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Multiple Distinct Subunits of the γ-Aminobutyric Acid-A Receptor Protein Show Different Ligand-Binding Affinities

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SUMMARY

The purified γ-aminobutyric acid/benzodiazepine receptor protein from mammalian brain contains at least four discrete polypeptides (M_r 51,000, 53,000, 55,000 and 58,000) by a variety of visualization techniques and in three species (rat, cow, and human). These polypeptide bands vary in their affinity for γ-aminobutyric acid analogs as shown by inhibition of [^{3}H]muscimol binding, demonstrated by photoaffinity labeling and gel electrophoresis in sodium dodecyl sulfate. One-dimensional peptide maps of proteolytic digests revealed that distinct fragments were produced, indicating that the four polypeptides represent discrete sequences. The four bands were identified by Western blotting with subunit-specific monoclonal antibodies as two species each of previously identified α and β subunits. [^{3}H]Muscimol photolabeled all four bands (β and α) to varying degrees not proportional to the extent of protein staining. The M_r 58,000 β subunit subtype showed a higher affinity for 4,5,6,7-tetrahydroisoxazolo-[5,4-c]pyridin-3-ol than the M_r 56,000 β subtype, whereas the M_r 56,000 β and M_r 51,000 α bands were more enhanced by pentobarbital than the M_r 58,000 band. Furthermore, the α subunit pattern revealed by photoaffinity labeling with [^{3}H]flunitrazepam was significantly different for three regions of bovine brain, showing only one major band in cerebellum at M_r 51,000, two major bands in cortex at M_r 53,000 and 51,000, and three bands in hippocampus at M_r 55,000 as well as M_r 53,000 and 51,000. Because the ratio of the amounts of the various polypeptides varies with brain region and the pharmacological properties of the peptides vary, it is likely that a family of oligomeric γ-aminobutyric acid/benzodiazepine receptors exists in the brain. This is consistent with the reported variable expression of different subunit subtype mRNAs and with brain region-dependent variation in pharmacology and binding behavior.

The $GABA_A$ receptors in mammalian brain are complex proteins composed of the GABA recognition site, a chloride ion channel, the binding sites for at least four categories of modulatory drugs, the picrotoxin-like convulsants (that inhibit GABA function) and BZDs, barbiturates, and steroid anesthetics [all of which enhance GABA-mediated inhibitory transmission (1)]. The receptor proteins from several mammalian species have been purified by BZD affinity column (2–7). The bovine receptor contained two major polypeptide subunit bands on polyacrylamide gel electrophoresis in SDS, at M_r 51,000–53,000 (α) and M_r 56,000–57,000 (β) (2). These two peptides have now been shown to be necessary and sufficient for GABA receptor-chloride channel function by molecular cloning and expression of this activity from genetically engineered mRNA for the two subunits (8). Multiple distinct α (9, 10) and β (11) sequences have now been cloned and expressed. The subtypes show about 70% amino acid sequence identity within a subunit family and about 30% identity between families (8, 9, 11). Two additional subunits, γ (12) and δ (13), were cloned and expressed with α and β cDNAs (12, 13). The γ subunit was shown to confer BZD sensitivity on receptor-chloride channels expressed in heterologous cells (12). The receptor subunit composition *in vivo* has not been determined for any tissue and the multiple cDNAs, especially γ and δ, have not been related to isolated polypeptide bands on a gel. The subunit subtype composition appears to vary with tissue, based on differential distribution of the α and β mRNA subtypes as well as γ and δ (9–14). Differential affinities for GABA were observed in oocyte expression of the various α clones (9), but it is unclear whether this applies to the situation *in vivo*.

The BZD ligand [^{3}H]flunitrazepam photoaffinity labels a M_r 51,000 band in crude brain homogenates (15) and the M_r 52,000 α subunit in purified preparations (2–7). The GABA ligand [^{3}H]muscimol photoaffinity labels a M_r 50,000–55,000 band in crude membranes (16, 17) and the M_r 56,000 β subunit in purified receptor (6, 18, 19). However, photoaffinity labeling of the purified receptor at high protein concentrations revealed that both major subunits (M_r 52,000 and 56,000) were significantly labeled by both ligands, indicating that each apparently carries binding sites for both ligands (20, 21); the possibility of

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ABBREVIATIONS: GABA, γ-aminobutyric acid; BZD, benzodiazepine; SDS, sodium dodecyl sulfate; THIP, 4,5,6,7-tetrahydroisoxazolo-[5,4-c] pyridin-3-ol.

microheterogeneity of α and β subunits was also suggested. [^{3}H]Flunitrazepam photolabels several additional minor species of BZD-binding polypeptides in various brain regions (22, 23) and these appear to vary in affinity for some ligands (24). In this study, we demonstrate that multiple subunits are present in the purified receptors, which differ in binding affinity for GABA agonists and probably in amino acid sequence.

Materials and Methods

Receptor protein purification. The purification of the BZD/GABA receptor proteins was carried out by our modification (6) of the methods of Sigel *et al.* (2). The activity of the washed membrane homogenates and solubilized and purified receptors was measured by the binding of [^{3}H]flunitrazepam and [^{3}H]muscimol, using polyethylene glycol precipitation for ligand binding in solution. Brain homogenate membranes at 0–4° were osmotically shocked, washed in buffer (Tris-HCl, 50 mM, pH 7.4), and solubilized in 2.5% Triton X-100, 1.0 M KCl for 75 min. Receptors were purified by affinity chromatography utilizing the BZD Ro7-1986 coupled to agarose (6). After the solubilized receptors from 100 g of brain were loaded, the affinity column was washed extensively in a high salt medium and eluted with free BZD (2 mM flurazepam) in 3 M urea and 0.5% Triton X-100. After dialysis, free BZD was removed and protein was concentrated on a small DEAE-Sephadex column, to be used for photoaffinity labeling, or the samples were concentrated by chloroform-methanol precipitation (25) adapted for large volumes.

Photolabeling. Photoaffinity labeling with [^{3}H]muscimol was performed as described in this laboratory (18), and photoaffinity labeling with [^{3}H]flunitrazepam was performed using a minor modification (6, 18) of the technique of Möhler *et al.* (15). Protein concentration in both cases was about 0.2 μM in binding sites, equivalent to 0.1 mg/0.5 ml. [^{3}H]Flunitrazepam (90 Ci/mmol) at 2 or 5 nM was preincubated with the purified receptor protein for 60 min at 4°, followed by irradiation for 35 min at 4 cm with an UV lamp of wavelength 365 nm. Photolabeling with [^{3}H]muscimol used 10 or 20 nM ligand, preincubated for 30 min at 4°, followed by irradiation for 10 min at 4.5 cm at a wavelength of 254 nm without wavelength filter. Where indicated, the preincubation and irradiation samples contained varying concentrations of nonradioactive analogs of the radiolabeled photoaffinity ligands or allosteric modulators of binding.

Gel electrophoresis. Polyacrylamide gel electrophoresis in SDS was carried out at 4° for approximately 6 hr at constant current (15 mA/gel during migration in the stacking gel and 30 mA/gel during migration in the separating gel). The stacking gel was 3.5% acrylamide and 0.1% SDS in 0.125 M Tris·HCl, pH 6.8; the separating gel was 5–15% acrylamide (linear gradient) and 0.1% SDS in a 0.375 M Tris·HCl, pH 8.8. When the peptides were to be subjected to amino acid analysis, the gel was prerun for 30 min with electrode buffer supplemented with 0.1 mM sodium thioglycolate. After electrophoresis, the gels were stained for 20 min at room temperature with 0.25% Coomassie brilliant blue R-250 and destained at 4° for 4 hr (with shaking) in acetic acid/methanol/water (50:165:785) or directly subjected to Western blotting.

Proteolytic digestion and one-dimensional peptide mapping. As described by Cleveland *et al.* (26), bands to be analyzed were separated by electrophoresis in SDS (see above). After a brief staining and destaining, the bands were rinsed with cold water, cut, trimmed to 5 mm, and soaked for 1 hr with occasional swirling in a buffer containing final concentrations of 0.125 M Tris·HCl, pH 6.8, 1% SDS, and 1 mM EDTA (minor modification of original procedure). The final sample wells of a second gel (15% acrylamide gel, 1.5-mm thick, containing 1 mM EDTA, with a 5-cm long stacking gel) were filled with the slices, overlaid with 10 μl of the soaking buffer containing 10% glycerol. Finally, 10 μl of this buffer containing 20% glycerol and 1 μg of *Staphylococcus aureus* V8 protease were overlaid into each slot, and electrophoresis was carried out (26).

Immunoblotting. The proteins were transferred (27) from SDS gels to nitrocellulose paper by submitting a "sandwich," prepared with a porous polyethylene sheet, No. 9 filter paper, the slab gel, another filter paper, and another polyethylene sheet, to a 2.2 V current for 3 hr, using an LKB 2117 Multiphor II Electrophoretic Transfer Kit. The transfer chamber was filled with 5 mM sodium borate, 20% methanol, and 0.02% β-mercaptoethanol, pH 8.0. Immediately after transfer, the nitrocellulose sheet was immersed in a blocking buffer [10 mM sodium phosphate, 0.9% sodium chloride, 3 mM sodium azide, and 5% nonfat dry milk powder (Carnation)] and incubated 2–12 hr at room temperature. The sheet was then incubated in the same solution containing the monoclonal antibody plus 2% milk proteins, at 37° for 1 h. It was washed three times for 10 min with fresh buffer and then incubated overnight with the secondary antibody (rabbit anti-mouse IgG) and Immunogold (Janssen), diluted 1:100 in 20 mM Tris·HCl (pH 8.2), 0.9% NaCl, 0.1% bovine serum albumin, 0.4% gelatin, 20 mM sodium azide. If necessary, the immunogold-stained transfer membrane was subjected to the silver enhancement procedure.

Electroelution. For electroelution (28), the individual stained bands were cut out and rinsed several times with deionized water over a 2–4-hr period. The gel pieces were cut into 1-mm cubes, rinsed with elution buffer (0.1% SDS in 0.05 M Tris·acetate, pH 7.8), placed in the well of an electroelution cell, covered with soaking buffer (2% SDS in 0.2 M Tris·acetate buffer, pH 7.8), and allowed to soak at 22° for 4 hr. The elution was performed for 16–20 hr at 100 V at 4°, followed by dialysis at 150 V for 36 hr in 0.02% SDS buffer.

Results

The GABA receptor proteins from bovine, rat, and human brain membranes were solubilized with Triton X-100 and purified over 1000-fold by BZD affinity column chromatography (6). Purified rat cortical receptor photolabeled with the GABA ligand [^{3}H]muscimol (18) showed four radioactive bands on SDS-polyacrylamide gel electrophoresis, with major incorporation corresponding to a M_r 55,000–58,000 doublet (Fig. 1A). Significant labeling of a M_r 51,000–53,000 doublet was also seen. A slightly greater extent of M_r 51,000–53,000 labeling could be achieved with a higher receptor concentration (1 μM) than employed here (0.2 μM) (20).

The four bands detected by photoaffinity labeling with muscimol corresponded to four bands detected by protein staining, shown for the bovine cerebral cortex preparation in Fig. 2C. The polypeptides had molecular weights of approximately 51,000, 53,000, 55,000, and 58,000, with the two middle bands staining darker than the other two. Rat and human preparations (not shown) were qualitatively similar. Western blotting showed that the bottom doublet (M_r 51,000/53,000) was stained with the α subunit-specific monoclonal antibody bd24 (6, 29) (Fig. 2A), and the M_r 55,000/58,000 doublet was stained with the β subunit-specific monoclonal antibody bd17 (6, 29) (Fig. 2B). There was no cross-reaction of antibody bd24 with the upper doublet or of bd17 with the lower doublet under these conditions. A minor band considered to be primarily a breakdown product of α subunits was visible with protein staining and bd24 at M_r 45,000. The four bands were electroeluted from one preparative gel and run in separate lanes of a second gel to demonstrate that the distinct mobility was reproducible and that none of the purified bands evidenced any additional observable microheterogeneity on electrophoresis (Fig. 2D).

None of the polypeptides produced any N-terminal sequences when subjected to microsequencing with up to 0.3 mg of protein, indicating that the N-termini were blocked, either biologically or during preparation. Therefore, the four distinct bands of the

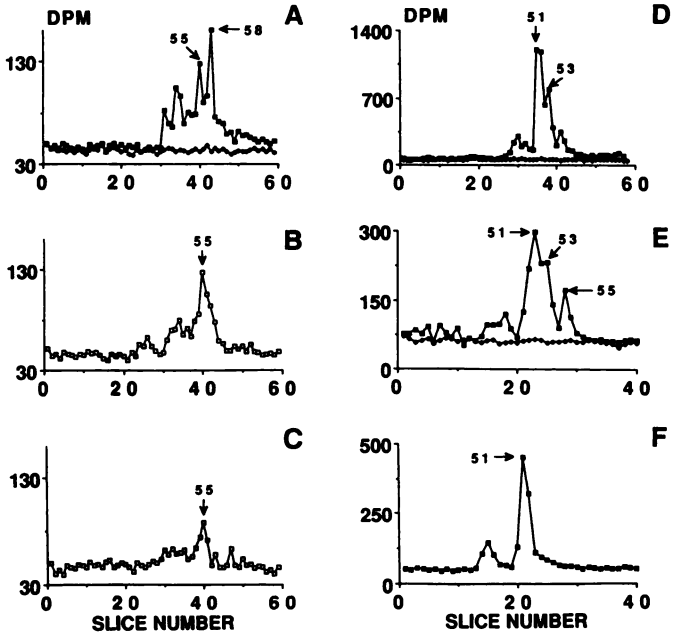

Fig. 1. Affinity column-purified receptor photoaffinity labeled with [^{3}H] muscimol or [^{3}H]flunitrazepam, followed by gel electrophoresis in SDS. The lanes were cut into 1-mm strips, digested, and counted for radioactivity. Nonspecific labeling was determined in the presence of excess nonradioactive GABA (10 μM) or diazepam (10 μM), respectively, for [^{3}H] muscimol and [^{3}H]flunitrazepam photolabeling and was found to be trivial. A–C, [^{3}H]Muscimol photolabeling of purified receptor from rat cerebral cortex. Before the UV exposure, all samples were identical during the preincubation with [^{3}H]muscimol (10 nM), except that they included THIP at five different concentrations and they were run on the same gel. The figure shows three of the six lanes including THIP at 0.1 (A), 0.3 (B), and 3 μM (C). The control photolabeling in the presence of 10 μM GABA is shown in A (♦). Identical results were observed in a repeat experiment. D–F, [^{3}H]Flunitrazepam (10 nM) photolabeling of purified receptor from cow brain regions, cerebral cortex (D), hippocampus (E), and cerebellum (F), with 10 μM GABA. The background, indicating samples labeled in the presence of nonradioactive diazepam (10 μM), is shown in D and E (♦). Similar results were observed on three occasions.

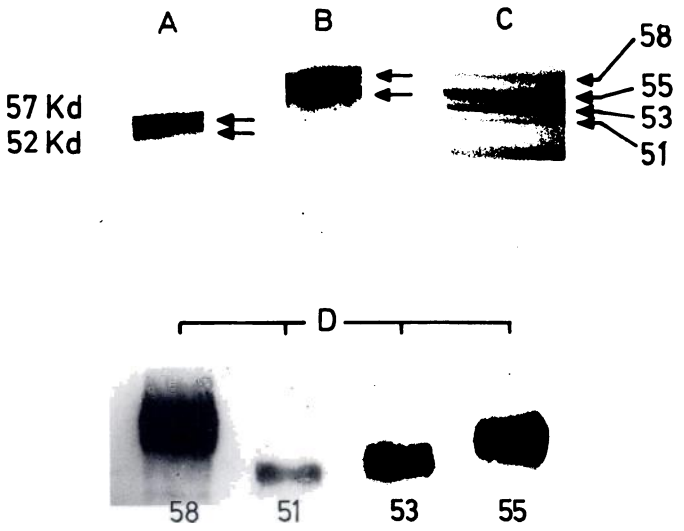

Fig. 2. Microheterogeneity of GABA/BZD receptor from cow cerebral cortex by gradient gel electrophoresis in SDS, visualized by immunoblotting (A and B) or protein staining (C and D). A, The subunit-specific monoclonal antibody bd24 staining the bottom doublet. In addition, a minor band at about M_r 45,000 has been identified as a breakdown product of α on the basis of variable stain levels, photolabeling, and immunoblots. B, The β subunit-specific monoclonal antibody bd17 staining the upper doublet. C, Coomassie blue stain of purified receptor, showing four discrete subunits at M_r 51,000, 53,000, 55,000 and 58,000. D, These four electroeluted bands have kept their distinct mobility after being run in separate lanes on a second gel, as described in Materials and Methods.

rat GABA receptor were separated on preparative gradient gels and subjected to proteolytic digestion with *S. aureus* V8 protease, followed by a second SDS gel electrophoresis as described by Cleveland *et al.* (26). Fig. 3 shows that all four bands have distinct proteolytic fragments. Both the M_r 51,000–53,000 doublet and the M_r 55,000–58,000 doublet differ from each other, e.g., bands at M_r 28,000–35,000 and M_r 11,000–15,000, indicating that this heterogeneity is due to different amino acid sequences. Partial proteolysis or glycosylation differences are unlikely to explain the microheterogeneity. Different amino acid compositions were also found for the four isolated polypeptides (not shown).

The pharmacological specificity of the [^{3}H]muscimol binding to the various bands was investigated by including other GABA analogs in the preincubation assays, at concentrations suitable for partial inhibition of [^{3}H]muscimol binding, before the UV exposure. Fig. 1A actually included a low concentration (0.1 μM) of the compound THIP [gift of P. Krogsgaard-Larsen, Copenhagen (30)] and this had no effect on the [^{3}H]muscimol incorporation, which was identical in the control (absence of THIP; not shown). Addition of increasing amounts of THIP led to selective inhibition of labeling. THIP at 0.3 μM (Fig. 1B) inhibited binding to the M_r 58,000 β polypeptide band by over 50% but did not inhibit binding to the M_r 55,000 β band at all. A higher concentration of THIP (3 μM; Fig. 1C) inhibited incorporation into the M_r 55,000 band by about 50%. The inhibition by THIP of muscimol binding to the M_r 58,000 band had an IC_{50} of 0.21 μM, whereas that for the M_r 55,000 band was 3.2 μM (a difference of 15-fold), shown in Fig. 4A). Fig. 4B demonstrates differential sensitivity of the different muscimol-labeled polypeptides to enhancement by pentobarbital (1, 31, 32). The M_r 51,000 and 55,000 bands were more enhanced than the M_r 58,000 and 53,000 bands. This is the first demonstration that the previously reported (20) binding of a GABA ligand (muscimol) to an α subunit can be modulated by other drugs like barbiturates. Other inhibitors and enhancers of [^{3}H]muscimol binding had differential effects on the binding to these multiple bands (31).[1]

This microheterogeneity was also apparent using the BZD ligand [^{3}H]flunitrazepam to photoaffinity label the purified receptors from different regions of cow brain (a species from which large amounts of tissue are readily available). In cerebral cortex, major incorporation corresponded to the M_r 51,000–53,000 doublet (Fig. 1D). Some labeling also was observed in the vicinity of the M_r 55,000/58,000 peptide regions (20). Minor labeling of a breakdown product at M_r 45,000 was variably observed. In comparison with the doublet at M_r 51,000/53,000 in cortex, the hippocampus showed the same doublet at M_r 51,000/53,000, plus another band at M_r 55,000 (Fig. 1E). The incorporation of [^{3}H]flunitrazepam in the cerebellum was primarily found in a sharp band at M_r 51,000, and this pattern of a single major peak was not changed by including an enhancing concentration (10 μM) of GABA (Fig. 1F).

[1] M. Bureau and R. W. Olsen, manuscript in preparation.

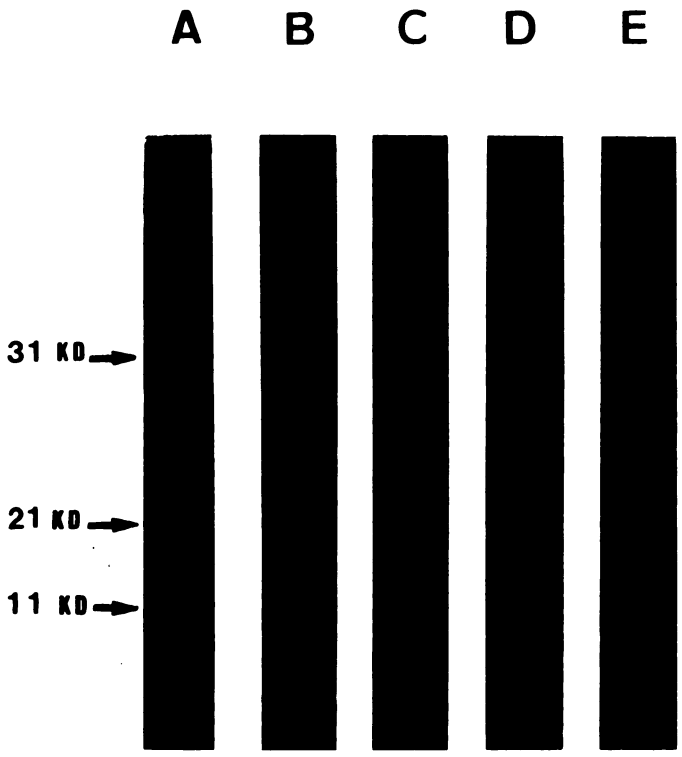

Fig. 3. One-dimensional peptide maps of the four bands of rat receptor, the M_r 51,000 band (B), M_r 53,000 band (C), M_r 55,000 band (D), and M_r 58,000 band (E), compared with standards (A), after digestion with *S. aureus* V8 protease and 15% gel electrophoresis. The four distinct bands of the rat GABA receptor were separated on a preparative gradient gel and stained. The gel slices containing the peptides were applied to a second SDS gel (15% acrylamide) in the presence of the *S. aureus* protease, as in Ref. 26, and visualized with Coomassie blue.

Discussion

This report presents biochemical evidence for at least five distinct polypeptides (three α and two β subtypes) in purified preparations of the $GABA_A$/BZD receptors from mammalian brain. These polypeptides differ in binding characteristics with respect to extent of photolabeling with [^{3}H]muscimol and in affinity for GABA analogs like THIP and allosteric modulators like pentobarbital. In cerebral cortex of cow, rat, and human, four polypeptide bands were observed on SDS-gel electrophoresis by protein staining, Western blotting with subunit-specific antibodies, and photoaffinity labeling with BZD and GABA ligands. These were identified as a "β" doublet at M_r 55,000 and 58,000 and an "α" doublet at M_r 51,000 and 53,000. The M_r 53,000 α was absent in bovine cerebellum and an extra α was present in bovine hippocampus at M_r 55,000, identified by [^{3}H]flunitrazepam photoaffinity labeling, as previously shown for crude homogenates (24). This ligand did not label the M_r 55,000 β band in cortex or cerebellum under identical conditions.

Although microheterogeneity of α subunits (7, 23, 33, 34) and β subunits (35) has been observed recently by photolabeling and/or immunoblotting, no prior clear evidence for multiple distinct stained polypeptide bands in purified preparations has been presented. In this report, we demonstrate the chemically distinct nature of four polypeptide bands by one-dimensional peptide mapping. The microheterogeneity is probably not due to posttranslational modification. In a similar manner, proteolytic fragmentation on crude homogenates has shown the heterogeneity of [^{3}H]flunitrazepam-labeled polypeptides (24). Consistent with the idea that the multiple GABA/BZD binding polypeptides represent distinct gene products, [^{3}H]flunitrazepam-binding peptides in purified receptor preparations show a brain regional variation. We have also found (36) that the two β bands (M_r 55,000 and 58,000) in our preparations show differential phosphorylation *in vitro* by protein kinases A and C, further supporting their distinct nature.

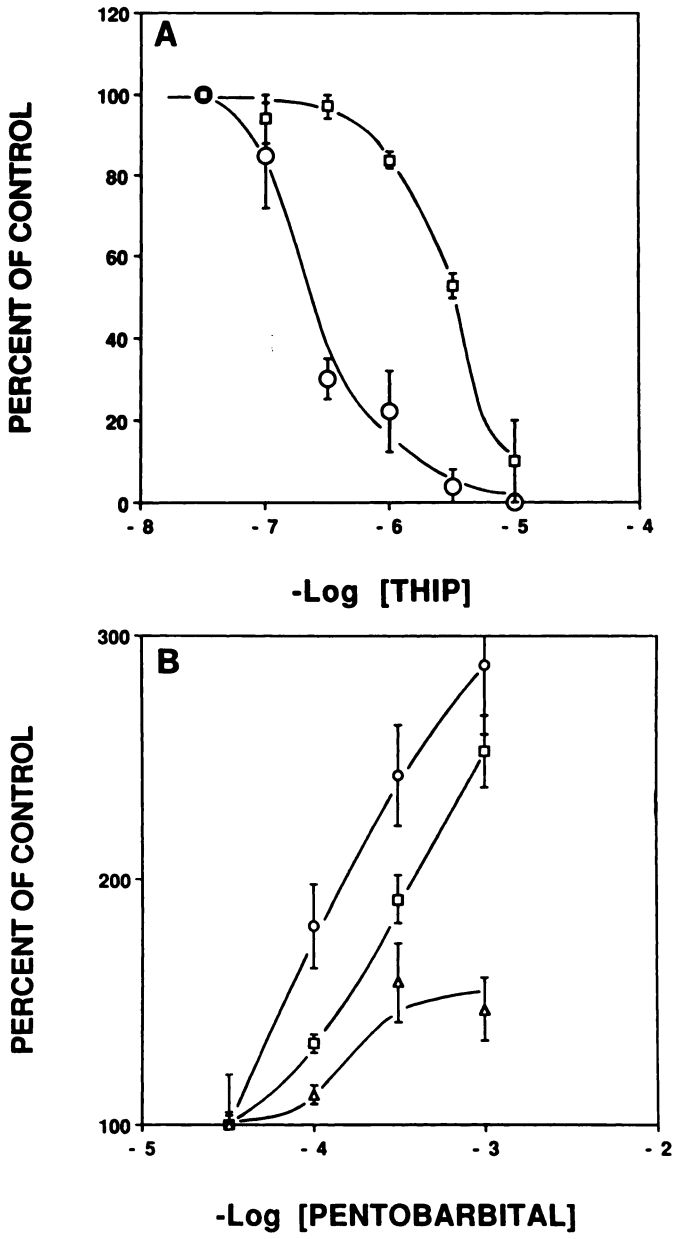

Fig. 4. Concentration dependence of THIP inhibition and pentobarbital enhancement of binding to various polypeptides. [^{3}H]Muscimol binding and photolabeling were carried out in the presence of various concentrations of THIP (A) or pentobarbital (B) and the samples were subjected to SDS-gel electrophoresis as in Fig. 1. The different traces in A represent the M_r 55,000 (□) and 58,000 (○) polypeptides. In B, these are the M_r 51,000 (○), 55,000 (□), and 58,000 (△) bands. The M_r 53,000 band was poorly enhanced but not clearly resolved in the gels containing pentobarbital. *Error bars* represent the range of two experiments. The values in A were calculated from areas under the curve for the two peaks. The values in B were from measured peak heights.

As many as 9–14 distinct cDNAs, coding peptides of M_r 47,000–55,000, have been described (8–13). This study shows that at least five polypeptides are present in reasonable abundance in brain and can be isolated by BZD affinity chromatography. Other polypeptides may be present in the preparations, depending on stability and purification yield. The major polypeptide bands vary in binding specificity and in brain regional distribution. This is consistent with observations of region-dependent heterogeneity in pharmacological properties (37, 38), ligand binding specificity (32, 38–43), and biochemical properties. Although binding heterogeneity might be related to multiple states of a single receptor (32, 44), some evidence for multiple biochemically distinct receptor proteins has been reported for crude homogenates (24, 45–48). Differential localization of GABA and BZD ligand binding (41, 43) suggested at least two types of $GABA_A$ receptor, as did heterogeneity of GABA ligand binding sites (32, 45–48). BZD binding sites could also be divided into at least two subtypes (38–40). GABA analogues showed variable abilities to enhance BZD binding, e.g., muscimol is very effective and THIP is not (30), suggesting that either GABA partial agonists exist or a heterogeneity of receptors exists with respect to GABA/BZD interactions (or both). Indeed, heterogeneity of GABA and pentobarbital interactions with BZD binding suggested at least three subtypes of BZD binding sites (42). We have recently described (43) the need for at least four subtypes to explain discrepancies in regional distributions for binding of seven different ligands of the $GABA_A$/BZD receptor complex, measured by quantitative autoradiography.

We have demonstrated that the multiple isolated polypeptides, especially M_r 55,000 and 58,000 β subtypes, exhibit a differential ability to be photoaffinity labeled with saturating concentrations of [^{3}H]muscimol, a differential affinity for THIP (Figs. 1 and 4) and other GABA analogs (31), a differential sensitivity to pentobarbital enhancement, and a differential regional localization of flunitrazepam-labeled polypeptides. This technique of photoaffinity labeling *in vitro* should be generally useful in analyzing receptor subtypes. These results indicate that the oligomers containing these polypeptides have different binding properties in the isolated state. Because relative amounts of these polypeptides vary with brain region, it is likely that variable binding and pharmacological specificity in different regions in the brain are related to this difference in subunit subtype composition in the oligomeric receptor.

The photolabeling of α subunits by muscimol might represent a proximity effect (portions of an α polypeptide situated in the three-dimensional structure near the muscimol binding site on a β subunit) but probably involves an actual GABA binding site on α (20). The differential enhancement by barbiturates of the M_r 51,000 band supports this notion, but it is not definitive without active site sequence information.

It is still possible that some of the binding heterogeneity may reside in different subunits of a single oligomer without differential localization, but the regional variation in polypeptide composition and binding properties is more consistent with multiple oligomers with variable pharmacological properties. Differential stability of purified oligomers or subunits, especially when associated with different oligomeric compositions including the γ and δ subunits, might produce differential binding properties *in vitro* that do not actually occur *in vivo*. We suggest that this is unlikely, in light of the reproducibility in the ratios of staining for the four bands in multiple cerebral cortex preparations and in the ratios of photoaffinity labeling with [^{3}H]muscimol of the four bands, using 10, 20, and 40 nM ligand concentrations. Furthermore, different polypeptides are sensitive to various ligands or modulators, e.g., the M_r 55,000 β is more sensitive to pentobarbital and taurine (31) and phosphorylation by protein kinase C (36), whereas the M_r 58,000 β is more sensitive to THIP and phosphorylation by protein kinase A (36).

The receptor subtypes demonstrated here on gels are strongly suggested to be related to the multiple subunit gene sequences recently demonstrated by cDNA cloning (9–13). Four different GABA receptor α subunit cDNA sequences, differing slightly from each other, express GABA/BZD receptor-chloride channels with distinct sensitivities to GABA and picrotoxin in chloride channel activation (9–13) and distinct BZD binding subtype characteristics (49). The different α, β, γ, and δ cDNAs show differential brain regional localization (9–14, 50). The α_1 clone is abundant, widespread, and the primary α present in the cerebellum (9, 10, 14, 50); we observed one principal α band by photolabeling in purified receptor from cerebellum. The cortex shows two major α bands (by three staining techniques) in our purified receptor and contains more than one α mRNA species observed by *in situ* hybridization [α_3 (9, 14) and α_4 (10), plus α_1]; the hippocampus shows three α polypeptide bands on our gels and multiple α mRNA species [α_2, α_3 (9, 14), and α_4 (10), plus α_1].

The subunit composition and number of subunits in an oligomer remains unknown at this point; a myriad of oligomeric combinations of subunits may exist *in vivo*. This is difficult to assess by the cloning and expression technique alone. The current study shows the presence of multiple protein subunits with different binding properties and varying with brain region. A combination of the approach of channel expression from mRNAs together with anatomical localization of subtypes using binding, protein chemistry, immunocytochemistry, and *in situ* hybridization is necessary for determining the native structure. Equally important to the demonstration of the existence of a $GABA_A$ receptor gene family is the evidence for multiple oligomeric protein species with different regional distribution in the brain, as well as distinct pharmacological properties. This pharmacological receptor heterogeneity suggests the potential for the development of subtype-specific drugs for use in humans, for example, for epilepsy or anxiety.

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