Physicochemical Characterization of Photoaffinity-Labeled Angiotensin II Receptors

Mary C. Carson*, Catherine M. Leach Harper, Albert J. Baukal, Greti Aguilera, and Kevin J. Catt

Endocrinology and Reproduction Research Branch
National Institute of Child Health and Human Development
National Institutes of Health
Bethesda, Maryland 20892

Specific receptor sites for angiotensin II (AII) were analyzed in the adrenal cortex and other target tissues including liver, anterior pituitary gland, and smooth muscle, after covalent labeling with the radioactive photoaffinity analog ^{125}I-[Sar^1,(4-N$_3$)Phe^8]-AII. The photoreactive AII derivative retained high affinity for adrenal receptors and full steroidogenic activity in adrenal glomerulosa cells. In bovine adrenal cortex, covalent labeling of AII receptors by the photoreactive analog was specifically inhibited by [Sar^1]AII with an IC$_{50}$ of about 5 nM. The M$_r$ of the covalent AII-receptor complex during polyacrylamide gel electrophoresis of the labeled protein under reducing conditions was 58,000. Under nonreducing conditions, a minor band with M$_r$ of 105,000 was also observed. Two labeled species were also found during gel permeation chromatography of the detergent-solubilized complex, with M$_r$s of 64,000 and 86,000. The pI of the solubilized photolabeled complex was absorbed to wheat germ lectin Sepharose 6MB and could be eluted by *N*-acetylglucosamine. The M$_r$s of specific AII-binding sites in several target tissues, determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, showed target tissues, determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, showed significant differences within and between species. The most striking differences were between rat adrenal cortex (79,000) and both rat liver (60,000) and bovine adrenal cortex (58,000). After enzymatic deglycosylation, the M$_r$ of the major component present in the bovine and rat adrenal cortex decreased by 40% and 55% to 35,000 and 34,000, respectively, suggesting that variations in carbohydrate content contribute to the physical heterogeneity of AII receptors in individual target tissues. (Molecular Endocrinology: 1: 147–153, 1987)

INTRODUCTION

Plasma-membrane receptors for angiotensin II (AII) have been characterized by radioligand binding analysis and pharmacological studies in several target tissues of the octapeptide. Most AII receptors possess similar binding affinities and comparable specificities for AII and its analogs, but individual tissues exhibit diverse biological responses to AII agonist and antagonist derivatives (1). The elucidation of mechanisms coupling AII binding to cellular activation will require a more complete understanding of the physicochemical and structural properties of the AII receptor in specific target cells, including adrenal, smooth muscle, liver, and nervous system. The physical characterization of AII receptor sites has been complicated by the difficulty of solubilizing the receptor in a sufficiently stable and active form to permit its analysis by standard fractionation procedures. For this reason, both photoaffinity labeling and radioligand cross-linking have been employed to investigate the properties of the solubilized AII receptor (2–8). In previous studies on canine adrenal and uterine AII receptors, azidobenzoyl-derivatized ^{125}I-AII was employed as the photoreactive radioligand to analyze receptor structure (2). In the present report, the carboxyterminal photoreactive analog [Sar^1,(4-N$_3$)Phe^8]AII (N$_3$-[Sar^1]AII) (3–6), is used to characterize the AII receptors of bovine and rat adrenal cortex, and to compare the receptors from individual target tissues in several species.

0888-8809/87/0147-0153$02.00/0
Molecular Endocrinology

RESULTS

Photolabeling of AII Receptors

In competitive binding studies, N$_3$-[Sar^1]AII inhibited ^{125}I-AII binding to bovine adrenal cortex particulate receptors with an IC$_{50}$ of 0.7 nM, similar to the IC$_{50}$ for [Sar^1]AII of 1.3 nM and significantly more potent than the AII IC$_{50}$ of 5.6 nM (Fig. 1). In rat zona glomerulosa cells, the azido derivative was equivalent to AII in its steroidogenic potency and efficacy, as indicated by stimulation of aldosterone production *in vitro* (data not shown). In direct binding studies, the radioiodinated azido-hormone retained high affinity and specificity for particulate AII receptors. During UV irradiation, up to 60% of the specifically bound hormone became covalently attached to adrenal membranes, as indicated by resistance to acid dissociation (Fig. 2). In the absence of photolysis,

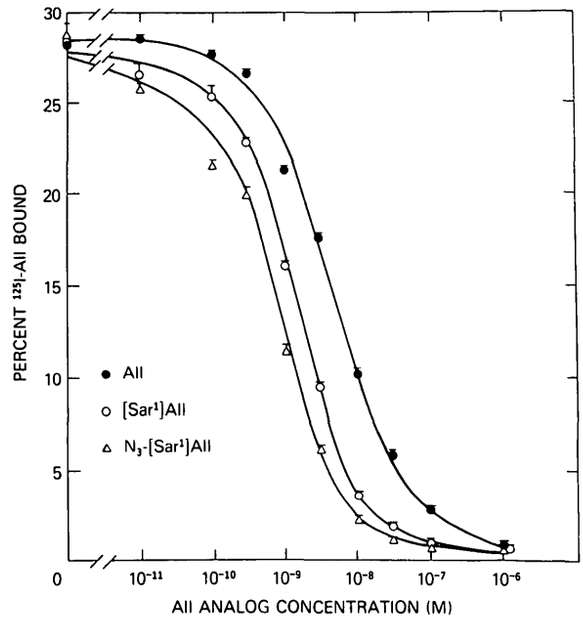

Fig. 1. Displacement of ^{125}I-AII Binding by AII, [Sar^1]AII, and N_3-[Sar^1]AII
Binding of ^{125}I-AII to bovine adrenal cortical membranes (90 μg) was determined in the dark as in *Materials and Methods* in the presence of increasing amounts of AII analogs. Data represent the mean ± SE of duplicate points and are typical of three similar experiments.

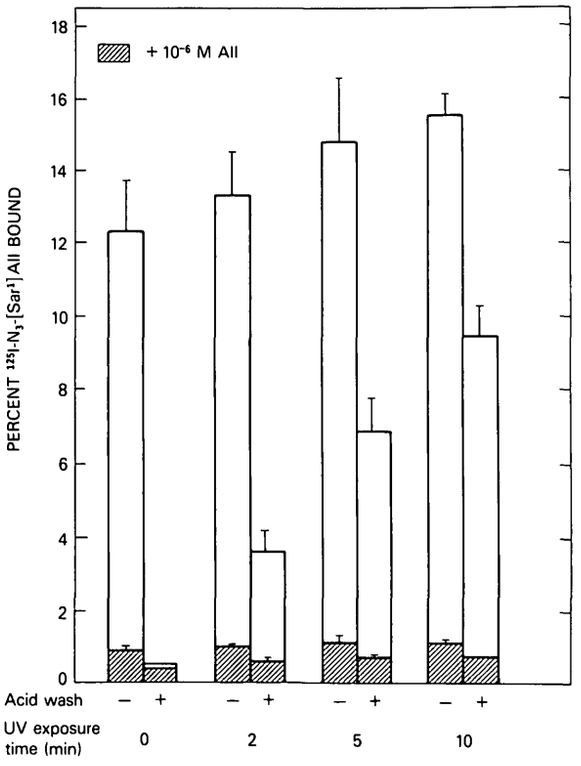

Fig. 2. Covalent Labeling of Bovine Adrenal AII Receptors
Membranes were incubated with ^{125}I-N_3-[Sar^1]AII in the presence or absence of 1 μM AII. The membranes were then irradiated for varying periods of time, treated with acid as indicated, and washed by filtration to remove free hormone. *Bars* indicate the mean ± SE of triplicate determinations, and the data are representative of three individual experiments.

95–100% of the specifically bound ligand was dissociated by acid treatment. A brief wash with Tris buffer immediately before photolysis was found necessary to free the membranes of BSA and unbound ^{125}I-N_3-[Sar^1]AII, which otherwise resulted in high nonspecific levels of covalent attachment. Under these conditions, nonspecific labeling was usually less than 15% of the total bound radioactivity.

To characterize the membrane component(s) labeled by the azido-hormone, bovine adrenal particulate samples were incubated with ^{125}I-N_3-[Sar^1]AII in the presence of increasing amounts of [Sar^1]AII. Analysis of the photolysis products by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis revealed progressive displacement of labeling ($IC_{50} \sim 5 \times 10^{-9}$ M) from the major radioactive band of M_r 55,000. Labeling experiments performed with the AII antagonist analog, ^{125}I-[Sar_1,D-(4-N_3)Phe^8]AII (6), gave similar results (Fig. 3). Omission of 2-mercaptoethanol during sample preparation caused no apparent change in the major labeled band, but did result in the appearance of a minor (<5%) band of approximate M_r 105,000 (data not shown).

Chromatography and Electrofocusing

During gel permeation chromatography of photolabeled SDS- or Triton-solubilized bovine adrenal membranes, the bulk of the radioactivity eluted as a peak with Stokes' radius corresponding to a protein of M_r 64,000 ± 3,000 (mean ± SE, n = 13) (Fig. 4). A minor peak of M_r 86,000 ± 2,000 (n = 8) was sometimes present, and represented up to 15% of the bound radioactivity. The elution profile of some preparations also showed a significant amount of radioactivity in the position of free AII. This may have originated from degradation of the covalent hormone-receptor complex, or from the pres-

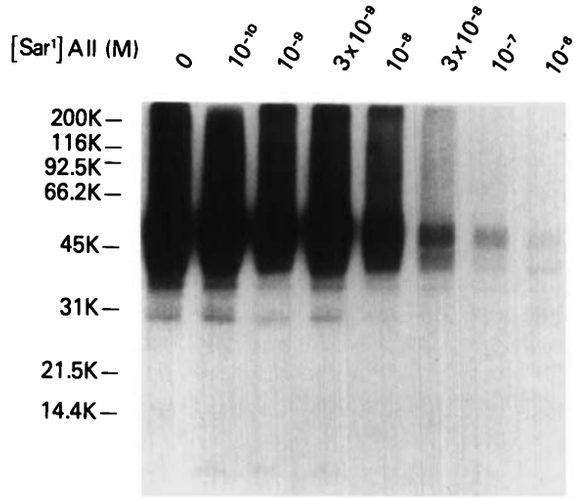

Fig. 3. Concentration-Dependent Inhibition of ^{125}I-N_3-[Sar^1]AII Labeling of Bovine Adrenal Membranes by [Sar^1]AII
Membrane preparations were incubated with ^{125}I-N_3[Sar^1]AII and increasing amounts of [Sar^1]AII, washed, and irradiated. The photolabeled membranes were analyzed by SDS-PAGE. The fluorogram of the gel is shown, with *bars* indicating the positions of Coomassie blue-stained molecular weight markers.

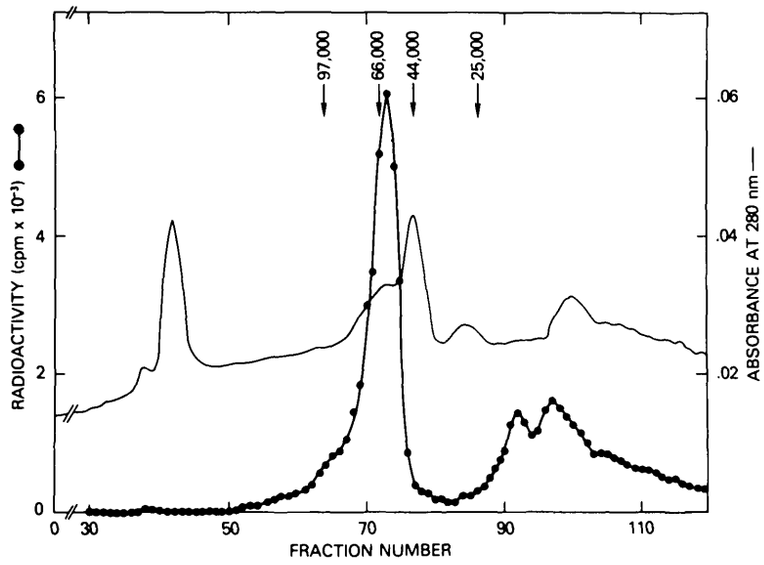

Fig. 4. HPLC Gel Permeation Chromatography of [^{125}I]N_3-[Sar^1]AII-Labeled Bovine Adrenal Membranes
The Triton-solubilized membranes were loaded onto Spherogel-TSK columns and eluted as described in *Materials and Methods*. The chromatogram shown is a representative profile of 13 separate experiments. *Arrows* indicate the elution positions of reference proteins.

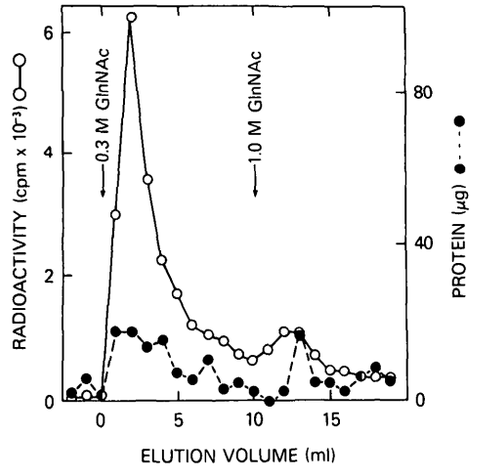

Fig. 5. Wheat Germ Lectin Affinity Chromotography of Solubilized ^{125}I-N_3-[Sar^1]AII-Labeled Bovine Adrenal Membranes
Photolabeled SDS-solubilized membranes were loaded onto 2 ml wheat germ lectin-Sepharose 6MB resin, the resin washed extensively, and specifically absorbed proteins eluted with the addition of *N*-acetylglucosamine at the indicated times.

ence of tightly bound, but not covalently linked, radioligand which dissociated during chromatography.

Wheat germ lectin Sepharose 6MB chromatography of SDS-solubilized, photolabeled adrenal membranes was performed in buffer containing 0.1% Triton X-100, after batch absorption to the resin. Under these conditions, 20–40% of the radioactive complex eluted in the initial wash, together with about 75% of the protein. Approximately one-half of the absorbed covalent hormone-receptor complex was then eluted with the specific sugar ligand for wheat germ lectin, *N*-acetylglucosamine (Fig. 5). Similar results were obtained in three experiments, indicating the presence of carbohydrate moieties on the AII-binding protein.

A typical chromatofocusing profile of the photolabeled, Triton-solubilized bovine adrenal membranes is shown in Fig. 6. The isoelectric point of the covalent hormone-receptor complex was 7.2 ± 0.1 (n = 6). This

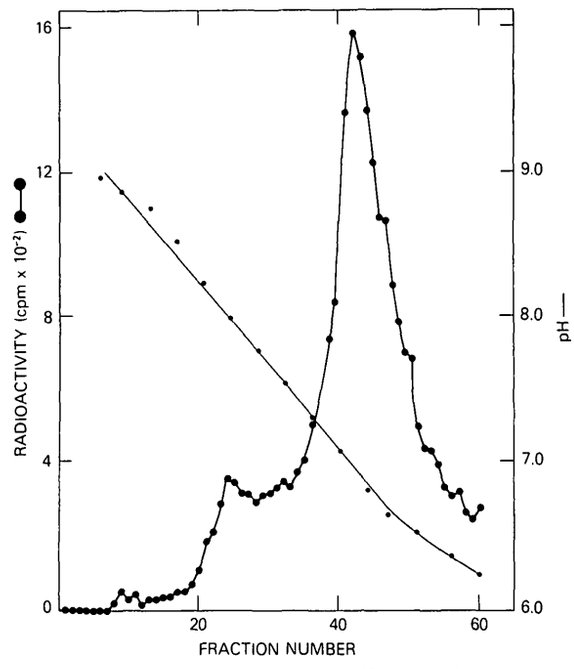

Fig. 6. FPLC Chromatofocusing of [^{125}I]N_3-[Sar^1]AII-Labeled Bovine Adrenal Membranes
Triton-solubilized membranes were loaded onto a Mono P column and eluted as described in *Materials and Methods*. The results are typical of six separate experiments.

pI was consistent with the distribution of the complex over the pH range from 7.0 to 7.5 on autoradiography of two-dimensional gels (data not shown) and also with the fact that the complex absorbs to diethylaminoethyl resins at pH 8.0 (9).

SDS-Polyacrylamide Gel Electrophoresis (PAGE) Analysis of AII Receptors

Membrane fractions from the adrenal cortex and liver of rat, rabbit, monkey, and cow, and also from the anterior pituitary, bladder, and mesenteric artery of the rat, were photolabeled with N_3-[Sar^1]AII and analyzed by SDS-PAGE followed by fluorography. As shown in Fig. 7, a single broad band was specifically labeled in all cases. However, the apparent molecular weights of these bands, as summarized in Tables 1 and 2, differed both between species for a given target tissue, and among different target tissues within a single species. A particularly striking difference was noted between the M_r of the AII-receptor complex of the rat adrenal gland (79,000) and those of the rat liver (60,000) and bovine adrenal (58,000).

Effects of Glycosidase Treatment

To further characterize the nature of the AII binding protein, photolabeled membranes were treated with the endoglycosidase, *N*-Glycanase, which cleaves *N*-linked saccharides. A marked decrease in M_r was evident in the glycosidase-treated adrenal cortical protein from cow (from 58,000 to 35,000) and rat (from 75,000 to 34,000), as shown in Fig. 8. These changes constitute reductions of 40% and 55% in M_r, respectively. The appearance of bands of intermediate size, presumably partial deglycosylation products, may have resulted from incomplete solubilization and/or denaturation before glycosidase treatment, rendering some linkages more accessible than others. Such intermediate bands were also observed after glycosidase treatment of labeled bovine adrenal membranes in the absence of SDS denaturation, which gave predominantly products of M_r 53,000 and 44,000, rather than 35,000 (data not shown).

Table 1. Molecular Weights of AII-Binding Proteins from Different Species

Tissue	$M_r \times 10^{-3}$			
	Rat	Rabbit	Monkey	Cow
Adrenal Cortex	79 ± 6 (8)	73 ± 8 (3)	74 ± 6 (4)	58 ± 5 (4)
Liver	60 ± 7 (9)	60 ± 6 (3)	78 ± 4 (2)	63 ± 5 (2)

Values indicate the mean ± SE of data from the numbers of experiments given in parentheses.

DISCUSSION

In previous studies on the physical characteristics of photoaffinity labeled AII receptors from canine adrenal gland and uterus (2), the presence of two binding species, of M_r 126,000 and 65,000 or 68,000, indicated that the AII holoreceptor could be a dimer of two subunits of about M_r 60,000. The M_r values derived for the binding subunits of dog adrenal (65,000) and uterus (68,000) were similar, suggesting that the AII receptor may be of comparable size in different target tissues. This possibility was supported by subsequent reports on the apparent molecular weights of AII receptors from bovine adrenal [60,000 (5)] and rabbit liver [68,000 (10)]. However, during the present studies on the properties of covalent AII-receptor complexes derived by photoaffinity labeling with ^{125}I-N_3-[Sar^1]AII, we observed several differences in the characteristics of AII receptors from a number of target tissues. The C-terminal azido AII derivative (3) employed in these studies has higher efficiency for labeling AII receptors than N-terminal azidobenzoyl AII derivatives (2) or chemical cross-linking methods (7, 8), and is the preferred reagent for the preparation of covalent AII-receptor complexes.

The molecular weight of the photolabeled AII receptor site was found to vary significantly with both the species and tissue of origin. The molecular weights of the bovine adrenal and rabbit liver sites were in agreement with those observed by Guillemette and Escher (5) and Sen *et al.* (10), respectively. However, the M_r of the rat adrenal AII receptor (79,000) was significantly smaller than that (116,000) reported by Paglin and Jamieson

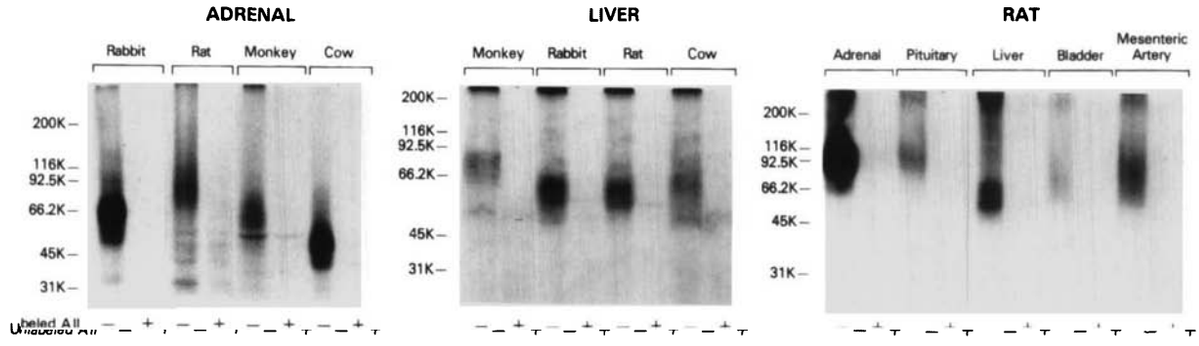

Fig. 7. Labeling of AII Receptors from Different Species and Target Tissues
Fresh particulate fractions prepared from individual tissues were incubated with either ^{125}I-N_3-[Sar^1]AII or ^{131}I-N_3-[Sar^1]AII in the absence (−) or presence (+) of 1 μM AII. The photolabeled membranes were solubilized and analyzed by SDS-PAGE followed by fluorography.

Table 2. Molecular Weights of AII-Binding Proteins from Rat Tissues

	Adrenal Glomerulosa	Liver	Anterior Pituitary	Mesenteric Artery	Bladder
$M_r \times 10^{-3}$	79 ± 6 (8)	60 ± 7 (9)	92 ± 2 (2)	71 ± 2 (3)	72 ± 3 (2)

Values indicate the mean ± SE of data from the numbers of experiments given in parentheses.

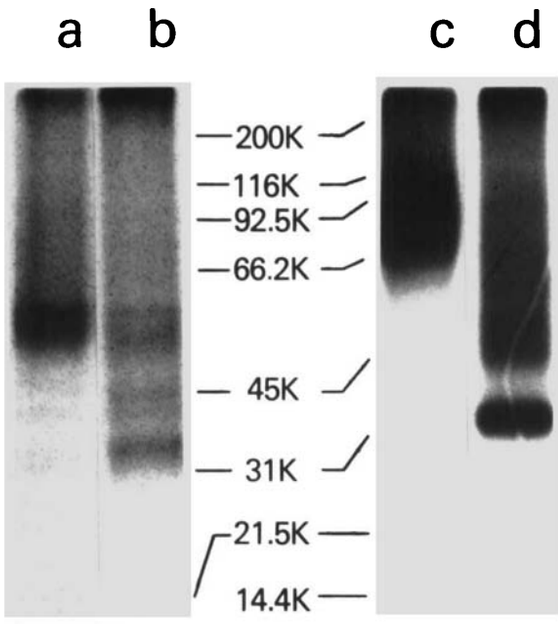

Fig. 8. Effects of *N*-glycanase Digestion on Electrophoretic Properties of ^{125}I-N_3-[Sar^1]AII-Labeled Adrenal Receptors
Bovine and rat adrenal membranes were photolabeled, solubilized, and denatured, then treated with *N*-glycanase and analyzed by SDS-PAGE. Lanes a and b, Bovine adrenal cortical membranes. Lanes c and d, Rat adrenal glomerulosa membranes. Lanes a and c, Controls. Lanes b and d, Membranes treated with *N*-glycanase.

(7). This difference suggests that chemical cross-linking of the particulate hormone-receptor complex may lead to overestimation of the molecular weight of the hormone-binding moiety, presumably by the cross-linking of adjacent proteins to the receptor, or of the receptor subunits if its native form is dimeric (3). Although the major photoaffinity-labeled protein in the bovine adrenal cortex had a relative molecular weight of approximately 60,000 as determined by HPLC gel permeation chromatography and SDS-PAGE, minor species of higher molecular weight were also detected in the absence of reducing agents. These data, together with the results of cross-linking studies, suggest that the AII holoreceptor is a dimer, or is closely associated with other membrane proteins.

The adrenal AII-receptor complex behaved as a glycoprotein, and was of almost neutral pI value as indicated by its properties during chromatofocusing and ion exchange chromatography (9). The presence of carbohydrate residues was shown by both specific absorption of immobilized wheat germ lectin and by the effects of glycosidase digestion. The carbohydrate moieties contributed significantly to the apparent M_r of the receptor-binding protein. This was most evident in the rat adrenal, where treatment with *N*-glycanase reduced the estimated size of the labeled protein by more than 50%. It is noteworthy that some of the smaller degradation products appearing in Fig. 3 coincide with the *N*-glycanase products in Fig. 8, suggesting that glycosidase digestion may occur during the extraction of receptors from their tissues of origin.

The labeled protein in rat adrenal glomerulosa was physically distinct from that in liver, suggesting the existence of at least two types of AII receptors. These results may be relevant to the suggestion by Peach (11) that receptors in various AII target tissues differ in their pharmacological properties. However, within any given tissue, only a single broad band was consistently labeled by the photoreactive AII derivative. The presence of lower molecular weight species in some gels was not reproducible, and was probably attributable to glycosidic or proteolytic digestion after photoaffinity labeling of the membranes receptor sites.

The broadness of the specifically labeled species observed by SDS-gel electrophoresis was consistent with the glycoprotein nature of the AII receptor. In some instances the labeled band appeared to be a doublet, but whether this represented two species of AII-binding sites, or was the product of minor receptor degradation, is not certain. It has been postulated that two types of angiotensin receptor are present in cardiac and vascular tissues (12). Whereas our observations neither support nor refute this possibility, it is interesting to note that the labeled bands from smooth muscle tissues, such as bladder and mesenteric artery, were particularly broad and diffuse.

The basis for the difference between the AII-binding sites in rat liver and rat adrenal, and whether their physical properties are relevant to functional differences between the two receptors, is not yet clear. It is possible that there is more than one gene for the AII receptor, and that these are differentially expressed in the various AII target tissues. A more likely possibility is that different posttranslational modifications of a single gene product occur within individual target tissues. In particular, variation in the degree of glycosylation, which contributes substantially to the molecular weights of the AII-binding sites, could be responsible for the differences among individual target tissues and between species. Thus, the rat adrenal receptor may be simply a more heavily glycosylated form of the corresponding liver receptor. This question is currently under investigation to clarify the role of carbohydrate components in the physical and functional properties of the AII receptor.

MATERIALS AND METHODS

Synthesis and Purification of Peptides

The photoreactive AII derivative N_3-[Sar^1]AII was prepared essentially as described (3) from [Sar^1,(4,NO_2)Phe^8]AII. The nitro peptide was prepared by solid phase synthesis from commerically available amino acids, with the exception of (4-NO_2)Phe, which was found to contain significant (>20%) contaminants that were not removable by recrystallization. This amino acid was therefore prepared by condensation of malonic acid ethyl ester with *p*-nitrobenzyl bromide (13). The L isomer was isolated in pure form from the resulting racemic mixture by acetylation, followed by treatment with Acylase I (Sigma, St. Louis, MO). After HF cleavage from the resin [Sar^1,(4-NO_2)Phe^8]AII was purified by Sephadex G-25 gel filtration and counter-current distribution. The subsequent reactions to yield N_3-[Sar^1]AII proceeded quantitatively. Purity of all peptides was confirmed by TLC, HPLC, and amino acid analysis. The azido-AII antagonist analog, [Sar^1,D-(4-N_3)Phe^8]AII, was the gift of Dr. E. Escher (University of Sherbrooke, Sherbrooke, Quebec, Canada), and [Sar^1]AII was the gift of Dr. M. C. Khosla (Cleveland Clinic, Cleveland, OH). Monoiodinated ^{125}I-AII analogs were prepared by a modified IODO-GEN (Pierce, Rockford, IL) method (14) (Meloy Laboratories, Springfield, VA) and had specific activities of about 1500 Ci/mmol.

Membrane Preparations

Particulate fractions from bovine, rat, rabbit, and monkey target tissues were prepared essentially as described previously (15), with the exception that all tissues were minced and homogenized in 20 mM $NaHCO_3$ containing 0.1 mM phenylmethylsulfonylfluoride, 50 μg/ml soybean trypsin inhibitor, 20 mM Na molybdate, 0.01% dimethylsulfoxide, and 0.5 mM dithiothreitol, before centrifugal fractionation at 4 C. The 35,000 × *g* membrane pellets were then washed twice with 20 mM Tris·HCl, pH 7.4, containing 0.02% NaN_3 (Tris buffer). Bovine adrenal cortical membranes were prepared in quantity and used fresh or stored in aliquots at −70 C. Before use, the frozen membranes were thawed and washed with Tris buffer. All other membranes were freshly prepared and kept on ice before binding studies and photoaffinity labeling.

Radioligand Binding Assays

Receptor-binding assays were performed by a modified version of a previously described procedure (16). Briefly, 50–100 μg membranes were incubated with approximately 0.1 nM radioiodinated hormone and varying amounts of unlabeled hormone in a total volume of 300 μl containing 20 mM Tris·HCl, pH 7.4, 100 mM NaCl, 5–10 mM $MgCl_2$, 0.2% BSA, and 0.02% NaN_3 (assay buffer). This buffer was used for binding studies involving bovine adrenal and liver from all species. The assay buffer for rat, rabbit, and monkey adrenal glomerulosa, and rat anterior pituitary, bladder, and mesenteric artery, also contained 2 mM EGTA and 0.5 mM dithiothreitol. Incubations were performed at 22 C for 45 min, and were stopped by the addition of 2 ml ice-cold PBS, followed by rapid filtration through Whatman GF/C filters, presoaked in 0.2% BSA, to isolate receptor-bound radioactivity.

Protein Determinations

In particulate samples, protein was measured by the method of Lowry *et al.* (17) after hydrolysis in 0.1 N NaOH. Protein in solubilized samples was determined by the method of Bradford (18) using Bio-Rad (Richmond, CA) Protein Dye reagent and standards containing equivalent detergent concentrations. In both assays, crystalline BSA was used as the protein standard.

Photoaffinity Labeling of Angiotensin II Receptors

Two methods were used according to the amount of protein to be labeled. For samples containing up to 1 mg protein, particulate fractions were incubated in the dark with ^{125}I-N_3-[Sar^1]AII (0.1–0.3 nM, 10^6 cpm) in the absence or presence of 1.0 μM AII for 45 min in 1.0 ml assay buffer. The membranes were then pelleted by centrifugation, resuspended in 1.0 ml Tris buffer, and transferred to a 12-well tissue culture plate. The plate was placed on ice and irradiated for 5–7 min with a Mineralight UVS-54 lamp placed directly on the plate.

For large scale preparation of labeled bovine adrenal cortex membranes, 50–100 mg particulate fraction were suspended in 100 ml assay buffer containing approximately 10^8 cpm ^{125}I-N_3-[Sar^1]AII and gently stirred in the dark for 45 min. The membranes were pelleted by centrifugation at 35,000 × *g* for 15 min, washed once with 100 ml Tris buffer, and resuspended with a Dounce homogenizer in 200 ml Tris buffer. The suspension was poured into a photochemical reaction vessel equipped with a 450 watt mercury vapor lamp in a water-cooled quartz immersion well (Ace Glass, Inc., Vineland, NJ). The lamp was warmed up for at least 15 min before use and was turned off 1 min after adding the membranes. To remove noncovalently bound hormone, the membranes were stirred for 15 min in 0.1 M acetic acid at room temperature, then pelleted and washed once more with acetic acid and twice with Tris buffer. Concentrated suspensions of labeled membranes were stored in aliquots at −70 C.

Solubilization of Bovine Adrenal Membranes

Labeled membranes were suspended in Tris buffer containing 1% SDS and mixed for 40 min at room temperature. The sample was then diluted to 0.1% SDS with Tris buffer, centrifuged at 35,000 × *g* for 20 min, and the supernatant solution passed through Extracti-Gel D (Pierce) to remove the SDS. The solubilized proteins were eluted from the gel with 0.1% Triton X-100 in Tris buffer, unless otherwise stated. Centrifugation at 100,000 × *g* for 60 min did not alter the recovery of solubilized material, as determined by both protein and radioactivity contents of the supernatant solution. For some experiments, labeled membranes were solubilized in 1% Triton X-100 for 90 min at 4 C, diluted to 0.1% Triton, and centrifuged as above. The yields of solubilization of the membrane AII-receptor complex with Triton X-100 and SDS were approximately 70% and 95%, respectively, and the specific activity of the soluble preparation was 100–300 dpm/μg protein.

SDS-Gel Electrophoresis

Labeled membranes were treated with 3% SDS and 1% 2-mercaptoethanol in 5% glycerol and analyzed on SDS-7.5–15% gradient polyacrylamide gels using the method of Laemmli (19). The gels were stained with Coomassie blue, dried, and exposed to Kodak XAR-5 film with Kodak Lanex intensifying screens for several days at −70 C.

Gel Permeation Chromatography

Labeled bovine adrenal membranes were solubilized in either SDS or Triton and eluted from Extracti-Gel D with Tris buffer containing 5 mM EDTA, 1 M NaCl, and 0.5% Brij 99. The solubilized preparation (200 μl, 100–200 μg protein) was applied to a Spherogel-TSK GSWP (Altex, Fullerton, CA) 7.5 × 75-mm precolumn connected to a series of three Spherogel-TSK columns (two 7.5 × 300 mm 3000s and a 7.5 × 600 mm 4000) attached to a Beckman HPLC system. Elution was performed with the above buffer at a flow rate of 0.5 ml/min and 1-ml fractions were collected. The chromatography system was calibrated with the standard proteins phosphorylase B, BSA, ovalbumin, and chymotrypsin.

Lectin Affinity Chromatography

Aliquots of the SDS-solubilized photolabeled adrenal membranes (0.5 ml, 2 mg protein, and 150,000 cpm) were added to 2 ml wheat germ lectin Sepharose 6MB (Pharmacia, Piscataway, NJ) equilibrated in Tris buffer containing 100 mM NaCl, 0.1% Triton X-100, and 1 mM each of $CaCl_2$, $MgCl_2$, and $MnCl_2$. The resin was agitated gently overnight in a closed column, then washed with the equilibration buffer at 0.4 ml/min until no further protein was detected in the eluate (10-column volumes). Specifically bound proteins were then eluted from the lectin by the addition of 0.3 M and 1 M *N*-acetylglucosamine to the buffer, and 1-ml fractions were collected.

Chromatofocusing

The isoelectric point of the AII-receptor complex was determined by analysis of Triton X-100-solubilized ^{125}I-N_3-[Sar^1]AII-labeled bovine adrenal membranes on a Mono-P-chromatofocusing column (Pharmacia). The detergent extract was diluted to 0.1% Triton with 75 mM Tris·HCl, pH 9.3. The Mono P column, attached to a Pharmacia FPLC unit, was equilibrated in 75 mM Tris·HCl, 0.1% Triton, pH 9.3. A 500-μl sample containing 250–500 μg protein was loaded onto the column and eluted with a 1:10 dilution of Polybuffer 96·HCl (Pharmacia), pH 6.0. The elution buffer flow rate was 0.25 ml/min, and 0.5-ml fractions were collected.

Glycosidase Treatment of Labeled Adrenal Membranes

Photoaffinity-labeled particulate samples (50–100 μg protein containing 8,000 to 20,000 cpm) were denatured and solubilized by boiling for 3 min in 0.5% SDS and 0.1 M 2-mercaptoethanol. Nonidet P-40 was added to 7.5-fold excess over the SDS, and sodium phosphate buffer, pH 8.6, and EDTA were added to final concentrations of 0.2 M and 5 mM, respectively. N-Glycanase (Genzyme, Boston, MA, 0.25 U) was added (or omitted in the case of controls) and the samples were incubated overnight at 30 C. The treated protein was then boiled again in the presence of 3% SDS and 1% 2-mercaptoethanol in 5% glycerol and analyzed by SDS-PAGE.

Acknowledgments

We are grateful to Dr. H. C. Chen and Mr. John Morell for valuable advice on peptide synthesis, and to Ms. J. Brown for amino acid analysis and HPLC analysis of peptides.

Received July 14, 1986.

Address correspondence and requests for reprints to: Dr. Kevin J. Catt, Endocrinology and Reproduction Research Branch, Building 10, Room 8C407, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892.

* Supported by National Research Service Award 1F32-HL-06723.

REFERENCES

1. Mendelsohn FAO 1985 Localization and properties of angiotensin receptors. J Hypertension 3:307–316
2. Capponi AM, Catt KJ 1980 Solubilization and characterization of adrenal and uterine angiotensin II receptors after photoaffinity labeling. J Biol Chem 255:12081–12086
3. Escher EHF, Nguyen TMD, Robert H, St-Pierre SA, Regoli DC 1978 Photoaffinity labeling of the angiotensin II receptor. 1. Synthesis and biological activities of the labeling peptides. J Med Chem 21:860–864
4. Escher EHF, Nguyen TMD, Regoli D 1978 Photoaffinity labeling of the angiotensin II receptor; pharmacology of the labeling peptides in the dark. Can J Physiol Pharmacol 56:956–962
5. Guillemette G, Escher E 1983 Analysis of the adrenal angiotensin II receptor with the photoaffinity labeling method. Biochemistry 22:5591–5596
6. Guillemette G, Guillon G, Marie J, Pantaloni C, Balestre MN, Escher E, Jard S 1984 Angiotensin-induced changes in the apparent size of rat liver angiotensin receptors. J Receptor Res 4:267–281
7. Paglin S, Jamieson JD 1982 Covalent crosslinking of angiotension II to its binding sites in rat adrenal membranes. Proc Natl Acad Sci USA 79:3739–3743
8. Rogers TB 1984 High affinity angiotensin II receptors in myocardial sarcolemmal membranes. J Biol Chem 259:8106–8114
9. De Lean A, Ong H, Gutkowska J, Schiller PW, McNicoll N 1984 Evidence of agonist-induced interaction of angiotensin receptor with a guanine nucleotide-binding protein in bovine adrenal zona glomerulosa. Mol Pharmacol 26:498–508
10. Sen I, Bull HG, Soffer RL 1984 Isolation of angiotensin II-binding protein from liver. Proc Natl Acad Sci USA 81:1679–1683
11. Peach MJ 1977 Renin-angiotensin system: biochemistry and mechanisms of action. Physiol Rev 57:313–370
12. Trachte GJ, Peach MJ 1983 A potent noncompetitive angiotensin II antagonist induces only competitive inhibition of angiotensin III responses. J Cardiovasc Pharmacol 5:1025–1033
13. Greenstein JP, Winitz M 1961 Chemistry of the Amino Acids. Wiley, New York, vol 1:2697
14. Markwell MAK, Fox CF 1979 Surface-specific iodination of membrane proteins of viruses and eucaryotic cells using 1,3,4,6-tetrachloro-3α,6α-diphenylaglycoluril. Biochemistry 17:4807–4817
15. Capponi AM, Catt KJ 1979 Angiotensin II receptors in adrenal cortex and uterus. J Biol Chem 254:5120–5127
16. Glossmann H, Baukal AJ, Catt KJ 1974 Properties of angiotensin II receptors in the bovine and rat adrenal cortex. J Biol Chem 249:825–834
17. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
18. Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
19. Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond) 227:680–685