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Molecular and Pharmacological Characterization of Native Cortical γ-Aminobutyric Acid$_A$ Receptors Containing Both α_1 and α_3 Subunits*

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We have investigated the existence, molecular composition, and benzodiazepine binding properties of native cortical α_1-α_3 γ-aminobutyric acid$_A$ (GABA$_A$) receptors using subunit-specific antibodies.

The co-existence of α_1 and α_3 subunits in native GABA$_A$ receptors was demonstrated by immunoblot analysis of the anti-α_1- or anti-α_3-immunopurified receptors and by immunoprecipitation experiments of the [^{3}H]zolpidem binding activity. Furthermore, immunodepletion experiments indicated that the α_1-α_3 GABA$_A$ receptors represented 54.7 ± 5.0 and 23.6 ± 3.3% of the α_3 and α_1 populations, respectively. Therefore, α_1 and α_3 subunits are associated in the same native GABA$_A$ receptor complex, but, on the other hand, these α_1-α_3 GABA$_A$ receptors from the cortex constitute a large proportion of the total α_3 population and a relatively minor component of the α_1 population.

The pharmacological analysis of the α_1- or α_3-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 Cl 218,872. These results indicate the existence of native GABA$_A$ receptors possessing both α_1 and α_3 subunits, with α_1 and α_3 subunits expressing their characteristic benzodiazepine pharmacology.

The molecular characterization of the anti-α_1-anti-α_3 double-immunopurified receptors demonstrated the presence of stoichiometric amounts of α_1 and α_3 subunits, associated with $\beta_{2/3}$, and γ_2 subunits. The pharmacological analysis of α_1-α_3 GABA$_A$ 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 [^{3}H]Ro15-4513-photolabeled peptides was 70:30. Therefore, the α_1 subunit is pharmacologically predominant over the α_3 subunit. These results indicate the existence of active and nonactive α subunits in the native α_1-α_3 GABA$_A$ receptors from rat cortex.

The neuropharmacological effects of benzodiazepines are mediated by the benzodiazepine (ω) binding sites associated with the GABA$_A$[1] 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, ω_1) displays high affinity for CL 218,872 (2), β-carboline derivates (3), and the imidazopyridine zolpidem (4, 5). Type II (benzodiazepine receptor 2, ω_2) displays low affinity for these compounds. A third benzodiazepine binding site with very low affinity for zolpidem (type II$_L$, ω_5) 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 GABA$_A$ receptor complex. Most of these families comprise several isoforms: α_1–α_6, β_1–β_3, γ_1–γ_3, δ, and ρ_1 and ρ_2 (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 GABA$_A$ 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 α_1-β_1–β_3-γ_2 combination confers type I pharmacology to the recombinant GABA$_A$ receptor (*i.e.* high affinity for, among others, zolpidem and Cl 218,872) (11). Type II properties are conferred by the presence of α_2, α_3, or α_5 subunits (11, 12).

Several approaches have been taken to identify which subunits co-exist in the native GABA$_A$ receptor complex. However, the subunit composition of the different native GABA$_A$ receptor complexes remains unsolved. Immunoprecipitations or immunoaffinity purifications using anti-α subunit antibodies (anti-α_1, -α_2, -α_3, -α_5, and -α_6 subunits) indicated that a significant proportion of native receptors are made by the association of two different α 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 α subunits (18, 19). On the other hand, the pharmacological properties of these GABA$_A$ 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 α subunits expressed in the rat cerebral cortex, the α_1 and α_3 subunits.

EXPERIMENTAL PROCEDURES

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

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[1] The abbreviations used are: GABA$_A$, γ-aminobutyric acid$_A$; 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 NH_3-α_3 (amino acids 1–10, pyroglutamyl-GESRRQEPG) and COO^--α_1 (amino acids 419–428, PQLKAPTPHQ) were synthesized and coupled to keyhole limpet hemocyanin, via an extra tyrosine located at the COOH or NH_2 terminus of α_3 or α_1 peptides, by Neosystem 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 α_3 and α_1 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-α_3 or anti-α_1 antisera (diluted 1/5 in PBS) were recirculated, overnight at 4 °C, in the corresponding 1-ml peptide column. After washing with 150 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-γ_2 and -γ_3 antibodies. These two polyclonal antibodies were produced using peptides from 2–10 or 1–15 amino acids of the NH_3^+ terminus of the γ_2 and γ_3 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-α_1 or anti-α_3 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 dithiothreitol, 1 mM benzamidine, 50 μg/ml trypsin inhibitor type II-S, and 50 μg/ml bacitracin.

The GABA$_A$ 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 represented 80–90% of the 5 nM [^{3}H]FMZ, 10 nM [^{3}H]FNZ, or 5 nM [^{3}H]zolpidem 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 Refs. 20–22). Ten or 75 μl of anti-α_1 or anti-α_3 antibodies/assay were incubated, for 2 h at 4 °C with agitation, with 50 μl of 10% protein A-Sepharose in a final volume of 300 μl of solubilization buffer. The IgG-protein A-Sepharose complexes were isolated by centrifugation, washed three times with 1.4 ml of solubilization buffer, and used for immunoprecipitation. On the other hand, preimmune sera was also absorbed to protein A-Sepharose and used in control experiments.

The immunoaffinity columns were synthesized as described (15). Briefly, 1–2 mg of each purified antibody were absorbed to 0.5 ml of protein A-Sepharose. The IgG-protein A-Sepharose complex was washed with 40 ml of PBS followed by 5 ml of 0.2 M triethylamine, pH 8.3. The column was then treated with 1.5 ml of 20 mM dimethylpimelimidate in 0.2 M triethylamine, pH 8.3, for 30 min at room temperature. After incubation, the medium was replaced by 1 ml of 0.2 M ethanolamine, pH 8.3, and incubated for 5 min. After coupling, the column was packed and washed, at 10 ml/h, with: 1) 40 ml of PBS; 2) 2 ml of 140 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 50 mM sodium phosphate, pH 11.5; and 3) 20 ml of PBS. The columns were pre-equilibrated with 10 ml of solubilization buffer.

For immunopurification, the solubilized GABA$_A$ receptor (30 pmol of [^{3}H]FMZ binding activity) was recirculated (10 ml/h), overnight at 4 °C through 0.5-ml columns. After absorption, the columns were washed (10 ml/h) with 20 ml of solubilization buffer. The retained material was eluted at 10 ml/h with 3 ml of 140 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% sodium deoxycholate, 0.5% CHAPS, and 50 mM sodium phosphate, pH 11.5. Fractions of 0.5 ml were collected and neutralized with 18 μl of 1 M sodium phosphate. The GABA$_A$ receptor was identified by determining the binding activity of 5 nM [^{3}H]FMZ. Positive fractions were pooled, and 0.5 mg of BSA plus protease inhibitors was added. Alternatively, the immunoaffinity columns were eluted by treatment with 2% SDS in 10 mM Tris-HCl, pH 6.8, for 30 min at room temperature.

For immunopurification in series, the anti-α_1-immunopurified receptor was recirculating through 0.1-ml anti-α_3 immunoaffinity columns. After washing, the immunobeads were either aliquoted for binding assays or eluted with SDS for immunoblot analysis of the retained material (23).

The immunopurification was quantified by determining the binding activity of [^{3}H]FMZ (5 nM) in the solubilized receptor, the column filtrate, and the final eluate.

The binding assays were done essentially as described previously (20–22, 24, 25). Nonspecific binding was determined using 5 μM clonazepam.

Pharmacological Properties of Immunopurified Receptors—Scatchard analysis of the immunopurified receptors was preformed as described (6, 24, 26). Briefly, aliquots of the immunopurified receptors were incubated in 50 mM Tris-HCl, pH 7.4, 0.05% sodium deoxycholate, and 0.05% CHAPS (0.75-ml final volume) with six different concentrations of [^{3}H]FNZ (1–20 nM) or [^{3}H]zolpidem (1–10 nM).

Displacement experiments were performed using 2 nM [^{3}H]FNZ or 1.2 nM [^{3}H]FMZ and 13 or 11 different concentrations of zolpidem (ranging from 0.5 nM to 100 μM) or CL 218,872 (ranging from 5 nM to 100 μM), respectively, as described previously (6, 24).

Other Methods—The affinity purification of the bovine GABA$_A$ receptor complex and immunoblots has been described elsewhere (27). Immunoblots were developed with luminol as recommended by the manufacturer (Boehringer Mannheim). Proteins were determined by the method of Lowry *et al.* (28). SDS-polyacrylamide gel electrophoresis was done according to the method of Laemmli (29). Photoaffinity labeling and fluorography were performed as described (20).

Data Analysis—Scatchard transformations of the saturation curves and the [^{3}H]FNZ displacement curves were adjusted using LIGAND (30), as described in detail elsewhere (6). The significance ($p < 0.05$) of the fit of the displacement curves to a one or two binding site model was determined by the F ratio test. The proportions of the different binding sites were calculated from the "maximal density of binding sites" corresponding to each affinity, determined by LIGAND. Densitometric analysis of both the immunoblots and the fluorographs was performed as described (26).

RESULTS

Anti-α_3 and -α_1 Antibodies—Specific polyclonal antibodies have been generated against peptides from the NH_2-terminal domain (amino acids 1–10) of the α_3 subunit or the C-terminal domain (amino acids 419–428) of α_1 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 [^{3}H]FMZ binding) was achieved with 5 or 25 μl of anti-α_1 or anti-α_3 antibody, respectively. Similar results were obtained with 10 nM [^{3}H]FNZ (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-α_3 or anti-α_1 antisera exclusively recognized the peptide used as antigen (NH_3^+-terminal from α_3 or COO^--terminal from α_1 subunits, respectively, Fig. 1*A*). Neither anti-α_3 nor anti-α_1 antisera immunoreacted with peptides from similar regions of other α subunits (Fig. 1*A*). 2) The immunoprecipitation of the native receptors was specifically inhibited by the peptide used as antigen but not by others corresponding to similar NH_3^+- or COO^--terminal regions of other α subunits (Fig. 1, *B* and *C*). 3) In immunoblots using the affinity-purified GABA$_A$ receptor (Fig. 1*D*) anti-α_3 antiserum immunoreacted with a faint band of M_r 59,000 (α_3 subunit), whereas anti-α_1 strongly reacted with a single band of M_r 51,000 (α_1 subunit). 4) In immunoblots using extracts from cortical membranes (Fig. 1*E*) anti-α_3 immunostained two bands of M_r 59,000 and 61,000 (see Ref. 15) and, on the other hand, anti-α_1 recognized a M_r 51,000 peptide. A nonspecific

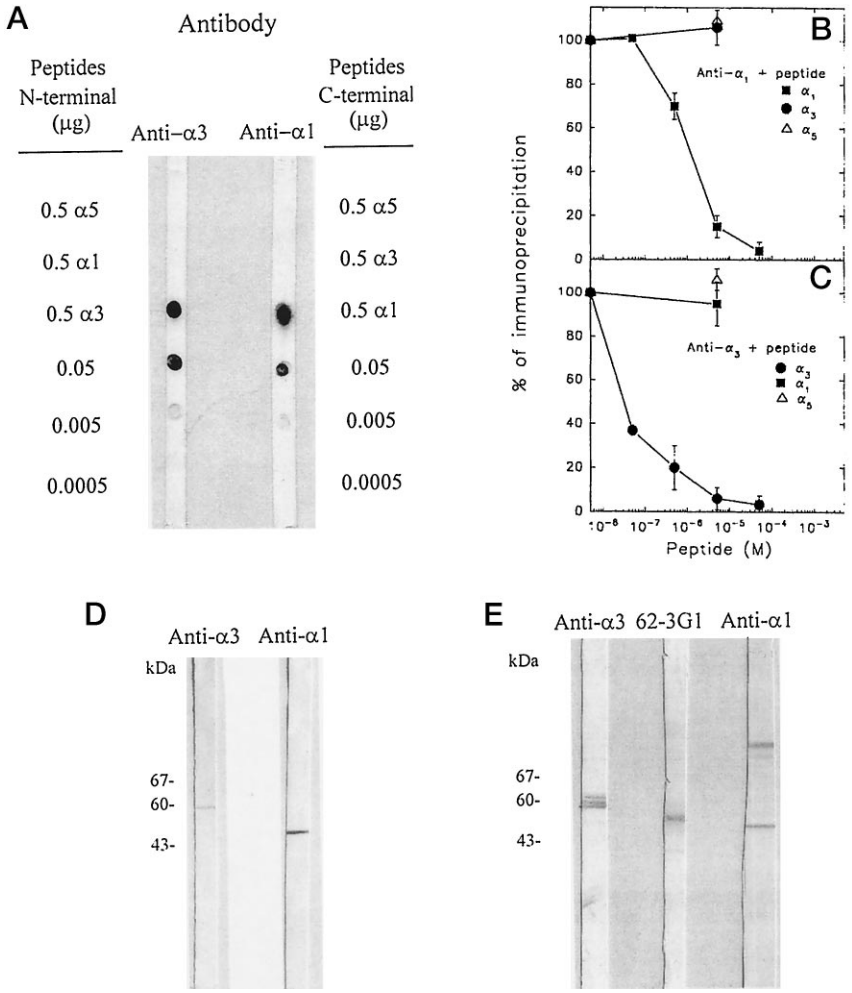

FIG. 1. **Specificity of anti-α_3 and anti-α_1 antisera.** *A,* different amounts of peptides from NH_2 termini (amino acids 1–10) of α_3, α_1, or α_5 subunits or COOH termini of α_1 (amino acids 419–428), α_3 (amino acids 459–465), or α_5 (amino acids 424–433) were blotted and immunostained with anti-α_3 (1/1000 dilution) and anti-α_1 (1/1000 dilution) antisera, respectively. *B* and *C*, solubilized receptor (0.1–0.2 pmol of [^{3}H]FMZ binding activity) was immunoprecipitated with 0.5 or 7.5 μl of anti-α_1 or anti-α_3 antisera, respectively, in the presence of increasing concentrations of the peptides specified above. *B,* COOH-terminal peptides α_1 (■), α_3 (●), and α_5 (△); *C,* NH_2-terminal peptides α_1 (■), α_3 (●), and α_5 (△). No inhibition was observed when anti-α_1 or anti-α_3 antisera were incubated in presence of NH_2-terminal α_1 or COOH-terminal α_3 peptides, respectively (not shown). Results are expressed as percentages from the [^{3}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-α_3 (1/500 dilution) or anti-α_1 (1/1000 dilution). Both antibodies show reactivity with single peptide bands of 59 and 51 kDa for anti-α_3 and anti-α_1, respectively. *E,* cortical membranes (75 μg of protein/lane) were processed as above and immunostained with 10 μg/ml of purified anti-α_3 or anti-α_1 antibodies. The mAb 62-3G1 (1/5 dilution), specific for β_2 and β_3 subunits, was included as a reference. The molecular sizes of the subunits are 58 and 60 kDa (α_3), 51 kDa (α_1) and 55–57 kDa (β_2 and β_3).

band of 100 kDa was also observed in some experiments (also see Fig. 2*B*). The mAb 62-3G1 (specific to β_2 and β_3, M_r 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 α_3 or α_1 subunits, determined by *in situ* hybridization or immunoprecipitation (18, 19, 32–35). As expected, the anti-α_1 antiserum immunoprecipitated most of the [^{3}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-α_3 antiserum immunoprecipitated a low proportion of receptors compared with anti-α_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 α_1 and α_3 Subunits—To determine the presence of α_1 subunits, co-assembled with α_3 subunits in the same receptor complex, we first quantified the [^{3}H]zolpidem binding activity immunoprecipitated by anti-α_3 antiserum. [^{3}H]Zolpidem binds with high affinity to α_1 subunit-containing GABA$_A$ receptors (type I benzodiazepine binding sites) (11, 12, 21, 22). Therefore, [^{3}H]zolpidem (5 nM) binding activity was used as a marker of the presence of α_1 subunits in the immunoprecipitated receptor (also see Ref. 21). The quantitative immunoprecipitation of [^{3}H]zolpidem binding was tested by two sequential incubations with anti-α_1 or anti-α_3 antibodies. The second incubation yielded 3.3 ± 2.8 and 3.8 ± 1.7% of immunoprecipitation for anti-α_1 and -α_3, respectively, indicating that the immunoprecipitation of the receptor was maximal.

As shown in Fig. 2*A*, anti-α_1 and -α_3 antibodies immunoprecipitated 90.0 ± 5.4 and 26.9 ± 3.6% of the [^{3}H]zolpidem binding activity, respectively. These results demonstrated that, in native GABA$_A$ receptors, the high affinity [^{3}H]zolpidem (5 nM) binding sites (type I benzodiazepine binding sites) are largely associated with the presence of an α_1 subunit (also see Refs. 21 and 22) and, importantly, that these sites can be immunoprecipitated in association with α_3 subunits.

To ascertain the co-assembling of α_1 and α_3 subunits in the

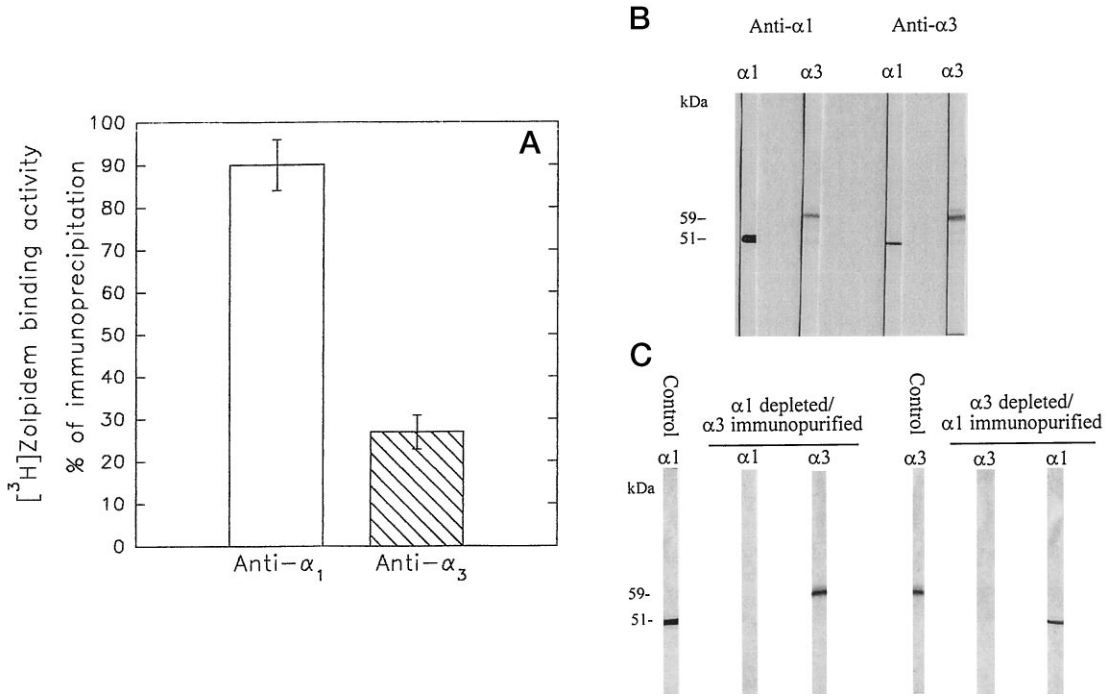

FIG. 2. **Co-localization of α_1 and α_3 subunits in the same GABA$_A$ complex demonstrated by cumulative immunoprecipitation (*A*), an immunoblot of the immunopurified receptors (*B*), and the absence of cross-reaction between the antibodies (*C*).** *A,* the solubilized receptors (1.0 pmol of [^{3}H]FMZ binding activity) were immunoprecipitated by sequential incubation with 85 and 75 μl or 15 and 15 μl of anti-α_3 or anti-α_1 antibodies, respectively. The [^{3}H]zolpidem (5 nM) binding were determined in the pellets and in the last supernatant. The results are expressed as percentages of the total specific binding for each ligand and are mean ± S.D. (*bars*) of six to eight experiments. *B,* the GABA$_A$ receptors were immunopurified by anti-α_1 (*lanes 1* and *2*) or anti-α_3 (*lanes 3* and *4*) immunoaffinity columns. After washing, the immunoaffinity columns were treated with SDS, and the eluted receptor was analyzed by immunoblot using 5 μg of purified anti-α_1 (*lanes 1* and *3*) or anti-α_3 (*lanes 2* and *4*) antibodies. *C,* the α_1 or α_3 subunits were immunodepleted from the solubilized receptor by three sequential incubations with anti-α_1 (15 μl each) and anti-α_3 (75 μl each), respectively. The α_1- or α_3-immunodepleted receptors were immunopurified by anti-α_3 (*lanes 2* and *3*) and anti-α_1 (*lanes 5* and *6*) immunoaffinity columns. *Lanes 1* and *4*, anti-α_1 and anti-α_3 control immunopurifications, respectively. The eluted receptor was analyzed by immunoblot using 5 μg of purified anti-α_1 (*lanes 1, 2,* and *6*) or anti-α_3 (*lanes 3–5*) antibodies. For all immunoblots, the affinity-purified antibodies were labeled with digoxigenin. *Numbers* on the *left, M*$_r$ values of the immunostained bands.

same receptor complex, the solubilized GABA$_A$ receptor was purified by anti-α_1 or anti-α_3 immunoaffinity columns. The results of the purification experiments are shown in Table I. Both immunoaffinity columns retained the GABA$_A$ receptor complex. The percentage of binding immunoabsorbed to the columns was similar to that determined by immunoprecipitation experiments. The anti-α_1 immunoaffinity column was efficiently eluted at pH 11.5 (21.3 ± 3.1% of the solubilized receptors or 33.0 ± 1.0% of the immunoabsorbed material). However, no significant benzodiazepine binding could be eluted from anti-α_3 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 immunoaffinity columns were eluted with SDS, and the purified material was subjected to immunoblot analysis. As shown in Fig. 2*B*, the presence of α_3 subunits (59- and 61-kDa peptides) could be detected in anti-α_1-immunopurified receptors, and, on the other hand, the α_1 immunoreactivity (51-kDa peptide) was identified in anti-α_3-immunopurified receptors.

The co-purification of both α subunits was not due to cross-reaction between the antibodies. As shown in Fig. 2*C* (*lane 2*), the anti-α_1 antibody produces absolutely no immunoreaction products in Western blots of the anti-α_3-immunopurified receptors that have been immunodepleted of the α_1 subunits. On the other hand, the anti-α_3 antibody immunoreacted with 59–61-kDa peptides (Fig. 2*C, lane 3*), demonstrating the presence of α_3-containing GABA$_A$ receptors. Conversely, no immunoreaction products were produced by the anti-α_3 antibody using the α_3 immunodepleted and anti-α_1-immunopurified receptor as an antigen (Fig. 2*C, lanes 5* and *6*). Furthermore, in membrane preparations of human embryonic kidney cells, transfected

TABLE I
Immunoaffinity purification of α_1- and α_3-containing GABA$_A$ receptors from rat cerebral cortex

The solubilized GABA$_A$ receptor was purified on 0.5-ml immunoaffinity columns, as described under "Experimental Procedures." The [^{3}H]FMZ (5 nM) binding activity was determined in each fraction. The results are mean ± S.D. of at least three independent experiments.

	[^{3}H]FMZ binding			
	Anti-α_1 column		Anti-α_3 column	
	pmol	*%*	*pmol*	*%*
Solubilized receptor	30 ± 6	100	30 ± 4	100
Bound	19.3 ± 0.5	64.3 ± 1.5	6.1 ± 1.5	20.5 ± 4.9
pH 11.5 elution	6.3 ± 0.9	21.3 ± 3.1	0.2 ± 0.1	0.7 ± 0.3

with the α_1-γ_2-β_2 combination, the anti-α_1 antibody immunostained a single 51-kDa peptide, whereas no immunoreaction products were detected using the anti-α_3 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_1 and α_3, was due to interactions between individual GABA$_A$ 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 II

Quantification of the association between α_1 and α_3 subunits by immunodepletion experiments

The immunodepletion of α_1 or α_3 subunits was done by two sequential incubations of the solubilized receptor (1 pmol of [^{3}H]FMZ binding sites) with either anti-α_1 (10 + 10 μl) and anti-α_3 (75 + 75 μl) antibodies, respectively. The remaining receptor was immunoprecipitated by incubation with 75 or 10 μl of anti-α_3 and -α_1 antisera, respectively. The binding activity was determined in pellets and supernatants. The results are expressed as pmol of [^{3}H]FMZ (5 nM) or [^{3}H]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.

Subunit depleted	Subunit immunoprecipitated	Specific binding activity			
		[^{3}H]FMZ		[^{3}H]zolpidem	
		pmol immunoprecipitated	*% of depletion*	*pmol immunoprecipitated*	*% of depletion*
	α_3	0.21 ± 0.04		0.05 ± 0.01	
α_1	α_3	0.10 ± 0.01	54.7 ± 5.0	0.005 ± 0.004	89.0 ± 7.8
	α_1	0.70 ± 0.05		0.24 ± 0.01	
α_3	α_1	0.54 ± 0.03	23.6 ± 3.3	0.17 ± 0.01	26.0 ± 5.0

[^{3}H]zolpidem binding activity immunoabsorbed to anti-α_3 immunobeads (26.3 ± 1.5 *versus* 32.5 ± 5.4 and 31.2 ± 3.7%, n = 3, for control and demecolcine- or cytochalasin D-treated membranes, respectively). Furthermore, after treatment with either drug, two [^{3}H]Ro15-4513 photoaffinity-labeled peptides of 51 and 59–61 kDa were immunopurified by anti-α_3 columns (not shown). On the other hand, results similar to those shown in Fig. 2*A* were obtained using purified synaptic membranes as starting material (not shown).

The co-assembling of α_1 and α_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-α_3 of the diazepam-insensitive [^{3}H]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 α_6 subunits (38, 39), and this subunit is not expressed in the cortex (32, 33, 39). The immunoprecipitation of diazepam-insensitive [^{3}H]Ro15-4513 binding activity by anti-α_3 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 antiserum. After depletion, the remaining GABA$_A$ receptor complex was immunoprecipitated by the other α subunit. As shown in Table II, depletion of α_1 subunits produced a significant decrease in the [^{3}H]FMZ binding activity immunoprecipitated by anti-α_3 antiserum (0.21 ± 0.04 *versus* 0.10 ± 0.01 pmol, respectively). Thus, 54.7 ± 5.0% of the benzodiazepine binding activity immunoprecipitated by anti-α_3 was depleted by preincubation with the anti-α_1 antiserum. On the other hand, most of the [^{3}H]zolpidem immunoprecipitated by the anti-α_3 antiserum was depleted by preincubation with the anti-α_1 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-α_3 antiserum were due to the presence of an α_1 subunit.

Reciprocally, depletion of α_3 subunits also affected the immunoprecipitation by the anti-α_1 antiserum. As shown in Table II, depletion of α_3 subunits produced a decrease in the [^{3}H]FMZ or [^{3}H]zolpidem binding activity immunoprecipitated by anti-α_1 (0.70 ± 0.05 or 0.24 ± 0.01 pmol *versus* 0.54 ± 0.03 or 0.17 ± 0.01 pmol for [^{3}H]FMZ and [^{3}H]zolpidem, respectively). Thus, 20–25% of the α_1 population is associated with an α_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 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-α_1- or Anti-α_3-immunopurified Receptors from Rat Cortex—The pharmacological properties of the anti-α_1- or anti-α_3-immunopurified receptors were determined by [^{3}H]zolpidem and [^{3}H]FNZ saturation studies and by displacement experiments using aliquots from the immunoaffinity columns.

As shown in Table III, in receptors immunopurified by anti-α_1 or anti-α_3, the Scatchard transformation of saturation experiments with [^{3}H]zolpidem or [^{3}H]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 [^{3}H]zolpidem or [^{3}H]FNZ were similar in both anti-α_3 or anti-α_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-α_3 and anti-α_1-immunopurified receptors also has been tested by displacement studies of [^{3}H]FNZ by type I-specific ligands (such as zolpidem or Cl 218,872). In both anti-α_1- and anti-α_3-immunopurified receptors and for both zolpidem and Cl 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_i, 5–8 and 40–70 nM for zolpidem and Cl 218,872, respectively) and low (K_i, 390–430 nM and 2–3.5 μM for zolpidem or CL 218,872, respectively) affinity (Table III). In contrast, diazepam displaced the [^{3}H]FNZ binding immunopurified by the anti-α_3 antibody, with a Hill coefficient of 1.2 and a single high affinity site (K_i, 7.1 nM). It is interesting that all the calculated K_i 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 α_3-α_1 GABA$_A$ Receptors—The association of α_3 and α_1 subunits and the pharmacological properties of the α_3-α_1 GABA$_A$ receptors were further tested by using sequential immunopurification (see Refs. 14 and 16). The GABA$_A$ receptor was first immunopurified by anti-α_1 immunoaffinity columns, and the eluted

TABLE III

Pharmacological characterization of the anti-α_1 and anti-α_3 immunopurified GABA$_A$ receptors from rat cortex

The solubilized receptor was immunoabsorbed to anti-α_3 or anti-α_1 affinity columns. For saturation or displacement experiments, aliquots of the anti-α_1 or anti-α_3 immunobeads (0.4–0.6 pmol of [^{3}H]FNZ binding activity/tube) were used. Saturation experiments were done by incubating the immunobeads with five or six different concentrations of [^{3}H]FNZ (1–20 nM) or [^{3}H]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 [^{3}H]FNZ and 13 or 10 different concentrations of zolpidem (ranging from 5×10^{-10}–10^{-4} M) or CL 218,872 (ranging from 5×10^{-9}–10^{-4} 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.

Immunoaffinity column	Ligand	Scatchard or displacement experiments				
		n_H	One binding site, K_D or K_i	Two binding sites		
				K_i	K_i	P(2S/1S)
Anti-α_3	[^{3}H]FNZ	0.94 ± 0.02	3.5 ± 2.0			
	[^{3}H]Zolpidem	0.96 ± 0.03	14.8 ± 2.3			
	Zolpidem	0.57 ± 0.05	204 ± 68	8.0 ± 7.1	392 ± 100	0.02
	Cl 218,872	0.55 ± 0.02	1,525 ± 220	67.5 ± 9.0	3,460 ± 900	0.03
Anti-α_1	[^{3}H]FNZ	0.94 ± 0.02	6.2 ± 1.9			
	[^{3}H]Zolpidem	0.99 ± 0.01	13.3 ± 2.8			
	Zolpidem	0.60 ± 0.03	88 ± 20	5.0 ± 4.9	424 ± 71	0.01
	Cl 218,872	0.58 ± 0.03	310 ± 84	39.6 ± 2.4	1,840 ± 360	0.03

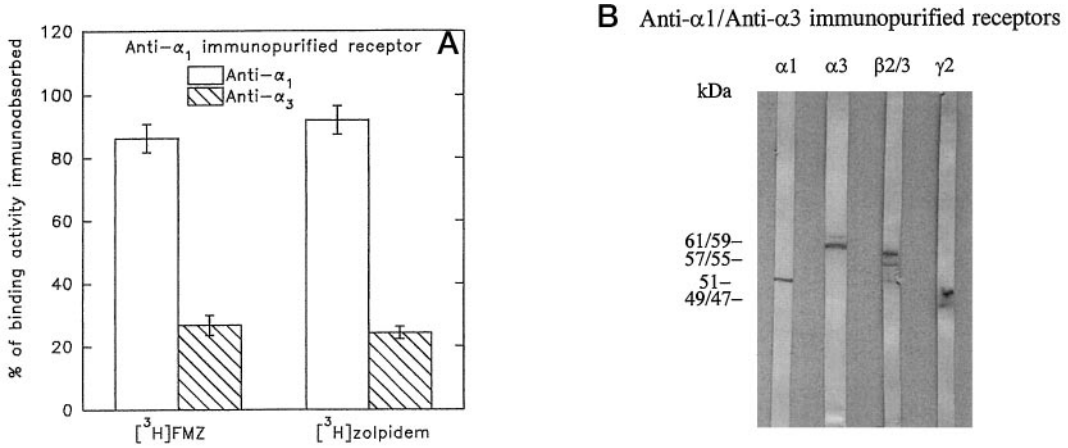

FIG. 3. **Binding activity and molecular composition of the α_1-α_3 GABA$_A$ receptor from rat cortex.** *A,* the anti-α_1-immunopurified GABA$_A$ receptors were immunoabsorbed to anti-α_1 (□) or anti-α_3 (▨) immunobeads, and the [^{3}H]FMZ (5 nM) or [^{3}H]zolpidem (5 nM) binding activity was quantified 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$_A$ receptors were immunopurified by anti-α_1 and anti-α_3 columns in series. The SDS-eluted material was blotted and incubated with 5 μg of purified anti-α_1 (*lane 1*) or anti-α_3 (*lane 2*) antibodies, a 1/5 dilution of the mAb 62-3G1 (specific for β_2 and β_3 subunits; *lane 3*), or 5 μg of purified anti-γ_2 antibody (*lane 4*). *Numbers* on the *left*, M_r values of the immunostained bands.

material was absorbed to anti-α_3 immunoaffinity columns. As was mentioned before (see Table I), no significant elution from the anti-α_3 immunoaffinity columns could be achieved. Therefore, the GABA$_A$ receptor retained by the anti-α_3 columns was analyzed by binding assays using aliquots of the anti-α_3 immunobeads (see Ref. 23) or by immunoblots of the SDS-eluted receptors.

We first quantified the presence of α_3 subunits in the anti-α_1-immunopurified receptors by testing the percentage of [^{3}H]FMZ and [^{3}H]zolpidem binding activity immunoabsorbed to anti-α_3 columns. Anti-α_1 immunobeads were used as control. As shown in Fig. 3*A*, the second anti-α_1 columns immunoabsorbed 90–95% of the previously anti-α_1-immunopurified receptors, whereas anti-α_3 columns immunoabsorbed 20–25% of either [^{3}H]FMZ and [^{3}H]zolpidem binding sites previously immunopurified by anti-α_1 columns. This indicated that 20–25% of the α_1 population also contained an α_3 subunit. These values agree with those calculated by depletion experiments (see Table II).

The presence of both α subunits, together with $\beta_{2/3}$ and γ_2 subunits, in the α_1- and α_3-immunopurified receptors was demonstrated by immunoblots. As shown in Fig. 3*B*, after sequential anti-α_1 and anti-α_3 immunopurifications, the anti-α_1 antibody immunoreacted with a single 51-kDa band, whereas the anti-α_3 antibody immunostained a doublet of 59 and 61 kDa. The mAb 62-3G1 and anti-γ_2 antibodies immunostained peptides of 55–57 and 47–49 kDa, respectively. The presence of γ_3 was not detected (not shown). These results demonstrated that both α_1 and α_3 subunits, $\beta_{2/3}$ and γ_2 are co-assembled in the same GABA$_A$ receptor complex.

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_3/α_1 ratio, 1.1 ± 0.1, n = 2; Fig. 4*B*).

Finally, we have tested the pharmacological properties of the α_1- and α_3-immunopurified GABA$_A$ receptors by displacement experiments with Cl 218,872 or zolpidem and also by [^{3}H]Ro15-4513 photoaffintiy labeling experiments of the double-immunopurified receptor. The results are shown in Table IV and Fig. 5. The displacement experiments of both [^{3}H]FNZ or [^{3}H]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-

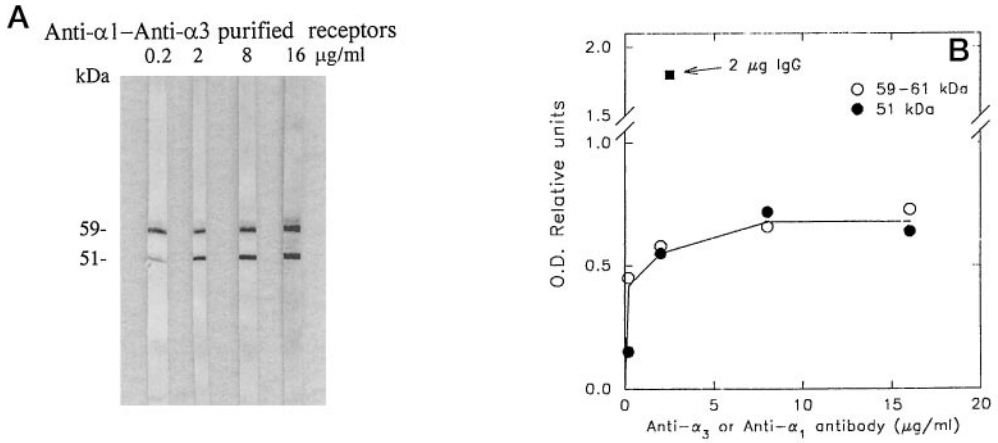

FIG. 4. **Stoichiometry of the α_1 and α_3 subunits in the α_1-α_3-immunopurified GABA$_A$ receptors from rat cortex.** *A,* a fixed amount of the double-immunopurified GABA$_A$ receptor was blotted and incubated with increasing amounts (0.2, 2, 8, and 16 μg/ml each antibody) of purified anti-α_1 and anti-α_3 antibodies in combination. *B,* densitometric analysis of the immunoblot shown in *A*. As a control of the staining protocol, a fixed amount of purified IgG (2 μg) was blotted, stained with the second antibody, and analyzed by densitometry. This experiment was repeated twice with similar results.

TABLE IV

Pharmacological properties of the α_1- and α_3 GABA$_A$ receptors purified by anti-α_1 and anti-α_3 immunoaffinity columns in series

The solubilized GABA$_A$ receptor (30 pmol of [^{3}H]FMZ binding) was immunopurified by anti-α_1 immunoaffinity column. The eluted receptor was immunoabsorbed to anti-α_3 columns. After washing, the anti-α_3 immunobeads were aliquoted (0.2 pmol of [^{3}H]FMZ binding/assay). Displacement experiments were performed as described in Table III. The proportion between the different binding sites, given in parentheses, was calculated from the maximal density of binding sites determined by LIGAND. The results are mean ± S.D. of two independent experiments.

Ligand	Displacement of [^{3}H]FNZ binding				
	n_H	One binding site, K_i	Two binding sites		p(2S/1S)
			K_i	K_i	
		nM	*nM*	*nM*	
Cl 218,872	0.69 ± 0.01	173.5 ± 68	46.3 ± 4.2 (69.5 ± 2.5%)	950 ± 200 (30.5 ± 2.5%)	<0.004
Zolpidem	0.73 ± 0.10	56.2 ± 6.8	13.0 ± 1.8 (69.0 ± 8.5%)	190 ± 89 (31.0 ± 8.5%)	<0.03

periments, was 70:30 for high and low affinity, respectively (see Table IV).

On the other hand, [^{3}H]Ro15-4513 (5 nM) photoaffinity-labeling experiments demonstrated the presence of two different labeled peptides of 51 and 59–61 kDa in the α_1-α_3 GABA$_A$ receptors (Fig. 5, *lane 3*). The molecular masses of these photoaffinity-labeled peptides corresponded to the major bands present in anti-α_3-immunopurified (Fig. 5, *lane 2*) and anti-α_1-immunopurified (Fig. 5, *lane 1*) receptors. The proportion between 51- and 59–61-kDa bands, determined by densitometric analysis of the fluorographs, was 70:30 (Fig. 5*B*). The proportion between both photolabeled peptides, in the double α_1- and α_3-immunopurified receptors, was further analyzed at two different degrees of saturation of the benzodiazepine binding sites. For these experiments, the membranes were photolabeled with 10 or 20 nM [^{3}H]Ro15-4513 (Fig. 5, *lanes 4* and *5*). As shown in Fig. 5*B*, the proportion between the 51- and 59–61-kDa photolabeled peptides was identical at all three [^{3}H]Ro15-4513 concentrations.

DISCUSSION

The molecular composition of native GABA$_A$ receptors is unknown. Evidence is accumulating for the existence of different α subunit combinations (such as $\alpha_1\alpha_3$, $\alpha_1\alpha_2$, $\alpha_1\alpha_5$, and $\alpha_1\alpha_6$) co-assembled in single native GABA$_A$ receptor complexes. However, other studies also indicated the absence of co-existence between different α 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 α subunits (10, 11, 12). Therefore, if two different α 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 α_1-α_3 GABA$_A$ receptors from the rat cortex.

The presence of α_1 and α_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 α_1 subunits. Other α subtypes (such as α_2, α_3, and α_5) confer low affinity for this ligand (type II benzodiazepine receptors) (11, 12). Therefore, the association of an α_1 with other α subunits could be estimated by immunoprecipitation of the [^{3}H]zolpidem binding activity. Our immunoprecipitation experiments (Fig. 2*A* 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 α_1 subunit in the GABA$_A$ receptor. Importantly, 25–30% of these [^{3}H]zolpidem binding sites were immunoprecipitated by anti-α_3 antibody, thus suggesting an α_1-α_3 association. Consistently, immunodepletion of the α_1 subunits suppress the immunoprecipitation of [^{3}H]zolpidem binding by the anti-α_3 antibody (Table II). Thus, α_1 binding properties could be immunoprecipitated in association with α_3 subunits, suggesting the presence of both α_1 and α_3 subunits in the same receptor complex.

The association between both subunits was confirmed by immunopurification experiments. The immunoblot analysis of the anti-α_1- or anti-α_3-immunopurified receptors (Fig. 2*B*) revealed the presence of α_3 immunoreaction product in the anti-

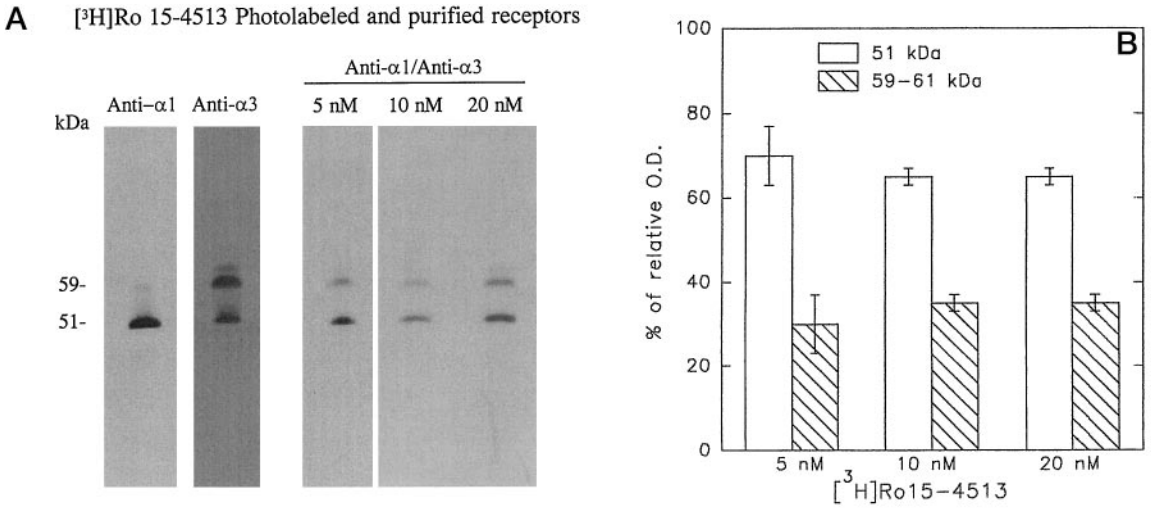

FIG. 5. **Fluorography (*A*) and desitometric analysis (*B*) of the [^{3}H]Ro15-4513-photolabeled and immunopurified GABA$_A$ receptor from rat cortex.** *A,* the cortical membranes were photoaffinity labeled with 5, 10, and 20 nM [^{3}H]Ro15-4513, solubilized, and immunopurified by anti-α_1, anti-α_3, or anti-α_1 and anti-α_3 immunoaffinity columns in series. The fluorographs were exposed for three different periods. Fluorographs shown were exposed for 20 days (anti-α_3) or 10 days (anti-α_1 or anti-α_1 and anti-α_3). *B,* densitometric analysis of the photolabeled peptides at 5, 10, and 20 nM [^{3}H]Ro15-4513, immunopurified by anti-α_1 and anti-α_3 columns in series. The data are mean ± S.D. (*bars*) of two independent experiments.

α_1-immunopurified receptors and, reciprocally, the presence of α_1 in anti-α_3-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 α_1-α_3 GABA$_A$ receptors from the rat cortex. Immunodepletion experiments indicated that the α_1-α_3 GABA$_A$ receptors constituted a relatively minor proportion of the total α_1-containing GABA$_A$ receptors (20–25% of this population) but 50–55% of the α_3 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 α_1-containing receptors but a high proportion of other α subunits, such as α_3.

The presence of different α subtypes, in combination with β_1–β_3 and γ_2 subunits, determines the benzodiazepine binding properties of recombinant GABA$_A$ receptors (10, 11, 12). As mentioned above, the α_1 subunit confers type I benzodiazepine binding properties (high affinity for zolpidem and Cl 218,872), whereas the α_3 subunit confers type II binding properties (low affinity for these ligands). Therefore, if two different α subunits, such as α_1 and α_3, are co-assembled in the same receptor complex, and both α_1 and α_3 subunits are pharmacologically active, two different benzodiazepine binding subtypes should be discriminated in either anti-α_1- and anti-α_3-immunopurified receptors. As shown in Table III, in anti-α_1- and anti-α_3-immunopurified receptors, two different binding sites were identified. The affinities for zolpidem (determined by Scatchard and displacement experiments) or Cl 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 α_1 subunits (high affinity binding sites) and α_3 subunits (low affinity binding sites) (11, 12). In consequence, these results suggest the presence of benzodiazepine binding sites in both α_1 and α_3 subunits co-assembled in a single GABA$_A$ receptor complex (also see Ref. 17).

To discern whether both α_1 and α_3 subunits, co-assembled in a single complex, display benzodiazepine binding activity, the GABA$_A$ receptor was immunopurified by anti-α_1 and anti-α_3 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-α_3 immunoaffinity columns retained 20–25% of the α_1-immunopurified GABA$_A$ receptors, corroborating the proportion of α_1 to α_3 GABA$_A$ receptors calculated by depletion experiments (compare Fig. 3*A* and Table II). Immunoblot analysis (Fig. 3*B*) indicates that α_1 and α_3 subunits are mainly associated with $\beta_{2/3}$ and γ_2 in the same receptor complex, consistent with previous experiments (21, 22). The β_1 subunits are a relatively minor component of the receptor (41), and, on the other hand, it has been demonstrated that γ_1 is not associated with γ_2-containing GABA$_A$ receptors (42). Thus, we propose a molecular composition of α_1, α_3, $\beta_{2/3}$ and γ_2 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-α_1 and anti-α_3 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 γ_2, β_2, and β_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 [^{3}H]Ro15-4513 to a similar extent. Indeed, the pharmacological analysis of the α_1-α_3 GABA$_A$ 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_i values for either ligand were similar to those of immunopurified α_1 or α_3 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 α_1 subunits are predominantly active over the α_3 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 α_1 and α_3 subunits for the

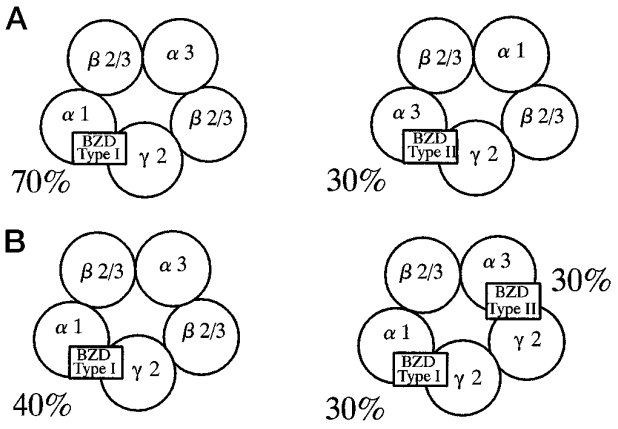

FIG. 6. **Model of the α_1-α_3 GABA$_A$ receptor and the benzodiazepine binding sites associated with the different α subunits.**

^{3}H-labeled ligand ([^{3}H]FNZ or [^{3}H]FMZ). However, these results were confirmed by [^{3}H]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 α_1 subunits) and 59–61 kDa (α_3 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, α_1 subunits are pharmacologically predominant over the α_3 subunits. It should be noted that [^{3}H]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 α_1-α_3 GABA$_A$ receptors (see Fig. 6 for a model). As shown in Fig. 6*A*, 70% of the α_1-α_3 GABA$_A$ receptors may be assembled by a functional α_1 subunit associated with an inactive α_3 subunit. The remaining 30% of the population may be constituted by a functional α_3 subunit associated with inactive α_1 subunits. Nevertheless, we cannot completely exclude the existence of α_1-α_3 GABA$_A$ receptors containing two benzodiazepine binding sites (Fig. 6*B*). In such a model, in which two functional α_1 and α_3 subunits are co-localized in the same receptor complex, 60% of the benzodiazepine binding sites should be conferred by GABA$_A$ receptors containing two functional α subunits and 30% by functional α_1 subunits associated with inactive α_3 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 γ_2 subunits in the pentameric GABA$_A$ receptor complex (10, 44). It has been proposed that both α and γ_2 subunits are implicated in the benzodiazepine binding sites (44, 45), and, on the other hand, the GABA$_A$ receptors may contain two α subunits, two β subunits, and a single γ_2 subunit (46, 47). Thus, the predominance of α_1 pharmacology (type I benzodiazepine binding sites or the 51-kDa photolabeled peptides) could be interpreted by the presence of a single γ_2 subunit properly associated with the α_1 subunit in the α_1-α_3 GABA$_A$ receptor complex (Fig. 6*A*). In these receptors, the α_3 subunits should lack the benzodiazepine binding sites (see Fig. 6). On the other hand, two different γ_2 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. 6*B*).

The physiological significance of GABA$_A$ receptors containing two different α subtypes, such as an α_1-α_3 combination, is unknown. The α_1 subunit is highly and uniformly expressed in all cortical layers, whereas the expression of the α_3 subunit is localized in layers V and VI (49). Therefore, the α_1- and α_3-containing GABA$_A$ receptors should be restricted to these cortical layers. Co-localization of α_1 and α_3 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 GABA$_A$ receptors, the co-expression of α_1, α_3, β_2, and γ_2 subunits confers unique functional properties, distinct from GABA$_A$ 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 GABA$_A$ receptor. The existence and pharmacological properties of $\alpha_1\alpha_3$-containing receptors increase the heterogeneity of the native GABA$_A$ receptor complex in the central nervous system.

In summary, our results demonstrate the existence of cortical GABA$_A$ receptors containing both α_1 and α_3 subunits in stoichiometric amounts. Furthermore, both α subunits retained their benzodiazepine binding properties. However, the α_1 subunit is pharmacologically predominant over α_3 subunits, indicating the existence of active and nonactive benzodiazepine binding sites associated with these α subunits.

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Molecular and Pharmacological Characterization of Native Cortical γ-Aminobutyric Acid $_A$ Receptors Containing Both α_1 and α_3 Subunits

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