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Cloning and expression of a novel rat $GABA_A$ receptor

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Two full-length cDNA clones encoding α- and β-subunits of a $GABA_A$ receptor have been isolated from a rat cerebral cortex cDNA library. The mature α-subunit protein consists of 428 amino acids with a calculated M_r of 48680. This protein is highly homologous (~99% amino acid identity) with the bovine brain α_1-subunit receptor [(1988) Nature 335, 76–79]. The mature rat β-subunit receptor is a 448 amino acid polypeptide and shares ~80% amino acid identity with the previously characterized bovine $GABA_A$ receptor β-subunit [(1987) Nature 328, 221–227]. Co-expression of the cloned DNA in *Xenopus* oocytes produces a functional receptor and ion channel with pharmacological characteristics of a $GABA_A$ receptor. $GABA_A$ α- and β-subunit mRNA is detectable in the cortex, cerebellum and hippocampus.

cDNA cloning; $GABA_A$ receptor; Sequence homology; (*Xenopus* oocyte; Rat cortex)

1. INTRODUCTION

γ-Aminobutyric acid (GABA), the major neurotransmitter underlying fast inhibition in the vertebrate central nervous system, binds to specific GABA/benzodiazepine ($GABA_A$) receptors that mediate the transient opening of integral chloride channels. The $GABA_A$ receptor has been purified by affinity chromatography from bovine, porcine, chick and rat brain [1–5]. The predominant $GABA_A$ receptor in cerebral cortex is comprised of β- and α-subunit polypeptides, which bind GABA (or $GABA_A$ agonists, muscimol and isoguvacine) and benzodiazepines (e.g. flunitrazepam and diazepam), respectively. Functional heterogeneity of the $GABA_A$ receptor complex has been suggested on the basis of pharmacological characterization of the receptor(s) by both electrophysiological and radioligand-binding analyses [6]. These conclusions are supported by the recent isolation of cDNAs encoding three α (designated α_{1-3}) and one β $GABA_A$ subunits from bovine brain [7,8]. There is considerable similarity between the deduced amino acid sequences of both subunits, particularly in the predicted four transmembrane regions of the two molecules. Here, we describe the cloning, sequencing and expression of cDNAs encoding the α- and β-subunits of a rat cortex $GABA_A$ receptor. While the deduced amino acid sequence of the rat α-subunit shows virtually 100% identity with its bovine counterpart (α_1), the β-subunit is considerably different in the two species.

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2. MATERIALS AND METHODS

2.1. *Isolation of rat $GABA_A$ receptor cDNA clones*

A cDNA library from adult rat cortex mRNA was constructed in pcDV1 vector according to Okayama and Berg [9]. The library contains ~7×10^6 recombinant transformants. Pools of recombinants were screened by Southern blot analysis [10] prior to isolation of individual α- and β-subunit clones. The library was screened by hybridization to nick-translated human cDNA probes (in preparation) for the α- (a 5′ 289 base pair *NsiI-BamHI* fragment) and β- (a 5′ 699 base pair *SalI-XhoI* fragment) subunits. Filters were hybridized to the probes and washed in 3 × SSC at 60°C. Six positive α clones and two positive β clones were subsequently isolated. Two clones, RC3a (α) and RCβ2a (β), were determined to be full length by restriction and sequence analysis. Overlapping fragments were

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isolated, subcloned into M13, and sequenced by the method of Sanger et al. [11] using the modified T_7 polymerase [12] ('Sequenase'; US Biochemical, Cleveland, OH).

2.2. *Expression in oocytes*

A 1.5 kb *Sma*I-*Eco*RI fragment and a 2.4 kb *Nru*I-*Xba*I fragment containing the entire coding sequences for the α- and β-subunits, respectively, were subcloned into pGEM-7Zf(+) plasmid vectors (Promega, Madison, WI). Capped RNA transcripts were obtained from linearized templates using SP6 polymerase and 7mG(5')ppp(5')G according to the manufacturer's instructions. The subunit-specific synthetic RNAs were injected into 8–10 mature *Xenopus* oocytes (defolliculated) at 250 ng/μl; total volume injected per oocyte was ~50 nl. Oocytes were maintained in modified Barth's solution [13] at 18–21°C for 48 h. $GABA_A$-evoked currents were recorded at a voltage-clamp potential of −60 mV. Drugs were applied by bath application until saturation of the response, typically 30 s.

2.3. *Northern blot analysis*

Total RNAs were extracted from adult rat cortex, cerebellum and hippocampus using guanidinium thiocyanate as in [14]. 10-μg samples were electrophoresed through denaturing 2.2 M formaldehyde/1% agarose gels and transferred to GeneScreen (NEN). The blots were baked for 90 min at 80°C, prehybridized at 37°C for 24 h in 4 × SSPE, 50% formamide, 5 × Denhardt's solution, 500 μg/ml single-stranded DNA, 250 μg/ml yeast tRNA and 0.1% SDS, and hybridized (in the same buffer) at 37°C for 18 h with 48-base oligodeoxynucleotide probes labeled on the 3'-end with terminal deoxynucleotidyl transferase and deoxyadenosine [^{32}P]triphosphate (NEN, >3000 Ci/mmol) [15]. The α- and β-subunit probes are complementary to sequence encoding amino acids −3 to 13 (α) and 127–142 (β) of the rat sequences. The blots were washed at 56°C in 1% SSPE/0.1% SDS, then autoradiographed for 3 days at −70°C with Dupont Lightning Plus intensifying screens.

3. RESULTS AND DISCUSSION

The rat cortex α-subunit cDNA clone isolated (RC3a) contains an open reading frame that encodes a 455 amino acid polypeptide (fig.1). The predicted amino acid sequence of this clone is virtually identical (~99%) to the bovine α_1 sequence [8], confirming that it is the rat homologue. There are only three amino acid substitutions; Pro ⟶ Arg and Thr ⟶ Ser at amino acids 5 and 22, respectively in the putative signal peptide, and Iso ⟶ Thr 152. In addition, Leu 4 is deleted in the rat sequence. The mature polypeptide of 428 amino acids, with a predicted M_r of 48680, has three potential glycosylation sites (Asn-X-Ser/Thr, where X is any amino acid) at Asn 10, 110 and 338. The sequences of the four (M1–M4) putative transmembrane domains are completely conserved between the two species.

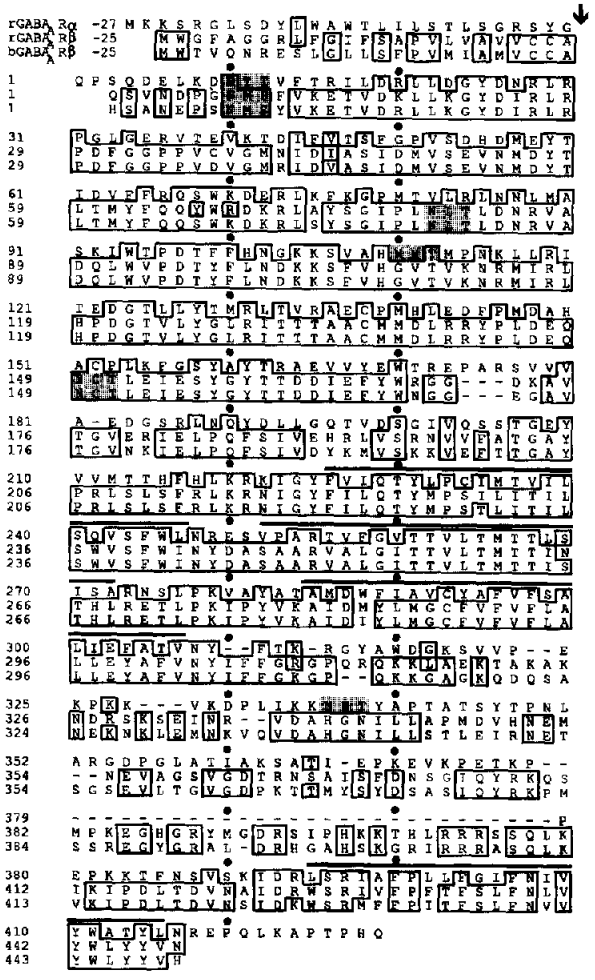

Fig.1. Comparison of the deduced amino acid sequences of the rat $GABA_A$ receptor α- and β-cDNAs with the previously characterized bovine $GABA_A$ β-subunit [7]. The overall sequence identity between the rat $GABA_A$ receptor subunits is ~35%. Dashes signify gaps introduced to align sequences maximally. Primary sequence identities are boxed. Potential signal cleavage sites are indicated by the large arrow. Amino acid sequence numbering begins at the proposed mature N-terminal residue, the presumptive signal sequences being indicated by negative numbering. The proposed membrane-spanning hydrophobic sequences [7,8] are indicated by solid lines, and the putative *N*-linked glycosylation sites by the hatched boxes.

The predicted amino acid sequence of the rat β-subunit (fig.1) shows that the protein is composed of 473 amino acids. The signal sequence of the mature protein was identified by the method of Von Heijne [16], and by its homology with the se-

quence of the amino-terminus of the bovine β-subunit [7]. The proposed mature rat β-subunit protein is preceded by a leader sequence of 25 residues, and is comprised of 448 amino acids with a calculated M_r of 51611. The rat β-subunit sequence differs from the bovine protein by 99 amino acid substitutions, 4 amino acid deletions, and 3 amino acid insertions. The majority (~50%) of the changes occur in the putative intracellular loop between M3 and M4, a region that is poorly conserved in the bovine α_{1-3}-subunit sequences [8]. Of the amino acid substitutions found in the M1–M4 transmembrane regions (7 over a stretch of 90 amino acids), 4 are present in M4, a feature that is also common to the distinct bovine α-subunit sequences. When conservative amino acid substitutions [17] are considered, the sequence homology between the β-subunits of both species increases from 79 to 90%. Three potential glycosylation sites occur at identical positions (Asn 8, 80 and 149) in the rat and bovine β-subunit proteins. Glycosylation at any, or all of these sites (and those in the α-subunit sequence) may account for the discrepancy between the predicted and observed M_r values of cDNA-derived [7,8] and biochemically isolated [1–6] $GABA_A$ receptor subunits, respectively.

The injection of mRNA(s) from tissues enriched in $GABA_A$ receptors [18] or synthetic mRNA from bovine cDNA clones [7,8] into *Xenopus* oocytes has been shown to produce functional, chloride channel complexes in the membrane. In the present study, GABA elicited a current response only in oocytes that were coinjected with α- and β-subunit synthetic mRNAs (fig.2), confirming that these rat cDNA clones encode a complete, functional $GABA_A$ receptor. Current responses to GABA were dose-dependent; the concentration of GABA eliciting half-maximal response was ~1.2 μM (not shown). The threshold for observable responses (10 nA) was ~10 nM. This pharmacological sensitivity to GABA is approx. 10-fold greater than that reported for the bovine α_1-β complex [8] and appears closer to that described for the bovine α_2-β complex [8]. The response to GABA was mimicked by isoguvacine, the $GABA_A$-specific agonist, and blocked by 5 μM bicuculline (fig.2) and picrotoxin (not shown). Rapid desensitization of the GABA response was observed at high (>1 μM) GABA concentrations. These results show that the rat α and β cDNAs are sufficient to encode an oligomeric receptor complex that possesses pharmacological properties similar to the native [6] and cloned bovine [7,8] $GABA_A$ receptor.

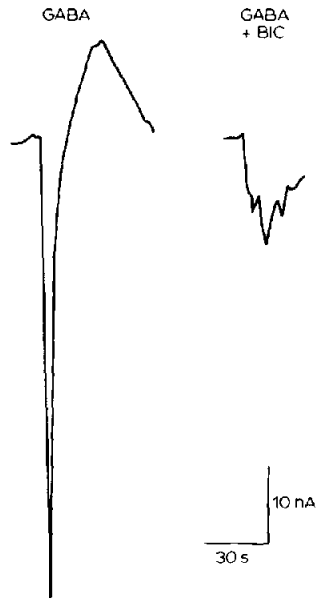

Fig.2. GABA-evoked current in *Xenopus* oocytes injected with synthetic rat $GABA_A$ α- and β-subunit mRNA. The response (downward deflection) to 0.1 μM GABA is shown in the left tracing. The right tracing shows the diminution of the response to GABA (0.1 μM) in the presence of 5 μM bicuculline; the latter was applied for 1.5 min before GABA application.

The expression of the α- and β-subunits was examined by Northern blot analysis of RNA from rat cortex, cerebellum and hippocampus (fig.3). A 4.2/4.6 kb doublet is recognized by the α probe in all three brain regions. One possible explanation for the presence of the two α-subunit bands is that the probe recognizes two different α-subunit mRNAs (analogous to the α-subunit heterogeneity in bovine brain [8]), although we note that the region to which the probe is directed is not highly conserved in the bovine α-subunit sequences. Alternatively, the two α-subunit mRNA species could represent transcripts of different length from the same gene, involving the usage of multiple poly(A) addition sites. The β probe binds to a single RNA species of 6.0 kb. The difference in size between this, and that reported for the β-subunit mRNA in bovine cerebellum (4.8 kb) [19] may reflect species differences, or support our se-

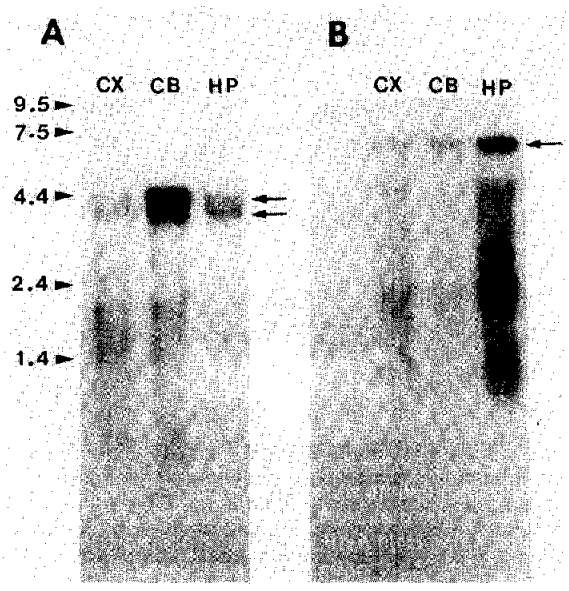

Fig.3. Distribution of $GABA_A$ α- (A) and β- (B) subunit mRNA in adult rat cortex (CX), cerebellum (CB) and hippocampus (HP) by Northern blot hybridization with α- and β-subunit oligodeoxynucleotide probes. The arrows indicate the specific hybridizing bands. An RNA ladder (BRL) was used for size markers.

quence data suggesting that this rat β-subunit clone encodes a novel $GABA_A$ receptor β-subunit.

Given the strong sequence similarity across species in the transmembrane regions of both α- and β-subunits and the lack of amino acid sequence identity in the region between transmembrane domains M3 and M4 in the bovine α-subunit subtypes, the striking dissimilarity in the M3–M4 loop sequence of the bovine and rat β-subunits suggests that these two β-subunit cDNA clones are not equivalent. Our recent cloning of another rat β-subunit cDNA which exhibits ~75% homology with the bovine (and rat; this report) β-subunit (in preparation) supports the concept of $GABA_A$ receptor β-subunit heterogeneity. This evidence, together with the bovine α_2-β-like pharmacological sensitivity of this rat α_1-β complex and the recent cloning of additional $GABA_A$ receptor components (e.g. γ-subunit subtypes [20]), suggests that the existence of diverse $GABA_A$ receptor responses may be mediated by the differential expression of multiple $GABA_A$ receptor subunits.

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