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Isolation of type I and type II $GABA_A$-benzodiazepine receptors by immunoaffinity chromatography

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Anti-peptide α_1 (1–9) and anti-peptide α_3 (459–467) antibodies coupled to Affigel-10 were used for the isolation of $GABA_A$ receptors containing the α_1- or α_3-subunit, respectively. Both types of $GABA_A$ receptors exhibited a high affinity for [^{3}H]flunitrazepam, and binding of [^{3}H]flunitrazepam was stimulated in the presence of GABA. $GABA_A$ receptors eluted from the anti-peptide α_1 (1–9) immunoaffinity column exhibited a high affinity and those from the anti-peptide α_3 (459–467) columns a low affinity for the type I benzodiazepine receptor-selective ligand Cl 218872, indicating the enrichment of type I and type II $GABA_A$-benzodiazepine receptors, respectively.

$GABA_A$-benzodiazepine receptor; Immunoaffinity chromatography; Type I and type II $GABA_A$ receptor; Flunitrazepam binding; Radioautography

1. INTRODUCTION

The $GABA_A$ receptor of mammalian brain is a ligand-gated chloride ion channel and the site of action of several important classes of drugs like benzodiazepines, barbiturates, convulsant compounds and some steroids [1]. This receptor has been purified to apparent homogeneity by benzodiazepine-affinity chromatography and seems to consist of several different protein subunits [2]. Molecular cloning studies not only have identified their amino acid sequence but also have demonstrated the existence of a significant heterogeneity of individual subunits. Thus, so far the existence of 6 α-, 4 β-, 2 γ- and 1 δ-subunit of the $GABA_A$ receptor has been demonstrated [3]. Studies on the expression in *Xenopus* oocytes and mammalian cells of the various subunits revealed that GABA-gated chloride ion channels modulated by benzodiazepines and β-carbolines can only be produced by the simultaneous presence of α-, β- and γ-subunit cDNAs in the cells [4]. This seems to indicate that at least three different subunits are necessary to reconstitute $GABA_A$-benzodiazepine receptors with correct pharmacology. In addition, it has been demonstrated that depending on the type of α-subunit cDNA used for the co-expression with β- and γ-subunit cDNAs, $GABA_A$-benzodiazepine receptors with different GABA and benzodiazepine binding properties are produced [5,6].

Recently, polyclonal antibodies directed against parts of the amino acid sequence of the α_1-, α_2- and α_3-subunits were raised [7–9]. These antibodies, each of which selectively recognized a single protein in purified $GABA_A$ receptor preparations not only were used for the identification of the corresponding α-subunits [7,8] but also for the separation of $GABA_A$-benzodiazepine receptors containing the respective α-subunits by immunoaffinity chromatography (Zezula et al., submitted). In this study, however, receptors were eluted from the affinity columns by a change of pH. This resulted in the inactivation of the eluted $GABA_A$ receptors. In the present study a different method was used for the elution of the receptors from the affinity columns. Receptors eluted by this method were still able to bind GABA or benzodiazepines. The properties of receptors eluted from different immunoaffinity columns were compared.

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2. MATERIALS AND METHODS

Anti-peptide α_1(1–9), anti-peptide α_2(416–424) and anti-peptide α_3(459–467) antibodies were isolated by affinity chromatography from sera of rabbits immunized with the respective peptides coupled to keyhole-limpet haemocyanin [8]. Purified antibodies selectively recognized their respective peptide and $GABA_A$ receptors purified from the brains of 5–10-day-old rats as measured by ELISA, immunoprecipitation and Western blot techniques [8].

Immunoaffinity columns were prepared by coupling of 10 mg purified anti-peptide antibodies to 5 ml of wet Affigel 10 using standard methods (BIO-RAD). The total binding capacity of these columns was about 30–60 pmol [^{3}H]flunitrazepam binding sites when the columns were freshly synthesized. However, since the columns had to be washed with a buffer containing 0.2% Triton X-100 and 0.1 M glycine-HCl, pH 2.4 after each run in order to completely remove $GABA_A$ receptors bound to the columns, and since the rabbit antibodies irreversibly bound to the columns leaked from the columns at acid pH, the binding capacity declined with time.

$GABA_A$ receptors were extracted from brain membranes of

5–10-day-old rats by constant agitation for 2 h at 4°C in a buffer containing 2% Triton X-100, 0.3 M KCl, 10 mM HEPES, pH 7.4, 0.5 mM dithiothreitol, 1 mM EDTA, 1 mM benzamidine, 0.1 mg/ml bacitracine and 0.3 mM phenylmethylsulfonylfluoride (PMSF). The suspension was centrifuged for 30 min at 200000 × g, and the supernatant was then applied to the immunoaffinity columns.

3. RESULTS AND DISCUSSION

$GABA_A$-benzodiazepine receptors present in extracts from the brain membranes of 5–10-day-old rats were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. As shown in Fig. 1, a mixture of anti-peptide α_1(1–9), anti-peptide α_2(416–424) and anti-peptide α_3(459–467) antibodies was able to recognize at least three proteins with apparent molecular weight 51000 (P_{51}), 53000 (P_{53}) or 59000 (P_{59}). The same proteins have been identified previously in $GABA_A$-benzodiazepine receptor preparations purified from the brains of 5–10-day-old rats and have been demonstrated to be the α_1-, α_2- and α_3-subunits of the $GABA_A$ receptors, respectively [8].

30–40 ml of the brain membrane extracts corresponding to a total of about 125 pmol reversible [^{3}H]flunitrazepam binding sites were applied to anti-peptide α_1(1–9), anti-peptide α_2(416–424) or anti-peptide α_3(459–467) immunoaffinity columns at a rate of 20 ml/h. 15–40% of the applied $GABA_A$ receptors were retained by the columns, depending on the type of antibody bound to the column and on the age of the columns as mentioned in section 2. The columns were washed with 15 times of their volume using the extraction buffer complemented with additional KCl to result in a total of 0.6 M KCl in order to remove unspecifically bound proteins and were subsequently equilibrated with 5 volumes of a low salt, low Triton X-100 buffer containing 10 mM HEPES, pH 7.4, 150 mM KCl, 0.2% Triton X-100, 1 mM EDTA, 0.5 mM dithiothreitol, 1 mM benzamidine and 0.3 mM PMSF.

In order to find conditions which result in the elution of $GABA_A$ receptors still able to bind benzodiazepines, several different chaotropic agents were investigated as eluents. It was found that a 0.2% Triton X-100 solution, pH 7.4, containing either 3 or 5 M $MgCl_2$, or 3 M urea plus 3 M $MgCl_2$, 3 M urea plus 5 M $MgCl_2$, 3 M urea alone, 3 M urea plus 5 M NaJ, 5 M NaJ alone, or 1 M NaSCN plus 1 M KCl were able to elute $GABA_A$-benzodiazepine receptors as demonstrated by a [^{3}H]-flunitrazepam binding assay in column eluates dialyzed overnight against 1000 vols of a buffer containing 10 mM HEPES, pH 7.4, 150 mM KCl, 0.2% Triton X-100, 1 mM EDTA, 0.5 mM dithiothreitol and 1 mM benzamidine. The relatively largest amount of $GABA_A$-benzodiazepine receptors was eluted by a 0.2% Triton X-100 solution containing 3 M urea plus 3 M $MgCl_2$. 5–20% of the $GABA_A$ receptor retained by the anti-peptide α_1(1–9) and anti-peptide α_3(459–467) column could be eluted under these conditions in a form still

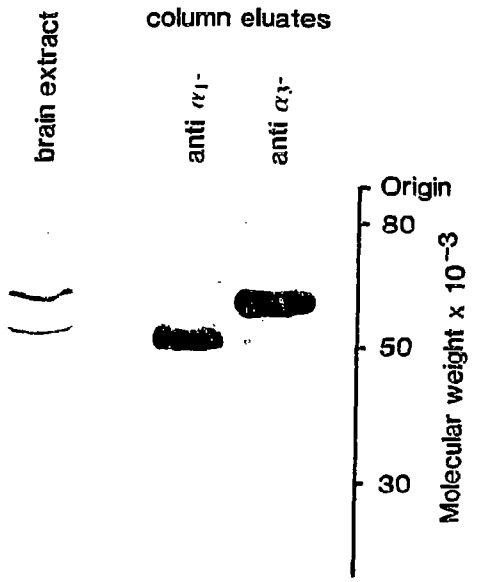

Fig. 1. Comparison of α-subunit proteins present in brain membrane extracts and eluates from two different immunoaffinity columns. Proteins in brain membrane extracts or in immunoaffinity column eluates were subjected to SDS-PAGE, transferred to nitrocellulose and probed with a mixture of anti-peptide α_1(1–9), anti-peptide α_2(416–424) or anti-peptide α_3(459–467) antibodies, an anti-rabbit IgG F(ab')$_2$ antibody conjugated to alkaline phosphatase (Jackson Immuno Research Labs, USA) and the alkaline phosphatase conjugate substrate kit (BIO-RAD). The experiment was performed 3 times with similar results.

able to bind [^{3}H]flunitrazepam. The elution of $GABA_A$-benzodiazepine receptors was therefore routinely performed with a 0.2% Triton X-100 solution, pH 7.4, containing 3 M urea and 3 M $MgCl_2$ in all subsequent experiments.

Interestingly, although $GABA_A$ receptors were retained by the antipeptide α_2(416–424) immunoaffinity column, in contrast to the anti-peptide α_1(1–9) or anti-peptide α_3(459–467) immunoaffinity columns a rather low amount of protein was eluted from this column using 0.2% Triton X-100, 3 M urea and 3 M $MgCl_2$. $GABA_A$ receptor proteins, however, were eluted from this column using a buffer containing 0.2% Triton X-100 and 0.1 M glycine-HCl, pH 2.4. In agreement with previous results (Zezula et al., submitted), $GABA_A$ receptors eluted by such a change of the pH no longer were able to bind [^{3}H]flunitrazepam even when the column eluate was immediately neutralized by addition of 3 M Tris-Cl buffer, pH 7.4. Thus, the elution method using a 0.2% Triton X-100 solution containing urea and magnesium chloride could not be used to isolate sufficient amounts of $GABA_A$ receptors from columns containing the anti-peptide α_2(416–424) antibody. Therefore all further experiments were performed with eluates from the anti-peptide α_1(1–9) or the anti-peptide α_3(459–467) column only.

$GABA_A$ receptors eluted from the anti-peptide $\alpha_1(1-9)$ or anti-peptide $\alpha_3(459-467)$ columns using a solution containing 0.2% Triton X-100, pH 7.4, 3 M urea and 3 M $MgCl_2$ were subjected to SDS-PAGE and Western blot analysis. For the detection of the respective subunits a mixture of anti-peptide $\alpha_1(1-9)$, anti-peptide $\alpha_2(416-424)$ and anti-peptide $\alpha_3(459-467)$ antibodies was used. As shown in Fig. 1 in the $GABA_A$ receptor fractions eluted from anti-peptide $\alpha_1(1-9)$ or anti-peptide $\alpha_3(459-467)$ columns, the α_1(Protein P_{51})- or α_3(Protein P_{59})-subunits of the $GABA_A$ receptors were strongly enriched. These results support a previous report [9] indicating that most, if not all $GABA_A$ receptors contain only a single type of α-subunit.

In addition to the α_1- or α_3-subunits predominantly retained by and eluted from the respective immunoaffinity columns other proteins were present in the column eluates in small amounts and were weakly labeled by the mixture of anti-peptide α_1, anti-peptide α_2 and anti-peptide α_3 antibodies (Fig. 1). Thus, these weakly labeled proteins are α_1-, α_2- or α_3-subunits of the $GABA_A$ receptor not completely removed from the α-subunits specifically retained by the immunoaffinity column. Since these contaminating α-subunits were detected in the column eluate even after extensive washing of the immunoaffinity columns, their presence in the column eluate might be due either to aggregated $GABA_A$ receptors containing other α-subunits or to a colocalization in a rather minor part of the receptors of two or more different α-subunits in the same $GABA_A$ receptor complex. A minor contribution of such receptors cannot be excluded by the data so far available [9].

In order to investigate the possible presence of β- and γ-subunits of the $GABA_A$ receptors in eluates from anti-peptide $\alpha_1(1-9)$ or anti-peptide $\alpha_3(459-467)$ immunoaffinity columns, these eluates were probed in immunoblots using the β-subunit-specific monoclonal antibody bd-17 [10] or a γ_2-subunit-specific polyclonal antibody provided by H. Möhler [11]. The same antibody specific protein staining pattern was observed in immunoblots from brain membrane extracts as from anti-peptide $\alpha_1(1-9)$ or anti-peptide $\alpha_3(459-467)$ column eluates (experiments not shown). These data indicate the presence of β- and γ_2-subunits in the immunoaffinity column eluates.

In order to compare the binding properties of $GABA_A$-benzodiazepine receptors before or after immunoaffinity chromatography, Scatchard analysis of [^{3}H]flunitrazepam binding (concentration range 0.5–10 nM) in the absence or presence of 2 μM diazepam was performed in membrane extracts and dialyzed immunoaffinity column eluates. As shown in Table I, $GABA_A$ receptors in membrane extracts or eluates from anti-peptide $\alpha_1(1-9)$ or anti-peptide $\alpha_3(459-467)$ columns exhibited a similar high affinity for [^{3}H]flunitrazepam. In addition, GABA was able to stimulate binding of [^{3}H]flunitrazepam to receptors from anti-peptide $\alpha_1(1-9)$ or anti-peptide $\alpha_3(459-467)$ column eluates. GABA stimulation of [^{3}H]flunitrazepam binding to membrane extracts was not determined due to the presence of endogenous GABA in those extracts. In order to investigate a possible differential affinity of $GABA_A$ receptors eluted from the different immunoaffinity columns, the potency of the type I benzodiazepine receptor-selective ligand Cl 218872 for displacement of [^{3}H]flunitrazepam binding was investigated in dialyzed eluates from anti-peptide $\alpha_1(1-9)$ and anti-peptide $\alpha_3(459-467)$ columns. In contrast to membrane ex-

Table I

Properties of $GABA_A$ receptors present in membrane extracts or in eluates from the immunoaffinity columns

100 μl membrane extracts or dialyzed eluates from the immunoaffinity columns were incubated for 90 min at 4°C either with 2.5 nM or with various concentrations (0.5–10 nM) of [^{3}H]flunitrazepam in the absence or presence of 2 μM diazepam, 100 μM GABA or various concentrations of Cl 218872 in 1 ml of a solution containing 50 mM Tris-citrate, pH 7.4, 150 mM NaCl, 50 μg γ-globulin and a total of 15% of polyethyleneglycol. Samples were filtered through Whatman GF/C filters and washed 3 times with a solution containing 50 mM Tris-citrate, pH 7.4 and 8% polyethyleneglycol and radioactivity on the filter was measured. Nonspecific binding (estimated in the presence of 2 μM diazepam) was subtracted from total binding to yield specific binding. Equilibrium binding data of [^{3}H]flunitrazepam were subjected to Scatchard analysis (linear regression). IC_{50} values were determined by linear least-squares fit of the data to the Hill-equation. Results are means ± SE with number of experiments performed in duplicates in parentheses. n.d. = not determined.

	Membrane extract	Anti-peptide α_1 column eluate	Anti-peptide α_3 column eluate
[^{3}H]Flunitrazepam: binding, K_d (nM)	2.3 ± 0.2 (5)	2.8 ± 0.2 (3)	2.4 ± 0.2 (4)
2.5 nM [^{3}H]Flunitrazepam ± 100 μM GABA (% of control)	n.d.	138 ± 6 (3)	139 ± 5 (3)
IC_{50} (nM) Cl 218872	1157 ± 26 (4)	317 ± 40 (4)	1405 ± 140 (4)

tracts, in which the potency of Cl 218872 was intermediate, the potency of Cl 218872 for inhibition of [^{3}H]flunitrazepam binding to eluates from the anti-peptide α_1(1–9) column was higher than that for eluates from the anti-peptide α_3(459–467) column. These data are consistent with the enrichment of type I and type II $GABA_A$-benzodiazepine receptors by the anti-peptide α_1(1–9)- and anti-peptide α_3(459–467) immunoaffinity columns, respectively.

Thus, in the present investigation for the first time type I and type II $GABA_A$-benzodiazepine receptor subtypes with retained binding properties were enriched and separated from other possible $GABA_A$-benzodiazepine receptor subtypes. The binding properties of the isolated type I and type II receptors were similar to but not identical with those of artificial receptors arising by the co-expression of α_1-, β_1- and γ_2- or α_3-, β_1- and γ_2-subunits in embryonic kidney cells [6]. One reason for this discrepancy might be the possibility that the artificially expressed receptors either by their subunit composition, their state of glycosylation or their lipid environment might not be identical with receptors expressed in vivo and isolated by immunochromatography. Other reasons could be the contamination of the α_1-subunit containing type I or the α_3-subunit containing type II receptors by small amounts of receptors containing different α-subunits as indicated by Fig. 1. And finally, the anti-peptide α_1(1–9) or anti-peptide α_3(459–567) columns presumably will not distinguish between $GABA_A$-benzodiazepine receptors containing different β- or γ-subunits but the same α-subunit. Thus, if such receptors existed in vivo, various $GABA_A$ receptors containing combinations of different β- or γ-subunits but the same α-subunit will be coenriched by the anti-peptide α_1 or anti-peptide α_3 columns and add to the overall binding properties measured in the separated receptors. Further experiments using antibodies highly specific for the various β- and γ-subunits (which are not available yet), will have to be performed to investigate the possible existence of different β- or γ-subunits in immunoaffinity purified $GABA_A$ receptors containing a single α-subunit.

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