Side-Chain Substitutions within Angiotensin II Reveal Different Requirements for Signaling, Internalization, and Phosphorylation of Type 1A Angiotensin Receptors

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ABSTRACT

Binding of the peptide hormone angiotensin II (AngII) to the type 1 (AT1A) receptor and the subsequent activation of phospholipase C-mediated signaling, involves specific determinants within the AngII peptide sequence. In contrast, the contribution of such determinants to AT1A receptor internalization, phosphorylation and activation of mitogen-activated protein kinase (MAPK) signaling is not known. In this study, the internalization of an enhanced green fluorescent protein-tagged AT1A receptor (AT1A-EGFP), in response to AngII and a series of substituted analogs, was visualized and quantified using confocal microscopy. AngII-stimulation resulted in a rapid, concentration-dependent internalization of the chimeric receptor, which was prevented by pretreatment with the nonpeptide AT1 receptor antagonist EXP3174. Remarkably, AT1A receptor internalization was unaffected by substitution of AngII side chains, including single and double substitutions of Tyr4 and Phe8 that abolish phospholipase C signaling through the receptor. AngII-induced receptor phosphorylation was significantly inhibited by several substitutions at Phe8 as well as alanine replacement of Asp1. The activation of MAPK was only significantly inhibited by substitutions at position eight in the peptide and specific substitutions did not equally inhibit inositol phosphate production, receptor phosphorylation and MAPK activation. These results indicate that separate, yet overlapping, contacts made between the AngII peptide and the AT1A receptor select/induce distinct receptor conformations that preferentially affect particular receptor outcomes. The requirements for AT1A receptor internalization seem to be less stringent than receptor activation and signaling, suggesting an inherent bias toward receptor deactivation.

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ABBREVIATIONS: AngII, angiotensin II; AT1A, type I angiotensin receptor; AT1B, type II angiotensin receptor; IP3, inositol trisphosphate; MAPK, mitogen-activated protein kinase; GPCR, G protein-coupled receptor; NHA, N-terminal hemagglutinin epitope tag; EGFP, enhanced green fluorescent protein; CHO-K1, Chinese hamster ovary cells; ARBB, angiotensin receptor binding buffer; WGA-TR, wheat germ agglutinin-Texas Red; HBSS, Hank’s buffered salt solution; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin.
phosphorylation, arrestin binding and receptor internalization (Thomas, 1999; Hunyady et al., 2000; Oakley et al., 2001). These processes are initiated soon after ligand-receptor interaction and may contribute to rapid receptor desensitization. The AT1 receptor is phosphorylated by both G protein-coupled receptor (GPCR) kinases and protein kinase C on specific carboxyl-terminal serine and threonine residues (Thomas, 1999, Hunyady et al., 2000). Phosphorylation by GPCR kinases presumably produces a high-affinity binding site for arrestins, which sterically hinder further G protein-coupling and act as adapters for the cellular internalization. The AT1 receptor carboxy terminus has been implicated in rapid agonist-induced endocytosis (Smith et al., 1998; Thomas et al., 1998), presumably via arrestin recruitment (Oakley et al., 2001).

Recent evidence indicates that multiple conformations exist for the AT1 receptor, some coupling the receptor to signaling pathways and others directing receptor phosphorylation or internalization (Thomas et al., 2000). The transition of the AT1 receptor through these various states may be induced or stabilized by specific AngII side chains during the docking of ligand onto receptor, because separate residues within the AngII peptide confer high-affinity binding and IP3 docking of ligand onto receptor, because separate residues during the internalization of the AT1 receptor and MAPK signaling.

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surface-bound ligand was stripped by acid-washing and collected. Radioactivity associated with the cells (internalized) was determined as a percentage of the total (acid wash plus cell-associated) (Thomas et al., 1998).

**Cytosensor Microphysiometer Signaling Assay.** CHO-K1 cells transiently transfected with NHA-AT1A or NHA-AT1A-EGFP were plated into 12-mm Transwells (3-μm pore diameter) at a density of 250,000 cells/well and grown overnight. Transwells with spaces and capsule inserts were placed in the sensor chambers and the cells were allowed to equilibrate for 1 to 2 h. When a steady state was achieved, AngII accumulative concentration-response curves were constructed over the concentration range 10⁻¹¹ to 10⁻⁶ M. Responses were measured as the rate of change of pH, both as a voltage change and as a percentage change from baseline voltage (normalized to 100%) using Cytosoft software.

**Receptor Internalization Measured by Confocal Microscopy.** CHO-K1 cells were seeded in eight-well chamber slides at a density of 100,000 cell/well, transfected as described above, incubated in complete media for 24 h, and serum-deprived overnight. Cells were equilibrated (37°C, 1 h) in 0.4 ml of angiotensin receptor binding buffer (ARBB): 50 mM Tris-HCl, pH 7.4, containing 120 mM NaCl, 4 mM KCl, 5 mM MgCl₂, 1 mM CaCl₂, 10 μg/ml bacitracin, 2 mg/ml α-glucose, and 0.25% bovine serum albumin. Ligands were added to give a final concentration of 100 times their respective published Kᵢ values (see Table 1) and slides were incubated at 37°C for 20 min. For AngII concentration-response experiments, cells were stimulated with 0, 10⁻¹¹, 10⁻¹⁰, 10⁻⁹, and 10⁻⁸ M AngII. Wells were aspirated and cells fixed with 4% paraformaldehyde in sodium phosphate buffer, pH 7.4 (0.5 ml), for 20 min at room temperature, washed with ARBB, incubated in ARBB with 10% fetal bovine serum and 100 mM glycine for 10 min, washed, and incubated with wheat germ agglutinin/Texas Red conjugate (2 μg/ml) for 30 min at room temperature. WGA-TR was cross-linked to cells by incubating in 4% paraformaldehyde for 20 min. Chambers were removed from the slide, washed twice with Hanks’ buffered salt solution (HBSS) and mounted in 90% glycerol/10% HBSS. All stimulations were performed coded and protocols withheld until after analysis of confocal imaging.

Slides were examined on an MRC1024-Zeiss Axioplan 2 confocal microscope with krypton/argon laser (Bio-Rad, Hercules, CA). All sections were scanned with a Plan Achromat 63×, 1.40 numerical aperture, oil-immersion objective and 3× digital zoom (box size, 58.8 × 58.8 μm). Green and red channel images were collected simultaneously using excitation at 488 nm for EGFP and 568 nm for Texas Red and laser intensity was kept constant for each experiment (10% or 30% power). Optical sections were taken through cells (nine cells/treatment) expressing a medium level of the NHA-AT1A-EGFP receptor. For each cell, a single optical section was collected at a point where the nucleus was largest and there was a clear outline of the plasma membrane. Images were collected by LaserSharp acquisition software and quantified as described previously (Southwell et al., 1998).

Location of AT1A receptors on the surface or in the cytoplasm was quantified (Optimas ver. 6.0) by comparing the fluorescence of EGFP (receptor) with that of WGA-TR (labels the cell surface). Although WGA-TR was visible as red fluorescence on the cell surface only, EGFP was visible in the green channel and was located on the cell surface and in the cytoplasm. Intracellular EGFP fluorescence was calculated by subtracting cell surface EGFP fluorescence, which colocalized with WGA-TR, from total EGFP fluorescence.

**MAPK Activation.** Serum-starved CHO-K1 cells expressing the AT1A receptor (in 12 well plates) were stimulated for 4 min with AngII or the substituted AngII analogs (Table 1), rapidly washed twice with ice-cold HBSS, and lysed in 250 μl of radioimmunoprecipitation buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM EDTA, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 μg/ml pepstatin) for 30 min at 4°C. Cell lysates were centrifuged at 14,000g for 15 min and 10 μl of supernatant was resolved on SDS-PAGE and Western blotted. Western blots were probed with a monoclonal antibody (E10) to phospho-44/42 MAPK (T202/Y204) to identify the phosphorylated (active) forms of MAPK and developed by enhanced chemiluminescence. Blots were subsequently reprobed with a rabbit polyclonal antibody (SC93) to detect total MAPK. Blots were quantified using Scion Image software.

**Receptor Phosphorylation Assay.** The procedure for receptor phosphorylation has been described previously (Thomas et al., 1998). [32P]-Loaded cells were stimulated (10 min, 37°C) with AngII and the various substituted analogs at a concentration 100 times that of the Kᵢ for the receptor. HA-tagged AT1A receptors were immunoprecipitated using anti-HA antibody (12CA5) and phosphorylated receptor resolved by 10% SDS-PAGE and a filmless autoradiographic system (Fuji Bio-imaging Analyzer BAS 1000; Fuji, Tokyo, Japan).

**Equipment and Reagents.** Equipment and reagents used and their source are as follows: CHO-K1 cells (American Type Culture Collection, Manassas, VA); cell culture media, additives, LipofectAMINE, pEGFP-N1 vector (Invitrogen, Melbourne, Australia); cell culture dishes and 12-mm transwells (Costar, Acton, MA); eight-well glass chamber slides (Nalge Nunc International, Naperville, IL); [32P]orthophosphate (ICN Biomedicals, Costa Mesa, CA); protein agarose (Roche Applied Science, Melbourne, Australia); wheat germ agglutinin/Texas Red conjugate (Molecular Probes, Eugene, OR); angiotensin II (Auspex, Melbourne, Australia); [125I]AngII and [125I]Sar₁Ile₈AngII (specific activity, ~2000 Ci/mmol; Austin Bio-medical Services, Melbourne, Australia); other AngII analogs were synthesized in the Cleveland Clinic Core synthesis facility (Cleveland, OH). The MAPK antibodies E10 and SC93 were from Cell Signaling Technologies (Beverly, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. All other chemicals were bought from Sigma (Sydney, Australia) or BDH (Kilsyth, Melbourne, Australia); Cytosensor Microphysiometer and Cytosoft software (Molecular Devices, Menlo Park, CA); LaserSharp Acquisition software (Bio-Rad, Hercules, CA); Optimas Image Analysis (Media Cybernetics, Del Mar, CA) and Scion Image software (Scion, Frederick, MD).

**Statistical Analysis.** Statistics performed were the unpaired t test and one-way analysis of variance (Newman-Keuls multiple-comparison test) calculated by Prism data analysis software (v.2.0; GraphPad, San Diego, CA). Figures are presented as mean ± S.E.

**Results**

**Comparison of the AT1A-EGFP and Wild-Type Receptors.** To examine the internalization of AT1A receptors, we generated a NHA-AT1A-EGFP chimera suitable for following receptor trafficking by confocal microscopy. Figure 1 shows a comparison of receptor binding, signaling and internalization for the wild-type (NHA-AT1A) and NHA-AT1A-EGFP receptors. Competition binding studies, using the radio-labeled peptide antagonist [125I]Sar₁Ile₈AngII, demonstrated that both receptors expressed equally well in CHO-K1 cells (~1000 fmol of receptor/mg of protein) and displacement experiments with AngII, EXP3174 (an AT1-selective nonpeptide antagonist), or PD123319 (an AT₂-selective nonpeptide antagonist) yielded identical binding profiles (Fig. 1A). AngII displaced the iodinated ligand with pKᵢ values of 8.13 ± 0.06 for the NHA-AT1A receptor and 8.10 ± 0.08 for the NHA-AT1A-EGFP receptor. pKᵢ values for the AT1 receptor selective nonpeptide antagonist, EXP3174, were calculated as 7.60 ± 0.08 for NHA-AT1A and 7.67 ± 0.07 for NHA-AT1A-EGFP. PD123319, an AT₂ receptor selective nonpeptide antagonist, did not significantly displace the labeled peptide as indicated by pKᵢ values <6.0 for both receptors.

The signaling capacity of the NHA-AT1A-EGFP receptor...
was compared with the wild-type (NHA-AT1A) receptor using a Cytosensor Microphysiometer, which measures the rate of change of pH as an index of overall cell activation. Concentration-response curves were constructed over the concentration range 10^{-11} to 10^{-6} M (Fig. 1B) and corresponding pEC_{50} values were determined. For the NHA-AT1A-EGFP receptor, a pEC_{50} value of 8.34 ± 0.10 was observed that was not significantly different from that of the wild-type NHA-AT1A (8.10 ± 0.16). The maximum response to AngII was similar for both receptors.

Fig. 1 shows a comparison of the time course of internalization for the NHA-AT1A and NHA-AT1A-EGFP receptors, measured by the radio-ligand {^{125}I}AngII method. The two receptors internalized rapidly and with similar kinetics, reaching a maximum (NHA-AT1A: 73.5 ± 2.3%, NHA-AT1A-EGFP: 70.8 ± 2.4%) with t_{1/2} of 4.3 ± 0.2 min for the wild-type and 4.6 ± 0.2 min for the EGFP-tagged receptor (n = 4). These results indicate that the addition of EGFP to the carboxyl terminus of the AT1A receptor does not influence the rate or degree of receptor endocytosis.

AngII also induced a similar magnitude and kinetics of receptor phosphorylation: a rapid 3-fold increase from basal was observed for both wild-type and EGFP-tagged receptors, peaking at 5 min, after which a steady level was maintained (data not shown).

**Internalization of NHA-AT1A-EGFP Receptor Measured by Confocal Microscopy.** AngII-induced internalization of the NHA-AT1A-EGFP receptor was examined by confocal microscopy in CHO-K1 cells that transiently expressed the receptor (see Fig. 2). Cells expressing a moderate level of receptor were chosen and optical sections were taken through cells at a point where the nucleus was largest. To differentiate between cell surface receptor and intracellular receptor, the plasma membrane of cells was defined by staining with a WGA-TR conjugate, which binds to carbohydrates on the cell surface of nonpermeabilized cells.

For each cell, images of the green fluorescence (AT1A-EGFP) and the red fluorescence (WGA-TR) were collected separately. In a computer overlay of the two images (Fig. 2A), yellow is generated where the two colors colocalize. By calculating the percentage of green fluorescence that is not coverted to yellow relative to the total green fluorescence signal, we were able to quantify intracellular (and thereby internalized) receptor.

Before stimulation, the majority of EGFP fluorescence was spread evenly across the cell surface, with some spots in perinuclear structures and diffuse staining throughout the cytosol. Thirty to forty percent of EGFP fluorescence was cytoplasmic in untreated cells (Fig. 2, B and C, 0 min). After AngII stimulation (10^{-7} M), fluorescence appeared in large spots in the cytoplasm—presumably endocytic vesicles (Fig. 2B). At 1 min, receptor aggregated on the membrane and spots appeared adjacent to the membrane. At 2 min, green fluorescent spots were deeper in the cytoplasm. At 5, 10, and 20 min, the fluorescent spots remained cytoplasmic. The proportion of green fluorescence in the cytoplasm was quantified after the initial rapid translocation of receptor from the cell surface (t_{1/2}: 2.1 ± 0.9, n = 3); receptor internalization reached a maximum of 70 to 75% at 5 min, then reached a plateau. For subsequent experiments, stimulation with AngII and analogs was for 20 min.

The effect of various concentrations of AngII on internalization is shown in Fig. 3A. Cells were stimulated with AngII for 20 min at concentrations ranging from 10^{-11} to 10^{-7} M. The pEC_{50} for this response was calculated as 9.27 ± 0.27, which is comparable with the affinity of AngII at the AT1A receptor (Chiu et al., 1993). Maximum internalization (~75%) was observed at 10^{-7} M, corresponding to a concentration 100 times the K_{d} of AngII at the AT1A receptor. This concentration of AngII was used to determine specificity.

Internalization of the NHA-AT1A-EGFP receptor was the result of a specific interaction between AngII and the recep-
tor (Fig. 3B). The selective AT₁ nonpeptide antagonist, EXP3174 (10⁻⁵ M), which binds with high affinity to residues within the transmembrane domains of the receptor, at a distinct, yet overlapping site to that of AngII (Hunyady et al., 1996), alone did not cause internalization. However, pre-treatment with the antagonist for 20 min (10⁻⁵ M) completely prevented the internalization induced by AngII stimulation (10⁻⁷ M, n = 3).

**Internalization by Substituted Analogs of AngII.**
Cells expressing the NHA-AT₁-EGFP receptor were stimulated for 20 min with analogs of AngII at a concentration equal to 100-fold the Kᵦ of that analog at the AT₁ receptor (Table 1). Saturating concentrations and the 20-min time-point were chosen to allow maximal internalization of each analog. Because substitutions in AngII at positions 4 and 8 lead to profound decreases in AT₁ receptor activation, as measured by inositol phosphate generation (see Table 1 (Miura et al., 1999; Miura and Karnik, 1999)), we measured the internalization caused by AngII with analogs substituted

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**Fig. 2.** Confocal imaging and time-dependence of internalization of the NHA-AT₁-EGFP receptor transiently expressed in CHO-K1 cells. A, single optical section of a transfected CHO-K1 cell taken with a confocal microscope. Left, NHA-AT₁-EGFP receptor fluorescence (green, labeled AT₁-EGFP) for an unstimulated cell. Scale bar, 2 μm. Middle, wheat-germ agglutinin-Texas Red (red, labeled WGA-TR) fluorescence bound to the cell membrane of the same cell. Right, an overlay of red and green images resulting in a yellow pseudocolor, where red and green colocalize. B, CHO-K1 cells expressing the AT₁-EGFP receptor were stimulated with AngII (10⁻⁷ M) for 0, 1, 2, 5, 10 and 20 min, fixed, and stained with WGA-TR. Representative overlay pictures are shown for each time-point. C, the proportion of intracellular receptor was quantified for nine cells at each time-point (as described under Materials and Methods). Data are mean ± S.E. of three experiments.

**Fig. 3.** Characterization of AT₁-EGFP receptor internalization. A, concentration-response curve for NHA-AT₁-EGFP receptor internalization. Cells expressing the receptor were stimulated with AngII at concentrations over the range 10⁻¹¹ to 10⁻⁷ M for 20 min. The proportion of intracellular receptor was measured by confocal microscopy and the concentration response-curve exhibited a pEC₅₀ of 9.27 ± 0.27. Data are mean ± S.E. of three experiments, nine cells per concentration in each experiment. B, the nonpeptide AT₁ receptor antagonist EXP3174 does not stimulate internalization of the NHA-AT₁-EGFP receptor and inhibits AngII-mediated internalization. Cells expressing the NHA-AT₁-EGFP receptor were stimulated with AngII (10⁻⁷ M, 20 min), EXP3174 (10⁻⁵ M, 20 min) or with AngII (10⁻⁷ M, 20 min) after pretreatment with EXP3174 (10⁻⁵ M, 20 min), and processed for confocal microscopy. (**, p < 0.01 of AngII; Con, unstimulated).
at these positions. As shown in Fig. 4A, both mono-substituted (Sar1Ala4AngII, Sar1Ala8AngII) and di-substituted analogs (Sar1Ile4Ile8AngII, Sar1Gly4Gly8AngII) induced full internalization of the NHA-AT1A-EGFP receptor.

Internalization of the NHA-AT1A-EGFP receptor caused by AngII analogs substituted at other positions was also examined (Table 1, Fig. 4B). AngIII is an aminopeptidase product of AngII (des-Asp1AngII), and an endogenous agonist at the AT1 receptor (Freeman et al., 1977; de Gasparo et al., 2000). Like AngII, it caused full internalization of the NHA-AT1A-EGFP receptor (Fig. 4B). Substitutions of Arg2 to glutamine, and the α-carboxyl of Phe8 to amide, which significantly reduce binding affinity (Table 1), were found to have a negligible effect on the amount of internalization compared with AngII. In addition, changing Asp1 to alanine, Val3 to alanine, His6 to alanine, or Pro7 to alanine, residues important for peptide conformation and stability (Regoli et al., 1974), had no observable effect on internalization.

**AT1A Receptor Phosphorylation by AngII Analogues.**

We examined the capacity of the various AngII analogs to cause phosphorylation of the AT1A receptor, expressed in CHO-K1 cells, using the N-terminal HA tag to immunoprecipitate the receptor. AngII caused a robust phosphorylation of the AT1A receptor (Fig. 5). Maximal receptor phosphorylation in response to AngII is observed at concentrations 10- to 100-fold higher than the KD and at 10 min after ligand stimulation (Thomas et al., 2000). In comparison to AngII, substitutions at Arg2 (G2), Val3 (A3), Tyr4 (A4), His6 (A6), and Pro7 (A7) or substitution of the α-carboxyl group of Phe8 (Am) had no significant effect on receptor phosphorylation. In contrast, alanine substitution of Asp1 (A1) led to a ~40% reduction in AT1A receptor phosphorylation, which was mirrored by a similar reduction with AngIII (data not shown). The most important residue for phosphorylation seems to be Phe8. Most substitutions at this position caused significant decreases in receptor phosphorylation. Single substitutions of Phe8 to alanine (A8), β-cyclohexylalanine (a nonaromatic, saturated ring side chain of equivalent size and hydrophobicity to phenylalanine/tyrosine) (C8), isoleucine (I8), or di-phenylalanine (a strongly aromatic side chain of increased size compared with phenylalanine/tyrosine) (D8) caused significant (45 to 70%) decreases in AT1A receptor phosphorylation compared with AngII. Double isoleucine substitution at

![](image1)

**Fig. 4.** Effect of substitution of AngII side chains on AT1A-EGFP receptor internalization. A, internalization of NHA-AT1A-EGFP receptor by analogs of AngII substituted at positions 4 and 8. Cells expressing the NHA-AT1A-EGFP receptor were stimulated for 20 min at a concentration of 100 × KD of the specified analog and intracellular receptor quantified by confocal microscopy. (A4, Sar1Ala4AngII; A8, Sar1Ala8AngII; I48, Sar1Ile4Ile8AngII; G48, Sar1Gly4Gly8AngII; Con: unstimulated). B, NHA-AT1A-EGFP receptor internalization by analogs of AngII substituted at positions 1, 2, 3, 6, 7, and the α-carboxyl group of Phe8 (see Table 1). Cells expressing the receptor were stimulated for 20 min at a concentration of 100 × KD of specified analogs. (A1, Ala1AngII; G2, Sar1Gly2AngII; A3, Sar1Ala3AngII; A6, Sar1Ala6AngII; A7, Sar1Ala7AngII; Am, Sar1AngII-CONH2). Quantification was performed by confocal microscopy from nine cells/analog/experiment. Data are mean ± S.E. of four experiments. AngII analog codes are from Table 1.

![](image2)

**Fig. 5.** Phosphorylation of the NHA-AT1A receptor transiently expressed in CHO-K1 cells stimulated with AngII analogs. A, CHO-K1 cells expressing the receptor were preloaded with 0.2 mCi/ml [32P]Pi and stimulated for 20 min with AngII or substituted analogs of AngII at concentrations 100 times their respective KD (see Table 1). The cells were lysed and the receptor immunoprecipitated and resolved on a 10% SDS-PAGE gel. Radioactively labeled receptors were visualized and quantified with a filmless autoradiographic system (Fujix Bio-imaging Analyzer BAS 1000) and presented as a percentage of the phosphorylation caused by AngII. The phosphorylated receptor migrates as a broad band running at 60 to 110 kDa. B, gels were quantified according to the band intensity and expressed as a percentage of the phosphorylation induced by AngII (100%). Data are mean ± S.E. of three experiments (*, p < 0.05; **, p < 0.01 compared with AngII). AngII analog codes are from Table 1; Con, unstimulated.
positions 4 and 8 (I48) decreased receptor phosphorylation similar to the single isoleucine substitution at position 8 (I8), consistent with a lack of effect of position 4 substituted analogs on receptor phosphorylation. Remarkably, the double substitution to glycine at position 4 and 8 (G48) did not significantly reduce receptor phosphorylation compared with AngII. So, although single substitutions at Phe8 can inhibit receptor phosphorylation somewhat, there was no clear preference for size, hydrophobicity, or aromaticity at this position. Moreover, based on the strong phosphorylation induced by G48, it would seem that the presence of both “agonism” defining residues, Tyr4 and Phe8, is not essential for AT1A receptor phosphorylation.

**Activation of MAP Kinase by AngII Analogs.** Previous studies on the structure-activity profiles of AngII analogs with respect to their ability to activate AT1 receptor signaling have focused principally on the Gαq/11-PLC/IP3 pathway. These studies have revealed strict requirements for position 4 and 8 in generating the active signaling form of the receptor (Miura et al., 1999; Miura and Karnik, 1999). To examine whether other signaling pathways activated by the AT1A receptor are similarly affected by substitutions in AngII, the activation of MAPK (p42/44; extracellular signal-regulated kinase) after stimulation by the series of AngII analogs was determined (Fig. 6). AngII stimulation (4 min at 37°C) of the AT1A receptor lead to a robust (~6-fold) increase in MAPK activation, primarily through activation of p42. Substitutions at position 1, 2, 3, 4, 6, and 7, the α-carboxyl of Phe8, and the substitution of Phe8 with diphenylalanine produced levels of MAPK activation similar to that of AngII. In contrast, substitution of Phe8 with alanine completely abolished activation. Single substitutions of Phe8 with β-cyclohexylalanine or isoleucine also significantly reduced MAPK activation compared with AngII. Similarly, double isoleucine and glycine substitutions of Tyr4 and Phe8 peptides weakly activated MAPK.

**Discussion**

A major initial aim of this study was to generate an AT1A-EGFP chimeric receptor, which would allow visualization and quantification of AT1A receptor endocytosis using confocal microscopy. Direct quantification was preferred because previous methods for examining AT1 receptor internalization have been predominantly indirect (e.g., acid-insensitive sequestration of radioactive AngII). The unobtrusive nature of the carboxyl-terminal EGFP tag on AT1A receptor binding affinity, selectivity, and receptor-mediated cellular activation was confirmed by competition binding experiments, CytoSensor measurements, and receptor phosphorylation assays. That these parameters were indistinguishable for EGFP-tagged and untagged AT1A receptors agrees with studies on other GFP-tagged GPCRs (Milligan, 1999) and with a recent report (Misery-Lenkei et al., 2001), in which an AT1A-EGFP receptor was used to investigate AT1A receptor trafficking in human embryonic kidney 293 cells.

AT1A-EGFP receptor internalization was quantified using confocal microscopy, using staining with WGA-TR to delineate the cell surface. Receptor endocytosis measured in this way was qualitatively equivalent to the traditional acid-insensitive [125I]AngII binding method: internalization was rapid (t1/2 = 2.1 min) and reached a steady state by 10 to 20 min, indicating that ligand sequestration correlates with receptor internalization. The pEC50 of AT1A-EGFP receptor internalization as determined by confocal microscopy (9.27 ± 0.27) approximated the Kd value of AngII at the AT1A receptor (~1 nM), which highlights the close relationship between ligand binding and internalization. The graded response to AngII demonstrates that the assay can distinguish different levels of receptor internalization. The nonpeptide antagonist EXP3174 caused no internalization of the AT1A-EGFP receptor, demonstrating that ligand binding to the receptor is not sufficient for internalization and that additional points of contact, or conformational changes in the receptor not provided by EXP3174, are required for this process. Importantly, the internalization in response to AngII was prevented by EXP3174, verifying that a specific interaction between AngII and the AT1A receptor causes internalization.

The principal, yet unexpected, finding of our study was that AT1A receptor internalization is unaffected by substitutions at any position within the AngII peptide sequence. This result contrasts with the accepted view that the two aromatic amino acid side-chains of AngII, Tyr4 and Phe8, are required for activating IP3 signaling through the AT1 receptor. Substitutions of Tyr4 and Phe8 peptides weakly activated MAPK.
substitution of these residues, reducing their bulk and removing their aromaticity, has been shown in vivo, isolated tissues, (Regoli et al., 1974; Samanen et al., 1989), and in cells expressing a recombinant AT1 receptor (Noda et al., 1996; Miura et al., 1999), to significantly reduce responses compared with AngII. Substitution of Phe8 or Tyr4 with isoleucine yields peptides with reduced ability to generate IP3 (20% and 80% of AngII, respectively) (Miura et al., 1999), yet we observed no effect of these two individual substitutions on the level of internalization. Even the di-substituted analogs (Sar1Ile6AngII and Sar1Gly6Gly8AngII) that display no agonism with respect to inositol phosphate signaling (see Table 1) gave full AT1A-EGFP receptor internalization. Consistent with our findings, previous studies have reported strong sequestration of [125I]Sar1Ile8AngII (Conchon et al., 1994; Thomas et al., 1996) and other Phe8 analogs of AngII also cause AT1A-EGFP receptor internalization (Miserelyenkei et al., 2001). This evidence shows that the AngII residues involved in coupling the receptor to inositol phosphate signaling are not required for receptor internalization, indicating that distinct ligand-receptor interactions and receptor conformations subserve PLC signaling and internalization.

Given that phosphorylation within the central region of the AT1A carboxyl terminus is associated with receptor internalization (Smith et al., 1998; Thomas et al., 1998), we investigated whether substitutions in AngII would affect receptor phosphorylation. Only changes to Asp1 (to alanine) and Phe8 phosphorylation. Only changes to Asp1 (to alanine) and Phe8 governed whether substitutions in AngII would affect receptor agonism (Smith et al., 1998; Thomas et al., 1998), we investigated with AngII. Substitution of Tyr4 with isoleucine, diphenylalanine, and Fmoc-alanine, which abrogates inositol phosphate signaling, reduces MAPK activation by only half.

Fig. 7. Comparison of individual receptor activities in response to stimulation by AngII analogs. Data for receptor internalization (Fig. 4), phosphorylation (Fig. 5), MAPK activation (Fig. 6), and inositol phosphate production (Table 1) were expressed as a percentage of AngII (100%). Values are corrected for basal values of the unstimulated control samples. This normalized data for the individual receptor processes was plotted to compare all receptor activities. A theoretical line (dashed), representing the position at which a particular substitution would affect two receptor functions to the same degree, is shown. For clarity, only analogs whose responses are shifted from full activity are labeled using the code described in Table 1.
duced (40% of AngII) MAPK activation (Fig. 7C), and completely fail to elicit inositol phosphate production (Fig. 7A). Ala\(^1\)AngII (A1) fully activates IP and MAPK, but shows a slight reduction (60% of AngII) in receptor phosphorylation (Fig. 7B). Sar\(^1\)Ala\(^8\)AngII (A8) elicits greatly reduced IP and MAPK responses (20% of AngII) and reduced capacity for phosphorylation (50% of AngII, Fig. 7B). Figure 7D highlights the structural requirements for position 8 with respect to receptor phosphorylation and MAPK activation. Although substitution Phe\(^8\) with isoleucine (I8) reduced phosphorylation (50% of AngII) and MAPK activation (40% of AngII), substitution with the strongly aromatic diphenylalanine (D8) causes reduced (50%) receptor phosphorylation but full MAPK activation. An AngII peptide carrying the nonaromatic ring side chain, \(\beta\)-cyclohexylalanine, at position 8 (C8) reduces receptor phosphorylation (30% of AngII) more than reducing MAPK activation (60% of AngII). These results suggest that aromaticity and bulk at position 8, although important for MAPK activation, are not crucial for phosphorylation. The bulky, but not aromatic, substitution at position 4 (Sar\(^1\)Cha\(^4\)AngII, C4) produces a ligand that promotes full phosphorylation (Fig. 7E) and MAPK (Fig. 7F) but only an intermediate inositol phosphate response. These data demonstrate differing structural and size requirements in the AngII peptide for AT\(_{1A}\) receptor activation, signaling, phosphorylation and internalization.

Multiple functional ligand-receptor contacts, variously referred to as agonist-receptor trafficking (Kenakin, 1995) or biased agonism (Jarpe et al., 1998), support the concept of separate receptor conformational states. Based on contemporary two- or three-state models for GPCR activation (Lefkowitz et al., 1993; Kenakin, 1995; Leff, 1995; Leff et al., 1997; Leurs et al., 1998; Scaramellini and Leff, 1998), receptor conformations that activate signaling (e.g., R\(^e\)) are also targeted for phosphorylation by GPK kinases, leading to arrestin binding, which inhibits further signaling and targets receptors for clathrin-mediated internalization. Thus, substitutions in the AngII peptide (specifically at Tyr\(^4\) and Phe\(^8\)) should equally affect signals emanating from the receptor, phosphorylation and internalization. Instead, we observed differences in the capacity of AngII analogs to promote receptor signaling, phosphorylation, and internalization. Most compelling was the observation that all substituted analogs stimulated robust AT\(_{1A}\) receptor internalization, indicating that internalization is more tolerant than signaling of modifications in the AngII peptide. These data argue strongly against a linear transition from an inactive to a signaling receptor, which is then phosphorylated, desensitized, and internalized. Instead, they support the idea of ligand-specific receptor states, each selected by unique contacts between peptide and receptor and potentially capable of coupling to one or more distinct receptor activities. A caveat to this interpretation is that we used saturating concentrations of ligand with single, maximal time-points of stimulation in one cell type (CHO-K1). Clearly, cellular environment (i.e., the complement and/or concentration of G proteins and other signaling and regulatory molecules) may affect the capacity of specific analogs to promote signaling, phosphorylation, and internalization. Also, experiments measuring initial rates of reactions may reveal subtle differences in the kinetics and/or efficacy of substituted AngII analogs not revealed by the current experimental approach.

Nevertheless, the concept of distinct AT\(_{1A}\) receptor states is consistent with AT\(_{1A}\) receptor mutants that display proclivity for specific receptor functions. For example, an Asp\(^74\)Asn AT\(_{1A}\) mutant undergoes full internalization yet does not signal (Conchon et al., 1994; Miserey-Lenkei et al., 2001); an AT\(_{1A}\) receptor carrying five tyrosine mutations is deficient in inositol phosphate and calcium signaling (Doan et al., 2001) but retains robust AngII-induced intracellular tyrosine kinase signaling and cell proliferation; and AngII causes internalization, but not phosphorylation, of constitutively active AT\(_{1A}\) receptor mutants (Thomas et al., 2000).

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References


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