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High Yield Photoaffinity Labeling of Angiotensin II Receptors

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SUMMARY

The angiotensin II analogues, [Sar^1, (4′-N_3)Phe^8]AII and [Sar^1, (4′-N_3)D-Phe^8]AII were synthetized and prepared in their iodinated and radioiodinated forms. On rat liver membranes ^{125}I-SarN_3PheAII and ^{125}I-SarN_3DPheAII labeled a single population of sites (maximal binding capacity, 1.13 ± 0.16 pmol/mg of protein) with high affinity (dissociation constants of 0.18 ± 0.05 and 0.37 ± 0.16 nM, respectively) and high specificity (nonspecific binding less than 10% of total binding for an 0.8 fractional receptor occupancy). ^{125}I-SarN_3PheAII and ^{125}I-SarN_3DPheAII allowed photoaffinity labeling of liver angiotensin receptors with high efficiency. The yield of photoaffinity labeling was independent of fractional receptor occupancy. ^{125}I-SarN_3PheAII and ^{125}I-SarN_3DPHeAII allow recovery in a soluble and covalently labeled form of about 40% of the total number of angiotensin receptors from rat liver membranes. For this reason they can be considered as potentially very useful tools for the purification of angiotensin receptors. The material covalently labeled with the antagonist ^{125}I-SarN_3DPHeAII was eluted from an Ultrogel ACA34 column as a homogeneous peak (Stoke's radius: 5.5 ± 0.2 nm). The material covalently labeled with the agonist ^{125}I-SarN_3PHeAII was more heterogeneous. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of ^{125}I-SarN_3PheAII- and ^{125}I-SarN_3DPHeAII-labeled material revealed a single component of 63,000 molecular weight.

A great deal of effort has been devoted to the development of methods allowing covalent labeling of angiotensin receptors from different tissular sources. Covalent cross-linking with a bifunctional reagent, and photoaffinity labeling were the two main approaches used. Among the successful attempts with a bifunctional reagent, Paglin and Jamieson (1), using disuccinimidyl suberate, specifically labeled a protein of M_r 116,000 in the rat adrenal cortex. Sen *et al.* (2), with the same covalent cross-linker, labeled a protein of M_r 68,000 in the rat liver, and Rogers (3), using dithiobis(succinimidyl propionate), labeled a protein of M_r 116,000 in the bovine myocardial sarcolemmal membranes. With the photoaffinity labeling method, Capponi and Catt (4), using an azidophenyl derivative on the first position of AII, labeled a protein of M_r 67,000 in the dog uterus and adrenal cortex. In all cases the yield of covalent labeling did not exceed 1–10%. Two of us (5), using an angiotensin analogue with an azido phenylalanine residue in position 8, labeled a protein of M_r 63,000 in the bovine adrenal cortex. This analogue was active in inducing rabbit and rat smooth muscle contraction. Its biological effect was maintained after photoactivation in experimental conditions where the effect of AII was rapidly reversed. This observation suggested that the yield of covalent labeling of angiotensin receptors by this new azido derivative might be much higher than that observed with other azido derivatives or bifunctional reagents. In the present work we demonstrate that two angiotensin analogues with an azido phenylalanyl residue in position 8 do allow a high yield covalent labeling of rat liver angiotensin receptors. We provide experimental evidence that these analogues might be useful tools for the purification of angiotensin receptors.

Materials and Methods

Chemicals. Chemicals and reagents used in the present study were purchased from the following sources: Na[^{125}I] from Amersham; [α-^{32}P] ATP and [^{3}H]cAMP from New England Nuclear; Iodogen from Pierce Chemical Co.; β-galactosidase (*Escherichia coli*), catalase (beef liver), and alcohol dehydrogenase (yeast) from Boehringer Mannheim; phage T4 (American Type Culture Collection, Rockville, MD) and cytochrome *c* (horse heart) from Sigma; Triton X-100 from Koch Light Laboratories; Ultrogel ACA 34 from LKB Instruments; and Bio-Gel P-30 from Bio-Rad.

Iodination and labeling of angiotensin analogues. The AII analogues SarAII, SarN_3PheAII, and SarN_3DPheAII were synthetized in the laboratory of E. Escher (Sherbrooke, Canada).

The tritiated peptide [^{3}H]SarAII was produced by catalytic tritiation of the halogenated equivalent peptide (6) and was purified by HPLC to a specific radioactivity of 132 Ci/mmol.

The iodinated peptides I-SarN_3PheAII and I-SarN_3DPheAII were

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ABBREVIATIONS: AII, [Val^5]angiotension II; SarAII, [Sar^1, Val^5]AII; SarN_3PheAII, [Sar^1, (4′-N_3)Phe^8]AII; SarN_3DPheAII, [Sar^1, (4′-N_3)D-Phe^8]AII; [^{3}H] SarAII, [Sar^1, (2′,3′,4′,5′,6′-[$^3H_5$])-Phe^8]AII; HPLC, high pressure liquid chromatography; I-SarN_3PheAII, [Sar^1, I-Tyr^4, (4′-N_3)Phe^8]AII; I-SarN_3DPheAII, [Sar^1, I-Tyr^4, (4′-N_3)D-Phe^8]AII; EDTA, ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate.

prepared as follows. Seven μl of 100 mM ICI dissolved in methanol were added to 700 μg of peptide dissolved in potassium phosphate buffer. 100 mM, pH 6.0. The reaction mixture was kept on ice for 5 min. The reaction was stopped by direct injection on an HPLC column (C18 μBondapak from Waters) and immediate elution with a linear gradient [10–55% of B in A; A: isopropanol/ammonium acetate, 0.2 M, pH 5.40 (5:95, v/v); B: acetonitrile (HPLC grade)]. The elution profile corresponding to the iodination of $SarN_3PheAII$ is shown in Fig. 1. Three peaks can be observed. The first peak corresponds to unreacted $SarN_3PheAII$. The other two were identified to the monoiodinated and the diiodinated species.

Radioiodinated azido derivatives of AII were obtained by the method of Fraker and Speck (7). One hundred μl of a solution of Iodogen (0.05 mg/ml in dichloromethane) were allowed to evaporate in a small polypropylene tube. Na[^{125}I] (1 mCi) diluted with nonradioactive iodide (28 μl of 0.1 mM KI), and 50 μl of a solution containing 1 mg/ml $SarN_3PheAII$ or $SarN_3DPheAII$ in 0.2 M acetic acid were introduced in the polypropylene tube. After 30 min at 22° the reaction was stopped by removing the mixture from the tube. Purification was performed by HPLC under the conditions used for nonradioactive iodinated peptides. The elution profile in Fig. 1 shows that most of the radioactivity migrated with the monoiodinated species. The radioactive peak was collected and rechromatographed under the same conditions. An identical procedure was used for the preparation of ^{125}I-$SarN_3DPheAII$. The dilution of Na[^{125}I] with nonlabeled KI was introduced in order to facilitate saturation of angiotensin receptors with reasonably low amounts of radioactivity. The specific radioactivity of the radioiodinated peptides was deduced from the determination of the dose-dependent specific ^{125}I binding to liver membranes (see below) using mixtures in fixed proportions of the radioiodinated peptide with known amounts of the homologous iodinated peptide in its unlabeled and purified form. Values obtained were close to the expected value of 300 Ci/mmol.

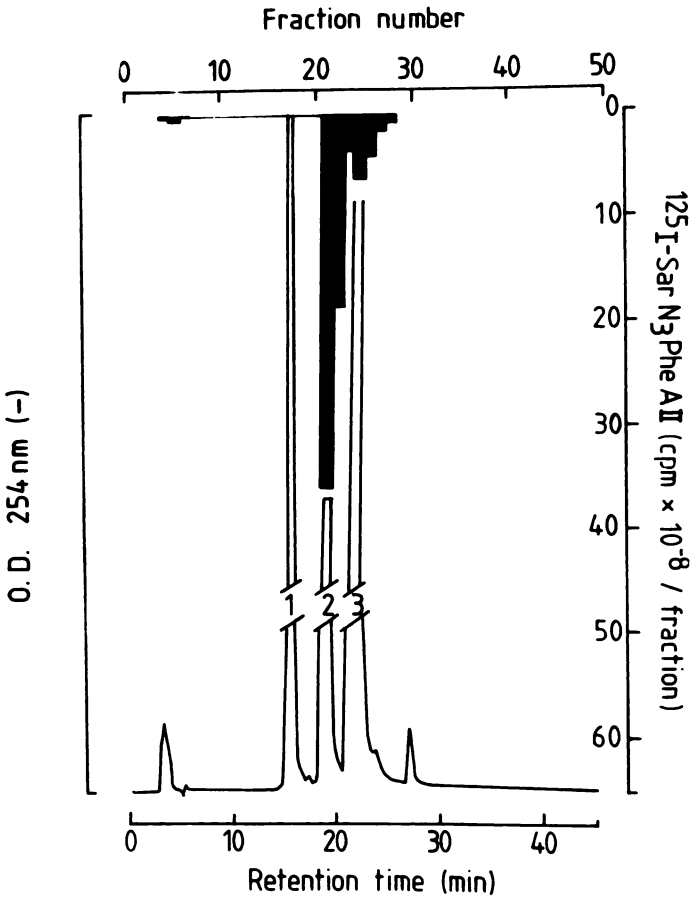

Fig. 1. Purification of iodinated azido derivatives of angiotensin. Unlabeled I-$SarN_3PheAII$ and ^{125}I-$SarN_3PheAII$ were prepared as described under Materials and Methods. The figure shows the elution profiles from the HPLC column (for details see the text) of the unlabeled peptide (detected by continuous UV absorption—*open profile*) and radioiodinated species (detected by ^{125}I radioactivity counting in 1-ml fractions of the column eluate—*solid profile*). Peaks 1–3 correspond to the nonreacted, monoiodinated, and dilodinated species, respectively. The major part of the radioiodinated peptide was eluted as monoiodinated $SarN_3PheAII$. The corresponding fractions were collected and submitted to HPLC chromatography under the same experimental conditions.

Rat liver membrane purification. Male Wistar albino rats (200–250 g body weight) fed *ad libitum* were from Iffa Credo (France). Purified plasma membranes were prepared from whole liver by the method of Neville (8) up to step 11. Membranes were stored in liquid nitrogen. They were thawed as needed just before each experiment, then washed by centrifugation at 27,000 × *g* for 10 min in 1 mM $NaHCO_3$ and taken up in 1 mM $NaHCO_3$.

Binding assays on membranes. Except when otherwise stated, binding assays were performed at 30° in 0.1 ml of a medium composed of triethanolamine/HCl, pH 7.4, 25 mM; $MgCl_2$, 10 mM; bovine serum albumin, 2 mg/ml; bacitracin, 0.5 mg/ml; and appropriate amounts of the different labeled and unlabeled ligands used. The reaction was initiated by the addition of washed membranes and stopped 30 min later by addition of 4 ml of ice-cold Tris-HCl buffer, 25 mM, pH 7.4, containing $MgCl_2$, 10 mM. The diluted samples were filtered and washed on Gelman filters (GA-3, 1.2 μm) as previously described (9). Nonspecific binding was determined in the presence of an excess of unlabeled ligand (200-fold the corresponding dissociation constant).

Determination of phosphorylase activity in isolated hepatocytes. Experiments were conducted as indicated (10). Briefly, hepatocytes were isolated from female Wistar rats (250–300 g) by collagenase treatment and incubated at a concentration of 3 × 10^6 cells/ml in Eagle's medium complemented with gelatin, (1.5%) and bacitracin (1 mg/ml). For the determination of phosphorylase activation by AII and AII analogues, cells were incubated for 1 min in the presence of the active peptide. The reaction was stopped by mixing 50 μl of the cell suspension with 200 μl of ice-cold buffer containing: sodium fluoride, 100 mM; EDTA, 20 mM; glycylglycine, pH 7.4, 50 mM; and glycogen, 0.5%. Samples were immediately frozen in liquid nitrogen and stored until they were assayed for phosphorylase *a* activity. When the effects of angiotensin antagonists were tested, cells were incubated for 2 min in the presence of antagonist before stimulation by AII.

Adenylate cyclase assay. Rat liver membranes (60 μg of protein) were incubated in a total volume of 100 μl of a medium containing: NaCl, 200 mM; GTP, 0.1 mM; cAMP, 1 mM; isobutylmethylxanthine, 1 mM; triethanolamine, pH 7.4, 50 mM; $MgCl_2$, 3 mM; creatine kinase, 1 mg/ml; creatine phosphate, 1.5 mg/ml; adenosine deaminase, 10 units/ml; myokinase, 36 units/ml; and [^{3}H]cAMP, 3–5 × 10^3 dpm. Membranes were incubated for 5 min in the presence of the agent to be tested. The reaction was then initiated by adding ATP (50 μM, final concentration) and [α-^{32}P]ATP (1 μCi/assay). The reaction was stopped 10 min later by adding 1 ml of a medium containing: Tris-HCl, pH 7.4, 50 mM; cAMP, 0.5 mM; ATP, 0.3 mM; and SDS, 2% (v/v). Labeled cAMP was separated by chromatography on Dowex and alumine columns as described previously (11).

Covalent labeling of angiotensin receptors with photoactivable ligands. Rat liver membranes (2–4 mg of protein/ml) were incubated with 5–15 nM ^{125}I-$SarN_3PheAII$ or ^{125}I-$SarN_3DPheAII$ at 30° in 1.0 ml of a medium composed of triethanolamine/HCl, pH 7.4, 25 mM; $MgCl_2$, 10 mM; bovine serum albumin, 2 mg/ml; and bacitracin, 0.5 mg/ml. The reaction was initiated by the addition of washed membranes and stopped 30 min later by cooling the sample in ice. Free labeled ligand was eliminated by centrifugation at 12,000 rpm for 1 min in an Eppendorf 5414 centrifuge. The loose membrane pellet was washed three times by the same method in a buffer (washing buffer) containing triethanolamine/HCl, pH 7.4, 25 mM; $MgCl_2$, 10 mM; bovine serum albumin, 2 mg/ml; and soybean trypsin inhibitor, 50 μg/ml. The

final pellet was resuspended in 3 ml of the washing buffer, layered in a 10-ml Petri dish, and irradiated for 30 min at 0° with UV light (mercury vapor lamp HPW 125 purchased from Phillips). The lamps were at 5 cm from the bottom of the uncovered Petri dish. After photolysis the membranes were washed by centrifugation. Nonspecific binding was determined in the presence of an excess of unlabeled ligand (1–10 μM) in the incubation medium. It was checked that the photoactivation procedure ensured complete photolysis of the ligand and did not cause damage to liver membranes. The UV absorption spectrum of Sar-N_3PheAII exhibited a peak at 250 nm. This peak was reduced in a time-dependent manner and was completely abolished after 30 min of irradiation. Membranes irradiated for 30 min in the absence of ligand and then incubated in the described binding assay conditions were found able to bind labeled [^{3}H]SarAII as efficiently as control nonirradiated membranes (results not shown).

Solubilization of liganded angiotensin receptors. After labeling angiotensin receptors and washing, the membranes were resuspended and incubated for 45 min at 0° in 1.5 ml of a medium containing triethanolamine/HCl, 25 mM, pH 7.4; $MgCl_2$, 10 mM; NaCl, 50 mM; bovine serum albumin, 2 mg/ml; soybean trypsin inhibitor, 50 μg/ml; and Triton X-100, 0.3% (v/v). After incubation with detergent, a nonsedimentable fraction was separated by centrifugation for 5 min at 12,000 rpm in an Eppendorf 5414 centrifuge. Two procedures were used to dissociate noncovalently bound radioactivity in solubilized samples. 1) The nonsedimentable fraction of solubilized membranes was incubated for 90 min in the presence of 20 mM EDTA and 50 mM Tris-maleate buffer, pH 6.0. The EDTA-treated material was boiled for 2 min in the presence of 2% SDS.

Separation of free from bound ligand in solubilized samples. In most experiments this separation was achieved by filtration through Bio-Gel P-30 columns (0.7 × 6 cm). Details of the separation procedure are as described by Roy *et al.* (12). In brief, 0.3 ml of solubilized material was layered on top of a Bio-Gel P-30 (100–200 mesh) column preequilibrated at 4° with a medium of the same composition except for the presence of membrane proteins. When the sample had entered the gel, 1.1 ml of elution buffer was added. The total amount of the effluent corresponding to the void volume of the column (1.4 ml) was collected directly in a counting vial. On these columns, free angiotensin began to elute with a volume of 1.8–2.0 ml. Therefore, this rapid procedure was efficient to separate hormone-receptor complexes from free hormone. However, we got confusing results when the filtration was performed after the aliquots were boiled 2 min in the presence of 2% SDS. A great proportion (65%) of the free ligand was eluted from the Bio-Gel with the void volume, probably due to association with SDS micella. In this case, the free ligand was eliminated by the use of dextran-coated charcoal. In brief, 1.0 ml of dextran-coated charcoal (0.07% dextran T-70, 0.7% Norit A) was added to 150 μl of solubilized membranes. After 10 min of incubation at 0°, the charcoal was spun at 2000 × *g* for 10 min. By this method 99% of the free ligand added to a sample of nonlabeled solubilized membranes was precipitated with the charcoal pellet. In samples derived from labeled membranes, it was checked that the radioactivity remaining in the supernatant of the charcoal precipitation was not SDS micella-associated free ligand. For this purpose, 1.0 ml of 0.1% γ-globulins and 1.2 ml of 24% polyethylene glycol were added to the supernatant of the charcoal precipitation step. After 30 min of incubation at 0° the mixture was spun at 2000 × *g* for 30 min. Nearly all of the radioactivity (88–90% corresponding to the yield of the method) was precipitated with polyethylene glycol. Under the same conditions the free ligand was not precipitated.

Gel filtration of soluble extracts. An Ultrogel ACA 34 column (1.6 × 40 cm) was equilibrated with an elution buffer containing triethanolamine/HCl, pH 7.4, 50 mM; $MgCl_2$; 10 mM; and Triton X-100, 0.1% (v/v). The solubilized material (1.0–1.5 ml) was layered on top of the column together with the following markers: β-galactosidase, catalase, cyctochrome *c*, alcohol dehydrogenase, tritiated water, and phage T4. The flow rate was 10 ml/hr. Fractions (0.8 ml) were collected and the amount of soluble hormone-receptor complex in each fraction was determined. The elution volumes of bound radioactivity and markers were expressed in terms of K_{av} according to the method of Laurent and Killander (13). Assay conditions and values for the hydrodynamic parameters of markers were as specified by Guillon *et al.* (14).

SDS-gel electrophoresis of covalently labeled angiotensin receptors. After photoaffinity labeling of angiotensin receptors and washing the membranes (see above), the membrane pellet was resuspended and incubated for 60 min at 30° in a medium containing 50 mM Tris-HCl, pH 6.8; 10% (v/v) SDS; 0 or 5% (v/v) β-mercaptoethanol; and 10% (v/v) glycerol. Gel electrophoresis under denaturating conditions was performed on 10% acrylamide gels in the discontinuous buffer system described by Laemmli (15). Staking gels were 5% acrylamide. Gels were stained for protein in 0.1% Coomassie brilliant blue, dissolved in 50% trichloroacetic acid, and destained in 10% (v/v) methanol, 7% (v/v) glacial acetic acid, and water. Autoradiograms (XAR5 films from Kodak) were obtained after 16 hr or more exposure at −80°.

Protein determination and radioactivity measurements. Proteins were estimated by the method of *Lowry et al.* (16) using bovine serum albumin as standard. Radioactivity measurements were performed by liquid scintillation spectrometry (^{3}H) and gamma spectrometry (^{125}I).

Results

The first series of experiments was designed to define the biological and binding properties of the iodinated forms of the two azido derivatives of angiotensin. I-SarN_3PheAII and I-SarN_3DPheAII. The labeled derivatives ^{125}I-SarN_3PheAII and ^{125}I-SarN_3DPheAII interacted with a single population of sites on rat liver membranes as revealed by linear Scatchard plots of the dose-binding curves (Fig. 2). The mean K_d values ± SD (number of experiments indicated in parentheses) were: 0.18 ± 0.05 (9) and 0.37 ± 0.16 nM (6) for ^{125}I-SarN_3PheAII and ^{125}I-SarN_3DPheAII, respectively. The maximal binding capacity was 1.13 ± 0.16 pmol/mg of protein (17 independent determinations). To ensure that the two ligands were interacting with the same population of receptor sites, inhibition experiments were performed using ^{125}I-SarN_3PheAII, ^{125}I-SarN_3DPheAII, [^{3}H]SarAII (a nonphotoactivable ligand of angiotensin receptors), and the corresponding unlabeled peptides. For each labeled ligand, dose-dependent inhibition by the homologous and heterologous unlabeled ligands was measured. As exemplified in Fig. 3, in the case of [^{3}H]SarAII and ^{125}I-SarN_3PheAII, the binding of each labeled ligand was inhibited to the same maximal extent by the corresponding and other unlabeled ligands. For ^{125}I-SarN_3PheAII and ^{125}I-SarN_3DPheAII, the nonspecific component of total binding was very low. It increased linearly with the concentration of free ligand in the incubation medium but never exceeded 10% for a concentration of free ligand leading to a fractional receptor occupancy of 0.8.

I-SarN_3PheAII activated phosphorylase from isolated hepatocytes and inhibited the adenylate cyclase activity from rat liver membranes in a dose-dependent manner (Fig. 4). Maximal responses elicited by I-SarN_3PheAII and AII were of similar magnitude. The apparent K_{act} values for adenylate cyclase inhibition and phosphorylase activation by I-SarN_3PheAII were 0.8 and 0.05 nM, respectively. I-SarN_3DPheAII produced a slight and dose-dependent activation of phosphorylase the maximal magnitude of which did not exceed 15% of the maximal response elicited by AII (Fig. 4). No detectable adenylate cyclase inhibition by I-SarN_3DPheAII could be observed. I-SarN_3DPheAII inhibited AII-induced responses in a competitive manner. The inhibition constants (K_i) derived from the experiments shown in Fig. 4 were 4 nM for both of the responses

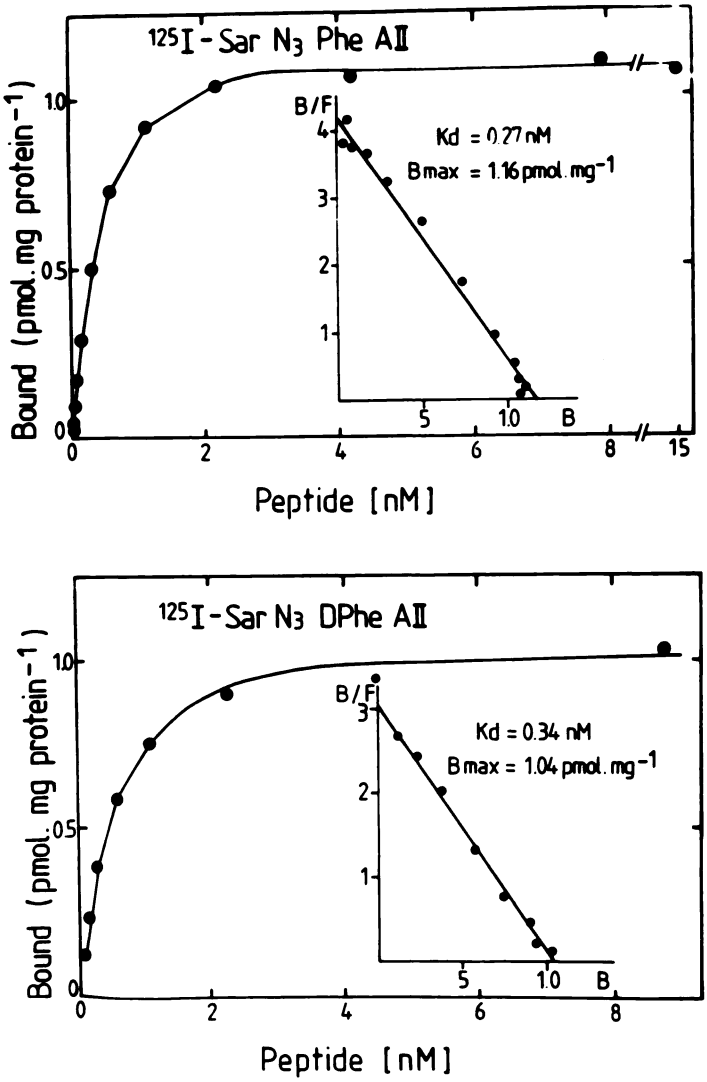

Fig. 2. Dose-dependent binding of ^{125}I-SarN$_3$PheAII and ^{125}I-SarN$_3$DPheAII to rat liver membranes. The binding experiments were conducted as indicated under Materials and Methods. Values on the graphs are means of three determinations. The Scatchard plots of the dose-binding curves are shown in the *insets*.

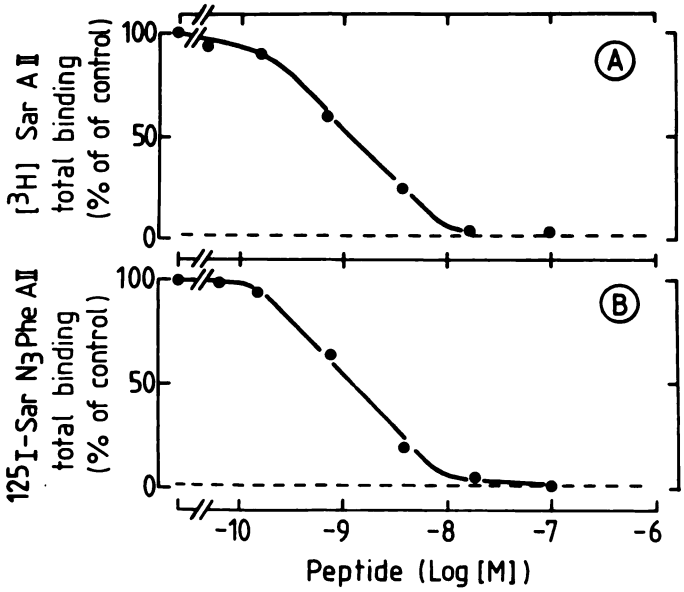

Fig. 3. Dose-dependent inhibition of ^{125}I-SarN$_3$PheAII and [^{3}H]SarAII binding by homologous and heterologous unlabeled ligands. A. Membranes (50 μg of protein) were incubated in the presence of a constant amount of [^{3}H]SarAII (0.5 nM) and of the indicated amounts of unlabeled I-SarN$_3$PheAII. - - - the nonspecific component of total binding determined in the presence of a large excess (2.5 μM) of unlabeled SarAII. B. A symmetrical experiment was performed using ^{125}I-SarN$_3$PheAII (0.2 nM) as the labeled ligand, unlabeled SarAII for the determination of the dose-dependent inhibition of binding, and unlabeled I-SarN$_3$PheAII for the determination of nonspecific binding. The amounts of ligand bound in the absence of competitor were 0.44 and 0.54 pmol/mg of protein in the experiments shown in A and B, respectively. The K_d values deduced from the dose-dependent inhibition experiments were 0.5 nM and 0.6 nM for I-SarN$_3$PheAII and SarAII, respectively.

studied. I-SarN$_3$DPheAII also behaved like an antagonist of the angiotensin-induced stimulation of calcium entry in isolated hepatocytes. I-SarN$_3$PheAII behaved like a full agonist in this biological test (not shown).

A second series of experiments was designed to establish the irreversible character of angiotensin receptor labeling with the photoactivable ^{125}I-SarN$_3$PheAII and ^{125}I-SarN$_3$DPheAII angiotensin analogues. The evolution of bound ^{125}I-SarN$_3$PheAII or ^{125}I-SarN$_3$DPheAII during the process of membrane solubilization with incubation in the presence of EDTA and EDTA plus SDS was studied as illustrated by Fig. 5. Using ^{125}I-SarN$_3$PheAII-labeled membranes, solubilization was accompanied by about a 25% dissociation of bound ligand whether or not membranes were irradiated before solubilization. With control nonirradiated membranes, incubation of the solubilized material at pH 6.0 in the presence of EDTA led to an almost complete and time-dependent dissociation of bound ^{125}I-SarN$_3$PheAII (Fig. 5A). In the case of irradiated membranes, dissociation plateaued at about 50%. Furthermore, boiling the sample for 2 min in the presence of 1% SDS did not lead to additional dissociation. Using ^{125}I-SarN$_3$DPheAII (Fig. 5B), an almost complete dissociation occurred upon solubilization of nonirradiated membranes. In irradiated membranes the dissociation was far less pronounced (about 40%) and was not markedly increased during the further incubation with EDTA and boiling in the presence of SDS. If one considers that SDS-resistant binding of ^{125}I-SarN$_3$PheAII and ^{125}I-SarN$_3$DPheAII does represent covalently bound ligand, the above-described results indicate that these two ligands allow the photoaffinity labeling and solubilization of liver membrane angiotensin receptors with an overall yield close to 50% (with reference to the amount of ligand initially bound to the membranes). Experiments similar to those described in Fig. 5 were performed using membranes exposed to various concentrations of ^{125}I-SarN$_3$PheAII and ^{125}I-SarN$_3$DPheAII in order to estimate the influence of receptor fractional occupancy on the yield of photoaffinity labeling. Results shown in Table 1 indicate that, for receptor fractional occupancy varying from 0.1 or less to 0.9, the overall yield of photoaffinity labeling with ^{125}I-SarN$_3$PheAII and ^{125}I-SarN$_3$DPheAII was independent of fractional occupancy and was close to 40%.

In order to further validate the photoaffinity labeling procedure, we determined some of the physicochemical properties of the soluble material covalently labeled with ^{125}I-SarN$_3$PheAII or ^{125}I-SarN$_3$DPheAII. Fig. 6 shows the elution profiles from the Ultrogel ACA 34 column of angiotensin receptors covalently labeled with ^{125}I-SarN$_3$PheAII or ^{125}I-SarN$_3$DPheAII. After labeling with the photoactivable antagonist, bound radioactivity was eluted as a major peak corresponding to a component with

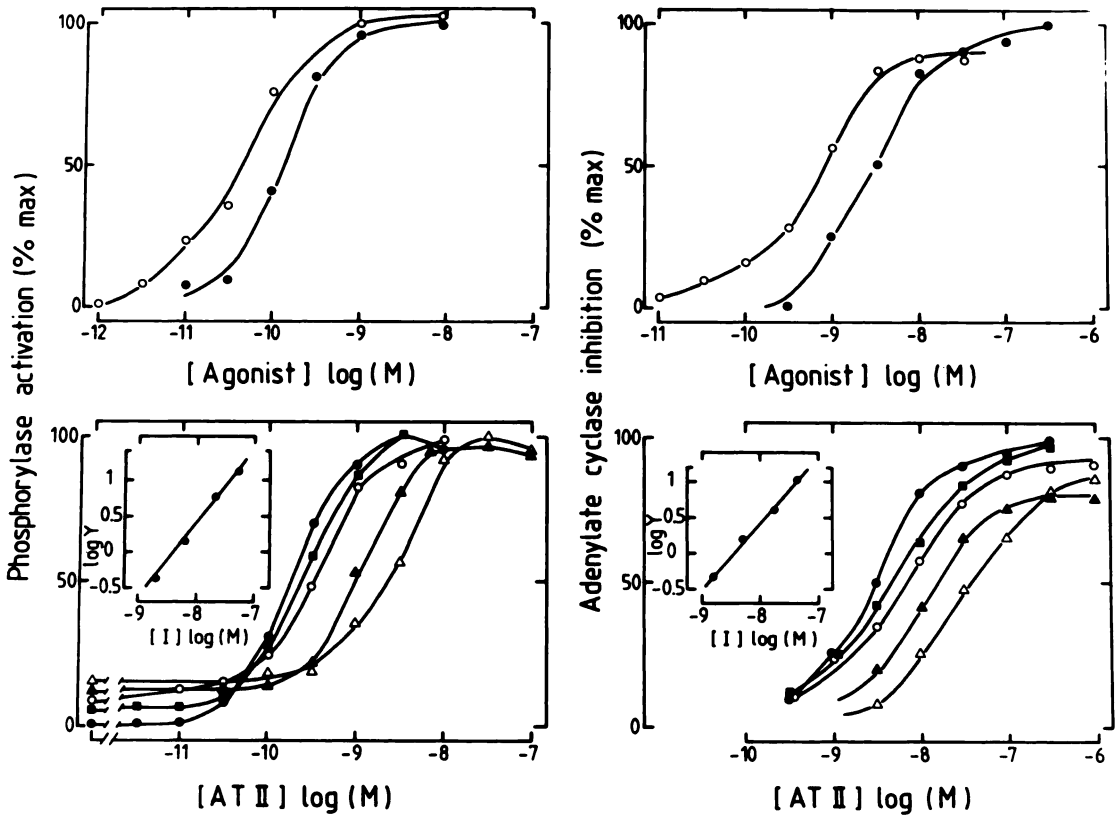

Fig. 4. Biological responses to I-SarN_3PheAII and I-SarN_3DPheAII. *Left upper panel.* Dose-dependent activation of phosphorylase in isolated hepatocytes by I-SarN_3PheAII (○) and AII (●). The responses are expressed as percentage of the maximal response induced by AII. Basal and maximally stimulated phosphorylase activities were 7.6 and 49.7 nmol/min/mg dry weight, respectively. *Right upper panel.* Dose-dependent inhibition of adenylate cyclase activity from rat liver membranes by I-SarN_3PheAII and AII. The results are expressed as percentage of the maximal inhibition induced by AII. Basal and maximally inhibited activities were 9.2 and 3.4 pmol of cAMP formed/min/mg of protein. *Lower panels.* Dose-dependent phosphorylase activation and adenylate cyclase inhibition by AII were determined in the absence (●) and the presence of increasing amounts of I-SarN_3DPheAII: 1.8 (■), 5.4 (○), 16.2 (▲), and 50 nM (△). In the *insets* $Y = [1 - (K_{app}/K_{act})]$, in which K_{app} and K_{act} are the AII concentrations eliciting half-maximal responses in the presence and absence of antagonist, respectively, is plotted as a function of the concentration of antagonist ([*I*]). For the experiment shown on the *lower left panel* *Y* was corrected by the factor, 50/(50 − 15.3) to take into account the intrinsic activity of the antagonist. All values in the figure are means of four determinations derived from two independent experiments.

a Stoke's radius very close to that found after labeling with a reversible antagonist. The mean value of 5.5 ± 0.2 nm (mean ± SD of three determinations) determined in the present study is not different from that of 5.6 nm derived from a previous study using the reversible angiotensin antagonist (Sar^{1}Ile^8)-angiotensin II (17). When using the photoactivable agonist ^{125}I-SarN_3PheAII, bound radioactivity was eluted as a broad and very probably heterogeneous peak.

The results of a typical SDS-gel electrophoresis of the covalently labeled material are shown in Fig. 7. Autoradiograms indicate that the major part of radioactivity migrated as a fairly large band. This band was not detectable on samples derived from membranes incubated in the presence of an excess of unlabeled ligand. The migration distance was the same whether the receptor was labeled with an agonist or with an antagonist. The mean values of the estimated molecular weight of the main radioactive component were 64,300 ± 2,300 (5) and 62,300 ± 2,800 (4), after labeling with ^{125}I-SarN_3PheAII and ^{125}I-SarN_3DPheAII, respectively.

Discussion

The labeled ^{125}I-SarN_3PheAII and ^{125}I-SarN_3DPheAII bind to liver membrane with high affinity. The observed dissociation constants are lower than that determined for angiotensin itself: 1.5 ± 0.7 nM (17). Clearly, ^{125}I-SarN_3PheAII and ^{125}I-SarN_3DPheAII interacted with the population of sites labeled with other well characterized ligands of angiotensin receptors. Importantly enough, the nonspecific binding of ^{125}I-SarN_3PheAII and ^{125}I-SarN_3DPheAII was very low (less than 10% for a fractional receptor occupancy of 0.8). This was indeed a prerequisite in an attempt to specifically and covalently label a large fraction of the total number of angiotensin receptors present on liver membranes. We found that the antagonist, ^{125}I-SarN_3DPheAII, labeled a single population of binding sites on liver membranes. This observation confirms the conclusions from previously published work (9) and the report by Campanile *et al.* (18) that the radioiodinated antagonist [^{125}I]-saralasin labeled a single population of sites on rat liver membranes. The results obtained with the radioiodinated agonist, ^{125}I-SarN_3PheAII, are at variance with those reported by Campanile *et al.* (18) and more recently by Gunther (19). These authors, using radioiodinated AII, found two categories of binding sites (of high and low affinity). Since agonist binding to angiotensin receptors has been shown (9, 18) to be sensitive to several effectors (guanyl nucleotides, sodium, magnesium, and other divalent cations), differences in the composition of the incubation media used might account for the apparent discrepancy between our results and those of Gunther (19) and Campanile *et al.* (18).

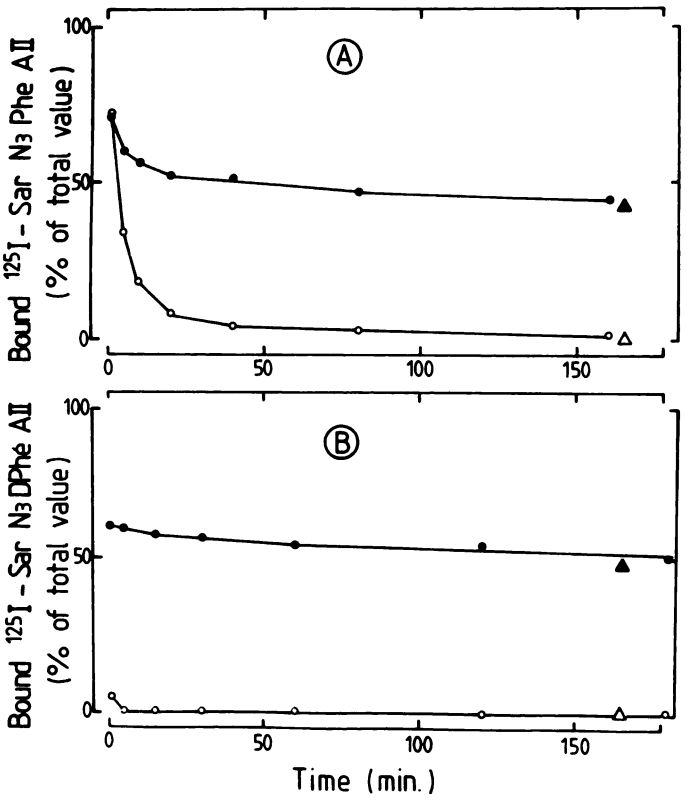

Fig. 5. Photoaffinity labeling of liver angiotensin receptors with ^{125}I-SarN$_3$PheAII and ^{125}I-SarN$_3$DPheAII. Rat liver membranes (5 mg) were incubated for 30 min at 30° in the presence of 0.6 nM ^{125}I-SarN$_3$PheAII (A) or ^{125}I-SarN$_3$DPheAII (B). Membranes were washed to eliminate unbound ligand and were separated into two samples, one of which was photolyzed for 30 min at 4°, and the other kept at 4° in the absence of UV light. The two samples were then solubilized with 0.3% Triton X-100 and centrifuged. The solubilization yields (percentage of the radioactivity initially bound to the membranes) were 77 (control) and 73 (photolyzed), and 84 (control) and 80% (photolyzed) for experiments shown in A and B, respectively. The supernatants were incubated at 30° in the presence of EDTA (20 mM) at pH 6.0. At the indicated times, triplicate aliquots were removed, and the amount of bound radioactivity was determined as indicated under Materials and Methods. The figure shows the decrease of bound radioactivity as a function of incubation time at pH 6.0 in the presence of EDTA for the photolysed (●) and non-photolysed (○) samples. All values are expressed as percentage of total radioactivity present in solubilized membranes, i.e., the sum of bound ligand and free ligand resulting from partial dissociation during the process of membrane solubilization. At the end of the experiments aliquots were removed and boiled for 2 min in the presence of 2% SDS, and the radioactivity remaining bound was determined. ▲, △, experimental values.

I-SarN$_3$PheAII and AII had similar efficacies in stimulating phosphorylase in isolated hepatocytes and inhibiting adenylate cyclase activity from liver membranes. Data shown in Fig. 4 confirm that dose-dependent phosphorylase activation by angiotensin agonists occurs in a lower concentration range than dose-dependent adenylate cyclase inhibition (18–22). The K_{act} values for adenylate cyclase inhibition and phosphorylase activation by I-SarN$_3$PheAII did not differ by more than a factor of 4 from the corresponding K_d value for binding to liver membranes. Taking into account that these parameters were determined under different experimental conditions, these results are compatible with the hypothesis that the specific binding sites which were labeled by ^{125}-SarN$_3$PheAII are the receptors mediating the biological responses to angiotensin in the liver.

TABLE 1

Solubilization and labeling yields of liver angiotensin receptors

Rat liver membranes (2–5 mg/assay) were incubated for 30 min at 30° in the presence of various amounts of ^{125}I-SarN$_3$PheAII or ^{125}I-SarN$_3$DPheAII (fractional occupancy ranging from 0.1 to 0.9). Membranes were washed and the membrane-bound radioactivity was determined. Membranes were then irradiated with UV light and solubilized by the use of Triton X-100 and centrifuged. Bound radioactivity present in the supernatant was measured after incubation for 1 hr at 30° in the presence of 20 mM EDTA at pH 6.0. Values are means ± standard deviations for the number of experiments indicated in parentheses.

	Photoaffinity labeling with:	
	^{125}I-SarN$_3$PheAII	^{125}I-SarN$_3$DPheAII
Bound to membranes (pmol/mg of protein)	0.07–0.9	0.2–1.3
Present in supernatant fraction of solubilized membranes (% of bound to membranes)[a]	79 ± 8 (9)	84 ± 5 (5)
Bound in EDTA-treated supernatant of solubilized membranes[b]		
% of supernatant	48 ± 4 (9)	50 ± 3 (5)
% of bound to membranes[c]	38 ± 3 (9)	42 ± 2 (5)

[a] Represents the sum of solubilized ligand-receptor complexes and free ligand resulting from partial dissociation during membrane irradiation and solubilization.
[b] Resistant to boiling in the presence of SDS (see Fig. 3).
[c] Represents the overall yield of the photoaffinity labeling procedure.

I-SarN$_3$DPheAII behaved as an almost pure competitive antagonist of angiotensin for the two well documented biological effects of angiotensin on liver, i.e., activation of the calcium-dependent regulatory pathway of glycogen phosphorylase (20, 21) and inhibition of liver membrane adenylate cyclase (22). This observation confirms the crucial role of phenylalanine in position 8 in the intrinsic biological activity of AII and AII analogues (23). Unexpected was the observation that the K_i values for inhibition of angiotensin-induced phosphorylase activation and adenylate cyclase inhibition were about 10 times higher than the K_d value determined on liver membranes. A possible explanation for this discrepancy is the fact that, under the experimental conditions used for measuring K_i values (short incubation periods in the presence of antagonist, see Materials and Methods), binding of I-SarN$_3$DPheAII probably did not reach its equilibrium value. In any case, experiments illustrated in Fig. 3 clearly indicate that I-SarN$_3$PheAII and I-SarN$_3$DPheAII labeled the same population of sites on liver membranes that was also labeled by SarAII.

The covalent character ^{125}I-SarN$_3$PheAII or ^{125}I-SarN$_3$DPheAII binding to the EDTA-treated solubilized material derived from labeled and irradiated membranes seems clearly established by the fact that it was preserved during denaturation at high temperature in the presence of SDS and resisted SDS-gel electrophoresis under reducing and nonreducing conditions.

The results derived from the gel filtration experiments illustrated in Fig. 6 deserve special comments. We previously showed (17) that the apparent molecular size of liver angiotensin receptors labeled with reversible agonists is higher than that of receptors labeled with a reversible antagonist (mean Stokes radii of 6.2 ± 0.1 and 5.5 ± 0.1 nM, respectively). The most likely explanation for this difference was to assume that agonists but not antagonists stabilize an interaction between the receptor and another membrane component. There is now

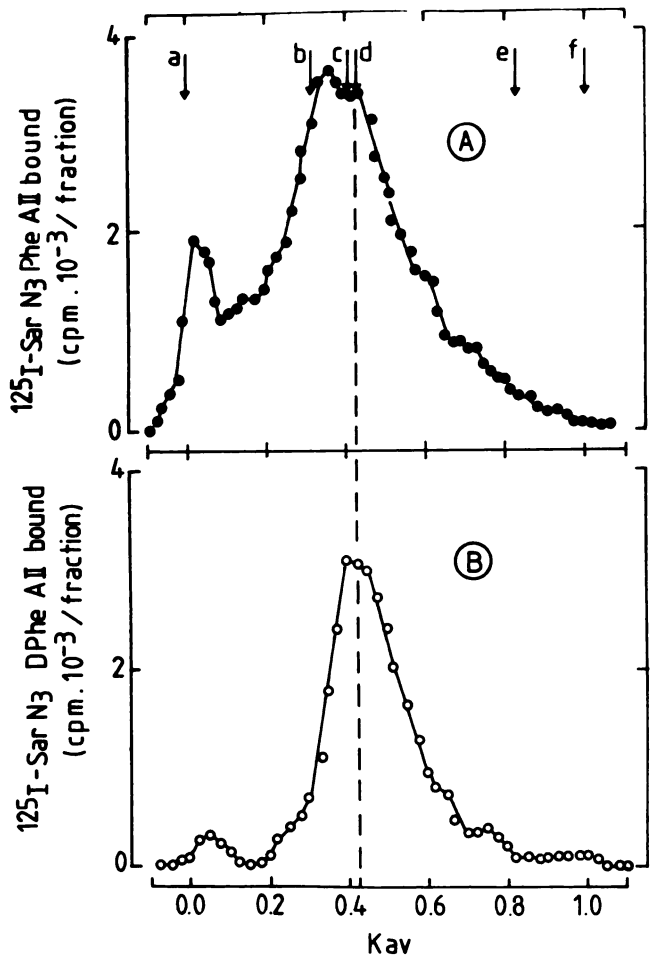

Fig. 6. Gel filtration of ^{125}I-SarN$_3$PheAII- and ^{125}I-SarN$_3$DPheAII-labeled angiotensin receptors. Rat liver membranes were preincubated in the presence of ^{125}I-SarN$_3$PheAII (A) or ^{125}I-SarN$_3$DPheAII (B) for 30 min at 30°, washed three times to eliminate free radioactivity, and irradiated for 30 min at 4° with UV light before solubilization with 0.3% Triton X-100. The solubilized extracts were incubated for 60 min at 30° in the presence of EDTA (20 mM) to dissociate noncovalently bound ligand. After elimination of the dissociated free ligand by dextran-coated charcoal precipitation, the solubilized extracts together with calibration markers were layered on the top of an Ultrogel ACA 34 column (1.6 × 40 cm). Gel filtration was performed at 4° for 9 hr at a flow rate of 10 ml/hr. The figure shows the elution profile of ^{125}I radioactivity. *Arrows* indicate the K_{av} values of the calibration markers used: *a*, phage T4; *b*, β-galactosidase; *c*, catalase; *d*, alcohol dehydrogenase; *e*, cytochrome *c*; and *f*, tritiated water.

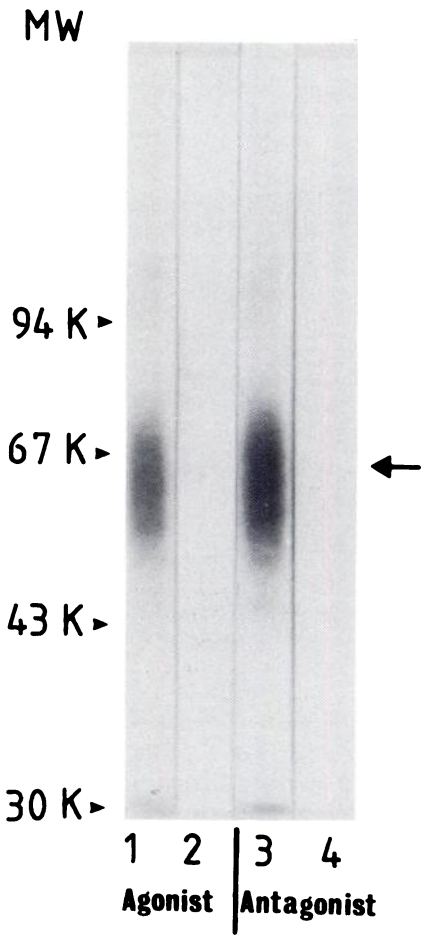

Fig. 7. SDS-gel electrophoresis of covalently labeled angiotensin receptors from rat liver. Four samples of rat liver membranes were incubated in the presence of 10 nM ^{125}I-SarN$_3$PheAII (samples *1* and *2*) or ^{125}I-SarN$_3$DPheAII (samples *3* and *4*) added alone (samples *1* and *3*) or together with unlabeled SarAII (1 μM) (samples *2* and *4*). All samples were irradiated and solubilized with SDS (2%). Electrophoresis was conducted as indicated under Materials and Methods. Protein standards (Pharmacia) of the indicated molecular weights were run in parallel. The figure shows the autoradiogram of the gel.

evidence (23)[2] that this component might be a GTP-binding protein involved in the coupling of angiotensin receptor to its effector, the catalytic component of adenylate cyclase, and/or the primary component involved in the process of angiotensin-induced calcium mobilization. We show in the present study that the antagonist ^{125}I-SarN$_3$DPheAII covalently labeled a macromolecular component of a size closely similar, if not identical, to that of the solubilized receptor prelabeled with a reversible antagonist. These results suggest that the two ligands label the same molecular species. In contrast, the photoactivable agonist ^{125}I-SarN$_3$PheAII labeled an apparently heterogeneous population of molecules. The elution profile obtained with ^{125}I-SarN$_3$PheAII-labeled angiotensin receptors is, in fact, very close to that which one might expect of a mixture of the light (antagonist-labeled) and heavy (agonist-labeled) forms of the receptor. This might indicate that the stability of the putative ternary complex, agonist-receptor-coupling protein, is only partially preserved during photoactivation, solubilization, EDTA treatment, and subsequent steps. On the basis of the observed apparent homogeneity of the ^{125}I-SarN$_3$DPheAII-labeled material, the use of this photoactivable antagonist for receptor purification might be preferred to that of the photoactivable agonist.

SDS-polyacrylamide gel electrophoresis of ^{125}I-SarN$_3$PheAII- and ^{125}I-SarN$_3$DPheAII-labeled angiotensin receptors revealed a single band of M_r 64,000. This value is close to those of 65,000 and 68,000 reported by Capponi and Catt (4) for uterine and adrenal angiotensin receptors, respectively. These authors also reported the existence of a heavy (Stoke's radius of 6.0 ± 0.5 nm) and light (Stoke's radius of 3.9 nm) forms of these receptors. They suggested that uterine and adrenal angiotensin receptors might have a dimeric structure. Our results are compatible with the hypothesis proposed by Capponi and Catt (4) that the AII receptor might be a dimer of 64,000 M_r binding

[2] G. Guillemette, G. Guillon, J. Marie, M.-N. Balestre, E. Escher, and S. Jard, manuscript in preparation.

units. They obviously do not exclude a heterologous polymeric structure.

The major contribution of the present work was to demonstrate that ^{125}I-SarN_3PheAII and ^{125}I-SarN_3DPheAII allow photoaffinity labeling of angiotensin receptors with a much higher yield than that obtained with other photoactivable angiotensin analogues or bifunctional reagents. The higher yield of photoaffinity labeling obtained with the N_3Phe^8 and N_3DPhe^8 derivatives of AII as compared to other azido derivatives could be related to the position at which the azido group was introduced. As already mentioned, the phenylalanyl residue in position 8 of the angiotensin molecule is very important for biological activity. Indeed, replacement of phenylalanine in position 8 by several amino acids such as valine, isoleucine, or D-phenylalanine (this study) confer antagonistic properties to the modified angiotensin molecule. This might indicate that the phenylalanine residue of bound angiotensin normally sits in very close vicinity to part of the receptor. Photogeneration of reactive intermediates from the *p*-azidophenyl groups has been the subject of several investigations (see, for instance, Ref. 24). These investigations showed that the singlet nitrenes, which are probably the primary photoproducts, rapidly rearrange to either benzazirines or cycloazaheptatetraenes. These nonradical species react within msec at nucleophilic sites of the substrates, e.g., with groups like =NH, −SH, or −OH. The presence of such a highly reactive group in the AII receptor site at the crucial locus corresponding to the eighth position of the hormone may probably explain the high labeling yield obtained.

Nevertheless, the described procedure allows the recovery of about 40% of the total number of angiotensin receptors present on liver membranes in a soluble, covalently labeled, and apparently homogeneous form. It is therefore conceivable that purification procedures of angiotensin receptors can be developed based on the purification of the labeled receptor molecules by standard biochemical techniques or indirect affinity chromatography using probes able to recognize the covalently bound ligand.

Acknowledgments

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