Molecular and Pharmacological Characterization of Native Cortical \(\gamma\)-Aminobutyric Acid\(\sub{A}\) Receptors Containing Both \(\alpha_1\) and \(\alpha_3\) Subunits*  

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We have investigated the existence, molecular composition, and benzodiazepine binding properties of native cortical \(\alpha_1-\alpha_3\) \(\gamma\)-aminobutyric acid\(\sub{A}\) (GABA\(\sub{A}\)) receptors using subunit-specific antibodies.

The co-existence of \(\alpha_1\) and \(\alpha_3\) subunits in native GABA\(\sub{A}\) receptors was demonstrated by immunoblot analysis of the anti-\(\alpha_1\) or anti-\(\alpha_3\)-immunopurified receptors and by immunoprecipitation experiments of the \([3H]\)zolpidem binding activity. Furthermore, immunodepletion experiments indicated that the \(\alpha_1-\alpha_3\) GABA\(\sub{A}\) receptors represented 54.7 ± 5.0 and 23.6 ± 3.3% of the \(\alpha_1\) and \(\alpha_3\) populations, respectively. Therefore, \(\alpha_1\) and \(\alpha_3\) subunits are associated in the same native GABA\(\sub{A}\) receptor complex, but, on the other hand, these subunits are not co-existent in the cortex. The results indicate the existence of active and nonactive GABA\(\sub{A}\) receptors was demonstrated by immunoblot analysis of the anti-\(\alpha_1\) or anti-\(\alpha_3\)-immunopurified receptors demonstrating 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 GABA\(\sub{A}\) receptors possessing both \(\alpha_1\) and \(\alpha_3\) subunits, with \(\alpha_1\) and \(\alpha_3\) subunits expressing their characteristic benzodiazepine pharmacology.

The molecular characterization of the anti-\(\alpha_1\)-anti-\(\alpha_3\) double-immunopurified receptors demonstrated the presence of stoichiometric amounts of \(\alpha_1\) and \(\alpha_3\) subunits, associated with \(\beta_2\)- and \(\gamma_2\) subunits. The pharmacological analysis of the \(\alpha_1-\alpha_3\) GABA\(\sub{A}\) receptors demonstrated that, despite the fact that each \(\alpha\) 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. The results indicate the existence of active and nonactive \(\alpha\) subunits in the native \(\alpha_1-\alpha_3\) GABA\(\sub{A}\) receptors from rat cortex.

The neuropharmacological effects of benzodiazepines are mediated by the benzodiazepine (\(\omega\)) binding sites associated with the GABA\(\sub{A}\) 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, \(\omega_1\)) displays high affinity for CI 218,872 (2), \(\beta\)-carboline derivates (3), and the imidazopyridine zolpidem (4, 5). Type II (benzodiazepine receptor 2, \(\omega_2\)) displays low affinity for these compounds. A third benzodiazepine binding site with very low affinity for zolpidem (type III, \(\omega_3\)) has also been identified in isolated rat brain membranes (6) and sections (7).

Molecular cloning experiments have demonstrated the existence of different families of subunits that are components of the GABA\(\sub{A}\) receptor complex. Most of these families comprise several isoforms: \(\alpha_1-\alpha_6\), \(\beta_1-\beta_5\), \(\gamma_1-\gamma_6\), \(\delta_1\) and \(\rho_1\) and \(\rho_2\) (for reviews, see Refs. 8 and 9). A minimum of \(\alpha\), \(\beta\), and \(\gamma\) subunits should be co-expressed in transfected cells to resemble all the pharmacological properties of native GABA\(\sub{A}\) receptors (10). On the other hand, the presence of different \(\alpha\) subunits determines the affinity of the different benzodiazepine binding sites. In this sense, the \(\alpha_1-\beta_1-\beta_2-\gamma_2\) combination confers type I pharmacology to the recombinant GABA\(\sub{A}\) receptor (i.e. high affinity for, among others, zolpidem and CI 218,872) (11). Type II properties are conferred by the presence of \(\alpha_2\), \(\alpha_3\), or \(\alpha_6\) subunits (11, 12).

Several approaches have been taken to identify which subunits co-exist in the native GABA\(\sub{A}\) receptor complex. However, the subunit composition of the different native GABA\(\sub{A}\) receptor complexes remains unsolved. Immunoprecipitations or immunooaffinity purifications using anti-\(\alpha\) subunit antibodies (anti-\(\alpha_1\), -\(\alpha_2\), -\(\alpha_3\), -\(\alpha_5\), and -\(\alpha_6\) subunits) indicated that a significant proportion of native receptors are made by the association of two different \(\alpha\) subunits (such as \(\alpha_1\alpha_2\), \(\alpha_1\alpha_3\), \(\alpha_1\alpha_5\), or \(\alpha_1\alpha_6\)) (13–17) in a single receptor complex. However, other authors have indicated the absence of association between different \(\alpha\) subunits (18, 19). On the other hand, the pharmacological properties of these GABA\(\sub{A}\) 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 \(\alpha\) subunits expressed in the rat cerebral cortex, the \(\alpha_1\) and \(\alpha_3\) 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).

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1 The abbreviations used are: GABA\(\sub{A}\), \(\gamma\)-aminobutyric acid; PBS, phosphate-buffered saline; FMZ, flumazenil; FNZ, flunitrazepam; mAb, monoclonal antibody; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonic acid.
mmol) were from DuPont 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-GESSRRQPEG) and COO-α1 (amino acids 419–428, PQLK-KAPT-PHQ) were synthesized and coupled to keyhole limpet hemocyanin, via an extra tyrosine located at the COOH or NH2 terminus of α1 or α1 peptides, by Neocetus System SA (Strasbourg, France). For immunizations, rabbits (New Zealand White) were subcutaneously injected with 200 μg of coupled 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.

The antibodies were purified through peptide affinity columns. The α1 and α1 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 anti-α1 or anti-α1 antisera (diluted 1/5 in PBS) were recirculated, overnight at 4°C, with 10% or 1/15 or 1/15 α1 peptides from similar regions of other membranes (also see Ref. 21). After washing with 1/15 ml of PBS, the antibodies were eluted with 50 mM glycine-HCl, pH 2.3, and the fractions (0.5 ml) were neutralized by 1 M Tris, pooled and dialyzed in 1 liter of PBS (overnight at 4°C).

Other antibodies used in this work were the mAb 63-3G1 and anti-γ1 and anti-γ2 antibodies. These two polyclonal antibodies were produced using rabbits immunized with 10–15 or 1–15 amino acids of the NH2-terminal and the NH2-terminal of the γ1 and γ2 subunits, respectively (to be published elsewhere).

For immunoblots, the purified antibodies were labeled with digoxigenin as recommended by the manufacturer (Boehringer Mannheim). The digoxigenin incorporated into anti-α1 or anti-α1 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 EGTA, 1 mM dihydrothreitol, 1 mM benzamidine, 50 μg/ml trypsin inhibitor type II-S, and 50 μg/ml bacitracin. The GABA receptor was solubilized at 4 μg of protein/ml at 4°C for 40 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 80–90% of the 5 nM [3H]FMZ, 10 nM [3H]Zol, or 5 nM [3H]Roche. The affinity and binding activity found in membranes (also see Ref. 21).

**Immunoprecipitation and Immunopurification**—For immunoprecipitation experiments, the different antisera were adsorbed to a suspension of protein A-Sepharose (10%, w/v, in solubilization buffer; also see Ref. 21).

**RESULTS**

**Anti-α1 and - α1 Antibodies**—Specific polyclonal antibodies have been generated against peptides from the NH2-terminal domain (amino acids 1–10) of the α1 subunit or the C-terminal domain (amino acids 419–428) of α1 subunit of the GABA(A) 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 μl of anti-α1 or anti-α1 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-α1 antisera exclusively recognized the peptide used as antigen (NH2-terminal from α1 or COO- terminus from α1 subunits, respectively, Fig. 1A). Neither anti-α1 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 regions of other α subunits (Fig. 1B and C). 3) In immunoblots using the affinity-purified GABA(A) receptor (Fig. 1D) anti-α1 antisem immunoreacted with a faint band of M, 59,000 (α1 subunit), whereas anti-α1 strongly reacted with a single band of M, 51,000 (α1 subunit). 4) In immunoblots using extracts from cortical membranes (Fig. 1E) anti-α1 immunoanised two bands of M, 59,000 and 61,000 (see Ref. 15) and, on the other hand, anti-α1 recognized a M, 51,000 peptide. A non-specific
Pharmacological Properties of α₁-α₃-containing GABAₐ Receptors

Fig. 1. Specificity of anti-α₂ and anti-α₃ antisera. A, different amounts of peptides from NH₂ termini (amino acids 1–10) of α₂, α₃, or α₅ subunits or COOH termini of α₁ (amino acids 419–428), α₃ (amino acids 459–465), or α₅ (amino acids 424–433) were blotted and immunostained with anti-α₂ (1/1000 dilution) and anti-α₃ (1/1000 dilution) antisera, respectively. B and C, solubilized receptor (0.1–0.2 pmol of [³H]FMZ binding activity) was immunoprecipitated with 0.5 or 7.5 μl of anti-α₂ or anti-α₃ antisera, respectively, in the presence of increasing concentrations of the peptides specified above. B, COOH-terminal peptides α₂ (●), α₃ (○), and α₅ (△); C, NH₂-terminal peptides α₁ (■), α₁ (●), and α₅ (△). No inhibition was observed when anti-α₁ or anti-α₅ antisera were incubated in presence of NH₂-terminal α₁ or COOH-terminal α₅ peptides, respectively (not shown). Results are expressed as percentages from the [³H]FMZ binding activity immunoprecipitated in absence of peptide and are mean ± S.D. (bars) of three independent experiments. D, GABAₐ receptors purified from adult bovine cerebral cortex (1 μg/lane) were subjected to SDS-polyacrylamide gel electrophoresis, blotted, and immunostained with anti-α₁ (1/500 dilution) or anti-α₃ (1/1000 dilution). Both antibodies show reactivity with single peptide bands of 59 and 51 kDa for anti-α₃ and anti-α₁, respectively. E, cortical membranes (75 μg of protein/lane) were processed as above and immunostained with 10 μg/ml of purified anti-α₁ or anti-α₃ antibodies. The mAb 62-3G1 (1/5 dilution), specific for β₁ and β₂ subunits, was included as a reference. The molecular sizes of the subunits are 58 and 60 kDa (α₁), 51 kDa (α₃), and 55–57 kDa (β₁ and β₂).

A band of 100 kDa was also observed in some experiments (also see Fig. 2B). The mAb 62-3G1 (specific to β₁ and β₃, M₅, 55,000–57,000 peptides; Refs. 27 and 31) was included as a control.5) In three brain regions studied (cortex, hippocampus, and cerebellum), the percentage of immunoprecipitation by these antibodies is consistent with the level of expression of α₂ or α₁ subunits, determined by in situ hybridization or immunoprecipitation (18, 19, 32–35). As expected, the anti-α₁, anti-α₃ antisera immunoprecipitated most of the [³H]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-α₃ antisera immunoprecipitated a low proportion of receptors compared with anti-α₁. 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 α₁ and α₃ Subunits—To determine the presence of α₁ subunits, co-assembled with α₃ subunits in the same receptor complex, we first quantified the [³H]zolpidem binding activity immunoprecipitated by anti-α₃ antisera. [³H]Zolpidem binds with high affinity to α₁ subunit-containing GABAₐ receptors (type I benzodiazepine binding sites) (11, 12, 21, 22). Therefore, [³H]zolpidem (5 nM) binding activity was used as a marker of the presence of α₁ subunits in the immunoprecipitated receptor (also see Ref. 21). The quantitative immunoprecipitation of [³H]zolpidem binding was tested by two sequential incubations with anti-α₁ or anti-α₃ antibodies. The second incubation yielded 3.3 ± 2.8 and 3.8 ± 1.7% of immunoprecipitation for anti-α₁ and anti-α₃, respectively, indicating that the immunoprecipitation of the receptor was maximal. As shown in Fig. 2A, anti-α₁ and -α₃ antibodies immunoprecipitated 90.0 ± 5.4 and 26.9 ± 3.6% of the [³H]zolpidem binding activity, respectively. These results demonstrated that, in native GABAₐ receptors, the high affinity [³H]zolpidem (5 nM) binding sites (type I benzodiazepine binding sites) are largely associated with the presence of an α₁ subunit (also see Refs. 21 and 22) and, importantly, that these sites can be immunoprecipitated in association with α₃ subunits.

To ascertain the co-assembling of α₁ and α₃ subunits in the
same receptor complex, the solubilized GABA<sub>A</sub> receptor was purified by anti-α<sub>1</sub> or anti-α<sub>3</sub> immunofinity columns. The results of the purification experiments are shown in Table I. Both immunofinity columns retained the GABA<sub>A</sub> receptor complex. The percentage of binding immunabsorbed to the columns was similar to that determined by immunoprecipitation experiments. The anti-α<sub>1</sub> immunofinity column was efficiently eluted at pH 11.5 (21.3 ± 3.1% of the solubilized receptors or 33.0 ± 1.0% of the immunosorbed material). However, no significant benzodiazepine binding could be eluted from anti-α<sub>3</sub> columns (0.7 ± 0.3%). Other treatments, such as low pH, did not improve the elution step (not shown). Therefore, to analyze the immunopurified receptor, both immunofinity columns were eluted with SDS, and the purified material was subjected to immunoblot analysis. As shown in Fig. 2B, the presence of α<sub>3</sub> subunits (59- and 61-kDa peptides) could be detected in anti-α<sub>1</sub>-immunopurified receptors, and, on the other hand, the α<sub>1</sub> immunoreactivity (51-kDa peptide) was identified in anti-α<sub>3</sub>-immunopurified receptors.

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-α<sub>1</sub> antibody produces absolutely no immunoreaction products in Western blots of the anti-α<sub>3</sub>-immunopurified receptors that have been immunodepleted of the α<sub>3</sub> subunits. On the other hand, the anti-α<sub>3</sub> antibody immunoreacted with 59–61-kDa peptides (Fig. 2C, lane 3), demonstrating the presence of α<sub>3</sub>-containing GABA<sub>A</sub> receptors. Conversely, no immunoreaction products were produced by the anti-α<sub>1</sub> antibody using the α<sub>3</sub> immunodepleted and anti-α<sub>3</sub>-immunopurified receptor as an antigen (Fig. 2C, lanes 5 and 6). Furthermore, in membrane preparations of human embryonic kidney cells, transfected with the α<sub>1</sub>-γ<sub>2</sub>-β<sub>2</sub> combination, the anti-α<sub>1</sub> antibody immunostained a single 51-kDa peptide, whereas no immunoreaction products were detected using the anti-α<sub>3</sub> 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 α<sub>1</sub> and α<sub>3</sub>, 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>[3H]FMZ binding</th>
<th>Anti-α&lt;sub&gt;1&lt;/sub&gt; column</th>
<th>Anti-α&lt;sub&gt;3&lt;/sub&gt; column</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubilized receptor</td>
<td>30 ± 6</td>
<td>100</td>
</tr>
<tr>
<td>Bound</td>
<td>19.3 ± 1.5</td>
<td>64.3 ± 1.5</td>
</tr>
<tr>
<td>pH 11.5 elution</td>
<td>6.3 ± 0.9</td>
<td>21.3 ± 3.1</td>
</tr>
</tbody>
</table>
Pharmacological Properties of \( \alpha_1\)-\( \alpha_3\)-containing GABA\(_A\) Receptors

**Table II**

Quantification of the association between \( \alpha_1 \) and \( \alpha_3 \) subunits by immunodepletion experiments

The immunodepletion of \( \alpha_1 \) or \( \alpha_3 \) subunits was done by two sequential incubations of the solubilized receptor (1 pmol of [\( ^3H \)]FMZ binding sites) with either anti-\( \alpha_1 \) (10 \( \pm \) 10 \( \mu \)l) and anti-\( \alpha_3 \) (75 \( \pm \) 75 \( \mu \)l) antibodies, respectively. The remaining receptor was immunoprecipitated by incubation with 75 or 10 \( \mu \)l of anti-\( \alpha_1 \) and -\( \alpha_3 \) 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 \( \pm \) 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>
<th>Specific binding activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha_1 )</td>
<td>( \alpha_3 )</td>
<td>0.21 ( \pm ) 0.04</td>
<td>54.7 ( \pm ) 5.0</td>
<td>0.05 ( \pm ) 0.01</td>
<td>89.0 ( \pm ) 7.8</td>
</tr>
<tr>
<td>( \alpha_3 )</td>
<td>( \alpha_1 )</td>
<td>0.10 ( \pm ) 0.01</td>
<td>0.065 ( \pm ) 0.004</td>
<td>0.17 ( \pm ) 0.01</td>
<td>26.0 ( \pm ) 5.0</td>
</tr>
<tr>
<td>( \alpha_3 )</td>
<td>( \alpha_3 )</td>
<td>0.54 ( \pm ) 0.03</td>
<td>23.6 ( \pm ) 3.3</td>
<td>0.11 ( \pm ) 0.01</td>
<td>89.0 ( \pm ) 7.8</td>
</tr>
</tbody>
</table>

[\( ^3H \)]zolpidem binding activity immunoabsorbed to anti-\( \alpha_3 \) immunobeads (26.3 \( \pm \) 1.5 versus 32.5 \( \pm \) 5.4 and 31.2 \( \pm \) 3.7%, \( n = 3 \), for control and demecolcine- or cytochalasin 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-\( \alpha_3 \) 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 co-assembling of \( \alpha_1 \) and \( \alpha_3 \) 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-\( \alpha_3 \) 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 \( \alpha_6 \) subunits (38, 39), and this subunit is not expressed in the cortex (32, 33, 39). The immunoprecipitation of diazepam-insensitive [\( ^3H \)]Ro15-4513 binding activity by anti-\( \alpha_3 \) was very low and similar in both solubilized preparations, pure cerebellar membranes, and a mixture of cerebellar and cortical membranes (0.01 \( \pm \) 0.01 and 0.01 \( \pm \) 0.01 pmol, \( n = 2 \), respectively), thus indicating that no apparent subunit redistribution takes place due to solubilization procedures.

The association between both \( \alpha \) subunits was quantified by immunodepletion experiments. In these experiments, a particular \( \alpha \) subunit was depleted by two sequential immunoprecipitations with the specific antisera. After depletion, the remaining GABA\(_A\) receptor complex was immunoprecipitated by the other \( \alpha \) subunit. As shown in Table II, depletion of \( \alpha_1 \) subunits produced a significant decrease in the [\( ^3H \)]FMZ binding activity immunoprecipitated by anti-\( \alpha_3 \) antisera (0.21 \( \pm \) 0.04 versus 0.10 \( \pm \) 0.01 pmol, respectively). Thus, 54.7 \( \pm \) 5.0% of the benzodiazepine binding activity immunoprecipitated by anti-\( \alpha_3 \) was depleted by preincubation with the anti-\( \alpha_1 \) antisera. On the other hand, most of the [\( ^3H \)]zolpidem immunoprecipitated by the anti-\( \alpha_3 \) antisera was depleted by preincubation with the anti-\( \alpha_1 \) antibody (89.0 \( \pm \) 7.8%; 0.05 \( \pm \) 0.01 versus 0.005 \( \pm \) 0.004 pmol). These results indicated that most, if not all, of the high affinity binding sites immunoprecipitated by the anti-\( \alpha_3 \) antisera were due to the presence of an \( \alpha_1 \) subunit.

Reciprocally, depletion of \( \alpha_3 \) subunits also affected the immunoprecipitation by the anti-\( \alpha_1 \) antisera. As shown in Table II, depletion of \( \alpha_3 \) subunits produced a decrease in the [\( ^3H \)]FMZ or [\( ^3H \)]zolpidem binding activity immunoprecipitated by anti-\( \alpha_1 \) (0.70 \( \pm \) 0.05 or 0.24 \( \pm \) 0.01 pmol versus 0.54 \( \pm \) 0.03 or 0.17 \( \pm \) 0.01 pmol for [\( ^3H \)]FMZ and [\( ^3H \)]zolpidem, respectively). Thus, 20–25% of the \( \alpha_1 \) population is associated with an \( \alpha_3 \) subunit in the same receptor complex.

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 \( \alpha \) 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-\( \alpha_1 \)- or Anti-\( \alpha_3 \)-immunopurified Receptors from Rat Cortex**—The pharmacological properties of the anti-\( \alpha_1 \) or anti-\( \alpha_3 \) immunopurified receptors were determined by [\( ^3H \)]zolpidem and [\( ^3H \)]FNZ saturation studies and by displacement experiments using aliquots from the immunofluorescence columns.

As shown in Table III, in receptors immunopurified by anti-\( \alpha_1 \) or anti-\( \alpha_3 \), the Scatchard transformation of saturation experiments with [\( ^3H \)]zolpidem or [\( ^3H \)]FNZ were linear, with Hill slopes close to unity, indicating the presence of a single high affinity binding population for each ligand. Affinities (\( K_D \)) for [\( ^3H \)]zolpidem or [\( ^3H \)]FNZ were similar in both anti-\( \alpha_3 \) or anti-\( \alpha_1 \)-immunopurified receptors and very close to \( K_D \) values obtained in crude cortical rat membranes for type I or total benzodiazepine binding sites (4, 6, 24, 40). The presence of two pharmacologically distinct receptors in the anti-\( \alpha_3 \) and anti-\( \alpha_1 \)-immunopurified receptors also has been tested by displacement studies of [\( ^3H \)]FNZ by type I-specific ligands (such as zolpidem or CI 218,872). In both anti-\( \alpha_1 \) and anti-\( \alpha_3 \)-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 (\( K_1 \), 5–8 and 40–70 nM for zolpidem and CI 218,872, respectively) and low (\( K_2 \), 390–430 nM and 2–3.5 \( \mu \)M for zolpidem or CI 218,872, respectively) affinity (Table III). In contrast, diazepam displaced the [\( ^3H \)]FNZ binding immunopurified by the anti-\( \alpha_3 \) antibody, with a Hill coefficient of 1.2 and a single high affinity site (\( K_1 \), 7.1 nM). It is interesting that all the calculated \( K_1 \) 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 \( \alpha_5\)-\( \alpha_7 \) GABA\(_A\) Receptors**—The association of \( \alpha_5 \) and \( \alpha_7 \) subunits and the pharmacological properties of the \( \alpha_5\)-\( \alpha_7 \) GABA\(_A\) receptors were further tested by using sequential immunopurification (see Refs. 14 and 16). The GABA\(_A\) receptor was first immunopurified by anti-\( \alpha_1 \) immunofluorescence columns, and the eluted
The solubilized receptor was immunosorbed to anti-α3 or anti-α1, affinity columns. For saturation or displacement experiments, aliquots of the anti-α1 or anti-α3 immunobeads (0.4–0.6 pmol of [3H]FMZ binding activity/tube) were used. Saturation experiments were done by incubating the immunobeads with five or six different concentrations of [3H]FMZ (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]FMZ and 13 or 10 different concentrations of zolpidem (ranging from $5 \times 10^{-10}$ to $10^{-8}$) or Cl 218,872 (ranging from $5 \times 10^{-10}$ to $10^{-8}$), 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>
<thead>
<tr>
<th>Immunoaffinity column</th>
<th>Ligand</th>
<th>$n_H$</th>
<th>$K_i$ (nM)</th>
<th>$K_i$ (nM)</th>
<th>$P(25/15)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-α3</td>
<td>[3H]FMZ</td>
<td>0.94 ± 0.02</td>
<td>3.5 ± 2.0</td>
<td>8.0 ± 7.1</td>
<td>392 ± 100</td>
</tr>
<tr>
<td></td>
<td>[3H]Zolpidem</td>
<td>0.96 ± 0.03</td>
<td>14.8 ± 2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl 218,872</td>
<td>[3H]FMZ</td>
<td>0.57 ± 0.05</td>
<td>204 ± 68</td>
<td>67.5 ± 9.0</td>
<td>3,460 ± 900</td>
</tr>
<tr>
<td>Cl 218,872</td>
<td>[3H]Zolpidem</td>
<td>0.99 ± 0.01</td>
<td>13.3 ± 2.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl 218,872</td>
<td>Zolpidem</td>
<td>0.60 ± 0.03</td>
<td>88 ± 20</td>
<td>5.0 ± 4.9</td>
<td>424 ± 71</td>
</tr>
<tr>
<td>Anti-α1</td>
<td>[3H]FMZ</td>
<td>0.94 ± 0.02</td>
<td>6.2 ± 1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl 218,872</td>
<td>[3H]Zolpidem</td>
<td>1.96 ± 0.30</td>
<td>13.3 ± 2.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl 218,872</td>
<td>Zolpidem</td>
<td>0.55 ± 0.02</td>
<td>310 ± 84</td>
<td>39.6 ± 2.4</td>
<td>1,840 ± 360</td>
</tr>
</tbody>
</table>

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 ($\alpha_3/\alpha_1$ ratio, 1.0 ± 0.1, n = 2; Fig. 4B).

Finally, we have tested the pharmacological properties of the α1- and α3-immunopurified GABA$_A$ 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]FMZ or [3H]Ro15-4513 (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<sub>A</sub> receptors is unknown. Evidence is accumulating for the existence of different α subunit combinations (such as α<sub>i</sub>α<sub>j</sub>, α<sub>i</sub>α<sub>j</sub>α<sub>i</sub>, α<sub>i</sub>α<sub>j</sub>α<sub>i</sub>, and α<sub>i</sub>α<sub>j</sub>α<sub>i</sub>) co-assembled in single native GABA<sub>A</sub> receptor complexes. However, other studies also indicated the occurrence of between different α subtypes (18, 19). On the other hand, it is currently accepted that the benzodiazepine binding properties of the GABA<sub>A</sub> receptors are mainly determined by the α subunits (10, 11, 12). Therefore, if two different α subunit subtypes are co-assembled in a single GABA<sub>A</sub> 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 α<sub>i</sub>-α<sub>j</sub> GABA<sub>A</sub> receptors from the rat cortex.

The presence of α<sub>i</sub> and α<sub>j</sub> subunits in the same GABA<sub>A</sub> receptor complex was demonstrated by immunoprecipitation and immunopurification experiments. It has been described that, in transfected GABA<sub>A</sub> receptors, the high affinity binding sites for zolpidem (type I benzodiazepine binding sites) are determined by the presence of α<sub>j</sub> subunits. Other α subtypes (such as α<sub>i</sub>α<sub>j</sub> and α<sub>i</sub>α<sub>j</sub>α<sub>i</sub>α<sub>j</sub>) confer low affinity for this ligand (type II benzodiazepine binding sites) are determined by the presence of α<sub>j</sub> subunits. Other α subtypes (such as α<sub>i</sub>α<sub>j</sub>, α<sub>i</sub>α<sub>j</sub>α<sub>i</sub>α<sub>j</sub>, and α<sub>i</sub>α<sub>j</sub>α<sub>i</sub>α<sub>j</sub>) confer low affinity for this ligand (type I benzodiazepine binding sites) are determined by the presence of α<sub>j</sub> subunits. Other α subtypes (such as α<sub>i</sub>α<sub>j</sub>, α<sub>i</sub>α<sub>j</sub>α<sub>i</sub>α<sub>j</sub>, and α<sub>i</sub>α<sub>j</sub>α<sub>i</sub>α<sub>j</sub>) confer low affinity for this ligand (type I benzodiazepine binding sites) are determined by the presence of α<sub>j</sub> subunits.

DISCUSSION

The molecular composition of native GABA<sub>A</sub> receptors is unknown. Evidence is accumulating for the existence of different α subunit combinations (such as α<sub>i</sub>α<sub>j</sub>, α<sub>i</sub>α<sub>j</sub>α<sub>i</sub>, α<sub>i</sub>α<sub>j</sub>α<sub>i</sub>, and α<sub>i</sub>α<sub>j</sub>α<sub>i</sub>α<sub>j</sub>) co-assembled in single native GABA<sub>A</sub> receptor complexes. However, other studies also indicated the occurrence of between different α subtypes (18, 19). On the other hand, it is currently accepted that the benzodiazepine binding properties of the GABA<sub>A</sub> receptors are mainly determined by the α subunits (10, 11, 12). Therefore, if two different α subunit subtypes are co-assembled in a single GABA<sub>A</sub> 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 α<sub>i</sub>-α<sub>j</sub> GABA<sub>A</sub> receptors from the rat cortex.

The presence of α<sub>i</sub> and α<sub>j</sub> subunits in the same GABA<sub>A</sub> receptor complex was demonstrated by immunoprecipitation and immunopurification experiments. It has been described that, in transfected GABA<sub>A</sub> receptors, the high affinity binding sites for zolpidem (type I benzodiazepine binding sites) are determined by the presence of α<sub>j</sub> subunits. Other α subtypes (such as α<sub>i</sub>α<sub>j</sub>, α<sub>i</sub>α<sub>j</sub>α<sub>i</sub>α<sub>j</sub>, and α<sub>i</sub>α<sub>j</sub>α<sub>i</sub>α<sub>j</sub>) confer low affinity for this ligand (type II benzodiazepine binding sites) are determined by the presence of α<sub>j</sub> subunits. Other α subtypes (such as α<sub>i</sub>α<sub>j</sub>, α<sub>i</sub>α<sub>j</sub>α<sub>i</sub>α<sub>j</sub>, and α<sub>i</sub>α<sub>j</sub>α<sub>i</sub>α<sub>j</sub>) confer low affinity for this ligand (type I benzodiazepine binding sites) are determined by the presence of α<sub>j</sub> subunits.

The association between both subunits was confirmed by immunopurification experiments. The immunoblot analysis of the anti-α<sub>i</sub> or anti-α<sub>j</sub>-immunopurified receptors (Fig. 2B) revealed the presence of α<sub>j</sub> immunoreaction product in the anti-
α1-immunopurified receptors and, reciprocally, the presence of α3 in anti-α3-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\textsubscript{A} receptors from the rat cortex. Immuno-depletion experiments indicated that the α1-α3 GABA\textsubscript{A} receptors constituted a relatively minor proportion of the total α1-containing GABA\textsubscript{A} receptors (20–25% of this population) but 50–55% of the α3 containing GABA\textsubscript{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 γ\textsubscript{2} subunits, determines the benzodiazepine binding properties of recombinant GABA\textsubscript{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\textsubscript{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\textsubscript{A} receptor was immunopurified by anti-α1 and anti-α3 affinity columns in series; therefore, the whole population of the isolated GABA\textsubscript{A} receptors should contain two different α subunits. It is noteworthy that anti-α3 immunoadfinity columns retained 20–25% of the α3-immunopurified GABA\textsubscript{A} receptors, corroborating the proportion of α1 to α3 GABA\textsubscript{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 β\textsubscript{2/3} and γ\textsubscript{2} in the same receptor complex, consistent with previous experiments (21, 22). The β\textsubscript{3} subunits are a relatively minor component of the receptor (41), and, on the other hand, it has been demonstrated that γ\textsubscript{1} is not associated with γ\textsubscript{2}-containing GABA\textsubscript{A} receptors (42). Thus, we propose a molecular composition of α1, α3, β\textsubscript{2/3} and γ\textsubscript{2} for these native GABA\textsubscript{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\textsubscript{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 semiquantiative. 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 γ\textsubscript{2}, β\textsubscript{2}, and β\textsubscript{3} 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\textsubscript{A} receptors indicated the presence of two different benzodiazepine binding sites. Both Cl 218,872 and zolpidem discriminated between two different binding sites with high (type I) and low affinities (type II). The calculated \(K_{D}\) 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 \(K_{D}\) values of α1 and α3 subunits for the
cannot completely exclude the existence of a pharmacologically different population of the presence of a single GABAA receptor complex (Fig. 6A). However, results were confirmed by [3H]Ro15-4513 photolabeling experiments at three different degrees of saturation. As expected, in the double-immunopurified receptors, two photolabeled peptides of 51 kDa (corresponding to α1 subunits) and 59–61 kDa (α2 subunits) were identified. However, despite the fact that both α 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, α1 and α2 subunits are pharmacologically predominant over the α3 subunits.

It should be noted that [3H]Ro15-4513 photolabeled most, if not all, the benzodiazepine binding sites from cerebral membranes (90% in this work; also see Ref. 43).

Our data could be explained by the existence of at least two pharmacologically different populations of α1-α3 GABAA receptor subunits (see Fig. 6 for a model). As shown in Fig. 6A, 70% of the α1-α3 GABAA receptors may be assembled by a functional α1 subunit associated with an inactive α3 subunit. The remaining 30% of the population may be constituted by a functional α3 subunit associated with inactive α1 subunits. Nevertheless, we cannot completely exclude the existence of α1-α3 GABAA receptor subunits containing two benzodiazepine binding sites (Fig. 6B). In such a model, in which two functional α1 and α3 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 α3 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-α3 GABAA receptor complex (Fig. 6A). In these receptors, the α3 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 α1-α3-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

Molecular and Pharmacological Characterization of Native Cortical γ-Aminobutyric Acid A Receptors Containing Both α1 and α3 Subunits
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