Interaction of Phe\(^8\) of Angiotensin II with Lys\(^{199}\) and His\(^{256}\) of AT\(_1\) Receptor in Agonist Activation*

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The acidic pharmacophores of selective ligands bind to Lys\(^{199}\) and His\(^{256}\) of the AT\(_1\) receptor (Noda, K., Saad, Y., Kinoshita, A., Boyle, T. P., Graham, R. M., Husain, A., and Karnik, S. (1995) J. Biol. Chem. 270, 2284-2289). In this report we examine how interactions between these residues and agonists activate inositol phosphate production in transiently transfected COS-1 cells. [Sar\(^1\)]angiotensin (Ang II) II and [Sar\(^1\)]Ang II-amide stimulated a 5-fold inositol phosphate response from wild-type AT\(_1\) receptor. The peptide agonist [Sar\(^1\),Ile\(^8\)]Ang II and the non-peptide agonist L-162,313 produced a partial but saturating response. Stimulation of wild-type receptor by [Sar\(^1\)]Ang II-amide and the mutant K199Q and K199A receptors by [Sar\(^1\)]Ang II demonstrates that AT\(_1\) receptor activation is not critically dependent on the ion-pairing of the \(\alpha\)-COOH group of Ang II with Lys\(^{199}\). The mutation of His\(^{256}\) produced diminished inositol phosphate response without commensurate change in binding affinity of ligands. The His\(^{256}\) side chain is critical for maximal activation of the AT\(_1\) receptor, although isosteric Gln substitution is sufficient for preserving the affinity for Phe\(^8\)-substituted analogues of [Sar\(^1\)]Ang II. Therefore, AT\(_1\) receptor activation requires interaction of Phe\(^8\) side chain of Ang II with His\(^{256}\), which is achieved by docking the \(\alpha\)-COOH group of Phe\(^8\) to Lys\(^{199}\). Furthermore, non-peptide agonists interact with Lys\(^{199}\) and His\(^{256}\) in a similar fashion.

Angiotensin II (Ang II)\(^1\) is a key hormone that influences blood pressure regulation. Two distinct classes of Ang II receptors, AT\(_1\) and AT\(_2\), mediate its function (1, 2). The AT\(_1\) receptor is responsible for mediating the potent vasoconstrictor effect of Ang II. Therefore, the AT\(_1\) receptor is a major target for drug design in the treatment of hypertension, congestive heart failure, and cardiac hypertrophy (1). Previous structure-activity evaluation has demonstrated that the Phe\(^8\) side chain and the \(\alpha\)-carboxyl group of Ang II are critical determinants of angiotensin’s biological potency (2). Several non-peptide agonists and antagonists of the AT\(_1\) receptor also preserve an acidic group attached to an aromatic function, suggesting that it is a crucial determinant of ligand specificity (1).

The binding pocket of the AT\(_1\) receptor for various ligands is not clearly defined. The structural model of the AT\(_1\) receptor contains seven transmembrane \(\alpha\)-helices with three interhelical loops on either side of the membrane. Structure-function studies of the AT\(_1\) receptor so far have indicated that the carboxyl terminus of Ang II and the non-peptide ligand bond within the transmembrane domain (3–11), but the binding of Ang II and other peptide analogues may also be influenced by the extracellular domain of the receptor (3–11). To identify points of interaction that are common to both peptide and non-peptide ligands on the AT\(_1\) receptor, we defined the subsite for binding the acidic pharmacophore that is present on all ligands (9). We showed that the carboxyl group of Ang II, tetrazole, and sulfonylamide groups of non-peptide antagonists bind to the \(\epsilon\)-amino group of Lys\(^{199}\) in the fifth transmembrane helix of the AT\(_1\) receptor. The role of this ion pair in the activation of AT\(_1\) receptor function is not clearly established. Aumelas et al. (12) showed that the \(\alpha\)-COOH group stabilizes the conformation of the Phe\(^8\) side chain in Ang II. Therefore, the interaction of Lys\(^{199}\) with the \(\alpha\)-COOH group of Ang II is likely very important for positioning the Phe\(^8\) within the pocket of the AT\(_1\) receptor. Molecular modeling studies indicate that Phe\(^8\) of Ang II might interact with His\(^{256}\) among other candidate residues. The functional role of His\(^{256}\) is not demonstrated as yet. Therefore, we investigated the following questions. Is the ion-pair interaction between Lys\(^{199}\) and \(\alpha\)-COOH of Ang II essential for the function of the receptor? What is the role of His\(^{256}\) in the AT\(_1\) receptor function? The results demonstrate that the ion-pair interaction of Ang II and Lys\(^{199}\) is not essential for receptor activation and that His\(^{256}\) plays an important role in receptor activation because it directly interacts with the Phe\(^8\) side chain of the ligand.

**EXPERIMENTAL PROCEDURES**

Materials—[Sar\(^1\),Ile\(^8\)]Ang II and Ang II were purchased from Bachem. Analogues of [Sar\(^1\),Ile\(^8\)]Ang II and Ang II were synthesized and purified by the peptide synthesis core facility of The Cleveland Clinic Foundation and characterized by mass spectrometry and peptide content analysis. [Sar\(^1\),Ile\(^8\)]Ang II was iodinated (specific activity 2200 Ci/mmol) by the lactoperoxidase method and purified (13). myo-[\(^{3}H\)-]inositol (20.5 Ci/mmol) was obtained from DuPont NEN. Losartan was a gift from DuPont-Merck. L-162,163 was a gift from Merck Sharp and Dohme.

Mutagenesis and Expression—A synthetic gene for rat AT\(_1\) receptor was used for expression and mutagenesis as described earlier (9, 10, 14). The expression level of receptor was determined by \(^{[125]I}\)-Sar\(^1\),Ile\(^8\)]Ang II saturation binding (9, 10, 15). The K, values (nanomolar) represent mean ± S.E., n = 3–10.

Inositol Phosphate (IP) Measurements—IP measurements were done as reported earlier (10). Total IP production was measured as described previously (16). Total IP production is expressed as a percentage of the maximum stimulation of wild-type AT\(_1\) receptor by [Sar\(^1\)]Ang II.

2 S. Sung and S. Karnik, unpublished observations.

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1 The abbreviations used are: Ang II or AII, the octapeptide hormone angiotensin II, NH\(_2\)-D-\(\beta\)-Y-I-H-P-F-COOH; L-162,163, [5,7-dimethyl-2-ethyl-3-{[4-[2-(n-butyl oxygen carbonyl sulfonyl amido)-isobutyl-thienyl] phenyl]methylimidazol}[4,5-J]pyridine (22); IP, inositol phosphate; HBSS, Hank’s balanced salt solution.

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Inositol phosphate production in COS-1 cells expressing wild-type and various mutants of the rat AT₁ receptor gene. Basal, shown as an open box, is the IP production in transfected cells without stimulation by [Sar¹]Ang II and the filled box is with stimulation by 10⁻⁶ M [Sar¹]Ang II. The concentration of other ligands is 10⁻⁶ M [Sar¹]Ang II, 10⁻⁵ M L-162,313, 10⁻⁶ M DUP753. The values represent mean ± S.E. of three or more independent transfection experiments performed in duplicate for each mutant. The level of expression of each of the mutant receptor proteins was within 3-fold of the wild-type receptor expression. The B₅₀ values estimated per mg of total membrane protein are as follows: wild type, 5.2 ± 0.2 pmol; K199Q, 2.8 ± 0.4; K199A, 2.4 ± 0.2; H256Q, 2.1 ± 0.3; H256A, 4.3 ± 0.2.

RESULTS

Stimulation of IP Production by the AT₁ Receptor—The signal transduction properties of transiently expressed wild-type and mutant AT₁ receptors were evaluated by measurement of IP response induced by treatment with [Sar¹]Ang II. In COS-1 cells expressing wild-type AT₁ receptors, 1 μM concentration of Ang II and [Sar¹]Ang II caused about a 5-fold increase in IP production over the unstimulated (basal) controls. The non-peptide agonist L-162,313 stimulated 2-3-fold responses at 1–10 μM concentration (Figs. 1A and 2). IP production stimulated in the mock-transfected COS-1 cells was below the basal IP production in cells transfected with AT₁ receptor genes without Ang II treatment. The AT₁ receptor-specific non-peptide antagonist DUP753 blocked (IC₅₀ = 10 nM) both the basal IP production and that stimulated by different agonists. Several different mutants at positions 199 and 256 showed partial to complete defect in stimulation of IP production at 1–10 μM concentration of [Sar¹]Ang II (Figs. 1B and 2). The expression level of these mutant receptors varied within 3-fold (see legend to Fig. 1).

The concentration dependence (EC₅₀ = 2 ± 0.1 nM) and level of stimulation (3- to 5-fold over the basal) of IP formation were similar to the previously reported characteristics of AT₁ receptor in the transfected COS-1 cells (Fig. 2; Refs. 8 and 10). [Sar¹]Ang II-amide (EC₅₀ = 73 ± 4 nM) produced nearly the same maximal level of response, although its affinity for the wild-type AT₁ receptor is 10-fold lower. Since [Sar¹]Ang II-amide could be converted to [Sar¹]Ang II because of spontaneous deamidation, fresh stock solutions were used each time. The molecular weight of the peptide in the assayed stock solution was confirmed by mass spectrometry. The antagonist [Sar¹,Ile⁶]Ang II (EC₅₀ = 1.5 ± 0.3 nM) produced <20% of the maximal [Sar¹]Ang II response. The maximal response to stimulation with the non-peptide agonist L-162,313 was 40 ± 5% of the [Sar¹]Ang II response with an EC₅₀ of 98 ± 26 nM, as reported earlier (8, 10). L-162,313 bound to wild-type receptors with an affinity of 14 ± 3 nM (Fig. 2).

Stimulation of IP Production by AT₁ Receptor Mutants—The Lys¹⁹⁹ and His²⁵⁶ mutants of AT₁ receptor harbored varying degrees of defects of receptor activation (Fig. 1). The IP production by the K199R (EC₅₀ = 1.3 nM) mutant is nearly identical with that stimulated by the wild-type. The maximal IP response is diminished by 20 ± 5% in K199Q (EC₅₀ = 32 nm) and 60 ± 5% in K199A (EC₅₀ = 48 nm) mutants. In both, the rightward shift of the dose-response curve (Fig. 3A) is apparently comparable with that of [Sar¹]Ang II-amide (Fig. 2). The affinity of [Sar¹]Ang II for K199Q and K199A mutants is, respectively, 10- and 30-fold lower than that of the wild-type AT₁ receptor (Kₐ = 0.2 nM) (see Ref. 9).

The receptor activation seems to be influenced by the kind of side chain at position 256. In the H256A and H256Q mutants, the maximal response produced is 20–40% of that for the wild-type (Fig. 3B). The EC₅₀ for this diminished response is 1.8 nM for H256Q and 5.4 nM for H256A. The affinity of [Sar¹]Ang II to both the mutants (0.6 nM and 0.8 nM) is nearly identical with that of the wild-type AT₁ receptor (see Ref. 9). The mutant H256R, K199A/H256A, and K199A/H256R receptors were completely defective, although they bound [Sar¹]Ang II with high affinity (Kₐ = 0.8 nM, 3.9 nM, and 1.6 nM, respectively) (Fig. 3C and Ref. 9).

As shown in Fig. 3D, the K199Q mutant is not stimulated by the non-peptide agonist L-162,313. This mutation caused an approximate 7-fold decrease of L-162,313 binding affinity (303 ± 8 nM). The stimulation of H256Q and the H256A mutants was diminished, respectively, to 10 ± 5% and 18 ± 10% of the wild-type receptor stimulation. The Kₐ of L-162,313 to mutants is 110 ± 11 nM for H256Q and 191 ± 14 nM for H256A mutants.

Influence of Modification of Phe⁶ Side Chain of Ang II on the Properties of Wild-type, H256Q, and H256A Receptors—Substitution of Leu or Ile side chains has little effect on binding affinity, suggesting that these side chains have no significant role in binding to the AT₁ receptor. To explore the effect on receptor affinity of side chain size at position 8 of Ang II, we examined the effect of substituting five different amino acid residues of varying sizes. As shown in Fig. 4, the change of Kₐ was influenced by the volume of the substituted amino acid side chain. Both decreases and increases of the side chain volume from that of Phe⁶ resulted in a decrease of binding affinity. The patterns of affinity change were identical for the wild-type and the H256Q mutant (data not shown).

In the H256A mutant, the affinity loss is larger for Ala and Thr side chains at position 8 of the Ang II than with wild-type AT₁ receptor. However, the affinity of the H256A mutant was increased toward the [Trp⁶]Ang II analogue in contrast to a decrease of affinity of wild-type receptor (Fig. 4A). However, the [Trp⁶]Ang II-stimulated IP responses from H256Q and
Interactions may collectively bind the Phe8 side chain, mutating changes in affinity for AngII. Furthermore, the Phe8 side chain does not replace the complementary interacting residue producing poor agonists without substantial change of affinity, and Asp281 predicted several potential sites for the interaction with wild-type and His256 mutants. The values represent (mean ± S.E.) from three independent experiments.

H256A mutants were approximately 40% of that from the wild-type AT1 receptor (Fig. 4B).

**Discussion**

The Phe8 side chain of Ang II plays a crucial role in the activation of the AT1 receptor (1, 2). Because several weak interactions may collectively bind the Phe8 side chain, mutation-induced loss of affinity may be difficult to measure. Thus, it was anticipated that the Phe8 binding site would be difficult to locate. We approached this problem by initially identifying the docking residues for the Arg2 and the Phe8 side chain. Since replacement of the Phe8 side chain of Ang II with alicyclic side chains such as Ile8, Ala8, and Thr8 produces poor agonists without substantial change of affinity, the replacement of the complementary interacting residue must also produce a functionally defective receptor with no change in affinity for AngII. Furthermore, the Phe8 side chain docking site is likely to be proximal to Lys199 because it binds the α-COOH group of Phe8. Topological location and the functional defect caused by the His256 mutations are consistent with this expectation.

The H256Q and H256A mutants cause only small changes in agonist affinity, but a substantial defect in IP response (Fig. 3B). As shown in Fig. 4A, reduction of the size of the His256 side chain correlates with a change of binding affinity for position 8 analogues of Ang II. For example, the alanine side chain of [Trp8]Ang II binds better to the His256 → Ala mutant receptor than to the His256 → Gln mutant receptor or the wild-type receptor. The Ala9, Thr9, and Ile8 analogues of Ang II lead to weaker binding to Ala256 receptor than to the His256 and Gln256 receptors. Schambye et al. (11) independently observed that increase of side chain size in a H256F mutant AT1 receptor improves affinity for [Sar1,Leu8]Ang II. The van der Waals contacts between His256 and the angiotensin position 8 side chain appears to be a critical factor for the differences in affinity of analogues shown in Fig. 4A. This presupposes that direct contacts of His256 with the Phe8 side chain is responsible for "transmitting" the agonist occupancy of the ligand pocket as a signal for receptor activation. Then one would expect that activation of the His256 → Ala mutant by Ang II should be similar to the level of activation of wild-type receptor by Ala8, Thr8, and Ile8 analogues of Ang II. Inconsistency in the observed response (Figs. 3B and 4B) is most likely due to the involvement of more than one residue making contact with the Phe8 side chain. It is possible that several residues are involved in stabilizing the bulkier Phe8 side chain, as has been commonly observed in protein structures (17). However, the interaction of Phe8 of Ang II with the His256 plays an important role in receptor activation. This cannot be explained by the simple contact between them, because substituting a Gln for His256 to provide isosteric hydrogen bonding properties also produced a 60% reduction of IP response. The histidine side chain has the unique protonation-tautomeration enabling it to act as a crucial bridging residue in a hydrogen-bonded network in the activated state (18). The Gln256 side chain may be very inefficient in this process because it lacks tautomeration. However, the ultimate chemical basis for the function of His256 needs high resolution structural evidence, which is currently not available for this receptor. The most significant conclusion from the present results, therefore, is that His256 is a point of contact between agonists and the AT1 receptor where the process of receptor activation is initiated.

Because the interaction of His256 with Ang II provides nearly insignificant binding energy, it is important to understand how this crucial interaction is achieved. Docking the α-COOH group of Phe8 to the Lys199 side chain is very important for positioning the Phe8 side chain of [Sar1]Ang II. The modification of the Phe8 side chain to Ile8, for example, reduces the affinity 2-fold, but modification of the α-COOH group reduces the affinity of both [Sar1]Ang II-amide and [Sar1,Ile8]Ang II-amide by 20-fold (9). It is very likely that loss of the docking interaction will affect positioning of the Phe8 side chain. Therefore, the effect of Lys199 mutation on receptor activation may be a direct consequence of the changes in positioning the Phe8 side chain of [Sar1]Ang II in the mutant receptors. The effects of ligand modification and complementary changes in the receptor confirm this. [Sar1]Ang II-amide activated the wild-type receptor.
with a rightward shift of the dose-response curve with nearly the same maximal IP response as did the [Sar1]Ang II. This observation is consistent with earlier bioassay results where Ang II amide demonstrated full potency, but at a higher concentration relative to Ang II (2, 18). The distance of interaction between Lys199 and the Ang II amide compared to Ang II must remain the same, but the modification replaces an ion-pair interaction by a neutral hydrogen bond interaction. Therefore, stimulation by [Sar1]Ang II does not require the negative charge of the α-COOH group. When Lys199 is mutated to Gin199, the effect is consistent with loss of charge-pair interaction and reduction of binding affinity (see Ref. 9). The decrease of maximal response (Fig. 3) correlates with a decrease of side chain length (17). If the Gin199 side chain forms a hydrogen bond with the α-COOH group of Ang II, then one would expect that the Ala199 mutant receptor should be poorly activated by [Sar1]Ang II. The 60% decrease in stimulation by the K199A mutant confirmed this. Basis for the defect is consistent with reduction in the volume of side chain combined with loss of hydrogen bonding ability (87 Å3 versus 169 Å3) (17). Therefore, we conclude that the loss of van der Waals interaction in the Gin199 or Ala199 receptors leads to the partial agonism with [Sar1]Ang II stimulation, presumably due to problems with positioning the Phe8 side chain.

To explain the putative function of Lys199 and His256, we propose that His256 interacts directly with the Phe8 side chain of [Sar1]Ang II when the α-COOH group of Phe8 is bound to Lys199. It has been demonstrated that the rotational entropy of the Phe8 side chain of Ang II is restricted by its interaction with the α-COOH group (12, 19). Therefore, interaction with Lys199 contributes most of the binding energy without requiring an additional contribution from the His256 interaction. A concerted interaction of α-COOH and the side chain of Phe8 with the receptor may be essential for potent activation of the receptor. This suggestion needs further confirmation, although it provides an explanation for the defect in double mutants where the Arg256 functions as a counterion for docking the α-COOH group of Ang II (9). Because Arg256 is utilized only for binding the α-COOH group of Ang II in the double mutant K199A/H256R, the Phe8 side chain cannot be positioned properly, resulting in an inactive phenotype.

The poor IP response of the two AT1 receptor mutants (Fig. 3) stimulated by the non-peptide agonist 1-L62,313 is also associated with no significant reduction of binding affinity. Since L162,313 contains a sulfonamide pharmacophore, it is likely to utilize Lys199 for docking, and His256 may stabilize its binding (see Ref. 9). Because 1-L62,313 has fewer contacts with the receptor, loss of any one contact may significantly affect its agonist function. Most likely, His256 functions as a counterion for the sulfonamide moiety of 1-L62,313 in the K199A and K199Q mutants. In that configuration, 1-L62,313 is likely to function as an antagonist, thus explaining the complete loss of activity (see discussion on H256R mutant above, for example). The partial defect in the H256Q and H256A mutants also suggests that there might be direct interaction of His256 with L162,313 that is critical for receptor activation in a fashion similar to the interaction of Ang II with His256. Perlman et al. (8) have suggested that the molecular interactions of the L162,313 may differ from both peptide and non-peptides that selectively bind to AT1 receptor. The results presented here suggest an overlap in the binding pocket for these two agonists, at least with regard to Lys199 and His256, which also form the subsite for the carboxyl-terminal fragment of Ang II. Therefore, L162,313 may truly be considered an analogue of the carboxyl-terminal fragment of Ang II.

It can now be argued that Lys199 and His256 make direct contacts with all classes of AT1 receptor-specific ligands (also discussed in Ref. 9). The type of interaction with these residues distinguishes agonists from antagonists. The position of His256 might be perturbed by antagonists and agonists differently. For example, both Lys199 and His256 interact with the tetrazole group of biphenyl antagonists, but only His256 interacts with the carboxyl group of imidazolyl-acrylic acid antagonists (9, 11). Presumably, these interactions stabilize an inactive conformation of the AT1 receptor. The activating conformation of the receptor might require specific interaction of the acidic group with Lys199 and weak electrostatic interaction with His256, each with considerably stringent stereospecificity. Both of these residues are conserved among all angiotensin receptors, and His256 corresponds to a well-defined ligand-binding residue in opsin and the rhodopsine receptors (20). Hence, we conclude that Lys199 and His256 constitute the critical component of the ligand pocket of the AT1 receptor that undergone stabilization/destabilization to initiate intramolecular events that are ultimately responsible for signal transduction by AT1 receptors.

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