Identification of $\alpha_2$- and $\alpha_3$-subunits of the GABA$_A$–benzodiazepine receptor complex purified from the brains of young rats

K. Fuchs, D. Adamiker* and W. Sieghart

Department of Biochemical Psychiatry, Psychiatrische Universitätssklinik Wien, Vienna and *Research Institute for Laboratory Animal Breeding, Himberg, Austria

Received 28 December 1989

Polyclonal antibodies were raised to synthetic amino acid sequences of the bovine GABA$_A$ receptor $\alpha_-$ and $\alpha_3$-subunits and purified by affinity chromatography on a column coupled with the respective peptide. Anti-peptide $\alpha_-$ (416-424) and anti-peptide $\alpha_3$ (459-467) antibodies immunoprecipitated GABA$_A$ receptors and recognized a protein of 53 kDa ($\alpha_-$) and 59 kDa ($\alpha_3$), respectively, in Western blots of GABA$_A$ receptors purified from the brains of 5-10 day old rats. $\alpha_-$ as well as $\alpha_3$ are specifically photolabeled by $[\text{H}]$flunitrazepam and are recognized by the $\alpha$-subunit specific monoclonal antibody bd 28.

Antibody; GABA$_A$-benzodiazepine receptor; Subunit; Heterogeneity

1. INTRODUCTION

$\gamma$-Aminobutyric acid (GABA) is one of the quantitatively most important neurotransmitters in the mammalian central nervous system. Most of the GABA actions are inhibitory and mediated by GABA$_A$ receptors. These receptors seem to consist of several protein subunits which form the GABA-gated chloride ion channel [1] and which possess allosteric binding sites for the anxiolytic benzodiazepines, for some barbiturates, steroids and some convulsant chloride channel ligands like picrotoxin and t-butylbicyclophosphorothionate (TBPS) [2]. The GABA$_A$-receptor has been purified to apparent homogeneity from several vertebrate species, and the molecular size of this receptor has been estimated to be 230–240 kDa [1]. Originally, it has been assumed that the purified receptor preparation contains only two polypeptide chains, an $\alpha$-subunit of 50–53 kDa and a $\beta$-subunit of 55–57 kDa [1]. Recently, however, using SDS-polyacrylamide gel electrophoresis with higher resolution, the existence of several different $\alpha$- and $\beta$-subunits of this receptor complex has been demonstrated [3]. The various $\alpha$-subunits exhibit molecular weights of 50–60 kDa, can be photolabeled by $[\text{H}]$flunitrazepam and be recognized by the $\alpha$-subunit specific monoclonal antibody bd 28. The $\beta$-subunits exhibit molecular weights in the same molecular weight range as the $\alpha$-subunits but can be photolabeled by the GABA$_A$ agonist $[\text{H}]$muscimol and be recognized by the $\beta$-subunit specific monoclonal antibody bd 17 [3]. Molecular cloning techniques supported these studies by revealing the primary structures of several different $\alpha$- and $\beta$-subunits of the GABA$_A$–benzodiazepine receptor complex from the corresponding complementary DNAs [4,5]. Coinjection into Xenopus oocytes of mRNAs of different $\alpha$- with that of the same $\beta$-subunit resulted in the expression of GABA-gated chloride ion channels with different GABA-sensitivity [4]. However, benzodiazepine sensitivity of expressed receptors was obtained only when the cDNAs of $\alpha$- and $\beta$-subunits were used together with that of a newly identified $\gamma$-subunit to transfect human embryonic kidney cells [6]. This seems to indicate that at least 3 different subunits are necessary to reconstitute GABA$_A$–benzodiazepine receptors with correct pharmacology.

In the present study the $\alpha_2$- and $\alpha_3$-subunit proteins of GABA$_A$–benzodiazepine receptors from the brains of 5–10 day old rats were identified by polyclonal antibodies directed against the C-terminal amino acid sequence of the bovine $\alpha_2$ or $\alpha_3$ primary structure [4].

2. MATERIALS AND METHODS

The C-terminal nonapeptides of the $\alpha_2$- and $\alpha_3$-subunits, peptides $\alpha_2$(416-424) (sequence CREPVLGVSP) and $\alpha_3$(459-467) (sequence CAIKGMIRKQ) were custom synthesized (Multiple Peptide Systems, San Diego, CA, USA) with an additional N-terminal cysteine each. Peptides were coupled to keyhole limpet hemocyanin (KLH) via the N-terminal cysteine [7] and peptide-KLH conjugates (100 µg protein) emulsified with Freund’s complete adjuvant at three-week intervals. Animals were bled one and two weeks after the fourth and all subsequent immunizations. All rabbits immunized produced antibodies recognizing the corresponding peptide or GABA$_A$ receptors as measured by an ELISA.
The GABAA receptor was extracted from the brain of 5-10 day old rats and partially purified by affinity chromatography on Affigel 15 (Bio-Rad, CA, USA) coupled to Ro 7-1986 as described previously [3]. In the partially purified receptor preparation the GABAA-benzodiazepine receptors were enriched at least 1000-fold compared to the original membrane fractions.

For immunoprecipitation [10], aliquots of the purified receptor preparations were incubated in the presence of antibody at 4°C overnight. After addition of Immunoprecipitin (Bethesda Res. Lab, MD, USA) and shaking for an additional 3 h at 4°C, the precipitate was washed three times with buffer, and the amount of precipitated receptor was measured by a [3H]flunitrazepam binding assay [11].

3. RESULTS AND DISCUSSION

Anti-peptide α2(416-424) and anti-peptide α3(459-467) antibodies were isolated from the sera of the rabbits by affinity chromatography on Thiopropyl-Sepharose 6B coupled to the cysteine residue of the respective peptides [12]. Purified antibodies selectively recognized their respective peptide and the GABAA-receptor purified from the brains of 5-10 day old rats (table 1). The anti-peptide α2(416-424) and the anti-peptide α3(459-467) antibodies were both able to recognize the receptor in a native conformation as demonstrated by immunoprecipitation experiments. Depending on the amount of purified antibody used, anti-peptide α2(416-424) or anti-peptide α3(459-467) were able to precipitate up to 54 or 44% of the GABAA receptor present in the incubation, respectively (experiments not shown).

In addition, each of these antibodies reacted with purified GABAA receptor in Western blots. As can be seen from the fluorography in fig.1 (lane 1), [3H]flunitrazepam specifically and irreversibly labeled at least 3 different proteins with apparent molecular weights 51 kDa (P51), 53 kDa (P53) and 59 kDa (P59) in receptor preparations purified from young animals [3,13]. In agreement with previous results [3] all 3 photolabeled proteins were recognized by the α-subunit specific monoclonal antibody bd 28 (fig. 1, lane 2). In contrast, proteins P51 or P59 were recognized selectively by the anti-peptide α3(416-424) (lane 3) or the anti-peptide α2(459-467) antibodies (lane 4) using the biotin-streptavidin horseradish peroxidase enhanced system from Amersham. The apparent molecular masses of the proteins were determined by calibrating the gel with standard proteins of known molecular weight. The arrows indicate proteins with apparent molecular weight 51 kDa (P51), 53 kDa (P53) or 59 kDa (P59).

The experiment was performed 3 times with similar results.

### Table 1

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>α2(416-424)</th>
<th>α3(459-467)</th>
<th>Purified receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-peptide α2(416-424)</td>
<td>1:100</td>
<td>224.4</td>
<td>n.d.</td>
<td>229.7</td>
</tr>
<tr>
<td></td>
<td>1:1000</td>
<td>24.5</td>
<td>n.d.</td>
<td>131.4</td>
</tr>
<tr>
<td></td>
<td>1:10000</td>
<td>4.0</td>
<td>n.d.</td>
<td>36.1</td>
</tr>
<tr>
<td>Anti-peptide α3(459-467)</td>
<td>1:100</td>
<td>n.d.</td>
<td>127.2</td>
<td>173.3</td>
</tr>
<tr>
<td></td>
<td>1:1000</td>
<td>n.d.</td>
<td>23.3</td>
<td>91.3</td>
</tr>
<tr>
<td></td>
<td>1:10000</td>
<td>n.d.</td>
<td>0.1</td>
<td>10.4</td>
</tr>
<tr>
<td>No primary antibody</td>
<td>–</td>
<td>0.5</td>
<td>0.1</td>
<td>0.4</td>
</tr>
</tbody>
</table>

GABAA receptor purified from the brains of 5-10 day old rats or peptides α2(416-424) or α3(459-467) were coated into microtiter wells as described [8,9]. The immobilized antigens were then probed by various dilutions of anti-peptide antibodies and a subsequent ELISA method [9] using horseradish peroxidase-conjugated anti-rabbit IgG (BIOMAKOR, Rehovot Israel) and the TMB EIA horseradish peroxidase substrate kit (BIORAD, Richmond, CA, USA). The experiment was repeated three times with similar results. n.d., not detectable.
anti-peptide \( \alpha_2(416-424) \) or the anti-peptide \( \alpha_3(459-467) \) antibody, respectively (fig.1, lanes 3 and 4).

Since the antibodies used in the present study had been purified before according to their ability to recognize the immunizing peptide (see above), these data provide a direct link between the amino acid sequence of the bovine \( \alpha_2 \)- or \( \alpha_3 \)-subunit of the GABA\(_A\) receptor and the proteins identified by the antibodies. Thus, the immunoprecipitation data indicate that epitopes are present in purified GABA\(_A\) receptor from rat brain which are identical with or rather similar to those of the C-terminal amino acid structures of the \( \alpha_2 \)- and \( \alpha_3 \)-subunits of the GABA\(_A\) receptor from bovine brain. These epitopes seem to be associated with proteins P\(_{53}\) and P\(_{59}\) since these proteins were the only proteins recognized by the anti-peptide \( \alpha_3(416-424) \) and anti-peptide \( \alpha_3(459-467) \) antibodies in this purified receptor preparation, respectively, and since these proteins are associated with the GABA\(_A\)-benzodiazepine receptor. This is indicated by the fact that proteins P\(_{53}\) and P\(_{59}\) are specifically photolabeled by \([3H]\)flunitrazepam and are recognized by the \( \alpha \)-subunit selective antibody bd 28. Thus, the present results strongly suggest that proteins P\(_{53}\) and P\(_{59}\) are the \( \alpha_2 \)- and \( \alpha_3 \)-subunits of the GABA\(_A\)-benzodiazepine receptor complex, respectively.

Whereas the present results provide the first identification of the \( \alpha_2 \)-subunit, a protein with an apparent molecular mass of 59–60 kDa has been identified recently in purified GABA\(_A\) receptors from bovine brain by a polyclonal antibody directed against the C-terminal 14 amino acids of the bovine \( \alpha_3 \)-subunit [14]. However, no attempt has been made to correlate this bovine protein with a protein photolabeled by \([3H]\)flunitrazepam as in the present study. Protein P\(_{59}\) which is photolabeled by \([3H]\)flunitrazepam and recognized by the anti-peptide \( \alpha_3(459-467) \) antibody, has been demonstrated previously to be associated with GABA\(_A\) receptors [13,15]. Its different regional distribution [15] and postnatal development [13] and the rather low potency of type I benzodiazepine receptor ligands for inhibition of \([3H]\)flunitrazepam binding to this protein [13] indicated that this protein is associated with a subtype of the GABA\(_A\)-benzodiazepine receptor complex.

Recent evidence [16] indicates that GABA\(_A\) receptor subtypes with different benzodiazepin binding properties can be constructed in human embryonic kidney cells transfected with \( \alpha_1 \), \( \beta \) and \( \gamma \)-cDNAs and that the \( \alpha \)- and not the other subunits largely determine the pharmacological properties of type I or type II benzodiazepine receptors. In agreement with the conclusion drawn above, receptors containing the \( \alpha_3 \)-subunits exhibit a much lower affinity for type I benzodiazepine receptor ligands than those containing the \( \alpha_1 \)-subunits.

In the present investigation no data are provided on the possible identity of protein P\(_{51}\). However, this protein has been shown previously to be associated with the type I benzodiazepine receptor [17] and GABA\(_A\) receptors containing the \( \alpha_1 \)-subunit exhibit benzodiazepine binding properties closely similar to type I receptors in transfected human embryonic kidney cells [16]. Since protein P\(_{51}\) [15] as well as the \( \alpha_1 \)-subunit [4] seem to be the most abundant \( \alpha \)-subunit of the GABA\(_A\) receptor in cerebellum, a brain tissue especially enriched in type I benzodiazepine receptors [17], protein P\(_{51}\) possibly is identical with the \( \alpha_1 \)-subunit of the GABA\(_A\) receptor. This conclusion, however, has to be confirmed by \( \alpha_1 \)-subunit selective antibodies.

In summary, the present data indicate that proteins P\(_{53}\) and P\(_{59}\) specifically and irreversibly labeled by \([3H]\)flunitrazepam are the \( \alpha_2 \)- and \( \alpha_3 \)-subunits of the GABA\(_A\)-benzodiazepine receptor, respectively. Using anti-peptide \( \alpha_2(416-424) \) and anti-peptide \( \alpha_3(459-467) \) antibodies, it should be possible to isolate and characterize the GABA\(_A\)-benzodiazepine receptor subtype associated with these subunits.

Acknowledgement: This work was supported by the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich.

REFERENCES