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Identification of the α_3-subunit in the $GABA_A$ receptor purified from bovine brain

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Polyclonal antibodies have been raised to synthetic amino acid sequences of the bovine $GABA_A$ receptor α_1 and α_3 subunits. Anti-α_1 subunit antibodies recognise a polypeptide of 53 kDa whereas anti-α_3 subunit antibodies recognise a polypeptide of 59–60 kDa, in Western blots of $GABA_A$ receptor purified from adult bovine cerebral cortex, cerebellum and 12-day calf cerebral cortex.

Benzodiazepine; Cl^- channel; Antibody; Receptor; Aminobutyric acid, γ-

1. INTRODUCTION

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system. It mediates its effects by the specific interaction with the $GABA_A$ receptor which is a ligand-gated Cl^- channel. The receptor is a complex multi-subunit protein which possesses allosteric binding sites for the anxiolytic benzodiazepines, the barbiturate drugs and a group of compounds called collectively the chloride channel ligands which include the convulsant compounds *t*-butylbicyclophosphorothionate (TBPS) and picrotoxin [1]. The $GABA_A$ receptor has been purified to apparent homogeneity from several vertebrate species and it has been shown to consist of two polypeptide chains, the α subunit of 53 kDa and the β subunit of 57–58 kDa [2–6] with a molecular size of 230–240 kDa [7]. The α subunit of the purified receptor is specifically photoaffinity-labelled by the benzodiazepine, [^{3}H]flunitrazepam [2,4,8], whereas it is predominantly the β subunit which is specifically photoaffinity-labelled by the GABA agonist, muscimol [9,10]. The primary structures of the α and β subunits have been deduced from the corresponding complementary DNAs and it was shown that the micro-injection of the α and β RNAs into the *Xenopus* oocyte is sufficient to form the GABA-gated Cl^- channel [11].

Extensive photoaffinity-labelling studies with [^{3}H]flunitrazepam however, have shown that in the membrane-bound form of the receptor from several species, multiple polypeptides are specifically labelled ([12] and review [1]). This phenomenon has been characterised in detail in the rat where it was demonstrated that each photolabelled subunit has a brain-regional distribution, a pattern of post-natal development and a pharmacological specificity of labelling [13–15]. Molecular cloning studies recently identified 3 forms of the α subunit, α_1, α_2 and α_3, respectively [16]. It was proposed that these may form the basis for the above-described observations with the rat receptor. We here report the identification of the α_3 polypeptide in the purified $GABA_A$ receptor using antibodies

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Abbreviations: GABA, γ-aminobutyric acid; KLH, keyhole limpet haemocyanin; ELISA, enzyme-linked immunoadsorbent assay; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TBPS, *t*-butylbicyclophosphorothionate

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raised against the C-terminal sequence of the deduced α_3 primary structure.

2. MATERIALS AND METHODS

[*N-methyl*-^{3}H]Flunitrazepam (85 Ci/mmol), [*methylene*-^{3}H]muscimol (9 Ci/mmol), biotinylated anti-(rabbit Ig), biotinylated anti-(mouse Ig) and streptavidin biotinylated peroxidase complex were purchased from Amersham International (England). Peptide α_3 479–492 was purchased from The Institute of Animal Physiology, Babraham, Cambridge, England. Flunitrazepam and Ro 7-1986/1 were gifts from Dr H. Mohler (Hoffman-La Roche, Basel, Switzerland) and chlorazepate was a gift from Boehringer Ingelheim (Bracknell, England). All other materials were as noted elsewhere [3,17].

The $GABA_A$ receptor was purified from adult bovine cerebral cortex, adult bovine cerebellum and 12-day calf cerebral cortex by benzodiazepine affinity chromatography as in [3]. GABA and benzodiazepine receptor-binding assays, and SDS-PAGE under reducing conditions, were carried out as before [3]. Western blotting was performed according to [17,18].

Peptides α_1 1–15 and α_1 413–429 and polyclonal antibodies to these peptides were prepared as previously described [18]. Peptides α_1 324–341, sequence PEKPKKVKDPLIKKNNT, and α_3 479–492 with an additional N-terminal cysteine, sequence CVNRESAIKGMIRKQ, were coupled to keyhole limpet haemocyanin (KLH) by either the glutaraldehyde procedure (α_1 324–341) or via the N-terminal cysteine (α_3 479–492), as described [19]. Peptide-KLH conjugates (0.2 μmol peptide) emulsified with Freunds complete adjuvant were injected at 2 sites intramuscularly into Dutch-belted rabbits (2 animals per peptide). Subsequent immunisations were in Freund's incomplete adjuvant at monthly intervals. The development of the immune response was measured by ELISA with the appropriate peptide or purified $GABA_A$ receptor as antigen, as described [18,20].

3. RESULTS AND DISCUSSION

We have previously shown that polyclonal antibodies raised against N- and C-terminal sequences of the α_1 subunit of the $GABA_A$ receptor recognise the receptor in both a native and denatured form [18]. Thus, following the discovery of the α_1, α_2 and α_3 cDNAs we synthesised a peptide which corresponded to the deduced C-terminal amino acid sequence of the α_3 polypeptide. Antibodies to this defined epitope would be predicted from our studies as above to react with the α_3 subunit if the protein is present in the purified receptor preparations. Antibodies to the α_3 479–492 peptide were detected in both rabbits in the sera obtained from the first bleed, which was made 7 days after the second immunisation. Positive antibody titres were obtained on bleed 3 in the ELISA assay, with the $GABA_A$ receptor purified from bovine cerebral cortex as antigen, again in both rabbits (not shown). The maximum respective antibody titre obtained to date with peptide as antigen is 1 in 90000 and with adult bovine cerebral cortical $GABA_A$ receptor as antigen 1 in 100. The antibody titre is defined here as the serum dilution which gives half the absorbance at 492 nm in the ELISA as a 1 in 10 dilution of that immune serum.

The anti-α_3 479–492 antibodies reacted with purified $GABA_A$ receptor in Western blots. Fig.1A shows the results of an immunoblot where the reactivities of the α_1 and α_3 subunit-specific antibodies with the $GABA_A$ receptor purified from adult bovine cerebral cortex are compared. It can be seen that in all cases, the α_1 subunit-specific antibodies react predominantly with a polypeptide of 53 kDa which is coincident with the subunit recognised by an anti-native $GABA_A$ receptor antibody and the anti-$GABA_A$ receptor monoclonal antibody 1A6. Weak immunoreactivity was also observed with a band at 68 kDa for the anti-α_1 peptide antibodies (fig.1). This band was also seen when non-GABA receptor immune sera were used and it was taken thus as a non-specific reaction. The anti-α_3 479–492 antibodies reacted with a polypeptide of 59–60 kDa (fig.1). When high-resolution SDS-PAGE was carried out with longer gels than previously employed and at low protein concentration it was possible to show that the β subunit was resolved into two bands of 57–58 and 59–60 kDa, respectively (fig.1B). Previous reports had described the β subunit in SDS-PAGE as a broad diffuse band [3,4] and this was attributed to the possibility that the β subunit was heavily and/or heterogeneously glycosylated. This appearance can now be ascribed to the presence of the two polypeptides.

The deduced primary structure of the α_3 subunit predicts a molecular mass of 52 kDa [16]. Within the sequence there are four potential *N*-glycosylation sites, the carbohydrate of which would contribute towards the size of the α_3 subunit in SDS-PAGE. It has been demonstrated that the α_3 subunit is sensitive to *N*-glycanase treatment as seen by a decrease in the apparent molecular mass of the polypeptide after the enzyme treatment of approx. 4 kDa (not shown). The molecular mass of the core polypeptide then agrees fairly well with

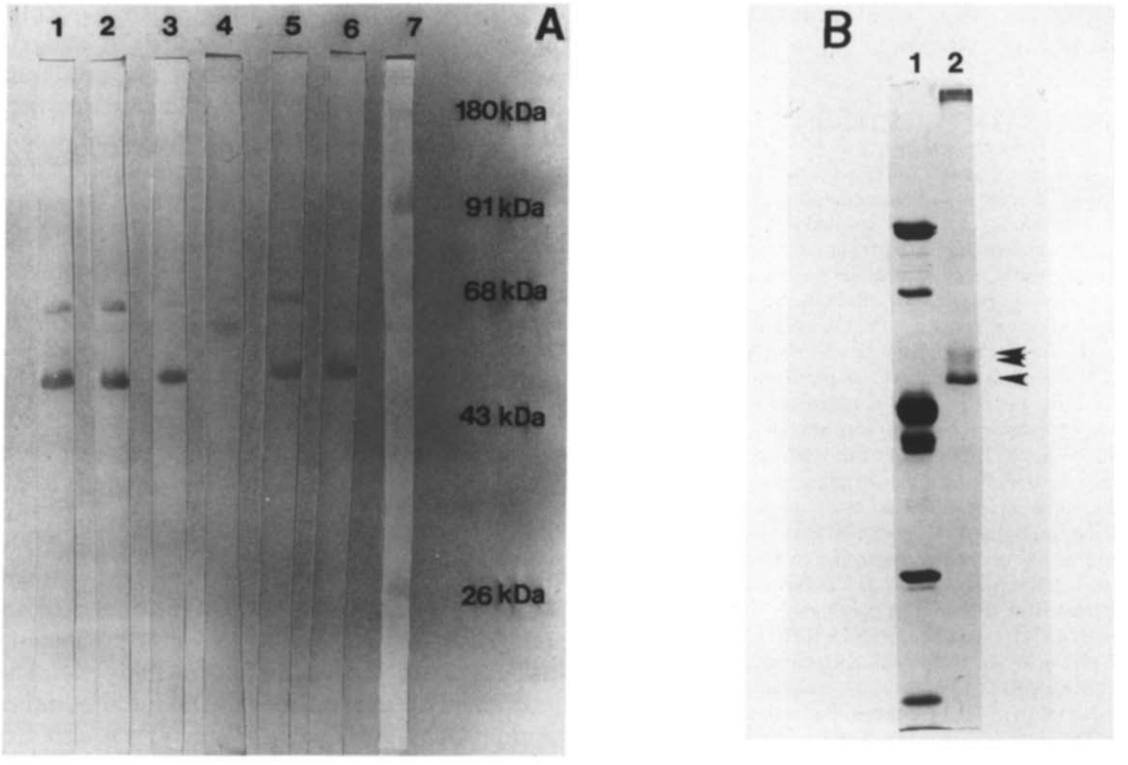

Fig.1. (A) Immunoblot with anti-$GABA_A$ receptor antibodies. $GABA_A$ receptor purified from adult bovine cerebral cortex was subjected to SDS-PAGE in 10% polyacrylamide slab gels under reducing conditions. Receptor was transferred to nitrocellulose membranes by Western blotting and probed with anti-$GABA_A$ receptor antibodies as described in section 2. Each lane corresponds to 2.5 pmol [^{3}H]flunitrazepam-binding sites which is approx. 2 μg protein. Antibodies: (1) anti-peptide α_1 1–15; (2) anti-peptide α_1 413–429; (3) anti-peptide α_1 324–341; (4) anti-peptide α_3 479–492; (5) anti-native $GABA_A$ receptor, α-subunit specific [17]; (6) monoclonal antibody 1A6 which at this receptor concentration recognises predominantly the α subunit [20] and (7) pre-stained protein standards with molecular masses as shown. (B) SDS-PAGE of purified bovine cerebral cortical $GABA_A$ receptor. $GABA_A$ receptor was purified from adult bovine cerebral cortex by benzodiazepine affinity chromatography, precipitated with trichloroacetic acid and subjected to SDS-PAGE under reducing conditions in 10% polyacrylamide slab gels all as described in [3]. Lanes: (1) silver stain of protein standards of molecular masses 93, 66, 45, 21 and 14 kDa; (2) silver stain of $GABA_A$ receptor purified from adult bovine cerebral cortex. An arrowhead denotes the position of the $GABA_A$ receptor subunits.

the value predicted from the deduced primary structure of α_3 once account is taken of the anomalous behaviour in SDS-PAGE of membrane proteins as seen for the α_1 and β subunits [11]. Interestingly, Kirkness and Turner [21] have recently shown that an anti-α_1 101–109 peptide antibody reacts with polypeptides of 51 kDa (α subunit) and 57 kDa from the $GABA_A$ receptor purified from porcine cerebral cortex receptor. The reactive band at 57 kDa is larger than the β subunit identified by photoaffinity labelling with [^{3}H]muscimol [21]. The sequences of the α_1 and α_3 subunits are identical in this region in the bovine species. Thus, it is conceivable these authors have detected the α_3 subunit, identified by us here using an antibody to a unique region of the α_3 sequence.

Previous studies with the rat receptor have demonstrated that in adult cerebellum, in contrast to other brain regions examined, one polypeptide only was predominantly photolabelled with [^{3}H]flunitrazepam [22]. Also, Northern blots using isoform-specific oligonucleotide probes have shown that the α_3 transcript is prominent at an early stage of bovine brain development [16]. Thus, we carried out an immunoblot study on the receptor purified from adult bovine cerebral cortex and cerebellum, and 12-day calf cortex. The results are shown in fig.2, where it can be seen that the α_1 and

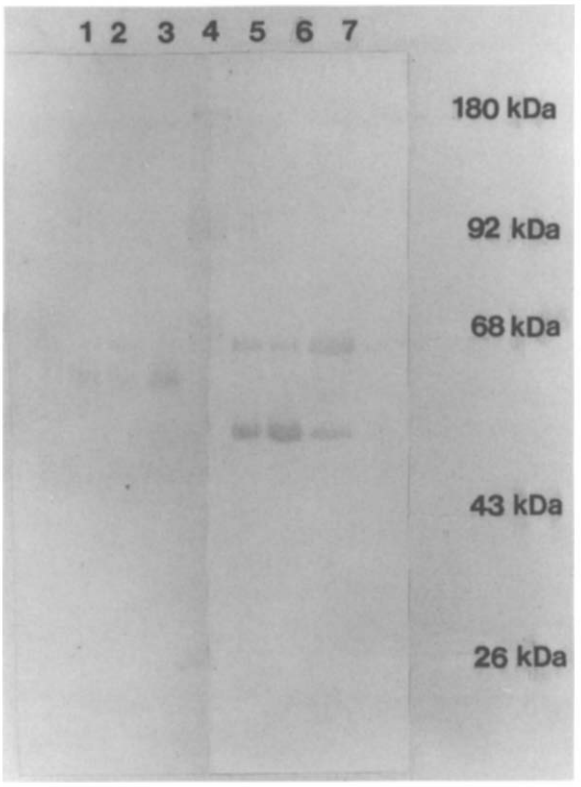

Fig.2. Immunoblot with anti-$GABA_A$ receptor antibodies against $GABA_A$ receptors purified from different brain regions. $GABA_A$ receptor purified from adult bovine cerebral cortex, adult bovine cerebellum and 12-day calf cerebral cortex was subjected to SDS-PAGE in 10% polyacrylamide slab gels under reducing conditions. Receptor was transferred to nitrocellulose membranes by Western blotting and probed with anti-peptide α_1 1–15 and anti-peptide α_3 479–492 antibodies as described in section 2. The amount of receptor applied per gel lane was 1, 2.5, 0.12 pmol [^{3}H]flunitrazepam-binding sites for adult cortex, 12-day calf cortex and adult cerebellum, respectively. Lanes: (1–3) blotting with anti-peptide α_3 479–492 antibodies; (5–7) blotting with anti-peptide α_1 1–15 antibodies; (1,5) adult cortex as antigen; (2,7) adult cerebellum as antigen; (3,6) 12-day calf cortex as antigen; (4) positions of pre-stained protein standards whose molecular masses are given on the right.

α_3 subunits are both present in the three types of preparation, with the same molecular masses as described for fig.1. In each case, the signal obtained for the α_1 subunit is qualitatively the strongest and also, the α_1 specific antibody was shown to recognise the non-specific band of approx. 66 kDa, as discussed above.

Thus, we have shown the presence of the α_3 subunit in the $GABA_A$ receptor purified from adult bovine cortex and cerebellum and 12-day calf cortex. Further work using the specific antibodies described herein should permit the quantitation of the respective isoforms present in different brain regions and perhaps an answer to the question as to whether the basis for the type I and II benzodiazepine receptors can be attributed to the individual subunits such as the α_1 and α_3 polypeptide.

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