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Characterisation of δ-subunit containing $GABA_A$ receptors from rat brain

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Abstract

Polyclonal antibodies have been raised in rabbits against the predicted cytoplasmic loop region of the δ-subunit of the $GABA_A$ receptor. These specifically identify the expressed fragment by Western blot but do not cross react with analogous polypeptides from the γ_1, γ_2 or γ_3-subunits. Polyclonal antisera immunoprecipitated [^{3}H]muscimol binding sites from several brain regions consistent with the reported distribution of δ-subunit mRNA and also detected the δ-subunit by Western blot, identifying a polypeptide of 55KDa. Receptors immunoprecipitated from rat brain with the δ-antisera exhibited an atypical profile with respect to their radioligand binding properties. Receptors immunoprecipitated from all regions tested bound [^{3}H]muscimol, but did not bind benzodiazepine site ligands [^{3}H]Ro 15,1788 or [^{3}H]flunitrazepam with high affinity. Receptors containing a δ-subunit accounted for 10.7 ± 2% of all $GABA_A$ receptors ([^{3}H]muscimol binding sites) in the rat central nervous system as deduced from quantitative immunoprecipitation experiments, the largest population being in the cerebellum where approximately 27% of all receptors contained a δ-subunit. The pharmacology of the GABA (γ-aminobutyric acid) binding site on receptors immunoprecipitated from cerebellum with γ_2 and δ-antisera was compared. The rank order of potency of a series of 6 compounds to compete for [^{3}H]muscimol binding sites was similar in these two populations, but muscimol had a significantly higher affinity for receptors containing the δ-subunit. These receptors therefore comprise a novel population of $GABA_A$ receptors which do not bind benzodiazepines but have a 5-fold higher affinity for muscimol. Coexistence of the δ-subunit with γ-subunits was investigated by purifying receptor on a γ_1, γ_2 or γ_3- immunoaffinity resins, separating the subunit components by polyacrylamide gel electrophoresis, transferring to nitrocellulose and probing with δ-subunit specific antibodies. The δ-subunit could not be detected in combination with any γ-subunit.

Keywords: GABA receptor δ-subunit; Polyclonal antiserum

1. Introduction

GABAergic neurotransmission in the central nervous system is mediated largely by activation of a family of GABA-gated chloride ion-channels, the $GABA_A$ receptors. Structurally, these are hetero-oligomeric pentamers whose five component subunits are selected from a possible repertoire of 16-six α-subunits, four β-subunits, three γ-subunits, one δ-subunit and two ρ-subunits (for review see Olsen and Tobin, 1990; Burt and Kamatchi, 1991; Wisden and Seeburg, 1992; Macdonald and Olsen, 1994). In addition to the agonist binding site, many of the $GABA_A$ receptors have other modulatory sites for steroids, barbiturates and benzodiazepines. The contribution of various subunits to these allosteric sites is not fully understood, although it is established that varying the α-or γ-subunit can produce $GABA_A$ receptors with differing benzodiazepine pharmacologies (Pritchett at al., 1989, Pritchett and Seeburg, 1990, Ymer et al., 1990, McKernan et al., 1991, Herb et al., 1992, Hadingham et al., 1993) and varying the β-subunit produces receptors with differing sensitivity to the anticonvulsant compound loreclezole (Wafford et al., 1994).

Experimental data surrounding the contribution of the δ-subunit to benzodiazepine sensitivity is contradictory. Replacement of the γ_2-subunit by a δ-subunit in expression studies in oocytes produces a $GABA_A$ receptor which is not potentiated by benzodiazepines (Shivers et al., 1989). In contrast, polyclonal antisera raised against the N-terminal 17 amino acids of the δ-subunit have been reported to immunoprecipitate [^{3}H]Ro 15-1788 binding sites (Benke et

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al., 1991) suggesting that δ-containing receptors have a benzodiazepine binding site which has a novel pharmacology (Mertens et al., 1993).

We have raised high titre polyclonal antisera against a long polypeptide derived from the cytoplasmic loop region of the δ-subunit (Asn^{318}-Leu^{400}) and have used this to immunoprecipitate δ-containing receptors from rat brain. Immunopurification and Western blot analysis have been carried out in an attempt to characterise further the subset of naturally-occurring $GABA_A$ receptors which contain this subunit and rationalise the conflicting information in the literature. Of particular importance is whether the δ-subunit exists in combination with any of the γ-subunits since γ_2 or γ_3 is necessary to confer benzodiazepine sensitivity (Pritchett et al., 1989; Knoflach et al., 1991; Herb et al., 1992; Horne et al., 1993; Luddens et al., 1994).

2. Materials and methods

2.1. Antibody production

Expression of the δ-subunit putative cytoplasmic loop was performed in a similar manner as previously described for the α-subunits (McKernan et al., 1991). The region encoding Asn^{318}-Leu^{400} was amplified from rat brain cDNA (1 μg) incorporating a BamH1 site on the senseprimer (5′-GCA TTT GGA TCC TTC AAT GCT GAC TAC AGG AAG-3′) and a HindIII site following a stop codon on the antisense primer (5′-TC GAT GGT GTC TGC AAG CTT GGG TTA GAG TCT G-3′). The PCR product was subcloned into pRSET 5a, a T7 polymerase expression vector, and the fusion protein comprising 14 amino acids from the gene 10 major capsid protein (Met-Ala-Ser-Met-Thr-Gly-Gly-Gln-Gln-Met-Gly-Arg-Gly-Ser) followed by 82 amino acids from the δ-subunit (Asn^{318}-Leu^{400}) was expressed after transformation into E Coli strain BL21 DE3 (LysS). Cells were grown in LB medium containing penicillin (20 μg/ml) to an A_{600} of 0.6. After 3 hours of induction with isopropyl-β-D-thiogalactoside, cells were harvested by centrifugation (1000 × g;10 min) and the expressed fusion protein was recovered from the insoluble fraction after homogenisation in 10mM Tris-HCl, 5mM EDTA, 0.1 mM Phenylmethyl sulphonlufluoride, pH 7.5 by centrifugation (20000 × g; 15 min) and purified by preparative 18% SDS-PAGE (sodium dodecyl sulphate polyacrilamide gel electrophoresis). The purified protein was emulsified with Freund's complete adjuvant (1 : 1) and rabbits were immunised with 100 μg aliquots subcutaneously, and boosted four times at monthly intervals after which time a maximal titre, as assesed by immunoprecipitation of [^{3}H]muscimol binding sites, was achieved. Antibodies to the γ_1-, γ_2-and γ_3-subunits were prepared as described previously (Quirk et al., 1994a,b)

2.2. Immunoprecipitation and radioligand binding

Immunoprecipitation of $GABA_A$ receptors solubilised from rat brain was carried out using antibodies bound to protein A-Sepharose as previously described (McKernan et al., 1991; Quirk et al., 1994a,b). Radioligand binding studies were carried out in a total volume of 0.5 ml in 10 mM Tris-HCl, 1 mM EDTA pH 7.4 for 1 h at room temperature prior to termination by filtration through Whatman GF/C filters followed by three times 3 ml washes with 5 mM Tris-HCl, pH 7.4, and scintillation counting. Non-specific binding was determined using 100 μM GABA for [^{3}H]muscimol binding and 10 μM Ro 15-4513 or 10 μM flunitrazepam for radioligands which bind at the benzodiazepine site.

2.3. Immunoaffinity purification and Western blot analysis

Immunoaffinity purification of individual subunit specific receptor populations was achieved using IgG-Sepharose immunoaffinity columns. IgG was first purified from 2 ml of each antiserum (δ, γ_1, γ_2 and γ_3) on a Protein A-Sepharose affinity resin (Quirk et al., 1994a) and coupled to cyanogen bromide activated Sepharose 4B (1 ml) using methods described by McKernan et al, 1991. Receptors were solubilised from whole rat brain and adsorbed onto the immunoaffinity resins batchwise overnight, put into a column and washed extensively with at least 50 column volumes of 0.5% triton X-100, 50 mM Tris-HCl, 1 mM EDTA, 120 mM NaCl pH 7.4 at 4°C. A small aliquot of immunoaffinity resin (10–50 μl) was retained for radioligand binding with [^{3}H]muscimol to determine more precisely the amount of receptor purified. Receptor was eluted from aliquots of resin with SDS/PAGE gel loading buffer (2% SDS, 20% glycerol, 160 mM Tris-acetate, pH 6.8, 0.01% bromophenol blue). Immediately prior to running the gel this was supplemented with 2 M urea and 2% β-mercaptoethanol. In preliminary experiments this elution procedure was found to elute the greatest yield of receptor, however, the immunoaffinity resins could not be reused. Eluted receptor was subjected to 10% SDS-PAGE, transferred to nitrocellulose and probed with crude antiserum or purified δ-specific antibodies (section 2.4) as indicated, using the protocol detailed elsewhere (McKernan et al., 1991). Detection of the primary antibody was achieved by incubating the blot with horse radish peroxidase-linked protein-A (1 : 500) followed by visualisation with TMBlue (fom TSI Center for Diagnostic products Milford MA) or by chemiluminescence using an ECL detection kit (Amersham International plc) as indicated, according to the manufacturers instructions.

2.4. Enrichment of the δ-subunit antiserum

Antibodies specific for δ-subunit were partially purified from crude antiserum for use in Western blot analysis.

Antibodies generated against proteins derived from the bacterial expression system were preabsorbed by incubating 0.5ml of crude antiserum for 1 h at room temperature with 10 mg of crude membranes prepared from the bacteria used for expression, BL21DE3(lysS), as described in reference 15. After centrifugation at 2000 × g for 20 min the supernatant was removed and exchanged into Tris buffered saline, TBS (50 mM Tris-HCl, 120 mM NaCl, pH 7.4 at 4°C) by gel filtration. The purified material contained 27–32% of the IgG present in the crude antiserum.

3. Results

3.1. Specificity of the δ-antiserum

The expressed fragment of the δ-subunit had an apparent molecular size after SDS/PAGE of 13.2 kDa in reasonable agreement with a predicted size of 11,973 derived from the published sequence (Shivers et al., 1989), as shown in Fig. 1A. Antiserum raised against the expressed cytoplasmic loop region of the δ-subunit was tested for specificity against other subunits of the $GABA_A$ receptor by Western blot analysis. As shown in Fig. 1B, the crude δ-antiserum was able to detect the cytoplasmic loop fragment from the δ-subunit, bacterially expressed in *E. coli*, at an antibody dilution of 1:20000. The amino acid sequence of the predicted cytoplasmic loop region of the δ-subunit shows no significant homology with the same region in other subunits. It is most similar to the γ-subunit family, although there are no regions of more than two identical residues making cross-reactivity with the γ-subunits or the more distant α-and β-subunits very unlikely. The δ-specific antiserum did not detect any signal in uninduced cells, nor in cells expressing γ_1, γ_2 or γ_3 putative cytoplasmic loop polypeptides (Fig. 1b). The antiserum is therefore specific and of high titre.

3.2. Immunoprecipitation of δ-containing receptors

The antiserum raised against the putative intracellular loop of the δ-subunit was tested for its ability to immunoprecipitate $GABA_A$ receptors from rat brain. Crude antiserum (up to 60 μl) was coupled to protein-A Sepharose (60 μl) and incubated with receptor solubilised from rat cerebellar membranes (0.4 ml). Cerebellum was used because of the high abundance of the δ-subunit in this tissue (Laurie et al., 1992; Benke et al., 1991). Under these conditions the capacity of protein A for IgG was not exceeded and the limiting factor in the incubation was the amount of solubilised receptor (approximately 1 pmol of [^{3}H]muscimol binding sites). This allowed determination of the maximal amount of receptor which could be immunoprecipitated by the antiserum. As shown in Fig. 2, solubilised receptor (equivalent to 3–4000 dpm of [^{3}H]muscimol binding sites) was maximally immunoprecipitated by 30 μl of antiserum. In control experiments where equivalent volumes of hyperimmune antiserum, raised against an irrelevant antigen, were used up to 300 dpm of [^{3}H]muscimol binding sites could be non-specifically immunoprecipitated. The δ-antiserum (30 μl) maximally immunoprecipitated 3–4 pmol of [^{3}H]muscimol binding sites giving a titre of 1×10^{-7} mol of [^{3}H]musci-

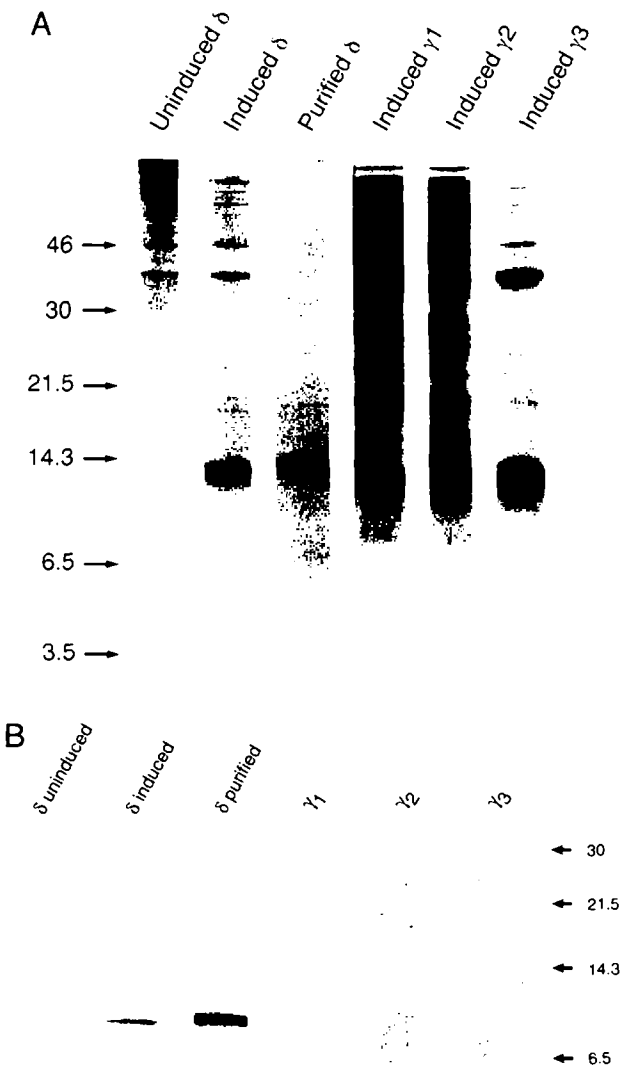

Fig. 1. Expression of the predicted cytoplasmic loop fragment of the δ-subunit in *E. coli*. A) cDNA encoding the cytoplasmic loop fragment of the δ-subunit (Asn^{318}-Leu^{400}) subcloned into the vector pRSET5a was transformed into *E. coli* (BL21DE3(LysS)) and induced with IPTG for 3 h. Bacterial lysates prepared before and after induction are shown in lanes 1 and 2 respectively. The expressed δ-subunit fragment, present at 13.2KDa in lane 2, was purified by preparative SDS/PAGE, lane 3, for use as an antigen. Lanes 4, 5 and 6 contained analogous cytoplasmic loop fragments for γ1(His^{320}-Tyr^{369}), γ_2(Leu^{317}-Tyr^{367}) and γ_3(Asn^{321}-Val^{388}) respectively, expressed in the same system. Proteins were separated on a 18% tricine gel and stained with Coomassie. Each lane contained 10–20 μg of protein, with the exception of the purified δ-subunit, lane 3, which contained 1 μg of protein. B) Proteins from a parallel gel were transferred onto nitrocellulose and probed with crude δ-antiserum at a dilution of 1:20000. Bands were visualised using the ECL detection system. Lanes on the gel were the same as in A.

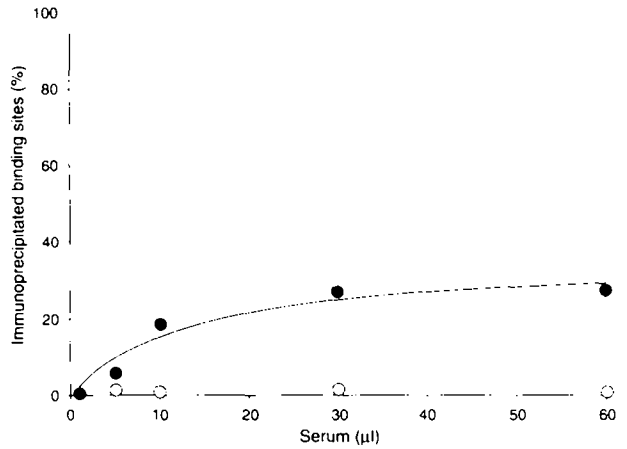

Fig. 2. Immunoprecipitation of $GABA_A$ receptors by δ-subunit specific antiserum. Protein A-Sepharose was incubated for 1 h with antiserum, washed extensively and then incubated overnight with $GABA_A$ receptors solubilised from rat cerebellar membranes, as described in Materials and methods. Binding of [^{3}H]muscimol (closed circles) and [^{3}H]Ro 15-1788 (open circles) was carried out to immunoprecipitated receptor. Data shown are the mean ± S.E.M. of three independent experiments, where no error bars are visible these lie within the symbols.

mol binding sites per litre of serum. Although receptors containing the δ-subunit represented 27% of receptors in the cerebellum, they were less abundant in whole brain. Receptors containing δ-subunits comprised 10.7 ± 2% of [^{3}H]muscimol binding sites in receptor preparations from whole rat brain. This represents a smaller proportion of $GABA_A$ receptors than were immunoprecipitated with a δ-antiserum raised against the N-terminal 17 amino acids by Benke et al. (1991).

When receptors were immmunoprecipitated from preparations solubilised from individual brain regions including cerebellum, hippocampus, striatum and cortex, the richest source of δ-subunit containing $GABA_A$ receptors was the cerebellum from which 27.6 ± 5.3% (n = 3) of [^{3}H]muscimol binding sites could be maximally immunoprecipitated (Fig. 2). Significant amounts of receptor could also be immunoprecipitated from cerebral cortex (8.3 ± 0.4%, n = 3), striatum (14.1 ± 1.8%, n = 3) and hippocampus (4.4 ± 0.8%, n = 3). This distribution is largely consistent with the observed distribution of mRNA by in situ hybridisation where the strongest signal is observed in the cerebellar granule cells with weaker signals in the hippocampus and striatum (Laurie et al., 1992; Benke et al., 1991; Shivers et al., 1989).

Although immunoprecipitation of [^{3}H]muscimol was observed in regions in which the δ-subunit has been demonstrated to be present, in no case could receptors immunoprecipitated with the δ-specific antiserum be labelled with a radioligand at the benzodiazepine binding site. As shown in Fig. 2, increasing the amount of antisera present did not allow immunoprecipitation of specific [^{3}H]Ro 15-1788 binding sites. [^{3}H]Ro 15-4513, [^{3}H]Ro5-4864 and [^{3}H]zolpidem were similarly unable to label $GABA_A$ receptors immunoprecipitated with antibodies against the δ-subunit. This is in contrast to results obtained by Mertens et al (1993) where binding of [^{3}H]Ro15-1788 was observed in receptors immunoprecipitated with antibodies raised against the N-terminal sequence of the δ-subunit (see discussion).

3.3. Identification of the δ-subunit in rat brain

The ability of the δ-specific antisera to detect the subunit from various regions of the rat brain by Western blot was also investigated. In preliminary experiments, the crude antiserum was employed. At dilutions of up to 1:250 it was barely able to detect a specific band in any brain region and additionally identified a non-specific band with a molecular size of approximately 80 kDa. However, as shown in Fig. 3, when antibodies, purified as detailed in the methods, were used it was possible to detect a specific band at 55 kDa in many brain areas. Additionally, the non-specific band at 80 kDa was not present in most brain regions. The quality of the Western blot was greatly improved although an additional band was detected at approximately 40 kDa in the hippocampus lane. This was not always present and may be a proteolytic fragment. The δ-subunit was present in the cerebellum, cortex and hippocampus. It was weakly detectable in striatum but not in liver. It was also not detected in the spinal cord or adrenal medulla (data not shown).

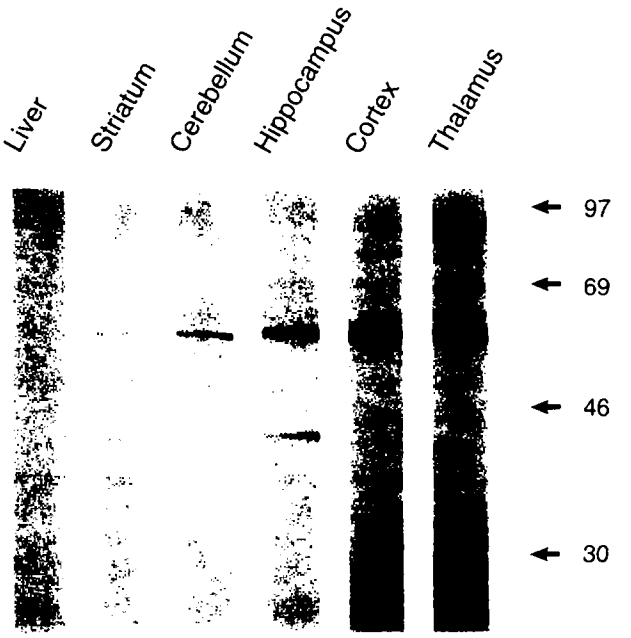

Fig. 3. Identification of the δ-subunit in different brain regions. Membranes (P2 pellet) were prepared from various brain regions or liver (as indicated). 30 μg of protein was applied to each lane and the resulting Western Blot was probed with purified δ-antiserum (30μg/ml). Bands were visualised using the ECL detection system.

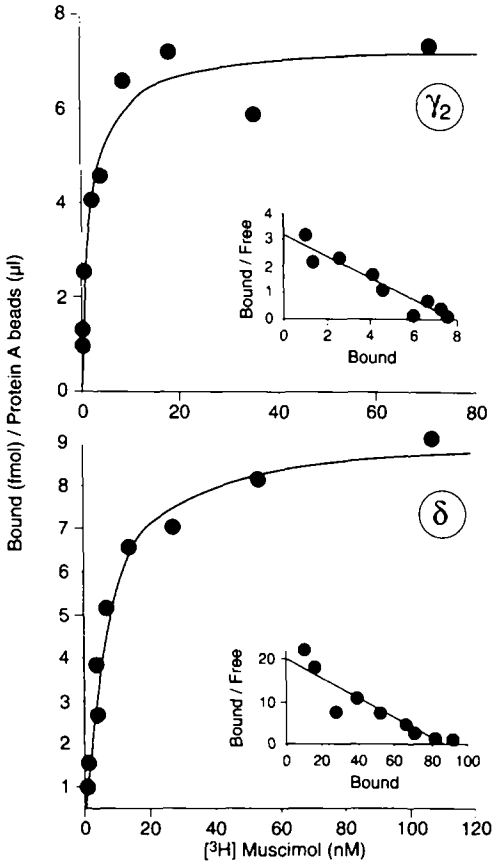

Fig. 4. Binding of [^{3}H]muscimol to the GABA site of receptors immunoprecipitated with antisera specific for the δ- or γ_2-subunits. $GABA_A$ receptors were immunoprecipitated from 3 ml of solubilised rat cerebellar membranes with 100 µl of crude antiserum immobilised on 100 µl of protein A-Sepharose. Saturation analysis and Scatchard plots were best fitted using ENZfit. Data shown are from a single experiment and are representative of four similar experiments.

3.4. Pharmacology of the GABA binding site of cerebellar δ-containing receptors

It was not possible to compare the pharmacology of the benzodiazepine binding site of δ-containing receptors with other better characterised subtypes because benzodiazepine site radioligands failed to bind this receptor subtype with high affinity. However it was possible to compare the pharmacology of the GABA binding site. The GABA site of δ-containing receptors was compared with that of receptors which contain a γ_2-subunit. Receptors were immunoprecipitated from solubilised cerebellar membranes with antisera specific for either the δ or γ_2-subunit. This region was chosen because detailed analysis of the $GABA_A$ receptor populations in this region predict that only one population of δ-containing receptors ($\alpha_6 \beta_n \delta$) exist (Quirk et al., 1994a).

[^{3}H]Muscimol binding to both γ- and δ- containing populations was saturable and of high affinity (Fig. 4). In paired experiments, receptors containing a δ-subunit had a significantly higher affinity for [^{3}H]muscimol (K_d for γ_2-containing receptors = 5.9 ± 0.8nM, $n = 4$; K_d for δ-containing receptors = 1.17 ± 0.25, $n = 4$). It was noted that there was some variation in the affinity of [^{3}H]muscimol for immunoprecipitated receptors. This may have been due either to residual GABA in individual preparations or, alternatively, variation in the stability or purity of different batches of radioligand. This was also reflected in the two fold higher affinity of unlabelled muscimol for GABA binding sites compared with [^{3}H]muscimol. The affinities of a series of compounds at the GABA site is presented in Table 1. Muscimol showed a higher affinity for the GABA site on δ-containing receptors and SR-95531 showed a small, but statistically significant, reduction in affinity for δ-containing receptors.

3.5. Are there $GABA_A$ receptors which contain both a δ- and a γ-subunit?

The possible presence of both γ and δ-subunits in a single receptor macromolecule was tested by purifying receptors from solubilised whole rat brain membranes by immunopurification on γ_1, γ_2, γ_3 and δ-immunoaffinity resins. After eluting the receptors, separating the subunits by SDS/PAGE and transferring to nitrocellulose, the Western blot was probed with IgG purified from the δ-specific antiserum (30 µg/ml). As shown in Fig. 5, there was a strong signal in the receptor preparation immunopurified on the δ-immunoaffinity resin. The polypeptide recognised had the same apparent molecular weight as observed in Fig. 3, although after the purification experiments the δ-subunit band was broader. No signal was observed in any other lane suggesting that the δ-subunit does not exist in the same macromolecular species as any of the γ-subunits to any great extent. This is consistent with the findings reported herein that receptors which contain a δ-subunit do not also have a high affinity binding site for benzodiazepines, since the presence of a γ-subunit has been observed to be obligatory to confer this property (Pritchett et al., 1989; Luddens et al., 1994; Herb et al. 1992; Knoflach et al., 1991). It is also consistent with experiments in which receptor was maximally immuno-

Table 1
A comparison of the pharmacology of the GABA site of receptors from cerebellum containing γ_2 or δ-subunits. Receptors were labelled with [^{3}H]muscimol (2 nM) and competition curves were carried out for each compound. K_i values were calculated from the IC_{50} values according to the Cheng-Prussof equation. Data shown are the mean ± S.E.M. of 3-5 independent determinations.

Compound	K_i (nM)	
	γ_2	δ
Muscimol	2.9 ± 1.06	0.49 ± 0.07
GABA	13.5 ± 1.1	9.1 ± 3
SR 95531	0.49 ± 0.08	1.28 ± 0.18
THIP	13.7 ± 1.0	24.1 ± 7
P-4S	106.8 ± 22.6	103.0 ± 1.5

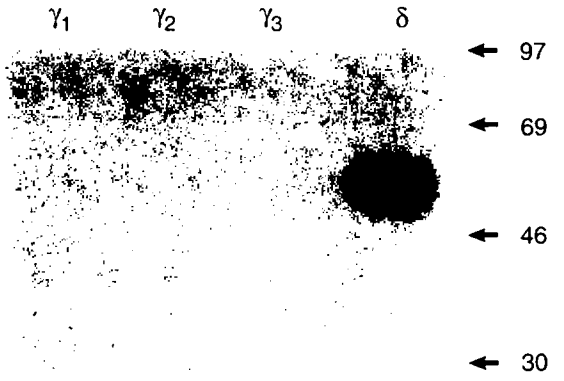

Fig. 5. The δ-subunit is not detected in receptors immunopurified on γ_1, γ_2 or γ_3 immunoaffinity resins. $GABA_A$ receptors solubilised from rat brain membranes were applied to columns coupled to the γ_1(His^{320}-Tyr^{369}), γ_2(Leu^{317}–Met^{403}), γ_3(Asn^{321}-Val^{388}) or δ-antibodies. After extensive washing receptor was eluted as described in Methods and 0.2 pmol of receptor ([^{3}H]muscimol binding sites) was applied per lane. Following separation of the subunits by SDS/PAGE and transfer to nitrocellulose the blot was probed with δ-specific antibodies purified as in methods at 30 μg/ml. Bands were visualised using the ECL detection system. Molecular size markers are shown to the right of the figure.

precipitated from cerebellum with the δ-subunit either alone, or in combination with the γ-subunits used here, where the δ and γ-subunits were concluded to reside on separate receptor molecules (Quirk et al, 1994a). The data presented here are in contradiction with the work of Mertens et al., (1993) who have proposed that the δ-subunit exists in combination with γ_2-subunit.

4. Discussion

We report here the generation of antisera with high titre and specificity for the δ-subunit of the $GABA_A$ receptor. As with other subunits for the $GABA_A$ receptor, the approach of immunising with a long sequence of amino acids derived from the intracellular loop has proved successful for the δ-subunit in addition to the α-and γ-subunits. The antiserum produced was able to immunoprecipitate [^{3}H]muscimol binding sites from rat brain and the δ-specific antibodies detected the subunit by Western blot both in crude rat brain membranes and in receptors immunopurified using the δ-specific antiserum. In the results reported here there is internal consistency between the different types of experiments.

The lack of benzodiazepine binding is entirely consistent with the δ-subunit not being detectable by Western Blot in the same receptor complex as the γ_2-subunit and also with other studies in which a population of cerebellar receptors containing both an α_6-and a δ-subunit have been proposed which do not posses a binding site for benzodiazepine ligands (Quirk et al., 1994a).

Both the α_6 and δ-subunits are expressed abundantly in cerebellar granule cells (Laurie et al., 1992; Bovolin et al., 1992), and therefore immunopurified $GABA_A$ receptors containing both these subunits originate from this region. It is likely that more than one population of $GABA_A$ receptors exist in cerebellar granule cells, since message for α_1, β_2, β_3 and γ_2 are also present (Laurie et al., 1992). Taking together information from the distribution of mRNA (Laurie et al., 1992; Bovolin et al., 1992), the pharmacological characteristics of cerebellar $GABA_A$ receptor populations (Quirk et al., 1994a) and the predominant pairing of the δ-subunit with α_6, but not α_1 or γ_2 (Quirk et al., 1994b) it is conceivable that cerebellar granule cells contain three types of $GABA_A$ receptors, $\alpha_6\beta_n\delta$, $\alpha_6\beta_n\gamma_2$ and $\alpha_1\beta_n\gamma_2$ (where $\beta_n = \beta_2$ or β_3), or as suggested by Laurie et al. (1992) the cerebellar granule cells are a heterogeneous pool expressing different receptor populations. There is circumstantial evidence that the α_4 and δ-subunits also co-exist in a single $GABA_A$ receptor population (Wisden et al., 1992). Unfortunately, the limitations of the antiserum raised against the α_4-subunit (data not shown) and low abundance of the protein in the brain prevented the possible pairing of α_4 and δ-subunits from being investigated directly.

A comparison of the GABA binding site of the γ_2 and δ-containing receptors from the cerebellum revealed some differences in [^{3}H]muscimol binding. The higher affinity for δ-containing receptors is consistent with previous observations of high affinity [^{3}H]muscimol binding sites in the cerebellum and thalamus where the density of benzodiazepine binding sites is low. (Bureau and Olsen, 1993; Leeb-Lundberg and Olsen, 1983). Given the localisation of δ-subunits in these two regions and the lack of benzodiazepine binding of δ-containing receptors it is likely that δ-containing receptors contribute significantly to this population of receptors which have high affinity for muscimol and poor benzodiazepine binding.

It is not possible to conclude which subunit of the receptor is responsible for the observed heterogeneity in the GABA binding site since there is conflicting evidence as to which subunit(s) form this site. It has been proposed that the principal site for binding of muscimol is the β-subunit (Bureau and Olsen, 1993; Macdonald and Olsen, 1994), whereas mutation in the α-subunit has been shown to modify the GABA affinity (Sigel et al., 1992) and in photoaffinity labelling studies [^{3}H]muscimol is irreversibly incorporated into the α-subunit (Smith and Olsen, 1994). Further experiments investigating the pharmacology of the GABA site in receptors transfected with receptors varying in the α, β or γ/δ subunit present will be required to clarify this issue.

With respect to the benzodiazepine sensitivity of δ-containing receptors, our findings are in accord with Shivers et al. (1989) and Laurie et al. (1992), in that receptors containing this subunit fail to bind benzodiazepines. The antisera raised here immunoprecipitate a smaller proportion of the $GABA_A$ receptors solubilised from whole rat brain compared with that of Mertens et al. (1993) employ-

ing antibodies directed against the N-terminal region of the subunit (10.7 ± 2% of all [^{3}H]muscimol binding sites and 0% of [^{3}H]Ro 15-1788 binding sites in this study versus 21% of [^{3}H]Ro 15-1788 binding sites immunoprecipitated by the N-terminal antibodies). Nevertheless, the distribution and size of the δ-subunit by Western blot in both studies is consistent and it is likely that both antisera are labelling the same protein. Since fewer receptors are immunoprecipitated in this study and these do not bind [^{3}H]Ro 15-1788 it is possible that, although both antisera recognise the same population of GABA receptors (i.e. those that only bind [^{3}H]muscimol), the N-terminal antisera immunoprecipitates (Benke et al., 1991) and immunopurifies (Mertens et al., 1993) an additional population of $GABA_A$ receptors which binds benzodiazepines. Such a conclusion is supported by the internal consistency of the immunopurification experiments in both studies. Mertens et al. (1993) report that receptors immunopurified on an immunoaffinity resin bearing an antibody raised against the N-terminal 17 amino acids of the δ-subunit have a high affinity benzodiazepine binding site, albeit with an atypical pharmacology. Consistently, they detect the γ_2-subunit in their immunopurified preparation. Similarly, neither the γ_2-subunit, nor specific [^{3}H]Ro 15-1788 binding sites are detected in the studies described herein. There are several possible explanations. Firstly, the cytoplasmic loop antisera described here may not detect populations of receptors which contain both a γ_2 and δ-subunit, perhaps because the epitopes are not accessible in receptors with this subunit combination. Secondly, receptors immunoprecipitated by the N-terminal antibody could be non-specifically bound to the antisera, allowing non-specific immunopurification of $GABA_A$ receptor populations which do not contain a δ-subunit. This would result in the purification of receptors with a complex benzodiazepine pharmacology since they may well contain more than one α-subunit and therefore would also have low Hill slopes. The benzodiazepine site pharmacology of receptors which contain both a γ_2 and δ-subunit has yet to be established and expression of these subunits together in a recombinant system and a detailed characterisation of their pharmacology would be critical in resolving these issues.

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