receptor α_4 and β_1 subunits in *Xenopus* oocytes. Membrane potential of the oocytes was held at −70 mV in a conventional 2-microelectrode voltage clamp. Downward deflections represent inward Cl^- currents following application of 10 μM GABA alone or together with indicated drugs. Each application was followed by washing with normal frog Ringer solution [13,23] for at least 3 min.

(0.3 ± 0.07, determined between 0.1 and 1 μM GABA; $n=4$) was significantly below the expected value of at least one for other recombinant $\alpha+\beta$ receptors [8]. This shallow slope could reflect a particularly rapid desensitization of $\alpha_4+\beta_1$ $GABA_A$ receptors.

The current response to GABA (10 μM) was blocked by the antagonist, bicuculline (10 μM), to 30% of initial amplitude (not shown) and potentiated two-fold upon application of the barbiturate pentobarbital (10 μM), indicating the expression of a barbiturate-sensitive site. However, no potentiation by the BZ-receptor agonists, diazepam (2–5 μM, $n=20$) and midazolam (10 μM, $n=2$) was observed at GABA concentrations ranging from 1 to 40 μM (fig.2). Thus the pharmacology displayed here differs from neuronal $GABA_A$ receptors. Recent results [11] suggest that a third subunit (γ_2) may be required in order to form channels displaying physiological responses to BZs [1,2].

The extent of α_4 expression was investigated by Northern blot analysis (fig.3). RNA samples were prepared from rat and bovine total brain as well as calf cerebellum, cortex and hippocampus. Northern blots of these RNAs were hybridized with a ^{32}P-end labelled oligonucleotide (60-mer) complementary to DNA sequence encoding part of the distinct intracellular domain of the α_4 subunit. In all 3 regions of the calf brain investigated, a single 4.0 kb mRNA was observed, being about equally abundant in the cortex and cerebellum and about one-fifth as abundant in the hippocampus (fig.3A). Probing with α subunit variant-specific oligonucleotides indicated the order of abundance in the bovine brain to be α_1, α_3, α_4 then α_2 (fig.3B). In the rat brain, α_4 expression was hardly detectable (not shown). No cDNA clones were identified in a rat forebrain cDNA library but a clone encoding an incomplete human α_4 subunit was isolated from a human brain cDNA library (unpublished). Hence, the α_4 subunit may be extremely rare in the adult rat brain but developmental regulation of this subunit needs investigating. Our results may also reflect the general observation that $GABA_A$ receptor subunits

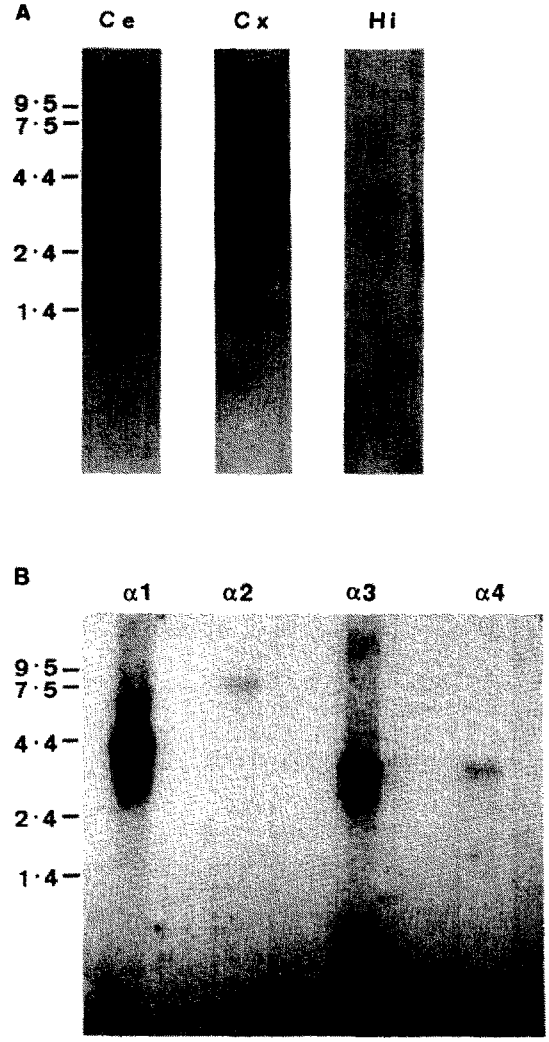

Fig.3. Expression of α_4 subunit mRNA in the bovine brain. (A) Northern blots of poly(A)$^+$ RNA from calf cerebellum (Ce), cortex (Cx) and hippocampus (Hi) probed with an α_4 subunit-specific oligonucleotide. (B) Northern blots of poly(A)$^+$ RNA from bovine total brain probed with α_1, α_2, α_3 and α_4 subunit-specific oligonucleotides. Size markers (kb) are indicated on the left.

have a lower cellular expression in the rat brain than the bovine brain [9].

In conclusion, the $GABA_A$ receptor α_4 subunit can be classified as such because it shares greatest sequence similarity to the α subunits and can induce GABA responses when co-expressed with a β subunit. It will be important to elucidate the physiological role of the α_4 subunit in $GABA_A$ receptors of central and peripheral neural tissue.

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