Characterization of AT_4 Receptor from Bovine Aortic Endothelium with Photosensitive Analogues of Angiotensin IV†

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ABSTRACT: Newly developed photosensitive analogues of AngIV were used to characterize the AT_4 receptor of bovine aortic endothelial cells. The photoactivatable AngIV analogues [N_3-Phe^6]AngIV and [Bpa^6]-AngIV displayed high affinities for AT_4 receptor, with IC_{50}'s of 3.7 ± 0.3 and 19.1 ± 3.5 nM, respectively. The radioiodinated ligands showed a good efficiency of photoaffinity labeling demonstrated by high proportions (60−75%) of acid-resistant binding. Covalently labeled receptor was solubilized under reducing or nonreducing conditions and subjected to SDS−PAGE. Under nonreducing conditions, autoradiographies revealed a major band of M_r 186 ± 2 kDa and a minor band of M_r 241 ± 6 kDa. The labeling of these bands was completely abolished in the presence of 10 μM AngIV. Under reducing conditions, only the low M_r 186 kDa band was revealed. After endoglycosidase digestion with an enzyme that cleaves N-linked saccharides, the M_r of the denatured AT_4 receptor was decreased by 31% to a value of 129 ± 10 kDa. Kinetic studies revealed a stepwise process of AT_4 receptor deglycosylation by endoglycosidase F, suggesting at least two different sites of N-linked saccharides. Mild trypsin treatment of photolabeled endothelial cell membranes released a large fragment of M_r 177 ± 3 kDa which accounts for about 95% of the whole receptor molecular mass. These results demonstrate that [N_3-Phe^6]AngIV and [Bpa^6]AngIV are very efficient tools for selective photoaffinity labeling of AT_4 receptor. We have shown that AT_4 receptor is a 186 kDa integral membrane glycoprotein with a very large extracellular domain. These properties are consistent with those of a growth factor or cytokine receptor.

Angiotensin II (AngII)[1] is an octapeptide hormone (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) that plays an important role in the regulation of the cardiovascular system. AngII produces its effects by activating two distinct classes of receptors designated AT_1 and AT_2 (*1*). Until a few years ago, fragments of angiotensin II (AngII) smaller than the heptapeptide (*2*−*8*) fragment (or AngIII) were thought to be biologically inactive and of little physiological significance. The C-terminal 3−8 hexapeptide fragment of AngII, with the amino acid sequence Val-Tyr-Ile-His-Pro-Phe (AngIV), was recently shown to possess a biological activity in the central nervous system by interacting with a unique binding site (*2*). These observations have now been extended, and high-affinity binding sites for AngIV have been described in many different tissues from various species (*3*−*7*).

Several potential functions have been attributed to AngIV. In the central nervous system AngIV is a modulator of memory acquisition and of exploratory behavior in rats, mice and crabs (*8*−*10*). AngIV was also shown to antagonize the angiotensin II-induced hypertrophy in cultured chick myocytes (*11*). This effect was independent of any interaction with AT_1 or AT_2 receptors for AngII. Although there are presently some controversies, AngIV was also implicated in the regulation of blood flow. On one hand, when infused into the renal artery, AngIV increased renal cortical blood flow (*12*), and when topically applied in the brain, AngIV potentiated L-arginine-dependent vasodilatation of rabbit cerebral arterioles (*13*). This latter effect was, however, inhibited by angiotensin AT_1 and AT_2 receptors antagonists (*14*). On the other hand, AngIV caused renal and mesenteric vasoconstrictor effects that were inhibited by losartan (*15*) and pulmonary vasoconstrictor effects that were also inhibited by an AT_1 receptor antagonist (*16*), suggesting that AngIV is a weak agonist of AT_1 receptor. A recent study demonstrated that AngIV stimulates endothelial expression of plasminogen activator inhibitor (*17*). This effect appeared to depend on the stimulation of a specific receptor for AngIV. Other recent studies showed that AngIV increases DNA and RNA synthesis in cultured rabbit cardiac fibroblasts (*18*) and enhances thymidine incorporation in bovine endothelial cells (*19*). These observations strongly suggest the existence of a specific receptor for AngIV which has now been designated the AT_4 receptor (*9*). Despite its high level of expression in several tissues and the numerous indications of its

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[1] Abbreviations: AngII, angiotensin II; AngIII, angiotensin III; AngIV, angiotensin IV; Bpa, benzoylphenylalanine; N_3-Phe, *p*-azidophenylalanine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin; ConA, concanavalin A; PNGase F, *N*-glycopeptidase F; TGFβ, transforming growth factor β; M_r, apparent molecular mass.

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physiological role, the biochemical and molecular properties of AT_4 receptor are still poorly characterized. In the present work, we used newly developed photosensitive analogues of AngIV to better characterize the AT_4 receptor of bovine aortic endothelial cells.

MATERIALS AND METHODS

Reagents. AngIV was purchased from Bachem (Torrance, CA). Nonidet P-40, soybean trypsin inhibitor, bovine serum albumin, concanavalin A-sepharose from *Canavalia ensiformis* (ConA), wheat germ agglutinin lectin from *Triticum vulgaris* (WGA), methyl α-D-mannopyranoside, *N*-acetyl-D-glucosamine, and trypsin (EC 3.4.21.4) were purchased from Sigma (St. Louis, MO). DuP 753 (2-*n*-butyl-4-chloro-5-(hydroxymethyl)-1-[((2′-1*H*-tetrazol-5-yl)biphenyl-4-yl)-methyl]imidazole potassium salt] and PD 123319 [1-[4-(dimethylamino)-3-methylphenyl]methyl-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo[4,5−6]pyridine-6-carboxylic acid] were generous gifts from DuPont Pharmaceuticals and Parke-Davis Warner Lambert, respectively. *N*-Glycopeptidase F (PNGaseF) (EC 3.5.1.52), *O*-glycodisase (EC 3.2.1.97), neuraminidase (EC 3.2.1.18), and phosphatidylinositol phospholipase C (PI-PLC) from *Bacillus cereus* were purchased from Boehringer Mannheim (Laval, PQ). [Bpa^6]-AngIV and [N_3-Phe^6]AngIV were synthesized in our laboratories by the solid phase method and purified by high-performance liquid chromatography as previously described (*20*). ^{125}I-[Bpa^6]AngIV and ^{125}I-[N_3-Phe^6]AngIV (specific radioactivities ∼ 1000 Ci/mmol) were prepared with IODOGEN as described by Fraker and Speck (*21*). Mercury vapor lamps (JC-Par-38, 100 W) were purchased from Westinghouse (Montréal, PQ) and the Raymaster black light filters (no. 5873) from Gates and Co. (Long Island, NY).

Cell Culture. Bovine thoracic aortas were taken out and flushed immediately with ice-cold sterile phosphate-buffered saline (pH 7.4, 3.5 mM NaH_2PO_4, 16.5 mM Na_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 3.5 mM KCl, and 135 mM NaCl). The aortas were cleaned of connective tissue under sterile conditions, opened longitudinally and fixed on a plate with the intimal face upward. The endothelial surface was carefully scraped and then incubated for 30 min at 37 °C in 30 mL (for 4−6 aortas) of M199 medium (pH 7.4) containing 25 mM HEPES, 27 mM $NaHCO_3$, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2.5 μg/mL fungizone, and 1 mg/mL collagenase. After centrifugation at 500*g* for 10 min, the cells were resuspended in 6 mL of Dulbecco's modified Eagle medium (D-MEM) supplemented with 10% fetal bovine serum, 50 U/mL penicillin, 60 μg/mL streptomycin, and 2 mM L-glutamine. The cells were plated in a cell culture flask (25 cm^2, Falcon). The cells were maintained in a 95% air−5% CO_2 humidified incubator at 37 °C. The culture medium was changed the day after seeding. Results presented in these studies were obtained with cells between the 5th and 20th passages.

The cells were identified as endothelial cells, based on their ability to form a typical cobblestone appearance at confluency and by their ability to take up fluorescently labeled acetylated low-density lipoprotein.

Preparation of Bovine Endothelial Cell Membranes. Bovine endothelial cells (grown in 150 mm diameter dishes) were scraped with a rubber policeman and homogenized with six strokes of a Dounce homogenizer (tight pestle) in a medium containing 100 mM NaCl, 5 mM $MgCl_2$, and 25 mM Tris-HCl, pH 7.4. After centrifugation at 35000*g* for 20 min at 4 °C, the pellet was resuspended in the same medium at a concentration of 5−10 mg protein/mL and then heated at 60 °C for 20 min. These preparations were stored at −70 °C.

Binding Assay. Bovine endothelial cell membranes (50 μg of protein) were incubated in a medium containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, and 1 mg/mL bovine serum albumin. Incubations were performed for 2 h at 37 °C in the presence of 0.6 nM ^{125}I-AngIV, ^{125}I-[Bpa^6]AngIV, or ^{125}I-[N_3-Phe^6]AngIV and selected concentrations of nonradioactive ligands, in a final volume of 500 μL. Nonspecific binding was determined in the presence of 10 μM AngIV. Incubations were terminated by vacuum filtration through glass fiber filters (Whatman GF/C) that had been presoaked for 2 h in the binding medium. Filters were washed twice with the same medium and receptor-bound radioactivity was determined by gamma spectrometry.

Photoaffinity Labeling Procedures. Bovine endothelial cell membranes (1 mg of protein) were incubated in the presence of 6 μCi ^{125}I-[Bpa^6]AngIV or ^{125}I-[N_3-Phe^6]AngIV in 1 mL of medium containing 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, and 0.5 mM EDTA. Nonspecific labeling was determined in the presence of 10 μM AngIV. After 90 min at room temperature in the dark, the membranes were washed by centrifugation and irradiated under filtered UV light (365 nm) for 30 min at 0 °C. Under reducing conditions, the irradiated membranes were washed and boiled for 5 min in a denaturing buffer containing 60 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate, 125 mM dithiothreitol, and 0.3% (w/v) bromophenol blue. Under nonreducing conditions, the irradiated membranes were washed and solubilized in a medium containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride and 1% (v/v) Nonidet P-40. After centrifugation at 13000*g* for 10 min (at 4 °C), 10 μL aliquots (corresponding to 50−75 μg of protein) were added to 10 μL of buffer containing 120 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate, and 0.6% (w/v) bromophenol blue. The material was analyzed by SDS−PAGE.

SDS−PAGE Analysis of Solubilized Photolabeled Proteins. Electrophoresis was carried out as described by Laemmli (*22*). Denatured proteins (25−40 μg) were subjected to electrophoresis on a 7.5% polyacrylamide gel at 200 V. Gels were stained with 0.05% Coomassie brilliant blue and dried before autoradiography on Kodak Biomax MS film. Exposures lasted for 2−24 h at −70 °C.

Endoglycosidase Digestion. After photoaffinity labeling, membranes were solubilized in a medium containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride, and 1% (v/v) Nonidet P-40. After centrifugation at 13000*g* for 10 min at 4 °C, 10 μL aliquots of supernatants (corresponding to 50−75 μg of protein) were added to 10 μL of deglycosylation buffer containing 50 mM sodium phosphate, pH 7.2, 0.5% (w/v) Nonidet P-40, 0.2 mg/mL soybean trypsin inhibitor, and 2 mM phenylmethanesulfonyl fluoride. *O*-Glycosidase (40 mU/mL) and neuramidase 30 mU/mL or PNGase F (40 U/mL) were added to

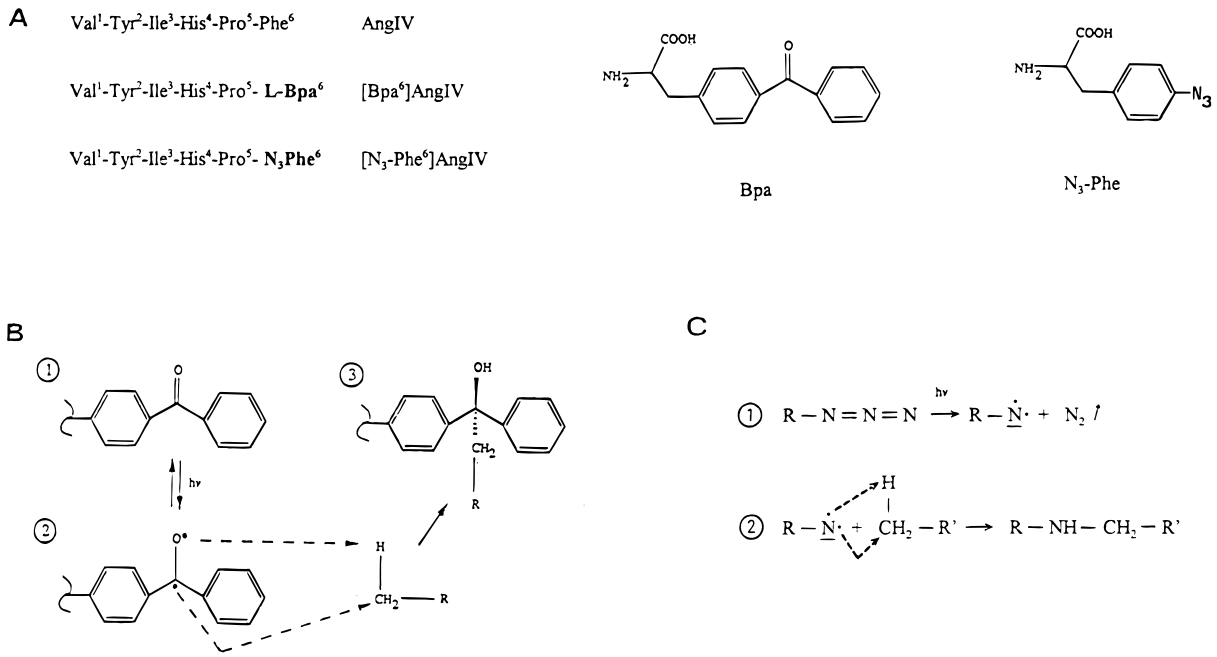

FIGURE 1: (A) Amino acid sequences of AngIV and photoreactive AngIV analogues. Residues represented in bold characters correspond to amino acid modifications. (B and C) Mechanisms of covalent modification following photoactivation. (B) The absorption of a photon at ~350 nm by the Bpa moiety (1) results in the promotion of one electron from a nonbonding sp^2-like n-orbital on oxygen to an antibonding π^*-orbital of the carbonyl group (2). In the diradicaloid triplet state (2), the electron-deficient oxygen n-orbital is electrophilic and therefore interacts with weak C−H bonds (2) to produce benzpinacol-type compounds (3) (*32*). (C) The absorption of a photon at ~350 nm by the azido group results in diradical nitrene generation (1), followed by an insertion reaction which the radical interacts with weak C−H bonds and forms two new covalent bonds (2) (*33*).

a final volume of 25 μL. Incubations were performed at 37 °C for 0−48 h. An equal volume (25 μL) of denaturing buffer (2×) was added before boiling for 5 min. The resulting material was analyzed by SDS−PAGE.

Adsorption to Lectins. Solubilized labeled receptors were incubated under gentle agitation for 18 h at 4 °C, with lectins (WGA or ConA) coupled to agarose beads (about 2 mg of settled protein-coupled gel) in a medium containing 25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 2 mM $MnCl_2$, 2 mM $CaCl_2$, and 0.1% (v/v) Triton X-100. The incubations were terminated by centrifugation at 200*g* for 1 min, and the radioactivity associated with the pellet was determined with a gamma counter. Specific adherence to ConA and to WGA was determined with *O*-D-mannopyranoside (0.5 M) and *N*-acetyl-D-glycosamine (0.5 M), respectively.

Tryptic Treatment of Photolabeled Bovine Endothelial Cell Membranes. Photolabeled membranes (150 μg of protein) were washed with ice-cold binding buffer and treated for different periods of time at 37 °C with different concentrations of trypsin in 100 μL of digestion buffer containing 25 mM Tris-HCl, pH 8.5, 10 mM $CaCl_2$, and 100 mM NaCl. The digestion was stopped with 10 μL of soybean trypsin inhibitor (1 mg/mL), and the samples were centrifuged at 1500*g* for 10 min at 4 °C. Identical results were obtained after centrifugation of the samples at 100000*g* for 1 h. An amount of 100 μL of supernatant (free of membranes) was mixed with 100 μL of denaturing buffer (2×) and boiled for 5 min. The denatured material was analyzed by SDS−PAGE.

PI-PLC Treatment of Photolabeled Bovine Endothelial Cell Membranes. Photolabeled membranes (150 μg of protein) were washed with ice-cold buffer containing 3.5 mM NaH_2PO_4 and 16.5 mM Na_2HPO_4, pH 7.4, and then treated for different periods of time at 37 °C in 100 μL of the same buffer containing 50 mU/mL PI-PLC (phosphatidylinositol phospholipase C). The aliquots were then centrifuged at 1500*g* for 10 min at 4 °C, and 100 μL of supernatants (free of membranes) was mixed with 100 μL of denaturing buffer (2×) and boiled for 5 min. The denatured material was analyzed by SDS−PAGE.

Statistical Analysis. Experimental data resulting from representative experiments are expressed as the mean ± SD of triplicate values. When the error bar is not seen, the symbol is larger than the experimental variation. When needed, the experimental data were analyzed with the Student's *t* test. $p < 0.05$ were considered to be statistically significant.

RESULTS

Figure 1 shows the primary structure of AngIV and photoreactive AngIV analogues used in this study. Phe⁶ was replaced by benzoylphenylalanine (Bpa) to give [Bpa⁶]AngIV or by *p*-azidophenylalanine (N_3-Phe) to give [N_3-Phe⁶]-AngIV. The potency of various peptide and nonpeptide ligands to inhibit ^{125}I-AngIV binding to bovine endothelial cell membranes is shown in Figure 2A. Dose−displacement experiments showed that ^{125}I-AngIV binding was completely abolished in the presence of 1 μM AngIV, [N_3-Phe⁶]AngIV, or [Bpa⁶]AngIV. The three peptides showed potent inhibitory effects, with IC_{50} values of 2.1 ± 0.5, 3.7 ± 0.3, and 19.1 ± 3.5 nM, respectively. DuP 753 and PD 123319, the selective ligands for AT_1 and AT_2 receptors for AngII, had no significant effect on ^{125}I-AngIV binding at concentrations as high as 10 μM. To demonstrate that the photosensitive analogues of AngIV interact exclusively with the AT_4

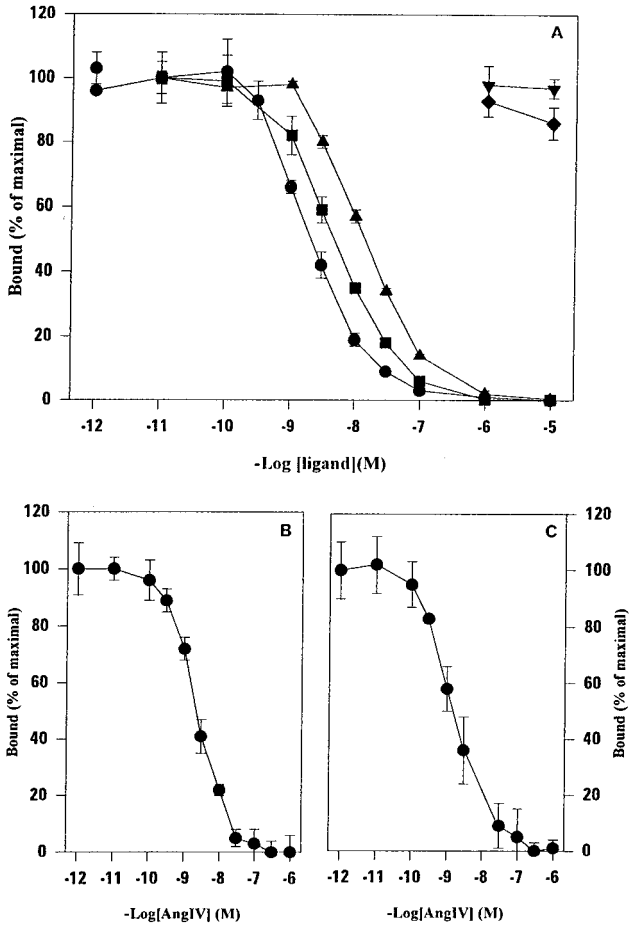

FIGURE 2: (A) Concentration-dependent inhibition of ^{125}I-AngIV binding with increasing concentrations of unlabeled ligands. Membranes (25 μg of protein) were incubated for 2 h at 37 °C with 0.6 nM ^{125}I-AngIV and increasing concentrations of unlabeled ligands: (●) AngIV; (■) [N_3-Phe^6]AngIV; (▲) [Bpa^6]AngIV; (▼) PD 123319; (◆) DuP 753. The data are expressed as values relative to the total binding observed in the absence of unlabeled ligand (26 160 ± 1158 cpm) and corrected for nonspecific binding (2018 ± 218 cpm) in the presence of 10 μM AngIV. (B) Concentration-dependent inhibition of ^{125}I-[N_3-Phe^6]AngIV binding by increasing concentrations of AngIV. Membranes (50 μg of protein) were incubated for 2 h at 37 °C in the presence of 0.6 nM ^{125}I-[N_3-Phe^6]-AngIV and increasing concentrations of unlabeled AngIV. The data are expressed as values relative to the total binding observed in the absence of AngIV (21 051 ± 1490 cpm) and corrected for nonspecific binding (4435 ± 500 cpm). (C) Concentration-dependent inhibition of ^{125}I-[Bpa^6]AngIV binding by increasing concentrations of AngIV. Membranes (50 μg of protein) were incubated for 2 h at 37 °C in the presence of 0.6 nM ^{125}I-[Bpa^6]-AngIV and increasing concentrations of unlabeled AngIV. The data are expressed as values relative to the total binding observed in the absence of AngIV (15 900 ± 946 cpm) and corrected for nonspecific binding (6784 ± 250 cpm). Each point represents the mean ± the standard deviation of data obtained in triplicate. Similar data were obtained in three independent experiments.

receptor, Figure 2, parts B and C shows that the binding of ^{125}I-[N_3-Phe^6]AngIV and ^{125}I-[Bpa^6]AngIV to endothelial cell membranes was completely inhibited by increasing concentrations of AngIV. The same potent inhibitory effect of AngIV (IC_{50} of 1.6 ± 0.5 nM) was observed with the three radioligands ^{125}I-AngIV (Figure 2A), ^{125}I-[N_3-Phe^6]AngIV (Figure 2B), and ^{125}I-[Bpa^6]AngIV (Figure 2C).

The irreversible character of AT_4 receptor labeling with the photosensitive analogues ^{125}I-[N_3-Phe^6]AngIV and ^{125}I-[Bpa^6]AngIV was verified. Figure 3 reveals that after UV

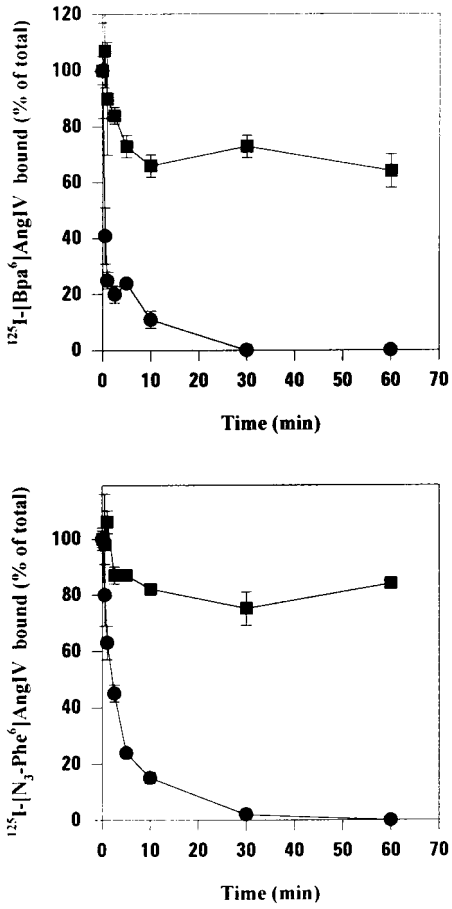

FIGURE 3: Acid-induced dissociation of ^{125}I-[Bpa^6]AngIV and ^{125}I-[N_3-Phe^6]AngIV from irradiated (■) and nonirradiated (●) bovine endothelial cell membranes. Membranes (1 mg of protein) were incubated with the indicated photoligand and irradiated as indicated in Materials and Methods. Half of the membranes were not irradiated. Acid-induced dissociation was started by the addition of citrate buffer, pH 5, to irradiated and nonirradiated membrane fractions at 4 °C. Nonspecific binding was determined in the presence of 10 μM AngIV. Data are expressed as values relative to the total binding (10 367 and 14 256 cpm for ^{125}I-[Bpa^6]AngIV and ^{125}I-[N_3-Phe^6]AngIV, respectively) observed before the addition of the acidic buffer and were corrected for nonspecific binding (2563 and 2463 cpm for ^{125}I-[Bpa^6]AngIV and ^{125}I-[N_3-Phe^6]AngIV, respectively). Each point represents a duplicate determination, and these experiments are representative of three similar observations.

irradiation of preformed photoligand−receptor complexes, only small proportions of ^{125}I- [N_3-Phe^6]AngIV (25−30%) (Figure 3B) and ^{125}I-[Bpa^6]AngIV(30−40%) (Figure 3A) were dissociable under acidic conditions. In the absence of UV irradiation, complete dissociation of bound ligand was rapidly achieved under acidic conditions. These results suggest that ^{125}I-[N_3-Phe^6]AngIV and ^{125}I-[Bpa^6]AngIV are very efficient ligands for covalent labeling of AT_4 receptor. The AT_4 receptor photolabeled with ^{125}I-[N_3-Phe^6]AngIV or ^{125}I-[Bpa^6]AngIV was further analyzed by SDS−PAGE under reducing and nonreducing conditions followed by autoradiography. Two radiolabeled bands of M_r 241 ± 6 kDa and M_r 186 ± 2 kDa were detected under nonreducing conditions (Figure 4, lanes 5 and 7), and the labeling was completely inhibited in the presence of 10 μM AngIV (Figure 4, lanes 6 and 8). Under reducing conditions the heavier band disappeared leaving only the low 186 kDa band (Figure 4, lanes 1 and 3) whose labeling was again completely abolished in the presence of 10 μM AngIV (Figure 4, lanes

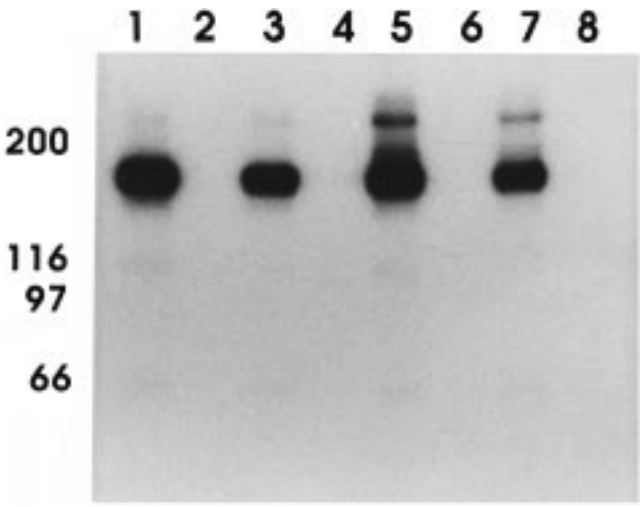

FIGURE 4: Specific photoaffinity labeling of AT_4 receptor from bovine endothelial cell membranes. Membranes (1 mg of protein) were incubated with 6 nM ^{125}I-[N_3-Phe^6]AngIV (lanes 1, 2, 5, and 6) or with 6 nM ^{125}I-[Bpa^6]AngIV (lanes 3, 4, 7, and 8) in the absence (lanes 1, 3, 5, and 7) or in the presence of 10 μM of AngIV (lanes 2, 4, 6, and 8) and were photolabeled as indicated in Materials and Methods. The material was resolved by 7.5% SDS−PAGE under reducing (lanes 1−4) or nonreducing conditions (lanes 5−8), followed by autoradiography. Protein standards of the indicated molecular masses (kDa) were run in parallel. These results are representative of at least three independent experiments.

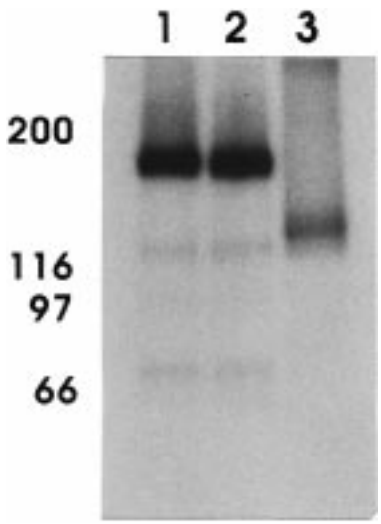

FIGURE 5: Deglycosylation of photolabeled AT_4 receptor. Membranes from bovine endothelial cells were photolabeled, solubilized, and incubated for 48 h at 37 °C in the absence of any glycosidase (lane 1) or in the presence of 40 U/mL PNGase F (lane 3) or 40 mU/mL *O*-glycosidase (lane 2), under conditions described in Materials and Methods. Aliquots were resolved by 7.5% SDS−PAGE under reducing conditions, 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.

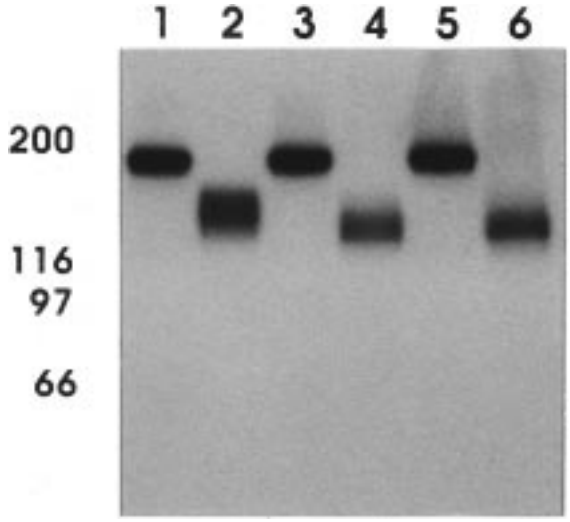

FIGURE 6: Time course of deglycosylation of photolabeled AT_4 receptor. Membranes from bovine endothelial cells were photolabeled, solubilized, and incubated in the presence (lanes 2, 4, and 6) or in the absence (lanes 1, 3, and 5) of 40 U/mL PNGase F for different periods of time (0.5 h, lanes 1−2; 6 h, lanes 3 and 4; 24 h, lanes 5 and 6) at 37 °C, under conditions described in Materials and Methods. Aliquots were resolved by 7.5% SDS−PAGE under reducing conditions, 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.

2 and 4). The labeling of the 186 kDa band was not affected by 10 μM PD 123319 or DuP 753 (data not shown).

Because many hormone receptors are glycoproteins, we evaluated the adsorption of photoaffinity-labeled solubilized AT_4 receptors to immobilized lectins. An important proportion of the radioactive material was retained on the WGA (30.9 ± 1.3%) and on the ConA (14.2 ± 1.9%). The specificity of this adsorption was assessed by the elution of the radioactive material with 0.5 M *N*-acetyl-D-glycosamine (in the case of WGA) and with 0.5 M *O*-D-mannopyranoside (in the case of ConA). To further characterize the glycoprotein nature of the AT_4 receptor, photolabeled membranes were treated with the endoglycosidase PNGase F, which cleaves N- linked saccharides (*23*), and with *O*-glycosidase, which cleaves O- linked saccharides (*24*). Figure 5 shows that treatment for 48 h of photolabeled bovine endothelial cell membranes with PNGase F reduced the apparent molecular mass of AT_4 receptor from 186 ± 2 kDa (lane 1, untreated receptors) to 129 ± 10 kDa (lane 3). Treatment of photolabeled membranes with *O*-glycosidase did not modify the migration of AT_4 receptor (Figure 5, lane 2). To further define the glycoprotein nature of AT_4 receptor from bovine endothelial cells, a time course of deglycosylation with PNGase F was performed. Figure 6 shows that deglycosylation occurred in at least two apparent steps. Figure 6, lane 2, reveals the production of a first deglycosylation product within the first few minutes of incubation (0−30 min). This product showed an apparent molecular mass of 148 ± 3 kDa, which represents a 20% size reduction, compared with the control receptor (molecular mass of 186 kDa) (Figure 6, lane 1). Figure 6, lane 4, reveals the appearance of a second deglycosylation product after a more prolonged incubation period (6 h). This second product showed an apparent molecular mass of 129 ± 10 kDa, corresponding to a size reduction of 31% compared with untreated receptor (Figure 6, lane 3). The stepwise process of deglycosylation is well illustrated in Figure 6 by the disappearance of the first deglycosylation product concomitant with the appearance of the second deglycosylation product. Prolonged incubation for up to 24 or 48 h with PNGase F did not reveal any further deglycosylation process (Figure 6, lane 6; Figure 5, lane 3). These results suggest that the band of M_r 129 kDa corresponds to a completely deglycosylated protein.

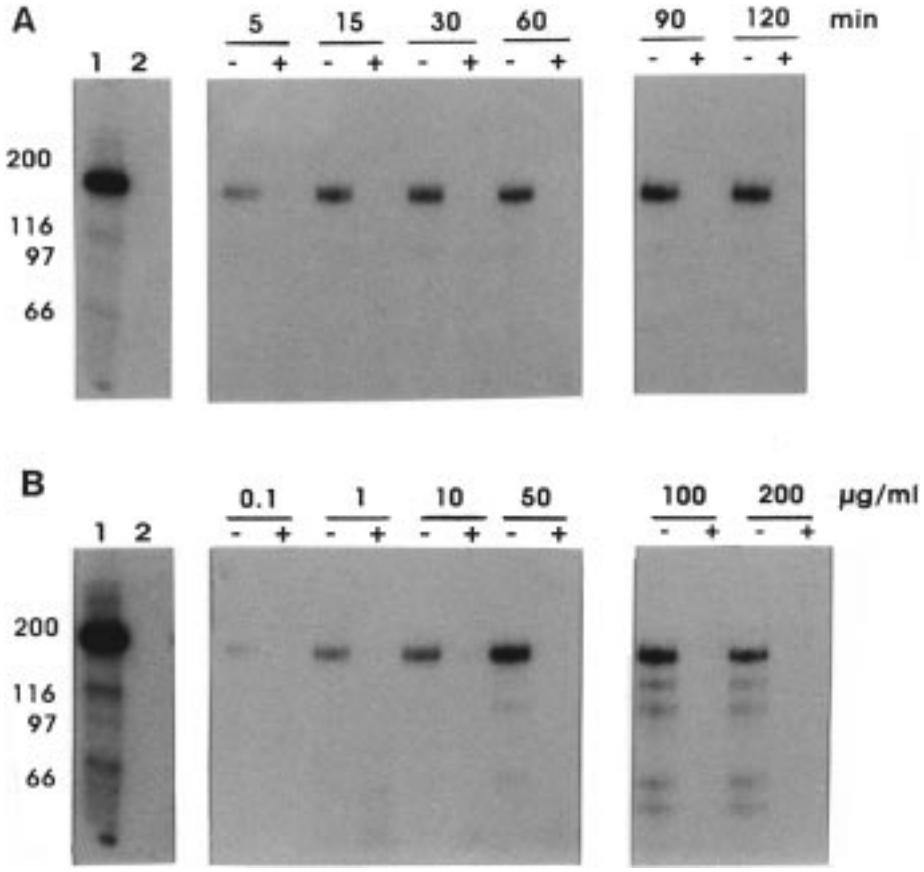

FIGURE 7: Trypsin treatment of AT_4 receptor from bovine endothelial cell membranes. (A) Left panel: membranes were photolabeled with ^{125}I-[N_3-Phe^6]AngIV in the absence (lane 1) or in the presence of 10 μM AngIV (lane 2) and incubated in the absence of trypsin for 1 h at 37 °C. After centrifugation the pellet was resolved by 7.5% SDS−PAGE under reducing conditions. Center and right panels: membranes were photolabeled with ^{125}I-[N_3-Phe^6]AngIV in the absence (−) or in the presence (+) of 10 μM AngIV and submitted to trypsin treatment for different periods of time at 37 °C. After centrifugation the supernatants (free of membranes) were mixed with an equal volume of 2× Laemmli's buffer under reducing conditions and boiled as described in Materials and Methods. (B) Left panel: membranes were photolabeled with ^{125}I-[N_3-Phe^6]AngIV in the absence (lane 1) or in the presence of 10 μM AngIV (lane 2) and incubated in the absence of trypsin for 1 h at 37 °C. After centrifugation, the pellet was resolved by 7.5% SDS−PAGE under reducing conditions. Center and right panels: membranes were photolabeled with ^{125}I-[N_3-Phe^6]AngIV in the absence (−) or in the presence (+) of 10 μM AngIV and digested with increasing trypsin concentrations for 1 h at 37 °C. After centrifugation the supernatants (free of membranes) were mixed with an equal volume of 2× Laemmli's buffer under reducing conditions and boiled as described in Materials and Methods. Samples were resolved by 7.5% SDS−PAGE, followed by autoradiography. Protein standards of the indicated molecular masses (kDa) were run in parallel. These results are representative of three separate experiments.

Additional biochemical characterization of the AT_4 receptor was undertaken by using the enzyme trypsin which cleaves after lysine or arginine residues. Intact endothelial cell membranes that had previously been labeled with the photosensitive analogues of AngIV were treated with different concentrations of trypsin (Figure 7B) for different periods of time (Figure 7A). Interestingly, treatment with a low concentration of trypsin (10 μg/mL) for different periods of time caused the slow accumulation of a high apparent molecular mass fragment of AT_4 receptor (177 ± 3 kDa) in the supernatant of the membranes suspension. This fragment showed a barely significant ($p < 0.05$) 4% size reduction as compared to intact AT_4 receptor solubilized from untreated membranes (lane 1). Treatment for 1 h with higher concentrations of trypsin (100−200 μg/mL) caused further fragmentations of photolabeled AT_4 receptor (Figure 7B). Similar results were obtained on trypsin digestion of photolabeled intact endothelial cells (data not shown). These results suggest that the AT_4 receptor is constituted of a very large extracellular single chain segment which represents the major portion of the protein. Phosphatidylinositol phospholipase C digestion of photolabeled endothelial cell membranes did not release any radioactive complex, eliminating the possibility that AT_4 receptor is a glycosyl−phosphatidylinositol-anchored protein (data not shown). Treatment of photolabeled membranes with 0.1 M Na_2CO_3, pH 11.0 (a condition known to disrupt protein−protein interaction; see ref *25*), did not release any radioactive complexes (data not shown). Altogether, these results suggest that the AT_4 receptor is an integral membrane protein.

DISCUSSION

We previously showed that bovine aortic endothelial cells express a high level of AT_4 receptors and no detectable amount of AT_1 or AT_2 receptors (*4*). This tissue constitutes thus an adequate model for studying the pharmacological and biochemical properties of AT_4 receptor. In the present study, we used two new photosensitive analogues of AngIV (^{125}I-[N_3-Phe^6]AngIV and ^{125}I-[Bpa^6]AngIV) for the covalent labeling and the biochemical characterization of AT_4 receptor from bovine aortic endothelial cells. We have shown that nonradioactive [N_3-Phe^6]AngIV and [Bpa^6]AngIV are potent inhibitors of ^{125}I-AngIV binding to bovine endothelial cell membranes. These results clearly demonstrated the specific

interaction of the photosensitive ligands with the AT_4 receptor. The slightly lower relative affinities of [N_3-Phe^6]-AngIV (IC_{50} of 3.7 nM) and [Bpa^6]AngIV (IC_{50} of 19.1 nM) compared to that of AngIV (IC_{50} of 2.1 nM) are consistent with previous structure−activity studies showing that the sixth position of AngIV can be occupied by a substituant larger than the Phe residue without important decrease of binding affinity (*26*). The radioactive photoligands ^{125}I-[N_3-Phe^6]AngIV and ^{125}I-[Bpa^6]AngIV specifically recognized the AT_4 receptor, as demonstrated by the potent and competitive binding inhibitory effect of AngIV. All together, these results demonstrate that the radioactive photoligands interact specifically and exclusively with the AT_4 receptor in bovine endothelial cell membranes.

Dissociation studies under acidic conditions revealed very high yields of covalent labeling of AT_4 receptor after UV irradiation in the presence of ^{125}I-[N_3-Phe^6]AngIV (70−75% covalent labeling) and ^{125}I-[Bpa^6]AngIV (60−70% covalent labeling). These high yields of covalent labeling are comparable to those obtained with ^{125}I-[Bpa^8]AngII for the labeling of AT_2 receptor of human myometrium (*27*) and with ^{125}I-[N_3−Phe^8]AngII for the labeling of AT_1 receptor of rat liver (*28*). Since so little information is available on the molecular properties of AT_4 receptor, it was important to use high-efficiency photoligands to ascertain that the results obtained with this approach are representative of the properties of the whole receptors population.

SDS−PAGE analysis of the photolabeled AT_4 receptor revealed a major band of M_r 186 kDa. The labeling of this protein was completely inhibited in the presence of an excess of AngIV, demonstrating, as previously discussed, the high specificity of the approach. A similar value of 186 kDa was previously reported by us (*4*), using the dissuccinimidyl suberate cross-linking approach, and also by Wright et al. (*9*), using the photoligand ^{125}I-[benzophenone^6]AngIV. Interestingly, our previous cross-linking approach labeled some minor bands of large molecular weight that were suggested to represent either different populations of AT_4 receptors or heterogeneous multimeric complexes between the receptor and putative coupling proteins involved in the signaling mechanism. The absence of these large molecular weight bands upon photoaffinity labeling (Figure 4) supports the second suggestion. It is important to note that, as previously observed by Wright et al. (*9*), SDS−PAGE analysis of photolabeled AT_4 receptor, under nonreducing conditions, revealed the presence of a larger molecular weight band, suggesting that AT_4 receptor may form a homodimer or a heterocomplex. The clarification of that point necessitates further investigation.

The glycoprotein nature of AT_4 receptor was demonstrated both by its adsorption to lectins columns and by its susceptibility to endoglycosidase digestion. On the basis of the stepwise size reduction observed during deglycosylation, we suggest that two sites of N-glycosylation are present on the AT_4 receptor. The carbohydrate moieties account for a significant proportion (around 30%) of the whole receptor apparent molecular mass. To our knowledge, this is the first study showing the glycoprotein nature of the AT_4 receptor. Further studies are needed to determine the role of carbohydrates in establishing the pharmacological properties of the AT_4 receptor. Carbohydrates could contribute to the stability, the intracellular trafficking, or the membrane targeting of the receptor.

Mild trypsin treatment of photolabeled endothelial cell membranes released a large fragment of M_r 177 kDa which represents about 95% of the whole receptor molecular mass. These results suggest that AT_4 receptor is an integral membrane protein constituted of a very large single chain extracellular segment and at least of one transmembrane domain. Considering that a transmembrane domain is composed of about 25 amino acid residues (M_r around 3 kDa), the 9−10 kDa fragment lost upon mild trypsin treatment cannot account for more than two transmembrane domains and one intracellular loop. Since such a topography has, to our knowledge, never been predicted for any known receptor, it is more likely that the AT_4 receptor is constituted of a single transmembrane domain and a short (5−6 kDa) intracellular tail. Such a topography is very frequently predicted for some cytokines receptors. For example, interleukin-11 receptor is constituted of a large extracellular domain (365 amino acid residues), a single transmembrane domain (25 residues), and a short intracellular tail (30 residues) (*29*); granulocyte-macrophage colony stimulating factor receptor is also constituted of a large extracellular domain (297 amino acid residues), a single transmembrane domain (27 residues), and a short cytosolic tail (54 residues) (*30*). Endoglin is part of a complex group of proteins that constitute the TGF-β receptor system. Endoglin is constituted of a large extracellular domain (561 amino acid residues), a single transmembrane domain (25 residues), and a short cytoplasmic tail (47 residues) (*31*). Taking into account the recently reported effects of AngIV on stimulation of DNA and RNA synthesis in cardiac fibroblasts (*18*), on expression of plasminogen activator inhibitor in endothelial cells (*17*), and on enhancement of DNA synthesis in endothelial cells (*19*), it is tempting to speculate that AT_4 receptor belongs to a family of growth factor or cytokine receptors.

In conclusion, we have used two new and efficient photoligands to characterize the AT_4 receptor of bovine aortic endothelial cells. We have shown that the AT_4 receptor is a 186 kDa integral membrane glycoprotein with a very large extracellular domain. These properties may be of interest in the design of specific strategies for the purification or the molecular cloning of AT_4 receptor.

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