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Antibodies directed against a nonapeptide sequence of the γ-aminobutyrate (GABA)/benzodiazepine receptor α-subunit

Detection of a distinct α-like subunit in pig cerebral cortex but not cerebellum

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A synthetic peptide, corresponding to amino acid residues 101–109 of the bovine γ-aminobutyrate/benzodiazepine receptor α-subunit, was used to raise a polyclonal antiserum. The reactivity of this antiserum towards polypeptides of both bovine and pig receptor preparations was established by immunoprecipitation and immunoblotting. Anti-peptide antibodies recognized the α-subunit (51 kDa) of receptor prepared from pig cerebellum or cerebral cortex. However, a polypeptide of 57 kDa was additionally recognized in cortical, but not cerebellar, preparations. This α-like polypeptide appeared larger than the band of polypeptides labelled irreversibly with [^{3}H]muscimol (β-subunit, 55–57 kDa) and corresponds to a polypeptide detected only in cortex after silver-staining or irreversible labelling with [^{3}H]flunitrazepam. These results support the idea that the distinct regional patterns of polypeptides labelled irreversibly with [^{3}H]flunitrazepam reflect the existence of heterologous distributions of distinct α-like subunits.

INTRODUCTION

The γ-aminobutyrate (GABA)/benzodiazepine receptor is a ligand-gated chloride-ion channel through which the inhibitory neurotransmitter, GABA, and the anxiolytic benzodiazepines mediate many of their effects. The receptor complex has been purified from several species (for a review, see [1]), and SDS/polyacrylamide-gel electrophoresis has, in most cases, indicated two major bands of polypeptides with apparent molecular masses 48–53 kDa (α-subunit) and 55–57 kDa (β-subunit). Complementary DNAs encoding bovine α- and β-subunits have been cloned, and co-expression of their respective RNAs in *Xenopus* oocytes produces a functional receptor and ion channel with the pharmacological properties of a GABA/benzodiazepine receptor [2].

Despite these minimal requirements for expression of an active receptor, two distinct lines of evidence suggest the existence of heterogeneous populations of receptor. Thus membrane binding studies, using the triazolopyridazine CL218872, predict multiple affinity states of the benzodiazepine receptor in cerebral cortex and hippocampus, but only high-affinity receptors in cerebellum (see e.g. [3]). Regional differences in affinity for CL218872 have also been observed in purified preparations of the receptor [4]. Also, when the photoaffinity ligand [^{3}H]-flunitrazepam was used, membrane preparations from rat cerebellum were found to incorporate the ligand into a single polypeptide (51 kDa), whereas in other brain regions additional polypeptides (53–57 kDa) were also labelled (see e.g. [5]). Similar results were obtained with preparations of the receptor purified from pig cerebellum and cerebral cortex, where, despite the apparent similarity of their polypeptide compositions, minor incorporation of [^{3}H]flunitrazepam into a 55 kDa subunit of the cortical, but not the cerebellar, preparation was observed [6]. This latter observation appeared consistent with the existence of β-subunit heterogeneity inferred from the relatively diffuse staining pattern in the 55 kDa range after single-dimensional gel electrophoresis [6–8], and the distinct polypeptides observed after two-dimensional electrophoresis [4]. However, receptor polypeptides which are labelled irreversibly with [^{3}H]flunitrazepam but exhibit a molecular mass corresponding to the β-subunit may represent α-like polypeptides which are derived from distinct α-like genes or are subjected to differential post-translational modification. In order to examine this possibility, we have used antibodies raised against a synthetic peptide corresponding to a region of the bovine α-subunit to probe for distinct α-like peptides in different regions of pig brain.

EXPERIMENTAL

Materials

Thiopropyl-Sepharose 6B, sodium cholate, L-α-phosphatidylcholine (type II-S), keyhole-limpet haemocyanin (KLH) and goat anti-(rabbit IgG) IgG (R5506) were from Sigma (Poole, Dorset, U.K.). The sources of all other materials were as listed previously [6,9].

Receptor preparation

Preparations of GABA/benzodiazepine receptor complex were purified from bovine cerebral cortex, pig cerebral cortex or pig cerebellum by a modification of methods described previously [6,10–12]. Briefly, washed membranes were treated with 2% (w/v) sodium cholate instead of Triton X-100 [6]. After application of the cholate-solubilized receptor complex to a 20 ml-volume 1012S-agarose affinity column [12], the column was washed with Column Wash A [20 mM-potassium phosphate (pH 7.4)/0.1 M-KCl/1% (w/v) sodium cholate/1 mM-EDTA/0.1% phosphatidylcholine/0.02% NaN_3] (200 ml). The column was then washed with the same

Abbreviations used: GABA, γ-aminobutyrate; KLH, keyhole-limpet haemocyanin.

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medium containing 1 M-urea (200 ml) and eluted by including 10 mM-chlorazepate in the latter washing medium. The chlorazepate eluate (40 ml) was concentrated to 1 ml by ultrafiltration in an Amicon cell. The concentrated sample was twice diluted to 10 ml with Column Wash A, followed by re-concentration to 1 ml.

The purified receptor was incorporated into phosphatidylcholine vesicles by the cholate-dialysis procedure described by Jones *et al.* [13]. The dialysis step, required to remove cholate, also served to remove residual chlorazepate and urea from the preparation. Incorporation of receptor into vesicles was confirmed by sucrose gradient centrifugation.

Typical yields of receptor from 100 g of bovine or pig cerebral cortex (~400 μg of protein) or 100 g of pig cerebellum (~50 μg) all exhibited specific [^{3}H]muscimol- and [^{3}H]flunitrazepam-binding activities in the range 700–1100 pmol/mg and 600–800 pmol/mg respectively.

Preparation of antibodies

The peptide, Cys-Phe-His-Asn-Gly-Lys-Lys-Ser-Val-Ala-amide, corresponding to the sequence Cys-(101–109) of the bovine α-subunit [2], was purchased from New Brunswick Scientific (Watford, Herts., U.K.) and characterized by reverse-phase h.p.l.c. and amino acid analysis. The peptide was coupled to KLH by reaction with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as described previously for the coupling of haemagglutinin to KLH [14].

Antisera were raised in rabbits against the native receptor complex purified from pig cerebral cortex (75 μg per injection) or peptide–KLH conjugate (200 μg per injection) using an immunization schedule described previously [9]. Anti-peptide antibodies were affinity-purified on a column of thiopropyl-Sepharose 6B to which the peptide had been coupled, and eluted with 0.2 M-glycine/HCl buffer, pH 2.3.

Immunoprecipitation

Samples of GABA/benzodiazepine receptor complex purified from bovine or pig cerebral cortex (each 50 μg) were labelled with 1 mCi of Na^{125}I by the chloramine-T method [15]. After dialysis, samples were denatured by boiling for 2 min in 0.25% (w/v) SDS, diluted 10-fold with 2 mM-NaH_2PO_4/20 mM-Na_2HPO_4/150 mM-NaCl/0.02% (w/v) NaN_3, pH 7.4 ('saline') containing 0.5% (w/v) Triton X-100, and centrifuged at 11000 *g* for 30 min to remove particulate matter. Preparations of 'dilute' rabbit preimmune serum or anti-peptide serum were prepared by diluting 1:9 with saline. Serial dilutions of the dilute anti-peptide antiserum were prepared in dilute preimmune serum. Aliquots of ^{125}I-labelled GABA/benzodiazepine receptor complex (5 μl; ~100000 c.p.m.; 5–50 ng) were incubated at 4 °C with dilute preimmune serum (5 μl) or serially diluted anti-peptide serum (5 μl) in a total volume of 50 μl of saline containing 0.5% (w/v) Triton X-100. After 8 h, 10 μl of goat anti-(rabbit IgG) IgG was added and incubation at 4 °C continued for 15 h. Precipitates were pelleted by centrifugation at 11000 *g* for 30 min, washed with 'saline'/0.5% (w/v) Triton X-100 (500 μl), re-centrifuged and the supernatant removed. The pellets were counted for radioactivity in a γ-radiation counter. Specific immunoprecipitation was determined by subtraction of radioactivity precipitated non-specifically (dilute preimmune serum only) from the total precipitated radioactivity.

Photoaffinity labelling

Preparations of purified receptor complex, reconstituted into liposomes (0.5 pmol of [^{3}H]flunitrazepam binding sites), were photolabelled with [^{3}H]flunitrazepam by the method of Mohler *et al.* [16] as described previously [6]. Photolabelling with [^{3}H]muscimol [17] was performed after incubation of reconstituted receptor complex (4.5 pmol of [^{3}H]muscimol binding sites) with 100 nM-[^{3}H]muscimol for 1 h at 0 °C in the presence of 1 mM-dithiothreitol. The samples were exposed to a u.v. light source (Rayonet RPR2537A, 254 nm) at a distance of 4 cm for 15 min at 0 °C. GABA was added to a final concentration of 1 mM, and incubation was continued for 1 h. Control experiments included GABA (1 mM) before addition of [^{3}H]muscimol. After sample concentration and SDS/polyacrylamide-gel electrophoresis (see below), gels were treated with sodium salicylate [18], dried, and subjected to fluorography at −70 °C on Fuji RX film for 14 days.

SDS/polyacrylamide-gel electrophoresis and immunoblotting

Samples were concentrated and prepared for electrophoresis as described previously [6]. SDS/polyacrylamide-gel electrophoresis was performed using the system of Laemmli [19] with a 7–13%-(w/v)-polyacrylamide gradient. After electrophoresis gels were silver-stained [10] or blotted on to nitrocellulose filters [9]. Filters were first treated with anti-(purified native receptor) antiserum (1:100) or affinity-purified anti-peptide antibody (1:1000), followed by ^{125}I-labelled goat anti-(rabbit IgG) IgG (10^6 c.p.m./ml) [9]. All washing and blocking steps were as described previously [9]. Autoradiography was performed at −70 °C on Fuji RX film.

RESULTS AND DISCUSSION

A synthetic peptide corresponding to a short region of the bovine α-subunit (amino acid residues 101–109; [2]) was conjugated to KLH via an *N*-terminal cysteine residue and used to raise a polyclonal antiserum. The ability of this antiserum to recognize full-length receptor subunits was established by immunoprecipitation of denatured ^{125}I-labelled receptor preparations. The antiserum immunoprecipitated both bovine and pig receptor polypeptides (Fig. 1) in a manner which was inhibited by free peptide [IC_{50} (concentration causing 50% inhibition) = 0.2 nM at 10^{-3} dilution of antiserum, $n = 2$]. The antiserum did not immunoprecipitate reversible [^{3}H]-muscimol and [^{3}H]flunitrazepam binding activities and immunoprecipitated [^{3}H]flunitrazepam-labelled receptor preparations only after denaturation with SDS (results not shown). These data indicate that the short bovine peptide sequence is similar to, or identical with, a region of the pig receptor, but is not accessible to antibodies in the native conformation of the receptor complex. However, the antiserum has proved useful for characterizing receptor preparations by immunoblotting (see below).

Previously we noted that there was little apparent difference between the polypeptide compositions of receptor preparations purified from pig cerebellum and cerebral cortex, despite differential labelling of 55 kDa subunits with [^{3}H]flunitrazepam [6]. Several reports have

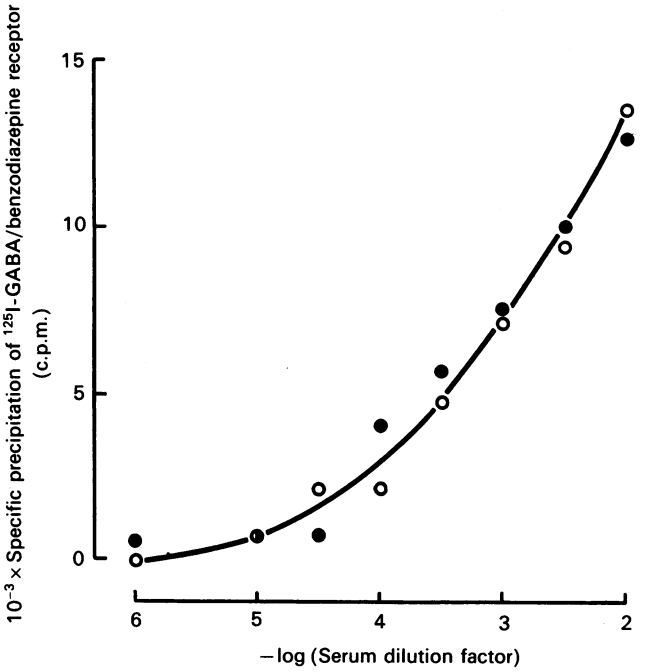

Fig. 1. Immunoprecipitation of ^{125}I-labelled GABA/benzodiazepine receptor purified from bovine (○) and pig (●) cerebral cortex

Immunoprecipitation of receptor preparations was performed by using dilutions of anti-peptide serum and goat anti-(rabbit IgG) IgG as described in the Experimental section. Data are from one experiment which was typical of three separate experiments. Average non-specific precipitation (in the presence of dilute preimmune serum only) was 2430 c.p.m. (bovine) and 2020 c.p.m. (pig). The serum titre was $\leqslant 2 \times 10^3$.

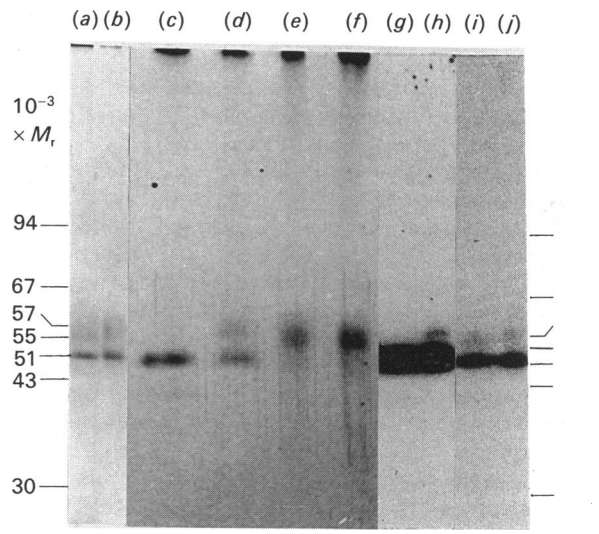

Fig. 2. Comparison of GABA/benzodiazepine receptor preparations purified from pig cerebellum and cerebral cortex after photoaffinity labelling and immunoblotting

Purified receptor (3 μg) from cerebellum (*a*) or cerebral cortex (*b*) was subjected to SDS/polyacrylamide-gel electrophoresis and silver-stained for protein. Purified receptor from cerebellum (*c*, *e*) or cerebral cortex (*d*, *f*) was photoaffinity-labelled with [^{3}H]flunitrazepam (*c*, *d*) or [^{3}H]-muscimol (*e*, *f*) as described in the Experimental section. Purified receptor (3 μg) from cerebellum (*g*, *i*) or cerebral cortex (*h*, *j*) was subjected to SDS/polyacrylamide-gel electrophoresis, transferred to nitrocellulose filters and treated with affinity-purified anti-peptide antibody (*g*, *h*) or anti-(purified native receptor) antiserum (*i*, *j*) as described in the Experimental section.

noted that the β-subunit region (55–57 kDa) appears relatively diffuse after electrophoresis [6–8]. By using a modified polyacrylamide gradient and by running the low-M_r markers off the end of the gel, it is now possible to obtain better resolution of this band. Under these conditions, differences between the polypeptide compositions of cerebellar and cortical preparations can be observed (Fig. 2, lanes *a* and *b*). The cerebellar receptor displays a 51 kDa polypeptide and a more diffuse band in the region of 55–56 kDa. In addition to this staining pattern, the cortical receptor displays a major polypeptide of 57 kDa which is not observed in the cerebellar preparation.

As shown previously [6], after photoaffinity labelling with [^{3}H]flunitrazepam, radioactivity is incorporated predominantly into the smallest polypeptide (51 kDa) in preparations from both brain regions, whereas the cortical receptor also displays labelling on a larger polypeptide (57 kDa) (Fig. 2, lanes *c* and *d*). After photoaffinity labelling with [^{3}H]muscimol, radioactivity has been reported to be incorporated into bovine [8,20] and rat [11] brain receptor preparations on the β-subunit. In both pig cerebellar and cortical preparations, [^{3}H]muscimol was incorporated predominantly into a band of 55–57 kDa (Fig. 2, lanes *e* and *f*). No incorporation of radioactivity was observed when labelling was performed in the presence of GABA. In cortical preparations, the [^{3}H]muscimol-labelled band migrated with a slightly lower apparent molecular mass than the 57 kDa polypeptide labelled with [^{3}H]flunitrazepam (Fig. 2, lanes *d* and *f*). However, the possibility of abnormal migration caused by covalent modification during u.v. illumination cannot be ignored. In common with a recent report [21], we have also detected significant incorporation of [^{3}H]muscimol into a smaller (51 kDa) polypeptide of soluble preparations of the purified receptor (results not shown). Labelling of a 51 kDa polypeptide by [^{3}H]muscimol was not detected using the reconstituted preparations described here, indicating that the lipid environment of the receptor may influence the pattern of peptide labelling, e.g. by masking or altering the conformation of amino acid sequences which are in intimate contact with [^{3}H]muscimol-binding sites.

Immunoblotting with the anti-peptide antibody revealed a strong reaction with a 51 kDa polypeptide in both cerebellar and cortical preparations (Fig. 2, lanes *g* and *h*). However, the antibodies also recognized a polypeptide of 57 kDa which is present only in cortical preparations (Fig. 2, lane *h*). A similar pattern of recognition (51 and 57 kDa) was obtained using bovine cortex receptor preparations (results not shown). In contrast, an antiserum raised against the native cortical receptor recognized a distribution of polypeptides (Fig. 2, lanes *i* and *j*) similar to that observed after silver-staining for protein (Fig. 2, lanes *a* and *b*).

Immunoblotting of crude membrane preparations with anti-peptide antibodies also revealed a 57 kDa polypeptide in pig cortex but not cerebellum, in addition

to a 49 kDa polypeptide and four polypeptides of < 35 kDa present in both preparations (results not shown). Attempts to identify these polypeptides as receptor subunits (and proteolytic fragments) or unrelated cross-reacting polypeptides have been initiated.

The anti-peptide antibodies reveal the presence of an α-like subunit in cerebral cortex but not cerebellum. This polypeptide exhibits an apparent molecular mass (57 kDa) slightly higher than the [^{3}H]muscimol-labelled band of polypeptides, but is similar in size to a polypeptide detected only in cortex by silver-staining or irreversible labelling with [^{3}H]flunitrazepam. Although it has been suggested that both α- and β-subunits of the receptor complex may possess benzodiazepine-binding sites [21], the data presented here are consistent with the idea that polypeptides which are labelled by [^{3}H]flunitrazepam are distinct α-like peptides. This possibility is supported by recent work [22] which has correlated three [^{3}H]flunitrazepam-labelled polypeptides (51, 53 and 59 kDa) in newborn rats with recognition by an α-, but not a β-subunit-specific antibody. Although the source of such α-like polypeptides (distinct genes or post-translational modification) is presently unknown, these studies should help to define the structural basis of benzodiazepine receptor subtypes.

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