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Identification of α_2- and α_3-subunits of the $GABA_A$–benzodiazepine receptor complex purified from the brains of young rats

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Polyclonal antibodies were raised to synthetic amino acid sequences of the bovine $GABA_A$ receptor α_2- and α_3-subunits and purified by affinity chromatography on a column coupled with the respective peptide. Anti-peptide α_2(416–424) and anti-peptide α_3(459–467) antibodies immunoprecipitated $GABA_A$ receptors and recognized a protein of 53 kDa (P_{53}) and 59 kDa (P_{59}), respectively, in Western blots of $GABA_A$ receptors purified from the brains of 5–10 day old rats. P_{53} as well as P_{59} are specifically photolabeled by [^{3}H]flunitrazepam and are recognized by the α-subunit specific monoclonal antibody bd 28.

Antibody; $GABA_A$-benzodiazepine receptor; Subunit; Heterogeneity

1. INTRODUCTION

γ-Aminobutyric acid (GABA) is one of the quantitatively most important neurotransmitters in the mamalian central nervous ystem. 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 α-subunit of 50–53 kDa and a β-subunit of 55–57 kDa [1]. Recently, however, using SDS-polyacrylamide gel electrophoresis with higher resolution, the existence of several different α- and β-subunits of this receptor complex has been demonstrated [3]. The various α-subunits exhibit molecular weights of 50–60 kDa, can all be photolabeled by [^{3}H]flunitrazepam and be recognized by the α-subunit specific monoclonal antibody bd 28. The β-subunits exhibit molecular weights in the same molecular weight range as the α-subunits but can be photolabeled by the $GABA_A$ agonist [^{3}H]muscimol and be recognized by the β-subunit specific monoclonal antibody bd 17 [3]. Molecular cloning techniques supported these studies by revealing the primary structures of several different α- and β-subunits of the $GABA_A$-benzodiazepine receptor complex from the corresponding complementary DNAs [4,5]. Coinjection into *Xenopus* oocytes of mRNAs of different α- with that of the same β-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 α- and β-subunits were used together with that of a newly identified γ-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 α_2- and α_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 α_2 or α_3 primary structure [4].

2. MATERIALS AND METHODS

The C-terminal nonapeptides of the α_2- and α_3-subunits, peptides α_2(416–424) (sequence CREPVLGVSP) and α_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 were injected at 3 sites subcutaneously into rabbits. Subsequent immunizations were in Freund's incomplete 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,

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using the peptides [8] or purified $GABA_A$ receptor [9] as the antigen.

The $GABA_A$ 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 $GABA_A$-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 [^{3}H]flunitrazepam binding assay [11].

3. RESULTS AND DISCUSSION

Anti-peptide α_2(416–424) and anti-peptide α_3(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 $GABA_A$-receptor purified from the brains of 5–10 day old rats (table 1). The anti-peptide α_2(416–424) and the anti-peptide α_3(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 α_2(416–424) or anti-peptide α_3(459–467) were able to precipitate up to 54 or 44% of the $GABA_A$ receptor present in the incubation, respectively (experiments not shown).

In addition, each of these antibodies reacted with purified $GABA_A$ receptor in Western blots. As can be seen from the fluorography in fig.1 (lane 1), [^{3}H]flunitrazepam specifically and irreversibly labeled at least 3 different proteins with apparent molecular weights 51 kDa (P_{51}), 53 kDa (P_{53}) and 59 kDa (P_{59}) 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 P_{53} or P_{59} were recognized selectively by the

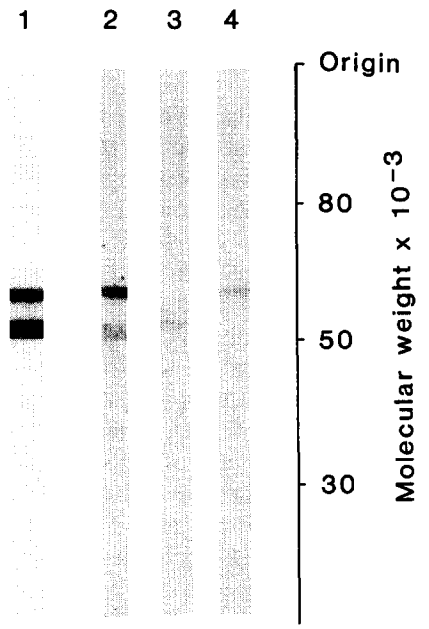

Fig.1. Comparison of proteins photolabeled by [^{3}H]flunitrazepam in a partially purified $GABA_A$ receptor preparation from young rats with those identified by several different antibodies. Partially purified $GABA_A$ receptors were specifically photolabeled by [^{3}H]flunitrazepam and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose and were either subjected to autoradiography (lane 1) or probed with the monoclonal antibody bd 28 (lane 2), the anti-peptide α_2(416–424) (lane 3) or the anti-peptide α_3(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 (P_{51}), 53 kDa (P_{53}) or 59 kDa (P_{59}). The experiment was performed 3 times with similar results.

Table 1

ELISA demonstrating the recognition of immobilized peptides or affinity purified $GABA_A$ receptors by antibodies raised against α-subunit specific peptides

Antibody (1 mg protein/ml)	Dilution	$E_{(450\ nm)} \times 100$		
		α_2(416–424)	α_3(459–467)	Purified receptor
Anti-peptide α_2(416–424)	1:100	224.4	n.d.	229.7
	1:1000	24.5	n.d.	131.4
	1:10000	4.0	n.d.	36.1
Anti-peptide α_3(459–467)	1:100	n.d.	127.2	173.3
	1:1000	n.d.	23.3	91.3
	1:10000	n.d.	0.1	10.4
No primary antibody	–	0.5	0.1	0.4

$GABA_A$ receptor purified from the brains of 5–10 day old rats or peptides α_2(416–424) or α_3(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 α_2(416–424) or the anti-peptide α_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 α_2- or α_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 α_2- and α_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 antipeptide α_2(416–424) and anti-peptide α_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 [^{3}H]flunitrazepam and are recognized by the α-subunit selective antibody bd 28. Thus, the present results strongly suggest that proteins P_{53} and P_{59} are the α_2- and α_3-subunits of the $GABA_A$-benzodiazepine receptor complex, respectively.

Whereas the present results provide the first identification of the α_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 α_3-subunit [14]. However, no attempt has been made to correlate this bovine protein with a protein photolabeled by [^{3}H]flunitrazepam as in the present study. Protein P_{59} which is photolabeled by [^{3}H]flunitrazepam and recognized by the anti-peptide α_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 [^{3}H]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 α-, β- and γ-cDNAs and that the α- 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 α_3-subunits exhibit a much lower affinity for type I benzodiazepine receptor ligands than those containing the α_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 α_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 α_1-subunit [4] seem to be the most abundant α-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 α_1-subunit of the $GABA_A$ receptor. This conclusion, however, has to be confirmed by α_1-subunit selective antibodies.

In summary, the present data indicate that proteins P_{53} and P_{59} specifically and irreversibly labeled by [^{3}H]flunitrazepam are the α_2- and α_3-subunits of the $GABA_A$-benzodiazepine receptor, respectively. Using anti-peptide α_2(416–424) and anti-peptide α_3(459–467) antibodies, it should be possible to isolate and characterize the $GABA_A$-benzodiazepine receptor subtype associated with these subunits.

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