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Subunit Composition and Quantitative Importance of Hetero-oligomeric Receptors: $GABA_A$ Receptors Containing α_6 Subunits

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In cerebellum, $GABA_A$ receptors containing α_6 subunits are expressed exclusively in granule cells. The number of α_6 receptor subtypes formed in these cells and their subunit composition presently are not known. Immunoaffinity chromatography on α_6 subunit-specific antibodies indicated that 45% of $GABA_A$ receptors in cerebellar extracts contained α_6 subunits. Western blot analysis demonstrated that α_1, β_1, β_2, β_3, γ_2, and δ subunits co-purified with α_6 subunits, suggesting the existence of multiple α_6 receptor subtypes. These subtypes were identified using a new method based on the one-by-one immunochromatographic elimination of receptors containing the co-purifying subunits in parallel or subsequent experiments. By quantification and Western blot analysis of α_6 receptors remaining in the extract, the proportion of α_6 receptors containing the eliminated subunit could be calculated and the subunit composition of the remaining receptors could be determined. Results obtained indicated that α_6 receptors in cerebellum are composed predominantly of $\alpha_6\beta_x\gamma_2$ (32%), $\alpha_1\alpha_6\beta_x\gamma_2$ (37%), $\alpha_6\beta_x\delta$ (14%), or $\alpha_1\alpha_6\beta_x\delta$ (15%) subunits. Other experiments indicated that 10%, 51%, or 21% of α_6 receptors contained homogeneous β_1, β_2, or β_3 subunits, respectively, whereas two different β subunits were present in 18% of all α_6 receptors. The method presented can be used to resolve the total number, subunit composition, and abundancy of $GABA_A$ receptor subtypes in the brain and can also be applied to the investigation of other hetero-oligomeric receptors.

Key words: $GABA_A$ receptor; composition, α_6 subunit; granule cell; cerebellum; antibodies; immunoaffinity chromatography; immunoprecipitation; [^{3}H]muscimol; [^{3}H]Ro 15-4513; binding studies

$GABA_A$ receptors are ligand-gated chloride ion channels and the site of action of various pharmacologically and clinically important drugs, such as benzodiazepines, barbiturates, steroids, anesthetics, and convulsants (Sieghart, 1995). So far six α, four β, three γ, one δ, one ϵ, and three ρ subunits have been cloned and sequenced from mammalian brain (Sieghart, 1995; Ogurusu and Shingai, 1996; Davies et al., 1997), and it is assumed that five subunits assemble to form functional $GABA_A$ receptors (Nayeem et al., 1994; Tretter et al., 1997). Expression studies have indicated that α, β, and γ subunits have to combine to form receptors closely resembling native receptors. Depending on the type of α, β, and γ subunits used for transfection of cells, however, recombinant receptors with different pharmacological properties do arise (Sieghart, 1995). The distinct but overlapping regional and cellular expression of the individual subunits (Persohn et al., 1992; Wisden et al., 1992) raises the possibility of the existence of an extremely large variety of $GABA_A$ receptor subtypes in the brain. So far the actual extent of $GABA_A$ receptor heterogeneity is not known.

$GABA_A$ receptors containing α_6 subunits are expressed in cerebellar granule cells and in the embryologically related granule cells of the cochlear nucleus only (Laurie et al., 1992; Persohn et al., 1992; Wisden et al., 1992; Varecka et al., 1994; Jones et al., 1997). Thus, all α_6 receptors from cerebellum are expressed in the same cell type. In addition, receptors consisting of $\alpha_6\beta_x\gamma_2$ subunits have special properties because they exhibit a high affinity for the inverse benzodiazepine agonist Ro 15-4513 but no affinity for the benzodiazepine agonist diazepam (Sieghart, 1995).

Several studies have investigated the subunit composition of $GABA_A$ receptors containing α_6 subunits. The results obtained, however, were partially conflicting. Whereas in one study (Quirk et al., 1994) α_6 subunits were not observed to occur in combination with other α subunits, other studies demonstrated a partial coexistence of α_6 and α_1 subunits in the same receptor (Pollard et al., 1993, 1995; Khan et al., 1994, 1996). Similarly, estimates of the abundancy of individual receptor subtypes differed between authors. Finally, because of the lack of suitable antibodies, not all α_6 subunit-containing receptors could be investigated.

The present study was performed to resolve these discrepancies. Using 13 highly specific antibodies directed against different $GABA_A$ receptor subunits, we demonstrated that only α_1, β_1, β_2, β_3, γ_2, and δ subunits significantly co-purified with α_6 subunits. To determine the identity and quantitative importance of receptors formed from these subunits, a generally applicable method was developed that is based on a one-by-one elimination by immunoaffinity chromatography of receptors containing the co-purifying subunits. Quantification of the remaining α_6 receptors allowed us to estimate the proportion of α_6 receptors containing the eliminated subunit. Repeating this subtractive purification by eliminating another co-purifying subunit in a parallel or a subsequent experiment finally allowed us to identify the subunit composition of α_6 receptors and to determine their quantitative importance.

MATERIALS AND METHODS

Generation and purification of antibodies. The antibodies anti-peptide α_1(1–9), anti-peptide α_2(416–424), and anti-peptide α_3(459–467)

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(Zezula et al., 1991), anti-peptide α_4(379–421) (Ebert et al., 1996), anti-peptide α_5(427–433) (Sieghart et al., 1993), anti-peptide β_3(345–408) (Slany et al., 1995), anti-peptide β_3(1–13), and anti-peptide γ_2(319–366) (Tretter et al., 1997), anti-peptide γ_1(324–366) (Mossier et al., 1994), anti-peptide γ_3(322–372) (Tögel et al., 1994), and anti-peptide δ(1–44) (Jones et al., 1997) were generated and affinity-purified as described previously. Polyclonal anti-peptide β_1(350–404) and anti-peptide β_2(351–405) antibodies were generated in a way similar to that described (Mossier et al., 1994).

The N-terminal peptide α_6(1–15) (sequence QLEDEGNFYS-ENVSR-) or the C-terminal peptide α_6(429–434) (sequence -VSSTVE) were custom-synthesized with an additional C- or N-terminal cysteine, respectively (piChem, Graz, Austria) and were coupled to keyhole limpet hemocyanin. These adducts were then used for the immunization of rabbits. The antibodies were isolated from the sera of the immunized rabbits by affinity chromatography on thiopropyl-Sepharose 6B coupled to the cysteine residue of the respective peptide according to the recommendations of Pharmacia LKB Biotechnology.

Cloning of α_1, β_1, β_2, β_3, or γ_2 subunits of $GABA_A$ receptors. A rat brain cDNA library was constructed in λZAP (Stratagene, La Jolla, CA) from poly A^+ mRNA isolated from the brains of 8- to 10-d-old rats as described in the protocol from Stratagene. α_1, β_1, β_2, β_3, and γ_2 subunits of $GABA_A$ receptors were cloned from this cDNA library (Fuchs et al., 1995; Slany et al., 1995), and their sequence proved to be identical to that of the respective sequence published previously.

Culture of human embryonic kidney (HEK) 293 cells and cDNA transfection. Transformed HEK 293 cells (CRL 1573; American Type Culture Collection, Rockville, MD) were grown in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS), 2 mM glutamine, 50 μM β-mercaptoethanol, 100 U/ml penicillin G, and 100 μg/ml streptomycin in 75 cm^2 petri dishes using standard cell culture techniques.

HEK 293 cells were transfected with rat α_1, β_1, β_2, β_3, or γ_2 subunit cDNAs subcloned individually into the expression vector pCDM8 (Invitrogen, San Diego, CA), using the calcium phosphate precipitation method (Chen and Okayama, 1988). The ratio for the α, β, and γ subunits used for transfection of 3×10^6 cells was 12:6:6 μg cDNA (Zezula et al., 1996). The cells were harvested 48 hr after transfection.

Extraction of $GABA_A$ receptors. Membranes from the cerebellum of adult rats or membranes from HEK 293 cells transfected with various $GABA_A$ receptor subunit cDNAs were extracted with a deoxycholate buffer containing 0.5% deoxycholate, 0.05% phosphatidylcholine, 10 mM Tris-chloride, pH 8.5, 150 mM NaCl, 500 μM benzamidine, 200 μg/ml bacitracin, and 300 μM phenylmethylsulfonylfluoride (PMSF).

Immunoaffinity chromatography of $GABA_A$ receptors and Western blot analysis. The anti-peptide α_6(429–434) antibody was the first α_6 antibody available in our laboratory and therefore was used to prepare an immunoaffinity column. Immunoaffinity columns were prepared by coupling 3–5 mg of the purified antibodies to 1 ml of protein A–agarose using the ImmunoPure IgG Orientation Kit (Pierce Europe, Oud-Beijerland, The Netherlands) as described previously (Mossier et al., 1994).

The immunoaffinity columns were equilibrated in the deoxycholate extraction buffer. Deoxycholate extracts of cerebellar membranes were applied to the immunoaffinity column at a rate of 2 ml/h. To completely eliminate the respective subunit and its associated receptors from the extract, the extract was cycled three times through the respective immunoaffinity column. The column was washed twice with 4 ml of deoxycholate extraction buffer, twice with 4 ml of IP–high buffer (0.5% Triton X-100, 50 mM Tris-chloride, pH 8.3, 600 mM NaCl, 1 mM EDTA, 500 μM benzamidine, 200 μg/ml bacitracin, and 300 μM PMSF) and then twice with 4 ml of IP–low buffer (0.2% Triton X-100, 50 mM Tris-chloride, pH 8.3, 150 mM NaCl, 1 mM EDTA, 500 μM benzamidine, 200 μg/ml bacitracin, and 300 μM PMSF). Proteins bound to the column were eluted with a buffer containing 0.1 M glycine-HCl, pH 2.45, 150 mM NaCl, and 0.1% Triton X-100. The eluted proteins were precipitated with methanol/chloroform (Wessel and Flügge, 1984) and subjected to Western blot analysis (Fuchs and Sieghart, 1989). Proteins transferred to polyvinylidene difluoride (PVDF) membranes were detected with digoxigenated primary antibodies (Tögel et al., 1994), as indicated, and the anti-digoxigenin-alkaline phosphatase Fab fragments (Boehringer Mannheim, Mannheim, Germany) and the chemiluminescence substrate CSPD (Tropix, Bedford, MA) according to the instructions of the manufacturer.

Immunoprecipitation and receptor binding assay. For immunoprecipitation, 300 μl of the clear deoxycholate membrane extract were mixed with 30 μl of antibody solution (0–20 μg of antibody), and the mixture was incubated under gentle shaking at 4°C overnight. Then 50 μl of immunoprecipitin (Life Technologies, Gaithersburg, MD) plus 150 μl of an IP–low buffer containing 5% dry milk powder were added, and incubation was continued for 2 hr at 4°C. The precipitate was centrifuged for 10 min at 10,000 × *g*, and the pellet was washed twice with 500 μl IP–high and once with 500 μl IP–low buffer.

For [^{3}H]Ro 15-4513 binding assays the precipitated receptors were suspended in 1 ml of a solution containing 0.1% Triton X-100, 50 mM Tris-citrate buffer, pH 7.1, 150 mM NaCl, and 10 or 20 nM [^{3}H]Ro 15–4513 (20.9 Ci/mmol; DuPont NEN, Dreieich, Germany) in the absence or presence of 100 μM Ro 15-1788 or various concentrations of diazepam, and were incubated for 90 min at 4°C. For [^{3}H]muscimol binding assays the precipitated receptors were suspended in 1 ml of a solution containing 0.1% Triton X-100, 50 mM Tris-citrate buffer, and 20 nM [^{3}H]muscimol (17.1 Ci/mmol; DuPont NEN) in the absence or presence of 10 μM GABA, and were incubated for 60 min at 4°C (Zezula and Sieghart, 1991). The suspensions were then filtered through Whatman GF/B filters, and the filters were washed twice with 5 ml ([^{3}H]Ro 15-4513 assay) or 3.5 ml ([^{3}H]muscimol assay) of a 50 mM Tris-citrate buffer, pH 7.1. When the percentage of α_6 receptors retained by an immunoaffinity column had to be determined, immunoprecipitation with the α_6(1–15) antibody and the subsequent [^{3}H]muscimol binding assays were performed in the same experiment with the original extract and the immunoaffinity column efflux.

Total [^{3}H]Ro 15-4513 binding in the extract before or after immunoprecipitation of α_6 subunit-containing $GABA_A$ receptors was measured using a polyethyleneglycol (PEG) precipitation assay as described (Zezula and Sieghart, 1991). For this, 100 μl of the deoxycholate extract (or of the supernatant from the immunoprecipitation with anti-α_6 antibodies) was incubated for 90 min at 4°C in a total volume of 1 ml with a buffer containing 50 mM Tris-citrate, pH 7.1, 150 mM NaCl, 50 μg γ-globulin, 15% (wt/vol) PEG, and 10 or 20 nM [^{3}H]Ro 15-4513 in the absence or presence of 100 μM Ro 15-1788. The suspension was then filtered through Whatman GF/B filters, and the filters were washed twice with 3.5 ml of an 8% PEG solution.

For the determination of total [^{3}H]muscimol binding in the extract, the PEG precipitation assay could not be used. This was attributable to the relatively high viscosity of the PEG solutions, prolonging the time needed for filtration of the samples, and the rapid dissociation of [^{3}H]muscimol from its binding site. Total [^{3}H]muscimol binding therefore was determined after all $GABA_A$ receptors present in the extract were precipitated with an antibody mixture containing 8 μg β_1(350–404), plus 8 μg β_2(351–407), plus 10 μg β_3(1–13) antibody, using the same assay as described above. The validity of this approach was demonstrated by the observation that [^{3}H]Ro 15-4513 binding data were identical whether receptors were precipitated with PEG or with this antibody mixture.

RESULTS

Anti-α_6 antibodies

The N- or C-terminal amino acid sequences α_6(1–15) or α_6(429–434) are unique for the α_6 subunit of $GABA_A$ receptors (Lüddens et al., 1990). Antibodies generated against these sequences were able to immunoprecipitate native $GABA_A$ receptors solubilized from rat cerebellar membranes in a dose-dependent manner (Fig. 1). Whereas anti-peptide α_6(1–15) antibodies precipitated up to 15 ± 4% (mean ± SD; $n = 4$) of all [^{3}H]Ro 15-4513 binding sites present in the extract, anti-peptide α_6(429–434) antibodies precipitated only 5 ± 1% (mean ± SD; $n = 4$) of these sites. Of the [^{3}H]Ro 15-4513 binding sites precipitated by these antibodies, 23 ± 2% were diazepam sensitive, whereas 77 ± 2% of these sites were diazepam insensitive.

Interestingly, however, it was demonstrated that in the same experiment the percentage of total [^{3}H]Ro 15-4513 binding sites eliminated from the supernatant was higher than that actually found in the precipitate. Thus, whether 15–20 μg of α_6(1–15) or α_6(429–434) antibodies was used for immunoprecipitation, the amount of [^{3}H]Ro 15-4513 binding sites in the supernatant was reduced by 30 ± 3% (mean ± SD; $n = 4$) (Fig. 1). These results

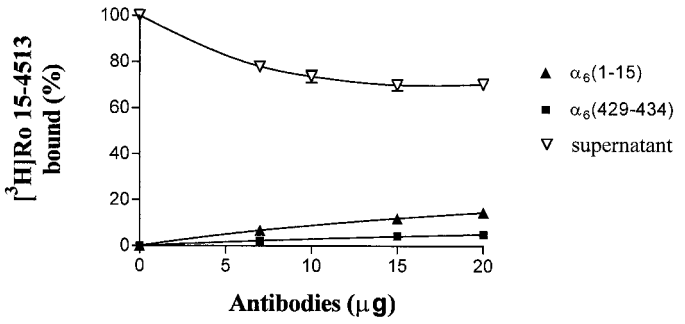

Figure 1. Immunoprecipitation of $GABA_A$ receptors solubilized from rat cerebellum. Solubilized receptors (470 fmol of [^{3}H]Ro 15-4513 binding sites) were incubated with increasing amounts of α_6(1–15) or α_6(429–434) antibodies in a final volume of 350 μl. Receptors present in the pellets (*solid symbols*) or the supernatant (*open symbols*) were determined by specific [^{3}H]Ro 15-4513 binding. Identical results were obtained when the supernatant from the α_6(1–15) or α_6(429–434) immunoprecipitation was investigated. The values are mean ± SD of four separate experiments performed in triplicates. SD bars that were smaller than the diameter of the symbols are not shown.

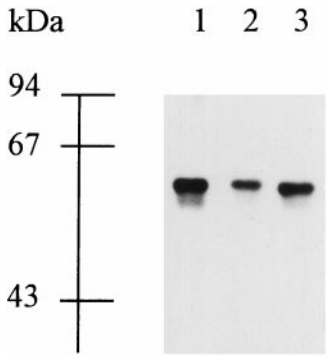

Figure 2. Identification of α_6 subunits in cerebellar membrane extracts. $GABA_A$ receptors were immunoprecipitated from cerebellar membrane extracts using α_6(1–15) antibodies. Receptors were then subjected to SDS-PAGE and Western blot analysis, using digoxigenated antibodies. *Lane 1*, α_6(429–434) antibodies; *lane 2*, α_6(1–15) antibodies from rabbit 15; *lane 3*, α_6(1–15) antibodies from rabbit 16. Antibodies bound to proteins were detected using anti-digoxigenin-alkaline phosphatase Fab fragments and a sensitive chemiluminescence detection system as described in Materials and Methods. The gel was calibrated with proteins of known molecular mass. The experiment was performed twice with similar results. The protein smear below the 56–57 kDa band was detected by all three antibodies and may have represented differentially glycosylated, partially degraded, or alternatively spliced α_6 subunits.

seem to indicate that each of these antibodies had a similar ability to bind to α_6 subunit-containing $GABA_A$ receptors, but that part of the receptors presumably were lost during washing of the precipitate.

In other experiments the ability of α_6(1–15) antibodies to immunoprecipitate [^{3}H]muscimol binding sites was investigated. As with [^{3}H]Ro 15-4513 binding, the amount of [^{3}H]muscimol binding sites retained in the pellet was smaller than that removed from the supernatant. Thus, α_6(1–15) antibodies precipitated 22 ± 3% (mean ± SD; $n = 3$) of all [^{3}H]muscimol binding sites present in cerebellar extracts but eliminated 42 ± 3% of these binding sites from the supernatant. Overall, these data indicated that more [^{3}H]muscimol than [^{3}H]Ro 15-4513 binding sites were precipitated from cerebellar extracts (or removed from the supernatant) by α_6(1–15) antibodies.

Proteins precipitated from cerebellar extracts by α_6(1–15) antibodies were then subjected to SDS-PAGE and Western blot analysis. The α_6(429–434) antibody as well as two α_6(1–15) antibodies purified from the sera of different rabbits were able to identify a single protein band with an apparent molecular mass of 56–57 kDa (Fig. 2). The observation that three antibodies directed against two distinct epitopes of the α_6 subunit specifically identified the same protein supports the conclusion that the protein with apparent molecular mass of 56–57 kDa was the α_6 subunit of $GABA_A$ receptors. This conclusion is in agreement with previous reports indicating that the α_6 subunit exhibits an apparent molecular mass of 56–57 kDa (Lüddens et al., 1990; Pollard et al., 1993; Quirk et al., 1994). The different signal intensity of the antibodies reacting with identical amounts of the immunoprecipitate indicates that these antibodies exhibited a differential affinity for α_6 subunits under Western blot conditions.

Isolation, subunit composition, and quantitative importance of $GABA_A$ receptors containing α_6 subunits

After solubilization of $GABA_A$ receptors from cerebellar membranes, 67.8% of the [^{3}H]Ro 15-4513 or [^{3}H]muscimol binding sites present in the membranes could be recovered in the extract. This corresponded to 92.5% of the binding sites identified in the extract and in the 100,000 × *g* pellet after extraction. Because there was no significant difference in the efficiency of solubilization by detergent between [^{3}H]muscimol binding sites or diazepam-sensitive or -insensitive [^{3}H]Ro 15-4513 binding sites, it can be concluded that the extracted receptors were representative of the entire functional α_6 subunit-containing $GABA_A$ receptor population.

To quantitatively isolate $GABA_A$ receptors containing α_6 subunits, cerebellar extracts were cycled three times through an immunoaffinity column containing anti-peptide α_6(429–434) antibodies. In the final efflux of this column, anti-peptide α_6(1–15) antibodies no longer were able to precipitate $GABA_A$ receptors, and α_6 subunits no longer could be demonstrated in Western blots, indicating that this procedure eliminated most if not all α_6 receptors from the extract. In the same efflux, [^{3}H]Ro 15-4513 binding was reduced by 31 ± 1% (mean ± SD; $n = 3$), and [^{3}H]muscimol binding was reduced by 45 ± 1% (mean ± SD; $n = 3$). These percentages correspond closely to the 30 ± 3% reduction of [^{3}H]Ro 15-4513 and 42 ± 3% reduction of [^{3}H]muscimol binding sites observed in cerebellar extracts after immunoprecipitation with α_6(1–15) antibodies (see above).

To identify $GABA_A$ receptor subunits co-purifying with α_6 subunits, receptors bound to the α_6(429–434) immunoaffinity column were eluted by a change in the pH value of the buffer and were probed with 13 different antibodies, each of which specifically recognized a distinct $GABA_A$ receptor subunit. As shown in Figure 3*A* (or Fig. 4*A*), in addition to the α_6 subunit, α_1, β_1, β_2, β_3, γ_2, and δ subunits were present in the α_6(429–434) column eluate. Thus, α_1(1–9), β_1(350–404), β_2(351–405), β_3(345–408), γ_2(319–366), and δ(1–44) antibodies identified proteins with apparent molecular mass of 51 kDa, 51–54 kDa, 50–53 kDa, 51–56 kDa, 41–44 kDa, and 53 kDa, respectively. Proteins with identical apparent molecular mass could be identified by these antibodies in parallel control experiments investigating recombinant $GABA_A$ receptors containing the respective subunits (experiments not shown). The β_3(345–408) antibody, in addition to the 51–56 kDa protein, identified a second protein with an apparent molecular mass of 42–47 kDa. The protein with lower molecular mass seemed to be a partially degraded β_3 subunit, because staining of this protein was variable in different experiments

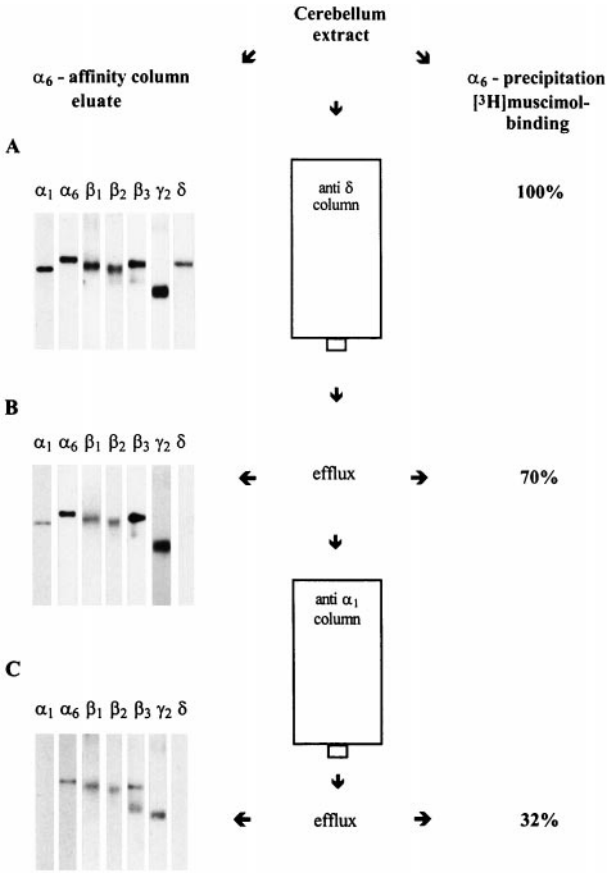

Figure 3. Subunit composition and quantification of α_6 receptors before or after elimination of δ or α_1 subunit-containing receptors. Cerebellar extracts were chromatographed on an α_6(429–434) immunoaffinity column either before (*A*) or after (*B*) chromatography on a δ(1–44) immunoaffinity column, or after (*C*) chromatography on both a δ(1–44) and an α_1(1–9) immunoaffinity column. α_6(429–434) column eluates were subjected to SDS-PAGE and Western blot analysis using the following digoxigenated antibodies: α_1(1–9), α_6(1–15), β_1(350–404), β_2(351–405), β_3(345–408), γ_2(319–366), and δ(1–44). Western blots are from a typical experiment that was performed three times with similar results. In parallel experiments, the original extract as well as the efflux from the δ(1–44) or the α_1(1–9) column were subjected to immunoprecipitation with α_6(1–15) antibodies and subsequent [^{3}H]muscimol binding assays. Data are presented as percentage of [^{3}H]muscimol binding sites precipitated in the original extract and are means of a single experiment performed in triplicate. The experiment was repeated three times with similar results.

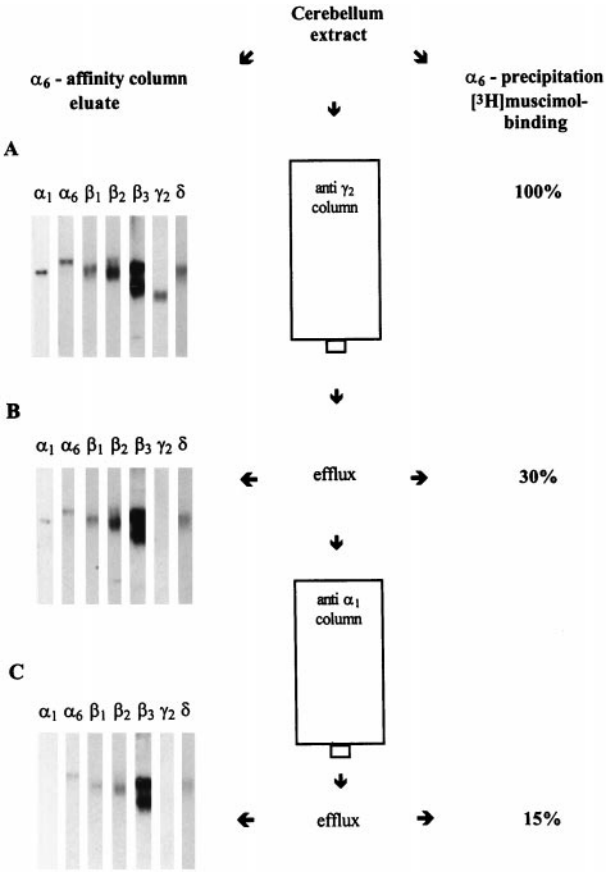

Figure 4. Subunit composition and quantification of α_6 receptors before or after elimination of γ_2 and α_1 subunit-containing $GABA_A$ receptors. Cerebellar extracts were chromatographed on an α_6(429–434) immunoaffinity column either before (*A*) or (*B*) after chromatography on a γ_2(319–366) immunoaffinity column, or after (*C*) chromatography on both a γ_2(319–366) and an α_1(1–9) immunoaffinity column. α_6(429–434) column eluates were subjected to SDS-PAGE and Western blot analysis using α_1, α_6, β_1, β_2, β_3, γ_2, and δ antibodies as described in Figure 3. Western blots are from a typical experiment that was performed three times with similar results. In parallel experiments, the original extract as well as the efflux from the γ_2(319–366) or the α_1(1–9) column were subjected to immunoprecipitation with α_6(1–15) antibodies and subsequent [^{3}H]muscimol binding assays. Data are presented as percentage of [^{3}H]muscimol binding sites precipitated in the original extract and are means of a single experiment performed in triplicate. The experiment was repeated three times with similar results.

(compare Figs. 3 and 4), and increased with increasing time needed for the isolation of $GABA_A$ receptors (compare Fig. 3*A–C*).

The co-purification of α_1, β_1, β_2, β_3, γ_2, and δ subunits together with α_6 subunits was not caused by a cross-reactivity of α_6(429–434) antibodies with these subunits, because neither α_6(429–434) nor α_6(1–15) antibodies were able to precipitate [^{3}H]muscimol binding sites or $GABA_A$ receptor subunits from extracts of forebrain membranes, which do contain α_{1-5}, β_{1-3}, γ_{1-3}, and δ, but no α_6 subunits (Persohn et al., 1992; Wisden et al., 1992). These data therefore indicate that any one of the α_1, β_1, β_2, β_3, γ_2, and δ subunits can be colocalized with α_6 subunits in the same $GABA_A$ receptor.

In contrast, the α_2, α_3, α_4, α_5, γ_1, and γ_3 subunits could not be detected in the eluate of the α_6(429–434) immunoaffinity column, although all of these subunits, except the α_3 and α_5 subunits, could be identified in cerebellar extracts by the respective antibodies (experiments not shown). This indicates that unspecific adsorption of receptors or an exchange of subunits during extraction was not a problem in this study.

Isolation, subunit composition, and quantitative importance of $GABA_A$ receptors containing α_6 and γ_2 subunits

Because $GABA_A$ receptors are composed of five subunits, the co-purification of a total of seven different subunits by the α_6(429–434) immunoaffinity column indicated that a mixture of $GABA_A$ receptor subtypes with different subunit composition was purified. To isolate $GABA_A$ receptors containing α_6, β_x, and γ_2 subunits, $GABA_A$ receptors containing any one of the other co-purifying subunits were quantitatively removed by immunoaffinity chromatography. In the first step, receptors containing δ subunits were eliminated from cerebellar membrane extracts us-

ing a δ(1–44) column (Fig. 3). The δ(1–44) antibody specifically recognized the δ but no other subunits of the $GABA_A$ receptor (Jones et al., 1997). Interestingly, in the pH 2.45 eluate of the δ(1–44) column, α_1, α_6, β_1, β_2, β_3, δ, and other subunits, but no γ_2 subunits, could be identified (R. Pelz, M. Jechlinger, and W. Sieghart, unpublished data).

To determine the composition of the remaining α_6 receptors, the efflux of the δ(1–44) column subsequently was chromatographed on the α_6(429–434) column. As shown in Figure 3*B*, δ subunits could no longer be identified in the eluate of this column, indicating that these subunits had been completely eliminated by the δ(1–44) column. The presence of six different subunits (α_1, α_6, β_1, β_2, β_3, γ_2) in the eluate of the α_6(429–434) column indicates that $GABA_A$ receptors retained by this column were still heterogeneous.

In the efflux of the δ(1–44) column, α_6(1–15) antibodies were able to precipitate 70% of the [^{3}H]muscimol binding sites that could be precipitated by these antibodies in the original extract (Fig. 3*B*). This indicates that 30% of the α_6 subunit-containing $GABA_A$ receptors were retained by the δ(1–44) column and contained the δ subunit.

In the next step, the efflux of the δ(1–44) column was chromatographed on an α_1(1–9) immunoaffinity column. The α_1(1–9) antibody has been demonstrated to selectively identify only α_1 but no other $GABA_A$ receptor subunits (Nusser et al., 1996; Zezula et al., 1991). The α_6 subunit-containing receptors remaining in the efflux of the α_1(1–9) column were then collected by the α_6(429–434) column. In the pH 2.45 eluate of this column, only α_6, β_1, β_2, β_3, and γ_2 subunits, but no α_1 subunits, could be detected (Fig. 3*C*). The five subunits present in this eluate still could have been combined in a variety of different ways, resulting in a multiplicity of pentameric $\alpha\beta$ or $\alpha\beta\gamma$ receptors with different subunit composition and stoichiometry. At this point, therefore, no conclusion on the identity and composition of the receptors isolated by this procedure could be made.

As expected, the intensity of the individual signals for α_6, β_1, β_2, β_3, and γ_2 subunits was lower in Figure 3*C* than in 3*A* or *B*. In the efflux of the α_1(1–9) column, 32 ± 3% (mean ± SD; n = 3) of the α_6 subunit-containing receptors present in the original extract could be precipitated by α_6(1–15) antibodies (Fig. 3*C*). Thus, 32% of α_6 receptors were composed of α_6, β_1, β_2, β_3, and γ_2 subunits. The observation that 70% of the α_6 receptors could be precipitated before and only 32% after the α_1(1–9) column additionally indicates that 38% of α_6 receptors were removed by the α_1(1–9) column and thus contained α_1 as well as α_6 subunits.

All of these percentages were obtained by investigating binding of [^{3}H]muscimol to the precipitated receptors. Because [^{3}H]muscimol binding sites can be demonstrated only on receptors containing α and β, or α, β, and γ subunits (Zezula et al., 1996), these experiments indicate that the 32% of α_6 and 38% of $\alpha_1\alpha_6$ receptors so far discussed must also have contained β subunits. Whether all or only some of these receptors additionally contained γ_2 subunits cannot be answered at this time.

Isolation, subunit composition, and quantitative importance of $GABA_A$ receptors containing α_6 and δ subunits

In another experiment (Fig. 4), $GABA_A$ receptors containing γ_2 subunits were eliminated from cerebellar membrane extracts using a γ_2(319–366) column. The high specificity of this immunoaffinity column has been demonstrated previously (Mossier et al., 1994). In the pH 2.45 eluate of the γ_2(319–366) column, α_1, α_6, β_1, β_2, β_3, γ_2, and other subunits, but no δ subunits, could be identified (experiments not shown). This again supports the conclusion that γ_2 and δ subunits, at least in the cerebellum, seem not to be present in the same $GABA_A$ receptors.

Receptors remaining in the efflux of the γ_2(319–366) column were then chromatographed on the α_6(429–434) column. In the eluate of this column, α_1, α_6, β_1, β_2, β_3, and δ subunits, but no γ_2 subunits, could be detected (Fig. 4*B*). Immunoprecipitation with α_6(1–15) antibodies in the efflux of the γ_2(319–366) column indicated that receptors composed of these subunits represented 30% of the α_6 receptors present in the original extract (Fig. 4*B*). All of these receptors contained the δ subunit, because 30% of all α_6-containing $GABA_A$ receptors could also be bound to the δ(1–44) immunoaffinity column, as discussed above (Fig. 3*B*).

The identification of only 30% of the α_6 receptors in the efflux of the γ_2(319–366) column indicates that 70% of these receptors were retained by this column and thus contained γ_2 subunits. Combined with the above observation (Fig. 3*B*) that 70% of all α_6 receptors could be precipitated in the efflux of the δ(1–44) column and were composed of α_1, α_6, β_1, β_2, β_3, and γ_2 subunits, these data suggest that α_6 receptors contain either γ_2 or δ subunits.

In the next step, the efflux of the γ_2(319–366) column was chromatographed on the α_1(1–9) column, and α_6 receptors remaining in the efflux of this column were then either collected by a subsequent α_6(429–434) immunoaffinity chromatography or precipitated by α_6(1–15) antibodies (Fig. 4*C*). In the eluate of the α_6(429–434) column, α_6, β_1, β_2, β_3, and δ subunits, but no α_1 subunits, could be identified. Immunoprecipitation experiments indicated that 15% of all α_6 subunit-containing $GABA_A$ receptors could still be precipitated in the efflux of the α_1(1–9) immunoaffinity column (Fig. 4*C*) and thus were composed of $\alpha_6\beta_x\delta$ subunits.

Because 30% of all α_6 (and δ) subunit-containing receptors could be precipitated before and only about 15% after chromatography on the α_1(1–9) column, these results additionally indicate that 15% of all α_6 subunit-containing receptors are composed of $\alpha_1\alpha_6\beta_x\delta$ subunits. Thus, the α_6 and δ subunit-containing receptors $\alpha_1\alpha_6\beta_x\delta$ and $\alpha_6\beta_x\delta$ obviously are present in cerebellum at a 1:1 ratio. As expected, the signal strength of the individual protein bands was reduced according to the receptors removed by the various immunoaffinity columns (compare Fig. 4*A–C*). In this experiment the staining of the β_3 subunit was quite prominent. Because staining intensity depends on the individual properties of the digoxigenated antibody batch used, different staining intensities obtained with different antibodies do not necessarily reflect differences in the amount of protein present in the extract.

Results so far presented indicate the existence of at least four α_6 subunit-containing $GABA_A$ receptor subtypes in cerebellum that are composed of $\alpha_6\beta_x\gamma_2$, $\alpha_1\alpha_6\beta_x\gamma_2$, $\alpha_6\beta_x\delta$, and $\alpha_1\alpha_6\beta_x\delta$ subunits. The same four α_6 receptor subtypes were also identified when the sequence of columns was changed, and an α_1(1–9) column was used before the γ_2(319–366) column to eliminate receptors containing the respective subunits from cerebellar extracts. In addition, the quantitative data obtained were consistent with each other and not dependent on the sequence of columns used (experiments not shown). These results strongly suggest that none of the antibodies used for immunochromatography exhibited a significant cross-reactivity and that the α_6(1–15) or α_6(429–434) antibodies were able to recognize or precipitate these four α6 subunit-containing $GABA_A$ receptor subtypes with comparable efficiency. The experiments described were repeated several

Table 1. Relative and absolute abundancy of α_6 receptor subtypes in rat cerebellum

Subunit composition	Percentage of α_6 receptors	Percentage of $GABA_A$ receptors
$\alpha_1\alpha_6\beta_x\gamma_2$	37 ± 3	16.7
$\alpha_6\beta_x\gamma_2$	32 ± 3	14.4
$\alpha_1\alpha_6\beta_x\delta$	15 ± 3	6.8
$\alpha_6\beta_x\delta$	14 ± 2	6.3

Data presented are calculated from three experiments performed as shown in Fig. 3 and from three experiments performed as shown in Fig. 4 and are means ± SD. Percentage of $GABA_A$ receptors was calculated from these data by taking into account that only 45 ± 1% of all $GABA_A$ receptors in cerebellum contain the α_6 subunit.

times, and the average proportion of the four $GABA_A$ receptor subtypes calculated from the individual experiments is given in Table 1. In addition, taking into account that only 45 ± 1% of all $GABA_A$ receptors in the cerebellum contained the α_6 subunit, the absolute contribution of the various α_6 receptors to total $GABA_A$ receptors present in cerebellum was calculated (Table 1).

Isolation, subunit composition, and quantitative importance of $GABA_A$ receptors containing α_6 and distinct β subunits

The low number of α_6 receptors remaining in the extract after complete removal of γ_2 and α_1 ($\alpha_6\beta_x\delta$, 15% of all α_6 receptors) or of δ and α_1 subunits ($\alpha_6\beta_x\gamma_2$, 32% of all α_6 receptors) prevented a direct investigation of the β subunit composition of these receptors, even more so because each immunoaffinity chromatography step is time consuming and enhances degradation and inactivation of receptors. Therefore, the β subunit-composition of α_6 receptors was investigated in the original extract from cerebellum only.

For this, cerebellum extracts were first chromatographed on a β_1(350–404) immunoaffinity column (Fig. 5*A*). In the efflux of this column, β_1 subunits no longer could be demonstrated (experiments not shown), indicating that receptors containing this subunit had been removed completely. Precipitation with α_6(1–15) antibodies indicated that 85 ± 1% (mean ± SD; $n = 4$) of the original α_6 receptors were still present after removal of the β_1 subunit-containing receptors and suggested that 15% of all α_6 receptors contained β_1 subunits (Fig. 5*A*).

The efflux of the β_1(350–404) column was then chromatographed on a β_2(351–405) immunoaffinity column (Fig. 5*B*). On this second column all receptors containing β_2 subunits were adsorbed, as indicated by the absence of β_2 subunits in the column efflux (experiments not shown). In the same efflux, however, 21 ± 7% (mean ± SD; $n = 3$) of the original α_6 receptors could be precipitated using α_6(1–15) antibodies. Because $GABA_A$ receptors containing β_1 as well as those containing β_2 subunits now had been completely removed from the extract, the remaining 21% of the α_6 receptors thus contained only β_3 subunits.

In other experiments, all receptors containing β_2 subunits were first removed from the cerebellum extract using a β_2(351–405) immunoaffinity column (Fig. 5*C*). In the efflux of this column, only 34 ± 2% (mean ± SD; $n = 4$) of the original α_6 receptors were present. From this it can be concluded that 66% of all α_6 receptors contained a β_2 subunit. A subsequent chromatography on a β_3(345–408) column (Fig. 5*D*) eliminated an additional 24% of the α_6 receptors. The remaining 10 ± 1% (mean ± SD; $n = 3$) of receptors thus contained only β_1 subunits.

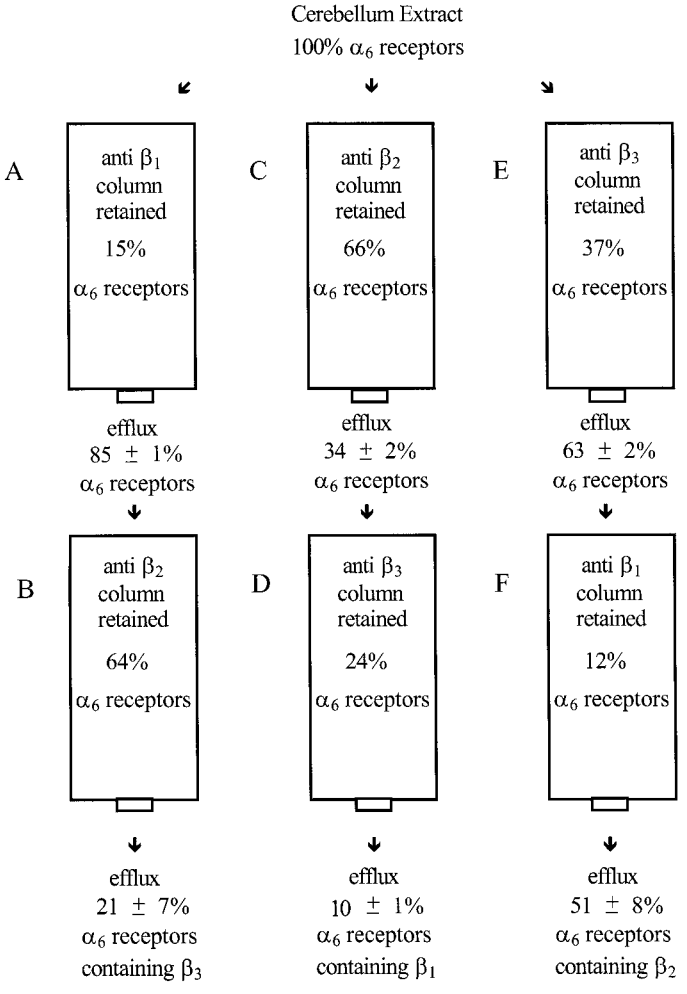

Figure 5. Quantification of α_6 receptors containing different β subunits. [^{3}H]muscimol binding to $GABA_A$ receptors immunoprecipitated with α_6(1–15) antibodies was determined in cerebellar membrane extracts before or after chromatography on a β_1(350–404), β_2(351–405), or β_3(345–408) immunoaffinity column as indicated. Data are presented as percentage of [^{3}H]muscimol binding sites precipitated by α_6(1–15) antibodies in the original extract and are mean values ± SD of three to four experiments performed in triplicate. The proportion of [^{3}H]muscimol binding sites retained on the initial anti-β immunoaffinity columns (*A, C, E*) was significantly different (Student's *t* test) from that remaining in the extract after the other two β subunits had been removed (*efflux, B, D, F*): *A*, efflux D ($p = 0.007$); *C*, efflux F ($p = 0.002$); *E*, efflux B ($p = 0.001$). [^{3}H]muscimol binding sites present in the original extract were significantly different ($p = 0.0001$) from the sum of the [^{3}H]muscimol binding sites retained on the initial anti-β immunoaffinity columns ($A + C + E$) and were also significantly different ($p = 0.007$) from the sum of the [^{3}H]muscimol binding sites found in the efflux of *B*, *D*, and *F*.

Finally, the cerebellum extract was chromatographed first on a β_3(345–408) column. In the efflux of this column, 63 ± 2% (mean ± SD; $n = 4$) of the α_6 receptors were still present (Fig. 5*E*), indicating that ~37% of all α_6 receptors contained a β_3 subunit. A subsequent chromatography on a β_1(350–404) column removed an additional 12% of α_6 receptors. The remaining 51 ± 8% (mean ± SD; $n = 3$) of α_6 receptors thus contained only β_2 subunits.

Interestingly, a comparison of the proportion of α_6 receptors retained by the β subunit-specific columns from the original extract with that remaining in the extract after removal of the other two β subunits revealed striking and statistically significant

differences (see legend to Fig. 5). Although 15% of all α_6 receptors were removed by the β_1 column from the original extract (Fig. 5*A*), only 10% of α_6 receptors were left after elimination of all β_2 and β_3 subunits (Fig. 5*D*). Although 66% of all α_6 receptors were removed by the β_2 column from the original extract (Fig. 5*C*), only 51% of these receptors were left after removal of β_1 and β_3 receptors (Fig. 5*F*). Finally, although 37% of all α_6 receptors were removed by the β_3 column from the original extract (Fig. 5*E*), only 21% of these receptors were left after removal of β_1 and β_2 subunits (Fig. 5*B*).

In addition, the sum of α_6 receptors retained by the β_1, β_2, and β_3 columns from the original extract was 118% (Fig. 5*A,C,E*), whereas the sum of the receptors remaining in the extract after two of the three β subunits had been removed was 82% (Fig. 5*B,D,F*). These differences could not be explained by a cross-reactivity of the antibodies, because β_1, β_2, or β_3 antibodies were unable to precipitate recombinant $\alpha_1\beta_x\gamma_2$ receptors containing the wrong β subunit (experiments not shown). These data therefore suggest that 18% of the α_6 receptors in cerebellum contain more than one type of β subunit. Because of the variability of binding data, however, a further calculation of the proportion of receptors containing $\beta_1\beta_2$, $\beta_1\beta_3$, or $\beta_2\beta_3$ subunit combinations does not provide reliable results.

DISCUSSION

Composition and quantitative importance of $GABA_A$ receptors containing α_6 subunits

In the present investigation, 13 antibodies, each one highly specific for a different $GABA_A$ receptor subunit, were used to investigate the subunit composition and quantitative importance of $GABA_A$ receptors containing α_6 subunits. Chromatography on an α_6(429–434) immunoaffinity column quantitatively removed α_6 subunits and 45 ± 1% of all $GABA_A$ receptors from cerebellar extracts, supporting previous conclusions (Khan et al., 1996; Jones et al., 1997) that 45% of all $GABA_A$ receptors in the cerebellum contain the α_6 subunit. In the eluate of this column, in addition to the α_6 subunit, only α_1, β_1, β_2, β_3, γ_2, and δ subunits of $GABA_A$ receptors could be demonstrated, suggesting that any one of these subunits can be colocalized with α_6 subunits in native $GABA_A$ receptors.

In contrast, α_2, α_3, α_4, α_5, γ_1, or γ_3 subunits did not co-purify with α_6 subunits. This is to be expected for α_2, α_3, α_5, or γ_1 subunits, which are not expressed in the granule cells of cerebellum (Persohn et al., 1992; Wisden et al., 1992). The existence of minor amounts of receptors containing γ_3 and α_6 subunits has been demonstrated previously after purification of $GABA_A$ receptors by a γ_3 subunit-specific immunoaffinity column (Tögel et al., 1994). The observation that α_4 subunits did not co-purify with α_6 subunits, although these subunits are expressed in cerebellar granule cells and could be identified in cerebellar extracts (E. Bencsits, V. Ebert, and W. Sieghart, unpublished data), indicates that receptors containing α_4 as well as α_6 subunits, if they exist at all, are quantitatively not important. Thus, the great majority of α_6 subunit-containing $GABA_A$ receptors is composed of α_6 and α_1, β_1, β_2, β_3, γ_2, or δ subunits.

A new strategy for the determination of the subunit composition and quantitative importance of hetero-oligomeric receptors

A random assembly of α_6 subunits with six other subunits into pentameric receptors (Nayeem et al., 1994; Tretter et al., 1997) would result in a total of 210 $GABA_A$ receptor subtypes with distinct subunit composition. It is impossible to isolate a single receptor subtype from an even much less heterogeneous mixture by immunoenrichment. In the present study, therefore, immunodepletion was used to purify and characterize $GABA_A$ receptors. Receptors containing one of the co-purifying subunits were eliminated from extracts by chromatography on subunit-specific antibodies. Quantification and Western blot analysis of α_6 receptors remaining in the extract then allowed us to estimate the proportion of α_6 receptors containing the eliminated subunit and to determine the composition of the remaining receptors. Repeating this procedure by eliminating all co-purifying subunits in parallel or subsequent experiments finally allowed us to identify the subunit composition of α_6 receptor subtypes and to determine their quantitative importance.

α_1, γ_2, or δ subunit-containing α_6 receptors

In agreement with previous studies (Khan et al., 1994, 1996; Pollard et al., 1995), 52% of the [^{3}H]muscimol binding sites precipitated by α_6(1–15) antibodies could be eliminated from cerebellar extracts by an α_1 subunit-specific column, indicating that $\alpha_1\alpha_6$ receptors are as abundant as receptors containing homogeneous α_6 subunits (Table 1). Other experiments indicated that 70% of α_6 receptors could be eliminated from cerebellar membrane extracts by a γ_2 subunit-specific (Fig. 4) and 30% by a δ subunit-specific column (Fig. 3). In addition, it was demonstrated that γ_2 and δ subunits did not co-purify with each other, supporting the conclusion that these subunits do not co-exist in the same $GABA_A$ receptor (Quirk et al., 1995).

Furthermore, the number of [^{3}H]Ro 15-4513 binding sites removed from cerebellar extracts by α_6(429–434) or α_6(1–15) antibodies was 69% or 71% of the [^{3}H]muscimol binding sites eliminated by these antibodies, respectively. Because [^{3}H]Ro 15-4513 binding sites are present on $GABA_A$ receptors containing $\alpha\gamma$ or $\alpha\beta\gamma$ subunits and [^{3}H]muscimol binding sites are present on receptors composed of $\alpha\beta$, $\alpha\beta\gamma$, and $\alpha\beta\delta$ subunits (Quirk et al., 1995; Sieghart, 1995; Zezula et al., 1996), these data agree with the conclusion that 70% of the α_6 receptors contained a γ_2 subunit. The observation that the [^{3}H]muscimol binding sites of γ_2 or δ subunit-containing α_6 receptors add up to 100% additionally indicates that all α_6 receptors contain either a γ_2 or a δ subunit. From this it can be concluded that receptors composed of $\alpha_6\beta_x$ subunits, and consequently also those composed of $\alpha_6\gamma_2$ subunits, which would contribute to [^{3}H]Ro 15-4513 but not to [^{3}H]muscimol binding sites, are not significantly expressed in cerebellum.

Further fractionation of the 70% α_6 receptors containing γ_2 subunits using an α_1 subunit-specific column indicated that 37 ± 3% of α_6 receptors are composed of $\alpha_1\alpha_6\beta_x\gamma_2$ and 32 ± 3% of $\alpha_6\beta_x\gamma_2$ subunits. $\alpha_1\alpha_6\beta_x\gamma_2$ receptors have been identified previously (Khan et al., 1994, 1996; Pollard et al., 1995), and quantification of these receptors led to comparable results (Khan et al., 1994).

Recombinant receptor studies have indicated that $\alpha_6\beta_x\gamma_2$ receptors, in contrast to $\alpha_1\beta_x\gamma_2$ receptors, exhibit a high affinity [^{3}H]Ro 15-4513 binding that could not be inhibited by diazepam (Lüddens et al., 1990; Sieghart, 1995). Other studies have indicated that in $GABA_A$ receptors containing α_6 and α_1 (Khan et al., 1996) or α_1 and α_3 subunits (Araujo et al., 1996), each one of the subunits expressed its characteristic benzodiazepine pharmacology. Because 32% of α_6 receptors are composed of $\alpha_6\beta_x\gamma_2$, whereas 37% are composed of $\alpha_1\alpha_6\beta_x\gamma_2$ subunits, these two receptor subtypes are responsible for 46.4% and 53.6% of all

[^{3}H]Ro 15-4513 binding sites precipitated by α_6(1–15) antibodies, respectively. Assuming that $\alpha_6\beta_x\gamma_2$ receptors contain two α_6 subunits (Im et al., 1995), these two receptor subtypes contain a total of 73% α_6 and 27% α_1 subunits. The present observation that 23 ± 2% of [^{3}H]Ro 15-4513 binding precipitated by α_6(1–15) antibodies could be inhibited by diazepam is supported by a recent study (Khan et al., 1996) and is in agreement with the conclusion that each one of the subunits expresses its characteristic benzodiazepine pharmacology.

Further fractionation of the 30% α_6 receptors containing δ subunits using an α_1 subunit-specific column indicated that 15 ± 3% of all α_6 receptors were composed of $\alpha_1\alpha_6\beta_x\delta$ and 14 ± 2% of $\alpha_6\beta_x\delta$ receptors. Although the existence of $\alpha_1\alpha_6\beta_x\delta$ receptors in cerebellum has been implicated previously (Pollard et al., 1995), their abundancy was not determined.

β Subunit composition of α_6 receptors

When β_1-, β_2-, and β_3-specific immunoaffinity columns were used to eliminate $GABA_A$ receptors from cerebellar extracts in parallel experiments, it was demonstrated that the total percentage of α_6 receptors removed was 118%. In the absence of a significant cross-reactivity of the β_1, β_2, or β_3 subunit-specific antibodies, these data suggested the colocalization of different β subunits in 18% of the α_6 receptors. This conclusion is supported by recent evidence indicating the colocalization of two different β subunits in native receptors (Li and De Blas, 1997). The proportion of α_6 receptors containing homogeneous β subunits was then determined by measuring α_6 receptors remaining in the extract after the removal of the other two β subunits. The results obtained indicated that 10, 51, or 21% of all α_6 receptors contained homogeneous β_1, β_2, or β_3 subunits, respectively. Because of the variability of binding data, a reliable estimation of the β subunit composition of the remaining 18% of α_6 receptors was not possible. The observation that β_1 and β_2 as well as β_3 subunits are co-purifying with α_6 and γ_2 (Fig. 3*C*) or α_6 and δ subunits (Fig. 4*C*), however, indicates that the $\alpha_6\beta_x\gamma_2$ or $\alpha_6\beta_x\delta$ receptor subtypes might exist in up to six isoforms containing different β subunit combinations (homogeneous β_1, β_2, or β_3 subunits, $\beta_1\beta_2$, $\beta_1\beta_3$, or $\beta_2\beta_3$). The same might be true for receptors consisting of $\alpha_1\alpha_6\beta_x\gamma_2$ or $\alpha_1\alpha_6\beta_x\delta$ subunits. Whether all of the resulting 24 α_6 receptors with different subunit composition actually exist cannot be answered by this study.

Subunit stoichiometry of native α_6 receptors

The present results, in agreement with studies investigating other receptors, indicate that native α_6 receptors can contain two different α (Sieghart, 1995) or two different β subunits (Li and De Blas, 1997), and in addition contain either a γ_2 or a δ subunit. Overall, these results suggest a subunit stoichiometry of two α, two β, and one γ (or one δ) subunit for native α_6 receptors. This is in agreement with studies investigating the subunit stoichiometry of $\alpha_6\beta_2\gamma_2$ (Im et al., 1995) or of other recombinant receptors (Chang et al., 1996; Tretter et al., 1997). The method of subtractive purification of $GABA_A$ receptors developed in the present study can be used to investigate whether all native α_6 receptors exhibit this stoichiometry or whether other stoichiometries also exist (Backus et al., 1993). In addition, this method can also be applied to the investigation of other hetero-oligomeric receptors.

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