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Diazepam-insensitive $GABA_A$ receptors in rat cerebellum and thalamus

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Abstract

Three major populations of $GABA_A$ receptor binding sites are present in cerebellar membranes: diazepam-sensitive [^{3}H]Ro15-4513 binding sites, diazepam-insensitive [^{3}H]Ro15-4513 binding sites and high-affinity [^{3}H]muscimol binding sites. All three populations contain a β subunit as shown by immunoprecipitation with antibodies that recognize all β subunits. The β3 subtype of β subunit is contained in all three populations, but only a similar low fraction (< 20%) in each. Thus, the majority contain β subunits other than β3 (β2 and β1) and β3 subunits are not selectively associated with nor lacking in any of the three binding populations. Antibodies to the γ2 subunit precipitated similar fractions of [^{3}H]Ro15-4513, [^{3}H]flunitrazepam and [^{3}H]muscimol binding sites, showing that γ2 subunits are present in high-affinity muscimol binding isoforms, as well as a significant fraction of the diazepam-insensitive [^{3}H]Ro15-4513 binding sites. Under conditions that identify the 56 kDa α6 subunit on SDS-PAGE as the diazepam-insensitive site of [^{3}H]Ro15-4513 binding in cerebellum, no polypeptide showing diazepam-insensitive binding of [^{3}H]Ro15-4513 could be photoaffinity-labeled in rat thalamus. These results suggest that α4 subunits in the thalamus participate primarily in subunit combinations which bind muscimol but not any benzodiazepine site ligands.

Keywords: $GABA_A$ receptor; $GABA_A$ receptor subunit; Heterogeneity; Cerebellum; Thalamus; Diazepam-insensitive

1. Introduction

$GABA_A$ receptors are ligand-gated chloride channels which are thought to have pentameric structures composed of different polypeptide subunits. Five major groups of subunits (α, β, γ, δ and ρ) were identified as constituent polypeptides for $GABA_A$ receptors, with sequence variants and also splice variants for each group (Olsen and Tobin, 1990; Burt and Kamatchi, 1991; Dunn et al., 1994; Stephenson, 1995). Clinically important drugs, such as benzodiazepines, barbiturates, anesthetic/neuroactive steroids, volatile anesthetics and, possibly, alcohol, have been shown to exert their physiological effects via modulation of $GABA_A$ receptors function (Olsen et al., 1991). The selectivity and also the degree of modulation of $GABA_A$ receptors by these agents are believed to be determined by different assemblies of the subunits (DeLorey and Olsen, 1992; Macdonald and Olsen, 1994; Stephenson, 1995; Lüddens et al., 1995). Anatomical selectivity of expression of these subunits suggests possible subunit combinations that occur in the brain and, more importantly, a significant functional diversity of the $GABA_A$ receptors.

In the cerebellum, the GABAergic neuronal pathways have been well characterized, due to relatively simple anatomical structures (Eccles et al., 1967). In situ hybridization studies have shown that α1, α6, β2, β3, γ2 and δ subunits are major $GABA_A$ receptors subunit variants expressed in the cerebellum, in a cell type-specific manner (Laurie et al., 1992; Persohn et al., 1992; Zdilar et al., 1992); different cells in the cerebellum show functional $GABA_A$ receptors with different pharmacological properties (Puia et al., 1994; Zempel and Steinbach, 1995). Three different pharmacological populations of $GABA_A$ receptors, of which the subunit components were partially matched, have been identified by radioligand binding as-

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says and photoaffinity labeling. These are benzodiazepine binding sites (commonly identified with [^{3}H]flunitrazepam binding or diazepam-sensitive [^{3}H]Ro15-4513 binding sites) and binding sites for [^{3}H]Ro15-4513 that are insensitive to diazepam. There are also high-affinity muscimol binding sites which are not coupled to either the diazepam-sensitive or -insensitive Ro15-4513 binding sites (Unnerstall et al., 1981; Sieghart et al., 1987; Olsen et al., 1990; Turner et al., 1991; Uusi-Oukari, 1992; Quirk et al., 1994; Khan et al., 1994). Diazepam-insensitive binding sites are defined as [^{3}H]Ro15-4513 binding, which cannot be displaced by 10 μM diazepam, and they appear to be insensitive to most benzodiazepine agonists (Turner et al., 1991; Korpi et al., 1992; Wong and Skolnick, 1992). Diazepam-insensitive Ro15-4513 binding sites are unique in that they are almost exclusively expressed in the cerebellar granule cell layer, with low levels in hippocampus, neostriatum, cerebral cortex and thalamus; a somewhat similar regional distribution is found for high-affinity muscimol binding sites that lack both diazepam-sensitive and -insensitive Ro15-4513 binding sites: cerebellum (granule cell layer) > thalamic nuclei > ≫ forebrain (Unnerstall et al., 1981; Sieghart et al., 1987; Olsen et al., 1990; Lüddens et al., 1990; Turner et al., 1991).

Although the physiological significance of these binding populations has not been directly established, some behavioral and biochemical evidence suggests that diazepam-insensitive Ro15-4513 binding sites might be related to the genetic susceptibility and intoxication effects of alcohol (Suzdak et al., 1986; Lüddens et al., 1990; Korpi et al., 1993). These sites have also been suggested to show not only unique benzodiazepine agonist selectivity but also possibly differential coupling or functional properties (Sieghart et al., 1987; Turner et al., 1991; Korpi et al., 1992).

In vitro heterologous expression with subunit combinations of α6β2γ2 reproduced the diazepam-insensitive Ro15-4513 binding property (Lüddens et al., 1990) and α6β3γ2 and α6β3δ isoforms can produce diazepam-insensitive and zinc-sensitive $GABA_A$ receptor channels as observed in cerebellum by electrophysiology (Saxena and Macdonald, 1996). Further, using subunit-specific antibodies, α6 and γ2 were verified as constituents of receptor protein isoforms in the cerebellum that exhibit diazepam-insensitive Ro15-4513 binding (Carlson and Olsen, 1993; Quirk et al., 1994; Khan et al., 1994). Further studies using subunit-specific antibodies demonstrated that α6δ and α2γ1 subunit combinations, probably also including β, are associated with high-affinity muscimol binding sites without benzodiazepine binding properties (Quirk et al., 1994).

In the cerebellum, mRNAs for all three β subunit variants are expressed, with abundance β2 > β3 > β1 (Laurie et al., 1992; Wisden et al., 1992; Persohn et al., 1992; Tyndale, Tobin and Olsen, unpublished). Immunoreactivity for β2 is high in cerebellum (Endo and Olsen, 1992; Machu et al., 1993). Even though the temporal and spatial specificity of the expression of these subunits implies the importance of β subunits as functional components of the $GABA_A$ receptors, functional and pharmacological signatures of these subunits have not been as well characterized as other subunit variants (Hadingham et al., 1993; Lüddens et al., 1994; Wafford et al., 1994). The α4 subunit mRNA is most abundantly expressed in the thalamus (Wisden et al., 1991, 1992). Recombinant a4β2γ2 combinations, like α6, have been shown to form $GABA_A$ receptors with diazepam-insensitive Ro15-4513 binding properties (Wieland and Lüddens, 1994) and these binding sites are detected by autoradiography in brain regions, such as thalamus, that express α4 (Turner et al., 1991). While the α4 subunit is probably a constitutent of benzodiazepine-insensitive $GABA_A$ receptors, either those binding [^{3}H]Ro15-4513 in a diazepam-insensitive manner, or not binding any benzodiazepines, correlation between the presence of $GABA_A$ receptors proteins that include the α4 subunit and these binding sites has not been made, in thalamus or elsewhere. Neither has the δ subunit polypeptide been identified in receptor proteins seen in thalamus evidencing high-affinity muscimol binding sites, even though δ mRNA is highly expressed (Wisden et al., 1991). In the present study, using β and γ subunit-specific antibodies, we made attempts to match specific subunit combinations, represented by their β subunit variants or the presence of γ2 subunits, with known pharmacological binding subtypes expressed in the cerebellum. Also, attempts to determine whether α4 subunits are components of thalamic diazepam-insensitive binding sites for [^{3}H]Ro15-4513 were made by photolabeling.

2. Materials and methods

2.1. Materials

[Methyl-^{3}H]Flunitrazepam (84.3 Ci/mmol), [7,9-^{3}H]Ro15-4513 (24.1 Ci/mmol) and [methylene-^{3}H-(*N*)]muscimol (20 Ci/mmol) were from DuPont-New England Nuclear (Boston, MA, USA). GABA and protein A Sepharose CL-4B beads were from Sigma (St. Louis, MO, USA). Ro15-4513 and diazepam were the kind gifts of Hoffmann-LaRoche (Nutley, NJ, USA). Triton X-100 was from Boehringer Mannheim (Indianapolis, IN, USA). Centriprep 30-Concentrator was from Amicon (Amicon, MA, USA). Polyvinylidene difluoride (PVDF) membranes were from Bio-Rad (Hercules, CA, USA). Biotinylated goat anti-rabbit immunoglobulin G (IgG) was from Chemicon (Temecula, CA, USA). 3,3′-Diaminobenzidine (DAB) was from Vector Laboratories (Burlingame, CA, USA).

2.2. Subunit-specific antibodies

Production and characterization of anti-β3 and βcyto antibodies are described elsewhere (Endo and Olsen, 1992;

DeLorey et al., 1992; Huh et al., 1995). Anti-γ2 antibodies were generated against the synthetic peptide sequence (#365–382) of the human γ2 subunit using methods previously described (Endo and Olsen, 1993). Antisera were purified using synthetic peptide-affinity columns. Some experiments where β3 subunit-specific antibodies were used were carried out only once, in triplicate. Insufficient amount of antisera, obtained from a single and now long deceased rabbit, did not enable us to perform multiple experiments.

2.3. Solubilization and immunoprecipitation

Membrane homogenates of the cerebellum from Sprague-Dawley rats were prepared as previously described (Endo and Olsen, 1992), substituting the following buffer: 20 mM KH_2PO_4/K_2HPO_4, pH 7.4, 50 mM KCl, containing 0.02% NaN_3, 0.5 mM dithiothreitol and various protease inhibitors (1 mM EDTA, 2 mM benzamidine chloride, 0.1 mM benzethonium chloride, 100 μg/l bacitracin, 0.3 mM PMSF, 10 mg/l soybean trypsin inhibitor, 10 mg/l ovomucoid trypsin inhibitor). Prepared membrane homogenates were stored at −70°C until use. Before solubilization, thawed membrane homogenates were washed twice in the same buffer and solubilized in a final detergent concentration of 1% TX-100 in the presence of 1 M KCl by incubating for 1 h at 4°C. Soluble extracts were desalted to a final salt concentration of 250 mM KCl using Centriprep 30-Concentrator.

Aliquots (500 μl) of desalted soluble extracts were added to protein A Sepharose beads to which antibodies were preattached (60 ml packed volume in a total volume 100 ml in 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.4, 150 mM NaCl, 1 mm EDTA, 0.02% NaN_3), followed by overnight incubation at 4°C. Receptor-antibody conjugates were separated by centrifugation and washed four times with 1 ml of 20 mM KH_2PO_4/K_2HPO_4, 0.5% TX-100, 200 mM KCl, 1 mM EDTA, 0.02% NaN_3 (pH 7.4) containing protease inhibitors, followed by resuspension in 600 μl total volume. To attach antibodies to protein A beads, various concentrations of affinity-purified antibodies were incubated with protein A beads (60 μl beads volume) for 4–6 h at 4°C, in a total volume 500 μl of PBS buffer (10 mM NaH_2PO_4/Na_2HPO_4, pH 7.4, 150 mM NaCl, 0.02% NaN_3). Total IgG concentrations were kept constant among different batches of various concentrations of antibodies by supplementing rabbit γ globulins. After incubation, protein A-bound antibodies were separated by a brief centrifugation and used for the immunoprecipitation.

2.4. Radioligand binding assays

For radioligand binding assays on immunoprecipitated fractions, 90-μl aliquots of the supernatant or the pellet suspension were incubated with appropriate ligands in a final volume 500 μl in 20 mM KH_2PO_4/K_2HPO_4, 100 mM KCl, 0.15% Triton X-100, 1 mM EDTA, 0.02% NaN_3. Diazepam-insensitive binding was determined with 30 nM [^{3}H]Ro15-4513 in the presence of 10 μM diazepam. [^{3}H]Flunitrazepam (20 nM) was used for the measurement of diazepam-sensitive binding. [^{3}H]Muscimol (25 nM) was used for determination of muscimol binding. Non-specific binding was determined by including 10 μM Ro15-4513 for diazepam-insensitive, 10 μM diazepam for diazepam-sensitive binding and 100 μM GABA for muscimol binding. After incubation for 30 min ([^{3}H]muscimol) or 90 min ([^{3}H]flunitrazepam and [^{3}H]Ro15-4513) at 4°C, incubation was terminated by adding 100 μl bovine γ globulin and 300 μl of polyethylene glycol. Free ligands were separated by centrifugation at 27 500 × g for 30 min for [^{3}H]muscimol binding assays or filtration through GF/B filters for [^{3}H]Ro15-4513 and [^{3}H]flunitrazepam binding assays. Binding assays for the membrane homogenates were performed in the same manner omitting the step of protein precipitation indicated above. Polyethylene glycol was included in the washing buffer at a 10% final concentration for soluble receptors.

2.5. Western blots

Purified $GABA_A$ receptors from bovine cortex, cerebellum and hippocampus were separated by 7.5% polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. Immunostaining was performed as previously described, using biotinylated anti-rabbit IgG, streptavidin-biotinylated horseradish peroxidase complex and 3,3′-diaminobenzidine as substrates (Endo and Olsen, 1992).

2.6. Photoaffinity labeling

Membrane homogenates of rat thalamus were prepared as described above. Thawed membrane homogenates were washed twice with the following buffer: 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA and resuspended in an appropriate volume. Aliquots of membrane homogenates were incubated with 10 nM [^{3}H]Ro15-4513 in the presence and absence of 10 μM Ro15-4513 or 10 μM diazepam for 90 min at 4°C, in a 500 μl total volume (final protein concentration of 300 μg/ml). Samples were irradiated on ice for 30 min at 366 nm wavelength (3.5 cm distance, Mineralight UVGL-25) for three 10-min periods with 5-min intervals. Irradiated membranes were washed three times with 10 mM Tris-HCl (pH 6.8) and resuspended in 200 μl of SDS sample buffer for gel electrophoresis as previously described (Sieghart et al., 1987; Turner et al., 1991). These conditions of photoincorporation were the most stringent (wavelength and time of ultraviolet exposure, protein and ligand concentration) of several tested, optimized for incorporation into diazepam-

insensitive and -sensitive sites (Sieghart et al., 1987; Stauber et al., 1987; Turner et al., 1991).

3. Results

3.1. Studies with antibodies to β subunits

The diazepam-insensitive component of [^{3}H]Ro15-4513 binding in rat cerebellum accounted for about 20% of the total Ro15-4513 binding activity in membrane homogenates and in Triton X-100 soluble extracts. Both diazepam-sensitive and -insensitive binding sites were readily solubilized using 1% Triton X-100 in the presence of high salt (1 M KCl), with comparable degrees of solubilization yields (70–80%) obtained for both.

Antibodies specific for the $GABA_A$ receptor β3 subunit immunoprecipitated 18 ± 4 and 15% of diazepam-insensitive and -sensitive Ro15-4513 binding sites in cerebellar extracts, respectively (Fig. 1). On the other hand, 65 ± 1.5 and 71 ± 0.8% of diazepam-insensitive and -sensitive binding sites were precipitated by anti-βcyto antibodies (Fig. 1) which have been suggested to recognize all $GABA_A$ receptors β subunit variants (Endo and Olsen, 1992).

The possibility that low precipitation of benzodiazepine binding sites by β3 antibodies might be due to preferential

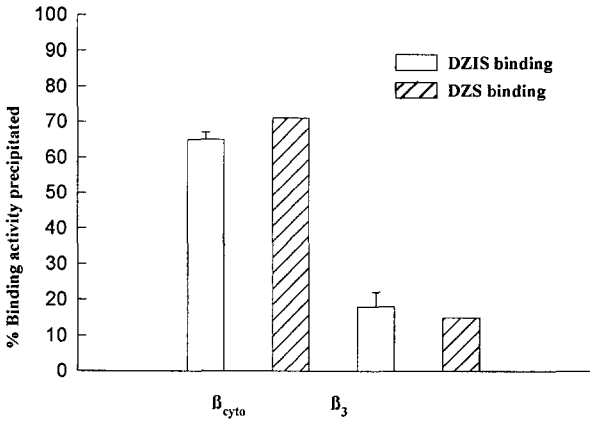

Fig. 1. Immunoprecipitation of benzodiazepine binding activity in soluble extracts of rat cerebellum by β subunit-specific antibodies. Diazepam-sensitive binding was assayed with [^{3}H]flunitrazepam, defining 'non-specific' binding with 10 μM diazepam. Diazepam-insensitive binding was assayed with [^{3}H]Ro15-4513 in the presence of 10 μM diazepam, using 10 μM Ro15-4513 to define 'non-specific' background. The % of soluble cerebellum extract binding (mean ± S.E.M.) that could be immunoprecipitated by 100 μg of affinity-purified βcyto or β3 antibodies was measured in triplicate in the supernatant or, in a separate experiment, in the pellet. In the absence of specific antibody attachment, non-specific association of binding with the pellet accounted for ≤ 3%. An example of the raw data is presented to indicate significant immunoprecipitation by anti-β3: in one experiment, the extract (treated with beads lacking antibodies) gave 4378 ± 177 DPM total, 1545 ± 172 non-specific, = 2833 DPM specific; supernatant after immunoprecipitation 3754 ± 144, = specific 2209 DPM, = 78% of control, so 22% precipitated.

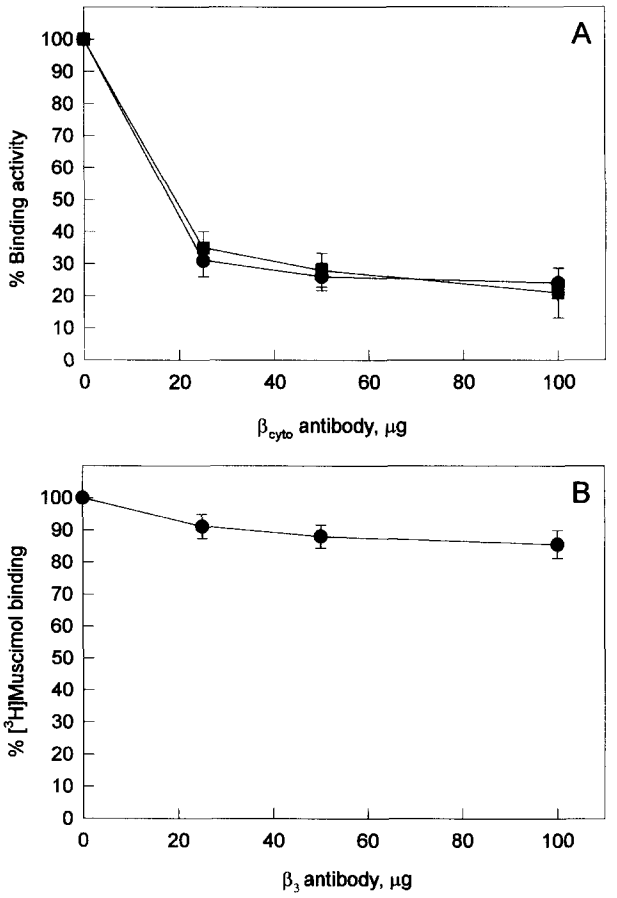

Fig. 2. Quantitative immunoprecipitation of [^{3}H]muscimol and [^{3}H]Ro15-4513 binding activities from soluble extracts of rat cerebellum by varying amounts of βcyto (A) and β3 (B) antibodies. [^{3}H]Muscimol (25 nM) binding (circles) was measured with 10 μM non-radioactive GABA as the blank in the soluble extract (treated with beads lacking antibodies) and in the supernatant fraction after immunoprecipitation. In addition, total specific binding of [^{3}H]Ro15-4513 (with 10 μM Ro15-4513 as blank, so includes both diazepam-sensitive and -insensitive sites) was measured in the soluble extract and in the immunopellets from the βcyto treated samples (Fig. 2A, squares). Each point is the mean of triplicate assays, S.E.M. indicated by the bars. An example of the raw data is presented to indicate significant immunoprecipitation by anti-β3: in 1 experiment, the extract gave 10542 ± 290 DPM total, 3987 ± 219 non-specific, = 6555 specific. The supernatant of the 25 μg antibody sample gave 9962 ± 108, = 5975 specific, 91.2 ± 3.7%. The 50 μg supernatant gave 9830 ± 90, = 5843 specific, 88.1 ± 3.6%. The 100 μg supernatant gave 9596 ± 184, = 5609 specific, 85.6 ± 4.4%.

association of these subunits with the high-affinity muscimol binding population which lacks benzodiazepine binding was tested by paired immunoprecipitation of [^{3}H]muscimol, [^{3}H]Ro15-4513 (total) and [^{3}H]flunitrazepam binding activities. Anti-β3 and anti-βcyto antibodies were able to quantitatively immunoprecipitate [^{3}H]-muscimol binding activity from the soluble extract of the rat cerebellum as shown in Fig. 2. Anti-β3 antibodies did not precipitate [^{3}H]muscimol binding activity in any higher abundance than that of [^{3}H]Ro15-4513 binding, precipitating 9.5 ± 6.9% of [^{3}H]muscimol and 14.4 ± 4.4% [^{3}H]Ro15-4513 binding sites (Fig. 2BFig. 3B). Also, βcyto

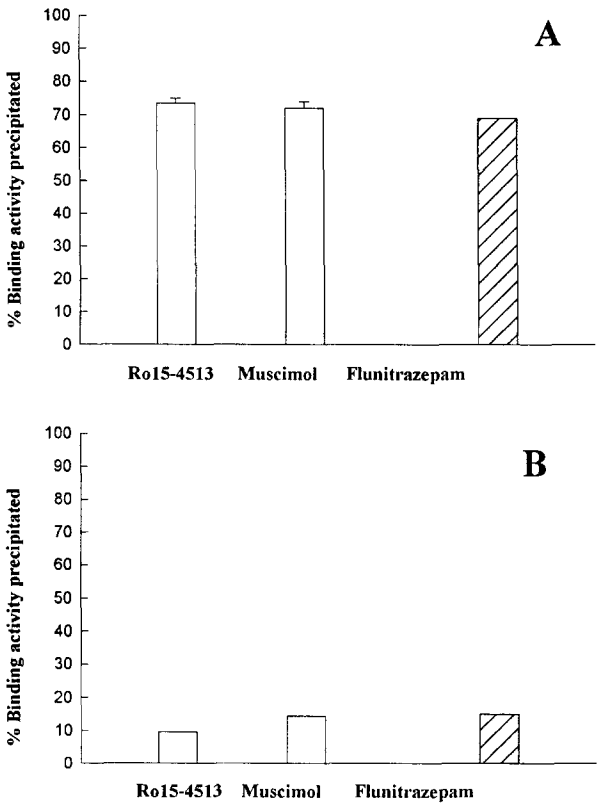

Fig. 3. Immunoprecipitation of muscimol, Ro15-4513 and flunitrazepam binding activities from soluble extracts of rat cerebellum. [^{3}H]Muscimol and total specific [^{3}H]Ro15-4513 binding activities were measured as in Fig. 2 in the supernatant fractions following immunoprecipitation by saturating amounts (100 μg) of βcyto (A) and β3 (B) antibodies; controls were treated with protein A beads only, lacking antibodies. [^{3}H]Flunitrazepam binding was also determined, using the immunopellets. Data are expressed as the mean ± S.E.M. from 2 experiments in triplicate (maximal variance 6.8%). An example of the raw data is presented to indicate similar immunoprecipitation by anti-β3 of muscimol and Ro15-4513 binding. The amount of muscimol binding precipitated is that shown in Fig. 2B, 14.4 ± 4.4%. The amount of Ro15-4513 binding precipitated was 9.5 ± 6.9% (mean of 2 measurements). Total binding in 1 experiment was 8887 ± 331 DPM, background 1424 ± 185, = 7463 specific; supernatant after anti-β3 treatment: 8041 ± 505, = 6617 specific, = 89 ± 7%, 11% precipitated.

antibodies precipitated both binding activities to the same extent, i.e. about 72% (Fig. 2AFig. 3A). In order to conserve antibodies, [^{3}H]flunitrazepam binding was measured in the untreated soluble extracts and immunopellets: 17 and 71% were immunoprecipitated by anti-β3 and βcyto, respectively (Fig. 3). Non-specific precipitation with beads lacking attached antibodies was ≤ 3% (data not shown).

3.2. Studies with antibodies to the γ2 subunit

The γ2 subunit is known to confer benzodiazepine sensitivity of $GABA_A$ receptors and, among the three γ subtypes, the γ2 subunit is the major variant expressed in the cerebellum and, indeed, in most regions (Wisden et al., 1992; Laurie et al., 1992). Quantitative immunoprecipitation indicates about 43% of [^{3}H]muscimol binding sites can be precipitated from the crude solubilizates of the rat cerebellum by anti-γ2, which may not be completely saturated at the high antibody concentration of 100 μg (Fig. 4). In Western blots using benzodiazepine affinity-purified bovine $GABA_A$ receptors, a major diffuse 45–47 kDa polypeptide band and a minor 49 kDa band were stained (Fig. 5) in all three regions. But, with the same amount of applied receptors, the 49 kDa peptide was immunostained with a weaker intensity than the peptide

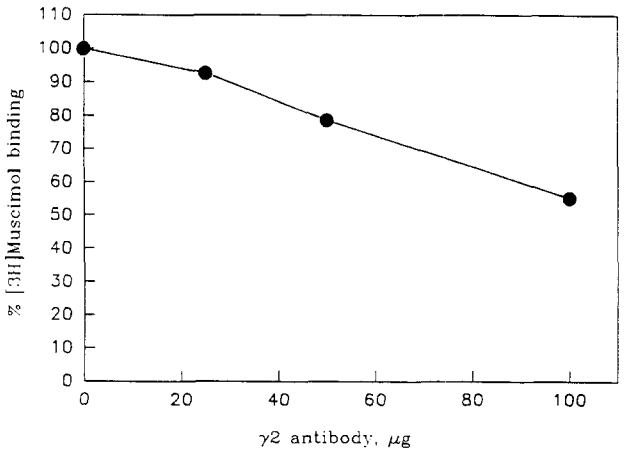

Fig. 4. Quantitative immunoprecipitation of muscimol binding activity in soluble extracts of rat cerebellum by varying amounts of anti-γ2 antibodies. Data are means of triplicate assays with maximal variance of 8.8%.

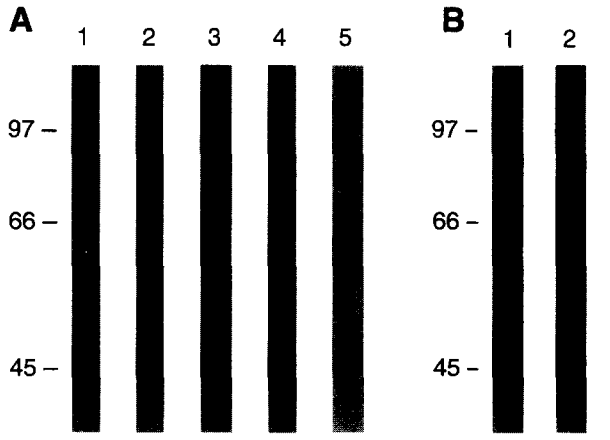

Fig. 5. Western blots of BZ affinity-purified $GABA_A$ receptors with anti-γ2 antibodies. Purified receptors from bovine cerebellum, cortex and hippocampus were subjected to SDS-PAGE and blotted onto polyvinylidene difluoride membranes, followed by immunostaining with γ2 subunit-specific antibodies at 1:200 dilution as described in Materials and methods. The following [^{3}H]muscimol binding activities were loaded on each lane: (A) Lane 1, hippocampus, 1.4 pmol; lane 2, cortex, 1.4 pmol; lane 3, hippocampus, 2.9 pmol; lane 4, cortex, 2.8 pmol. (B) Lane 1, cerebellum, 3.0 pmol; lane 2, cortex, 2.0 pmol. Densitometric analysis showed virtually identical amounts of the 46 kDa band in all regions: A1 vs. A2 and A3 vs. A4; B1 vs. B2, while the 49 kDA band showed about 3-fold lower intensity in hippocampus than cortex, A1 vs. A2 and A3 vs. A4.

with lower molecular weight in the hippocampus, suggesting lower abundance of this antigenic species in this brain region relative to others rather than lower affinity of antibodies to this peptide (measured by semi-quantitative densitometric analysis of bands; Fig. 5).

These γ2 antibodies did not precipitate more benzodiazepine binding activity measured with [^{3}H]Ro15-4513 or [^{3}H]flunitrazepam than that of [^{3}H]muscimol in rat cerebellum (Fig. 6). Even though immunoprecipitation with anti-γ2 antibodies may not be completely saturated with respect to the antibody concentration and thus direct quantitative interpretation of the paired immunoprecipitation might not be reliable, the observation that similar degrees of [^{3}H]muscimol and [^{3}H]flunitrazepam binding sites are precipitated is likely to hold and agrees with a previous study (Duggan et al., 1992).

3.3. Photoaffinity labeling of rat thalamus membranes with [^{3}H]Ro15-4513

Compared to the cerebellum, the abundance of the diazepam-insensitive component of Ro15-4513 binding in the thalamus appears to be low (Sieghart et al., 1987; Turner et al., 1991). Reversible diazepam-insensitive binding accounted for $\leq 5\%$ of the total [^{3}H]Ro15-4513 binding (data not shown). In attempts to identify a polypeptide associated with this diazepam-insensitive binding, we photoaffinity-labeled thalamic membranes with [^{3}H]Ro15-4513, using conditions which were successful in the cerebellum, i.e., the α6 polypeptide at 56 kDa on SDS-PAGE showed incorporation that was not inhibited by diazepam (data not shown). Covalent diazepam-sensitive specific labeling (inhibited by Ro15-1788 and diazepam) was observed in thalamic polypeptides at M_r 51 kDa and at M_r 58–61 kDa on SDS-PAGE. These appear to represent α1 and α3 subunits, respectively (Duggan et al., 1992; Endo and Olsen, 1993; Mertens et al., 1993). No diazepam-insensitive labeling (inhibited by Ro15-1788 but not diazepam) was detected on the gel (Fig. 7), including the region between the M_r range of 50 and 70 kDa where most of the known $GABA_A$ receptors subunits migrate and the 67 kDa region expected for the α4 subunit from Western blotting using α4 subunit-specific antibodies (Kern and Sieghart, 1994; Weng et al., 1994). A minor labeling which was resistant to displacement by diazepam was consistently found at the molecular mass of 55 kDa, but this appears to be non-specific photoincorporation, since it was not blocked by high concentrations of Ro15-1788.

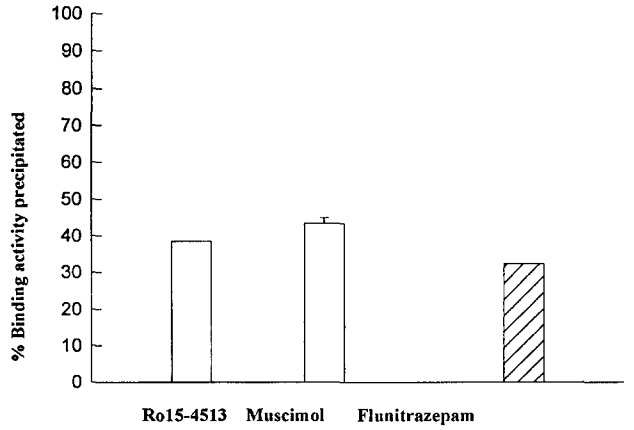

Fig. 6. Immunoprecipitation of muscimol, Ro15-4513 and flunitrazepam binding activities from soluble extracts of rat cerebellum by anti-γ2 antibodies. Binding of [^{3}H]muscimol and total specific [^{3}H]Ro15-4513 binding were measured in the crude extract (supernatant after precipitation with beads lacking antibody) and the supernatant fraction after immunoprecipitation with 100 μg of anti-γ2, and [^{3}H]flunitrazepam binding was measured in the extract and the immunopellets. Data are the mean ± S.E.M. from 2 experiments in triplicate.

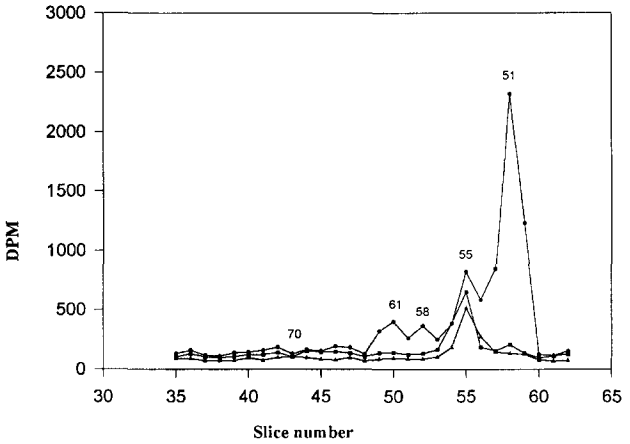

Fig. 7. Photoaffinity labeling of crude membrane homogenates of rat thalamus using [^{3}H]Ro15-4513 binding to diazepam-sensitive (inhibited by 10 μM diazepam) or diazepam-insensitive sites (not inhibited by 10 μM diazepam, but inhibited by 10 μM Ro15-4513). Photolabeling was carried out as described in Materials and methods. Photolabeled samples were run on SDS-PAGE and each lane was cut into 1-mm slices which were measured for radioactivity. Total covalent binding (closed circles); labeling in presence of 10 μM diazepam (open triangles); or 10 μM non-radioactive Ro15-1788 (closed triangles). The apparent molecular masses of the peaks were estimated from a plot of migration distance vs. mass for standard polypeptides run in another lane of the gel.

4. Discussion

In order to relate pharmacological subtypes defined by binding or functional studies in animals, cells, tissue sections, or homogenates, with biochemical isoforms isolated from detergent-extracted tissues, one would ideally like to have 100% solubilization yield, and no effect of detergent solubilization on $GABA_A$ receptor properties. We obtained a solubilization yield for both diazepam-sensitive and insensitive binding sites for Ro15-4513 in rat cerebellum of 75%, similar to our previous results for benzodiazepine and GABA binding (Stephenson and Olsen, 1982). There

is no reason to believe that the sites not recovered have properties different from those recovered, but it is possible. Most GABA or benzodiazepine binding site heterogeneity and allosteric properties are retained in detergent-solubilized preparations, and can be studied using subunit-specific antibody separations (McKernan et al., 1991; Benke et al., 1991; Mertens et al., 1993; Khan et al., 1994; Quirk et al., 1994; Huh et al., 1995). However, some activities, such as barbiturate enhancement of benzodiazepine and GABA binding, as well as TBPS binding, are lost in Triton X-100 but not in 3-[(3-cholamidopropyl)dimethylammonio] propane sulfonate (CHAPS) detergent (Stephenson and Olsen, 1982); functional channels can be reconstituted into membranes more efficiently from CHAPS than from Triton (Dunn et al., 1994). This suggests that the attempts to study native subtypes in detergent-solubilized extracts should be approached with caution.

The current results agree with our previous observation in cortex using βcyto immunoprecipitation, that the majority of benzodiazepine and muscimol binding sites appear to contain β subunits in their oligomeric structures. The present study demonstrates that β3 subunits are constituents of both diazepam-sensitive and insensitive components of Ro15-4513 binding sites in the cerebellum. More importantly, both populations of binding sites, each of which is apparently homogeneous in its pharmacology, are in fact heterogeneous protein populations, being composed of receptor isoforms with different β subunits. The low abundance of β3 subunits present in $GABA_A$ receptors that bind benzodiazepines was not due to the preferential association of β3 subunits with the high-affinity muscimol binding sites which lack benzodiazepine binding (Fig. 3B). Therefore, the majority of $GABA_A$ receptors in the cerebellum appear to have β subunits other than β3 subunits, β2 and β1, and the ratio of β3 to other β subunits is roughly 1 : 3.

The results agree with previous observations. Subunit mRNAs for β2 are abundantly expressed in the cerebellum (Wisden et al., 1992; Laurie et al., 1992; Persohn et al., 1992) and we identified the β2 subunit as a 54 kDa polypeptide band which is predominantly immunostained by both β2-specific antibodies (Machu et al., 1993) and generic β (βcyto) antibodies in the cerebellum (Endo and Olsen, 1992; DeLorey et al., 1992). Furthermore, the β3 subunit is not preferentially associated with or excluded from the three populations of $GABA_A$ receptor binding sites studied, within experimental error of immunoprecipitation. The β3 subunit thus associates equally well with different combinations of other subunits that have greater effects on the pharmacological specificity of the isoforms produced. This is consistent with observations that β3 associates 'promiscuously' with any α subunits (Pollard et al., 1991) and that the nature of the β subunit has little (Hadingham et al., 1993; Lüddens et al., 1994) but not zero (Wafford et al., 1994) effect on pharmacological specificity. A reasonable hypothesis suggests that β subunits differ in sensitivity to phosphorylation control mechanisms (e.g. Browning et al., 1990).

Autoradiographic studies have shown that [^{3}H]muscimol labels the granule layer of the cerebellum much more densely than the molecular layer, which is opposite to the labeling pattern for [^{3}H]flunitrazepam (Unnerstall et al., 1981; Olsen et al., 1990). A portion of these high-affinity muscimol binding sites might be explained by α6 subunit-containing diazepam-insensitive Ro15-4513 binding sites. However, the B_{max} for such sites appears to be too low to explain all the high-affinity muscimol sites. Recently, the subunit compositions of these high-affinity muscimol binding sites were identified and the majority of such binding sites did not bind Ro15-4513: GABAR containing α6,δ or α2,γ1 subunits were demonstrated to be insensitive to [^{3}H]Ro15-4513 binding (Quirk et al., 1994). Presumably, these isoforms can contain a β subunit, but not a γ2. However, our results show a large fraction of high-affinity muscimol binding isoforms contain γ2.

Antibodies to γ2 precipitated similar amounts, but not 100%, of benzodiazepine and GABA binding activities in cerebellum. The presence of γ2 in both diazepam-sensitive and -insensitive Ro15-4513 binding sites is expected from recombinant expression studies (Wieland and Lüddens, 1994), but precipitation of similar degrees of muscimol and benzodiazepine binding activities by γ2 antibodies was not expected (Fig. 6). The results suggest that diazepam-insensitive binding sites for Ro15-4513, primarily isoforms containing α6β2γ2, may contribute to a portion of the high-affinity muscimol binding population. Because the anti-γ2 immunoprecipitations were not saturated, even when precipitation with 100 μg of purified antibodies was repeated, we could not determine whether γ2 was present in some isoforms that bind muscimol but not Ro15-4513, but the high content of γ2 suggest that this might be the case. The evidence for γ2 in most cerebellar muscimol binding isoforms agrees with the study by Duggan et al. (1992) where about 60% of both [^{3}H]muscimol and [^{3}H]flunitrazepam binding sites were precipitated by anti-γ2 antibodies from soluble extracts of bovine cerebellum. The δ subunit may replace γ in many of these muscimol binding isoforms that lack benzodiazepine binding, but this may not be true for all of them. Further, anti-δ antibodies immunoprecipitated some benzodiazepine binding and co-purified some γ2 subunit (Mertens et al., 1993). Recombinant isoforms containing both γ and δ subunits can be expressed and may account for zinc sensitivity seen in some brain $GABA_A$ receptors (Saxena and Macdonald, 1994).

Anti-γ2 antibodies in the present study recognized diffuse polypeptide bands of 44–47 and 49 kDa in SDS-PAGE of the benzodiazepine affinity-purified receptors from bovine cortex, hippocampus and cerebellum (Fig. 5). Similar multiple peptides immunostained by γ2 antibodies were also detected by others (Khan et al., 1994; Duggan et al., 1992; Quirk et al., 1994; Benke et al., 1991). However,

a brain regional difference of these multiple peptides has not been reported previously. The nature of the 49 kDa polypeptide, which is present in lower abundance in the hippocampus than other regions, is not clear. It probably can be interpreted as cross-reactivity of our antiserum with other isoforms, based on high homology of our antigen peptide sequence with corresponding peptide sequences of other γ subunits (Ymer et al., 1990; Knoflach et al., 1991), e.g. 83% with γ1 and 78% sequence identity with γ3 subunits of the rat. The lack of complete immunoprecipitation of binding activity by our anti-γ2 antibodies might be due merely to low-affinity antibodies to the relatively small peptide antigen employed. It could also result from steric hindrance in that this antigen in the cytoplasmic loop of the subunit contains a consensus tyrosine phosphorylation site (Y365). Further studies are needed to identify the exact subunit composition and stoichiometry of γ2-containing $GABA_A$ receptors in cerebellum.

Like the cerebellum, the thalamus contains a high degree of muscimol binding lacking benzodiazepine binding (Olsen et al., 1990), and a high level of expression of the δ subunit, and a benzodiazepine-insensitive subunit, α4 (Wisden et al., 1992). The negative photolabeling results in thalamic membranes presented here suggest that, even though α4 mRNAs are highly expressed in most of the nuclei of the thalamus, either mRNA levels do not reflect protein levels, or most receptors containing α4 subunits do not produce diazepam-insensitive Ro15-4513 binding site subunit combinations, contrary to earlier suggestions (Wisden et al., 1991; Yang et al., 1995). Even if a combination of α4αx were present, it does not appear to be labeled on the α4. This is based on the assumption that all of the subunits are irreversibly labeled with an equal efficiency. Alternatively, α4 subunits might not be efficient substrates for photolabeling.

The labeled bands on the gel are presumably α subunit constituents of the usual diazepam-sensitive type, i.e. α1 and α3. The α5 subunits migrates in the molecular weight region of 55–60 kDa (McKernan et al., 1991; Mertens et al., 1993; Endo and Olsen, 1993 [called α4 at that time]), but is not expressed in adult rat thalamus based on in situ hybridization (Wisden et al., 1992; Persohn et al., 1992). Thus, the nature of one of the 58 and 61 kDa polypeptides is not clear. Some anti-α3 antibodies have been shown to detect a doublet around M_r 59 kDa (McKernan et al., 1991; Mertens et al., 1993), so these two peaks might represent the two polypeptides detected by anti-α3 antibodies. It is also possible that one of these polypeptides could be the α4 subunit photolabeled by [^{3}H]Ro15-4513, but only if the binding of Ro15-4513 is diazepam-sensitive. In conclusion, it seems that the α4 subunit might form muscimol binding isoforms lacking benzodiazepine sites in other brain regions, much as the α6 does in cerebellum, but that α4 and α6 are not strictly homologous in the formation of other isoforms, such as diazepam-insensitive sites for [^{3}H]Ro15-4513.

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