Residues Val^{254}, His^{256}, and Phe^{259} of the Angiotensin II AT$_1$ Receptor Are Not Involved in Ligand Binding but Participate in Signal Transduction

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The role of the external third of helix VI of the angiotensin II (AII) AT$_1$ receptor for the interaction with its ligand and for the subsequent signal transduction was investigated by individually replacing residues 252–256 by Ala, and residues 259 or 261 by Tyr, and permanently transfecting the resulting mutants to Chinese hamster ovary (CHO) cells. Binding experiments showed no great changes in affinity of any of the mutants for AII, [Sar^1]-AII, or [Sar^1, Leu^8]-AII, but the affinity for the nonpeptide antagonist DuP753 was significantly decreased. The inositol phosphate response to AII was remarkably decreased in mutants V254A, H256A, and F259Y. These results indicate that AT$_1$ residues Val^{254}, His^{256}, and Phe^{259} are not involved in ligand binding but participate in signal transduction. Based in these results and in others from the literature, it is suggested that, in addition to the His^{256} imidazole ring, the Phe^{259} aromatic ring interacts with the AII's Phe^8, thus contributing to the signal-triggering mechanism. (Molecular Endocrinology 12: 810–814, 1998)

INTRODUCTION

Over the years, a great deal of data have been collected on the structural requisites for the potent effects of the octapeptide hormone angiotensin II (AII: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) upon different biological systems (for a review see Ref. 1). These findings showed that, among other features, the agonist molecule must have a Phe residue at the C-terminal position and a free C-terminal carboxylate to exert its biological activity. In particular, the Phe^8 side-chain, although not needed for binding to the receptor, is very important for triggering the cellular response. Thus, when Phe^8 is replaced by alanine or leucine, for instance, high-affinity analogs with low activity are obtained, which act as competitive antagonists of AII (2, 3).

More recently, two AII receptor subtypes (AT$_1$ and AT$_2$) have been cloned and sequenced and shown to belong to the family of rhodopsin-like G protein-coupled receptors (GPCRs), characterized by having seven transmembrane α-helices (TM-I–TM-VII) linked by three extracellular and three intracellular loops (4). The AT$_1$ receptor has been shown to be responsible for most of the known biological effects of AII, and a great deal of information was recently reported about the importance of its amino acid residues for ligand binding (5–13). As a result, some interactions have been proposed between the AII molecule and different portions of the AT$_1$ receptor, as modeled with basis on the rhodopsin seven-helix configuration (6, 14, 15). Among these interactions, it was proposed that the ϵ-ammonium group of Lys^{199}, at the external third of AT$_1$'s TM-V, is the counterion for the C-terminal carboxylate of AII (5–7). This interaction would pull the C-terminal His-Pro-Phe sequence of AII to a deeper position in the locus of the receptor central cavity surrounded by helices III–VI (6, 11). The possibility therefore arises that other residues placed at the external third of helices IV–VI, and pointing to the receptor central cavity, might also be crucial for AII binding. Previous attempts to confirm this hypothesis experimentally have been unsuccessful. Thus, whereas the K199A mutant presented a remarkable loss of affinity (increase in binding constant by at least 2 orders of magnitude) (5–7), not more than 30-fold losses were observed in mutants modified in residues of helices III–VI, which are thought to be close to Lys^{199} in the seven-helix bundle configuration (6, 7, 9–11). However, a single but quite significant finding was that the

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H256A mutation does not affect ligand binding but impairs the signal transduction triggered by AII through the activation of phospholipase C (11). This finding was interpreted as indication that the His^{256} side chain might be interacting with AII's Phe^8 aromatic ring, thus stabilizing a productive peptide-receptor complex. According to this model, the cavity surrounded by helices III–VI might be the receptor locus at which the signal transduction is originated after agonist binding.

In the present work we have investigated this hypothesis by studying the effect of point mutations in different residues of the AT_1 receptor's TM-VI upon the phospholipase C response of permanently transfected Chinese hamster ovary (CHO) cells. Our results indicate that mutations at residues Val^{254} and Phe^{259} of the AT_1 receptor also cause impairment of signal transduction without remarkable loss of affinity for the agonist. These results led us to suggest that AT_1's Phe^{259} side chain might also be interacting with the AII's Phe^8 aromatic ring, playing a role that does not depend on His^{256} but alone is able to elicit the receptor's physiological response.

RESULTS

AT_1 Mutants

The mutants were obtained from point mutations carried out on the amino acid sequence S^{252} W^{253} V^{254} P^{255} H^{256}. . . . F^{259}. . . F^{261}, corresponding to the extracellular third of TM-VI of the AT_1 receptor. AT_1 residues 252–256 were mutated to Ala, residues 259 and 261 were mutated to Tyr, and the resulting mutants were permanently transfected in CHO cells. The transfected cells were assayed for their affinity toward different ligands and for receptor-mediated phospholipase C activation.

Binding Experiments

IC_{50} values were estimated from binding experiments in which competition curves were obtained for the displacement of the respective iodinated peptide by AII as well as by the agonist peptide analog [Sar^1]-AII, the antagonist peptide [Sar^1, Leu^8]-AII, and for the displacement of the nonpeptide antagonist DuP753 by its ^3H-labeled analog. With the exception of an increased affinity observed in the S252A mutant, the IC_{50} values obtained for AII with all the mutants were not much different from those obtained with the wild-type receptor (Table 1). This also occurred with [Sar^1]-AII and [Sar^1, Leu^8]-AII, with the exception of the mutant F261Y, which did not bind these two ligands. This intriguing but reproducible finding may reflect a difference in the interaction of the Sar^1 analog with AT_1's helix VI and will require further investigation. With the nonpeptide antagonist DuP753, significant binding impairment was observed with most of the receptor mutants. Thus, with the exception of the P255A mutant, which showed only an 8-fold decrease in affinity, the other mutants showed a 20- to 30-fold loss. These changes in affinity are in agreement with the idea that the binding of nonpeptide ligands involves sites on transmembrane helices VI and VII (16).

Table 1. IC_{50} Values (nM) for the Binding of Different Ligands to AT_1 Receptors with Single-Site Mutations in the External Third of TM-VI

Mutant	AII	[Sar^1]-AII	[Sar^1,Leu^8]-AII	DuP753
Wild-type	5 (1)	0.9 (0.1)	0.7 (0.5)	3 (1)
Ser252Ala	0.1 (0.1)	1.3 (0.1)	0.6 (0.3)	66 (7)
Trp253Ala	7.0 (0.4)	9.9 (0.2)	1.0 (0.1)	90 (20)
Val254Ala	5.0 (0.1)	11.4 (0.4)	0.5 (0.04)	55 (16)
Pro255Ala	9.0 (0.3)	6.8 (0.2)	0.9 (0.1)	23 (9)
His256Ala	10.0 (0.9)	5.3 (0.4)	0.8 (0.1)	68 (4)
Phe259Tyr	2.0 (0.3)	5.8 (0.3)	0.20 (0.04)	58 (4)
Phe261Tyr	7 (2)	NB	NB	59 (10)

Values inside parentheses are SDs. NB, No detectable binding.
Data represent the means from at least two separate measurements made in triplicate.

Table 2. All-Induced Inositol Phosphate Production in CHO Cells Expressing AT_1 Mutants

Mutant	ED_{50} (nM)	E_{max} (pM/cell/h)	B_{max} (fmol/cell)	Specific Activity (pM/fmol/h)
Wild-type	0.8 (0.3)	2.4 (0.2)	0.06 (0.01)	40 (7)
S252A	1.3 (0.5)	0.9 (0.1)	0.03 (0.01)	30 (10)
W253A	1.7 (0.8)	2.3 (0.2)	0.05 (0.02)	50 (20)
V254A	ND	ND	0.06 (0.02)	ND
P255A	0.6 (0.1)	3.5 (0.3)	0.06 (0.02)	60 (20)
H256A	ND	ND	0.06 (0.01)	ND
F259Y	1.0 (0.6)	0.30 (0.05)	0.05 (0.01)	6 (2)
F261Y	1.1 (0.4)	1.3 (0.1)	0.03 (0.01)	40 (20)

ND, Low response level [not amenable to Wilkinson's treatment of data (22)].
Values inside parentheses are SDs. B_{max}, Maximum binding values determined by Scatchard analysis of saturation binding. E_{max}, Maximum inositol phosphate response. ED_{50}, 50% effective ligand concentration. Data represent the means (and SDs) of at least two separate measurements made in triplicate.

Inositol Phosphate Responses

The cells transfected with the wild-type and mutant receptors were stimulated with AII and their response, in terms of inositol phosphate accumulation, was measured. Table 2 shows that, except for V254A and H256A, which presented no detectable response, the ED_{50} values obtained for the other mutants were not significantly different from that of the wild-type receptor. The values for the maximum effects (E_{max}), however, show that, in addition to V254A and H256A, other mutants showed significant losses in their ability to activate the inositol phosphate response. Thus, the

S252A, F259Y, and F261Y mutants had E_{max} values that were, respectively, 38%, 13%, and 54% of that of the wild-type receptor. To determine whether this diminished response could be due to a lower receptor concentration, we used the same cell batches used for the inositol phosphate measurements to perform saturation binding curves with ^{125}I-labeled AII. The maximum binding (B_{max}) and specific activity (E_{max}/B_{max}) values obtained in mutant and wild-type receptors are shown in the last two columns of Table 2. These values indicate that the smaller capacity of the S252A and F261Y mutants to induce response (38% and 54% of the wild type, respectively) may be attributed, at least in part, to the smaller receptor concentrations (~50% relative to wild type) as denoted from the wild-type levels of specific activity. Mutants V254A, H256A, and F259Y, however, induced wild-type-like B_{max} values but still presented a low response to AII, indicating that the activity of these receptor species was really affected by the corresponding mutations.

DISCUSSION

Our results show that the amino acid residues Val^{254}, His^{256}, and Phe^{259}, located on the external third of TM-VI of the AT_1 receptor, which are not important for ligand binding, are involved in receptor-mediated G protein activation. In the case of His^{256}, this agrees with the previous finding (11) that mutation in this residue causes diminished inositol phosphate response without commensurate change in binding affinity of ligands. Mutations at the 254 position have not yet been reported, whereas at position 259 only binding studies were reported for the F259A mutant, which showed approximately 15-fold decreased affinity for AII (6). This is significantly larger than the 2-fold loss of affinity found for F259Y (Table 1), suggesting that the benzene ring of the Phe^{259} side chain has contributed in part to the free energy of receptor binding.

Noda *et al.* (11) attributed an important role to His^{256} in the signal-triggering mechanism of the AT_1 receptor, proposing that the imidazole ring of this residue interacts with AII's Phe^8 aromatic ring while the peptide's terminal carboxyl is salt-bridged to the receptor's Lys^{199} ammonium group. In addition, experimental findings obtained with Lys^{199} receptor mutants show that this residue can modulate the effect of His^{256} side chain on receptor binding. Whereas the H256A mutation does not affect binding, the loss of affinity of the [K199A;H256A] double mutant is significantly larger than that of the K199A mutant (7), suggesting that, in the AT_1 structure, the Lys^{199} ammonium group is able to keep the His^{256} imidazole ring at a specific position, perhaps by forming an intramolecular hydrogen bond. This interaction might not contribute to ligand-receptor affinity, as shown experimentally (6, 7), but could be important to form a more productive receptor-agonist binding mode. This might involve a relay-like structure (17) consisting of AII's C-terminal carboxylate, the receptor's Lys^{199} ammonium group, and the His^{256} imidazole ring.

Table 2 shows that the F259Y mutation significantly impairs the signaling in AT_1 receptors, allowing us to suggest, in addition to a previously drawn picture (11), that the receptor's Phe^{259} side chain also interacts with AII's Phe^8 aromatic ring, but places it at a different position than that of His^{256}. In a schematic representation of the AII-AT_1 complex (Fig. 1), the His^{256} and Phe^{259} side chains are shown at the same side of helix VI, pointing at different levels toward AII's C terminus and flanking the agonist's Phe^8 benzene ring. In this configuration one of the His^{256} imidazole nitrogens may point toward the salt bridge between the Lys^{199} ammonium and the AII carboxyl groups, a more favorable position for the histidine side chain, which is more commonly found forming bridges between hydrogen-

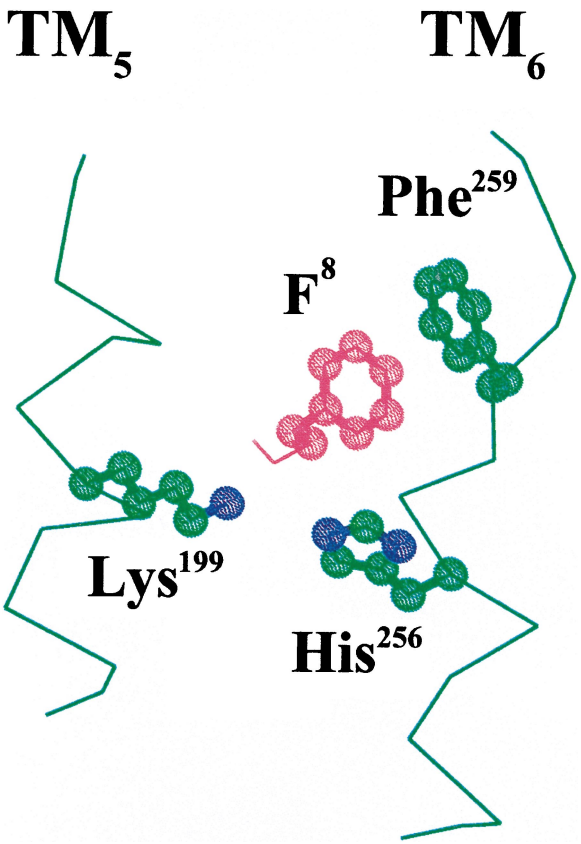

Fig. 1. A Computer-Aided Representation of the Interaction of AII's C Terminus with the External Third of TM-V and TM-VI of the AT_1 Receptor

Using the program WHAT IF (23), the side chains corresponding to the AT_1 receptor sequences were added to a backbone template taken from the corresponding regions of the bacteriorhodopsin 3D structure (24). Based on previous models (6, 7, 13), the Lys^{199} (TM-V) and His^{256} (TM-VI) side chains were set close to each other and at bond distances of the AII's Phe^8 (F8) side chain and the C-terminal carboxylate. The aromatic rings of the receptor's Phe^{259} and the peptide's Phe^8 are in close proximity, allowing a double aromatic ring side-coupling to be admitted in the structure.

donor and -acceptor groups than in hydrophobic clusters (17). Inoue *et al.* (13) propose a model showing also a clustering of His^{256}, Phe^{259}, and AII's Phe^8 side chains. This role of Phe^{259} has not yet been experimentally verified by observing the effect of nonaromatic replacement of this residue on AT_1 activation. Nevertheless, a plausible explanation for the deleterious effect of the F259Y mutation on signaling would be a possible interaction of the Tyr^{259} phenoxyl with some other side chain which would shift its aromatic ring to a position removed from AII's Phe^8 and the receptor's His^{256}.

MATERIALS AND METHODS

Materials

^{125}I-labeled and ^{3}H-labeled DuP753 were purchased from New England Nuclear (Boston, MA), and DuP753 was kindly provided by the DuPont/Merck Pharmaceutical Co. (Wilmington, DE). The peptides AII, [Sar^1]-AII, and [Sar^1,Leu^8]-AII were synthesized and labeled with ^{125}I (18) using the iodo-beads method (Pierce Chemical Co., Rockford, IL) in our laboratory. Myo-[2-^{3}H]inositol was purchased from Amersham International (Little Chalfont, Buckinghamshire, UK) and Dowex-AG-1 $\times$ 8 resin (100–200 mesh in formate form) from Bio-Rad (Richmond, CA).

Site-Directed Mutagenesis of AT_1 Receptor

A new AT_1 receptor expression vector (pAT1R-NF), containing an epitope ("Flag") with the hydrophilic amino acid sequence Asp-Tyr-Leu-Asp-Asp-Asp-Asp-Leu at the N terminus (19), was generated using the expression vector pTEJ8 (20), carrying the rat AT_{1A} receptor cDNA and the neo^R resistance marker, which was kindly provided by Dr T. W. Schwartz. The construction of mutants with pAT1R-NF was done with the PCR overlap extension technique using Vent polymerase (New England Biolabs, Inc. Beverly, MA) for amplification. For identification of mutant receptor cDNA, the silent restriction sites introduced during the synthesis of oligonucleotides were first verified. Later, the region of DNA corresponding to the PCR-amplified cassette was sequenced by Sanger's dideoxynucleotide sequencing method (20).

Cell Culture and Transfection

Chinese hamster ovary cells were cultured in DMEM containing 10% FBS, penicillin, and streptomycin in a humidified atmosphere of 5% CO_2 and 95% air. Cell transfection was performed by calcium phosphate coprecipitation with plasmid DNA purified on Qiagen columns (QIAGEN Inc., Chatsworth, CA). Resistant cells were selected with 0.8 mg/ml geneticin (GIBCO/BRL, Gaithersburg, MD) for about 2 weeks, until antibiotic-resistant clones were obtained. The expressed receptors were visualized by immunocytochemistry using anti-Flag M2 (Eastman Kodak Co., Rochester, NY) as primary antibody and rabbit antimouse IgG labeled with fluorescein isothiocyanate (Sigma Chemical Co., St. Louis, MO) as second antibody, as described elsewhere (19). Northern blot analysis of nontransfected CHO cells showed no detectable signal for AT1 receptor expression. After the cells were grown in the selective medium, they were frozen in liquid nitrogen still in the presence of geneticin. For each experiment, only one vial of the cell stock was used, and cell amplification was carried out, in most cases with less than eight passages. Binding experiments done with and without geneticin in the medium showed no significant differences.

Binding Experiments

The cells 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% CO_2, 95% air. The culture medium was siphoned off, and the cells were washed twice with cold PBS. They were then suspended in cold binding buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 4 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 μg/ml bacitracin, and 2 mg/ml glucose) in the presence of a fixed concentration of the radioligands [^{125}I]AII, or [^{125}I-Sar^1]AII, or [^{125}I-Sar^1,Leu^8]AII, or [^{3}H]DuP753 and of different concentrations of the respective unlabeled compounds (1 pM to 1 μM). The plates were incubated overnight at 4 C with gentle shaking. The binding buffer was siphoned off and the cells were lysed with 2% NP-40 solution containing 8 M urea and 3 M acetic acid. Receptor-bound radiolabels were counted on a γ-counter (Packard Instrument Co., Downers Grove, IL), and the results were treated by nonlinear regression analysis using the Inplot software (Graph-Pad Software, San Diego, CA) to determine kinetic constants.

No evidence of the presence of AT_1 receptors was found in the untransfected CHO cells, since no signal was detected in Northern blots, and no AII binding was seen in control experiments using untransfected cells.

Inositol Phosphate Turnover

Confluent cells (1–2 $\times$ 10^6 cells) expressing the wild-type rat AT_1 receptor or mutants were cultivated for 18 h in inositol-free medium (199 Dulbecco with $NaHCO_3$ supplemented with 10% FCS, 2 mM glutamine, 0.1 mg/ml streptomycin, and 100 U/ml penicillin) in 3.5-ml culture plates containing 10 μCi [^{3}H]myoinositol (Amersham). The cells were washed twice with Tyrode solution (137 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, 0.49 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 5.6 mM D-glucose) and subsequently incubated for 30 min with the same solution containing 10 mM LiCl. AII dose-response experiments were performed with 30-min incubation time, and the reaction was terminated by siphoning off the medium and adding a mixture of 1 ml 0.1 M NaOH and 1 ml of chloroform-methanol (1:1, vol/vol). After centrifugation, 0.5 ml H_2O and 0.5 ml chloroform were added to the aqueous phase containing the inositol phosphates, the mixture was centrifuged, and the aqueous phase was applied to a column containing 0.5 ml of AG1-X8 anion-exchanger resin (Bio-Rad, Richmond, CA) (21). The resin was washed three times with 5 mM myoinositol and the [^{3}H]inositol phosphates were eluted by adding 1 ml of 1.0 M ammonium formate in 0.1 M formic acid. The data were analyzed by Wilkinson's treatment (22). Maximum binding values were determined on the same batch of the cells used in the inositol phosphate assays, by Scatchard analyses of saturation binding curves with [^{125}I]AII (specific activity, 2,000 Ci/mmol).

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