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N-Deglycosylation and immunological identification indicates the existence of β-subunit isoforms of the rat $GABA_A$ receptor

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β_2- and β_3-subunits of $GABA_A$ receptors purified from the brains of 5–10-day-old rats were investigated in the intact or completely *N*-deglycosylated state using the β-subunit-specific monoclonal antibody bd-17 and polyclonal antibodies directed against synthetic amino acid sequences specific for the $GABA_A$ receptor β_2- or β_3-subunits. The present results seem to indicate the existence of two different isoforms of the β_3-subunit and several different isoforms of the β_2-subunit of the $GABA_A$ receptor which probably are produced by alternative splicing.

$GABA_A$ receptor; *N*-Deglycosylation; β-Subunit; Antibody

1. INTRODUCTION

The γ-aminobutyric acid(A) ($GABA_A$) receptor is a ligand-gated ion channel and the major inhibitory neurotransmitter receptor in the vertebrate brain. Molecular biological studies have demonstrated the existence of a variety of different receptor subunits, each encoded by a single gene [1]. Thus, so far 6α-, 4β-, 2γ-, and 1δ-subunit of the $GABA_A$ receptor have been cloned and sequenced, and expression studies have indicated that at least three different subunits (α, β and γ) are necessary to reproducibly reconstitute $GABA_A$ receptors with correct pharmacology [2]. These studies were supported by biochemical studies indicating the existence of several different α-subunit proteins of the $GABA_A$ receptor [3], some of which recently have been identified using polyclonal antibodies selectively recognizing individual α-subunits [4,5]. In a subsequent study, the existence of several different β-subunit proteins with apparent molecular weights between 50 and 60 kDa was demonstrated [6]. Each of these proteins was photolabeled by the $GABA_A$ agonist [^{3}H]muscimol and recognized by the monoclonal antibody bd-17, which has been demonstrated to selectively recognize the β_2- and β_3-subunit of the $GABA_A$ receptor [7]. Interestingly, however, the published amino acid sequences indicated that the β_1-, β_2- and β_3-subunits of the $GABA_A$ receptors consist of 449, 450 and 448 amino acid residues respectively, and thus should exhibit a quite similar molecular weight of about 52 kDa [8]. In the present study, the apparent molecular weight of β-subunits of the $GABA_A$ receptor was investigated before or after exhaustive *N*-deglycosylation using the β-subunit-selective monoclonal antibody bd-17 and polyclonal antibodies directed against specific amino acid sequences of the β_2- or β_3-subunit.

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2. MATERIALS AND METHODS

The $GABA_A$ receptor was extracted from the brains of 5–10 day-old-rats and purified by affinity chromatography on Affigel 15 (Bio-Rad, CA, USA) coupled to Ro 7-1986 as described previously [6]. In the purified receptor preparation the $GABA_A$-benzodiazepine receptors were enriched at least 1000-fold compared to the original membrane fractions.

For *N*-deglycosylation purified receptor preparations (about 25 μg protein in 170 μl of a solution containing 0.4 M Tris-HCl, pH 8.6, 0.5% SDS and 50 mM β-mercaptoethanol) was denatured at 65°C for 10 min. After cooling to room temperature several additions were made resulting in 500 μl of a solution containing 25 μg receptor protein, 0.13 M Tris-HCl, pH 8.6, 0.17% SDS, 17 mM β-mercaptoethanol, 1.25% Nonidet P-40, 0.1 μM aprotinin, 1 μM leupeptin, 1 mM phenylmethylsulfonylfluoride (PMSF) and 10 mM EDTA. This solution was incubated with 3.7 U recombinant *N*-Glycanase (Genzyme, Boston, MA, USA) for 20 h at 37°C. After deglycosylation, receptor proteins were precipitated with methanol/chloroform [9], were then resuspended in a solution containing 2% SDS, 62.5 mM Tris-HCl, pH 6.8, 5% mercaptoethanol, 1 mM EDTA and 10% glycerol and subjected to SDS-PAGE and Western blot analysis as described previously [6].

Peptides β_2 (191–197) (sequence YKLITKK), β_2 (382–389) (sequence AGLPRHSF) or β_3 (190–197) (sequence EHRLVSRN) were custom synthesized (Multiple Peptide Systems, San Diego, CA, USA) with an additional C-terminal cysteine each. Peptides were coupled to keyhole limpet haemocyanine (KLH) via the C-terminal cysteine [10] and peptide KLH-conjugates (100 μg protein) emulsified with Freund's complete adjuvant were injected at three sites subcutaneously into rabbits. Subsequent immunizations were in Freund's incomplete adjuvant at 3 week intervals. Animals were bled one and two weeks after the 4th and all subsequent immunizations. All rabbits immunized produced antibodies recognizing the corresponding peptide

or $GABA_A$ receptors as measured by an ELISA using the peptides or purified $GABA_A$ receptor as the antigen [5].

Anti-peptide β_2 (191–197), anti-peptide β_2 (382–389) and anti-peptide β_3 (190–197) antibodies were isolated from the sera of the rabbits by affinity chromatography on thiopropyl-Sepharose 6B (Pharmacia, Fine Chemicals, 1983) coupled to the cysteine residue of the respective peptides.

For immunoprecipitation aliquots of the purified receptor preparations were incubated in the presence of antibody at 4°C overnight. After addition of Immunoprecipitin (Bethesda Research Laboratories, 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

$GABA_A$ receptors purified by affinity chromatography from the brains of 5–10-day-old rats were subjected to SDS-PAGE. The proteins were then transferred to nitrocellulose and probed with the monoclonal antibody bd-17. This antibody has been demonstrated to specifically recognize the β_2- and β_3-subunits of the $GABA_A$ receptors [7]. In agreement with previous results [6], bd-17 labeled at least 4 different proteins in the apparent molecular weight range between 50 und 60 kDa and a protein smear between 40 and 47 kDa (Fig. 1). Since the published amino acid sequences of the β_2- and β_3-subunit indicated that these subunits in the deglycosylated state should exhibit a rather similar apparent molecular weight [8], one possible explanation for the existence of a variety of different proteins identified by bd-17 was the existence of β_2- and β_3-subunits in several different states of glycosylation. Complete deglycosylation of these subunits should then result in a single protein band identified by the antibody bd-17. $GABA_A$ receptors purified from the brains of 5–10-day-old rats were therefore exhaustively treated with *N*-Glycanase. This enzyme (peptide: *N*-glycosidase F) hydrolyzes all classes of *N*-linked oligosaccharides at the asparaginyl residue and results in completely *N*-deglycosylated proteins [12]. $GABA_A$ receptors were then again subjected to SDS-PAGE and Western blot analysis. As shown in Fig. 1, the monoclonal antibody bd-17 after complete *N*-deglycosylation of the purified $GABA_A$ receptor still recognized a variety of different proteins. Thus, two proteins with apparent molecular weights 51 and 53 kDa were strongly and at least three other proteins with apparent molecular weight 42, 44 and 46 kDa were weakly labeled. The same pattern of labeling by bd-17 was observed whether purified $GABA_A$ receptors were incubated with either half or twice the concentration of *N*-glycanase or whether receptors were incubated with *N*-glycanase for 20 or 72 h. Furthermore, a second and third addition of fresh *N*-Glycanase 19 and 48 h after the first addition, and incubation for an additional 24 h (resulting in a total of 72 h of incubation with increasing concentrations of *N*-Glycanase) did not result in an immunolabeling pattern

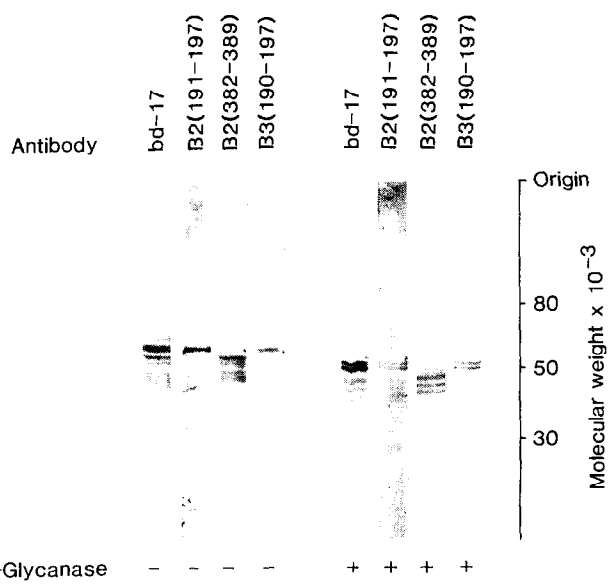

Fig. 1. Comparison of β-subunits of the $GABA_A$ receptor identified by four different antibodies before or after complete *N*-deglycosylation. $GABA_A$ receptors purified from the brains of 5–10-day-old rats before or after complete *N*-deglycosylation with *N*-Glycanase were subjected to SDS-PAGE and Western blot analysis using the antibodies as indicated. Specifically bound antibodies were detected with biotinylated anti-mouse Ig from sheep (Amersham, UK; for bd-17) or biotinylated anti-rabbit Ig from goat (Bethesda Res. Laboratories, MD, USA; for the polyclonal antibodies), the streptavidin alkaline phosphatase conjugate (Amersham) and the alkaline phosphatase conjugate substrate kit (Bio-Rad, Richmond, CA). The experiment was performed 6 times with similar results.

different from that shown in Fig. 1. These results argue in favour of a complete *N*-deglycosylation of the $GABA_A$ receptor proteins and confirm the absence of a significant proteolytic action of the *N*-glycanase preparation under the conditions used. Since isolation and purification of $GABA_A$ receptors was performed in the presence of a variety of protease inhibitors and the same pattern of labeling by the monoclonal antibody bd-17 was observed whether $GABA_A$-receptors freshly purified by affinity chromatography at 4°C or receptors incubated for 20 h at 37°C in the absence of *N*-glycanase were investigated (experiments not shown), the production by proteolytic degradation of β_2- or β_3-subunits of some of the protein bands identified by bd-17 in receptor preparations before *N*-glycanase treatment is also rather unlikely (additional arguments see below) although it cannot completely be excluded.

In order to identify the β_2- or β_3-subunit of the $GABA_A$ receptor, polyclonal antibodies were raised against specific amino acid sequences of these subunits and purified from the sera of rabbits by affinity chromatography. These antibodies, anti-peptide β_2 (191–197), anti-peptide β_2 (382–389) or anti-peptide β_3 (190–197), specifically recognized their immunizing peptide and purified $GABA_A$ receptors as demonstrated by ELISA's using the peptides or purified receptors as

antigens, and were able to precipitate 7.2%, 12% or 17% of the $GABA_A$ receptor present in the incubation, respectively. As shown in Fig. 1, anti-peptide β_2 (191–197) and anti-peptide β_3 (190–197) each predominantly identified a single protein band with apparent molecular weight 56 kDa in $GABA_A$ receptors purified from 5–10-day-old rats. The apparent molecular weight of this protein band was identical to that of a protein band predominantly identified by the β-subunit-specific monoclonal antibody bd-17. These data indicate that β_2- and β_3-subunits exhibit an identical apparent molecular weight of 56 kDa, a result expected if oligosaccharide chains of similar apparent molecular weight are assumed to be associated with these subunits. After exhaustive *N*-deglycosylation, however, the anti-peptide β_2 (191–197) and anti-peptide β_3 (190–197) antibody each identified the same two protein bands with apparent molecular weights of 51 and 53 kDa which also were most prominently labeled by bd-17. The apparent molecular weight of these proteins fit quite well compared to the theoretical molecular weight of about 52 kDa derived from the amino acid sequences of both the β_2- or β_3-subunit [8], indicating a complete deglycosylation of these proteins. This conclusion is further supported by a previous report [13] indicating that similar apparent molecular weights for β-subunits were obtained whether purified $GABA_A$ receptors were treated with endoglycosidase F, which similar to *N*-Glycanase removes *N*-linked carbohydrates from glycoproteins or with trifluoromethane sulfonic acid, which removes *N*-linked as well as *O*-linked carbohydrate groups from glycoproteins. Thus, these data seem to indicate that at least two β_2- and two β_3-subunit proteins with slightly different apparent molecular weight exist. In the intact $GABA_A$ receptor these proteins presumably are *N*-glycosylated to a different degree and thus exhibit a rather similar apparent molecular weight. An alternative explanation of a cross-reactivity of the antibodies with a different $GABA_A$ receptor subunit or a protein not associated with the $GABA_A$ receptor but present in the purified receptor preparations is highly unlikely because it then has to be assumed that 3 different antibodies, each directed against a different epitope present on the β_2- and/or β_3-subunit crossreacted with the same protein.

In order to further verify the identity of the β_2-subunit of the $GABA_A$ receptor, a β_2-subunit-specific antibody directed against a different epitope of the β_2-subunit than the anti-peptide β_2 (191–197) antibody was used. As shown in Fig. 1, surprisingly this antibody, anti-peptide β_2 (382–389), only weakly labeled the protein band with apparent molecular weight 56 kDa which was labeled predominantly by bd-17, anti-peptide β_2 (191–197) or anti-peptide β_3 (190–197). In contrast, this antibody predominantly labeled at least 4 different protein bands with apparent molecular weights 45, 47, 51 and 53 kDa. These protein bands seem to consist of β-subunits since protein bands with identical apparent molecular weights were labeled by the β-subunit-specific monoclonal antibody bd-17. After exhaustive *N*-deglycosylation, the anti-peptide β_2 (382–389) antibody predominantly labeled 3 different protein bands with apparent molecular weights 42, 44 and 46 kDa. Again protein bands with identical apparent molecular weights were labeled by the β-subunit specific monoclonal antibody bd-17. These proteins cannot have been produced by a proteolytic digestion of the β_2-subunits identified by the anti-peptide β_2 (191–197) antibodies, since loss by proteolytic digestion of the epitope identified by the anti-peptide β_2 (191–197) antibody which is localized close to the middle of the amino acid sequence of the β_2-subunit [8] would have reduced the apparent molecular weights of the β_2-subunits much more dramatically than shown in Fig. 1. Furthermore, in this case the anti-peptide β_2 (382–389) antibody should then be able to strongly recognize the protein with apparent molecular weight 56 kDa, identified by the anti-peptide β_2 (191–197) antibody. Thus, the fact that two different antibodies directed against two different epitopes of the same β_2-subunit predominantly recognize different proteins in purified $GABA_A$ receptors can only be explained by the assumption that these epitopes are not associated together in the same protein. This however is in apparent contradiction to the published amino acid sequence of the β_2-subunit [8] which indicates the existence of both epitopes together in the same molecule. A possible resolution of this discrepancy is indicated in Fig. 1. In the intact as well as in the *N*-deglycosylated $GABA_A$ receptor, the anti-peptide β_2 (191–197) antibody in addition to the strongly recognized protein bands seems to weakly identify the proteins recognized by the anti-peptide β_2 (382–389) antibody. Similarly, the anti-peptide β_2 (382–389) antibody weakly recognizes the proteins identified by the anti-peptide β_2 (191–197) antibody. It is thus possible that in a significant part of the β_2-subunits both the epitopes comprising the amino acids 191–197 and the amino acids 382–389 are coexisting. In addition, there have to exist, however, β_2-subunits in which either the amino acids 191–197 or the amino acids 382–389 are absent. Furthermore, there have to be β_2-subunits lacking other parts of the published amino acid sequence. Only then could the existence of several proteins strongly recognized by bd-17 or the anti-peptide β_2 (382–389) antibody be explained. Alternatively, these lower molecular weight proteins could be degradation products of a β_2-subunit which doesn't contain the amino acids 191–197. This, however, is not very probable since, as shown in Fig. 1, these proteins are weakly identified by the anti-peptide β_2 (191–197) antibody and as mentioned above, a series of protease inhibitors was used throughout the isolation and purification of the receptor and the *N*-deglycosylation experiments. And

finally since, for instance, the proteins identified by the anti-peptide β_2 (191–197) or the anti-peptide β_3 (190–197) antibodies did not show such a significant heterogeneity. All these results, however, could be explained by the possibility that the expression of several parts of the β_2-subunit, amongst them parts containing the amino acids 191–197 or 382–389, is regulated by alternative splicing. Recently, two different isoforms of γ_2- [14,15] or β_4-subunit [16] messenger RNAs have been identified which are produced by alternative splicing. The present results are in agreement with these reports and indicate that possibly a variety of β_2-subunit isoforms exist differing only in a small part of their molecules. The unequivocal identification of these $GABA_A$ receptor isoforms, the investigation of their regional and cellular distribution, the regulation of their expression and the investigation of their function is a big challenge for the future of $GABA_A$ receptor research.

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