We have investigated the existence, molecular composition, and benzodiazepine binding properties of native cortical α₁-α₃ γ-aminobutyric acid₃ (GABA₃) receptors using subunit-specific antibodies.

The co-existence of α₁ and α₃ subunits in native GABA₃ receptors was demonstrated by immunoblot analysis of the anti-α₁- or anti-α₃-immunopurified receptors and by immunoprecipitation experiments of the [³H]zolpidem binding activity. Furthermore, immunodepletion experiments indicated that the α₁-α₃ GABA₃ receptors represented 54.7 ± 5.0 and 23.6 ± 3.3% of the α₃ and α₁ populations, respectively. Therefore, α₁ and α₃ subunits are associated in the same native GABA₃ receptor complex, but, on the other hand, these α₁-α₃ GABA₃ receptors from the cortex constitute a large proportion of the total α₃ population and a relatively minor component of the α₁ population.

The pharmacological analysis of the α₁- or α₃-immunopurified receptors demonstrated the presence of two different benzodiazepine binding sites in each receptor population with high (type I binding sites) and low (type II binding sites) affinities for zolpidem and CI 218,872. These results indicate the existence of native GABA₃ receptors possessing both α₁ and α₃ subunits, with α₁ and α₃ subunits expressing their characteristic benzodiazepine pharmacology.

The molecular characterization of the anti-α₁-antι-α₃ double-immunopurified receptors demonstrated the presence of stoichiometric amounts of α₁ and α₃ subunits, associated with β₂₂ and γ₂ subunits. The pharmacological analysis of α₁-α₃ GABA₃ receptors demonstrated that, despite the fact that each α subunit retained its benzodiazepine binding properties, the relative proportion between type I and II binding sites or between 51- and 59-61-kDa [³H]Ro15-4513-photolabeled peptides was 70:30. Therefore, the α₁ subunit is pharmacologically predominant over the α₃ subunit. These results indicate the existence of active and nonactive α subunits in the native α₁-α₃ GABA₃ receptors from rat cortex.

The neuropharmacological effects of benzodiazepines are mediated by the benzodiazepine (ω) binding sites associated with the GABA₁ receptor complex (for reviews, see Refs. 1 and 2). Based on their affinity for different drugs, two different benzodiazepine binding sites have been identified in the central nervous system. Type I (benzodiazepine receptor 1, ω₁) displays high affinity for CI 218,872 (2), β-carboline derivates (3), and the imidazopyridine zolpidem (4, 5). Type II (benzodiazepine receptor 2, ω₂) displays low affinity for these compounds. A third benzodiazepine binding site with very low affinity for zolpidem (type II, ω₃) has also been identified in isolated rat brain membranes (6) and sections (7).

Molecular cloning experiments have demonstrated the existence of five different families of subunits that are components of the GABA₃ receptor complex. Most of these families comprise several isoforms: α₁-α₆, β₁-β₃, γ₁-γ₃, δ, and ρ₁ and ρ₂ (for reviews, see Refs. 8 and 9). A minimum of α, β, and γ subunits should be co-expressed in transfected cells to resemble all the pharmacological properties of native GABA₃ receptors (10). On the other hand, the presence of different α subunits determines the affinity of the different benzodiazepine binding sites. In this sense, the α₁-β₁-β₂-γ₂ combination confers type I pharmacology to the recombinant GABA₃ receptor (i.e. high affinity for, among others, zolpidem and CI 218,872) (11). Type II properties are conferred by the presence of α₂, α₃, or α₄ subunits (11, 12).

Several approaches have been taken to identify which subunits co-exist in the native GABA₃ receptor complex. However, the subunit composition of the different native GABA₃ receptor complexes remains unsolved. Immunoprecipitations or immunofluorescence purifications using anti-α subunit antibodies (anti-α₁, -α₂, -α₃, -α₅, and -α₆ subunits) indicated that a significant proportion of native receptors are made by the association of two different α subunits (such as α₁α₂, α₁α₅, α₁α₆, or α₁α₅) (13-17) in one single receptor complex. However, other authors have indicated the absence of association between different α subunits (18, 19). On the other hand, the pharmacological properties of these GABA₃ receptors are also unknown.

In the present article we have addressed these questions by determining the molecular and pharmacological properties of the immunopurified receptors using subunit-specific antibodies to the major α subunits expressed in the rat cerebral cortex, the α₁ and α₃ subunits.

**EXPERIMENTAL PROCEDURES**

Materials—[³H]Zolpidem (58.0 Ci/mmol), [³H]flumazenil (75.2 Ci/mmol), [³H]Ro15-4513 (24.1 Ci/mmol), and [³H]flunitrazepam (84.0 Ci/mmol).

* The abbreviations used are: GABA₃, γ-aminobutyric acid; PBS, phosphate-buffered saline; FMZ, flumazenil; FNB, flunitrazepam; mAb, monoclonal antibody; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonic acid.

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mmol) were from Du Pont NEN. Zolpidem was synthesized in the preclinical research department of Synthélabo Recherche. Cl 218,872 was from Cyanamid. All other benzodiazepines were from Hoffmann-La Roche.

Antibody Preparation—Peptides NH2–α1 (amino acids 1–10, pyroglutamyl-GESRRQEPG) and COO–α1 (amino acids 419–428, PQKLKAPT-PHQ) were synthesized and coupled to keyhole limpet hemocyanin, via an extra tyrosine located at the COOH or NH2 terminal of α1 or α2 peptides, by N-acryloyl cysteamine coupling (1:2) in Freund’s complete adjuvant followed 20 days later by a booster injection of conjugate with incomplete adjuvant (1:1). Rabbits were then boosted every 2–3 weeks. The animals were bled 10 days after each booster injection. Development of an immune response was followed by immunoprecipitation of the solubilized receptor.

The antibodies were purified through peptide affinity columns. The α1 and α2 peptides were coupled to adipic acid dihydrazide-agarose (sigma) or CNBr-activated Sepharose 4B (Pharmacia Biotech), respectively, as recommended by the manufacturer. Two ml of α1- or α2-antiserum (diluted 1/5 in PBS) were recirculated, overnight at 4 °C, by 1-ml cartridges (150 ml of Sepharose) with 10 ml of α1 or α2 peptides. After coupling, the column was washed three times with 1.4 ml of solubilization buffer, and used for IgG-protein A-Sepharose complexes were isolated by centrifugation, using peptides from 2–10 or 1–15 amino acids of the NH2-terminus of α1 or α2, by Neosystem SA (Strasbourg, France). For immunizations, rabbits (New Zealand White) were subcutaneously injected with 200 μg of peptide emulsified (1:2) in Freund’s complete adjuvant followed 20 days later by a booster injection of conjugate with incomplete adjuvant (1:1). Rabbits were then boosted every 2–3 weeks. The animals were bled 10 days after each booster injection. Development of an immune response was followed by immunoprecipitation of the solubilized receptor.

For immunoblots, the purified antibodies were labeled with digoxigenin as recommended by the manufacturer (Boehringer Mannheim). The digoxigenin incorporated into anti-α1 or anti-α2 antibodies was determined by enzyme-linked immunosorbent assay or dot blot. Both antibodies displayed a similar activity (not shown).

Membrane Preparation and Receptor Solubilization—Membranes from a 3-month-old Wistar rat cerebral cortex were prepared as described elsewhere (6, 20) in presence of protease inhibitors: 1 mM EDTA, 1 mM dithiothreitol, 1 mM benzamidine, 50 mM NaCl, and 10 mM Tris-HCl, pH 7.5 (solubilization buffer), containing 1 mM EGTA, 1 mM dithiothreitol, 1 mM benzamidine, 50 μg/ml trypsin inhibitor type II-S, and 50 μg/ml bacitracin.

The GABA receptor was solubilized at 4 mg of protein/ml at 4 °C for 60 min with 0.5% (w/v) sodium deoxycholate, 0.5% (w/v) CHAPS, 140 mM NaCl, and 10 mM Tris-HCl, pH 7.5 (solubilization buffer), containing the same protease inhibitors as above. After centrifugation at 100,000 g for 60 min at 4 °C, the supernatant was collected. The recovery of the benzodiazepine binding activity in the solubilized material was determined spectrophotometrically (6, 20). The binding assays were done essentially as described previously (26) with the following modifications. The incubation time was extended to 60 min at 4 °C, the medium was replaced by 1 ml of 0.2 M ethanolamine, pH 8.3, and the fractions (0.5 ml) were neutralized by 1 M Tris, pooled and dialyzed in 1 liter of PBS (overnight at 4 °C).

For immunopurification, the native receptors were solubilized from rat cortical membranes in a dose-dependent manner (not shown). The maximal immunoprecipitation (64 ± 2 or 22 ± 4% of the [3H]FMZ binding) was achieved with 5 or 25 μg of anti-α1 or anti-α2 antibody, respectively. Similar results were obtained with 10 nm [3H]FMZ (not shown). A second round of incubation with saturating amounts of either antibody immunoprecipitated less than 5% of either binding activity (not shown).

The specificity of these polyclonal antibodies (Fig. 1) has been tested by different criteria. 1) In dot blot experiments using immobilized peptides, anti-α1 or anti-α2 antisera exclusively recognized the peptide used as antigen (NH2-terminal peptide 1–10). 2) In immunoblots using the affinity-purified GABAα receptor (Fig. 1D) anti-α3 antisera immunoreacted with a faint band of Mr 59,000 (α3 subunit), whereas anti-α1 strongly reacted with a single band of Mr 51,000 (α1 subunit). 3) In immunoblots using extracts from cortical membranes (Fig. 1E) anti-α3 immunostained two bands of Mr 59,000 and 61,000 (see Ref. 15) and, on the other hand, anti-α1 recognized a Mr 51,000 peptide. A nonspecific

RESULTS

Anti-α3 and -α1 Antibodies—Specific polyclonal antibodies have been generated against peptides from the NH2-terminal domain (amino acids 1–10) of the α3 subunit or the C-terminal domain (amino acids 419–428) of α1 subunit of the GABAα receptor complex. Both polyclonal antibodies immunoprecipitated the native receptors solubilized from rat cortical membranes in a dose-dependent manner (not shown). The maximal immunoprecipitation (64 ± 2 or 22 ± 4% of the [3H]FMZ binding) was achieved with 5 or 25 μg of anti-α1 or anti-α3 antibody, respectively. Similar results were obtained with 10 nm [3H]FMZ (not shown). A second round of incubation with saturating amounts of either antibody immunoprecipitated less than 5% of either binding activity (not shown).

The specificity of these polyclonal antibodies (Fig. 1) has been tested by different criteria. 1) In dot blot experiments using immobilized peptides, anti-α3 or anti-α1 antisera exclusively recognized the peptide used as antigen (NH2-terminal from α3 or COO-terminus from α1 subunits, respectively, Fig. 1A). Neither anti-α3 nor anti-α1 antisera immunoreacted with peptides from similar regions of other α subunits (Fig. 1A). 2) The immunoprecipitation of the native receptors was specifically inhibited by the peptide used as antigen but not by others corresponding to similar NH2- or COO-terminus of other α subunits (Fig. 1A, B, and C). 3) In immunoblots using the affinity-purified GABAα receptor (Fig. 1D) anti-α3 antisera immunoreacted with a faint band of Mr 59,000 (α3 subunit), whereas anti-α1 strongly reacted with a single band of Mr 51,000 (α1 subunit). 4) In immunoblots using extracts from cortical membranes (Fig. 1E) anti-α3 immunostained two bands of Mr 59,000 and 61,000 (see Ref. 15) and, on the other hand, anti-α1 recognized a Mr 51,000 peptide. A nonspecific
Pharmacological Properties of $\alpha_1$-$\alpha_3$-containing GABA$_A$ Receptors

**Fig. 1.** Specificity of anti-$\alpha_3$ and anti-$\alpha_1$ antisera. A, different amounts of peptides from NH$_2$ termini (amino acids 1–10) of $\alpha_3$, $\alpha_1$, or $\alpha_2$ subunits or COOH termini of $\alpha_1$ (amino acids 419–428), $\alpha_3$ (amino acids 459–465), or $\alpha_2$ (amino acids 424–433) were blotted and immunostained with anti-$\alpha_3$ (1/1000 dilution) and anti-$\alpha_1$ (1/1000 dilution) antisera, respectively. B and C, solubilized receptor (0.1–0.2 pmol of [H]$FMZ$ binding activity) was immunoprecipitated with 0.5 or 7.5 µl of anti-$\alpha_3$ or anti-$\alpha_1$ antisera, respectively, in the presence of increasing concentrations of the peptides specified above. B, COOH-terminal peptides $\alpha_1$ (●), $\alpha_3$ (○), and $\alpha_2$ (△); C, NH$_2$-terminal peptides $\alpha_1$ (■), $\alpha_3$ (●), and $\alpha_2$ (△). No inhibition was observed when anti-$\alpha_1$ or anti-$\alpha_3$ antisera were incubated in presence of NH$_2$-terminal $\alpha_1$ or COOH-terminal $\alpha_3$ peptides, respectively (not shown). Results are expressed as percentages from the [H]$FMZ$ binding activity immunoprecipitated in absence of peptide and are mean ± S.D. (bars) of three independent experiments. D, GABA$_A$ receptors purified from adult bovine cerebral cortex (1 µg/lane) were subjected to SDS-polyacrylamide gel electrophoresis, blotted, and immunostained with anti-$\alpha_3$ (1/500 dilution) or anti-$\alpha_1$ (1/500 dilution). Both antibodies show reactivity with single peptide bands of 59 and 51 kDa for anti-$\alpha_3$ and anti-$\alpha_1$, respectively. E, cortical membranes (75 µg of protein/lane) were processed as above and immunostained with 10 µg/ml of purified anti-$\alpha_3$ or anti-$\alpha_1$ antibodies. The mAb 62-3G1 (1/5 dilution), specific for $\beta_3$ and $\beta_2$ subunits, was included as a reference. The molecular sizes of the subunits are 58 and 60 kDa ($\alpha_3$), 51 kDa ($\alpha_1$) and 55–57 kDa ($\beta_3$ and $\beta_2$).

A band of 100 kDa was also observed in some experiments (also see Fig. 2B). The mAb 62-3G1 (specific to $\beta_3$ and $\beta_2$, M. 55,000–57,000 peptides; Refs. 27 and 31) was included as a control. 5) In the three brain regions studied (cortex, hippocampus, and cerebellum), the percentage of immunoprecipitation by these antibodies is consistent with the level of expression of $\alpha_3$ or $\alpha_1$ subunits, determined by in situ hybridization or immunoprecipitation (18, 19, 32–35). As expected, the anti-$\alpha_1$ antisemum immunoprecipitated most of the [H]$FMZ$ binding activity from the cerebellum (85.3 ± 7.5%), followed by the cortex (71.0 ± 5.3%) and hippocampus (52.2 ± 2.0%). Anti-$\alpha_3$ antisemum immunoprecipitated a low proportion of receptors compared with anti-$\alpha_1$. The maximal immunoprecipitation was obtained in the cortex (25.8 ± 4.7%), followed by the hippocampus (19.1 ± 3.2%) and cerebellum (9.8 ± 3.5%). In conclusion, by all these criteria both antibodies are specific for their corresponding subunits.

Association between $\alpha_1$ and $\alpha_2$ Subunits—To determine the presence of $\alpha_3$ subunits, co-assembled with $\alpha_1$ subunits in the same receptor complex, we first quantified the [H]$zolpidem binding activity immunoprecipitated by anti-$\alpha_3$ antisemum. [H]$zolpidem binds with high affinity to $\alpha_1$ subunit-containing GABA$_A$ receptors (type I benzodiazepine binding sites) (11, 12, 21, 22). Therefore, [H]$zolpidem (5 nM) binding activity was used as a marker of the presence of $\alpha_3$ subunits in the immunoprecipitated receptor (also see Ref. 21). The quantitative immunoprecipitation of [H]$zolpidem binding was tested by two sequential incubations with anti-$\alpha_1$ or anti-$\alpha_3$ antibodies. The second incubation yielded 3.3 ± 2.8 and 3.8 ± 1.7% of immunoprecipitation for anti-$\alpha_1$ and -$\alpha_3$, respectively, indicating that the immunoprecipitation of the receptor was maximal.

As shown in Fig. 2A, anti-$\alpha_2$ and -$\alpha_3$ antibodies immunoprecipitated 90.0 ± 5.4 and 26.9 ± 3.6% of the [H]$zolpidem binding activity, respectively. These results demonstrated that, in native GABA$_A$ receptors, the high affinity [H]$zolpidem (5 nM) binding sites (type I benzodiazepine binding sites) are largely associated with the presence of an $\alpha_3$ subunit (also see Refs. 21 and 22) and, importantly, that these sites can be immunoprecipitated in association with $\alpha_3$ subunits.

To ascertain the co-assembling of $\alpha_1$ and $\alpha_3$ subunits in the
Preparations of human embryonic kidney cells, transfected were treated with SDS, and the eluted receptor was analyzed by immunoblot using 5-mol % pmol % and anti-α3 antibodies, respectively. The α3 subunit was depleted from the solubilized receptor by three sequential incubations with anti-α1 (15 μl each) and anti-α3 (75 μl each), respectively. The α1, or α3-immunodepleted receptors were immunopurified by anti-α3 (lanes 2 and 3) and anti-α3 (lanes 5 and 6) immunofinity columns. Lanes 1 and 4, anti-α1, and anti-α1 control immunopurifications, respectively. The eluted receptor was analyzed by immunoblot using 5 μg of purified anti-α1 (lanes 1 and 3) or anti-α3 (lanes 2 and 4) antibodies. C, the α1 or α3 subunits were immunodepleted from the solubilized receptor by three sequential incubations with anti-α1 (15 μl each) and anti-α3 (75 μl each), respectively. The α1, or α3-immunodepleted receptors were immunopurified by anti-α1 (lanes 2 and 3) and anti-α3 (lanes 5 and 6) immunofinity columns. Lanes 1 and 4, anti-α1, and anti-α1 control immunopurifications, respectively. The eluted receptor was analyzed by immunoblot using 5 μg of purified anti-α1 (lanes 1, 2, and 6) or anti-α3 (lanes 3–5) antibodies. For all immunoblots, the affinity-purified antibodies were labeled with digoxigenin. Numbers on the left, M, values of the immunostained bands.

The co-purification of both α subunits was not due to cross-reaction between the antibodies. As shown in Fig. 2C (lane 2), the anti-α1 antibody produces absolutely no immunoreaction products in Western blots of the anti-α3-immunopurified receptors that have been immunodepleted of the α3 subunits. On the other hand, the anti-α3 antibody immunoreacted with 59–61-kDa peptides (Fig. 2C, lane 3), demonstrating the presence of α3-containing GABA<sub>A</sub> receptors. Conversely, no immunoreaction products were produced by the anti-α3 antibody using the α1 immunodepleted and anti-α1-immunopurified receptor as an antigen (Fig. 2C, lanes 5 and 6). Furthermore, in membrane preparations of human embryonic kidney cells, transfected with the α1-γ2-β2 combination, the anti-α1 antibody immunostained a single 51-kDa peptide, whereas no immunoreaction products were detected using the anti-α3 antibody (not shown). These results clearly demonstrate the absence of cross-reaction between the antibodies and confirm the co-purification of both α subunits in the same receptor complex.

It could be argued that the co-purification of two different α subunits, such as α1 and α3, was due to interactions between individual GABA<sub>A</sub> receptor complexes through cytoskeletal elements (36) or, on the other hand, to the presence of anomalous receptors (partially assembled receptors) due to the solubilization of intracellular stores in our membrane preparations (37). Additional control experiments were performed to test these possibilities. Treatment of the cortical membranes, prior to solubilization and purification, with 5 μg/ml demecolcine (a tubulin-depolymerizing agent) or 10 μg/ml cytochalasin D (an actin-depolymerizing agent) did not modify the percentage of

Table I

<table>
<thead>
<tr>
<th>Solubilized receptor</th>
<th>Anti-α1 column</th>
<th>Anti-α3 column</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bound</td>
<td>19.3 ± 0.9</td>
<td>64.3 ± 1.5</td>
</tr>
<tr>
<td>19.3 ± 0.9</td>
<td>64.3 ± 1.5</td>
<td>6.1 ± 1.5</td>
</tr>
<tr>
<td>pH 11.5 elution</td>
<td>6.3 ± 0.2</td>
<td>21.3 ± 3.1</td>
</tr>
<tr>
<td>6.3 ± 0.2</td>
<td>21.3 ± 3.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>6.3 ± 0.2</td>
<td>21.3 ± 3.1</td>
<td>0.7 ± 0.3</td>
</tr>
</tbody>
</table>
Pharmacological Properties of α₁-α₆-containing GABAA Receptors

TABLE II

Quantification of the association between α₁ and α₃ subunits by immunodepletion experiments

The immunodepletion of α₁ or α₃ subunits was done by two sequential incubations of the solubilized receptor (1 pmol of [³H]FMZ binding sites) with either anti-α₁ (10 + 10 μl) and anti-α₃ (75 + 75 μl) antibodies, respectively. The remaining receptor was immunoprecipitated by incubation with 75 or 10 μl of anti-α₁ and -α₃ antisera, respectively. The binding activity was determined in pellets and supernatants. The results are expressed as pmol of [³H]FMZ (5 nM) or [³H]zolpidem (5 nM) specific binding activity immunoprecipitated in each condition. The percentage of depletion was calculated from the specific binding activity immunoprecipitated before and after depletion. Data are mean ± S.D. of three to six independent experiments.

<table>
<thead>
<tr>
<th>Subunit depleted</th>
<th>Subunit immunoprecipitated</th>
<th>[³H]FMZ</th>
<th>Specific binding activity</th>
<th>[³H]zolpidem</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pmol</td>
<td>% of depletion</td>
<td>pmol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>immuno-</td>
<td></td>
<td>immuno-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>precipitated</td>
<td></td>
<td>precipitated</td>
</tr>
<tr>
<td>α₁</td>
<td>α₃</td>
<td>0.21 ± 0.04</td>
<td>54.7 ± 5.0</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>α₂</td>
<td>α₃</td>
<td>0.10 ± 0.01</td>
<td>0.005 ± 0.004</td>
<td>0.05 ± 0.01</td>
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<tr>
<td>α₃</td>
<td>α₁</td>
<td>0.70 ± 0.05</td>
<td>23.6 ± 3.3</td>
<td>0.17 ± 0.01</td>
</tr>
</tbody>
</table>

As mentioned above, the second immunoprecipitation with either antibody or for either binding site yielded a residual 3–4% of immunoprecipitation. Therefore, after two rounds of immunoprecipitation, a particular α subunit should be completely depleted from the solubilized material. However, no attempts were made to detect the depleted subunit remaining in the supernatants. We are aware that some residual amounts of the depleted subunit could persist in the solubilized receptor. Therefore, these results could be, in some extent, underestimated.

Pharmacological Properties of Anti-α₁- or Anti-α₆-immunopurified Receptors from Rat Cortex—The pharmacological properties of the anti-α₁- or anti-α₆-immunopurified receptors were determined by [³H]zolpidem and [³H]FMZ saturation studies and by displacement experiments using aliquots from the immunopurified membrane preparations.

The co-assembling of α₁ and α₃ in the same receptor complex also could be due to redistribution of subunits during solubilization. This possibility was tested by determining the immunoprecipitation by anti-α₃ of the diazepam-insensitive [³H]Ro 15-4513 binding sites in solubilized receptors from cerebellar membranes or from the mixture (1:1) of cerebellar plus cortical membranes. The diazepam-insensitive binding sites are associated with the presence of α₂ subunits (38, 39), and this subunit is not expressed in the cortex (32, 33, 39). The immunoprecipitation of diazepam-insensitive [³H]Ro 15-4513 binding activity by anti-α₃ was very low and similar in both solubilized preparations, pure cerebellar membranes, and a mixture of cerebellar and cortical membranes (0.01 ± 0.01 and 0.01 ± 0.01 pmol, n = 2, respectively), thus indicating that no apparent subunit redistribution takes place due to solubilization procedures.

The association between both α subunits was quantified by immunodepletion experiments. In these experiments, a particular α subunit was depleted by two sequential immunoprecipitations with the specific antisera. After depletion, the remaining GABAA receptor complex was immunoprecipitated by the other α subunit. As shown in Table II, depletion of α₁ subunits produced a significant decrease in the [³H]FMZ binding activity immunoprecipitated by anti-α₁ antisera (0.21 ± 0.04 versus 0.10 ± 0.01 pmol, respectively). Thus, 54.7 ± 5.0% of the benzodiazepine binding activity immunoprecipitated by anti-α₁ was depleted by preincubation with the anti-α₁ antisera. On the other hand, most of the [³H]zolpidem immunoprecipitated by the anti-α₃ antisera was depleted by preincubation with the anti-α₃ antibody (89.0 ± 7.8%; 0.05 ± 0.01 versus 0.005 ± 0.004 pmol). These results indicated that most, if not all, of the high affinity binding sites immunoprecipitated by the anti-α₃ antisera were due to the presence of an α₁ subunit.

Reciprocally, depletion of α₃ subunits also affected the immunoprecipitation by the anti-α₁ antisera. As shown in Table II, depletion of α₃ subunits produced a decrease in the [³H]FMZ or [³H]zolpidem binding activity immunoprecipitated by anti-α₁ (0.70 ± 0.05 or 0.24 ± 0.01 pmol versus 0.54 ± 0.03 or 0.17 ± 0.01 pmol for [³H]FMZ and [³H]zolpidem, respectively). Thus, 20–25% of the α₁ population is associated with an α₃ subunit in the same receptor complex.

[³H]zolpidem binding activity immunoabsorbed to anti-α₃ immunohanes (26.3 ± 1.5 versus 32.5 ± 5.4 and 31.2 ± 3.7%, n = 3, for control and demecolcine- or cytchalasin D-treated membranes, respectively). Furthermore, after treatment with either drug, two [³H]Ro15-4513 photoaffinity-labeled peptides of 51 and 59–61 kDa were immunopurified by anti-α₁ columns (not shown). On the other hand, results similar to those shown in Fig. 2A were obtained using purified synaptic membranes as starting material (not shown).

The association between both α₁ and α₃ subunits was quantified by immunodepletion experiments in these experiments, a particular α subunit was depleted by two sequential immunoprecipitations with the specific antisera. After depletion, the remaining GABAA receptor complex was immunoprecipitated by the other α subunit. As shown in Table II, depletion of α₁ subunits produced a significant decrease in the [³H]FMZ binding activity immunoprecipitated by anti-α₁ antisera (0.21 ± 0.04 versus 0.10 ± 0.01 pmol, respectively). Thus, 54.7 ± 5.0% of the benzodiazepine binding activity immunoprecipitated by anti-α₁ was depleted by preincubation with the anti-α₁ antisera. On the other hand, most of the [³H]zolpidem immunoprecipitated by the anti-α₃ antisera was depleted by preincubation with the anti-α₃ antibody (89.0 ± 7.8%; 0.05 ± 0.01 versus 0.005 ± 0.004 pmol). These results indicated that most, if not all, of the high affinity binding sites immunoprecipitated by the anti-α₃ antisera were due to the presence of α₁ subunit.

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Pharmacological Properties of Anti-α₁- or Anti-α₆-immunopurified Receptors from Rat Cortex—The pharmacological properties of the anti-α₁- or anti-α₆-immunopurified receptors were determined by [³H]zolpidem and [³H]FMZ saturation studies and by displacement experiments using aliquots from the immunopurified membrane preparations.

The presence of two pharmacologically distinct receptors in the anti-α₃ and anti-α₁-immunopurified receptors also has been tested by displacement studies of [³H]FMZ by type I-specific ligands (such as zolpidem or CI 218,872). In both anti-α₁- and anti-α₆-immunopurified receptors and for both zolpidem and CI 218,872, the Hill slope of the displacement curves was lower than unity (Table III), indicating the existence of a heterogeneous population of binding sites. Furthermore, in every case, displacement curves were better fitted (based on the extra sum of squares using the program LIGAND, three of three experiments; p < 0.05) to a two binding site model with high (Kₕ, 5–8 and 40–70 nM for zolpidem and CI 218,872, respectively) and low (Kₕ, 390–430 nM and 2–3.5 μM for zolpidem or CI 218,872, respectively) affinity (Table III). In contrast, diazepam displaced the [³H]FMZ binding immunopurified by the anti-α₃ antibody, with a Hill coefficient of 1.2 and a single high affinity site (Kₕ, 7.1 nM). It is interesting that all the calculated Kₕ values were very similar to those determined in crude cortical membrane preparations for type I and II benzodiazepine receptors (6, 24).

Immunopurification and Pharmacological Properties of α₅-α₆ GABAA Receptors—The association of α₅ and α₁ subunits and the pharmacological properties of the α₅-α₁ GABAA receptors were further tested by using sequential immunopurification (see Refs. 14 and 16). The GABAA receptor was first immunopurified by anti-α₁ immunoaffinity columns, and the eluted
Pharmacological Properties of α1-α2-containing GABA<sub>A</sub> Receptors

The solubilized receptor was immunoadsorbed to anti-α<sub>1</sub> or anti-α<sub>3</sub> affinity columns. For saturation or displacement experiments, aliquots of the anti-α<sub>1</sub> or anti-α<sub>3</sub> immunobeads (0.4–0.6 pmol of [3H]FNZ binding activity/tube) were used. Saturation experiments were done by incubating the immunobeads with five or six different concentrations of [3H]FNZ (1–20 nM) or [3H]zolpidem (1–10 nM). The Scatchard transformation of the data was performed by LIGAND. Displacement experiments were performed by determining the binding activity of 2 nM [3H]FNZ and 13 or 10 different concentrations of zolpidem (ranging from 5 × 10<sup>−8</sup> to 10<sup>−10</sup> M), respectively. Displacement curves were fit (LIGAND) to a one or two binding site model. Results, mean ± S.D. of three experiments, are expressed in nM.

### Table III

<table>
<thead>
<tr>
<th>Immunoaffinity column</th>
<th>Ligand</th>
<th>Scatchard or displacement experiments</th>
<th>One binding site, K&lt;sub&gt;i&lt;/sub&gt; or K&lt;sub&gt;a&lt;/sub&gt;</th>
<th>Two binding sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
<td>K&lt;sub&gt;a&lt;/sub&gt;</td>
</tr>
<tr>
<td>Anti-α&lt;sub&gt;3&lt;/sub&gt;</td>
<td>[3H]FNZ</td>
<td>0.94 ± 0.02</td>
<td>3.5 ± 2.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[3H]Zolpidem</td>
<td>0.96 ± 0.03</td>
<td>14.8 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>Zolpidem</td>
<td>0.57 ± 0.05</td>
<td>204 ± 68</td>
<td>80 ± 7.1</td>
<td></td>
</tr>
<tr>
<td>CI 218,872</td>
<td>0.55 ± 0.02</td>
<td>1,525 ± 220</td>
<td>67.5 ± 9.0</td>
<td></td>
</tr>
<tr>
<td>Anti-α&lt;sub&gt;1&lt;/sub&gt;</td>
<td>[3H]FNZ</td>
<td>0.94 ± 0.02</td>
<td>6.2 ± 1.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[3H]Zolpidem</td>
<td>0.99 ± 0.01</td>
<td>13.3 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>Zolpidem</td>
<td>0.60 ± 0.03</td>
<td>88 ± 20</td>
<td>5.0 ± 4.9</td>
<td></td>
</tr>
<tr>
<td>CI 218,872</td>
<td>0.58 ± 0.03</td>
<td>310 ± 84</td>
<td>39.6 ± 2.4</td>
<td></td>
</tr>
</tbody>
</table>

B. Anti-α1/Anti-α3 immunopurified receptors

![Diagram](image)

**Fig. 3.** Binding activity and molecular composition of the α<sub>1</sub>-α<sub>3</sub> GABA<sub>A</sub> receptor from rat cortex. A, the anti-α<sub>1</sub>-immunopurified GABA<sub>A</sub> receptors were immunoadsorbed to anti-α<sub>1</sub> (I) or anti-α<sub>3</sub> (III) immunobeads, and the [3H]FMZ (5 nM) or [3H]zolpidem (5 nM) binding activity was quantified in pellets and supernatants. The results, expressed as percentages of binding activity retained by the columns, are mean ± S.D. (bars) of three experiments. B, the GABA<sub>A</sub> receptors were immunopurified by anti-α<sub>1</sub> and anti-α<sub>3</sub> columns in series. The SDS-eluted material was blotted and incubated with 5 μg of purified anti-α<sub>1</sub> (lane 1) or anti-α<sub>3</sub> (lane 2) antibodies, a 1/5 dilution of the mAb 62-3G1 (specific for β<sub>2</sub> and β<sub>3</sub> subunits; lane 3), or 5 μg of purified anti-γ<sub>2</sub> antibody (lane 4). Numbers on the left, M<sub>r</sub> values of the immunostained bands.

The stoichiometry between both α subunits was estimated by densitometric analysis of semiquantitative immunoblots (Fig. 4; also see Ref. 16). For these experiments, a fixed amount of receptor was immunoblotted and incubated with increasing concentrations of both antibodies in combination. After 4 h of incubation, the medium was aspirated and replaced by a new batch of antibodies. The immunoreaction products were quantified by densitometry. As shown in Fig. 4, A and B, at saturating concentrations, both antibodies yielded similar amounts of immunoreaction products. Thus, these results indicated a stoichiometry of approximately 1:1 (α<sub>3</sub>/α<sub>1</sub> ratio, 1.1 ± 0.1, n = 2; Fig. 4B).

Finally, we have tested the pharmacological properties of the α<sub>1</sub>- and α<sub>2</sub>-immunopurified GABA<sub>A</sub> receptors by displacement experiments with Cl 218,872 or zolpidem and also by [3H]Ro15-4513 photoaffinity labeling experiments of the double-immunopurified receptor. The results are shown in Table IV and Fig. 5. The displacement experiments of both [3H]FNZ or [3H]FMZ (not shown) binding activity by both zolpidem or Cl 218,872 demonstrated the presence of two different binding sites with high (type I) and low (type II) affinities. The proportion between both binding sites, calculated from displacement ex-
The molecular composition of native GABA\(_A\) receptors is unknown. Evidence is accumulating for the existence of different \(\alpha\) subunit combinations (such as \(\alpha_1\alpha_2\), \(\alpha_1\alpha_3\), \(\alpha_3\alpha_2\), and \(\alpha_1\alpha_3\)) co-assembled in single native GABA\(_A\) receptor complexes. However, other studies also indicated the absence of coexistence between different \(\alpha\) subtypes (18, 19). On the other hand, it is currently accepted that the benzodiazepine binding properties of the GABA\(_A\) receptors are mainly determined by the \(\alpha\) subunits (10, 11, 12). Therefore, if two different \(\alpha\) subunit subtypes are co-assembled in a single GABA\(_A\) receptor, two pharmacologically different benzodiazepine binding sites could co-exist in a single complex. In the present article we have investigated the possible existence and the pharmacological properties of native \(\alpha_1\alpha_3\)-GABA\(_A\) receptors from the rat cortex.

The presence of \(\alpha_1\) and \(\alpha_3\) subunits in the same GABA\(_A\) receptor complex was demonstrated by immunoprecipitation and immunopurification experiments. It has been described that, in transfected GABA\(_A\) receptors, the high affinity binding sites for zolpidem (type I benzodiazepine binding sites) are determined by the presence of \(\alpha_1\) subunits. Other \(\alpha\) subtypes (such as \(\alpha_2\), \(\alpha_3\), and \(\alpha_5\)) confer low affinity for this ligand (type II benzodiazepine receptors) (11, 12). Therefore, the association of an \(\alpha_3\) with other \(\alpha\) subunits could be estimated by immunoprecipitation of the \(^3\text{H}\)zolpidem binding activity. Our immunoprecipitation experiments (Fig. 2A and Refs. 21 and 22) demonstrate that most, if not all (90.0 ± 5.4%), of the high affinity binding sites for zolpidem are due to the presence of an \(\alpha_1\) subunit in the GABA\(_A\) receptor. Importantly, 25–30% of these \(^3\text{H}\)zolpidem binding sites were immunoprecipitated by anti-\(\alpha_3\) antibody, thus suggesting an \(\alpha_1\alpha_3\) association. Consistently, immunodepletion of the \(\alpha_1\) subunits suppress the immunoprecipitation of \(^3\text{H}\)zolpidem binding activity. Our immunoprecipitation experiments (Fig. 2A and Refs. 21 and 22) demonstrate that most, if not all (90.0 ± 5.4%), of the high affinity binding sites for zolpidem are due to the presence of an \(\alpha_1\) subunit in the GABA\(_A\) receptor. Importantly, 25–30% of these \(^3\text{H}\)zolpidem binding sites were immunoprecipitated by anti-\(\alpha_3\) antibody, thus suggesting an \(\alpha_1\alpha_3\) association. Consistently, immunodepletion of the \(\alpha_1\) subunits suppress the immunoprecipitation of \(^3\text{H}\)zolpidem binding activity. Our immunoprecipitation experiments (Fig. 2A and Refs. 21 and 22) demonstrate that most, if not all (90.0 ± 5.4%), of the high affinity binding sites for zolpidem are due to the presence of an \(\alpha_1\alpha_3\) association. Consistently, immunodepletion of the \(\alpha_1\alpha_3\) subunits, suggesting the presence of both \(\alpha_1\) and \(\alpha_3\) subunits in the same receptor complex.

The association between both subunits was confirmed by immunopurification experiments. The immunoblot analysis of the anti-\(\alpha_1\) or anti-\(\alpha_3\)-immunopurified receptors (Fig. 2B) revealed the presence of \(\alpha_3\) immunoreaction product in the anti-

**Table IV**

Pharmacological properties of the \(\alpha_1\)- and \(\alpha_3\)-GABA\(_A\) receptors purified by anti-\(\alpha_1\) and anti-\(\alpha_3\) immunoaffinity columns in series

<table>
<thead>
<tr>
<th>Ligand</th>
<th>(n_m)</th>
<th>One binding site, (K_i)</th>
<th>Two binding sites</th>
<th>(p(28/18))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl 218,872</td>
<td>0.69 ± 0.01</td>
<td>173.5 ± 68</td>
<td>46.3 ± 2</td>
<td>950 ± 200</td>
</tr>
<tr>
<td>Zolpidem</td>
<td>0.73 ± 0.10</td>
<td>56.2 ± 6.8</td>
<td>13.0 ± 1.8</td>
<td>190 ± 89</td>
</tr>
</tbody>
</table>

DISCUSSION

The molecular composition of native GABA\(_A\) receptors is unknown. Evidence is accumulating for the existence of different \(\alpha\) subunit combinations (such as \(\alpha_1\alpha_2\), \(\alpha_1\alpha_3\), \(\alpha_3\alpha_2\), and \(\alpha_1\alpha_3\)) co-assembled in single native GABA\(_A\) receptor complexes.
α2-immunopurified receptors and, reciprocally, the presence of α3 in anti-α2-immunopurified receptors. Furthermore, the association between both α subunits was not due to interactions with cytoskeletal elements. Taken together, these results demonstrated the existence of α1-α3 GABA_A receptors from the rat cortex. Immunodepletion experiments indicated that the α1-α3 GABA_A receptors constituted a relatively minor proportion of the total α1-containing GABA_A receptors (20–25% of this population) but 50–55% of the α3 containing GABA_A receptors. Thus, and in partial agreement with previous reports (13–15), the association between two different α subunits represents a minor population from the total α1-containing receptors but a high proportion of other α subunits, such as α3.

The presence of different α subtypes, in combination with β1–β3 and γ2 subunits, determines the benzodiazepine binding properties of recombinant GABA_A receptors (10, 11, 12). As mentioned above, the α1 subunit confers type I benzodiazepine binding properties (high affinity for zolpidem and CI 218,872), whereas the α3 subunit confers type II binding properties (low affinity for these ligands). Therefore, if two different α subunits, such as α1 and α3, are co-assembled in the same receptor complex, and both α1 and α3 subunits are pharmacologically active, two different benzodiazepine binding subtypes should be discriminated in either anti-α1- and anti-α3-immunopurified receptors. As shown in Table III, in anti-α1- and anti-α3-immunopurified receptors, two different binding sites were identified. The affinities for zolpidem (determined by Scatchard and displacement experiments) or CI 218,872 (determined by displacement experiments) were similar in both immunopurified receptors and similar to those reported for type I and II benzodiazepine binding sites in cortical membranes (6, 7). Furthermore, the affinities for both ligands corresponded to those reported for recombinant receptors containing α1 subunits (high affinity binding sites) and α3 subunits (low affinity binding sites) (11, 12). In consequence, these results suggest the presence of benzodiazepine binding sites in both α1 and α3 subunits co-assembled in a single GABA_A receptor complex (also see Ref. 17).

To discern whether both α1 and α3 subunits, co-assembled in a single complex, display benzodiazepine binding activity, the GABA_A receptor was immunopurified by anti-α1 and anti-α3 affinity columns in series; therefore, the whole population of the isolated GABA_A receptors should contain two different α subunits. It is noteworthy that anti-α3 immunopurification columns retained 20–25% of the α1-immunopurified GABA_A receptors, corroborating the proportion of α1 to α3 GABA_A receptors calculated by depletion experiments (compare Fig. 3a and Table II). Immunoblot analysis (Fig. 3B) indicates that α1 and α3 subunits are mainly associated with β2/3 and γ2 in the same receptor complex, consistent with previous experiments (21, 22). The β3 subunits are a relatively minor component of the receptor (41), and, on the other hand, it has been demonstrated that γ1 is not associated with γ2-containing GABA_A receptors (42). Thus, we propose a molecular composition of α1, α3, β2/3 and γ2 for these native GABA_A receptor complexes from rat cortex.

A relevant question to ascertain the pharmacological activity of the α subunits, co-assembled in a single native GABA_A receptor, is the stoichiometry between both subunits in the complex. Thus, we have estimated the stoichiometry between both α subunits by quantifying the immunoreaction products of anti-α1 and anti-α3 antibodies in immunoblots. We are aware that immunoblots are only semiquantitative. However, within the limitations of the technique, the results (Fig. 4) indicated the presence of stoichiometric amounts of each α subunit (ratio 1:1; also see Ref. 16 for discussion). The stoichiometry of γ2, β2, and β3 subunits was not determined.

If both α subunits display benzodiazepine binding sites, the double-immunopurified receptors should display type I and II binding properties in similar proportions, and two peptides should be photoaffinity labeled by [3H]Ro15-4513 to a similar extent. Indeed, the pharmacological analysis of the α1-α3 GABA_A receptors indicated the presence of two different benzodiazepine binding sites. Both CI 218,872 and zolpidem discriminated between two different binding sites with high (type I) and low affinities (type II). The calculated Ki values for either ligand were similar to those of immunopurified α1 or α3 receptors (compare Tables III and IV) and to cerebral membranes (6, 7). However, the proportion between both binding sites (70:30 for high and low affinity, respectively) demonstrates that the α1 subunits are predominantly active over the α3 subunits. It could be argued that the different proportions between both binding sites, determined by displacement experiments, is due to differences in the Ki values of α1 and α3 subunits for the benzodiazepine ligands.
Benzodiazepine binding sites of the α₁-α₅ GABAₐ receptors from rat cortex

3H-labeled ligand ([3H]Fluzol or [3H]Mol). However, these results were confirmed by [3H]Ro15-4513 photoaffinity-labeling experiments at three different degrees of saturation. As expected, in the double-immunopurified receptors, two photolabeled peptides of 51 kDa (corresponding to α₂ subunits) and 59–61 kDa (α₃ subunits) were identified. However, despite the fact that both α₂ subunits are assembled in stoichiometric amounts in the same receptor complex, the proportion between both photolabeled peptides (at all three concentrations) was 70:30 for 51 and 59–61 kDa, respectively (Fig. 5). Thus, α₂ subunits are pharmacologically predominant over the α₃ subunits. It should be noted that [3H]Ro15-4513 photolabeled sites could be determined by the distribution of the benzodiazepine binding sites (44, 45), and, on the other hand, two different subunits containing two benzodiazepine binding sites (Fig. 6 for a model). As shown in Fig. 6A, 70% of the α₁-α₂ GABAₐ receptors may be assembled by a functional α₁ subunit associated with an inactive α₂ subunit. The remaining 30% of the population may be constituted by a functional α₂ subunit associated with inactive α₁ subunits. Nevertheless, we cannot completely exclude the existence of α₁-α₂ GABAₐ receptors containing two benzodiazepine binding sites (Fig. 6B). In such a model, in which two functional α₁ and α₂ subunits are co-localized in the same receptor complex, 60% of the benzodiazepine binding sites should be conferred by GABAₐ receptors containing two functional α subunits and 30% by functional α₁ subunits associated with inactive α₂ subunits. Our results do not allow discrimination between these two models.

The presence or absence of active benzodiazepine binding sites could be determined by the distribution of the α and γ₂ subunits in the pentameric GABAₐ receptor complex (10, 44). It has been proposed that both α and γ₂ subunits are implicated in the benzodiazepine binding sites (44, 45), and, on the other hand, the GABAₐ receptors may contain two α subunits, two β subunits, and a single γ₂ subunit (46, 47). Thus, the predominance of α₁ pharmacology (type I benzodiazepine binding sites or the 51-kDa photolabeled peptides) could be interpreted by the presence of a single γ₂ subunit properly associated with the α₁ subunit in the α₁-α₂ GABAₐ receptor complex (Fig. 6A). In these receptors, the α₂ subunits should lack the benzodiazepine binding sites (see Fig. 6). On the other hand, two different γ₂ subunits could also co-exist in the same receptor complex (46, 48). If this is the case, both α subunits could display benzodiazepine binding properties (Fig. 6B).

The physiological significance of GABAₐ receptors containing two different α subtypes, such as an α₁-α₃ combination, is unknown. The α₁ subunit is highly and uniformly expressed in all cortical layers, whereas the expression of the α₃ subunit is localized in layers V and VI (49). Therefore, the α₁- and α₃-containing GABAₐ receptors should be restricted to these cortical layers. Co-localization of α₁ and α₃ subunits has been also observed in other discrete brain regions (such as mitral cells of the olfactory bulb and the medial septum; Ref. 49). On the other hand, in recombinant GABAₐ receptors, the co-expression of α₁, α₂, β₂, and γ₂ subunits confers unique functional properties, distinct from GABAₐ receptors containing a single α subtype (50, 51). Therefore, the presence and pharmacological activity of two different α subunit subtypes in native receptor complexes, localized in discrete brain areas and/or cellular regions, could influence the functional and pharmacological properties of the GABAₐ receptor. The existence and pharmacological properties of α₁α₃-containing receptors increase the heterogeneity of the native GABAₐ receptor complex in the central nervous system.

In summary, our results demonstrate the existence of cortical GABAₐ receptors containing both α₁ and α₃ subunits in stoichiometric amounts. Furthermore, both α subunits retained their benzodiazepine binding properties. However, the α₁ subunit is pharmacologically predominant over α₃ subunits, indicating the existence of active and nonactive benzodiazepine binding sites associated with these α subunits.

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REFERENCES
Pharmacological Properties of $\alpha_1\alpha_3$-containing GABA$_A$ Receptors