Subunit Composition and Quantitative Importance of Heterooligomeric Receptors: GABA<sub>A</sub> Receptors Containing α<sub>6</sub> Subunits

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In cerebellum, GABA<sub>A</sub> receptors containing α<sub>6</sub> subunits are expressed exclusively in granule cells. The number of α<sub>6</sub> receptor subtypes formed in these cells and their subunit composition presently are not known. Immunoaffinity chromatography on α<sub>6</sub> subunit-specific antibodies indicated that 45% of GABA<sub>A</sub> receptors in cerebellar extracts contained α<sub>6</sub> subunits. Western blot analysis demonstrated that α<sub>1</sub>, β<sub>1</sub>, β<sub>2</sub>, β<sub>3</sub>, γ<sub>2</sub>, and δ subunits co-purified with α<sub>6</sub> subunits, suggesting the existence of multiple α<sub>6</sub> 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<sub>6</sub> receptors remaining in the extract, the proportion of α<sub>6</sub> receptors containing the eliminated subunit could be calculated and the subunit composition of the remaining receptors could be determined. Results obtained indicated that α<sub>6</sub> receptors in cerebellum are composed predominantly of α<sub>6</sub>β<sub>2</sub>γ<sub>2</sub> (32%), α<sub>1</sub>α<sub>6</sub>β<sub>2</sub>γ<sub>2</sub> (37%), α<sub>6</sub>β<sub>3</sub>δ (14%), or α<sub>6</sub>α<sub>6</sub>β<sub>3</sub>δ (15%) subunits. Other experiments indicated that 10%, 51%, or 21% of α<sub>6</sub> receptors contained homogeneous β<sub>1</sub>, β<sub>2</sub>, or β<sub>3</sub> subunits, respectively, whereas two different β subunits were present in 18% of all α<sub>6</sub> receptors. The method presented can be used to resolve the total number, subunit composition, and abundance of GABA<sub>A</sub> receptor subtypes in the brain and can also be applied to the investigation of other heterooligomeric receptors.

Key words: GABA<sub>A</sub> receptor; composition, α<sub>6</sub> subunit; granule cell; cerebellum; antibodies; immunoaffinity chromatography; immunoprecipitation; [3H]muscimol; [3H]Ro 15-4513; binding studies

GABA<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> receptor subtypes in the brain. So far the actual extent of GABA<sub>A</sub> receptor heterogeneity is not known.

GABA<sub>A</sub> receptors containing α<sub>6</sub> 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<sub>6</sub> receptors from cerebellum are expressed in the same cell type. In addition, receptors consisting of α<sub>6</sub>β<sub>2</sub>γ<sub>2</sub> 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<sub>A</sub> receptors containing α<sub>6</sub> subunits. The results obtained, however, were partially conflicting. Whereas in one study (Quirk et al., 1994) α<sub>6</sub> subunits were not observed to occur in combination with other α subunits, other studies demonstrated a partial coexistence of α<sub>6</sub> and α<sub>1</sub> subunits in the same receptor (Pollard et al., 1993, 1995; Khan et al., 1994, 1996). Similarly, estimates of the abundance of individual receptor subtypes differed between authors. Finally, because of the lack of suitable antibodies, not all α<sub>6</sub> subunit-containing receptors could be investigated.

The present study was performed to resolve these discrepancies. Using 13 highly specific antibodies directed against different GABA<sub>A</sub> receptor subunits, we demonstrated that only α<sub>1</sub>, β<sub>1</sub>, β<sub>2</sub>, β<sub>3</sub>, γ<sub>2</sub>, and δ subunits significantly co-purified with α<sub>6</sub> 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<sub>6</sub> receptors allowed us to estimate the proportion of α<sub>6</sub> 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<sub>6</sub> receptors and to determine their quantitative importance.

MATERIALS AND METHODS

Generation and purification of antibodies. The antibodies anti-peptide α<sub>1</sub>(1–9), anti-peptide α<sub>2</sub>(416–424), and anti-peptide α<sub>3</sub>(459–467)
described previously. Polyclonal anti-peptide antibodies were custom-synthesized with an additional C- or N-terminal cysteine, and were coupled to keyhole limpet hemocyanin. These adducts were then used for the immunization of 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 α1, β1, β2, or γ2 subunits of GABA A receptors. A rat brain cDNA library was constructed in λZAP (Strategen, La Jolla, CA) from poly A+ mRNA isolated from the brains of 8- to 10-old rats as described in the protocol from Strategen. α1, β1, β2, and γ2 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 μg/ml streptomycin, 50 μg/ml gentamicin, 2 ml of 10% dry milk powder were added, and incubated reduced by 30% in the supernatant was found in the precipitate. Thus, whether 15–20% of these sites were diazepam sensitive, whereas 77 ± 2% were diazepam resistant (Zezula and Sieghart, 1991). For this, 100 μl of the deoxycholate extract (or of the supernatant from the immunoprecipitation with anti-α2 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 [3H]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 5 ml of 1% Triton X-100, 50 mM Tris-citrate buffer, pH 7.1. When the percentage of α2 receptors retained by an immunoaffinity column had to be determined, immunoprecipitation with the anti-α2 (1–15) antibody and the subsequent [3H]muscimol binding assays were performed in the same experiment with the original extract and the immunoaffinity column efflux.

Total [3H]Ro 15-4513 binding in the extract before or after immunoprecipitation of α2 subunit-containing GABA A receptors was measured using a polyethylene glycol precipitation kit (Pierce, Rockford, IL), and the validity of this approach was demonstrated by the observation that [3H]Ro 15-4513 binding data were identical whether receptors were precipitated with PEG or with this antibody mixture.

RESULTS

Anti-α6 antibodies

The N- or C-terminal amino acid sequences α6(1–15) or α6(429–434) are unique for the α6 subunit of GABA A receptors (Lüddens et al., 1990). Antibodies generated against these sequences were able to immunoprecipitate native GABA A receptor subunits from rat cerebellar membranes in a dose-dependent manner (Fig. 1). Whereas anti-peptide α6(1–15) antibodies precipitated up to 15 ± 4% (mean ± SD; n = 4) of all [3H]Ro 15-4513 binding sites present in the extract, anti-peptide α6(429–434) antibodies precipitated only 5 ± 1% (mean ± SD; n = 4) of these sites. Of the [3H]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 [3H]Ro 15-4513 binding sites eliminated from the supernatant was higher than that actually found in the precipitate. Thus, whether 15–20 μg of α6(1–15) or α6(429–434) antibodies was used for immunoprecipitation, the amount of [3H]Ro 15-4513 binding sites in the supernatant was reduced by 30 ± 3% (mean ± SD; n = 4) (Fig. 1). These results

Immunoprecipitation and receptor binding assay. Whereas 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 [3H]Ro 15-4513 (20.9 Ci/mmol; Du Pont 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 [3H]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 [3H]muscimol (17.1 Ci/mmol; Du Pont 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 of 1% Triton X-100, 50 mM Tris-citrate buffer, pH 7.1. When the percentage of α6 receptors retained by an immunoaffinity column had to be determined, immunoprecipitation with the anti-α6 (1–15) antibody and the subsequent [3H]muscimol binding assays were performed in the same experiment with the original extract and the immunoaffinity column efflux.

Immunoprecipitation and receptor binding assay. For immunoprecipitation, 30 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.

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Immunoprecipitation of GABA<sub>A</sub> receptors solubilized from rat cerebellum. Solubilized receptors (470 fmol of [3H]Ro 15-4513 binding sites) were incubated with increasing amounts of α<sub>6</sub>(1–15) or α<sub>6</sub>(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 [3H]Ro 15-4513 binding. Identical results were obtained when the supernatant from the α<sub>6</sub>(1–15) or α<sub>6</sub>(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 1.

### Isolation, subunit composition, and quantitative importance of GABA<sub>A</sub> receptors containing α<sub>6</sub> subunits

After solubilization of GABA<sub>A</sub> receptors from cerebellar membranes, 67.8% of the [3H]Ro 15-4513 or [3H]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 [3H]muscimol binding sites or diazepam-sensitive or -insensitive [3H]Ro 15-4513 binding sites, it can be concluded that the extracted receptors were representative of the entire functional α<sub>6</sub> subunit-containing GABA<sub>A</sub> receptor population.

To quantitatively isolate GABA<sub>A</sub> receptors containing α<sub>6</sub> subunits, cerebellar extracts were cycled three times through an immunoaffinity column containing anti-peptide α<sub>6</sub>(429–434) antibodies. In the final eluX of this column, anti-peptide α<sub>6</sub>(1–15) antibodies no longer were able to precipitate GABA<sub>A</sub> receptors, and α<sub>6</sub> subunits no longer could be demonstrated in Western blots, indicating that this procedure eliminated most if not all α<sub>6</sub> receptors from the extract. In the same eluX, [3H]Ro 15-4513 binding was reduced by 31 ± 1% (mean ± SD; n = 3), and [3H]muscimol binding was reduced by 45 ± 1% (mean ± SD; n = 3). These percentages correspond closely to the 30 ± 3% reduction of [3H]Ro 15-4513 and 42 ± 3% reduction of [3H]muscimol binding sites observed in cerebellar extracts after immunoprecipitation with α<sub>6</sub>(1–15) antibodies (see above).

To identify GABA<sub>A</sub> receptor subunits co-purifying with α<sub>6</sub> subunits, receptors bound to the α<sub>6</sub>(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<sub>A</sub> receptor subunit. As shown in Figure 3A (or Fig. 4A), in addition to the α<sub>6</sub> subunit, β<sub>2</sub>, β<sub>3</sub>, γ<sub>2</sub>, and δ subunits were present in the α<sub>6</sub>(429–434) column eluate. Thus, α<sub>6</sub>(1–9), β<sub>1</sub>(350–404), β<sub>2</sub>(351–405), β<sub>3</sub>(345–408), γ<sub>2</sub>(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<sub>A</sub> receptors containing the respective subunits (experiments not shown). The β<sub>3</sub>(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<sub>3</sub> subunit, because staining of this protein was variable in different experiments.
together with 1992). These data therefore indicate that any one of the GABA A receptor subtypes with different subunit composition can 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 α6 and γ2 subunits**

Because GABA A receptors are composed of five subunits, the co-purification of a total of seven different subunits by the α6(429–434) immunoaffinity chromatography indicated that a mixture of GABA A receptor subtypes with different subunit composition was purified. To isolate GABA A receptors containing α6, βx, and γ2 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<sub>A</sub> receptor (Jones et al., 1997). Interestingly, in the pH 2.45 eluate of the δ(1–44) eluate, α<sub>1</sub>, α<sub>6</sub>, β<sub>1</sub>, β<sub>2</sub>, β<sub>3</sub>, δ, and other subunits, but no γ<sub>2</sub> subunits, could be identified (R. Pelz, M. Jechlinger, and W. Sieghart, unpublished data).

To determine the composition of the remaining α<sub>6</sub> receptors, the efflux of the δ(1–44) column subsequently was chromatographed on the α<sub>6</sub>(429–434) column. As shown in Figure 3B, 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<sub>1</sub>, α<sub>6</sub>, β<sub>1</sub>, β<sub>2</sub>, β<sub>3</sub>, γ<sub>2</sub>) in the eluate of the α<sub>6</sub>(429–434) column indicates that GABA<sub>A</sub> receptors retained by this column were still heterogeneous.

In the elux of the δ(1–44) column, α<sub>6</sub>(1–15) antibodies were able to precipitate 70% of the [3H]muscimol binding sites that could be precipitated by these antibodies in the original extract (Fig. 3B). This indicates that 30% of the α<sub>6</sub> subunit-containing GABA<sub>A</sub> 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<sub>1</sub>(1–9) immunoaffinity column. The α<sub>1</sub>(1–9) antibody has been demonstrated to selectively identify only α<sub>1</sub> but no other GABA<sub>A</sub> receptor subunits (Nusser et al., 1996; Zezula et al., 1991). The α<sub>6</sub> subunit-containing receptors remaining in the efflux of the α<sub>1</sub>(1–9) column were then collected by the α<sub>6</sub>(429–434) column. In the pH 2.45 eluate of this column, only α<sub>6</sub>, β<sub>1</sub>, β<sub>2</sub>, β<sub>3</sub>, and γ<sub>2</sub> subunits, but no α<sub>1</sub> subunits, could be detected (Fig. 3C). The five subunits present in this eluate still could have been combined in a variety of different ways, resulting in a multiplicity of pentameric αβ or αβγ 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<sub>6</sub>, β<sub>1</sub>, β<sub>2</sub>, β<sub>3</sub>, and γ<sub>2</sub> subunits was lower in Figure 3C than in 3A or B. In the efflux of the α<sub>1</sub>(1–9) column, 32 ± 3% (mean ± SD; n = 3) of the α<sub>6</sub> subunit-containing receptors present in the original extract could be precipitated by α<sub>6</sub>(1–15) antibodies (Fig. 3C). Thus, 32% of α<sub>6</sub> receptors were collected as α<sub>6</sub>, β<sub>1</sub>, β<sub>2</sub>, β<sub>3</sub>, and γ<sub>2</sub> subunits. The observation that 70% of the α<sub>6</sub> receptors could be precipitated before and only 32% after the α<sub>1</sub>(1–9) column additionally indicates that 38% of α<sub>6</sub> receptors were removed by the α<sub>1</sub>(1–9) column and thus contained α<sub>1</sub> as well as α<sub>6</sub> subunits.

All of these percentages were obtained by investigating binding of [3H]muscimol to the precipitated receptors. Because [3H]muscimol binding sites can be demonstrated only on receptors containing α and β, or α and γ subunits (Zezula et al., 1996), these experiments indicate that the 32% of α<sub>6</sub> and 38% of α<sub>6</sub> receptors so far discussed must also have contained β subunits. Whether all or only some of these receptors additionally contained γ<sub>2</sub> subunits cannot be answered at this time.

**Isolation, subunit composition, and quantitative importance of GABA<sub>A</sub> receptors containing α<sub>6</sub> and δ subunits**

In another experiment (Fig. 4), GABA<sub>A</sub> receptors containing γ<sub>2</sub> subunits were eliminated from cerebellar membrane extracts using a γ<sub>2</sub>(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<sub>2</sub>(319–366) column, α<sub>1</sub>, α<sub>6</sub>, β<sub>1</sub>, β<sub>2</sub>, β<sub>3</sub>, γ<sub>2</sub>, and other subunits, but no δ subunits, could be identified (experiments not shown). This again supports the conclusion that γ<sub>2</sub> and δ subunits, at least in the cerebellum, seem not to be present in the same GABA<sub>A</sub> receptors.

Receptors remaining in the elux of the γ<sub>2</sub>(319–366) column were then chromatographed on the α<sub>6</sub>(429–434) column. In the eluate of this column, α<sub>1</sub>, α<sub>6</sub>, β<sub>1</sub>, β<sub>2</sub>, β<sub>3</sub>, and δ subunits, but no γ<sub>2</sub> subunits, could be detected (Fig. 4B). Immunoprecipitation with α<sub>6</sub>(1–15) antibodies in the elux of the γ<sub>2</sub>(319–366) column indicated that receptors composed of these subunits represented 30% of the α<sub>6</sub> receptors present in the original extract (Fig. 4B). All of these receptors contained the δ subunit, because 30% of all α<sub>6</sub>-containing GABA<sub>A</sub> receptors could also be bound to the δ(1–44) immunoaffinity column, as discussed above (Fig. 3B).

The identification of only 30% of the α<sub>6</sub> receptors in the elux of the γ<sub>2</sub>(319–366) column indicates that 70% of these receptors were retained by this column and thus contained γ<sub>2</sub> subunits. Combined with the above observation (Fig. 3B) that 70% of all α<sub>6</sub> receptors could be precipitated in the elux of the δ(1–44) column and were composed of α<sub>1</sub>, α<sub>6</sub>, β<sub>1</sub>, β<sub>2</sub>, β<sub>3</sub>, and γ<sub>2</sub> subunits, these data suggest that α<sub>6</sub> receptors contain either γ<sub>2</sub> or δ subunits.

In the next step, the elux of the γ<sub>2</sub>(319–366) column was chromatographed on the α<sub>1</sub>(1–9) column, and α<sub>6</sub> receptors remaining in the elux of this column were then either collected by a subsequent α<sub>6</sub>(429–434) immunoaffinity chromatography or precipitated by α<sub>6</sub>(1–15) antibodies (Fig. 4C). In the eluate of the α<sub>6</sub>(429–434) column, α<sub>1</sub>, α<sub>6</sub>, β<sub>1</sub>, β<sub>2</sub>, β<sub>3</sub>, and δ subunits, but no α<sub>1</sub> subunits, could be identified. Immunoprecipitation experiments indicated that 15% of all α<sub>6</sub> subunit-containing GABA<sub>A</sub> receptors could still be precipitated in the elux of the α<sub>1</sub>(1–9) immunoaffinity column (Fig. 4C) and thus were composed of α<sub>6</sub>βδ subunits.

Because 30% of all α<sub>6</sub> (and δ) subunit-containing receptors could be precipitated before and only about 15% after chromatography on the α<sub>1</sub>(1–9) column, these results additionally indicate that 15% of all α<sub>6</sub> subunit-containing receptors are composed of α<sub>1</sub>α<sub>6</sub>βδ subunits. Thus, the α<sub>6</sub> and δ subunit-containing receptors α<sub>1</sub>α<sub>6</sub>βδ and α<sub>6</sub>βδ 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. 4A–C). In this experiment the staining of the βδ 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<sub>6</sub> subunit-containing GABA<sub>A</sub> receptor subtypes in cerebellum that are composed of α<sub>1</sub>α<sub>6</sub>βγ<sub>2</sub>, α<sub>1</sub>α<sub>6</sub>βγ<sub>2</sub>δ, α<sub>1</sub>α<sub>6</sub>βδ, and α<sub>1</sub>α<sub>6</sub>βδγ<sub>2</sub> subunits. The same four α<sub>6</sub> receptor subtypes were also identified when the sequence of columns was changed, and an α<sub>1</sub>(1–9) column was used before the γ<sub>2</sub>(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<sub>6</sub>(1–15) or α<sub>6</sub>(429–434) antibodies were able to recognize or precipitate these four α<sub>6</sub> subunit-containing GABA<sub>A</sub> receptor subtypes with comparable efficiency. The experiments described were repeated several times.
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 cerebellum contain 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 (α_6β_1δ, 15% of all α_6 receptors) or of δ and α_1 subunits (α_6β_2γ_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. 5A). 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. 5A).

The efflux of the β_1(350–404) column was then chromatographed on a β_2(351–405) immunoaffinity column (Fig. 5B). 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_2, 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_1 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. 5C). 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. 5D) eliminated an additional 24% of the α_6 receptors. The remaining 10 ± 1% (mean ± SD; n = 3) of receptors thus contained only β_3 subunits.

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. 5E), indicating that ~37% of all α_6 receptors contained a β_3 subunit. A subsequent chromatography on a β_2(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

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

<table>
<thead>
<tr>
<th>Subunit composition</th>
<th>Percentage of α_6 receptors</th>
<th>Percentage of GABA_A receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>α_6α_6β_1γ_2</td>
<td>37 ± 3</td>
<td>16.7</td>
</tr>
<tr>
<td>α_6α_6β_1δ</td>
<td>32 ± 3</td>
<td>14.4</td>
</tr>
<tr>
<td>α_6α_6β_2γ_2</td>
<td>15 ± 3</td>
<td>6.8</td>
</tr>
<tr>
<td>α_6α_6β_3δ</td>
<td>14 ± 2</td>
<td>6.3</td>
</tr>
</tbody>
</table>

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.

**Figure 5.** Quantification of α_6 receptors containing different β subunits. [3H]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 [3H]muscimol binding sites precipitated by α_6(1–15) antibodies in the original extract and in the sum of the [3H]muscimol binding sites in the efflux of the other two β subunits retained in 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).

[3H]Muscimol binding sites present in the original extract were significantly different (p = 0.001) from the sum of the [3H]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 [3H]muscimol binding sites found in the efflux of B, D, and F.
differences (see legend to Fig. 5). Although 15% of all $\alpha_x$ receptors were removed by the $\beta_3$ column from the original extract (Fig. 5A), only 10% of $\alpha_x$ receptors were left after elimination of all $\beta_2$ and $\beta_3$ subunits (Fig. 5D). Although 66% of all $\alpha_x$ receptors were removed by the $\beta_2$ column from the original extract (Fig. 5C), only 51% of these receptors were left after removal of $\beta_1$ and $\beta_2$ receptors (Fig. 5F). Finally, although 37% of all $\alpha_x$ receptors were removed by the $\beta_3$ column from the original extract (Fig. 5E), only 21% of these receptors were left after removal of $\beta_1$ and $\beta_2$ subunits (Fig. 5B).

In addition, the sum of $\alpha_x$ receptors retained by the $\beta_1$, $\beta_2$, and $\beta_3$ columns from the original extract was 118% (Fig. 5A,C,E), whereas the sum of the receptors remaining in the extract after two of the three $\beta$ subunits had been removed was 82% (Fig. 5B,D,F). These differences could not be explained by a cross-reactivity of the antibodies, because $\beta_1$, $\beta_2$, or $\beta_3$ antibodies were unable to precipitate recombinant $\alpha_x\beta_x\gamma_x$ receptors containing the wrong $\beta$ subunit (experiments not shown). These data therefore suggest that 18% of the $\alpha_x$ receptors in cerebellum contain more than one type of $\beta$ 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$\text{A}$ receptors containing $\alpha_x$ subunits**

In the present investigation, 13 antibodies, each one highly specific for a different GABA$\text{A}$ receptor subunit, were used to investigate the subunit composition and quantitative importance of GABA$\text{A}$ receptors containing $\alpha_x$ subunits. Chromatography on an $\alpha_x(429–434)$ immunoaffinity column quantitatively removed $\alpha_x$ subunits and 45 ± 1% of all GABA$\text{A}$ receptors from cerebellar extracts, supporting previous conclusions (Khan et al., 1996; Jones et al., 1997) that 45% of all GABA$\text{A}$ receptors in the cerebellum contain the $\alpha_x$ subunit. In the eluate of this column, in addition to the $\alpha_x$ subunit, only $\alpha_x$, $\beta_1$, $\beta_2$, $\beta_3$, $\gamma_x$, and $\delta$ subunits of GABA$\text{A}$ receptors could be demonstrated, suggesting that any one of these subunits can be colocalized with $\alpha_x$ subunits in native GABA$\text{A}$ receptors.

In contrast, $\alpha_x$, $\alpha_4$, $\alpha_5$, $\gamma_1$, or $\gamma_3$ subunits did not co-purify with $\alpha_x$ subunits. This is to be expected for $\alpha_2$, $\alpha_3$, $\alpha_5$, or $\gamma_1$ 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 $\gamma_1$ and $\alpha_x$ subunits has been demonstrated previously after purification of GABA$\text{A}$ receptors by a $\gamma_1$ subunit-specific immunoaffinity column (Tögel et al., 1994). The observation that $\alpha_x$ subunits did not co-purify with $\alpha_x$ 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 $\alpha_x$ as well as $\alpha_x$ subunits, if they exist at all, are quantitatively not important. Thus, the great majority of $\alpha_x$ subunit-containing GABA$\text{A}$ receptors is composed of $\alpha_x$ and $\alpha_1$, $\beta_1$, $\beta_2$, $\beta_3$, $\gamma_x$, or $\delta$ subunits.

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

A random assembly of $\alpha_x$ subunits with six other subunits into pentameric receptors (Nayem et al., 1994; Tretter et al., 1997) would result in a total of 210 GABA$\text{A}$ 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$\text{A}$ 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 $\alpha_x$ receptors remaining in the extract then allowed us to estimate the proportion of $\alpha_x$ 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 $\alpha_x$ receptor subtypes and to determine their quantitative importance.

$\alpha_1$, $\gamma_2$, or $\delta$ subunit-containing $\alpha_x$ receptors

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

Furthermore, the number of $[^3\text{H}]$Ro 15-4513 binding sites removed from cerebellar extracts by $\alpha_x(429–434)$ or $\alpha_x(1–15)$ antibodies was 69% or 71% of the $[^3\text{H}]$muscimol binding sites eliminated by these antibodies, respectively. Because $[^3\text{H}]$Ro 15-4513 binding sites are present on GABA$\text{A}$ receptors containing $\alpha_1\gamma_2$ or $\alpha_2\gamma_2$ subunits and $[^3\text{H}]$muscimol binding sites are present on receptors composed of $\alpha_2\beta_x\gamma_x$ and $\alpha_3\delta$ subunits (Quirk et al., 1995; Sieghart, 1995; Zecula et al., 1996), these data agree with the conclusion that 70% of the $\alpha_x$ receptors contained a $\gamma_x$ subunit. The observation that the $[^3\text{H}]$muscimol binding sites of $\gamma_x$ or $\delta$ subunit-containing $\alpha_x$ receptors add up to 100% additionally indicates that all $\alpha_x$ receptors contain either a $\gamma_x$ or a $\delta$ subunit. From this it can be concluded that receptors composed of $\alpha_x\beta_x$ subunits, and consequently also those composed of $\alpha_x\gamma_x$ subunits, which would contribute to $[^3\text{H}]$Ro 15-4513 but not to $[^3\text{H}]$muscimol binding sites, are not significantly expressed in cerebellum.

Further fractionation of the 70% $\alpha_x$ receptors containing $\gamma_x$ subunits using an $\alpha_1$ subunit-specific column indicated that 37 ± 3% of $\alpha_x$ receptors were composed of $\alpha_x\beta_x\gamma_x$ and 32 ± 3% of $\alpha_x\beta_x\gamma_x$ subunits. $\alpha_x\beta_x\gamma_x$ 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 $\alpha_x\beta_x\gamma_x$ receptors, in contrast to $\alpha_x\beta_x\gamma_x$ receptors, exhibit a high affinity $[^3\text{H}]$Ro 15-4513 binding that could not be inhibited by diazepam (Lüddens et al., 1990; Sieghart, 1995). Other studies have indicated that in GABA$\text{A}$ receptors containing $\alpha_x$ and $\alpha_1$ (Khan et al., 1996) or $\alpha_1$ and $\alpha_3$ subunits (Araujo et al., 1996), each one of the subunits expressed its characteristic benzodiazepine pharmacology. Because 32% of $\alpha_x$ receptors are composed of $\alpha_x\beta_x\gamma_x$, whereas 37% are composed of $\alpha_x\beta_x\gamma_x$ subunits, these two receptor subtypes are responsible for 46.4% and 53.6% of all.
[3H]Ro 15-4513 binding sites precipitated by α6(1-15) antibodies, respectively. Assuming that αβγδ receptors contain two α6 subunits (Im et al., 1995), these two receptor subtypes contain a total of 75% α6 and 27% α1 subunits. The present observation that 23 ± 2% of [3H]Ro 15-4513 binding precipitated by α6(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% α6 receptors containing δ subunits using an α subunit-specific column indicated that 15 ± 3% of all α6 receptors were composed of α1αβδδ and 14 ± 2% of α6ββδ receptors. Although the existence of α1αβδδ receptors in cerebellum has been implicated previously (Pollard et al., 1995), their abundance was not determined.

**β Subunit composition of α6 receptors**

When β1-, β2-, and β3-specific immunoaffinity columns were used to eliminate GABAA receptors from cerebellar extracts in parallel experiments, it was demonstrated that the total percentage of α6 receptors removed was 118%. In the absence of a significant cross-reactivity of the β1, β2, or β3 subunit-specific antibodies, these data suggested the colocalization of different β subunits in 18% of the α6 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 α6 receptors containing homogeneous β subunits was then determined by measuring α6 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 α6 receptors contained homogeneous β1, β2, or β3 subunits, respectively. Because of the variability of binding data, a reliable estimation of the β subunit composition of the remaining 18% of α6 receptors was not possible. The observation that β1 and β2 as well as β3 subunits are co-purifying with α6 and γ2 (Fig. 3C) or α6 and δ subunits (Fig. 4C), however, indicates that the αβγδ or αβδδ receptor subtypes might exist in up to six isoforms containing different β subunit combinations (homogeneous β1, β2, or β3 subunits, β1β2, β1β3, or β2β3). The same might be true for receptors consisting of α1αβδδ or α1αβδδ subunits. Whether all of the resulting 24 α6 receptors with different subunit composition actually exist cannot be answered by this study.

**Subunit stoichiometry of native α6 receptors**

The present results, in agreement with studies investigating other receptors, indicate that native α6 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 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 α6 receptors. This is in agreement with studies investigating the subunit stoichiometry of αβγδ (Im et al., 1995) or of other recombinant receptors (Chang et al., 1996; Tretter et al., 1997). The method of subtractive purification of GABA<sub>A</sub> receptors developed in the present study can be used to investigate whether all native α6 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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