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Promiscuity of $GABA_A$-receptor $\beta 3$ subunits as demonstrated by their presence in $\alpha 1$, $\alpha 2$ and $\alpha 3$ subunit-containing receptor subpopulations

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Polyclonal antibodies were raised in rabbits against the $GABA_A$-receptor $\beta 3$ subunit peptide sequence, KQSMPREGHGRHMDR-NH_2 coupled to keyhole limpet haemocyanin. These anti-$\beta 3$ 379–393 antibodies immunoprecipitated in a dose-dependent manner specific benzodiazepine agonist binding sites from Na^+ deoxycholate extracts of bovine cerebral cortex. In immunoblots, anti-$\beta 3$ 379–393 antibodies recognised two species with M_r 59 900 and M_r 57 200 in all preparations tested, which included crude detergent-solubilised, benzodiazepine affinity chromatography-purified receptor, anti-$\alpha 1$ 324–341 antibody, anti-Cys $\alpha 2$ 414–424 antibody and anti-Cys $\alpha 3$ 454–467 antibody immunoaffinity-purified $GABA_A$-receptor subpopulations. These results provide evidence for the ubiquity and promiscuity of the $GABA_A$-receptor $\beta 3$ subunit.

$GABA_A$; Receptor; Benzodiazepine; Antibody; Immunoaffinity; Purification

1. INTRODUCTION

The γ-aminobutyric $acid_A$ ($GABA_A$)-receptors of mammalian brain are chloride ion channels whose function can be allosterically regulated by benzodiazepines, barbiturates and neurosteroids [1]. Molecular cloning has revealed an extensive family of $GABA_A$-receptor subunit genes whose products, on the basis of their deduced amino acid sequence similarities, can be divided into five distinct subunit types designated α, β, γ, δ and ρ. Within each subunit class, isoforms exist i.e. $\alpha 1$–$\alpha 6$, $\beta 1$–$\beta 4$ and $\gamma 1$–$\gamma 3$ which share at least 75% amino acid sequence identity [2]. Two examples of alternative splicing of $GABA_A$-receptor subunit genes have been described, revealing an additional dimension to $GABA_A$-receptor complexity [3,4]. The subunit complements of native $GABA_A$-receptors are not known but it is thought that different combinations of polypeptides assemble to form pentameric structures analogous to the other members of the ligand-gated ion channel superfamily. In order to approach this problem, we have recently described the immunoaffinity purification of natural $GABA_A$-receptor subpopulations using antibodies specific for the $\alpha 1$, $\alpha 2$ and $\alpha 3$ subunits and the coexistence of the $\gamma 2$ polypeptide with each of these respective subunit-enriched preparations [5]. We now report the co-purification of the $\beta 3$ subunit with the different receptor subpopulations using a novel anti-$\beta 3$ subunit antibody.

Abbreviations: ELISA, enzyme linked immunoadsorbent assay; GABA, γ-aminobutyric acid; KLH, keyhole limpet haemocyanin; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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2. MATERIALS AND METHODS

Peptides $\beta 2$ 381–395, amino acid sequence KAGLPRHSFWR-NALE-NH_2, and $\beta 3$ 379–393, amino acid sequence KQSM-PREGHGRHMDR-NH_2, were purchased from Multiple Peptide Systems, San Diego, California, USA. Both peptides were coupled to keyhole limpet haemocyanin (KLH) by the glutaraldehyde method and polyclonal antibodies raised in rabbits as in [6]. Anti-peptide antibody production was monitored by an enzyme linked immunoadsorbent assay (ELISA) and antibodies were purified on the respective peptide-CH Sepharose 4B affinity column [6,7]. All other materials and methods, including the immunoaffinity purification of $GABA_A$-receptors, were as previously described [5–8].

3. RESULTS AND DISCUSSION

We have previously shown that antibodies raised against the N- or C-terminal amino acid sequences of the $GABA_A$-receptor $\alpha 1$, $\alpha 2$ and $\alpha 3$ subunits are useful probes in that these regions of the polypeptides are unique to each of the aforementioned subunits and that these antibodies recognise the native conformation of receptors in solution [6,7,9]. In selecting candidate amino acid sequences for the production of β subunit isoform-specific antibodies the N- and C-terminal regions are not useful, since the C-terminus does not extend beyond the fourth transmembrane domain and is therefore probably inaccessible for antibody binding; the respective N-terminal sequences have a high degree of identity. Indeed, even the putative intracellular loop, a divergent region between α subunit isoforms, has ~40% amino acid sequence identity thus restricting the selec-

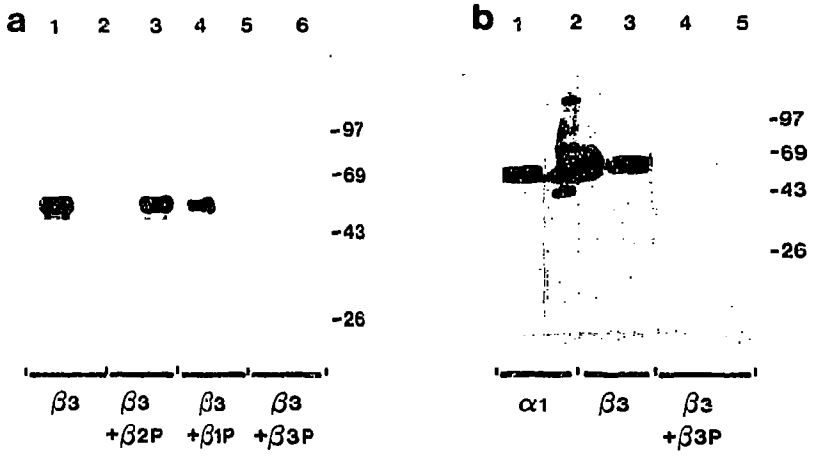

Fig. 1. Immunoblots using anti-β3 379–393 $GABA_A$-receptor subunit antibodies. Samples were subjected to SDS-PAGE in 10% polyacrylamide slab mini-gels followed by immunoblotting using affinity-purified antibodies as described [8]. (a) immunoblotting with anti-β3 379–393 antibodies (15 μg/ml) with benzodiazepine affinity chromatography-purified receptor as antigen (1 pmol [^{3}H]flunitrazepam binding sites, lanes 1,3,4 and 6) or prestained protein standards with molecular weights as shown on the right (lanes 2,5); (lanes 1,2) antibody alone; (lanes 2,3) antibody pre-incubated overnight at 4°C with β2 381–395, 11.6 μM final concentration; (lanes 4,5) antibody pre-incubated overnight at 4°C with β1 329–341, 11.6 μM final concentration; (lanes 5,6) antibody pre-incubated overnight at 4°C with β3 379–393, 11.6 μM final concentration. (b) immunoblotting with Na^+ deoxycholate-solubilised receptor as antigen (0.1 pmol [^{3}H]flunitrazepam binding sites, lanes 1,3 and 4) and benzodiazepine affinity chromatography purified receptor (1 pmol [^{3}H]flunitrazepam binding sites, lanes 2 and 5); (lanes 1,2) blotting with anti-α1 324–341 antibody, 5 μg/ml; (lanes 2,3,4 and 5) blotting with anti-β3 379–393, 15 μg/ml where, in lanes 4 and 5, antibody was pre-incubated with β3 379–393, 11.6 μM final concentration; (lanes 2,5) prestained standards with molecular weights as shown. Note that in (a), lanes 2 and 5 and in (b), lane 2, were cut in half.

tion of peptides to sequences β2 381–395 and β3 379–393. Immunisation with both peptide-KLH conjugates induced the production of anti-peptide antibodies with maximum antibody titres, 1:12 500 (β2 381–395) and 1:40 000 (β3 379–393) but reactivity with $GABA_A$- receptors was found with anti-β3 379–393 antibodies only, thus this antibody was used for further characterisation.

Fig. 1 shows the results obtained for immunoblotting with anti-β3 379–393 antibodies using Na^+ deoxycholate detergent-solubilised bovine cerebral cortex or benzodiazepine affinity chromatography-purified $GABA_A$-receptor as antigen. It can be seen that in both cases anti-β3 379–393 antibodies recognise specifically two polypeptides with approximately equal intensity with M_r 59 900 ± 500 (n=7) and M_r 57 200 ± 500 (n=7). The molecular weight for the non-glycosylated β3 mature subunit predicted from the cDNA sequence is 52 kDa. The β3 subunit contains three consensus sequences for N-glycosylation thus once account is taken for the contribution by weight of the carbohydrate, there is good agreement between the predicted and the experimental value. The same two immunoreactive species were present in anti-α1 324–341, anti-Cys α2 414–424 and anti-Cys α3 454–467 immunoaffinity-purified $GABA_A$-receptors (Fig. 2). Buchstaller et al. [10] reported recently that anti-β3 190–197 antibodies recognised a single polypeptide with M_r 56 000 in $GABA_A$-receptors purified from 5–10-day-old rat brain. Following exhaustive de-

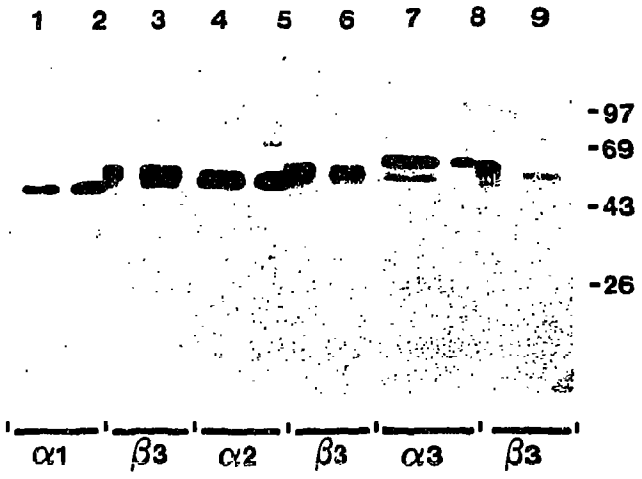

Fig. 2. Western blotting of immunoaffinity-purified $GABA_A$ receptor subpopulations. Samples were subjected to SDS-PAGE in 10% polyacrylamide mini-slab gels followed by immunoblotting with respective affinity-purified antibodies as described [8]. Antigen used was benzodiazepine affinity chromatography-purified receptor (1 pmol [^{3}H] flunitrazepam binding sites; lanes 2,5 and 8); anti-α1 324–341 immunoaffinity-purified receptor (50 μl; lanes 1 and 3); anti-Cys α2 414–424 immunoaffinity-purified receptor (35 μl; lanes 4 and 6); anti-Cys α3 454–467 immunoaffinity-purified receptor (200 μl; lanes 7 and 9). Affinity-purified antibodies used were: anti-α1 324–341, 5 μg/ml, lanes 1 and 2; anti-Cys α2 414–424, 7 μg/ml, lanes 4 and 5; anti-Cys α3 454–467, 15 μg/ml, lanes 7 and 8; and anti-β3 379–393, 20 μg/ml, lanes 2,3,5,6,8 and 9. The molecular weights of prestained protein standards are shown on the right. Note that lanes 2,5 and 8 were cut in half.

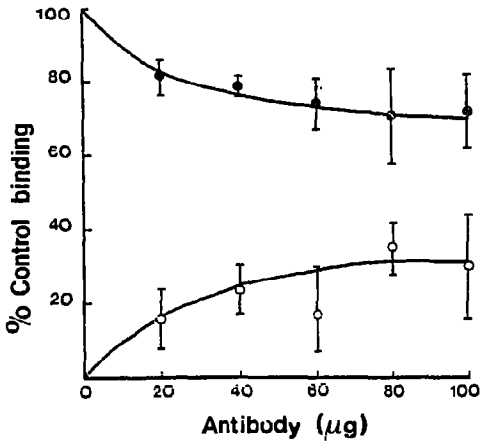

Fig. 3. Dose-dependent immunoprecipitation of [^{3}H]flunitrazepam-specific binding sites from Na^+ deoxycholate extracts of bovine cerebral cortex by anti-β3 379–393 antibodies. A Na^+ deoxycholate extract was prepared from adult bovine cerebral cortex and the soluble extract (200 μl) was incubated with increasing concentrations of affinity-purified anti-β3 379–393 antibodies diluted with normal rabbit Ig to give a fixed protein concentration per tube. Immune complexes were precipitated by the addition of Immunoprecipitin and [^{3}H]flunitrazepam binding to the resultant supernatants and pellets was carried out as described [7]. The results are expressed as the percentage decrease (supernatants, ●) or percentage increase (pellets, ○) of specific [^{3}H]flunitrazepam binding sites present following immunoprecipitation with anti-β3 379–393 antibodies with respect to control samples which contained an equivalent concentration of protein A-purified nonimmune rabbit Ig. The results are the mean ± standard deviations of at least four independent experiments.

glycosylation, anti-β3 190–197 antibodies recognised two immunoreactive species with M_r 53 000 and M_r 51 000, which was interpreted as evidence for the existence of alternative spliced products of the β3 subunit gene. The results obtained here would be in agreement with this, with the two immunoreactive species being resolved in our SDS-PAGE system before deglycosylation. Alternative explanations for our results would be that the lower molecular weight species is a proteolytic product, β3′, or that the sequence recognised by the antibody is common to an as yet unidentified bovine β subunit. Although we cannot distinguish between these two possibilities, we would argue against proteolysis since the intensity of the two bands is approximately equal in all preparations tested and, furthermore, there is no evidence in these for the α1′ product, a well-characterised C-terminal-delted proteolytic fragment (Figs. 1 and 2; [11]).

Anti-β3 379–393 antibodies recognised $GABA_A$-receptors in their native detergent-solubilised state as demonstrated by their dose-dependent immunoprecipitation of specific [^{3}H]flunitrazepam binding sites from extracts of bovine cerebral cortex (Fig. 3). The percentage increase in the binding sites immunoprecipitated was always accompanied by a concomitant decrease in the [^{3}H]flunitrazepam binding sites in the resultant supernatants (Fig. 3). It was found that the maximum percentage of binding sites immunoprecipitated was 28 ± 10% (n=4) which is in the same range, i.e. 17%, reported by [10]. We have previously found, using anti-γ2 1–15 Cys antibodies, that although dose-dependent immunoprecipitation experiments reached a plateau value, re-immunoprecipitation with a second batch of affinity-purified antibody resulted in increased precipitation of receptor compared to the use of anti-α1 324–341 antibodies, where a maximum value was obtained for a single immunoprecipitation [8]. Successive immunoprecipitations with anti-β3 379–393 antibodies did yield an increased immunoprecipitation with 33 ± 10% (n=5) and 49 ± 4% (n=5) [^{3}H]flunitrazepam binding sites pelleted after one and two immunoprecipitations, respectively. Control experiments using Protein A purified non-immune Ig and anti-α1 324–341 antibodies gave no increased immunoprecipitation.

In conclusion, these studies have shown that the β3 subunit of the $GABA_A$-receptor is both abundant and promiscuous in bovine cerebral cortex, being present in α1, α2 and α3 $GABA_A$-receptor-containing populations. Additionally we have provided supportive evidence in agreement with [10] for the existence of alternative splice variants of the β3 subunit gene.

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