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THE GLYCOPROTEIN NATURE OF A_1 ADENOSINE RECEPTORS

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SUMMARY. A_1 adenosine receptors from different tissues and species were photoaffinity labelled and then the carbohydrate content was examined by both enzymatic and chemical treatment. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the labelled membrane receptors shows that neuraminidase treatment alters the electrophoretic mobility of the receptor band indicating the presence of terminal neuraminic acids. Neuraminidase digestion does not influence the binding characteristics of the receptor. The totally deglycosylated receptor protein obtained by chemical treatment has an apparent molecular weight of 32,000.

INTRODUCTION. Membrane-bound adenosine receptors have been demonstrated in the central nervous system as well as in peripheral tissues (for review see ref. 1). On the basis of their coupling to adenylate cyclase two subtypes have been distinguished with the A_1 subtype mediating an inhibition and the A_2 subtype a stimulation of adenylate cyclase (2, 3). Recently we have developed a photoaffinity label for the A_1 adenosine receptor (4) which enables us to undertake further biochemical characterization of the receptor protein. For many membrane bound proteins including various receptors (5, 6, 7) it has been shown that they are glycoproteins. In the present study we demonstrate the glycoprotein nature of the A_1

ABBREVIATIONS. ^{125}I-AHPIA, R-2-azido-N^6-^{125}I-p-hydroxyphenylisopropyladenosine; R-PIA, R-N^6-phenylisopropyladenosine; TFMS, trifluoromethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS-PAGE, sodiumdodecyl sulfate-polyacrylamide gel electrophoresis.

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adenosine receptor by both enzymatic and chemical treatment of the photoaffinity labelled receptor.

MATERIAL AND METHODS

Materials. Trifluoromethanesulfonic acid, α-mannosidase (EC 3.2.1.24) and neuraminidase (EC 3.2.1.18) were purchased from Sigma. ^{125}I-AHPIA was synthesized as described (4). All other materials were from sources described previously (4, 8).

Preparation of membranes. Membranes from rat and hamster brain were prepared as described previously (9). Rat fat cells were isolated according to Rodbell (10), and hamster fat cells as described by Hittelman et al. (11) with minor modifications. Fat cell membranes were prepared from the respective fat cells by the method of McKeel and Jarett (12).

Binding assay. Brain membranes (0.4 mg/ml protein) were incubated with [^{3}H]PIA (1 nM) in 50 mM Tris/HCl pH 7,4 for 2 h at 25°C as described (8). Nonspecific binding was determined in the presence of 1 mM theophylline.

Photoaffinity labelling protocol. The respective membranes (0.4 mg/ml) were incubated with 70 pM ^{125}I-AHPIA (300 pM for labelling samples for chemical deglycosylation) as described (4). The incubation buffer for photoaffinity labelling contained 0.1% CHAPS to avoid attachment of the radioligand to the incubation vial. After 2 h at 25°C samples were centrifuged to remove unbound ligand. The membranes were resuspended in icecold incubation buffer and subsequently UV-irradiated for 3 min for photoincorporation of the bound ligand. Membranes were then centrifuged and the membrane pellet was prepared for enzymatic or chemical treatment or for SDS-PAGE.

Enzymatic treatment. Photoaffinity labelled membranes were resuspended in 100 mM sodium acetate buffer pH 5 containing neuraminidase (165 mU/ml, unless stated otherwise) to a protein concentration of 0.5 mg/ml. After 30 min incubation at 37°C the membranes were separated by centrifugation and prepared for SDS-PAGE. For digestion with α-mannosidase, labelled membranes were resuspended in 50 mM sodium citrate buffer pH 4.5 with 6 U/ml of the enzyme and incubated for 18 h at 25°C.

Chemical deglycosylation. Chemical deglycosylation was performed according to Herzberg et al. (13) with some modifications. Photoaffinity labelled membranes (300 µg of protein) were solubilized in 0.1 ml 1% SDS and lyophilized. 150 µl of a mixture of anisole and TFMS (1+2), cooled on ice, was added and the reaction vial (teflon) was flushed with N_2 and sealed. After shaking the reaction vial for 3 h at 0°C about 500 µl of ether (cooled in acetone/dry ice) and subsequently 300 µl pyridine/H_2O (1+1, icecold) were added. The ether phase was discarded and the aqueous phase was washed four times with approximately 1 ml of cooled ether. Then the aqueous phase was dialyzed against H_2O, lyophilized and prepared for SDS-PAGE.

Electrophoresis. SDS-PAGE was performed with 10% gels according to Laemmli (14) as described (4). Gels were usually stained with Coomassie Blue and then dried for autoradiography.

RESULTS

Photoaffinity labelling of A_1 adenosine receptors is demonstrated in different tissues and species in Fig. 1. The binding subunit of the A_1 receptor of rat brain membranes has an apparent molecular weight of 35,000. In all other tissues tested (Fig 1. B) including hamster, bovine and sheep brain (not shown) the same molecular weight has been found with the exception of the hamster fat cell receptor with 36,000.

To determine whether non-protein moieties are present in the receptor protein we constructed Ferguson plots (15) from SDS-PAGE runs in gels with different concentrations of acrylamide. As shown in Fig. 2 the receptor protein behaves differently from some molecular weight markers and a non-specifically labelled band (40,000) of photoaffinity labelled rat brain membranes. The anomalous behaviour is indicated by a different y-axis intercept and refers to an altered SDS-binding which can be caused by glycosylation of a protein. Therefore, treatment of glycoproteins with glycosidases alters their

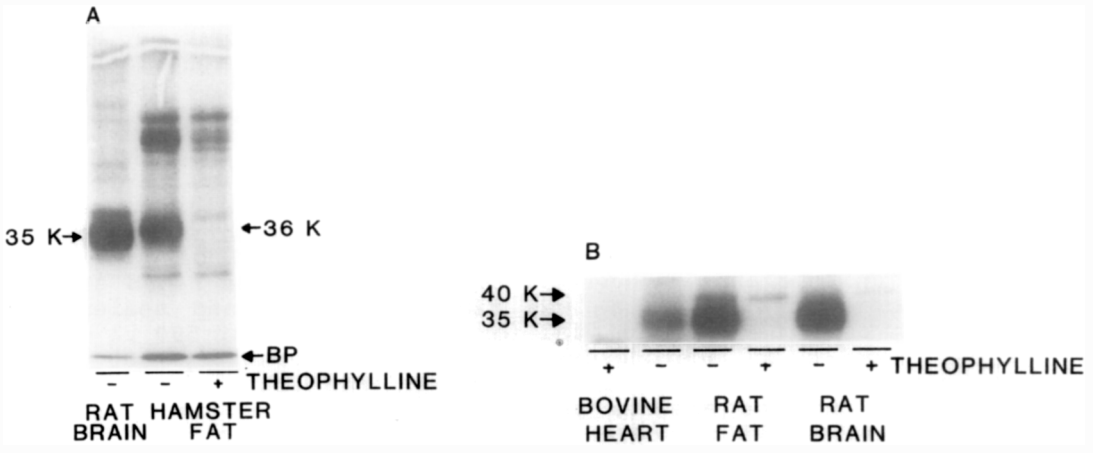

Figure 1. Photoaffinity labelling of A_1 adenosine receptors from different tissues. Shown are the autoradiograms of SDS-PAGE gels. A difference in the molecular weight between the hamster fat cell receptor and the rat brain receptor is demonstrated (A), while receptors from other tissues are not different (B).

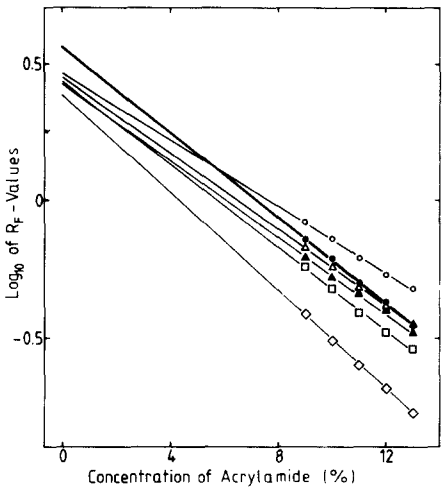

Figure 2. Ferguson plots of different molecular weight marker proteins and the A_1 adenosine receptor. SDS-PAGE was done in gels with different concentrations of acrylamide. The relative mobilities of several proteins were plotted versus the concentration of acrylamide of the respective gel. The different proteins are carbonic anhydrase (○), glyceraldehyde-3-phosphate dehydrogenase (△), ovalbumin (□), bovine serum albumin (◇), the non-specifically labelled 40K-band (▲) and the A_1 adenosine receptor (●). The receptor protein shows a different y-axis intercept indicating non-protein moieties.

electrophoretic mobilities. This effect is shown in Fig. 3 for treatment of rat brain and hamster fat cell membranes with neuraminidase after photoaffinity labelling. Neuraminidase treatment slightly enhances the electrophoretic mobility of the receptor bands corresponding to an apparent molecular

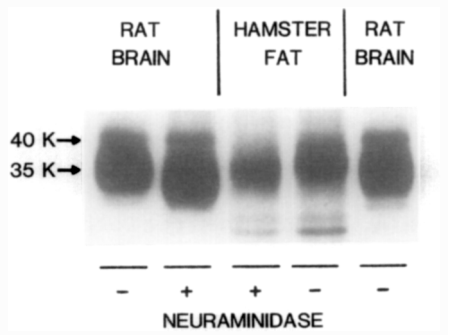

Figure 3. Treatment of A_1 adenosine receptors with neuraminidase. The autoradiogram shows the effect of neuraminidase digestion on the electrophoretic mobility of rat brain and hamster fat cell receptors.

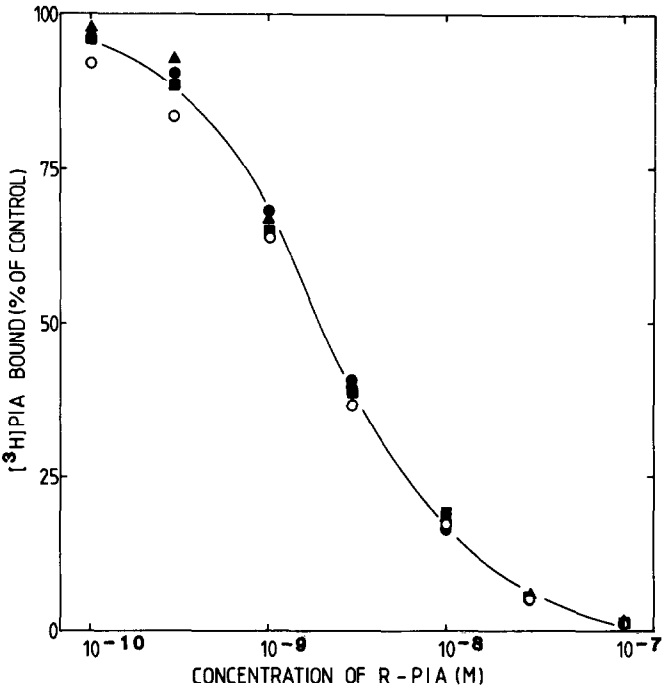

Figure 4. Binding of [^{3}H]PIA to neuraminidase treated rat brain membranes. The digestion was performed at pH 6 for 1 h at 37°C. The neuraminidase concentrations used were 0 mU/ml (○), 50 mU/ml (●), 150 mU/ml (■) and 300 mU/ml (▲). Subsequently competition of R-PIA for [^{3}H]PIA binding to treated and control membranes was measured. The K_i- and B_{max}-values were estimated by non-linear curve-fitting (8): 0.62 nM, 700 fmol/mg (○); 0.87 nM, 790 fmol/mg (●); 0.68 nM, 730 fmol/mg (■); 0.85 nM, 760 fmol/mg (▲).

weight decrease of about 1000. The difference in molecular weight between the hamster fat cell receptor and the other receptors was not abolished by this enzymatic treatment, indicating that it does not result from a different neuraminic acid content. α-Mannosidase had no effect on rat brain A_1 receptors, just as α- and ß-galactosidase and α- and ß-glucosidase (not shown).

Agonist binding to A_1 adenosine receptors does not seem to be dependent on the presence of neuraminic acids since the binding characteristics of [^{3}H]PIA to rat brain membranes were not changed by digestion of the membranes with increasing concentrations of neuraminidase (Fig. 4).

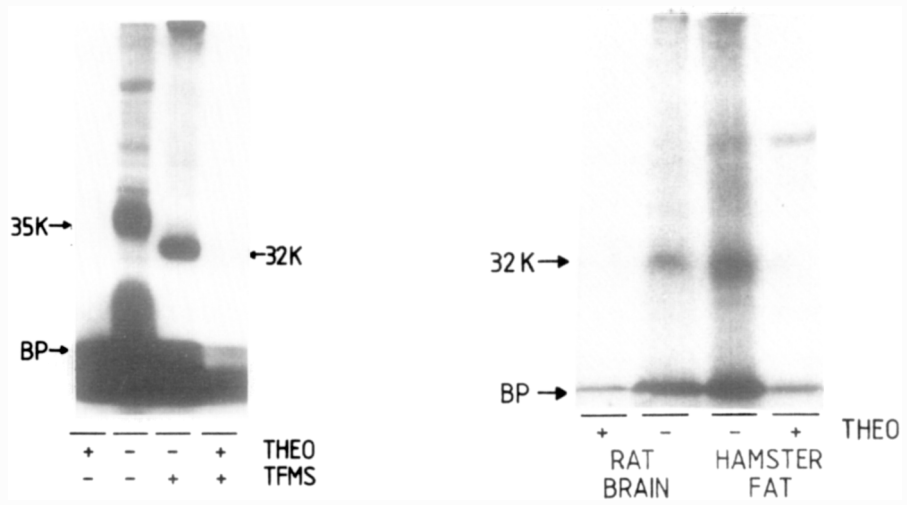

Figure 5. Chemical deglycosylation of A_1 adenosine receptors. The photoaffinity labelled receptor from TFMS treated rat brain membranes were compared with untreated control membranes (left). Totally deglycosylated receptors from hamster fat cells are compared with those from rat brain (right). The low molecular weight radioactivity in the left autoradiogram represents free ligand which was not washed out, because fixation of the gel before drying was omitted.

Adenosine receptors from rat brain and hamster fat cells were completely deglycosylated by treatment with anhydrous TFMS. The resulting core protein has an apparent molecular weight of 32,000 (Fig. 5). Comparison of the deglycosylated receptors from rat brain and hamster fat cells demonstrates that the differences between these two receptors are abolished, indicating that their different molecular weight is due to differences in their carbohydrate moieties.

DISCUSSION

The present study clearly demonstrates the glycoprotein nature of A_1 adenosine receptors. From the effect of neuraminidase on the photoaffinity labelled receptor it can be concluded that the carbohydrate moieties are occupied by terminal neuraminic acids. This and the insensitivity of the receptor to digestion with α-mannosidase indicates the presence of a complex type

rather than a high mannose carbohydrate chain. The terminal neuraminic acids do not seem to be involved in the ligand binding reaction as the binding characteristics are not influenced by neuraminidase treatment. Similar results have been obtained for the insulin receptor (16).

A different content of neuraminic acids cannot account for the difference in the apparent molecular weight between the hamster fat cell receptor and the receptors from other tissues as neuraminidase treatment of the respective membranes does not abolish this molecular weight difference. By chemical deglycosylation with TFMS an apparent molecular weight of 32,000 was estimated for the protein backbone for both the hamster fat cell and the rat brain receptor. This clearly demonstrates differences in the carbohydrate content other than in the terminal neuraminic acids. Such differences in the carbohydrate moiety have also been observed for the insulin receptor from different tissues (17).

Taken together, our data indicate the presence of carbohydrate moieties in the A_1 adenosine receptor from different tissues. A common core protein with the apparent molecular weight of 32,000 is differently glycosylated as demonstrated for the hamster fat cell and the rat brain receptor. The occurence of terminal neuraminic acids suggests a complex type carbohydrate chain, but the neuraminic acids not involved in the ligand binding reaction.

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