0026-895X/95/040666-10$3.00/0

MOLECULAR PHARMACOLOGY, 48:666–675 (1995).

Pharmacological Subtypes of the γ-Aminobutyric Acid$_A$ Receptors Defined by a γ-Aminobutyric Acid Analogue 4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridin-3-ol and Allosteric Coupling: Characterization Using Subunit-Specific Antibodies

KYUNG-HYE HUH, TIMOTHY M. DELOREY, SHUICHI ENDO, and RICHARD W. OLSEN

Department of Molecular and Medical Pharmacology (K.-H.H., T.M.D., S.E., R.W.O.), Brain Research Institute (R.W.O.), Mental Retardation Research Center, School of Medicine (T.M.D., R.W.O.), and Molecular Biology Institute (R.W.O.), University of California, Los Angeles, California 90024

Received February 16, 1995; Accepted July 12, 1995

SUMMARY

Various α and β3 subunit-specific antibodies were used to characterize some of the heterogeneous ligand-binding properties of γ-aminobutyric acid$_A$ receptors. Polyclonal antibodies that were raised against the cytoplasmic amino acid sequence (380–392) of the rat β3 subunit recognized a single polypeptide of molecular mass of 58 kDa in Western blots with Ro7–1986 affinity-purified GABA$_A$ receptors from the rat brain, and a doublet of molecular mass of 54 kDa and 56 kDa in receptors from the bovine cortex, hippocampus, and cerebellum. Deglycosylation of purified receptors from the bovine cortex with *N*-glycanase resulted in a single band immunostained at molecular mass of 52 kDa. These anti-β3 subunit antibodies immunoprecipitated ~50% of [^{3}H]flunitrazepam binding sites from soluble extracts of bovine cortex, whereas βcyto antibodies, which probably recognize all β subunit isoforms, precipitated almost 100% of benzodiazepine binding sites. These results indicate heterogeneity of GABA$_A$ receptor subunit composition with respect to the nature of β subunits. The GABA analogue 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol (THIP), like GABA, shows heterogeneous binding affinities in brain homogenates. The higher affinity sites were previously suggested as corresponding to a 58-kDa polypeptide in rat that is photoaffinity-labeled with [^{3}H]muscimol, a band that comigrates with the one stained by anti-β3 antibodies. However, THIP affinity was not significantly different between receptors containing β3 subunits and those lacking β3, as demonstrated by similar affinities in receptors that ere immunoprecipitated by anti-β3 antibodies and those that were not. Also, THIP displaced [^{3}H]muscimol binding with similar multiple affinities across brain regions where different β subunit variants are expressed with varying abundances. These observations suggest that the property of high affinity THIP binding cannot be explained solely by β3 subunits. The coupling efficiency between GABA and benzodiazepine binding sites appears to be determined by the nature of α subunits rather than of β subunits. GABA enhanced [^{3}H]flunitrazepam binding with different efficacies and potencies in receptors immunoprecipitated by anti-α1, -α2, and -α3 subunit antibodies. In contrast, β3 subunit-enriched and disenriched receptors did not differ in this property. [^{3}H]Flunitrazepam binding in GABA$_A$ receptors containing α2 and α3 subunits was enhanced to a significantly greater extent than were those with α1. In addition, receptors containing α1 and α3 subunits had higher potencies of enhancement than did those with α2 subunits. These binding results suggest that benzodiazepines are likely to show differential efficacies at different subtypes of GABA$_A$ receptors in the brain, as suggested by studies on recombinant receptor expression.

GABA$_A$ receptors are chloride ion channels with heterooligomeric structures, probably pentameric. As major inhibitory neurotransmitter receptors in the vertebrate central nervous system, they also serve as binding sites for clinically useful drugs such as BZs, barbiturates, and anesthetic steroids and for convulsants, which are represented by *t*-butyl bicyclophosphorothionate and picrotoxin (1). These binding sites are functionally coupled with each other and with the GABA binding sites (2). So far, 17 cDNA clones have been identified as GABA$_A$ receptor polypeptide subunits and grouped by degree of sequence identity into five subunit families: 6 α, 4 β, 4 γ, 1 δ, and 2 ρ (3–8). These can evidently be combined in a variety of subunit combinations to produce functionally and pharmacologically different receptor subtypes.

Ever since the molecular heterogeneity of GABA$_A$ receptors was first suggested by displacement of [^{3}H]flunitrazepam binding by the triazolopyridazine Cl-218872 with two

This work was supported by National Institutes of Health Grant NS28772.

ABBREVIATIONS: GABA, γ-aminobutyric acid; BZ, benzodiazepine; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol; Cl-218872, 3-methyl-6-[(3-trifluoromethyl)phenyl]-1,2,4-triazolo[4,3-*b*]pyridazine; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

different affinities (9, 10), the structural basis of binding subtypes so defined has been sought in terms of unique subunit combinations. Aided by clinical importance and the availability of subtype-specific ligands, $GABA_A$ receptor heterogeneity in relation to the BZ binding site has been relatively well characterized. The nature of α and γ subunits was shown to determine the affinity and/or efficacy of BZ ligands (11–15). In addition to BZs, the structural and functional heterogeneity of $GABA_A$ receptors is apparent in heterogeneous sensitivity to allosteric modulators such as barbiturates or steroids and compounds that bind to the GABA binding sites such as the agonist THIP and the antagonist bicuculline (16–19). However, the subunit specificity that can explain these pharmacological subtypes has not been characterized as well as it has been for BZs.

With BZ affinity-purified receptors from the rat brain, it has been shown that photoincorporation of [^{3}H]flunitrazepam and [^{3}H]muscimol into multiple polypeptide bands can be differentially modulated by GABA, steroids, and barbiturates (18). In addition, THIP inhibited [^{3}H]muscimol photoincorporation into a 58-kDa polypeptide with higher affinity than a 55-kDa band, whereas taurine preferentially inhibited photolabeling of [^{3}H]muscimol to the 55-kDa band (16, 20). The 58-kDa band is tentatively identified as β3 and the 55-kDa band as the β2 subunit, with subunit-specific antibodies (21, 22). Thus, $GABA_A$ receptors containing different β subunit polypeptides appear to be distinct molecules that can be distinguished by their heterogeneous pharmacological properties. Considering that BZ pharmacological subtypes have not been correlated with the β subunit heterogeneity and that GABA binding sites are believed to reside on these subunits, it is interesting that receptors with different β subunits apparently have different affinities for THIP and taurine.

As a bicyclic analogue of muscimol, THIP was originally developed for greater therapeutic selectivity with the strategy of conformational restriction (23). The possibility that THIP might be selective for certain subclasses of $GABA_A$ receptors has been suggested from both functional and radioligand binding assays. In the spinal cord and in CA1 pyramidal cells of the hippocampus, THIP had higher affinity for depolarizing responses rather than hyperpolarizing GABA responses (24, 25). In addition, these two receptors were shown to have different sensitivities for blockade by GABA antagonists and for enhancement of the response by BZ and barbiturates, further suggesting that these two $GABA_A$ receptors are functionally distinct entities. In autoradiographic studies with cultured cerebellar and spinal neurons of rats, it has been observed that [^{3}H]THIP labels a considerably smaller population of $GABA_A$ receptors than [^{3}H]isoguvacine, another GABA agonist (26). Also, a separate study showed the density of binding sites for [^{3}H]THIP in membrane homogenate binding assays is much lower than that for [^{3}H]GABA and [^{3}H]piperidine-4-sulfonate (27). All of these observations suggest the possibility that THIP binds with detectably high affinity to a subpopulation of $GABA_A$ receptors, although it is unknown whether THIP binds with low affinity or does not bind to other $GABA_A$ receptors. Heterogeneity in THIP affinity is supported by an autoradiography study in which THIP displaced [^{3}H]muscimol binding with different affinities depending on brain regions (18). Based on these observations, we investigated whether receptors containing different β subunit variants might confer a different sensitivity for THIP with β3 subunit-specific antibodies in attempts to separate a high affinity THIP binding population. Allosteric coupling efficiency between GABA and BZ binding sites in relation to subunit specificity was also studied with the same approach. Some of the results have been presented in preliminary form (21, 29).

Experimental Procedures

Materials. [Methyl-^{3}H]flunitrazepam (84.3 Ci/mmol) and [methylene-^{3}H]muscimol (19.5–20 Ci/mmol) were from DuPont-New England Nuclear (Boston, MA). THIP was the kind gift of Dr. Povl Krogsgaard-Larsen (Royal Danish School of Pharmacy, Copenhagen, Demark). GABA, diazepam, Sephadex G-25 fine, protein A Sepharose CL-4B beads, rabbit γ-globulin, and diethylaminoethyl-Sephacel were obtained from Sigma Chemical Co. (St. Louis, MO). *Staphylococcus aureus* cell suspension (Pansorbin) was obtained from Calbiochem Corp. (San Diego, CA). Triton X-100 and *N*-glycosidase F (recombinant) were purchased from Boehringer Mannheim Corp. (Indianapolis, IN). Western blot analysis was performed with the Vectastin ABC Kit from Vector Laboratories (Burlingame, CA).

Preparation of subunit-specific antibodies. Anti-β3 subunit antibodies were raised in rabbits against a synthetic peptide sequence (380–390) of rat β3 subunits of the $GABA_A$ receptors as previously described (28). The generic βcyto antibody was raised against a bovine β1 subunit sequence of 413–438. Synthesis and characterization of βcyto, α1, α2, and α3 subunit-specific antibodies were reported previously (28, 30). Antisera were purified with Sepharose 4B columns, to which respective synthetic peptides were attached in the same way as described previously (28).

Preparation of membrane homogenates. Tissues from different areas of the cow brain, obtained fresh from the slaughterhouse, were dissected and stored in 0.32 M sucrose at −70° until use. Membrane homogenates for synaptosomal fractions (P2 pellets) were prepared as described previously (31), with the substitution of the following buffer: 20 mM KH_2PO_4/K_2HPO_4, pH 7.4, 50 mM KCl, containing 0.02% NaN_3, 0.5 mM dithiothreitol and various proteinase inhibitors (1 mM EDTA, 2 mM benzamidine chloride, 0.1 mM benzethonium chloride, 100 μg/l bacitracin, 0.3 mM phenylmethylsulfonyl fluoride, 10 mg/l soybean trypsin inhibitor, 10 mg/l ovomucoid trypsin inhibitor). Prepared membrane homogenates were stored at −70° until use. For the ligand binding assays, thawed membrane homogenates were washed twice with potassium phosphate buffer (pH 7.4) containing 150 mM KCl, 1 mM EDTA, and 0.02% NaN_3 by repeated centrifugation and homogenization. Final suspension was made at a protein concentration of 1–1.5 mg/ml. Aliquots of the membrane homogenates (300 μl) were incubated with 4 nM [^{3}H]muscimol in the presence of varying concentrations of THIP. Nonspecific binding was measured with 100 μM GABA.

Immunoprecipitation of purified $GABA_A$ receptors. $GABA_A$ receptors from bovine cortex (~100 g) or hippocampus (~60 g) were purified with the Ro7–1986 affinity column as described previously (30) with the following changes. For solubilization, 1% Triton X-100 was used instead of 0.5%. $GABA_A$ receptors were dialyzed after elution from the affinity column, concentrated, and freed of flurazepam with a 2-ml diethylaminoethyl-Sephacel anion exchange column (31). High salt (800 mM KCl), which was used to elute receptors from the anion exchange column, was removed by dialysis against PBS buffer containing 0.2% Triton X-100, 10% glycerol, and various protease cocktails. Purified receptors were stored at −70° until use. Under this storage condition, ~80% of [^{3}H]muscimol binding activity was recovered after 5 weeks.

Immunoprecipitation of receptors was performed as described previously (28) with slight modifications. Aliquots of purified receptors (~320 fmol of muscimol binding sites) were incubated with varying concentrations of antibodies in a total volume 500 μl of PBS buffer

(10 mM NaH_2PO_4/Na_2HPO_4, 150 mM NaCl, pH 7.4). Extra Triton X-100 and EDTA were added as supplements to the incubation medium to adjust concentrations of Triton X-100 and EDTA to 0.1% and 1 mM, respectively. Total immunoglobulin concentrations were kept constant by including rabbit γ-globulins. After overnight incubation at 4°, antibody/receptor conjugates were separated by incubation for 1 hr with *S. aureus* cell suspensions or protein A Sepharose beads at 4°, followed by centrifugation for 15 min at 10,000 × *g*. Immunopellets of protein A beads gave negligible nonspecific binding (<5%) in comparison to that of cell suspensions (15–20%). For binding assays in immunopellets, pellets were washed four times with 500 μl of PBS buffer containing 0.1% Triton X-100 (or 0.5%), 1 mM EDTA, and 0.02% NaN_3 and resuspended in 500 μl of the same buffer. Typically, 20–50 μl of antibody-treated supernatant or immunopellet suspension was used for the ligand binding assays.

Immunoprecipitation of $GABA_A$ receptors from soluble extracts. Quantitative immunoprecipitation of [^{3}H]flunitrazepam binding sites was carried out as described above for purified receptors but with 100-μl aliquots of crude solubilized receptors (~30 fmol of [^{3}H]flunitrazepam binding sites).

To measure GABA enhancement of [^{3}H]flunitrazepam binding, endogenous GABA was removed from the solubilizates with a Sephadex G-25 gel filtration column (1.5 cm × 47 cm). Soluble extracts (6 ml) were applied to the gel filtration column that were preequilibrated with 20 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.4) containing 0.5% Triton X-100, 150 mM KCl, 1 mM EDTA, 0.02% NaN_3, and protease inhibitors, which were used for solubilization. Void volume fractions containing $GABA_A$ receptors, as measured by 10 nM [^{3}H]flunitrazepam binding, were pooled (~9 ml). Desalted soluble extracts (4.5 ml) were incubated with protein A beads to which 150 μg of antibodies was preattached. After overnight incubation at 4°, protein A beads were collected by centrifugation at 10,000 × *g* for 2–3 min and washed four times with 1 ml of 20 mM KH_2PO_4/K_2HPO_4 (pH 7.4) containing 150 mM KCl, 0.5% Triton X-100, 0.02% NaN_3, and various protease inhibitors. Final suspension was made in 1.2 ml of the same buffer, and 50-μl aliquots of the bead suspension were used for the binding assays. To attach antibodies to protein A beads, 150 μg of affinity purified antibodies was incubated with protein A beads (~120 μl bead volume) for 4–6 hr at 4° in a total volume of 500 μl of the sodium phosphate buffer as mentioned. After overnight incubation, bound and unbound antibodies were separated by a brief centrifugation. Pellets were washed four times with 1 ml of 20 mM KH_2PO_4/K_2HPO_4, 0.5% Triton X-100, 200 mM KCl, 1 mM EDTA, and 0.02% NaN_3, pH 7.4, containing protease inhibitors.

Deglycosylation of the BZ affinity-purified receptors. Purified $GABA_A$ receptors were *N*-deglycosylated according to methods of Buchstaller *et al.* (32) with minor modifications. Briefly, 25 μg purified $GABA_A$ receptor protein in 170 μl (0.4 M Tris·HCl buffer, pH 8.6, containing 0.5% SDS and 50 mM β-mercaptoethanol) was denatured at 65° for 10 min in a water bath. On cooling to room temperature, 330 μl of 15% octylglucoside (nonionic detergent), 15 mM EDTA, and 3.7 units recombinant *N*-glycanase were added. This mixture was incubated at 37° for 13 hr. Receptor protein was precipitated with MeOH/$CHCl_3$ (18, 32). The pellet was resuspended in SDS-digestion buffer (2.5% SDS, 10 mM Tris·HCl, 5% mercaptoethanol, 1 mM EDTA, 0.01% Bromophenol blue, pH 8.0) and heated to ~95° for 5 min. Samples were subjected to SDS-PAGE separation and detection by Western blot analysis.

Western blots. Purified $GABA_A$ receptor preparations were subjected to SDS-PAGE with 7.5% acrylamide and subsequent electrophoretic transfer to polyvinylidene difluoride membrane for immunostaining. Immunostaining was accomplished by first incubating the membrane with a blocking solution of 5% BSA, 0.05% Tween-20 in PBS, pH 7.2, for 3 hr at room temperature. This was followed by overnight incubation at 4° with a $GABA_A$ receptor subunit-specific polyclonal antibody in PBS containing 1% BSA and 0.05% Tween-20. The membrane was washed three times with PBS for a total time of 15 min. The washed membrane was exposed to biotinylated chicken anti-rabbit IgG in PBS containing 1% BSA and 0.05% Tween-20 for 3 hr at room temperature and washed as before. The membrane was then exposed to the streptavidin-biotinylated alkaline phosphatase complex in PBS with 1% BSA for 30 min and washed. Detection of peptide bands sensitive to the subunit antibodies was accomplished with a BCIP/NBT substrate kit for alkaline phosphatase and took ~10 min for a dark-blue color to develop.

Radioligand binding assays. For experiments of THIP displacement of [^{3}H]muscimol binding in crude soluble extracts, membrane homogenates were dialyzed, before solubilization, against PBS buffer containing protein inhibitors to remove endogenous GABA.

Aliquots of soluble or immunoprecipitated antibody-bound receptors were incubated with the respective radioligand in a total of 500 μl PBS buffer containing 0.02% NaN_3 and 1 mM EDTA, in the presence of final 0.1% concentration of Triton X-100. Concentrations of 10 nM of [^{3}H]flunitrazepam or 25 nM [^{3}H]muscimol were used for assays of quantitative immunoprecipitation. Other concentrations used in experiments in the presence of modulatory agents are indicated in figure legends or tables. Nonspecific binding was determined in the presence of 100 μM GABA for [^{3}H]muscimol and 10 μM diazepam for [^{3}H]flunitrazepam binding. After incubation for 30 min ([^{3}H]muscimol) or 90 min ([^{3}H]flunitrazepam) at 4°, incubation was terminated by the addition of 100 μl bovine γ-globulin and 300 μl of polyethylene glycol. Free ligands were separated by centrifugation at 27,500 × *g* for 30 min for [^{3}H]muscimol binding assays or filtration through GF/B filters for [^{3}H]flunitrazepam binding assays. Polyethylene glycol was included in the washing buffer at a 10% final concentration. Binding assays for the membrane homogenates were performed in the same manner but omitting the step of protein precipitation indicated earlier and with the washing buffer without polyethylene glycol.

Data analysis. Saturation isotherms of [^{3}H]muscimol binding, GABA enhancement of [^{3}H]flunitrazepam binding, and THIP displacement curves were analyzed by nonlinear least-squares regression analysis with BMDP-PAR computer program, and parameters for the fits were simultaneously obtained from the nonlinear regression analysis. Formulas that were used to fit data sets for one- or two-site models are as follows:

One-site model:

$$\text{Bound} = \frac{B_{max} \times \text{Free}}{K_d + \text{Free}}$$

Two-site model:

$$\text{Bound} = \frac{B_{max\,1} \times \text{Free}}{K_{d1} \times \text{Free}} + \frac{B_{max\,2} \times \text{Free}}{K_{d2} \times \text{Free}}$$

where B_{max} is the maximum binding sites (fmol), maximal enhancement (percentage) or proportion of the binding sites (percentage), and K_d is the dissociation constant for the saturation isotherms or IC_{50} for the THIP displacement experiments or EC_{50} for GABA enhancement experiments. Better fit for one- or two-site models was determined with residual sum of squares, followed by *F* test, as described by Motulsky and Ransnas (33). To examine the significance between different parameter values, pooled data sets were fitted into the the respective functions, and the parameter of interest was forced to be shared. By running the BMDP-PAR nonlinear regression program, a residual sum of squares was obtained for the pooled data set. Residual sum of squares from the pooled data set and those from separate data sets were used for the *F* test to examine significance of the difference between parameter values as described. Hill coefficients and IC_{50} values for β3 antibody-separated fractions were obtained with BMDP nonlinear regression analysis by fitting data to the following logistic function: $f = 100/(1 + conc./X_0)^n$, where *conc.* is the concentration of the displacing agent, X_0 is percentage of displacement, and *n* is a Hill coefficient. Significance tests between different IC_{50} values were done in the same way as described.

Results

Antibodies specific for the $GABA_A$ receptor β3 subunit were raised against the cytoplasmic peptide sequence (380–392) of the rat β3 subunit. Low amino acid sequence homology (≤30%) of the epitope peptide region with those of β2 or β1 subunits suggests relative specificity of these antibodies toward β3 subunits (Fig. 1). In Western blots of Ro 7–1986 affinity-purified receptors, anti-β3 subunit antibodies recognized a 58-kDa polypeptide band for the whole rat brain and two polypeptides of of 54 and 56 kDa for the bovine cortex (Fig. 2). The 56-kDa polypeptide was immunostained in much weaker intensity in comparison to the 54-kDa polypeptide. Deglycosylation of purified $GABA_A$ receptors from the bovine cortex with *N*-glycanase resulted in immunostaining of a single polypeptide of 52 kDa (Fig. 3). Doublet polypeptides were also detected in $GABA_A$ receptors that were purified from the bovine hippocampus (Fig. 4). However, receptors from the bovine cerebellum appeared to contain the 54-kDa subunit as the predominant species as shown in Fig. 4. Anti-β3 antibodies quantitatively immunoprecipitated 50% of [^{3}H]flunitrazepam binding sites from crude Triton X-100-soluble extracts of the bovine cortex; 100 μg of antibodies reduced the supernatant to 53 ± 10%, with the pellet containing 74 ± 20%. On the other hand, almost 100% of the binding activity was precipitated by βcyto antibodies, which are believed to recognize all of the β subunit variants (Fig. 5, A and B) (30). Thus, most if not all BZ affinity-purified $GABA_A$ receptors appear to contain β subunits in their receptor complexes, and they show structural heterogeneity in relation to their β subunits, i.e., some oligomeric isoforms contain β3, whereas others contain β1 or β2 subunits.

The GABA analogue THIP displaced [^{3}H]muscimol binding with multiple affinities in membrane homogenates of the bovine cortex, as indicated by a curvilinear Eadie-Hofstee plot and a Hill coefficient of <1 (Fig. 6A and Table 1). Binding parameters were obtained from a computer-fitted nonlinear regression analysis. The two-site binding model with IC_{50} values differing by at least 10-fold with similar abundances of the binding populations gave a significantly better fit than the one-site model. This heterogeneous binding profile was preserved in Triton X-100-soluble extracts and in the BZ affinity-purified receptors (Fig. 6, B and C). Estimated binding parameters are given in Table 1.

Receptors containing β3 subunits were immunoprecipitated from BZ affinity-purified receptors of the bovine cortex with the β3 subunit-specific antibodies. Displacement of [^{3}H]muscimol binding by THIP was performed on immunopellets, the antibody-treated supernatant, and untreated soluble receptors. Even though it is unlikely that the binding of antibodies to the cytoplasmic epitope between TM3 and TM4 changes the affinity for [^{3}H]muscimol, which is believed to bind to the amino-terminal extracellular domain, this possibility was tested with βcyto antibodies. The K_d values of [^{3}H]muscimol binding in βcyto immunopellets and the receptors not treated with antibodies were identical (5.1 and 5.7 nM, respectively; Fig. 7). Anti-β3 antibodies precipitated

Rat	β1	P	L	S	S	R	E	G	Y	G	K	-	G	L
Bovine	β1	P	M	S	S	R	E	G	Y	G	K	-	A	L
Rat	β2	A	G	I	P	R	H	S	F	G	K	N	A	L
Bovine	β2	A	G	L	P	R	H	S	F	W	K	N	A	L
Rat	β3	Q	S	M	P	K	E	G	H	G	R	Y	M	Q
Bovine	β3	Q	S	M	P	R	E	G	H	G	K	H	M	-

Fig. 1. Comparison of the epitope peptide sequence of β3 subunits of the rat (380–392, *shaded*) with the corresponding peptide sequences of the β1 and β2 subunits from various species. Homologous amino acid residues to those of the β3 peptide sequence are shaded.

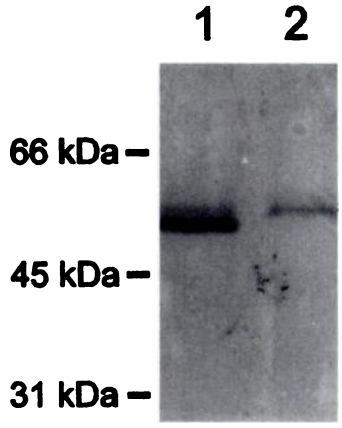

Fig. 2. Western blots of $GABA_A$ receptors purified from brains of adult rat and bovine cortex using affinity purified β3 antibodies (dilution 1:500). Purified receptors were subjected to SDS-PAGE and Western blot. The receptor protein was detected by immunostaining with the β3 antibody as indicated in Experimental Procedures. Each lane contains 1 pmol of the purified receptor. Experiments were performed five times with the same results. *Lane 1*, bovine cortex; *lane 2*, rat brain.

Fig. 3. Comparison of purified $GABA_A$ receptors from the bovine cerebral cortex before and after deglycosylation. After complete *N*-deglycosylation by *N*-glycanase, receptors were subjected to SDS-PAGE and Western blot analysis and detected with affinity-purified β3 antibodies (dilution 1:500). *Lane 1*, after deglycosylation; *lane 2*, before deglycosylation. Experiments were performed four times with similar results.

Fig. 4. Comparison of bovine brain regions with the affinity-purified β3 antibody for the $GABA_A$ receptor. Purified $GABA_A$ receptors from bovine cerebellum (*lane 1*, 1 pmol), hippocampus (*lane 2*, 1 pmol), and cerebral cortex (*lane 3*, 2 pmol) were subjected to SDS-PAGE and Western blot analysis. Receptor protein was detected by immunostaining with the β3 antibody at a dilution of 1:500. Experiments were repeated six times with similar results.

~50% of [^{3}H]muscimol binding sites from BZ affinity-purified receptors of bovine cortex (Fig. 8A). From this quantitative immunoprecipitation experiment, the immunopellet obtained after incubation with 100 μg of antibodies was used for determination of THIP sensitivity. In this experiment, β3 subunit-containing receptors did not have higher affinity for THIP, as indicated by similar IC_{50} values among antibody-separated fractions (Fig. 8B). Those values together with Hill coefficients are given in Table 2. Comparable IC_{50} values were obtained when protein A beads, which have lower nonspecific binding (<5%), were used for immunoprecipitation instead of *S. aureus* cells (15–20% nonspecific binding) (data not shown).

Correlation between GABA binding site heterogeneity demonstrated by THIP and the nature of β subunits was further investigated with membrane homogenates from different regions of the bovine brain. Thalamus and hypothalamus are regions where β2 and β3 subunits are expressed as predominant β subunit variants, respectively, as indicated by *in situ* hybridization histochemistry in the rat (34, 35). If the nature of β subunits were a major determinant for THIP sensitivity, enrichment of high or low affinity populations for THIP would be expected in these brain regions. However, neither of the THIP affinity states played a dominant role in these regions, as indicated in Table 3. Cortex and hippocampus, where β2 and β1 isoforms are abundantly expressed together with β3 subunits (34), had similar binding profiles to other brain regions tested. In the cerebellum, an additional high affinity population was found, giving K_d = 11 nM and B_{max} = 7%, and a three site-binding model gave a significantly better fit than that of two sites. These results suggest that high affinity binding sites for THIP, which were demonstrated by the autoradiographic study and the photolabeling experiment, cannot be explained solely by $GABA_A$ receptors containing β3 subunits.

In addition to the role of β subunits as a determinant of GABA binding site heterogeneity, the possibility of their involvement in the allosteric coupling property between GABA and BZ binding sites was investigated. With the same approach, GABA enhancement of [^{3}H]flunitrazepam binding was measured in antibody-separated fractions of the affinity-purified receptors from bovine cortex. Values of maximal enhancement of [^{3}H]flunitrazepam binding by GABA determined by the nonlinear curve-fitting analysis were similar among antibody-separated fractions, i.e., 38% and 46% for antibody-treated supernatant and anti-β3 immunopellets, respectively (Fig. 9). This result suggests that β subunits do not appear to play a dominant role in determining coupling efficiency between GABA and BZ binding sites compared with α subunits (see below).

Heterogeneity of GABA enhancement of BZ binding was further characterized with α subunit-specific antibodies. $GABA_A$ receptors containing α1, α2, or α3 subunits were immunoprecipitated from crude soluble extracts of the rat brain after endogenous GABA was removed by gel filtration. GABA enhanced [^{3}H]flunitrazepam binding with signifi-

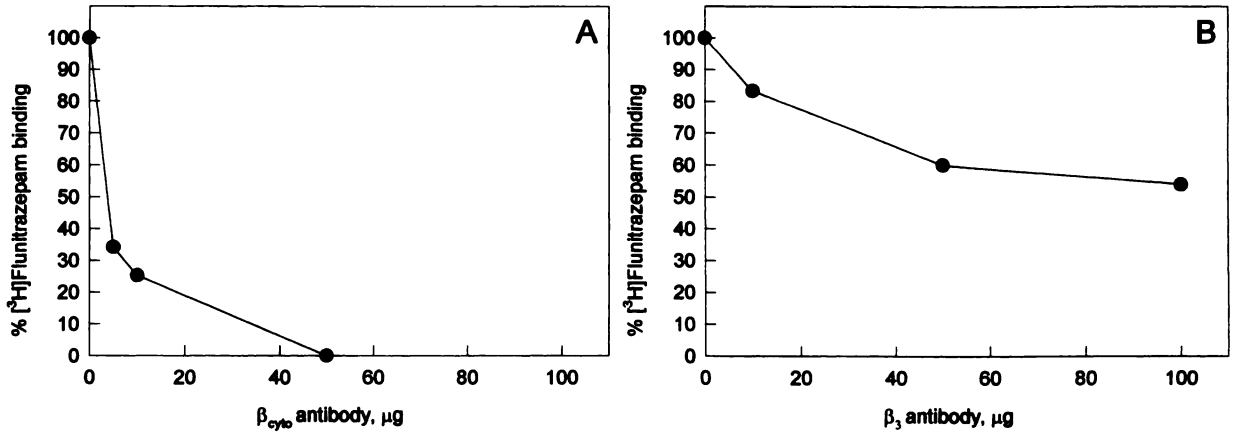

Fig. 5. Quantitative immunoprecipitation of [^{3}H]flunitrazepam binding sites from crude soluble extracts of the bovine cortex with βcyto (A) and β3 (B) subunit-specific antibodies. After immunoprecipitation, remaining binding activity in the supernatant was determined with 30 nM [^{3}H]flunitrazepam and expressed as percentage of the control binding that was determined in the absence of antibodies. Data were expressed as the mean of triplicate experiments from a single experiment.

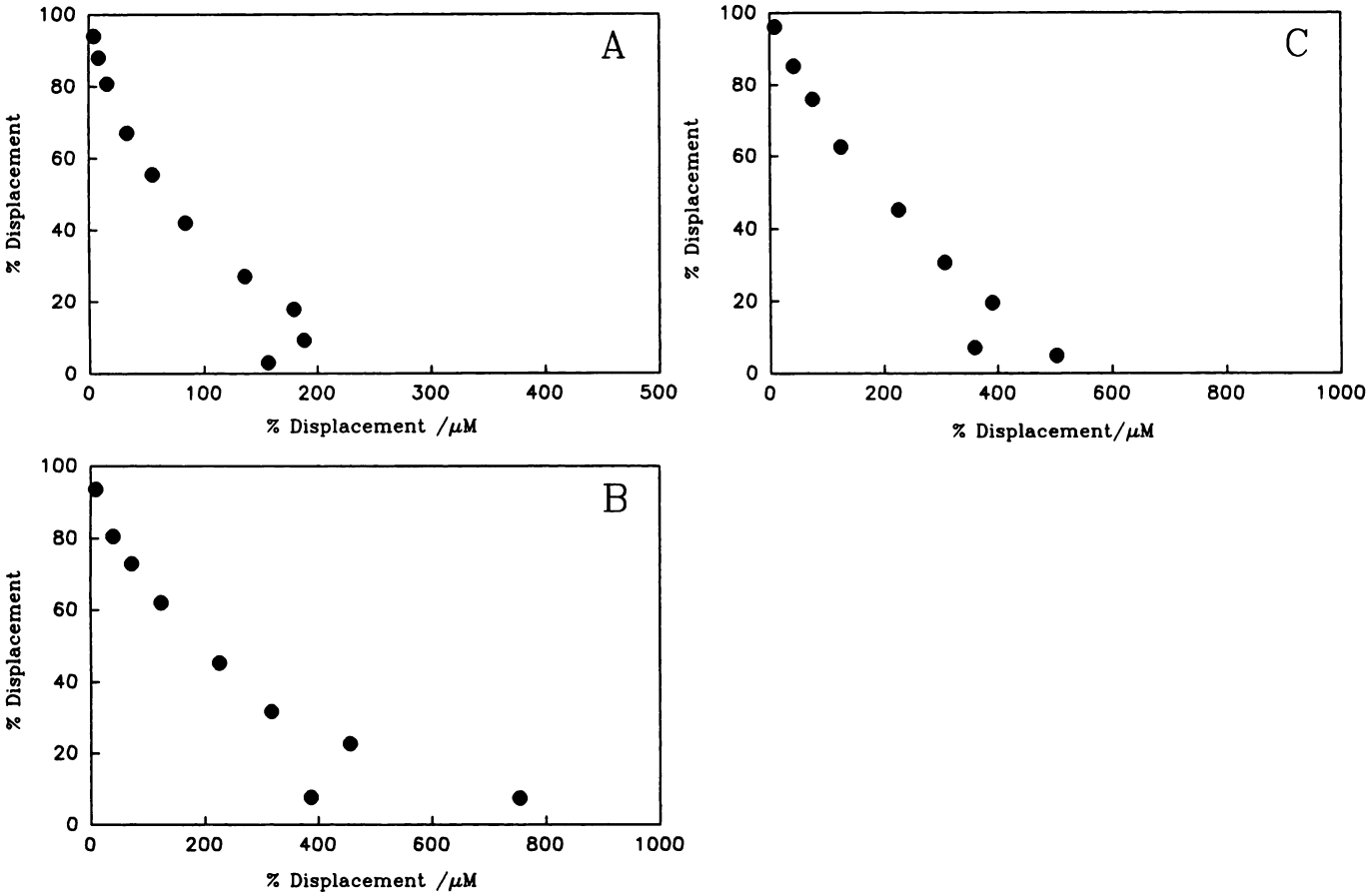

Fig. 6. Eadie-Hofstee plots of THIP displacement of [^{3}H]muscimol binding in the membrane homogenates (A), soluble extracts (B), and BZ affinity-purified receptors (C) of the bovine cortex. Concentrations of THIP that were used to displace 4 nM [^{3}H]muscimol binding were in the range of 5 nM to 20 μM. Each set of data is representative of two or three separate determinations. Data are mean of triplicate assays.

TABLE 1
Estimated binding parameters for Fig. 6

Receptor preparation	IC_{50} (1)	B 1	IC_{50} (2)	B 2	Hill coefficient
	μM	%	μM	%	
Membrane homogenate	0.32 ± 0.1	59 ± 2	3.7 ± 0.8	42 ± 2	0.8 ± 0.02
Soluble extract	0.1 ± 0.02	60 ± 6	1.1 ± 0.3	40 ± 8	0.78 ± 0.01
Purified receptor	0.14 ± 0.03	59 ± 2	1.2 ± 0.5	41 ± 12	0.87 ± 0.01

Values of IC_{50}, B and Hill coefficients were obtained by computer-fitted nonlinear regression analysis as described in Experimental Procedures. For the membrane homogenates and the purified receptors, values are mean ± standard error from three independent experiments. For crude soluble extract, values are mean ± standard error from two separate determinations. B1 and B2 refer to the percentage of total binding to two populations of sites that showed IC_{50} values (1) and (2), respectively.

cantly higher affinities in α1 and α3 subunit-containing receptors than in α2 subunit-containing receptors. On the other hand, receptors with α2 and α3 subunits had higher maximal enhancement than did those with α1 subunits, suggesting that different α subunit-containing receptors are not identical in their conformational change induced by GABA as measured by coupling with BZ binding sites (Fig. 10 and Table 4).

Discussion

Expression of $GABA_A$ receptors from cloned subunit cDNAs and examination of pharmacological properties by electrophysiology and binding have been partially successful in reproducing some of the heterogeneity observed in nature (2, 4, 11–13, 19, 36). Further information on which subunit combinations occur in nature, their localization, and their binding and pharmacological specificity is needed, and conclusions on these matters cannot be produced solely from recombinant studies. Attempts to examine receptor isoforms by biochemical separation have been aided by the use of subunit-specific antibodies (14, 15, 22, 30, 37),[1] especially because separations based on binding specificity have been mostly unsuccessful. Furthermore, antibodies to subunits involved in GABA receptors that do not bind benzodiazepines, e.g., α6 (14), can be used to purify receptors that are not isolated by the traditional BZ affinity chromatography.

Anti-β3 antibodies immunostained a single polypeptide band at 58 kDa in Western blots of BZ affinity-purified re-

[1]K. H. Huh, S. Endo, and R. W. Olsen, Diazepam-insensitive $GABA_A$ receptors in rat cerebellum and thalamus analyzed by subunit-specific antibodies and photolabeling, submitted for publication.

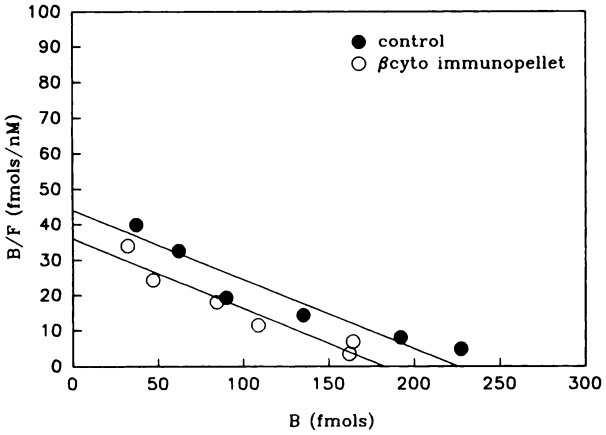

Fig. 7. Scatchard analysis of [^{3}H]muscimol binding in antibody-bound and the soluble form of $GABA_A$ receptors. $GABA_A$ receptors purified from bovine hippocampus were immunoprecipitated with βcyto antibodies as described in Experimental Procedures. Binding activities were determined in protein A bead immunopellets and in the receptors that were not treated with antibodies. Values of K_d were 5.7 nM for the soluble form of receptors and 5.1 nM for receptors in immunopellets. Data are from one experiment with triplicate assays.

TABLE 2
Estimated binding parameters for Fig. 8B

Separated fraction	Hill coefficient	IC_{50}
		nM
Control	0.84	447
β_3 immunopellet	0.93	320
β_3 supernatant	1.07	430

To precipitate antibody-receptor conjugates, *S. aureus* cell suspensions were used. Hill coefficients and IC_{50} values were determined using BMDP nonlinear curve-fitting program.

ceptors from the rat whole brain, whereas a doublet of 54- and 56-kDa polypeptides were stained with receptors from the bovine cortex (Fig. 2). The same bands, plus others, were recognized by our "generic" anti-β antiserum, β cyto (28), and a different band at 56 kDa in rat brain was recognized by anti-β2 antiserum (22) (results not shown). These results agree reasonably well with studies in which β3 subunit-specific antibodies against the cytoplasmic peptide sequence recognized a 56-kDa band in 5–10-day-old rat brain and two polypeptides of 58 and 60 kDa in the bovine cortex, with BZ affinity-purified receptors (32, 37). Two polypeptides in cow that are detected by the β3 subunit-specific antibodies appear to represent differential glycosylation products rather than splice variants, as indicated by *N*-deglycosylation experiments, producing the 52-kDa polypeptide (Fig. 3). The production of a single deglycosylated product from these two polypeptides with a molecular mass (52 kDa) predicted from the cDNA (36, 39) argues against the assumption that the lower-molecular-weight polypeptide is produced from the higher-molecular-weight polypeptide by proteolysis. These two polypeptide bands were also found in the BZ affinity-purified receptors from the bovine hippocampus. However, in the cerebellum, only the 54-kDa polypeptide was detected (Fig. 4). This result could indicate selective association of the 56-kDa polypeptide with the high affinity muscimol binding sites that were not retained in the BZ-affinity column or the presence of heterogeneous glycosylation machinery in different brain regions. However, considering the relatively low abundance of β3 subunits expressed in the rat cerebellum (~20% of GABA and BZ binding can be immunoprecipitated; results not shown), the possibility that the present immunostaining method is not sufficiently sensitive to detect the relatively fainter 56-kDa polypeptide cannot be totally disregarded. Anti-β3 antibodies immunoprecipitated 50% of [^{3}H]flunitrazepam binding activity from crude soluble extracts of the bovine cortex (Fig. 5A). On the other hand, generic β subunit antibodies precipitated almost all of the BZ binding sites (Fig. 5B). These results demonstrate that β3 subunits are constituents of native $GABA_A$ receptors and suggest the structural heterogeneity of $GABA_A$ receptors in their β subunit, consistent with differential expression of β subunit mRNAs across the brain (34, 35, 39, 40). Immunoreactivity in Western blotting and immunoprecipitation with the anti-β3 varied with brain region consistent with the variable mRNA expression.

High affinity THIP binding sites were not enriched or disenriched in the immunopurified $GABA_A$ receptors with anti-β3 antibodies, as shown by similar IC_{50} values in the antibody-separated fractions (Fig. 8B and Table 2). This result does not agree with the previous observation in which THIP displaces [^{3}H]muscimol photoincorporation in rat receptors to a 58-kDa polypeptide with higher affinity than to a 56-kDa peptide band (IC_{50} of 0.21 μM versus 3.2 μM). Previous (21, 29) and current results suggest that these bands represent β3 and β2 subunits of the $GABA_A$ receptors, respectively. Pollard *et al.* (37) reported that β3 subunit-specific antibodies made against a cytoplasmic peptide sequence precipitated 49% of [^{3}H]flunitrazepam binding sites from soluble extracts of bovine cortex by sequential immunoprecipitations; this closely agrees with the value obtained in the present study. In Western blots of BZ affinity-purified receptors for the bovine cortex, their antibodies immunostained two polypeptides with molecular masses of 60 and 58 kDa, which are similar to those recognized by β3 antibodies used in the present study. Therefore, the lack of correspondence between THIP affinities and the nature of the β subunit does not appear to be due to an incomplete immunoseparation of receptors containing β3 subunits from those lacking the β3 subunit. Also, similar affinities of [^{3}H]muscimol between antibody-bound and nonbound forms of receptors (Fig. 7) provide validity for comparison of binding parameters between the immunoprecipitated and a soluble form of the receptors, excluding the possibility of a conformational change of GABA binding sites induced by antibody binding to the receptors.

Further attempts to match the subunit populations with high affinity THIP binding were made with membrane homogenates from different regions of the bovine brain (Table 3). In autoradiographic studies, high affinity THIP binding sites have been located in CA1 and CA3 regions of the hippocampus, caudate putamen, and cerebellar granule cells, in comparison with the low affinity sites found in some layers of the cortex, hypothalamus, substantia nigra, and cerebellar molecular layer (18). In these brain regions, mRNAs of different β subunit variants are found in variable abundance (34, 35, 40). However, the present study shows that THIP displaced [^{3}H]muscimol binding with two similar affinities and abundances across the brain regions (Table 3). In areas such as thalamus and hypothalamus where a single type of the β subunit is relatively enriched, i.e., β2 and β3, respec-

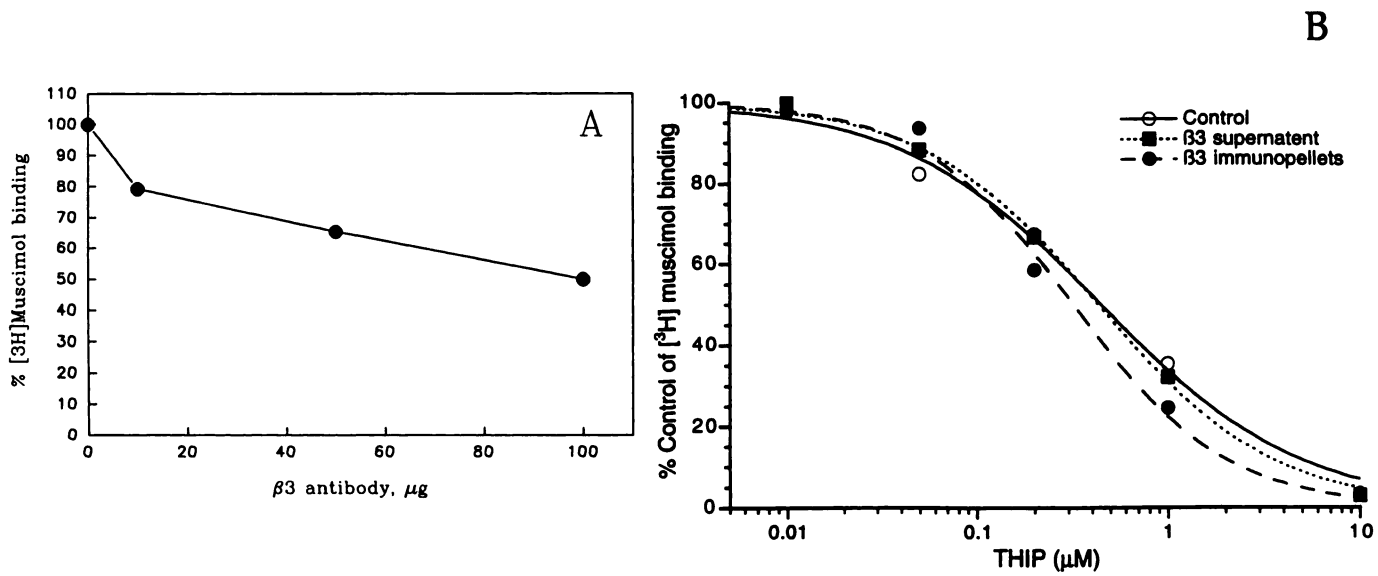

Fig. 8. A, Immunoprecipitation of [^{3}H]muscimol binding sites from the purified $GABA_A$ receptors of bovine cortex with β3 antibodies. Degree of immunoprecipitation was determined by measuring [^{3}H]muscimol (25 nM) binding activity in the supernatant after immunoprecipitation. B, Displacement of 4 nM [^{3}H]muscimol binding by THIP in β3 antibody-separated fractions. Binding activities were determined in immunopellets of *S. aureus* cells (after incubation with 100 μg of antibodies), supernatant, and the control supernatant, to which antibodies were not added. Data are the mean of one experiment of triplicate (A) or duplicate (B) assays. Use of protein A beads gave similar results (see Results).

TABLE 3

Binding parameters for displacement of [^{3}H]muscimol binding by THIP in the membrane homogenates of the bovine brain

Membrane homogenate	IC_{50} (1)	B 1	IC_{50} (2)	B 2	IC_{50} (3)	B 3	Hill coefficient
	μM	%	μM	%	μM	%	
Cortex (n = 3)			0.32 ± 0.1	59 ± 2	3.7 ± 0.8	42 ± 2	0.8 ± 0.02
Cerebellum (n = 1)	0.011	7	0.23	60	2.2	32	0.75
Hippocampus (n = 1)			0.22	54	3.5	45	0.72
Thalamus (n = 2)			0.31 ± 0.04	56 ± 3	7.3 ± 1.7	44 ± 2	0.68 ± 0.03
Hypothalamus (n = 2)			0.38 ± 0.02	51 ± 4	8 ± 0.1	50 ± 11	0.69 ± 0.1

Values were obtained by nonlinear regression analysis as described in Experimental Procedures. B1, B2, and B3 refer to the percentage of total bindng to three populations that showed IC_{50} values (1), (2), or (3), respectively; n refers to number of separate measurements.

tively, similar binding profiles for THIP were observed. Therefore, it appears that high affinity THIP binding sites cannot be matched solely with the expression of β3 subunits. Considering the diverse expression pattern of different $GABA_A$ receptor subunits in different brain areas, it is possible that certain subunit assemblies among β3 subunit-containing receptors might account for high affinity THIP binding, i.e., β3 plus some specific α, γ, or δ; such an idea was suggested recently by a comparison of various recombinant subunit combinations (19).

In the present study, the apparent K_i of THIP for its high affinity binding sites in the purified $GABA_A$ receptors is approximately 50–150 nM, as corrected from the IC_{50} values according to the Cheng and Prusoff equation (41). This value agrees with the K_d of the low affinity binding sites for THIP obtained from saturation isotherms with [^{3}H]THIP, where three different affinities were identified: 4–6 nM, 40–80 nM, and >5 μM (27). Because [^{3}H]muscimol labels predominantly nM high affinity GABA binding sites with autoradiography (42), THIP heterogeneity demonstrated in the above autoradiographic study might represent the microheterogeneity of nM high affinity THIP binding sites that cannot be resolved by the present displacement experiment.

It should also be noted that identification of polypeptide bands labeled with [^{3}H]muscimol by immunoblots is not conclusive due to comigration of other subunits, including α, in a similar molecular weight range. Microsequencing of a chymotryptic peptide fragment demonstrated the α subunits as one of two major substrates for [^{3}H]muscimol photolabeling (43), and separate studies of site-directed mutagenesis showed that both α and β subunits can affect the affinity of GABA binding (44, 45).

Another complicating factor is the heterogeneity in affinity for GABA-site ligands (46). These binding subpopulations may correspond to multiple conformational states of each individual receptor isoform, possibly agonist- and antagonist-preferring states, or different protein isoforms with variable subunit composition, so far undefined. Available evidence suggests that most of the heterogeneity is due to multiple protein isoforms. In particular, the nM affinity agonist sites show a different regional location from the μM sites that are coupled to BZ sites (42, 47). There is an additional layer of microheterogeneity of both these populations with respect to both other GABA ligands and allosteric modulators. GABA analogues differ in efficacy to enhance BZ binding *in vitro* (48, 49); this property has not yet been related to any functional heterogeneity for GABA site ligands. The micromolar concentration of GABA required to modulate BZ binding is close to that required to activate chloride channels (51, 52).

The mode and degree of allosteric coupling between GABA

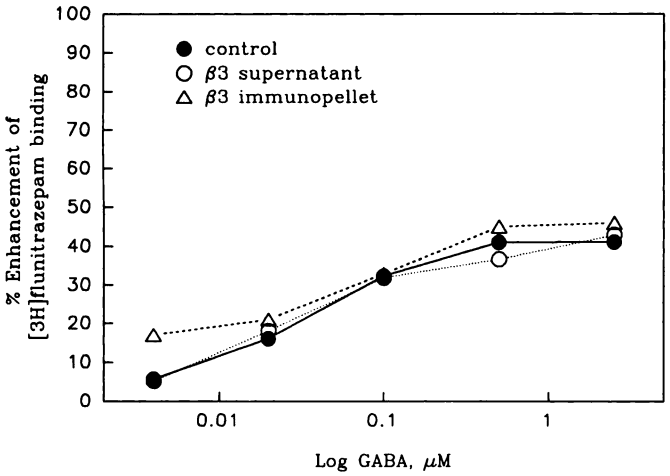

Fig. 9. [^{3}H]Flunitrazepam binding enhanced by GABA in β3 antibody-separated fractions of purified $GABA_A$ receptors of bovine cortex. [^{3}H]flunitrazepam concentration of 1 nM was used for binding assays. EC_{50} and percent E_{max} values were determined with the nonlinear curve-fitting computer program as described in Experimental Procedures. These are: control, 32 nM and 43%; supernatant, 33 nM and 38%; and immunopellets, 18 nM and 46%. Data are from triplicates of a single experiment.

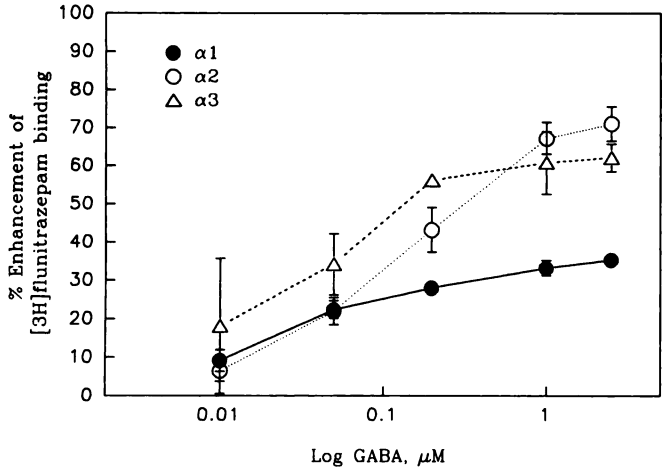

Fig. 10. GABA enhancement of [^{3}H]flunitrazepam binding in immunopellets of α1, α2, and α3 subunit-specific antibodies. Soluble extracts of the rat brain from which endogenous GABA was removed with the Sephadex G-25 gel filtration column were used for immunoprecipitation. Binding activities were determined with 0.7 nM [^{3}H]flunitrazepam in the presence of varying concentrations of GABA. Data are mean ± standard error of two or three separate determinations.

and BZ sites, i.e., GABA modulation of BZ binding, have been interpreted as an indication of the functional efficacy of BZs (50–52). The results from the present study appear to indicate that the nature of α subunit variants is the major determinant for the GABA/BZ coupling efficiency rather than β subunits (Figs. 9 and 10 and Table 4). Significantly higher maximal enhancement of [^{3}H]flunitrazepam binding by GABA in α2 and α3 subunit-containing receptors than in α1 subunit-containing receptors provides a molecular explanation for the observation that brain regions enriched with type 2 BZ binding have higher coupling efficiency (52, 53). This also agrees with the photolabeling study in which irreversible labeling of [^{3}H]flunitrazepam to the α2 subunit polypeptide is more sensitive to GABA than the α1 polypeptide (18).

TABLE 4
Binding parameters for Fig. 10

Immunopellet	EC_{50}	E_{max}
	nM	%
α1	32 ± 10	35 ± 2
α2	142 ± 27[a]	75 ± 4[b]
α3	36 ± 20	63 ± 1[b]

Values were determined by nonlinear curve-fitting analysis as described in Experimental Procedures.
[a] $p < 0.01$, EC_{50} is significantly different than that of α1 or α3 immunopellets.
[b] $p < 0.005$, E_{max} values are significantly different than that of α1 immunopellets.

Recombinant receptors with the α3β1γ2 combination had a greater efficacy for GABA to enhance diazepam binding than the α2 or α1 combinations (50); α2β1γ2 or α3β1γ2 combinations produced greater potentiation of GABA-elicited currents by diazepam than α1 or α5 counterparts (13, 51, 52), providing functional relevance to the present results. Furthermore, the lower potency found in receptors immunoprecipitated with α2 subunit-specific antibodies than those immunoprecipitated with α1 and α3 subunit-specific antibodies provide more evidence for microheterogeneity of type 2 BZ receptors *in situ*.

Although BZs such as flunitrazepam and diazepam have a full spectrum of anxiolytic, anticonvulsant, and sedative-hypnotic effects, other BZ site-acting agonists such as abecarnil and bretazenil have been characterized to have more selective therapeutic effects with reduced side effects, e.g., sedation, ataxia, and dependence (52). It is feasible that different efficacies of BZs in different subunit combinations serve as structural bases for their therapeutic selectivities.

In conclusion, our study indicates that high affinity THIP binding sites in the brain cannot be explained solely by the presence of the β3 subunit. Considering the diversity of $GABA_A$ receptor subunit expression, more discrete subunit combinations need to be characterized to define GABA site selectivity. Differential coupling efficiency between GABA and BZ binding sites is determined by the nature of α subunits in native receptor isoforms, as suggested from recombinant receptor expression studies. Our results from studies on receptors present in brain tissue also provide a partial molecular explanation for previous reports with brain tissue and recombinant receptor expression in which type 2 BZ binding receptors have a higher degree of coupling than those of type 1 BZ binding and in which type 2 BZ receptors are actually heterogeneous.

Acknowledgments

The authors thank Geoff Smith for helpful advice.

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Send reprint requests to: Dr. Richard W. Olsen, Department of Molecular and Medical Pharmacology, UCLA School of Medicine, Room CHS23–120, 10833 LeConte Avenue, Los Angeles, CA 90024-1735.