Analysis of the role of N-glycosylation in cell-surface expression and binding properties of angiotensin II type-2 receptor of rat pheochromocytoma cells

Guy SERVANT,* David T. DUDLEY,† Emanuel ESCHER* and Gaétan GUILLEMETTE*‡

*Department of Pharmacology, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Québec, J1H 5N4, Canada, and †Department of Signal Transduction, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor, Michigan 48105, U.S.A.

We previously demonstrated that the AT₂ receptor is a glycoprotein containing N-linked oligosaccharide side chains and that the marked disparity between the sizes of AT₂ receptors from different tissues was related to different degrees of N-glycosylation. In the present study, we used an inhibitor of N-glycosylation, tunicamycin, as well as an endoglycosidase, glycopeptidase-F, to examine the contribution of carbohydrate moieties to the ligand-binding properties, cell-surface expression and apparent molecular mass of AT₂ receptors of rat pheochromocytoma cells (PC-12 cells). Photoaffinity labelling of cell-surface AT₂ receptors revealed that PC-12 cells grown in the presence of tunicamycin expressed, in addition to the previously described 140 kDa receptor, lower-molecular-mass receptors of 63 kDa, 47 kDa and 32 kDa. Lectin affinity chromatography revealed that the 63 kDa and the 47 kDa receptors are partially glycosylated and that the 32 kDa receptor is completely deglycosylated. Competitive binding experiments were carried

out on tunicamycin-treated cells that expressed predominantly the 63 kDa or the 47 kDa receptors. Both receptor forms exhibited a high affinity for angiotensin II, although a slight decrease (of about 2-fold) was consistently observed on tunicamycin-treated cells as compared with control cells. Endoglycosidase digestion of AT₂ receptors of PC-12 cells also yielded smaller receptor forms of 47 kDa and 32 kDa. Similarly, angiotensin II showed a high but slightly decreased binding affinity (of about 2-fold) for deglycosylated membranes as compared with control membranes. In conclusion, the stepwise action of tunicamycin suggests the presence of at least three N-linked oligosaccharide side chains on the AT₂ receptor of PC-12 cells. These oligosaccharide side chains have a minor contribution to the affinity of the receptor. Interestingly, glycosylation is not an essential requirement for the expression of AT₂ receptor at the surface of PC-12 cells.

INTRODUCTION

Angiotensin II (AngII) is the active component of the renin-angiotensin system and produces a wide variety of physiological effects, including vascular contraction, aldosterone secretion, catecholamine release, glycogenolysis, and decreased renal filtration [1,2]. AngII has also recently been shown to promote myoproliferation in heart and vascular tissues [3]. The recent development of selective ligands permitted the identification of two AngII receptor subtypes, designated AT₁ and AT₂ [4]. The AT₁ receptor is preferentially recognized by DuP 753 (Losartan), whereas the AT₂ receptor is preferentially recognized by PD 123177 [5–7]. Losartan antagonizes all of the known physiological effects of AngII, indicating that these actions are related to the activation of AT₁ receptor [8].

AT₂ receptor is present in a wide variety of tissues, including rat adrenal medulla, rat brain, rat ovarian granulosa cells, rabbit and human uterus, mesenchymal tissues of rat fetus, and in a large number of cell lines including Swiss 3T3 cells and PC-12 cells [8]. Although most of the physiological actions of AngII are mediated by the AT₁ receptor, some recent data suggest a physiological role for the AT₂ receptor. Cultured neurons from neonatal rat brain contain almost exclusively AT₂ receptor that mediates a reduction in basal cyclic GMP level [9]. This reduction is blocked by PD 123177 or CGP 42112A but not by DuP 753. Furthermore, it was reported that particulate guanylate cyclase

activity of rat adrenal glomerulosa cells and PC12W cells was inhibited by AngII [10]. This effect, which could not be reproduced by two independent groups [11,12], was shown to depend on the activation of a phosphotyrosine phosphatase that was not blocked by DuP 753, suggesting that it was mediated through AT₂ receptors. Interestingly, a recent study showed that the AT₂ receptor of PC-12 cells inhibits protein tyrosine phosphatase [13]. In human astrocytes and mouse adipocytes, prostaglandin synthesis appears to be partly related to the activation of AT₂ receptors [14,15]. It was also recently shown that AngII stimulates, via the AT₂ receptor, the delayed rectifier K⁺ current in cultured neurons from neonatal rat brain [16]. The demonstration that many neonatal tissues, including brain, express high levels of AT₂ receptors has also led to the suggestion that these sites are somehow involved in development [17].

The potent photolabel ¹²⁵I-[Sar¹,Val⁵,p-benzoyl-Phe⁸]AngII (¹²⁵I-AngII-Bpa) was very useful for the biochemical characterization of AT₂ receptors from different tissues [18,19]. In a recent report, we showed that the AT₂ receptor, like several other membrane receptors, is a glycoprotein. We also demonstrated that the size variability of AT₂ receptors from different tissues was related to different degrees of N-glycosylation [19]. The rat, mouse and human AT₂ receptors were recently cloned. The sequences revealed that the AT₂ receptor belongs to the G-protein-coupled class of receptor with seven transmembrane domains [13,20–22]. The three sequences contain five conserved

Abbreviations used: AngII, angiotensin II; AngII-Bpa, [Sar¹,Val⁵,p-benzoyl-Phe⁸]angiotensin II; PNGase-F, glycopeptidase-F; WGA, wheat germ agglutinin; ConA, concanavalin A; Endo H, endoglycosidase H.

‡ To whom correspondence should be addressed.

asparagine residues in their N-termini which are potential sites for N-glycosylation (consensus sequence Asn-Xaa-Ser/Thr, where Xaa can be any amino acid except proline or aspartate [23]). The presence of such consensus sequences in the N-termini of most members of the G-protein-coupled receptor superfamily suggests that N-glycosylation may play important roles in receptor expression and/or functions. The purpose of the present study was to assess the role of N-glycosylation in the expression and the ligand-binding properties of the AT₂ receptor. The effect of an inhibitor of N-glycosylation, tunicamycin [24], and of an endoglycosidase, glycopeptidase-F (PNGase-F) [25], were evaluated on cultured PC-12 cells and on crude membrane preparations from these cells. Our results reveal that N-glycosylation has only a minor influence on the binding properties and is not essential for the targeting of AT₂ receptors to the cell surface.

EXPERIMENTAL

Cell culture

PC-12 cells (2×10^6 cells or 4×10^6 cells) were plated into 75 cm² culture dishes and grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin, 60 µg/ml streptomycin and 2 mM L-glutamine. Cells were kept in culture at 37 °C, in a CO₂ incubator, and the medium was changed daily or every other day depending on the state of confluency.

Tunicamycin treatment

Two different protocols were used. Tunicamycin (0.3 µg/ml) was added 24 h after plating and the cells were grown for 3 days. Alternatively, tunicamycin (1 µg/ml) was added 48 h after plating and the cells were grown for 2 days. Viability of tunicamycin-treated cells was determined by Trypan Blue exclusion and was always similar to that of control cells. However, tunicamycin significantly reduced the rate of cell growth. When similar cell densities were required at the end of a specific treatment, tunicamycin-treated cells were usually plated at twice the density of control cells. Our tunicamycin preparation contained A, B, C and D isomers [24] in proportions of 3%, 41%, 34% and 20% respectively.

Preparation of PC-12 cell membranes

Cell culture medium was removed and the cells were washed twice with 25 mM Tris/HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl₂. The cells were submitted to three cycles of freezing and thawing followed by homogenization with five strokes of a Dounce homogenizer (tight pestle) in the same medium. The homogenate was centrifuged at 34000 g for 20 min and the resulting pellet was resuspended in the same medium at a final concentration of 1–2 mg of protein/ml, and stored at –70 °C. All procedures were done at 0 °C. The protein content was determined by the method of Lowry et al. [26].

AngII binding assay with PC12 cell membranes

PC12 cell membranes (5–10 µg of protein) were incubated in medium containing 25 mM Tris/HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 2 mg/ml BSA, 0.1 mg/ml bacitracin and 50 µg/ml soybean trypsin inhibitor. ¹²⁵I-AngII and selected concentrations of AngII were added in a final incubation volume of 500 µl. Incubations were performed at 37 °C for 180 min. Non-specific binding was determined in the presence of 1 µM AngII. Incubations were terminated by vacuum filtration through

presoaked glass-fibre filters (Whatman GF/C) and three rapid washes with 2 ml of cold medium. The receptor-bound radioactivity was analysed by gamma counting.

AngII binding assay with intact PC-12 cells

PC-12 cells were washed twice and gently scraped with a rubber policeman. The cells were then submitted to five strokes of a Dounce homogenizer (loose pestle) to disperse the majority of cell clusters. After this treatment, the cell viability determined by Trypan Blue exclusion was greater than 80%. The cells were diluted to a final concentration of 500–1000 cells/µl in a medium containing 25 mM Tris/HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 2 mg/ml BSA, 0.1 mg/ml bacitracin and 50 µg/ml soybean trypsin inhibitor. The binding assay was performed as already described.

Photoaffinity labelling

PC-12 cell membranes or PC-12 cells were incubated with 5 nM ¹²⁵I-AngII-Bpa in 1 ml of medium containing 25 mM Tris/HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 2 mg/ml BSA, 0.1 mg/ml bacitracin and 50 µg/ml soybean trypsin inhibitor. After 45 min at room temperature, the samples were washed by centrifugation (cells, 200 g; membranes, 13000 g) and irradiated for 60 min at 0 °C under filtered UV light (365 nm) [the mercury vapour lamp (JC-Par-38) were purchased from Westinghouse and the Raymaster black light filters (no 5873) were purchased from Gates and Co., Inc. (Long Island, NY, U.S.A.)]. Irradiated membranes were incubated for 60 min at 37 °C, in denaturing buffer containing 60 mM Tris/HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 125 mM dithiothreitol and 3% (w/v) Bromophenol Blue. The material was then divided into aliquots and stored at –20 °C until required for further use. For irradiated cells, cell membranes were prepared prior to denaturation.

Endoglycosidase digestion of solubilized material

For PNGase-F digestion, photoaffinity labelled membranes (2 mg of protein) were solubilized for 1 h at 37 °C in a medium containing 100 mM sodium phosphate, pH 7.2, 25 mM EDTA, 5 mM NaN₃, 1% (w/v) SDS, 0.2 mg/ml bacitracin, 0.2 mg/ml soybean trypsin inhibitor and 2 mM PMSF. Aliquots (10 µl) of the solubilized material (corresponding to 130 µg of protein) were added to a deglycosylation buffer containing 100 mM sodium phosphate, pH 7.4, 25 mM EDTA, 5 mM NaN₃, 1% (w/v) Nonidet P-40, 0.1 mg/ml bacitracin, 0.1 mg/ml soybean trypsin inhibitor and 1 mM PMSF. Different amounts of PNGase-F were added to a final volume of 50 µl. Incubations were performed at 37 °C for 3 to 6 h. For endoglycosidase H (Endo H) digestion, photoaffinity labelled membranes (1 mg of protein) were solubilized for 1 h at 37 °C in a medium containing 50 mM sodium citrate, pH 5.5, 100 mM NaCl, 1 mM EDTA, 1% (w/v) SDS, 0.2 mg/ml bacitracin, 0.2 mg/ml soybean trypsin inhibitor and 2 mM PMSF. Aliquots (8 µl) of the solubilized material (corresponding to 100 µg of protein) were added to a deglycosylation buffer containing 50 mM sodium citrate, pH 5.5, 100 mM NaCl, 1 mM EDTA, 0.1 mg/ml bacitracin, 0.1 mg/ml soybean trypsin inhibitor and 1 mM PMSF. Endo H (50 m-units) was added to a final volume of 100 µl. Incubations were performed at 37 °C for 3 to 20 h. Endoglycosidase digestions were terminated by addition of an equal volume of double-strength denaturing buffer (previously described) and incubation at 37 °C for an additional hour. The resulting material was immediately analysed by SDS/PAGE or was stored at –20 °C.

Endoglycosidase digestion of membranes

PC-12 membranes (700 μ g of protein) were resuspended in a medium containing 100 mM sodium phosphate, pH 7.2, 25 mM EDTA, 5 mM Na₂S₂O₃, 0.1 mg/ml bacitracin, 0.1 mg/ml soybean trypsin inhibitor, 1 mM PMSF and 20 units of PNGase-F, in a final incubation volume of 100 μ l. Control membranes were resuspended in the same buffer in the absence of PNGase-F. Membranes were incubated for 20–24 h at 37 °C. At the end of incubation, membranes were centrifuged and resuspended in the binding buffer. Aliquots were removed for photoaffinity labelling and the remaining membranes were used immediately in the binding assay.

SDS/PAGE

Electrophoresis was carried out as described by Laemmli [27]. Denatured proteins (25–50 μ g) were subjected to electrophoresis on 9% or 12% polyacrylamide gels and were run at 95 V for approx. 2–3 h. Gels were stained with 0.05% (w/v) Coomassie Brilliant Blue and dried before autoradiography on Kodak X-Omat AR films. Exposures lasted for 1–4 weeks.

Lectin affinity chromatography

Lectin affinity chromatography was performed according to the protocol of Boege et al. [28] with slight modifications. Wheat germ agglutinin (WGA)–Sephacrose and concanavalin A (ConA)–Sephacrose (700 μ l–1000 μ l wet bed volume) were equilibrated with 12 ml of 25 mM Tris/HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 2 mM CaCl₂, 2 mM MnCl₂ and 0.1% Triton X-100. Photoaffinity labelled membranes were solubilized in the same medium containing 1% Triton X-100, 0.1 mg/ml bacitracin and 0.1 mg/ml soybean trypsin inhibitor, for 1 h at 0 °C. Detergent extracts were diluted to a final concentration of 0.1% Triton X-100 and 1–2 mg of solubilized protein were incubated with the lectin at 4 °C for 16–20 h under gentle agitation. The lectin was then transferred into a Poly-Prep chromatography column (Bio-Rad) and the pass-through fraction was collected and kept on ice. The lectin were washed at room temperature with 4 to 8 ml of the same buffer until all unbound radioactivity was washed out. Specific elution was initiated with 20 mM sodium borate, pH 8.0, 1 M NaCl, containing 0.5 M *N*-acetyl-D-glucosamine for WGA and 0.5 M α -methyl D-mannopyranoside for ConA. Eluted proteins were precipitated with 7.2% (w/v) trichloroacetic acid and centrifuged at 13000 *g* for 10 min at 4 °C. The proteins were dissolved in denaturing buffer and subjected to SDS/PAGE and autoradiography.

Materials

BSA, bacitracin, soybean trypsin inhibitor, WGA–Sephacrose 6MB, ConA–Sephacrose 4B, *N*-acetyl-D-glucosamine, α -methyl D-mannopyranoside and tunicamycin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Tunicamycin (1 mg/ml) was dissolved in 0.1 M NaOH, sterile-filtered and stored at 4 °C. PNGase-F (EC 3.5.1.52) and Endo H (EC 3.2.1.96) (lyophilized) were from Boehringer Mannheim. AngII-Bpa was synthesized in our laboratories. ¹²⁵I-AngII and ¹²⁵I-AngII-Bpa (specific radioactivity ~ 1000 Ci/mmol) were prepared with Iodogen as described by Fraker and Speck [29]. Briefly, 50 μ l of the peptide solution (0.2 mM) was incubated with 5 μ g of Iodogen (Pierce Chemical), 1 mM Na¹²⁵I (2200 Ci/mmol), 10 μ l of acetic acid (2 M) and 30 μ l of water for 30 min at room temperature. The labelled hormones were purified by HPLC on a C-18 column

(Allteck Associates Inc.; no. 29004) with a 20–40% acetonitrile gradient. The specific radioactivity of the labelled hormones was determined by self-displacement and saturation binding analysis.

RESULTS

Treatment with tunicamycin

PC-12 cells were grown for different periods of time in the presence of tunicamycin, an antibiotic which blocks the formation of the first lipid intermediate (*N*-acetylglucosamine-phosphoryldolichol biphosphate) of the dolichol pathway of protein glycosylation [24]. Figure 1(A), lane 1, shows that the AT₂ receptor of untreated cells migrated as a single broad band with an apparent molecular mass of 140 kDa, as previously described [19]. After 24 h of treatment with 0.3 μ g/ml tunicamycin, three additional thinner bands with apparent molecular masses of 63 kDa, 47 kDa and 32 kDa appeared in addition to the original 140 kDa receptor band (Figure 1A, lane 3). Similar results were obtained with PC-12 cells adherent to culture dishes, eliminating the possibility of labelling intracellular receptors (Figure 1C). After 48 h of treatment, a major proportion of the 140 kDa receptor form had disappeared and was apparently replaced by the 63 kDa and 47 kDa receptor forms and to a lesser extent, the 32 kDa receptor form (Figure 1A, lane 4). Densitometric analysis of the bands revealed that, under these conditions, the 63 kDa receptor form was predominant at the surface of PC-12 cells. This receptor form represented 52% of the cell-surface labelling whereas the 47 kDa and the 32 kDa receptor forms represented 30% and 2.4% of the cell-surface labelling respectively. The 140 kDa receptor form represented 16% of the cell-surface labelling under these conditions. After a 72 h exposure to tunicamycin, the proportion of the 140 kDa receptor form decreased to almost undetectable levels, whereas the proportions of the other receptor forms slightly increased (Figure 1A, lane 5). Figure 1(A), lane 2, shows that the specific labelling of the native AT₂ receptor of PC-12 cells was completely

Figure 1 Effect of tunicamycin on the cell-surface expression of AT₂ receptors

(A) PC-12 cells were grown in the presence of 0.3 μ g/ml tunicamycin for 0 h (untreated cells; lanes 1 and 2), 24 h (lane 3), 48 h (lane 4), and 72 h (lane 5). Cells were then gently dispersed and photoaffinity labelled with ¹²⁵I-AngII-Bpa (5 nM) as described in the Experimental section. Non-specific labelling was evaluated in the presence of 1 μ M AngII (lane 2). Labelled proteins were separated on a 12% separating gel, followed by autoradiography. Protein standards of the indicated molecular masses (kDa) were run in parallel. These results are representative of three separate experiments. (B) PC-12 cells were treated with 1 μ g/ml tunicamycin for 0 h (untreated cells; lane 1), 4.5 h (lane 2), 8 h (lane 3), 21 h (lane 4), and 32 h (lane 5). Labelled proteins were then analysed as described in (A). These results are representative of three separate experiments. (C) PC-12 cells were treated with 0.3 μ g/ml tunicamycin for 24 h, after which adherent intact cells were photolabelled with ¹²⁵I-AngII-Bpa (5 nM). Photoaffinity labelling was carried out in the absence (lane 1) or in the presence (lane 2) of 1 μ M AngII. Labelled proteins were then analysed as described in (A). These results are representative of three separate experiments.

Figure 2 WGA-Sepharose affinity chromatography of AT₂ receptor forms

PC-12 cells were grown in the presence of tunicamycin (1 μ g/ml) for 27.5 h. Cell membranes were then prepared and AT₂ receptors were photolabelled with ¹²⁵I-AngII-Bpa, solubilized with 1% Triton X-100 and adsorbed on WGA-Sepharose before elution with 0.5 M *N*-acetyl-D-glucosamine as described in the Experimental section. The eluates were then precipitated with trichloroacetic acid and subjected to SDS/PAGE and autoradiography. Lane 1 corresponds to proteins that did not adhere to the WGA-Sepharose (pass-through). Lane 2 corresponds to material recovered after a wash with 8 ml of incubation buffer and lane 3 corresponds to the proteins eluted with *N*-acetyl-D-glucosamine. Arrowheads indicate the molecular masses (kDa) of the different AT₂ receptor forms. Identical results were obtained in three independent experiments.

abolished in the presence of a high concentration of AngII (1 μ M). Figure 1(C), lane 2, shows that the specific labelling of the AT₂ receptor in 24 h-tunicamycin-treated cells was also abolished in the presence of 1 μ M AngII.

When PC-12 cells were grown in the presence of a higher dose of tunicamycin (1 μ g/ml), the rate of appearance of the AT₂ receptor forms and their relative proportions at the cell surface were modified. Figure 1(B), lane 2, shows that after 4.5 h of treatment under these conditions, PC-12 cells expressed only the mature 140 kDa receptor form, which was also expressed by untreated cells (Figure 1B, lane 1). After 8 h of treatment with 1 μ g/ml tunicamycin, the previously described 63 kDa, 47 kDa and 32 kDa receptor forms were already apparent in addition to the 140 kDa receptor form (Figure 1B, lane 3). When the treatment was prolonged to 21 h, approximately half of the 140 kDa receptor form was replaced by the 63 kDa, 47 kDa and 32 kDa receptor forms (Figure 1B, lane 4). These receptor forms represented respectively 5%, 31% and 3% of the total labelling as revealed by densitometric analysis. When the treatment was prolonged to 32 h, the proportion of the 140 kDa receptor form decreased to 31% with a concomitant increase of the 63 kDa, 47 kDa and 32 kDa receptor forms to 15%, 45% and 9% respectively. These results demonstrate that PC-12 cells, grown in the presence of tunicamycin, express at their surface AT₂ receptors of different apparent molecular masses. Furthermore, the relative proportions of these different receptor forms vary depending on the dose of tunicamycin used.

The glycoprotein nature of the different AT₂ receptor forms was verified by WGA-Sepharose affinity chromatography. Figure 2 (lane 3) shows that the 140 kDa and the 47 kDa receptor forms were retained by the lectin and recovered on specific elution with *N*-acetyl-D-glucosamine, confirming their glyco-

Figure 3 Endoglycosidase digestions of photoaffinity labelled AT₂ receptor

(A) Photoaffinity labelled membranes were solubilized and incubated with PNGase-F for 6 h at 37 °C, under conditions described in the Experimental section. Aliquots were then denatured and subjected to gel electrophoresis on a 12% separating gel, followed by autoradiography. Lane 1, control; lane 2, PNGase-F 1 unit/ml; lane 3, PNGase-F 3.33 units/ml; lane 4, PNGase-F 10 units/ml; lane 5, PNGase-F 33 units/ml; lane 6, PNGase-F 100 units/ml. (B) Photoaffinity labelled membranes were solubilized and incubated at 37 °C, in the presence (+) or in the absence (–) of Endo H (50 m-units) for different periods of time: lanes 1 and 2 (3 h); lanes 3 and 4 (6 h); lanes 5 and 6 (10 h); lanes 7 and 8 (20 h). Aliquots were then denatured and subjected to gel electrophoresis on a 10% separating gel, followed by autoradiography. Protein standards of the indicated molecular masses (kDa) were run in parallel. These experiments are representative of two similar observations.

protein nature. The 32 kDa receptor form did not bind to the lectin and appeared exclusively in the pass-through fraction, suggesting that this receptor form is totally deglycosylated (Figure 2, lane 1). Trace amounts of the 47 kDa receptor form were also discernible in the pass-through fraction, reflecting a weaker interaction with the lectin, probably due to its lower saccharide content. The 63 kDa receptor form, produced in large amounts after 72 h of treatment with 0.3 μ g/ml tunicamycin, was also retained by the lectin, confirming the glycoprotein nature of this receptor form (results not shown). These results suggest that the 32 kDa receptor form corresponds to the completely deglycosylated protein and that the larger receptor forms of apparent molecular masses of 47 kDa and 63 kDa are partially glycosylated forms of the mature 140 kDa AT₂ receptor. Additional lectin-affinity chromatography experiments showed that native AT₂ receptor does not adhere to ConA-Sepharose, indicating that this receptor does not contain a high-mannose type of carbohydrate chain. This conclusion is supported by the results shown in Figure 3(B), indicating that AT₂ receptor is

Figure 4 Photoaffinity labelling of cell-surface AT₂ receptor after treatment with 1 µg/ml tunicamycin

Untreated PC-12 cells (lane 2) and 48 h-tunicamycin-treated cells (lane 1) were photolabelled with ¹²⁵I-AngII-Bpa as described in the legend of Figure 1. Cell membranes were prepared and the material was solubilized in denaturing buffer and subjected to electrophoresis on a 12% separating gel. Protein standards of the indicated molecular masses (kDa) were run in parallel. Similar results were obtained in four separate experiments.

insensitive to Endo H treatment, under conditions where it is sensitive to PNGase-F treatment (Figure 3A).

Binding studies on tunicamycin-treated cells and deglycosylated membranes

PC-12 cells were first treated with 1 µg/ml tunicamycin, a condition in which the 47 kDa receptor form was predominant at their surface. Figure 4 (lane 1) shows that the 47 kDa receptor form represented 46% of the total cell-surface labelling. The 140 kDa, 63 kDa and 30 kDa receptor forms represented respectively 30%, 17% and 6% of the total cell-surface labelling, as previously described (Figure 1B). Untreated cells expressed only the mature 140 kDa receptor form (Figure 4, lane 2). Competitive binding experiments were carried out with ¹²⁵I-AngII and increasing concentrations of AngII. Figure 5(A) shows that ¹²⁵I-AngII binding was slightly reduced on treated cells as compared with untreated cells, with binding values of 2300 c.p.m. and 3800 c.p.m. respectively. Increasing concentrations of unlabelled AngII effectively inhibited ¹²⁵I-AngII binding to both treated and untreated cells to a similar non-specific binding value of about 500 c.p.m. In Figure 5(B), the same binding data were transformed into values relative to the total binding observed in the absence of unlabelled AngII. The half-maximal inhibitory concentrations (IC₅₀) were 0.65 nM for treated cells and 0.27 nM for untreated cells. Scatchard analysis of the same binding data (shown in Figure 5C) yielded K_d values of 0.54 nM and 0.21 nM for the AT₂ receptor of

Figure 5 Binding properties of AT₂ receptor after treatment with 1 µg/ml tunicamycin

(A) PC-12 cells were grown in the presence of 1 µg/ml tunicamycin for 48 h. At the end of the treatment, tunicamycin-treated cells (○) and control cells (●) were incubated at 37 °C in medium containing ¹²⁵I-AngII (58 966 c.p.m.; 0.07 nM) and increasing concentrations of unlabelled AngII. After 3 h, the incubation was stopped by filtration. Each binding value corresponds to the mean ± S.D. of a triplicate determination. This experiment is representative of four similar observations. (B) The binding data were transformed into values relative to the total binding observed in the absence of unlabelled AngII and were corrected for non-specific binding. (C) Scatchard analysis of the same binding data.

treated and untreated cells respectively. Both curves converged towards a similar maximal binding capacity (B_{max}) of ~ 60000 receptors/cell. These results suggest that the discrepancy in ¹²⁵I-AngII binding observed between tunicamycin-treated cells and untreated cells is due to a mild 2-fold decrease in binding affinity. Similarly, competitive binding experiments were carried out on PC-12 cells that expressed predominantly the 63 kDa receptor form. Figure 6 (lane 2) shows that after a treatment of 72 h with 0.3 µg/ml tunicamycin, the 63 kDa receptor form accounted for 67% of the cell-surface labelling. The 47 kDa and 32 kDa receptor forms accounted for the remaining 33% of the cell-surface labelling. Untreated cells expressed only the mature 140 kDa receptor (Figure 6, lane 1). Figure 7 shows that in

Figure 6 Photoaffinity labelling of cell-surface AT_2 receptors after treatment with 0.3 μ g/ml tunicamycin

Untreated cells (lane 1) and 72 h-tunicamycin-treated cells (lane 2) were photolabelled with ^{125}I -AngII-Bpa as described in the legend of Figure 1. Cell membranes were prepared and the material was solubilized in denaturing buffer and subjected to electrophoresis on a 9% separating gel. Protein standards of the indicated molecular masses (kDa) were run in parallel. Similar results were obtained in at least three separate experiments.

Figure 7 Binding properties of AT_2 receptor after treatment with 0.3 μ g/ml tunicamycin

PC-12 cells were grown in the presence of 0.3 g/ml tunicamycin for 72 h. At the end of the treatment, tunicamycin-treated cells ($\circ$) and untreated control cells ($\bullet$) were incubated at 37 $^{\circ}C$ in medium containing ^{125}I -AngII (51 754 c.p.m.; 0.06 nM) and increasing concentrations of unlabelled AngII. After 3 h, the incubation was stopped by filtration. Data are expressed as values relative to the total binding observed in the absence of unlabelled AngII and were corrected for non-specific binding. Each binding value corresponds to the mean $\pm$ S.D. of a triplicate determination. This experiment is representative of three similar observations.

competitive binding experiments, AngII binding to both treated and untreated cells was of high affinity. However, the AT_2 receptor of treated cells exhibited a slightly weaker affinity for AngII (IC_{50} of 0.37 nM) than that of untreated cells (IC_{50} of 0.23 nM).

Figure 8 Enzymic deglycosylation of the AT_2 receptor

PC-12 cell membranes (700 μ g) were incubated with (lane 2) or without (lane 1) PNGase-F (200 units/ml) for 24 h at 37 $^{\circ}C$ in a medium containing 100 mM sodium phosphate, pH 7.2, 25 mM EDTA, 5 mM NaN_3 , 0.1 mg/ml bacitracin, 0.1 mg/ml soybean trypsin inhibitor and 1 mM PMSF. Aliquots corresponding to approx. 100 μ g–200 μ g of membrane protein, were photolabelled with ^{125}I -AngII-Bpa and subjected to gel electrophoresis on a 9% separating gel, followed by autoradiography. Protein standards of the indicated molecular masses (kDa) were run in parallel. This experiment is representative of at least three similar observations.

We also verified the effect of endoglycosidase on the AT_2 receptor binding affinity. PC-12 cell membranes were submitted to PNGase-F digestion. Aliquots were photolabelled with ^{125}I -AngII-Bpa and subjected to SDS/PAGE, while the remaining membranes were immediately used in the binding assay. Figure 8 (lane 2) clearly shows that after a 24 h treatment with PNGase-F, PC-12 cell membranes contained almost exclusively the 47 kDa receptor form, which accounted for 73 % of the labelling. Trace amounts of the 32 kDa form were also found. Control membranes contained only the mature 140 kDa AT_2 receptor (Figure 8, lane 1). Figure 9(A) shows that ^{125}I -AngII binding was higher on control membranes than on PNGase-F-treated membranes, with binding values of 4500 c.p.m. and 2700 c.p.m. respectively. Increasing concentrations of AngII effectively inhibited ^{125}I -AngII binding to a similar non-specific binding value of about 700 c.p.m. Figure 9(B) shows that AngII binding was of high affinity on both treated and control membranes. However, deglycosylated membranes displayed a weaker affinity (IC_{50} of 0.9 nM) for AngII than control membranes (IC_{50} of 0.45 nM). Scatchard analysis of the binding data confirmed that the loss of ^{125}I -AngII binding on treated membranes was essentially due to a 2-fold decrease of binding affinity (Figure 9C). Scatchard plots yielded K_d values of 0.45 nM and 0.9 nM for control and treated membranes respectively. The two curves converged towards a similar B_{max} value around 1 pmol/mg of protein.

DISCUSSION

Membrane-bound receptors for hormones and growth factors are generally glycosylated. Of particular interest is the conservation of consensus sequences for N-glycosylation among

Figure 9 Binding properties of deglycosylated AT₂ receptors

(A) PC-12 cell membranes were deglycosylated as described in the legend of Figure 7. Control membranes (●) and deglycosylated membranes (○) (8 μg of protein) were incubated at 37 °C in medium containing [¹²⁵I]-AngII (133 784 c.p.m.; 0.16 nM) and increasing concentrations of unlabelled AngII. After 3 h, the incubation was stopped by filtration. Each binding value represents the mean ± S.D. of a triplicate determination. This experiment is representative of at least three similar observations. (B) The binding data were transformed into values relative to the total binding observed in the absence of unlabelled AngII and were corrected for non-specific binding. (C) Scatchard analysis of the same binding data.

many of the G-protein-coupled receptors, in their extracellular N-termini, suggesting that the asparagine-linked carbohydrates may play a general role in receptor expression and function. The role of N-glycosylation has been studied mainly by using inhibitors of N-linked glycosylation and by using specific enzymes that can remove all or most of the protein-linked carbohydrates, and more recently by using site-directed mutagenesis of the consensus sequences for N-glycosylation. In the present study, an inhibitor of N-glycosylation, tunicamycin [24], as well as an endoglycosidase, PNGase-F [25], were utilized to verify the role of N-glycosylation on cell-surface expression and ligand-binding properties of the AT₂ receptor of PC-12 cells.

The AT₂ receptor is a glycoprotein of 68 kDa to 140 kDa containing N-linked oligosaccharide chains [19]. The glyco-

protein nature of the AT₂ receptor was previously confirmed by its sensitivity to N-endoglycosidase treatment and its specific adsorption to immobilized lectins [19,30]. In the present study, the effect of tunicamycin further confirmed the glycoprotein nature of the AT₂ receptor of PC-12 cells. Photoaffinity labelling of cell-surface AT₂ receptor revealed that in addition to the mature 140 kDa receptor, PC-12 cells grown in the presence of tunicamycin expressed lower-molecular-mass receptor forms of 63 kDa, 47 kDa and 32 kDa. Lectin affinity chromatography revealed that the 63 kDa and the 47 kDa receptor forms correspond to partially glycosylated AT₂ receptors. The 32 kDa receptor form corresponds to the completely deglycosylated AT₂ receptor.

Since tunicamycin treatments produced two different partially glycosylated forms of the AT₂ receptor in addition to the completely deglycosylated 32 kDa receptor, it is suggested that at least three of the five putative N-glycosylation sequences of the AT₂ receptor [13,20] are effectively occupied by oligosaccharide chains in PC-12 cells. We recently reported that the disparity between the sizes of AT₂ receptors from different tissues, including human myometrium and PC-12 cells, is related to different degrees of N-glycosylation [19]. Interestingly, the photo-labelled AT₂ receptor of human myometrium, which is approximately half the size of the AT₂ receptor of PC-12 cells on denaturing polyacrylamide gels (68 kDa) [18,19], was also shown to contain at least three N-linked oligosaccharide chains [19]. Taken together, these results suggest that the variation of apparent AT₂ receptor size may be due either to various degrees of occupation of putative glycosylation sites or to occupation of these sites by different carbohydrate chains.

The AT₂ receptor is a 363-amino-acid protein with a deduced molecular mass of 41 kDa [13,20]. This value is slightly larger than that observed after complete deglycosylation of the AT₂ receptor by endoglycosidase, where a value of 31 kDa to 33 kDa was estimated ([19,30]; this study). A simple explanation for this discrepancy would be proteolytic cleavage of the AT₂ receptor occurring during the deglycosylation experiments. However, the consistent observation of a lower-molecular-mass protein after either *in vitro* or *in vivo* deglycosylation of the AT₂ receptor argues against this possibility. Rather, we believe that this discrepancy is merely due to a limitation of the electrophoresis technique, which does not always provide the actual molecular masses of proteins. A similar phenomenon was observed in the case of AT₁ receptor when Carson et al. [31] revealed a molecular mass of 34 kDa for the deglycosylated AT₁ receptor of bovine adrenal cortex, whereas Sasaki et al. [32] revealed a molecular mass of 41 kDa after cloning and sequencing the protein. Our results show that PC-12 cells grown in the presence of tunicamycin express different AT₂ receptor forms that are targeted to the plasma membrane.

Interestingly, the use of different doses of tunicamycin significantly changed the rate of appearance of the different AT₂ receptor forms and their relative proportions at the surface of PC-12 cells. The lower dose produced predominantly the 63 kDa receptor form, whereas the higher dose produced predominantly the 47 kDa receptor form. It may reflect a different susceptibility of the putative N-glycosylation sites, to the size of lipid-linked oligosaccharide pools. The action of tunicamycin is to diminish the pool of lipid-linked oligosaccharides that are transferred to the nascent protein by a glycosyltransferase [24]. Herein, it has been proposed that glycosyltransferase *K_m* values may be site-dependent on the nascent protein [33].

N-glycosylation is required for transport and function of some secreted and integral membrane proteins but not for others and this requirement cannot yet be predicted for any given protein.

Partial glycosylation is required for processing and/or hormone-binding activity of insulin receptor [34,35], epidermal-growth-factor receptor [36,37], nicotinic acetylcholine receptor [38], and members of the G-protein-coupled class of receptors, including the vasoactive intestinal peptide receptor [39], somatostatin receptor [40] and β -adrenergic receptor [41,42]. The main purpose of the present study was to verify the role of N-glycosylation in the cell-surface expression and the ligand-binding properties of the AT₂ receptor. Our results clearly showed that partially and totally deglycosylated AT₂ receptors are effectively targeted to the plasma membrane of PC-12 cells. Further studies are needed to assess the role of N-glycosylation in the rates of synthesis, translocation and the stability of the AT₂ receptor. Our results also showed that high binding affinity is not lost after *in vivo* deglycosylation of the AT₂ receptor to the 63 kDa and the 47 kDa receptor forms. High binding affinity was also maintained after enzymic deglycosylation of the AT₂ receptor to the 47 kDa and 32 kDa receptor forms. Similarly, other groups also showed that N-glycosylation was not a requirement for cell-surface expression and high binding affinity of some G-protein-coupled receptors, including the AT₁ receptor [43] and the m2 muscarinic receptor [44].

Although high binding affinity was maintained after deglycosylation *in vivo* or *in vitro*, a slight (2-fold) decrease of binding affinity was consistently observed on deglycosylated AT₂ receptor populations. This decrease in binding affinity could result from a deficient coupling of the deglycosylated AT₂ receptor with its G-protein or with a putative effector. However, the direct coupling of the AT₂ receptor with a G-protein or with an intracellular effector pathway is still a subject of controversy. A simpler explanation would be that carbohydrates play a modest role in the binding conformation of the AT₂ receptor. It is likely that the functional significance of this minor effect may not be important. Similarly, partial deglycosylation of the vasoactive intestinal peptide receptor resulted in a 3-fold reduction of the agonist binding affinity. It was suggested that deglycosylation-induced alteration of the binding affinity occurred at the receptor level, since the deglycosylated receptor exhibited a normal capability to interact with the stimulatory G-protein G_s [39].

In conclusion, we have demonstrated that N-glycosylation of the AT₂ receptor is not a requisite for its cell-surface expression or its ligand-binding properties. Further studies are needed to determine the significance of N-glycosylation on the AT₂ receptor. Some interesting hypotheses are that it could contribute to the stability of the receptor, to its coupling to intracellular effector pathways or to its intracellular transit time.

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REFERENCES

- Peach, M. J. (1981) *Biochem. Pharmacol.* **80**, 2745–2751
- Vallotton, M. B. (1987) *Trends Pharmacol. Sci.* **8**, 69–74
- Powell, J. S., Müller, R. K. M., Rouge, M., Kuhn, H., Hefti, F. and Baumgartner, H. R. (1990) *J. Cardiovasc. Pharmacol.* **16** (Suppl.4), S42–S49
- Bumpus, F. M., Catt, K. J., Chiu, A. T., de Gasparo, M., Goodfriend, T., Husain, A., Peach, M. J., Taylor, D. G. and Timmermans, P. B. M. W. M. (1991) *Hypertension* (Dallas) **17**, 720–723
- Chang, R. S. L. and Lotti, V. J. (1989) *Pharmacologist* **31**, 150
- Whitebread, S., Mele, M., Kamber, B. and de Gasparo, M. (1989) *Biochem. Biophys. Res. Commun.* **163**, 284–291
- Chiu, A. T., Herblin, W. F., McCall, D. E., Ardecky, R. J., Carini, D. J., Duncia, J. V., Pease, L. J., Wong, P. C., Wexler, R. R., Johnson, A. L. and Timmermans, P. B. M. W. M. (1989) *Biochem. Biophys. Res. Commun.* **165**, 196–203
- Timmermans, P. B. M. W. M., Wong, P. C., Chiu, A. T., Herblin, W. F., Renfield, P., Carini, D. J., Lee, R. J., Wexler, R. R., Saye, J. M. and Smith, R. D. (1993) *Pharmacol. Rev.* **45**, 205–251
- Summers, C., Tang, W., Zelezna, B. and Raizada, M. K. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 7567–7571
- Bottari, S. P., King, I. N., Reichlin, S., Dahlstroem, I., Lydon, N. and de Gasparo, M. (1992) *Biochem. Biophys. Res. Commun.* **183**, 206–211
- Webb, M. L., Liu, E. C. K., Cohen, R. B., Hedberg, A., Bogosian, E. A., Monshizadegan, H., Molloy, C., Serafino, R., Moreland, S., Murphy, T. J. and Dickinson, K. E. J. (1992) *Peptide* **13**, 499–508
- Leung, K. H., Roscoe, W. A., Smith, R. D., Timmermans, P. B. M. W. M. and Chiu, A. T. (1992) *Eur. J. Pharmacol. (Mol. Pharmacol. Sect.)* **227**, 63–70
- Kambayashi, Y., Bardhan, S., Takahashi, K., Tsuzuki, S., Inui, H., Hamakubo, T. and Inagami, T. (1993) *J. Biol. Chem.* **268**, 24543–24546
- Jaiswal, N., Tallant, E. A., Diz, D. I., Khosla, M. C. and Ferrario, C. M. (1991) *Hypertension* **17**, 1115–1120
- Darimont, C., Vassaux, G., Ailhaud, G. and Negrel, R. (1994) *Endocrinology* **135**, 2030–2036
- Kang, J., Summers, C. and Posner, P. (1993) *Am. J. Physiol.* **265**, C607–C616
- Grady, E. F., Sechi, L. A., Griffin, C. A., Schambelan, M. and Kalinyak, J. E. (1991) *J. Clin. Invest.* **88**, 921–933
- Servant, G., Boulay, G., Bossé, R., Escher, E. and Guillemette, G. (1993) *Mol. Pharmacol.* **43**, 677–683
- Servant, G., Dudley, D. T., Escher, E. and Guillemette, G. (1994) *Mol. Pharmacol.* **45**, 1112–1118
- Mukoyama, M., Nakajima, M., Horiuchi, M., Sasamura, H., Pratt, R. E. and Dzau, V. J. (1993) *J. Biol. Chem.* **268**, 24539–24542
- Nakajima, M., Mukoyama, M., Pratt, R. E., Horiuchi, M. and Dzau, V. J. (1993) *Biochem. Biophys. Res. Commun.* **197**, 393–399
- Tsuzuki, S., Ichiki, T., Nakakubo, H., Kitami, Y., Guo, D.-F., Shirai, H. and Inagami, T. (1994) *Biochem. Biophys. Res. Commun.* **200**, 1449–1454
- Kornfeld, P. and Kornfeld, S. (1985) *Annu. Rev. Biochem.* **54**, 631–664
- Elbein, A. D. (1984) *Crit. Rev. Biochem.* **16**, 21–49
- Tarentino, A. L., Gómez, C. M. and Plummer, T. H., Jr. (1985) *Biochemistry* **24**, 4665–4671
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–683
- Boege, F., Ward, M., Jurss, R., Hekman, M. and Helmreich, E. J. M. (1988) *J. Biol. Chem.* **263**, 9040–9049
- Fraker, P. J. and Speck, J. C. (1978) *Biochem. Biophys. Res. Commun.* **80**, 849–857
- Lazard, D., Villageois, P., Briand-Sutren, M. M., Cavallé, F., Bottari, S., Strosberg, A. D. and Nahmias, C. (1994) *Eur. J. Biochem.* **220**, 919–926
- Carson, M. C., Leach Harper, C. M., Baukal, A. J., Aguilera, G. and Catt, K. J. (1987) *Mol. Endocrinol.* **1**, 147–153
- Sasaki, K., Yamano, Y., Bardhan, S., Iwai, N., Murray, J. J., Hasegawa, M., Matsuda, Y. and Inagami, T. (1991) *Nature (London)* **351**, 230–232
- Carver, J. P., Michnick, S. W., Imberty, A. and Cumming, D. A. (1989) in *Carbohydrate Recognition in Cellular Function*, pp. 6–26, J. Wiley and Sons, Chichester
- Collier, E., Carpentier, J. L., Beitz, L., Caro, L. H. P., Taylor, S. I. and Gorden, P. (1993) *Biochemistry* **32**, 7818–7823
- Ronnett, G. V., Knutson, V. P., Kohanski, R. A., Simpson, T. L. and Lane, M. D. (1984) *J. Biol. Chem.* **259**, 4566–4575
- Soderquist, A. M. and Carpenter, G. (1984) *J. Biol. Chem.* **259**, 12586–12594
- Slieker, L. J. and Lane, M. D. (1985) *J. Biol. Chem.* **260**, 687–690
- Merlie, J. P. and Smith, M. M. (1986) *J. Membr. Biol.* **91**, 1–10
- El Battari, A., Forget, P., Fouchier, F. and Pic, P. (1991) *Biochem. J.* **278**, 527–533
- Rens-Domiano, S. and Reisine, T. (1991) *J. Biol. Chem.* **266**, 20094–20102
- Rands, E., Candelore, M. R., Cheung, A. H., Hill, W. S., Strader, C. D. and Dixon, R. A. F. (1990) *J. Biol. Chem.* **265**, 10759–10764
- Cervantes-Olivier, P., Delavie-Klutcho, C., Durieu-Trautmann, O., Kaveri, S., Desmadril, M. and Strosberg, A. D. (1988) *Biochem. J.* **250**, 133–143
- Yamano, Y., Ohyama, K., Chaki, S., Guo, D.-F. and Inagami, T. (1992) *Biochem. Biophys. Res. Commun.* **187**, 1426–1431
- van Koppen, C. J. and Nathanson, N. M. (1990) *J. Biol. Chem.* **265**, 20887–20892