Biochemical Evidence for the Existence of γ-Aminobutyrate Receptor Iso-oligomers*

(Received for publication, September 25, 1989)

Michael J. Duggan and F. Anne Stephenson‡
From the Department of Pharmaceutical Chemistry, The School of Pharmacy, 29-39 Brunswick Square, London WC1N 1AX, United Kingdom

Polyclonal antibodies were raised against synthetic peptides whose sequences were from unique regions of the bovine γ-aminobutyrate, receptor α1, α2, and α3 subunits. The anti-α1 324-341, anti-Cys α2 414-424, and anti-Cys α3 454-467 antibodies all specifically immunoprecipitated [3H]flunitrazepam and [3H]muscimol binding activities in parallel from Na⁺ deoxycholate extracts of bovine cerebral cortex. The maximum number of benzodiazepine binding sites immunoprecipitated by each antibody in three brain regions, cerebral cortex, cerebellum, and hippocampus, was investigated. Differences were found between the maximum number of sites immunoprecipitated by each antibody in one brain region and for the percentage of benzodiazepine binding sites immunoprecipitated by one specific antibody between the different brain regions. Furthermore, it was found that co-immunoprecipitation with either anti-α1 324-341, anti-Cys α2 414-424, and anti-Cys α3 454-467 or anti-α1 324-341 and anti-Cys α3 454-467 antibodies resulted in an increase in the percentage of benzodiazepine binding sites immunoprecipitated, the sum of which was equal to the percentages pelleted by the individual antibodies. These results demonstrate for the first time the existence of mammalian brain of γ-aminobutyrate receptor α subunit iso-oligomers.

The GABA₄ receptor of mammalian brain is a ligand-gated chloride ion channel and the site of action of several important classes of drugs, notably the benzodiazepines, the barbiturates, cage convulsant compounds, and some steroids (reviewed in Ref. 1). The receptor protein has been purified to apparent homogeneity by benzodiazepine affinity chromatography (reviewed in Ref. 2). These isolation studies showed that the GABA₄ receptor from several vertebrate species is a heterologous membrane glycoprotein with a molecular mass of 240,000–250,000 daltons consisting of two subunit types, α with M, 53,000 and β with M, 57,000 (2, 3). The bovine α and β subunits have been cloned (4), and subsequently isoforms of one subunit type coexist within one oligomer or however are not yet established. It is not known even if isoforms of one subunit type coexist within one oligomer or whether each receptor protein contains only one isoform of each subunit type. Northern blots (5, 6) and in situ hybridization studies (13) have demonstrated a brain regional distribution of the different α and β subunit isorot mRNAs which would support the existence of the iso-oligomers, e.g. β1 + β1, α2 + β1, etc.

In order to study the subunit compositions of the GABA₄ receptor proteins, we have made antibodies specific for each of the α1, α2, and α3 subunits. We report in this paper the use of these antibodies to demonstrate the existence of GABA₄ receptor α subunit iso-oligomers.

MATERIALS AND METHODS

[N-methyl-³H]Flunitrazepam (86 Ci/mmol), [methylene-³H]muscimol (9 Ci/mmol), biotinylated anti-rabbit Ig and streptavidin-biotinylated peroxidase complex were from Amersham International (Bucks, United Kingdom). Immunoprecipitin was from Bethesda Research Laboratories. Reagents for peptide synthesis were purchased from Cambridge Research Biochemicals (Cambridge, UK). The peptide Cys α2 414-424 was from Multiple Peptide Systems (San Diego, CA), and the peptide Cys α3 454-467 was from the Institute of Animal Physiology and Genetics Research Station (Cambridge, UK). Activated CH-Sepharose 4B and activated thiol-Sepharose 4B were from Pharmacia (Uppsala, Sweden). Flunitrazepam was a gift from Hoffmann-La Roche (Basel, Switzerland). All other materials were as noted elsewhere (14, 15).

Antibody Production and Characterization—The peptides α1 324-341, sequence PEKPKKKVDPK, and Cys α3 454-467, sequence CYNRRESAIGMIRQ, were coupled to keyhole limpet hemocyanin (KLH), and polyclonal antibodies against the respective peptide-KLH conjugates were raised in Dutch-belted rabbits as previously described (14, 15). The peptide Cys α2 414-424, sequence CYNRRESAIGMIRQ, was coupled to KLH via the N-terminal cysteine by the method of Stephenson et al. (15), and the peptide-KLH conjugate was used as antigen for the production of polyclonal antibodies as before (14). For each of the peptide-KLH conjugates, the immune response was measured by ELISA with either the respective peptide or the GABA₄ receptor purified from adult bovine cerebral...
cortex as antigen as described (14). The maximum antibody titers as defined by the serum dilution which gives the half-maximal absorbance at \( \lambda = 490 \) nm in the ELISA were for (the three peptides employed here with respective peptide as antigen) 1 in 41,000 (\( \alpha_1 \) 324–341), 1 in 80,000 (Cys \( \alpha_3 \) 454–467), and 1 in 56,000 (Cys \( \alpha_2 \) 414–424). With GABA \(_x\) receptor as antigen the maximum titers were 1 in 2,900 (\( \alpha_2\) 324–341), 1 in 100 (Cys \( \alpha_3 \) 454–467), and 1 in 300 (Cys \( \alpha_2 \) 414–424).

Affinity Purification of Anti-peptide Antibodies—The peptide \( \alpha_1 \) 324–341 was coupled to activated CH-Sepharose. The affinity resin (0.35 g) was swollen in \( H_2O \) and washed with 1 mM HCl (300 ml) at 4°C and equilibrated in 0.1 M NaHCO\(_3\), 0.3 M NaCl, pH 8.0, in a final volume of 1 ml. The \( \alpha_1 \) 324–341 was dissolved in the equilibration buffer at a concentration of 5 mg ml\(^{-1}\) and then incubated with the washed gel for 1 h at room temperature. The reaction was terminated by washing the gel with equilibration buffer (25 ml), and the remaining activated groups were blocked by incubation of the resin with 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.0, for 1 h at room temperature. The gel was washed four times each and alternately with 0.1 M CH\(_3\)COOH, 0.5 M NaCl, pH 4.0, and 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.0, and equilibrated with phosphate-buffered saline.

The peptides Cys \( \alpha_3 \) 454–467 and Cys \( \alpha_2 \) 414–424 were both coupled to activated thiol-Sepharose 4B. The affinity resin (0.35 g for each peptide) was swollen in \( H_2O \) and then washed with 0.1 M Tris-HCl, 0.3 M NaCl, 1 mM EDTA, pH 8.0. The peptides were dissolved in this buffer at a concentration of 5 mg ml\(^{-1}\) and then incubated with the respective washed activated thiol-Sepharose 4B for 2 h at room temperature. The reactions were terminated by washing the gel with 0.1 M Tris-HCl, 0.3 M NaCl, 1 mM EDTA, pH 8.0 (25 ml) followed by 100 mM citric acid, pH 4.5 (10 ml). The remaining activated groups were blocked by incubation of the gel with 1 mM \( \beta\)-mercaptoethanol in citrate buffer (2 ml) for 45 min at room temperature. The gel was washed with 100 mM citric acid, pH 4.0 (20 ml) and equilibrated with phosphate-buffered saline.

For affinity purification, the respective immune sera (1–5 ml) were recirculated through the appropriate peptide affinity resin (1 ml) for 2 h at 40 ml h\(^{-1}\) at room temperature or overnight at 4°C. The filtrate was collected and the columns washed with phosphate-buffered saline at 40 ml h\(^{-1}\) (100 ml). The antibody was eluted with 10 ml of 50 mM glycine HCl, pH 2.3, at 10 ml h\(^{-1}\) and fractions of 1 ml collected. Each fraction was neutralized immediately following elution by the addition of 1 ml Tris (20 \( \mu \)l) to give pH 7.4. The antibody activity was determined by the measurement of the optical density at \( \lambda = 280 \) nm and by ELISA with the appropriate peptide as antigen (14).

Immunoprecipitation of Solubilized \( \alpha \)GABA \(_x\) Receptors—Immunoprecipitation of solubilized GABA \(_x\) receptors from the three brain regions were carried out as previously described using Immunoprecipitin for the precipitation of the antigen-antibody complex (14). In all cases, affinity-purified antibodies (300 \( \mu \)l) were used for immunoprecipitation at protein concentrations up to 200 \( \mu \)g ml\(^{-1}\). Control samples were incubated with rabbit immunoglobulin purified by protein A-Sepharose chromatography (15) from a nonimmune animal and used at the same protein concentrations as the affinity-purified anti-peptide antibodies. As before, following immunoprecipitation the respective supernatant and pellet fractions were assayed for both GABA and benzodiazepine-specific binding activities (14).

**RESULTS AND DISCUSSION**

The primary structures of the GABA \(_x\) receptor \( \alpha \) subunit isoforms as deduced from their corresponding cDNAs have \( \approx 80\% \) amino acid sequence identity. In order to develop antibodies specific for each of these isoforms, we have made antibodies to synthetic peptides whose sequences are from divergent and unique regions of the respective bovine \( \alpha_1 \), \( \alpha_2 \), and \( \alpha_3 \) subunit isoforms. The amino acid sequence \( \alpha_1 \) 324–341 is from the putative cytoplasmic loop of the polypeptide which in the alignment of the primary structures of the \( \alpha \) subunits (5) has identical amino acids with the \( \alpha_2 \) and \( \alpha_3 \) subunits in 3- and 2-positions, respectively, out of a total of 17 used for the antigen. The sequences Cys \( \alpha_2 \) 414–424 and Cys \( \alpha_3 \) 454–467 are the C-terminal amino acid sequences of the \( \alpha_2 \) and \( \alpha_3 \) subunits each with an additional N-terminal cysteine for coupling to the carrier protein. For these peptides, the terminal 7 amino acids for the \( \alpha_2 \) subunit and the terminal 10 amino acids for the \( \alpha_3 \) subunit are unique to the corresponding polypeptides.

All three specificity antibodies were shown to recognize GABA \(_x\) receptors purified from adult bovine cerebral cortex in ELISAs (see “Materials and Methods” (14, 15)), immunoblots (14, 15), and soluble immunoprecipitation assays (see below), thus they recognize both denatured and native conformations of the proteins. No immuno-cross-reactivity was found between the anti-\( \alpha_1 \) 324–341 and anti-Cys \( \alpha_3 \) 454–467 nor between the anti-Cys \( \alpha_2 \) 414–424 and anti-Cys \( \alpha_3 \) 454–467 antibodies since in immunoblots, the molecular weight of subunits recognized by the anti-Cys \( \alpha_3 \) 454–467 antibodies was 58,000–59,000 compared with the apparent single subunit of M, 53,000 which was recognized by both the anti-\( \alpha_1 \) 324–341 and the anti-Cys \( \alpha_2 \) 414–424 antibodies (15).

From the deduced amino acid sequence of the \( \alpha_2 \) subunit, it would be predicted to comigrate with the \( \alpha_1 \) polypeptide in one-dimensional SDS-PAGE in contrast to the predicted size of the \( \alpha_3 \) subunit (5). The possibility of immuno-cross-reactivity between the anti-\( \alpha_1 \) 324–341 antibodies and the anti-Cys \( \alpha_2 \) 414–424 antibodies with the corresponding \( \alpha_1 \) and \( \alpha_2 \) subunits is unlikely because of the amino acid sequences chosen for antibody production (see above) and the properties of the respective antibodies in the quantitative immunoprecipitation assays (see below).

The ability of these antibodies to recognize the native receptor enabled the quantification of the various receptor polypeptide isofoms by immunoprecipitation. All three affinity-purified antibodies specifically immunoprecipitated \( [\text{H}] \) flunitrazepam binding activity from a soluble extract of adult bovine cerebral cortex in a dose-dependent manner (Fig. 1). For each specificity antibody, the decrease in benzodiazepine binding in the supernatant following immunoprecipitation was concomitant with an increase in specific benzodiazepine binding in the pellet (results not shown). In agreement with previous results using anti-\( \alpha_1 \) 1–15 and anti-\( \alpha_1 \) 413–429 antibodies (14), anti-\( \alpha_1 \) 324–341 antibodies immunoprecipitated a maximum of 57 ± 5% (\( n = 14 \)) benzodiazepine binding sites from Na’ deoxycholate extracts of adult bovine cerebral cortex (Fig. 1 and Table I). In contrast anti-Cys \( \alpha_2 \) 414–424 antibodies immunoprecipitated a maximum of 8 ± 8% (\( n = 7 \)), and anti-Cys \( \alpha_3 \) 454–467 antibodies immunoprecipitated 27 ± 10% (\( n = 11 \)) central benzodiazepine binding sites from adult bovine cerebral cortex.

We have previously shown that anti-\( \alpha_1 \) 1–15 and anti-\( \alpha_1 \) 413–429 antibodies immunoprecipitated in parallel \( [\text{H}] \) flunitrazepam and \( [\text{H}] \) muscimol binding sites from both purified and detergent extracts of adult bovine cerebral cortex (14). Similarly, anti-Cys \( \alpha_3 \) 454–467 antibodies immunoprecipitated a maximum of 24 ± 9% (\( n = 3 \)) \( [\text{H}] \) muscimol binding sites from Na’ deoxycholate extracts of cerebral cortex and anti-Cys \( \alpha_2 \) 414–424 antibodies immunoprecipitated a maximum of 41 ± 5% (\( n = 3 \)) \( [\text{H}] \) muscimol binding sites from Na’ deoxycholate extracts of hippocampus. Both values agree with the maximum number of benzodiazepine binding sites immunoprecipitated for each respective antibody (Table I) showing that each \( \alpha \) subunit isoform is associated with a binding site for the benzodiazepines and for GABA. The maximum number of benzodiazepine binding sites immunoprecipitated by the three specificity antibodies varied

\( ^{2} M. J. Duggan and F. A. Stephenson, results in preparation. \)
Identification of GABA<sub>a</sub> Receptor Iso-oligomers

FIG. 1. Concentration dependence for the immunoprecipitation of [³H]flunitrazepam binding sites from Na<sup>+</sup> deoxycholate extracts of bovine brain by anti-α subunit antibodies. A Na<sup>+</sup> deoxycholate extract was prepared from bovine brain and the soluble extract (200 µl) incubated with increasing concentrations of either affinity-purified anti-α<sub>1</sub> 324-341, anti-Cys α<sub>2</sub> 414-424, or anti-Cys α<sub>3</sub> 454-467 antibodies diluted with normal rabbit IgG to give a fixed protein concentration per tube. Immune complexes were precipitated by the addition of Immunoprecipitin, and [³H]flunitrazepam binding to the resultant supernatants was carried out as described under "Materials and Methods." The results are expressed as the percentage decrease of specific [³H]flunitrazepam binding sites remaining in the supernatant after immunoprecipitation with respect to samples which were incubated with an equivalent protein concentration of normal protein A-purified rabbit IgG. A is immunoprecipitation with anti-α<sub>1</sub> 324-341 antibodies from extracts of adult bovine cerebral cortex; B is immunoprecipitation with anti-Cys α<sub>2</sub> 414-424 antibodies from extracts of adult bovine hippocampus, and C is immunoprecipitation with anti-Cys α<sub>3</sub> 454-467 antibodies from extracts of adult bovine cerebral cortex.

TABLE I

Immunoprecipitation of [³H]flunitrazepam-specific binding sites by α subunit isoform-specific antibodies

Immunoprecipitation was carried out as described under "Materials and Methods" using saturating concentrations of either anti-α<sub>1</sub> 324-341, anti-Cys α<sub>2</sub> 414-424, anti-Cys α<sub>3</sub> 454-467, or combinations of anti-α<sub>1</sub> 324-341 + anti-Cys α<sub>3</sub> 454-467 or anti-α<sub>1</sub> 324-341 + anti-Cys α<sub>2</sub> 414-424 + anti-Cys α<sub>3</sub> 454-467 antibodies (see also Fig. 1). The results are expressed as the percentage decrease of [³H]flunitrazepam binding sites following immunoprecipitation. In each case, these percentages were calculated against control samples where immunoprecipitation was carried out using an equivalent protein concentration of protein A-purified normal rabbit IgG. * refers to the antibody or combination of antibodies that was used for immunoprecipitation and % is the percentage of the benzodiazepine binding sites predicted to be immunoprecipitated by the summation of the experimental results for the individual antibodies. The values are the mean ± S.D. for at least six independent determinations for the single antibody experiments and for at least three for the experiments where combinations of antibodies were used.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>[³H]Flunitrazepam binding sites immunoprecipitated</th>
<th>α&lt;sub&gt;1&lt;/sub&gt;*</th>
<th>α&lt;sub&gt;2&lt;/sub&gt;*</th>
<th>α&lt;sub&gt;3&lt;/sub&gt;*</th>
<th>α&lt;sub&gt;1&lt;/sub&gt; + α&lt;sub&gt;3&lt;/sub&gt;*</th>
<th>Theoreticalα</th>
<th>α&lt;sub&gt;1&lt;/sub&gt; + α&lt;sub&gt;2&lt;/sub&gt; + α&lt;sub&gt;3&lt;/sub&gt;*</th>
<th>Theoreticalβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>57 ± 5</td>
<td>8 ± 8</td>
<td>27 ± 10</td>
<td>75 ± 8</td>
<td>84 ± 11</td>
<td>76 ± 13</td>
<td>92 ± 14</td>
<td></td>
</tr>
<tr>
<td>Cerebellum</td>
<td>82 ± 8</td>
<td>7 ± 9</td>
<td>8 ± 6</td>
<td>88 ± 13</td>
<td>90 ± 10</td>
<td>90 ± 10</td>
<td>97 ± 15</td>
<td></td>
</tr>
<tr>
<td>Hippocampus</td>
<td>35 ± 9</td>
<td>42 ± 5</td>
<td>24 ± 8</td>
<td>54 ± 14</td>
<td>59 ± 12</td>
<td>86 ± 18</td>
<td>102 ± 13</td>
<td></td>
</tr>
</tbody>
</table>

Between brain regions (Table I). It was found that the α<sub>1</sub> subunit was the most abundant of the isoforms in Na<sup>+</sup> deoxycholate extracts of cerebellum where anti-α<sub>1</sub> 324-341 antibodies immunoprecipitated a maximum of 82 ± 8% (n = 13) of the total benzodiazepine binding sites; the α<sub>2</sub> subunit antibody was most efficacious in Na<sup>+</sup> deoxycholate extracts of hippocampus where 43 ± 9% (n = 13) of the total benzodiazepine binding sites were immunoprecipitated. Anti-Cys α<sub>3</sub> 454-467 antibodies immunoprecipitated similar percentages of sites from both cerebral cortex and hippocampus of the order 24-27% (Table I). These results agree with the brain regional distribution of the various α subunit mRNA transcripts in that the α<sub>1</sub> subunit mRNA is the most abundant whereas the α<sub>2</sub> subunit mRNA is the least abundant of the α subunit isoforms (5), but it is enriched in the hippocampus (6). Agreement is also found with our previous qualitative observations in immunoblots with anti-α<sub>1</sub> 1-15 and anti-α<sub>1</sub> 413-429 antibodies where we showed that the α<sub>1</sub> subunit is enriched in cerebellum and in cerebral cortex compared with its relative abundance in the hippocampus and the striatum (14).

In a second series of experiments, immunoprecipitation of
benzodiazepine binding sites from detergent extracts of the three brain regions was studied following the addition of either anti-\(\alpha_1\) 324–341 plus anti-Cys \(\alpha_3\) 454–467 antibodies or the addition of anti-\(\alpha_1\) 324–341 plus anti-Cys \(\alpha_3\) 454–467 plus anti-Cys \(\alpha_2\) 414–424 antibodies to the incubation mixture. In these cases, the amount of each respective affinity-purified antibody added to each assay was that which immunoprecipitated the maximum number of benzodiazepine binding sites (Fig. 1). Control immunoprecipitations with each individual antibody were always carried out in parallel. The results obtained from these experiments are summarized in Table I where all values are expressed as a percentage of the benzodiazepine sites not immunoprecipitated nonspecifically by an equivalent protein concentration of nonimmune protein A-purified IgG. It can be seen that in all three brain regions immunoprecipitation with both anti-\(\alpha_1\) 324–341 and anti-Cys \(\alpha_3\) 454–467 antibodies combined in result in an increase in the percentage of benzodiazepine binding sites precipitated compared with those precipitated by either anti-\(\alpha_1\) 324 341 or anti-Cys \(\alpha_3\) 454–467 alone. Within the limits of experimental error, the percentage of sites immunoprecipitated by both anti-\(\alpha_1\) subunit and anti-\(\alpha_3\) subunit antibodies was the sum of the sites immunoprecipitated by the individual anti-\(\alpha_1\) 324–341 or the anti-Cys \(\alpha_3\) 454–467 antibodies (Table I). Similarly in the hippocampus, addition of the anti-\(\alpha_1\) 324–341, anti-Cys \(\alpha_3\) 454–467, and anti-Cys \(\alpha_2\) 414–424 antibodies together in the immunoprecipitation assay resulted in an increase in the specific benzodiazepine binding sites pelleted compared with those immunoprecipitated by anti-\(\alpha_1\) 324–341 and anti-Cys \(\alpha_3\) 454–467 antibodies combined. Again, within the limits of experimental error, the percentage of the sites immunoprecipitated by the three specificity antibodies together was in agreement with the sum of the percentages of sites immunoprecipitated by each individual antibody (Table I). In the cerebral cortex and cerebellum, the addition of the anti-Cys \(\alpha_2\) 414 424 antibody did not increase significantly the percentage of benzodiazepine binding sites immunoprecipitated compared with the sum of the anti-\(\alpha_1\) 324–341 and the anti-Cys \(\alpha_3\) 454–467 antibodies.

As described previously (see the Introduction) early protein purification studies showed that the GABA\(_A\) receptor was composed of two polypeptides, \(\alpha\) (with \(M, 53,000\)) and \(\beta\) (with \(M, 57,000\)) (3). Densitometric scans of SDS-PAGE of purified receptor showed that these apparent \(\alpha\) and \(\beta\) subunits were present in a ratio of 1:1, and a model of a heterologous tetramer \(\alpha_2\beta_2\) was proposed (12). More recently, it was shown using anti-peptide antibodies that the \(\alpha_3\) subunit comigrates with the \(\beta\) subunit in one-dimensional SDS-PAGE with a molecular weight of 58,000–59,000 which is in good agreement with the molecular weight predicted from the cDNA sequence (5, 15). Kirkness et al. (16) were able to separate the \(\alpha_3\) and \(\beta\) subunits (57,000 band) by extended SDS-PAGE, and they showed that the ratio of the \(\alpha\) (53,000 band), \(\beta\) (57,000 band), and \(\alpha_3\) polypeptides in the GABA\(_A\) receptor purified from porcine cerebral cortex is 2:1:1. These results, however, did not address the question as to whether all these polypeptides are integral to the same oligomeric structure. In the experiments that we have described herein, in agreement with Kirkness et al. (16) we have shown by quantitative immunoprecipitation of benzodiazepine binding sites that the ratio of the \(\alpha_1\) to \(\alpha_3\) polypeptides in adult cerebral cortex is indeed approximately 2 to 1. The maximum number of benzodiazepine binding sites immunoprecipitated under native conditions by either anti-\(\alpha_1\) 324–341 or anti-Cys \(\alpha_3\) 454–467 antibodies was significantly different, and importantly the percentage of benzodiazepine sites immunoprecipitated by both antibodies together was the sum of the individual \(\alpha_1\) and \(\alpha_3\) components; thus the \(\alpha_1\) and \(\alpha_3\) polypeptides must not form part of the same macromolecular complex. This is evidence for the existence of GABA\(_A\) receptor \(\alpha\) subunit iso-oligomers.

The same phenomenon was found using the anti-Cys \(\alpha_2\) 414–424 antibodies in combination with the antibodies specific for the \(\alpha_1\) and \(\alpha_3\) isoforms of the \(\alpha\) subunit in the hippocampus (Table I). In experiments where all three antibodies were used in immunoprecipitation experiments from Na\(^+\) deoxycholate extracts of cerebral cortex and cerebellum, there was no significant increase in the number of benzodiazepine sites immunoprecipitated by anti-\(\alpha_1\) 324–341, anti-Cys \(\alpha_2\) 414–424, and anti-Cys \(\alpha_3\) 454–467 antibodies together compared with the combination of anti-\(\alpha_1\) 324–341 and anti-Cys \(\alpha_3\) 454–467 antibodies. While we cannot exclude the possibility that the different \(\alpha\) subunits, i.e. \(\alpha_1 + \alpha_2\) or \(\alpha_3 + \alpha_2\), may co-exist within the same oligomer, in both these brain regions the \(\alpha_2\) subunit is low in abundance compared with the hippocampus (Table I). It may be therefore that the increase in the benzodiazepine sites immunoprecipitated by the anti-Cys \(\alpha_2\) 414–424 antibodies together with the anti-\(\alpha_1\) and anti-\(\alpha_3\) antibodies is beyond the limits of sensitivity in these experiments. It was apparent that in the three brain regions studied most significantly in the cortex, the number of benzodiazepine binding sites immunoprecipitated was not 100%. Other isoforms of the \(\alpha\) subunit have recently been described, namely \(\alpha_4, \alpha_5,\) and \(\alpha_6\) (17), which may account for the remaining benzodiazepine sites which are not associated with the \(\alpha_1, \alpha_2,\) or \(\alpha_3\) subunits.

In this paper, we have demonstrated the distribution and quantitation of the \(\alpha_1, \alpha_2,\) and \(\alpha_3\) subunit isoforms by the immunoprecipitation of the specific \(^{1}\H\)flunitrazepam binding activities from different brain regions. Biochemical experiments showed that the \(\alpha\) subunit was specifically photo-affinity-labeled by \(^{1}\H\)flunitrazepam (2, 3, 18); however, more recent studies on the expression of recombinant GABA\(_A\) receptors showed that the \(\gamma_2\) polypeptide was required to give a large benzodiazepine response to these GABA-gated channels and for the measurement of benzodiazepine binding activity to the expressed proteins (7). From the deduced primary structure and thus molecular weight of the \(\gamma_2\) subunit, it is predicted to co-migrate with the \(\alpha_1\) polypeptide in SDS-PAGE, and it calls into question the roles played by the \(\alpha\) and \(\gamma_2\) subunits in benzodiazepine binding. At this time, the evidence supports the \(\alpha\) subunits as the sites of photo-affinity labeling although mapping studies have shown that this site does not lie within the sequences \(\alpha_1\) 1–58 and \(\alpha_1\) 149–429 (19). Further a correlation has previously been noted between the distribution of the \(\alpha_1\) mRNA, the \(\alpha_1\) polypeptide, and the type I benzodiazepine pharmacological subclass (5, 20, 21) and between the \(\alpha_2\) and \(\alpha_3\) mRNA and the type II benzodiazepine binding site (5, 13). The results here further substantiate this correlation in that in the cerebellum where binding studies in crude and purified GABA\(_A\) receptor preparations have shown an apparently homogeneous benzodiazepine receptor population in displacement studies with benzodiazepine site-discriminating drugs, e.g. the \(\beta\)-carbolines, there is a preponderance of \(\alpha_1\) subunit (80% (20)). Type I sites are enriched in cerebral cortex which has 57% \(\alpha_1\) subunit whereas in contrast type II sites are found in highest concentrations in the hippocampus which we now report is enriched in the \(\alpha_2\) subunit isoform (Table I and Ref. 20). The \(\alpha_3\) subunit is present at approximately equal concentrations in the cortex and hippocampus which may suggest that this isoform of the \(\alpha\) subunit may also confer the type II benzodiazepine receptor pharmacology. Indeed, recently it has been
Identification of GABA<sub>A</sub> Receptor Iso-oligomers

shown that when recombinant α variant GABA<sub>A</sub> receptors, i.e. α1β1γ2, α2β1γ2 or α3β1γ2, are expressed in mammalian cells, the α1-containing receptor shows selective binding to those compounds known to have high affinity for type I receptors whereas the α2- or α3-containing receptors have the pharmacological specificity of type II receptors (22).

In conclusion, the existence of GABA<sub>A</sub> receptor α subunit iso-oligomers has been shown, but the remaining subunit complement of these proteins must await the development of additional isoform-specific probes for use in immunoaffinity purification and characterization, in particular probes for the γ2 and δ polypeptides (17).

Acknowledgments—We are grateful to Mihajlo Milic for technical assistance and Gill Patterson for word processing.

REFERENCES
GABA Receptors and Chloride Channels: Structural and Func-
tional Properties, Alan R. Liss, Inc., New York
(1985) J. Biol. Chem. 258, 6965–6971
4. Schofield, P. R., Darlison, M. G., Fujita, N., Burt, D. R., Step-
henson, F. A., Rodriguez, H., Rhee, L. M., Ramachandran, J., 
Reale, V., Glencorse, T. A., Seeburg, P. H., and Barnard, E. A. 
5. Levitan, E. S., Schofield, P. R., Burt, D. R., Rhee, L. M., Wisden, 
W., Kohler, M., Fujita, N., Rodriguez, H., Stephenson, F. A., 
Nature 335, 76–79
6. Ymer, S., Schofield, P. R., Draguhn, A., Werner, P., Kohler, M., 
and Seeburg, P. H. (1989) EMBO J. 8, 1665–1670
7. Pritchett, D. B., Sontheimer, H., Shivers, B. D., Ymer, S., Ket- 
338, 582–585
8. Shivers, B. D., Killisch, L., Sprengel, R., Sontheimer, H., Kohler, 
373
9. Grenningloh, G., Gundelfinger, E. D., Schmitt, B., Betz, H., 
Darlison, M. G., Barnard, E. A., Schofield, P. R., and Seeburg, 
Sci. U. S. A. 85, 7394–7398
EMBO J. 6, 561–565
13. Wischen, W., Morris, B. J., Darlison, M. G., Hunt, S. P., and 
129–139
FEBS Lett. 243, 358–362
17. Pritchett, D. B., Schofield, P. R., Sontheimer, H., Ymer, S., 
Abstr. 14, 641
Biol. Chem. 261, 15013–15016
139–206
154, 293–298
45, 1389–1392
Biochemical evidence for the existence of gamma-aminobutyrateA receptor iso-oligomers.
M J Duggan and F A Stephenson


Access the most updated version of this article at http://www.jbc.org/content/265/7/3831

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/7/3831.full.html#ref-list-1