

Ligand-induced endocytosis and nuclear localization of angiotensin II receptors expressed in CHO cells

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Abstract

A construct (AT1R-NF) containing a "Flag" sequence added to the N-terminus of the rat AT₁ receptor was stably expressed in Chinese hamster ovary cells and quantified in the cell membrane by confocal microscopy after reaction with a fluorescein-labeled anti-Flag monoclonal antibody. Angiotensin II bound to AT1R-NF and induced endocytosis with a half-time of 2 min. After 60-90 min, fluorescence accumulated around the cell nucleus, suggesting migration of the ligand-receptor complex to the nuclear membrane. Angiotensin antagonists also induced endocytosis, suggesting that a common step in the transduction signal mechanism occurring after ligand binding may be responsible for the ligand-receptor complex internalization.

Key words

- Angiotensin II
- Agonist-induced receptor internalization
- Endocytosis
- Fluorescence-tagged receptor
- Nuclear angiotensin receptors
- Renin-angiotensin system

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Introduction

Two receptor subtypes (AT₁ and AT₂) have been described for the octapeptide hormone angiotensin II, the AT₁ receptor being responsible for most of the classical actions of this peptide (for a review, see Ref. 1).

As is the case with other G protein-coupled receptors, interaction of the AT₁ receptor with its specific ligand, besides triggering the release of second messengers that leads to cellular responses (2), also triggers endocytosis (for a review, see Ref. 3) and processing of the ligand-receptor complex which occurs in endosomes, part of the receptors being returned to the plasma membrane (4). Internalization and recycling are important factors in determining the number of free receptors on the cell surface available

for interaction with the ligand (5).

The structural requirements for ligand-induced internalization of G protein-coupled receptors have been investigated, and different motifs were found to be involved in this process. Usually these motifs are located in the intracellular domains of the receptors and contain aromatic (mainly tyrosine) or hydrophobic amino acid residues. One such motif is the highly conserved NPXXY sequence located in the intracellular end of the seventh transmembrane domain, containing a tyrosine residue that was shown to be essential for internalization of the β_2 -adrenergic receptor (6). However, mutations in the corresponding motif in the angiotensin AT₁ receptor did not affect ligand-induced internalization (7-9). On the other hand, the C-terminal cytoplasmic domain of AT₁ was

found to be important for internalization (8, 10,11), particularly residues L316, Y319 (6), T332, S335, T336 and L337 (7). Furthermore, residues 215-220 at the N-terminal portion of the third intracellular loop, but not other segments of this loop, were found to be important for AT₁ endocytosis (12).

Since AT₁ ligand-induced internalization is independent of the triggering of the cellular responses by angiotensin II, it is possible that the structural features responsible for agonist-receptor internalization may differ from those involved in binding and signal transduction. In this respect, the available data are somewhat conflicting. Conchon et al. (13) report that the agonist [Sar¹]-angiotensin II and the antagonist [Sar¹,Ile⁸]-angiotensin II are equally potent in inducing internalization of AT₁ receptors, whereas the nonpeptide antagonist DuP735 is far less potent. Other authors, however, found that peptide antagonists are much less capable (14) or incapable (15) of promoting receptor internalization. These findings, however, were obtained with radio-iodinated ligands, and it would be important to compare them with measurements made with non-iodinated ligands. Furthermore, previous studies of internalization were done with the "acid wash" technique, in which the amount of internalized radiolabeled ligand is estimated by the amount of radioactivity that remains on the cell after treatment with an acidic solution, which is thought to selectively remove non-internalized ligand. It would be desirable to investigate this phenomenon with a more direct method for the assessment of receptor internalization. Therefore, in the present study we have examined the ability of non-iodinated agonists and antagonists to induce internalization of wild-type rat AT_{1A} receptors stably expressed in Chinese hamster ovary (CHO-K1) cells. To estimate internalization we used a method based on the introduction of an epitope ("Flag") at the amino terminus of the receptor, and detection of the Flag-tagged receptor (AT1R-NF)

in the cells by immunofluorescence and confocal microscopy.

Material and Methods

Ligands

Angiotensin II, [Sar¹]-angiotensin II and [Sar¹,Ala⁸]-angiotensin II were synthesized by the solid phase method using Boc-chemistry, as previously described (16). The peptides were purified by HPLC and their purity and molecular weight were confirmed by mass spectrometry. DuP753 was kindly provided by the DuPont Merck Pharmaceutical Co. (Wilmington, DE, USA). Angiotensin II was labeled in our laboratory with ¹²⁵I (2200 Ci/mmol) (Amersham, Little Chalfont, Buckinghamshire, UK) using the iodo-beads method (Pierce Chemical Co., Rockford, IL, USA), as previously described (17).

Construction of pAT1R-NF

A vector coding for a construct containing the Flag epitope (DYLDLDDL) added to the N-terminus of the rat AT_{1A} receptor (18) was generated. Initially, the AT₁ cDNA was cut from the p171 vector (19) by hydrolysis with the restriction enzymes *Nco*I and *Bam*HI. The vector pGEM3z-FlagβH6 (containing the sequence: signal peptide-endoropeptidase cleavage site-Flag-β-adrenergic receptor) was also hydrolyzed by the same restriction enzymes to remove the adrenergic receptor sequence and the AT₁ cDNA was inserted and ligated in its place. The resulting vector was hydrolyzed with *Bam*HI and *Hind*III to release Flag-AT1R and placed in p171 at the same restriction sites. The final construct, named pAT1R-NF, contains the AT₁ receptor sequence with the addition of the octapeptide Flag sequence at the N-terminus.

Transfection of pAT1R-NF into CHO-K1 cells

The cells were seeded at the density of

10⁴ cells/cm² in 80-cm² culture bottles (Corning Costar Corp., Cambridge, MA, USA) and maintained in RPMI medium (Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C with 5% CO₂ and 95% air. After 24 h the cells were transfected with 20 µg DNA by co-precipitation with calcium phosphate (20), and 48 h later were submitted to antibiotic selection with 0.8 mg/ml G-418 (Gibco). The selection process was maintained for about 2 weeks, until antibiotic-resistant clones were obtained. To isolate the clones, the cells were trypsinized and diluted to a single cell per well on a 96-well plate. The cells were maintained under the same conditions and, as confluence was attained, they were successively transferred to 24- and 6-well plates.

Northern blot assays

The cells were cultured in 80-cm² culture bottles until confluence was reached. Total RNA was extracted by lysis of the confluent cells with Trizol solution (Gibco), and purified under the conditions and according to the procedures recommended by the manufacturer. After electrophoresis on agarose gel with 1.2% formaldehyde (20), the RNA was transferred from the gel to a nylon membrane (Bio-Rad Laboratories, Hercules, CA, USA) by capillarity for 6 h, with saline-sodium citrate buffer (Sigma, St. Louis, MO, USA). Hybridization was done at 42°C under the conditions suggested by the membrane manufacturer, using as probes the AT₁ receptor cDNA or the region corresponding to the Flag sequence labeled with ³²P-dCTP (Amersham). The time for autoradiography varied from 3 to 4 days at -80°C with an intensifying screen.

Immunocytochemistry

Transfected cells grown on coverslips

were rinsed in 100 mM PBS, pH 7.4, and then incubated in RPMI medium without serum (Gibco) at 37°C for 30 min. Ligands were added to the cells and incubated for specific time periods in the humidified incubator. The cells were washed twice with 0.5 ml PBS and incubated with 1% BSA in PBS at 37°C for 30 min to block nonspecific binding. Two different procedures were used to visualize the receptors by immunofluorescence. To detect the receptors present only on the cell surface, the ligand-treated cells were first incubated with 10 µg/ml of primary antibody (mouse anti-Flag M2 monoclonal antibody, Eastman Kodak Co., Rochester, NY, USA) for 30 min at room temperature. The cells were washed with 0.5 ml PBS and incubated with the secondary antibody, rabbit anti-mouse IgG labeled with fluorescein isothiocyanate (FITC) (Sigma), and left for 30 min at room temperature. The coverslips were washed and the cells fixed with methanol for 3 min and then mounted in glycerol-buffered PBS (1:9). To reveal intracellularly located receptors as well, the fixation step preceded the treatment with the primary and secondary antibodies. Previous fixation using methanol allowed the antibody to reach the intracellular compartment, a fact that did not occur in the post-fixed cells. The cells were examined with a Bio-Rad 1024-UV confocal system attached to a Zeiss Axiovert 100 microscope, using a 63 x NA 1.4-Plan-Apochromatic (DIC) oil immersion objective and a 2-mm pinhole. Kalman averaging was used to collect all images. The fluorescent images were printed using an NP-1600 printer from Codonics. After localizing an area with homogeneously spread cells, the fluorescence contained on a defined surface was measured using the Quant Laser Sharp software, version 1.02 (Bio-Rad). With this program it is possible to eliminate the background and quantify the fluorescence emitted by cells. For each experiment, repeated four times, the measurements were made with the same number of

cells (100). In cells treated with different ligand concentrations, the rate of internalization was estimated by the disappearance of the fluorescence associated with the membranes. Groups of cells not treated with the ligand, in which the fluorescence was restricted to the cell membranes, were used as controls for internalization.

Receptor internalization

Agonist-induced receptor endocytosis was estimated by measuring the disappearance of the AT₁R-NF receptor from the cell membrane, as visualized by immunocytochemistry, and also by the "acid wash" method, which is commonly used to distinguish between cell surface and intracellular receptors (21). The cells were incubated with 100 nM angiotensin II for different times and incubations were stopped by washing with PBS. The cells were washed for 3 min with 50 mM acetic acid in 150 mM NaCl, pH 3.0, followed by two washes with PBS. The cells were incubated with BSA, and then with the primary and secondary anti-

bodies, post-fixed with methanol and examined for fluorescence as described above.

Binding experiments

CHO-K1 cells transfected with pAT₁R-NF were seeded at 5×10^4 cells per well in 24-well plates, and left for 24 h at 37°C in a humidified incubator with 5% CO₂ and 95% air. The culture medium was siphoned off and the cells were washed twice with cold PBS and then incubated in "binding buffer" (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 4 mM KCl, 5 mM MgCl₂, 1 mM CaCl₂, 10 µg/ml bacitracin and 2 mg/ml glucose) in the presence of 30 pM [¹²⁵I]-angiotensin II and of different concentrations of unlabeled angiotensin II (1 pM to 10 µM). After overnight incubation at 4°C with gentle shaking, the binding buffer was siphoned off, the cells were washed three times with "washing buffer" (25 mM Tris-HCl, 140 mM NaCl, 5 mM MgCl₂, 0.1% BSA) and then lysed with 2% NP-40 solution containing 8 M urea and 3 M acetic acid. Receptor-bound radiolabel was counted with a gamma counter (Packard) and the results were treated by nonlinear regression analysis using the "GraphPad Prism" (Graph-Pad Software, San Diego, CA, USA) to determine the kinetic constants.

Results

Characterization of the AT₁R-NF receptor expressed in CHO-K1 cells

The expression of the AT₁ receptor, alone or linked with the Flag epitope, was analyzed by Northern blot using total RNA extracted from stably transfected CHO-K1 cells. Figure 1 shows that the untransfected cells expressed undetectable levels of AT₁ receptor mRNA, whereas those transfected with AT₁R-NF showed a positive signal with both probes of a size corresponding to about 18S ribosomal RNA. When a ³²P-labeled oligonucleotide complementary to the Flag

Figure 1. Northern blot of total RNA extracted from CHO-K1 cells. Lanes 1 and 4, Nontransfected cells; lanes 2 and 5, cells transfected with AT₁R-NF; lanes 3 and 6, cells transfected with the wild-type receptor. Blots were probed with a ³²P-labeled oligonucleotide complementary to the Flag sequence (lanes 1, 2 and 3) or with full-length wild-type cDNA (lanes 4, 5 and 6). The positions of 28S and 18S ribosomal RNA are indicated.

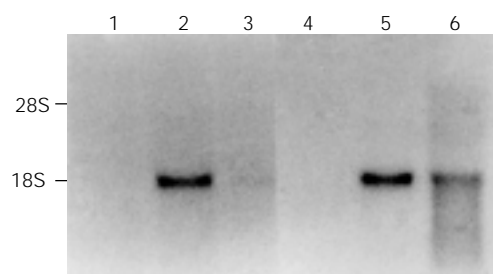
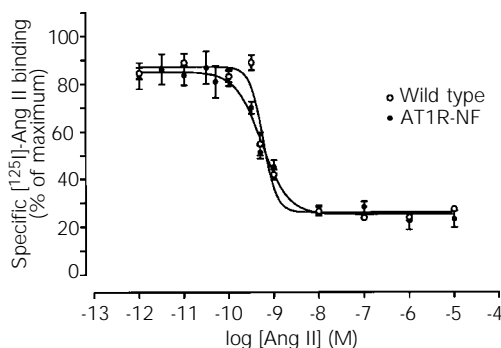


Figure 2. Competition binding of angiotensin II to CHO-K1 cells stably expressing AT₁R-NF and wild-type receptors. The cells were incubated overnight with 30 pM [¹²⁵I]-angiotensin II ([¹²⁵I]-Ang II) at 4°C in the presence of different concentrations of unlabeled angiotensin II. Each point represents the mean \pm SD from 6-9 determinations.



sequence was used as a probe, only cells transfected with AT1R-NF showed the corresponding band, confirming the presence of the Flag label in the receptor.

Figure 2 shows that the ligand-binding properties of the CHO-K1 cells expressing the Flag-labeled receptor were similar to those of the wild-type AT₁, with EC₅₀ values of 6.2 nM and 5.8 nM, respectively.

Immunocytochemistry

In cells expressing the AT1R-NF mutant, strong fluorescence signals were observed after treatment with 100 nM angiotensin II and incubation with the anti-Flag antibody at room temperature. When incubation with the antibody preceded fixation, a strong fluorescence was observed on the cell surface (Figure 3A), which gradually disappeared at 37°C (Figure 3B-D). When incubation was performed at 4°C, the fluorescence remained on the cell surface after 90 min (Figure 3E). When the antibody was applied to angiotensin II-treated cells after fixation with methanol, the fluorescence initially appeared on the cell surface (Figure 4A), and then gradually moved to the intracellular space (Figure 4B-F). At the end of the 90-min observation period, all fluorescence had disappeared from the cell surface and tended to be distributed around the cell nuclei (Figure 4F). In nontransformed CHO cells and in cells transfected with wild-type AT₁ receptors no fluorescence was observed after up to 90-min incubation using either protocol (data not shown).

A half-time of internalization of 2 min was found, which was confirmed by the acid

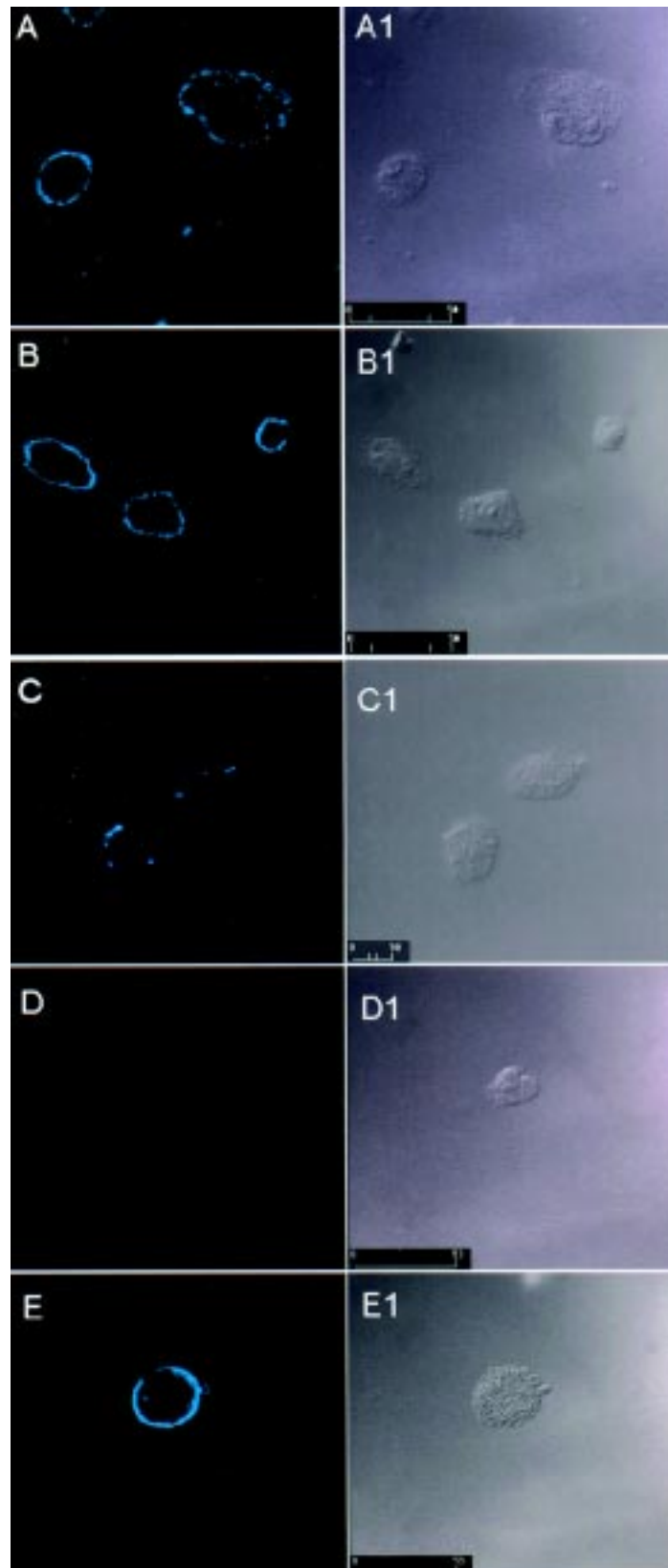


Figure 3. Agonist-induced AT1R-NF internalization visualized by reaction with the anti-Flag M2 antibody before fixation with methanol. CHO-K1 cells expressing the AT1R-NF receptor were incubated with 100 nM angiotensin II at 37°C for 0 (A), 10 (B), 30 (C) and 90 (D) min or at 4°C for 24 h (E). The cells' immunofluorescence is shown on the left and the respective no-mask images are shown on the right.

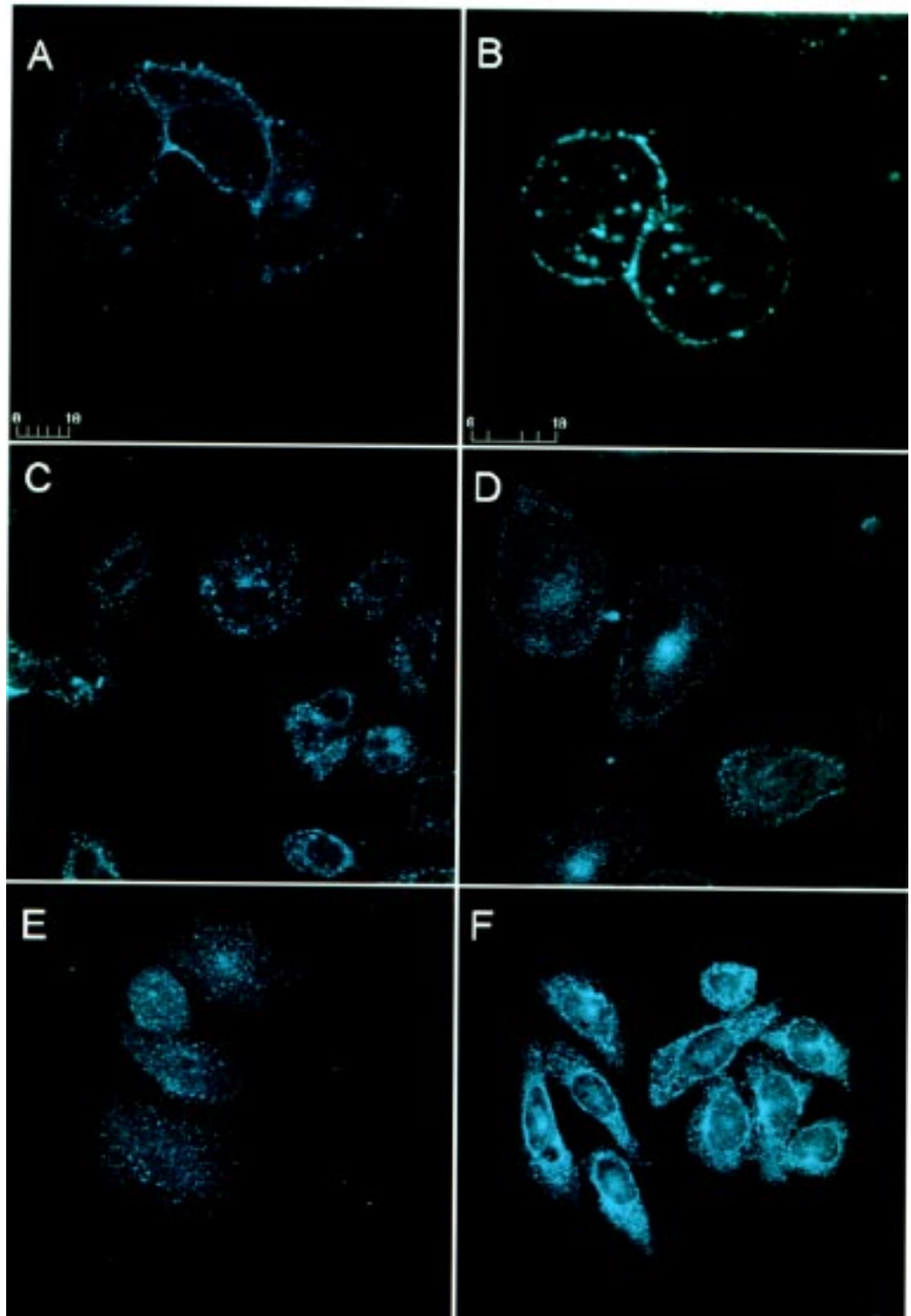


Figure 4. Agonist-induced AT1R-NF internalization visualized by reaction with the anti-Flag M2 antibody after fixation with methanol. CHO-K1 cells expressing the AT1R-NF receptor were incubated with 100 nM angiotensin II at 37°C for 0 (A), 10 (B), 20 (C), 40 (D), 60 (E) and 90 (F) min.

wash method (data not shown) and was similar to the values described in the literature for angiotensin endocytosis in different cells (21). Figure 5 shows that angiotensin II induced internalization of the AT₁R-NF receptor with an EC₅₀ of 2 nM, which is close to the 6.2 nM value obtained in the binding experiments (Figure 2). Similar results were obtained with other ligands, namely the agonistic peptide analogue [Sar¹]-angiotensin II and the antagonistic peptide analogue [Sar¹,Ala⁸]-angiotensin II, whereas the non-peptide antagonist DuP753 was internalized with about 50% of the degree of endocytosis observed with angiotensin II. As a control we employed bradykinin, a peptide that does not interact with angiotensin II receptors and was not internalized.

Staurosporine, an inhibitor of protein kinases C and A, which is thought to inhibit receptor desensitization (22), had no significant effect on the internalization induced by high angiotensin II concentrations (Figure 5).

Discussion

Our results show that CHO-K1 cells stably transfected with the Flag-tagged angiotensin II receptor bind angiotensin II with an affinity similar to that of cells expressing the wild-type AT₁, confirming that the modified receptor is properly expressed in the cell membrane (3).

We have demonstrated that the expression of the AT₁R-NF receptor in the cell membrane may be quantitatively determined by integration of the fluorescence signal associated with the membrane after treatment with the mouse anti-Flag antibody followed by the fluorescent-labeled rabbit anti-mouse IgG. This quantitation of the receptors present on the cell membrane allows a more direct procedure to measure angiotensin II receptor endocytosis. The internalization was specific for AT₁ receptor ligands, since it was not observed with bradykinin, which does not bind to angiotensin II receptors. In

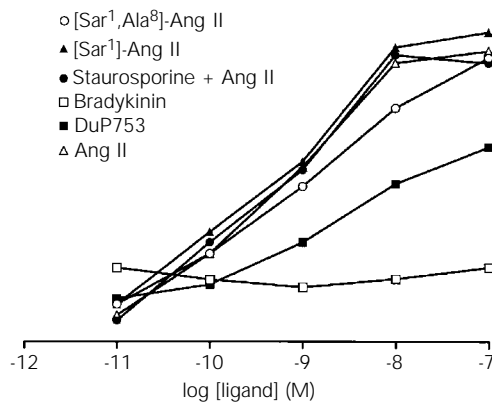


Figure 5. Effect of different ligands on the rate of angiotensin II (Ang II) receptor internalization. The CHO-K1 cells expressing the AT₁R-NF receptor were incubated with different concentrations of the ligands at 37°C for 30 min and fixed with methanol after labeling with the anti-Flag/FITC-anti-mouse IgG system. The fluorescence signal associated with the cell membrane was integrated and expressed as a percentage of the fluorescence emitted by cells incubated in the absence of ligand.

addition, the protein kinase inhibitor staurosporine did not prevent internalization, in agreement with the hypothesis that desensitization is caused by the rapid uncoupling of the receptor from G protein and not necessarily by receptor internalization (22,23).

Although less is known about the sites of the extracellular domains of AT₁ that are important for triggering receptor internalization, some information may be inferred from ligand structure-activity studies. The peptide analogue [Sar¹]-angiotensin II, which has a high agonistic activity (24), and the peptide antagonist [Sar¹,Ala⁸]-angiotensin II both caused internalization with the same efficiency as angiotensin II (Figure 5). This is in agreement with the previous finding that [Sar¹]-angiotensin II and [Sar¹,Ile⁸]-angiotensin II are equally capable of promoting receptor internalization in CHO cells expressing AT₁ (13), but contrasts with reports that angiotensin II antagonists are less capable (14) or incapable (25) to induce endocytosis in cultured adrenal glomerulosa cells.

It is noteworthy that even the nonpeptide antagonist DuP753 was able to induce endocytosis, albeit with less efficiency than the peptide analogues, also in agreement with previous findings with transfected CHO cells (14). Since previous work has shown that different receptor sites are responsible for binding peptide and nonpeptide ligands

(26,27), the fact that both types of ligands cause internalization suggests that a common step in the transduction chain of events occurring after binding may be responsible for ligand-induced endocytosis. Further studies are needed to investigate this possibility and to characterize this hypothetical step.

Receptor-mediated sequestration and endocytosis have been shown to be the first steps in receptor processing and recycling, involving capture of both ligand and receptor by endosomes, followed by diversion of angiotensin II to lysosomes and recycling of the receptors to the plasma membrane (4). In our experiments, however, we detected an accumulation of immunofluorescence around the cell nuclei, indicating that part of the receptor molecules are able to reach the nucleus, possibly bound to their specific ligand.

Some evidence has been reported that angiotensin II, besides its better known effect on plasma membrane receptors triggering second-messenger cascades, may be transported to the nucleus, where it may interact directly with receptors on the nuclear membrane. Thus, early autoradiographic experiments (28) showed that tritium-labeled angiotensin II accumulated around the nuclei of smooth and cardiac muscle cells, and

more recent work showed that an angiotensin II-colloidal gold conjugate also tended to locate around the nuclei (29). Furthermore, high-affinity specific angiotensin II receptors of type I were shown to be present in the nuclear membrane and to induce transcription of renin and angiotensinogen RNA (30,31).

A high-affinity soluble angiotensin II-binding protein has been described in the cytosol of liver cells (32), which may play a role in transporting angiotensin II molecules from the cell membrane to the perinuclear space where it may interact with nuclear membrane receptors. Another possibility, however, suggested by our results, is that the AT₁ receptor itself, through ligand-induced internalization, may transport the hormone to the nucleus, where it might deliver it to the nuclear receptors.

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