We have shown previously that the octapeptide angiotensin II (Ang II) activates the AT$_1$ receptor through an induced-fit mechanism (Noda, K., Feng, Y. H., Liu, X. P., Saad, Y., Husain, A., and Karnik, S. S. (1996) Biochemistry 35, 16435–16442). In this activation process, interactions between Tyr$^4$ and Phe$^8$ of Ang II with Asn$^{111}$ and His$^{256}$ of the AT$_1$ receptor, respectively, are essential for agonism. Here we show that aromaticity, primarily, and size, secondarily, of the Tyr$^4$ side chain are important in activating the receptor. Activation analysis of AT$_1$ receptor position 111 mutants by various Ang II position 4 analogues suggests that an amino-aromatic bonding interaction operates between the residue Asn$^{111}$ of the AT$_1$ receptor and Tyr$^4$ of Ang II. Degree and potency of AT$_1$ receptor activation by Ang II can be recreated by a reciprocal exchange of aromatic and amide groups between positions 4 and 111 of Ang II and the AT$_1$ receptor, respectively. In several other bonding combinations, set up between Ang II position 4 analogues and receptor mutants, the gain of affinity is not accompanied by gain of function. Activation analysis of position 256 receptor mutants by Ang II position 8 analogues suggests that aromaticity of Phe$^8$ and His$^{256}$ side chains is crucial for receptor activation; however, a stacked rather than an amino-aromatic interaction appears to operate at this switch locus. Interaction between these residues, unlike the Tyr$^4$:Asn$^{111}$ interaction, plays an insignificant role in ligand docking.

A central question in characterizing the biochemistry and action of the octapeptide hormone angiotensin II (Ang II)$^3$ is the mechanism by which the Tyr$^4$ and Phe$^8$ residues mediate the biological functions of Ang II. In vivo, Ang II is an important regulator of mean arterial pressure, water-electrolyte balance, and cardiovascular homeostasis. A clear understanding of the molecular mechanism of Ang II activation of cell surface receptors is necessary for the development of therapeutic agents to treat disorders such as high blood pressure and cardiac hypertrophy. A recently proposed model, based on NMR constraints of locked Ang II analogues, suggests that a very large pharmacophore spanning the entire structure of Ang II is presented to the receptor. Every other Ang II residue is involved in receptor contact. Only the Tyr$^4$ and Phe$^8$ side chains are considered agonist “switches” because analogues of Ang II function as agonists in vivo if the position 4 residue is tyrosine and the position 8 residue is a phenylalanine. Modifications of Tyr$^4$ and Phe$^8$ in Ang II give rise to antagonists in vivo that display high affinity for the Ang II receptor but at higher concentrations elicit partial receptor agonism (1–5).

Ang II type I (AT$_1$) and type II (AT$_2$) receptors belonging to the G-protein-coupled receptor (GPCR) superfamily are mediators of Ang II effects. AT$_1$ receptor is necessary and sufficient for regulating blood pressure, and activates intracellular inositol phosphate (IP) production via coupling to a pertussis toxin-insensitive G protein (3). Ang II-binding pocket consists of transmembrane domain and the extracellular loops. Two salt-bridge interactions, one between the α-COO$^–$ group of Phe$^8$ of Ang II and Lys$^{139}$ of the AT$_1$ receptor and the other between the Arg$^5$ of Ang II and the Asp$^{254}$ of the AT$_1$ receptor, have been assigned. These salt-bridge interactions are not critical for receptor activation. Thus, a charge-separation mechanism described for the light activation of rhodopsin and the agonist activation of structurally related monoamine receptors is not a valid paradigm in the Ang II activation of the AT$_1$ receptor. Instead, the interactions of the Tyr$^4$ and Phe$^8$ residues of Ang II initiate the AT$_1$ receptor activation process (6–10).

We previously obtained evidence for interaction of the Tyr$^4$ and Phe$^8$ side chains of Ang II, respectively, with Asn$^{111}$ and His$^{256}$ residues of the AT$_1$ receptor (10, 11). Asn$^{111}$ also plays a critical role in stabilizing the basal “inactive” conformation of the native AT$_1$ receptor (11–13). His$^{256}$ is important for coupling agonist occupancy to G-protein activation (10). However, the nature of the bonds between Ang II and the receptor that switch the AT$_1$ receptor to its active state conformation is not clearly defined. In this report, we examine the hypothesis that amino-aromatic bonding between the agonist “switches” Tyr$^4$ and Phe$^8$ of Ang II and the respective agonist switch-binding residues Asn$^{111}$ and His$^{256}$ of the AT$_1$ receptor is responsible for initiating receptor activation. The absence of amino-aromatic interaction should primarily affect the receptor activation process. Analogues bearing saturated unnatural amino acid analogue β-cyclohexylalanine (Cha) substituents at the X$^4$ and X$^8$ positions of Ang II were synthesized and tested with wild-type and AT$_1$ receptor mutants with amino acid replacements at position Asn$^{111}$ or His$^{256}$. Cha does not have a negatively charged planar aromatic ring but through its saturated ring provides nearly the same size and hydrophobicity as the aromatic rings of Tyr and Phe. We show that Cha replacements at either position 4 or position 8 of Ang II principally hinder ligand-dependent activation of the receptor.
Activation of a Peptide Hormone Receptor

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were obtained from the oligonucleotide synthesis core facility of the Lerner Research Institute, The Cleveland Clinic Foundation. [Sar1,16Ile6]Ang II and Ang II were purchased from Bachem. [Sar1,16Ile6]Ang II was iodinated by the lactoperoxidase method and purified, as described previously (8). The specific activity of the 125I-[Sar1,16Ile6]Ang II was 2200 Ci/mmol. Losartan was a gift from DuPont Merck Co., Wilmington, DE.

Analogues of Ang II—Analogues of [Sar1]Ang II were synthesized and reverse phase high performance liquid chromatography purified by the peptide synthesis core facility at the Lerner Research Institute. The accuracy of synthesis was confirmed by electrospray mass spectrometry of the pure analogues using a PE-Sciex model API III spectrometer. Concentration of the peptide in stock solutions was estimated by mo- learity of individual amino acids determined against known standards on an amino acid analyzer.

To evaluate the role of the aromaticity of Tyr4 and Phe8 rings without a change of residue size, β-cyclohexylalanine, an unnatural analogue of Phe, was substituted at these positions to obtain [Sar1,Cha4]Ang II and [Sar1,Cha4]Ang II. In addition, we synthesized three analogues of [Sar1]Ang II containing position 4 side chain size modifications to evaluate the influence of size. [Sar1,Phe8]Ang II serves as a control for the size difference between Tyr and Cha. [Sar1,di-I-Tyr4]Ang II analogue retains the aromatic ring but bulkier than Tyr. The smaller side chain substituted Ang II analogues, [Sar1,Ala4]Ang II and [Sar1,Ile8]Ang II both lack an aromatic ring. The surface area accessible for interaction with the receptor, defined as the area accessible to a sphere of water molecule of 1.4 Å in diameter (14), was estimated in each case as described below. The pharmacological studies on the wild-type and mutant receptors using various position 4 analogues is shown in Figs. 1–6.

Molecular Graphics and Estimation of Side Chain Surface Area—To model the surface area values of Cha, mono-I-Tyr, and di-I-Tyr, we modeled the different molecules using bond lengths and angles extracted from a small molecule structure data base in an interactive graphics program called “O” (15). We then used the program “GRASP” to calculate the surface areas (16). The surface area values for the natural amino acid residues are from Ref. 14.

Mutagenesis and Expression of the AT1 Receptor—The synthetic rat AT1 receptor gene, cloned in the shuttle expression vector pMT-2, was used for expression and mutagenesis, as described previously (8–11, 17). Mutants were prepared either by the restriction fragment replacement method or by the polymerase chain reaction method, and DNA sequence analysis was done to confirm the mutations. To express the AT1 receptor protein, 10 μg of purified plasmid DNA/107 cells was used in transfection. COS1 cells (American Type Culture Collection, Rockville, MD), cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, were transfected by the DEAE-dextran method. Transfected cells cultured for 72 h were harvested, and sonicates were prepared by the nitrogen parbomb disruption method. The receptor expression was assessed in each case by immunoblot analysis (data not shown) and by 125I-[Sar1,16Ile6]Ang II saturation binding analysis.

Radioligand Binding Studies—125I-[Sar1,16Ile6]Ang II binding experiments were carried out under equilibrium conditions, as described previously (8–11, 17). For competition binding studies, membranes expressing the wild-type receptor or the mutants were incubated at room temperature for 1 h with 300 pM 125I-[Sar1,16Ile6]Ang II and various concentrations of the agonist or antagonist. All binding experiments were carried out at 25 °C in a 250-μl volume. Nonspecific binding of the radioligand and measured in the presence of 10 μM 125I-[Sar1,16Ile6]Ang II was <5–5% of the total binding. After equilibrium was reached, the binding experiments were stopped by filtering the binding mixture through Whatman GPC glass fiber filters, which were extensively washed further with binding buffer to wash the free radioligand. The bound ligand fraction was determined from the counts per minute (cpm) remaining on the membrane. Equilibrium binding kinetics were determined using the computer program Ligand (17). The Ki values represent the mean ± S.E. of three to five independent determinations.

Formation of COS1 Cells for Cell Surface Studies—The COS1 cells (cultured in DMEM containing 10 mM LiCl for 20 min; agonists were added and incubation continued for another 45 min at 37 °C. At the end of incubation, the medium was removed, and total solubl IP was extracted from the cells by the perchloric acid extraction method, as described previously (8–11). The amount of [3H]HIP eluted from the column was counted and a concentration-response curve generated using iterative nonlinear regression analysis (see Refs. 8–11 and 17 for additional details).

RESULTS

The Experimental System—Transiently transfected COS1 cell model system was used for analysis as described previously (8–11, 17). Immunoblotting experiments (data not shown) indicated that the expression of the mutant AT1 receptors described in this report were ≥20% of the level of the wild-type receptor expression. This level of variability in receptor polyepptide expression did not cause significant variation in cell surface receptor numbers (1.4–1.6 × 107 sites/cell), which was determined from acid-labile 125I-[Sar1,16Ile6]Ang II binding in intact cells. The B MAX estimated varied ~2-fold (see Table I). Statistical analysis of 125I-[Sar1,16Ile6]Ang II binding kinetics was best fit to a one-site model, which indicated that a homogeneous population of wild-type and mutant receptors were produced in COS1 cells. Affinity of the wild-type AT1 receptor to the radioligand was 0.77 ± 0.02 nm. The Ki values for the agonist [Sar1]Ang II and the native hormone Ang II were 0.33 ± 0.02 and 1.48 ± 0.05 nm, respectively. The measured affinities did not change significantly in the presence of analogues of GTP, since our membrane preparations have been EDTA washed to uncouple G-proteins. Hence, the Ki values in Table I represent the intrinsic affinity of the receptor in the absence of G-protein coupling. The expressed AT1 receptor bound the nonpeptide antagonist losartan with high affinity (Ki, 10 ± 2 nm) and did not bind the AT2 receptor-selective antagonist PD123319 (Ki, <10−7 m). The ability of the AT1 receptor to activate IP production in COS1 cells is shown in Fig. 1. [Sar1]Ang II-stimulated IP production in COS1 cells varied with the plasmid DNA transfected (see Fig. 1A). Expression plasmid concentrations, >4 μg of DNA/dish, did not further increase the maximal IP produced, as well as cell surface receptor number (see above). All IP measurements shown in this report were carried out at 10 μg of expression plasmid DNA/dish. Therefore, the maximal IP values given in each case truly represent maximal signal transduction and not the differences in cell surface receptor numbers. The basal IP production without [Sar1]Ang II treatment is <5 ± 0.3% when compared with the maximal IP response (taken as 100%) elicited by [Sar1]Ang II concentrations >10−7 m in transfected COS1 cells.

Pharmacological Characterization of Tyr4-modified [Sar1] Ang II Analogues—Interaction of [Xα]Ang II analogues with the

| Receptor | Expression (Bmax) | Kd | Pmoll/mg
|---------|------------------|---|---------|
| [Sar1]Ang II | [Sar1,Cha4]Ang II | [Sar1,Cha4]Ang II | [Sar1,Cha4]Ang II |
|           |                  |    |          |
| Wild type | 5.4 ± 0.10       | 0.33 ± 0.02 | 75.9 ± 1.6 | 0.47 ± 0.04 |
| N111K    | 5.8 ± 0.16       | 0.77 ± 0.11 | 69.3 ± 1.6 | ND          |
| N113Q    | 5.0 ± 0.03       | 0.24 ± 0.01 | 31.3 ± 3.4 | ND          |
| N111H    | 5.1 ± 0.07       | 0.24 ± 0.01 | 34.3 ± 2.8 | ND          |
| N111I    | 3.1 ± 0.13       | 0.52 ± 0.02 | 1.98 ± 0.10 | ND         |
| N111F    | 3.8 ± 0.77       | 1.67 ± 0.21 | 2.18 ± 0.14 | ND         |
| N111Y    | 4.3 ± 0.41       | 1.09 ± 0.11 | 3.06 ± 0.20 | ND         |
| H256A    | 4.3 ± 0.20       | 0.36 ± 0.08 | 1.69 ± 0.24 | ND         |
| H256Q    | 4.1 ± 0.50       | 0.58 ± 0.10 | 1.08 ± 0.17 | ND         |
| H256Y    | 4.4 ± 0.14       | 0.13 ± 0.02 | 0.30 ± 0.04 | ND         |
wild-type AT₁ suggests that aromaticity and the size provided by Tyr⁴ in Ang II are critical for function. The affinity of the AT₁ receptor for [Sar¹,Cha⁴]Ang II and [Sar¹,Phe⁴]Ang II (the size of Phe is nearly the same as Cha) was 230- and 2-fold lower, respectively, than the affinity of [Sar¹]Ang II (see Fig. 2A), indicating that the aromatic ring is an important determinant of affinity. Size restriction in the interaction of aromatic group with the receptor is evident from 328-fold reduction of affinity for [Sar¹,di-I-Tyr⁴]Ang II (with an 40-Å increase of accessible area). It is known that the addition of iodine atom on the Tyr⁴ ring alters the interaction with the receptor. [Sar¹,mono-I-Tyr⁴]Ang II is a full agonist with slightly improved affinity, while [Sar¹,di-I-Tyr⁴]Ang II is a partial agonist with lower affinity. Samanen et al. (18) and Guillemette et al. (19) also reported that different halide modifications of the position 4 aromatic ring of Ang II increase the electronegativity associated with a reduction of affinity for the receptor. The 458- and 710-fold reduced affinity, respectively, for aliphatic amino acid-substituted analogues [Sar¹,Ala⁴]Ang II and [Sar¹,Ile⁴]Ang II is also consistent with size and aromaticity constraints.

The binding energy contributions for the interactions were calculated as described in legend to Table II. The energy of interactions indicates that the aromatic ring in Tyr⁴ contributed ~2.8 kcal mol⁻¹, whereas the hydroxyl group in Tyr⁴ contributed ~0.4 kcal mol⁻¹ and the surface area (based on comparison between Cha⁴ and Ile⁴) accounted for ~0.6–1.0 kcal mol⁻¹ (see Table II).

Data in Table II indicate that AT₁ receptor activation is specifically hindered by Cha⁴ substitution in Ang II. The [Sar¹,Cha⁴]Ang II analogue partially activated 48% of the receptor function at concentration 1320-fold lower, respectively, than the affinity of [Sar¹]Ang II and Ang II are identical; this was used as 100%. The values shown are average ± S.E. of three to five independent determinations in each case.

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group of Tyr^4 in Ang II is crucial for activation (see below). Taken together, these observations indicate that activation of receptor by [X^4]Ang II analogues is not directly dependent on their binding to the AT_1 receptor (Fig. 2B).

Effect of Size Substitution of Asn^{111} on Interaction with Different [X^4]Ang II Analogues—Interaction of the [X^4]Ang II analogues with the residue 111 mutant receptors suggests that the interaction between the Asn^{111} of the AT_1 receptor and the Tyr^4 of Ang II is a side chain size-dependent amino-aromatic bonding, which is essential for effective hormone-receptor coupling (Fig. 3). Amide group bearing substitution mutants N111Q and N111H bound [Sar^1]Ang II and [Sar^1,Phe^4]Ang II with 1.3-fold better affinity when compared with the wild-type AT_1 receptor. These mutants also bound [Sar^1,Cha^4]Ang II and [Sar^1,Ile^4]Ang II analogues with slightly improved affinity over that of the wild-type receptor. The N111K mutant affinity for these analogues is comparable to that exhibited by the wild-type receptor (Fig. 3).

In contrast, the affinity of [Sar^1]Ang II was decreased 1.6-, 5.1-, and 3.3-fold, respectively, in N111I, N111F, and N111Y mutants. Substitution of a larger residue at position 111 that did not provide an amide group cost 0.2–1.2 kcal mol\(^{-1}\).

**Table II**

| Receptor residue | [X^4]Ang II residue | \(\Delta G\) | Function |
|------------------|---------------------|-------------|----------|
| Asn^{111}        | Tyr^4               | 0.00        | 100%     |
| Asn^{111}        | Phe^4               | 0.41        | 81%      |
| Phe^{111}        | Asn^4               | 0.19        | 78%      |
| Asn^{111}        | Cha^4               | 3.19        | 48%      |
| Asn^{111}        | Ile^4               | 3.85        | 80%      |

The \(\Delta G\) value for each interaction was calculated using the formula:

\[
\Delta G = \Delta G_{\text{mut}} - \Delta G_{\text{WT}} = -RT \ln \left( \frac{K_{d,\text{mut}}}{K_{d,\text{WT}}} \right) = -RT \ln (K_{d,\text{mut}}) + \Delta (\ln K_{d})
\]

for each interaction.

**Fig. 3.** Influence of different position 111 mutations on the affinity and activation by various [X^4]Ang II analogues. The side chain size in each case is shown in scale using Chem Office®. The values shown in each case are: \(K_d\) (nm; top row), relative activation (IP%; middle row) and the EC_{50} (nm; bottom row). The values are derived from concentration-response curves in which ligand concentrations were varied from 10^{-15} to 10^{-11} M. The IP response produced by the wild-type receptor by 10 \mu M [Sar^1]Ang II (see Fig. 1 for cpm values) was taken as 100%. In each experiment, this control was included for comparison and values are normalized for variations in IP formation in basal and mock-transfected cells. Relative activation value close to 100% indicates that activation of the mutant by the analogue and [Sar^1]Ang II are nearly identical. Note that substitution with smaller residues, such as Gly, Ala, Ser, and Cys for Asn^{111}, gives rise to a partially activated receptor that binds almost all Ang II analogues with high affinity, and maximal stimulation of these mutant receptors requires Ang II, but not its agonist side chains. Hence, those mutations are not included in the current analysis (32).

The gain of affinity, however, is not accompanied by gain of function in most bonding combinations set up between Ang II position 4 analogues and receptor mutants (see Fig. 3) to evaluate the critical requirement for amino-aromatic interaction in signal transduction. The maximal IP production stimulated by [Sar^1]Ang II and [Sar^1,Xaa^4]Ang II in most of the mutants shown in Fig. 3 was ~50% reduced in comparison to the wild-type AT_1 receptor. In several combinations the binding interaction was preserved, for instance Tyr^4-Gln^{111}, Tyr^4-His^{111}, Tyr^4-Lys^{111}, Tyr^4-Ile^{111}, Phe^4-Gln^{111}, Phe^4-His^{111}, and Phe^4-Ile^{111}, but the activation was inadequate (see Fig. 3). The \(K_d\) and EC_{50} in these ligand-receptor combinations were close to (~2-fold) that of a wild-type situation, but the maximal IP response was impaired. Thus, the increased size of the residue 111 affected receptor activation much more than the binding affinity (\(K_d\) and potency (EC_{50}). An exception is the Phe^4-Tyr^{111} interaction, in which the measured \(K_d\), EC_{50}, and maximal IP values are comparable to that of Phe^4-Asn^{111} interaction. The reasons for this are unknown.

Reversal of Amino-aromatic Interaction—An efficient functional interaction is reproduced by substitution of an Asn residue at position 4 in [Sar^1]Ang II and a Phe residue at position 111 in the receptor (see Fig. 4, also see Table II). The binding affinity of [Sar^1,Asn^4]Ang II for the wild-type AT_1 receptor is 230-fold reduced. High affinity for [Sar^1,Asn^4]Ang II was regained in the N111F (\(K_d = 0.49 \pm 0.05\) nM) and N111Y (\(K_d = 0.32\) nM)
The most impressive effect of the reversal of the bonding was gain of function: the IP production stimulated by [Sar, Asn]Ang II in the N111F receptor was identical (maximum 57.6% and EC₅₀ = 3.8 ± 0.7 nM) to that elicited by [Sar, Phe]Ang II in the wild-type receptor. Although the maximum IP production stimulated by [Sar, Phe]Ang II in N111F mutant receptor was nearly the same, the EC₅₀ was distinct (see Fig. 4C). Rather surprisingly, in the N111Y mutant, the IP stimulation by [Sar, Asn]Ang II did not reach the wild-type level. Activation of function was impaired in this mutant in response to several other Ang II analogues as well. Reasons for this discrepancy are not known.

Molecular dynamic simulations indicate that Tyr111 hydroxyl group is oriented toward transmembrane domain II (Asn 69 is 2.1 Å from Tyr 111 hydroxyl group), and may have interfered with interactions between transmembrane helices II, III, and VII. N111F does not have this effect, and with regard to the aromaticity Phe and Tyr are equivalent. This might partly explain lower IP stimulation in the N111Y mutant.

In addition we examined several additional analogues. Binding of [Sar, Gln]Ang II to the N111F (K_d = 24 nM) mutant was not a high affinity interaction and was not associated with a gain of function. The affinity of N111F, N111Y, and N111K mutants toward [Sar, Asp]Ang II was 91, 20, and 468 nM and toward [Sar, Glu]Ang II was 290, 198, and 2980 nM, respectively. These observations suggest that providing an electro-negative group at the X₄ position is insufficient. The fact that Asn¹¹¹/Phe⁸ and Phe¹¹¹/Asn⁸ combinations produced identical affinity and potency demonstrates that an amino-aromatic interaction is essential for the fidelity of AT₁ receptor-Ang II coupling. The wild-type receptor interaction with Ang II yields ~20% more IP, suggesting that the critical interaction provided by the Tyr⁴ hydroxyl group is not mimicked in Asn¹¹¹/Phe⁸ and Phe¹¹¹/Asn⁸ combinations. If conventional electrostatic/hydrogen bonding occurred between Asn¹¹¹ and Tyr⁴, our analysis would have been expected to restore affinity and potency (equal to that of the wild-type receptor) in several different combinations.

Pharmacological Characterization of Phe⁸-modified [Sar] Ang II Analogues—Substitution of Cha⁸ for Phe⁸ did not significantly affect the binding affinity, but reduced the level of AT₁ receptor activation (Table I, Fig. 5). The maximal IP response elicited by [Sar, Cha]Ang II was reduced by 60 ± 5%, which appears to be caused by diminished AT₁ receptor activation by the bound analogue. Size alteration at the Phe⁸ position has a nearly insignificant effect on binding. The change of K_d resulting from the substitution of Phe⁸ with Gly⁸, Ala⁸, Thr⁸, and Ile⁸ was within 6-fold. Replacement with Glu⁸ and Trp⁸ produced lower affinity, 89 ± 9 and 13.4 ± 2 nM, respectively, indicating that charged groups and large hydrophobic groups are not accommodated at the putative Phe⁸ binding site on the receptor. Preservation of the aromatic character, as in [Sar, Trp]Ang II, yielded nearly full receptor activation, suggesting that the reduction of binding affinity at the Phe⁸ binding pocket did not affect the ability to activate. Thus, the aromaticity of Phe⁸ is required for receptor activation, but essentially plays no role in the receptor-binding step.

Effect of Different Substitutions of His²₅₆ on Activation by Ang II—Ang II-Phe⁸-mediated functional activation is specifically and uniquely dependent on His²₅₆ (10). Substitution of His²₅₆ did not affect Ang II binding affinity, but caused receptor to lose the ability to be activated by Ang II. We examined which residue combination would mimic the type of functional
interaction between Phe^8 and His^{256}, employing three substitution mutants of His^{256} (Fig. 6). The binding affinity of H256A, H256Q, and H256Y mutants for [Sar^1]Ang II was within 2-fold of the binding affinity for the wild-type. The receptor activation was reduced ~60% in the H256Q and ~70% in the H256A mutant receptors. In contrast, substitution of His^{256} with a Tyr (H256Y) led to a ~6% reduction of maximal IP response. Activation by the [Sar^1,Cha^8]Ang II was relatively less affected by various mutations (see Fig. 6). The [Sar^1,Cha^8]Ang II evoked ~40% response in the wild-type receptor, ~52% in the H256Y mutant, ~35% in the H256Q mutant, and ~28% in the H256A mutant receptor. The most surprising outcome in this analysis was the ability of a tyrosine substituted for His^{256} to display full activation, which suggests that a stacking interaction involving the aromatic rings of His^{256} and Phe^8 might be responsible for AT_1 receptor activation.

**DISCUSSION**

Like a large variety of hormone GPCRs, the molecular mechanism involved in Ang II-mediated AT_1 receptor activation remains unclear. Some insights into the mechanism have come from structure-function studies and the discovery of mutations that uncouple receptor activation from agonist binding (8–13, 20–22). Two most important such mutations in AT_1 receptor affect amide group bearing residues. Asn^{111} and His^{256}, both interact with the agonist switches, Tyr^4 and Phe^8 of Ang II. Since aromatic rings are able to participate in amino-aromatic bonding (23–25) in addition to the obvious hydrophobic, hydrogen bonding and van der Waals interactions because of an accessible center of negative charge (from the δ − π electrons), our goal was to distinguish potential bonding interactions of Tyr^4 and Phe^8 in Ang II. Because the information available regarding Ang II function was obtained from in vivo functional studies prior to the cloning of the receptor, and also before radioligand binding assay came into routine use (5), our study is justified. Primary comparison involved Cha-substituted Ang II analogues but the aliphatic-substituted analogues served mostly to calibrate size effects. Cha lacks a negative charge, lacks hydrogen bonding potential, and provides hydrophobicity. The volume effect from Cha modification is not significant because the space-filling models indicate that the overall sizes of chair and boat configurations of the cyclohexyl ring and the planar configuration of aromatic ring are substantially similar (23). Since the functional significance of the highly directional and significantly attractive interaction between amide groups of Asn, Gln, His, and Lys and the aromatic rings of Tyr, Phe, and Trp has been questioned in several proteins (23–25), we evaluated whether the amino-aromatic bonding interactions are essential in Ang II-AT_1 receptor coupling. Our results indicate that the aromaticity of both Tyr^4 and Phe^8 is crucial, but an amino-aromatic bonding operates at the Tyr^4 switch of Ang II and a stacked rather than an amino-aromatic interaction appears to operate at the Phe^8 switch locus.

The complex role of Tyr^4-Asn^{111} amino-aromatic bonding in Ang II-AT_1 receptor coupling is suggested by the observation that both affinity and potency of receptor activation is recreated by reversal of interaction (Fig. 4), but not in several other combinations that restore binding affinity. Functional equivalent of this bonding is recapitulated in Phe^4-Phe^{111}, Phe^4-Tyr^{111}, and Tyr^4-Tyr^{111} interactions. An edge-to-face aromatic-aromatic interaction that might be operating in all three instances would be similar to an amino-aromatic interaction based on past examples (23, 25–27). The enthalpic contribution
of this interaction to Ang II binding is estimated at 3.2 kcal/mol (see Table II) which is comparable to the estimated energy (3.3 kcal/mol) of amino-aromatic hydrogen bonds in the protein structure data base (26). This interaction is superimposed with size and hydrophobicity constraints. For instance, the reduction in binding affinity resulting from Tyr4 → Ile4 change in [Sar1]Ang II could be overcome by substitution of Asn111 in the receptor with larger hydrophobic residues Ile, Phe, and Tyr but not by larger hydrophilic residues Lys, Gln, and His (Fig. 3). Increasing the accessible surface area from ~160 Å (Asn111) to ~180 Å (Gln111), and ~195 Å (His111) appeared to increase affinity for [Sar1]Ang II, and [Sar1,Phe4]Ang II. The gain of affinity in both instances, however, is without gain in receptor activation, indicating that Tyr4 is the most complex switch in Ang II because the receptor-binding and agonism-specifying elements are structurally integrated. The size chiefly influences the Kₐ, but receptor activation requires stringent conservation of size and aromaticity.

The binding and agonism-specifying elements are structurally separate in the Phe₈ switch. The α-COO− in Phe₈ is the docking group and the benzyl-alanyl moiety is the agonist switch, modifications of which generated potent antagonists without compromising the binding affinity (also seen in Fig. 5). Classical structure-activity relationship studies portrayed that any modification of the Phe₈ that prevents the planar arrangement of benzyl side chain over the Pro²-Phe₈ amide bond and the α-COO− group will disrupt agonist potential (1, 2, 5). Such modifications include aliphatic substitutions, and aromatic groups in β-configurations (β-Phe, α-MePhe) or with bulky ring substitutions (Trp, Ind) (1−6). These evidences indicate that rigid planar configuration is a restriction in the interactions of Phe₈ switch with the receptor. Proposed stacking interaction between the aromatic rings of His²⁵⁶ and Phe₈ is consistent with this requirement. When His²⁵⁶ is replaced with isosteric Gln or of Phe₈ with isosteric Cha, an inefficient hormone-receptor coupling occurs because Gln and Cha do not participate in the planar stacking interaction with an aromatic ring. Since the Tyr²⁵⁶ mutant is fully active, this suggests that the His²⁵⁶-Phe₈ interaction is not an ion-quadrupole type of interaction. In the protein structure data bank, stacked geometry is far more abundant for His-Phe interactions than typical amino-aromatic interactions (29). A true stacking interaction (with 1R₃ dispersion force between two strategically placed planar rings nearly parallel) is a weaker interaction than an amino-aromatic interaction, consistent with the insignificant contribution of Phe₈ to the binding affinity of Ang II. Underwood et al. have previously suggested an aromatic stacking interaction model for AT₁ receptor agonism involving the nonpeptide agonist L-162,313 (30). Evidences suggest that His²⁵⁶ makes contact with AT₁ receptor specific non peptide antagonists also (10, 31). Therefore, we propose that actuation of the His²⁵⁶ switch of the receptor is a common step for AT₁ receptor activation by both peptide and nonpeptide agonists and the site for antagonist action. Preservation of planar benzyl imidazole or benzyl acrylic acid ring structure is crucial in these ligands, suggesting that a stacking interaction with His²⁵⁶ is likely in the mechanism of receptor antagonism as well.

Based on these results, we speculate that the functional coupling of Ang II binding to receptor activation requires a structural coupling through stringent bonding between the agonist switches and respective switch-binding residues. In the wild-type receptor, because Asn¹¹¹ is smaller, a conformational change might be required to facilitate the amino aromatic Tyr⁴-Asn¹¹¹ interaction. The proposed conformational change is the basis for efficient activation of function. Ang II binding, in all likelihood, does not trigger a similar conformational change in the N11Q, N111H, N111K, N111L, and N111F mutants, which forms the basis for defect. Although an amino-aromatic bonding is expected in N111Q mutant (also N111H), the defect in IP formation suggests that one methylene unit increase in size is detrimental and perhaps uncouples binding and activation. In contrast, the stacking Phe₈-His²⁵⁶ interaction is not accompanied by a large conformational change, but likely exposes sites within the receptor for the crucial interactions. Numerous studies have described partial agonist effects of modifying Tyr⁴ and Phe₈ in Ang II. For the most part these reports indicated that conservation of these two side chains is critical for Ang II function. Our results extend these observations in that highly efficient receptor activation of Ang II analogues is lost, leaving high affinity binding intact, suggesting the analogue-receptor docking occurs, but due to lack of the required bonding interactions, functional response of the receptor is incomplete. This finding has important implications for drug discovery.

CONCLUSIONS

Different roles played by the agonist switch residues Tyr⁴ and Phe₈ of Ang II in the AT₁ receptor activation are pointed out. The conclusion that two critical coupling contacts between Ang II and the AT₁ receptor involve the transmembrane helices III and VI of the receptor suggests that the receptor activation by Ang II may involve motion of these helices, a phenomenon also observed in other GPCRs (28). Conformational changes induced by the two distinctly different types of bonding interactions could orient helices and loops on the receptor so as to enhance the efficiency of receptor-G-protein coupling. Such conformational switching could also serve as the basis for agonist potency because the ability of a full agonist analogue to properly align helices far exceeds that of partial agonist analogues. The working model suggested is based on the assumption that different peptide analogues induce non-identical conformation of the activated receptor that lead to differences in the kinetics and the magnitudes of responses. Whether this induced-fit mechanism is unique to the subfamily of peptide hormone GPCRs or is a more general mechanism that has not been considered in prototypical GPCRs is unclear at present.

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REFERENCES

1. Marshall, G. R., Bosshard, H. E., Vine, W. H., Glickson, J. D., and Needleman, P. (1974) in Recent Advances in Renal Physiology and Pharmacology (Wesson, L. G., and Fanelli, G. M., Jr., eds) pp. 215–256, University Park Press, Baltimore
2. Bumpus, F. M., and Khosla, M. C. (1977) in Hypertension: Physiology and Treatment (Genest, J., Kuw, E., and Kuchel, O., eds) pp. 183–201, McGraw-Hill, New York
3. Timmermans, P. B. M. W. M., Wong, P. C., Chiu, A. T., Herblin, W. F., Benefeld, P., Carini, D., Lee, J. R., Wexler, R. R., Saye, J. M., and Smith, R. D. (1995) Pharmacol. Rev. 47, 265–297
4. Nikiforovich, G. V., Kao, J. L., Fuchs, K., Zhang, W. J., and Marshall, G. R. (1994) Biochemistry 33, 3391–3398
5. Samolenen, J., and Regoli, D. (1984) in Angiotensin II Receptors: Medicinal Chemistry (Rufolo, R., ed) Vol. 2, pp. 11–97, CRC Press, Boca Raton, FL
6. Karnik, S. S., Husain, A., and Graham, R. M. (1996) Clin. Exp. Pharm. Physiol. Suppl. 3, S55–S66
7. Yamano, Y., Ohyama, K., Chaki, S., Guo, D.-F., and Inagami, T. (1992) Biochem. Biophys. Res. Commun. 187, 1426–1431
8. Noda, K., Saad, Y., Kinoshita, A., Boyle, T. P., Graham, R. M., Husain, A., and Karnik, S. S. (1995) J. Biol. Chem. 270, 25265–25269
9. Feng, Y.-H., Noda, K., Saad, Y., Liu, X., Husain, A., and Karnik, S. S. (1995) J. Biol. Chem. 270, 12846–12850
10. Noda, K., Saad, Y., and Karnik, S. S. (1995) J. Biol. Chem. 270, 12851–12854
11. Noda, K., Feng, Y.-H., Liu, X. P., Saad, Y., Husain, A., and Karnik, S. S. (1996) Biochemistry 35, 16435–16442
12. Balmforth, A., Lee, A. J., Wardburton, P., Donnelly, D., and Ball, S. C. (1997) J. Biol. Chem. 272, 4245–4251
13. Grohlewski, T., Maigret, B., Larguier, R., Lombard, C., Bonnafoos, J.-C., and Marie, J. (1987) J. Biol. Chem. 272, 1822–1836
14. Creighton, T. E. (1984) in Proteins: Structural and Molecular Principles, pp.
2–60, Freeman and Co., New York

15. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. A 47, 110–119

16. Nicholls, A. J. (1993) GRASP Manual, Columbia University, New York

17. Noda, K., Saad, Y., Graham, R. M., and Karnik, S. S. (1994) J. Biol. Chem. 269, 6743–6752

18. Samanen, J., Cash, T., Narindray, D., Brandies, E., Yellin, T., and Regoli, D. (1989) J. Med. Chem. 32, 1366–1370

19. Guillemette, G., Bernier, M., Parent, P., Leduc, R., and Escher, E. (1984) J. Med. Chem. 27, 315–320

20. Bihoreau, C., Monnot, C., Davis, E., Teutsch, B., Bernstein, K. E., Corvol, P., and Clauser, E. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5133–5137

21. Marie, J., Maigret, B., Joseph, M. P., Larguier, R., Nouet, S., Lombard, C., and Bonnafous, J. C. (1994) J. Biol. Chem. 269, 20815–20818

22. Monnot, C., Bihoreau, C., Conchon, S., Curnow, K. M., Corvol, P., and Clauser, E. (1996) J. Biol. Chem. 271, 1507–1513

23. Armstrong, K. M., Fairman, R., and Baldwin, R. L. (1993) J. Mol. Biol. 230, 284–291

24. Walksman, G., Kominos, D., Robertson, S. C., Pant, N., Baltimore, D., Birg, R. B., Cowburn, D., Hanafusa, H., Mayer, B. J., Overduin, M., Rios, C. B., Silverman, L., and Kurian, J. (1992) Nature 358, 646–653

25. Strader, C. D., Fong, T. M., Tota, M. R., and Underwood, D. (1994) Annu. Rev. Biochem. 63, 101–132

26. Levitt, M., and Perutz, M. F. (1988) J. Mol. Biol. 201, 751–754

27. Dougherty, D. A., and Stauffer, D. A. (1990) Science 250, 1558–1560

28. Gether, U., and Kobilka, B. K. (1998) J. Biol. Chem. 273, 17979–17982

29. Mitchell, J. B. O., Nandi, C. L., McDonald, I. K., and Thornton, J. M. (1994) J. Mol. Biol. 239, 315–331

30. Underwood, D. J., Strader, C. D., Rivero, R., Patchett, A. A., Greenlee, W., and Prendergast, K. (1994) Chem. Biol. 1, 211–221

31. Perlman, S., Schambye, H. T., Rivero, R. A., Greenlee, W. J., Hjorth, S. A., and Schwartz, T. W. (1995) J. Biol. Chem. 270, 1493–1496

32. Feng, Y.-H., Miura, S.-I., Husain, A., and Karnik, S. (1998) Biochemistry 45, 15791–15798