The Active State of the AT_1 Angiotensin Receptor Is Generated by Angiotensin II Induction†

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ABSTRACT: In the current model of receptor activation, the given hormone is not involved in the conversion of the inactive receptor (R) to the fully active state (R*). Rather, it preferentially selects the activated receptor conformation, thereby shifting the equilibrium toward R*. The hormone angiotensin II (Ang II) contains two residues, Tyr^4 and Phe^8, that are essential for agonism. We show that the conserved Asn^{111} in transmembrane helix III of the AT_1 angiotensin receptor directly interacts with the Tyr^4 side chain. A decrease in the size of the Asn^{111} side chain induces an intermediate activated receptor conformation (R′). The Ang II analogue [Sar^1,Ile^4,Ile^8]Ang II fully activates the N111G mutant, indicating that either the transition from R′ to R* or the stabilization of the R* state requires binding by Ang II but not its Tyr^4 and Phe^8 side chains. In contrast, [Sar^1,Ile^4,Ile^8]Ang II binds to but does not activate the wild-type AT_1 receptor (R), suggesting that in the wild-type receptor spontaneous occurrence of R′ and R* states is rare. Thus, Ang II through interactions involving Tyr^4 and Phe^8 induces a transition from R to R′ and through unspecified interactions induces transition from R′ to R* states rather than stabilizing the spontaneously generated R* state by "conformational selection".

The octapeptide hormone angiotensin II (Ang II;[1] DRVYIHPF) plays an important role in regulating hydromineral balance and arterial blood pressure in species as diverse as fish and humans. Most of the cellular effects attributed to Ang II are mediated by the AT_1 receptor (Peach, 1977; Timmermans et al., 1993). The 359-residue rat AT_1 receptor is thought to consist of seven hydrophobic transmembrane α-helical segments connected by three loop regions on both the cytoplasmic and extracellular sides of the embedding membrane (Figure 1). Ang II-dependent activation of the AT_1 receptor causes intracellular inositol phosphate (IP) production through the activation of a G-protein that is pertussis toxin insensitive (Murphy et al., 1991; Sasaki et al., 1991).

The C-terminal pentapeptide region of Ang II has been proven important for receptor activation (Bumpus & Khosla, 1977). Substitution of the Phe^8 side chain with an aliphatic group produces an agonist-to-antagonist transition without a change in binding affinity. Hydrophobic aliphatic substitutions at position 4 weaken agonist activity and reduce binding affinity for the receptor (Marshall et al., 1974; Bumpus & Khosla, 1977). The remaining hormone residues are not considered to be crucial for agonist activity. Unlike the Tyr^4- and Phe^8-modified analogues of Ang II that are partial agonists, the recently developed nonpeptide ligands of the AT_1 receptor, e.g., losartan and EXP3174, are pure antagonists; these compounds completely inhibit Ang II-dependent IP production by the AT_1 receptor (Timmermans et al., 1993).

Several electrostatic and hydrophobic interactions were proposed to be required for the specific high-affinity binding of Ang II to the AT_1 receptor (Marshall et al., 1974; Bumpus & Khosla, 1977). Recently, salt-bridge interactions between Ang II and the AT_1 receptor have been assigned using group-specific modifications of Ang II in combination with AT_1 receptor mutagenesis. Studies by Yamano et al. (1992) and Noda et al. (1995a) suggest a direct interaction between the α-COO^- group of the Ang II Phe^8 and Lys^{199} of AT_1 (Figure 1). Lys^{199} is located in transmembrane helix V of the AT_1 receptor. Feng et al. (1995) have additionally provided evidence for a direct salt-bridge interaction between Arg^2, located near the N-terminus of Ang II, and Asp^{281}, located near the extracellular border of the AT_1 receptor's transmembrane helix VII (Figures 1 and 2). Molecular modeling using these anchor points indicates that the Phe^8 side chain of Ang II can interact with AT_1 residues Asn^{200}, Gly^{203}, His^{256}, and Thr^{260}. We recently showed that an interaction between Phe^8 of Ang II and His^{256} of the AT_1 receptor is necessary for Ang II-dependent receptor activation (Noda et al., 1995b).

Because this family of receptors couples diverse extracellular modulators, including hormones, neurotransmitters, and sensory stimuli, to intracellular signal transducers, the receptors' function is thought to be governed by a fundamentally similar mechanism. The key step in transmembrane signal transduction is the transition of the receptor from the inactive (R) to the active (R*) state. In current models of receptor activation, intramolecular constraint is proposed to control the equilibrium between R and R* states (Lefkowitz,

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[1] Abbreviations: Ang II, angiotensin II (DRVYIHPF); IP, inositol phosphate; Sar, sarcosine; DEAE, diethylaminoethyl; GTPγS, guanosine 5′-(γ-thio)triphosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HBSS, Hanks' balanced salt solution; R, inactive receptor conformation; R′, unconstrained receptor conformation; R*, activated receptor conformation; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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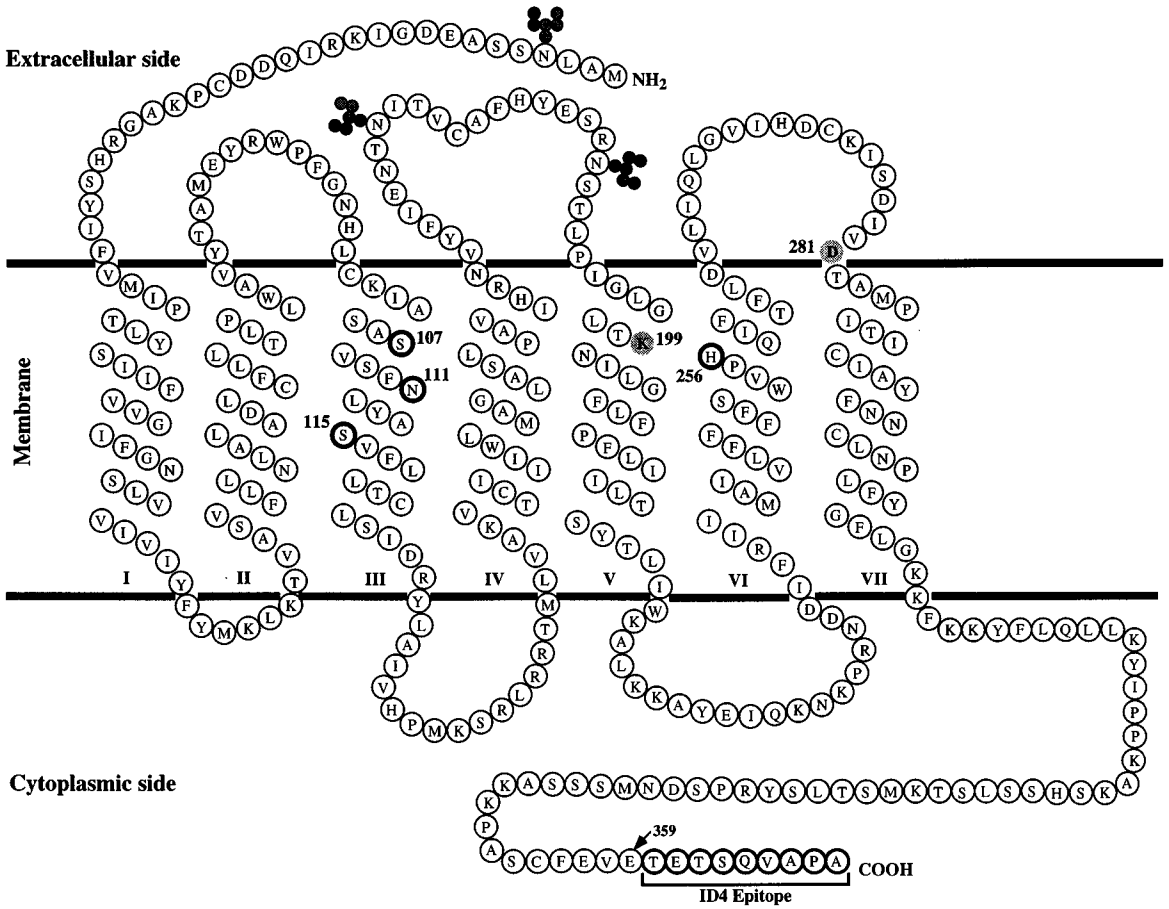

FIGURE 1: Secondary structure model of the rat AT_1 angiotensin II receptor. Transmembrane segments I−VII are putative α-helices forming the ligand pocket. The residues examined by mutagenesis in this study are indicated. The shaded residues, Lys^{199} and Asp^{281}, have previously been shown to form salt-bridge interactions with the α-COO^- group of Phe^8 and the guanidinium group of Arg^2 of Ang II (Yamano et al., 1993; Noda et al., 1995a; Feng et al., 1995). The potential glycosylation sites are shown. The epitope tag attached at the C-terminal end for detection by the 1D4 monoclonal antibody is underlined. Attachment of this sequence does not alter the properties of the AT_1 receptor. The membrane interface boundaries for all seven helices are tentative.

1993). Some investigators theorize that the receptor is in a state of flux between R and R* and that agonists simply capture the active state conformation of the receptor by stabilizing R* (Black & Shankley, 1995). This theory implies that agonists are not involved in generating R*. Alternatively, studies on the activation of rhodopsin suggest that R* is generated following a loss of constraint; in the ground state of this receptor, a salt bridge is chiefly responsible for keeping the receptor in a constraint conformation (Robinson et al., 1992). This theory implies that once the constraining interaction is removed, other intramolecular interactions within the receptor by themselves generate and stabilize R*. The mechanism by which the receptors for the small peptide hormones are activated is unclear at this time.

In this report, we have identified Asn^{111} in transmembrane helix III of the AT_1 receptor as the residue that plays a critical role in stabilizing the "inactive" basal conformation in the native AT_1 receptor; further, the interaction of Asn^{111} with the Tyr^4 side chain of Ang II is necessary to activate the receptor. Reduction of the Asn^{111} side chain size causes partial activation of the AT_1 receptor. We now show that activation of the AT_1 receptor involves at least two Ang II-dependent steps: an initial release of constraint produced by the agonist-specifying Ang II side chains causes a conformational change in the ligand-binding pocket such that it favors agonists and partial agonists but not antagonists; next, a hormone-dependent stabilization of R* occurs, in which the agonism-specifying side chains of Ang II are not necessary. These findings indicate that, in the main, the R → R* transition and the stabilization of R*, once constraint has been removed, are not passive processes but rather that the agonist coordinates the transition of R to R*.

EXPERIMENTAL PROCEDURES

Materials. The monoclonal antibody 1D4, oligonucleotides, Ang II, and various analogues were obtained as reported earlier (Noda et al., 1994, 1995a). The specific activity of the ^{125}I-[Sar^1,Ile^8]Ang II was 2200 Ci/mmol. Losartan was a gift from DuPont-Merck, Wilmington, DE. Methods for mutagenesis, expression, characterization of mutant genes, and cloning of the synthetic rat AT_1 receptor gene in the shuttle expression vector pMT-2 have been described earlier (Noda et al., 1994, 1995a).

Transfection and Immunoblotting of the AT_1 Receptor. COS-1 cells were transfected using the DEAE-dextran method, and transfected cell membranes were prepared as described earlier (Noda et al., 1994). CsCl-purified DNA (10 μg) was used to transfect COS-1 cells (obtained from American Type Culture Collection, Rockville, MD, and passaged routinely) in a 100 mm plate containing approximately 5×10^6 cells by the DEAE-dextran method (Noda et al., 1994). About 72 h after the completion of transfection, the cells were harvested by scraping and then suspended in buffer containing 50 mM HEPES, pH 7.2, 5 mM EDTA, 0.25 M sucrose, and proteinase inhibitor. The membranes were prepared by the nitrogen cavitation−Parr

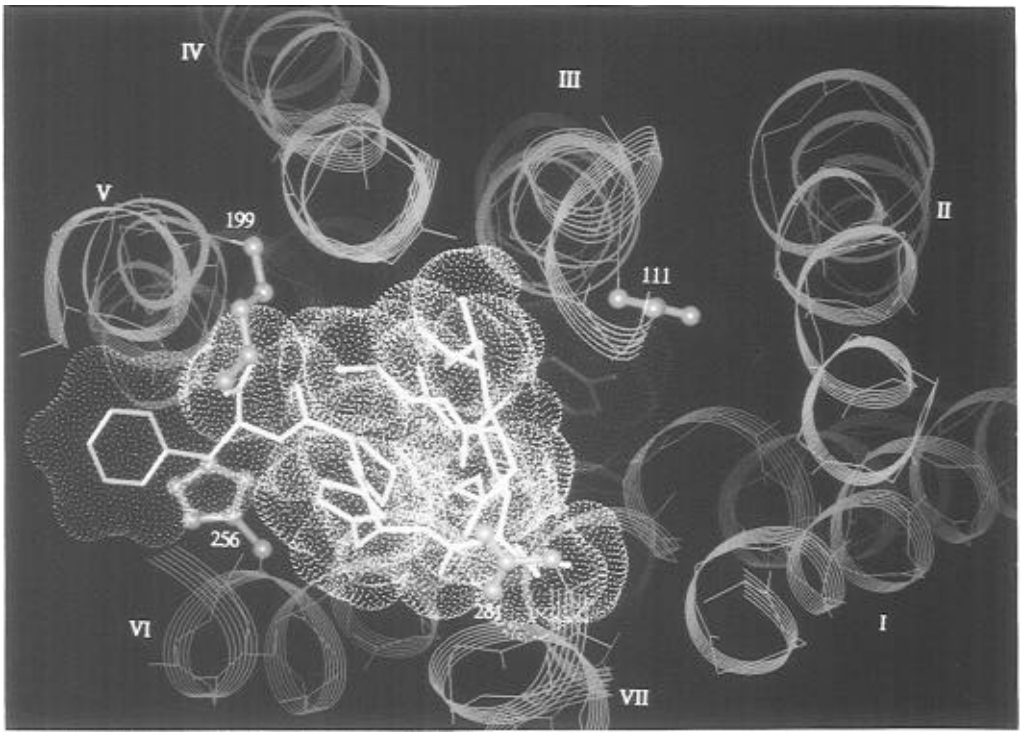

FIGURE 2: Model of Ang II bound to the AT_1 receptor viewed from the extracellular side. The transmembrane helical bundle shown in green is based on the proposed arrangement of helices for rhodopsin (Baldwin, 1993) and the determined structure of bacteriorhodopsin (Henderson et al., 1990). Ang II was modeled using the nuclear magnetic resonance coordinates (Nikiforovich et al., 1994). The side chains of Lys^{199} and Asp^{281} docked to the ends of Ang II are shown from above the plane of Ang II in the pocket. The residues His^{256} and Asn^{111} interact respectively with aromatic side chains of Phe^8 (colored yellow) and Tyr^4 (colored red) of Ang II from below. From classical structure−activity relationship studies, it is known that positioning of these two groups within the pocket of the receptor is the critical step in receptor activation.

bomb disruption method. The disrupted suspension was spun at 2000*g* for 10 min and the postnuclear supernatant at 40000*g*. The pellet was washed with membrane suspension buffer, and the final membrane pellet was resuspended in 50 mM HEPES, pH 7.2, 12.5 mM $MgCl_2$, 1.5 mM EGTA, and 10% glycerol. Alternatively, COS-1 cells from a single plate were solubilized for 2 h with shaking at 4 °C in 400 μL of 50 mM Tris-HCl, pH 6.8, 1% CHAPS, 50 μg/mL protease inhibitor PMSF dissolved in absolute ethanol, and 5 mM EDTA, pH 8.0. The solubilized cells were centrifuged in a TLA-100.3 rotor at 40 000 rpm for 15 min using a Beckman TL-100 ultracentrifuge. The supernatant was transferred into a new tube and the protein stored at −70 °C. The protein concentration was estimated (Bensadoun & Weinstein, 1976), and 50 μg proteins were resolved by SDS−polyacrylamide gel electrophoresis (PAGE) and transferred onto an Immobilion-P membrane (Millipore, Bedford, MA) for Western blot analysis (Burnette, 1981). The membrane was incubated overnight in 50 mL of 2.5 μg/mL mouse monoclonal antibody 1D4 and then incubated in 50 μCi of ^{125}I-anti-mouse Ig (Amersham, Arlington Heights, IL), as described earlier (Noda et al., 1994).

Equilibrium Binding Studies. The ligand-binding experiments were carried out under equilibrium conditions. Membranes expressing wild-type receptors were incubated with 0.03−3 nM ^{125}I-[Sar^1-Ile^8]Ang II in 50 mM sodium phosphate, pH 7.2, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 0.2% bovine serum albumin (ICN Biomedicals, Inc., Costa Mesa, CA), and 10 μg/mL bacitracin (Sigma, St. Louis, MO) at 22 °C for 1 h. Nonspecific binding to the membranes was determined from ^{125}I-[Sar^1-Ile^8]Ang II binding in the presence of 10^{-5} M ^{127}I-[Sar^1-Ile^8]Ang II. The binding reaction was stopped by filtering (Brandel Type M-24R) on FP-200 GF/C filters (Whatman Inc., Fairfield, NJ). Filter-bound ^{125}I-[Sar^1-Ile^8]Ang II was quantitated in a γ counter (Packard). For competition binding studies, membranes expressing the wild-type receptor or the mutants were incubated at room temperature for 1 h with 300 pM ^{125}I-[Sar^1-Ile^8]Ang II and various concentrations of the agonist Ang II or the antagonist [Sar^1-Ile^8]Ang II (Bachem). Equilibrium binding kinetics were determined using the computer program Ligand. The K_i values represent the mean ± SEM of three to five independent determinations (Cheng & Prussoff, 1973).

IP Formation Studies. Semiconfluent COS-1 cells transfected in 60 mm dishes by the DEAE-dextran method were labeled for 24 h at 37 °C in a CO_2 incubator with [^{3}H]myoinositol (Amersham) in DMEM containing 10% bovine calf serum. After labeling, cells were washed with HBSS and exposed with 10 mM LiCl in HBSS for 30 min. Cells were treated with or without various concentrations of angiotensin ligand in HBSS containing 20 mM sodium phosphate, pH 7.4, for 45 min. Cells were lysed with 0.4 M perchloric acid, and total IP production was measured (Feng et al., 1995; Noda et al., 1995b).

RESULTS AND DISCUSSION

The Molecular Model of Ang II Docked to the AT_1 Receptor Predicts Interaction between Tyr^4 of Ang II and Asn^{111} in the Transmembrane Helix III of the Receptor. We created a model of the AT_1 receptor with Ang II bound within the ligand-binding pocket to identify candidate residues interacting with the agonism-specifying Tyr^4 side chain of Ang II. The model shown in Figure 2 was constructed from a separate AT_1 receptor model built by homology modeling methods (Trump-Kallmeyer et al., 1992) and an Ang II model

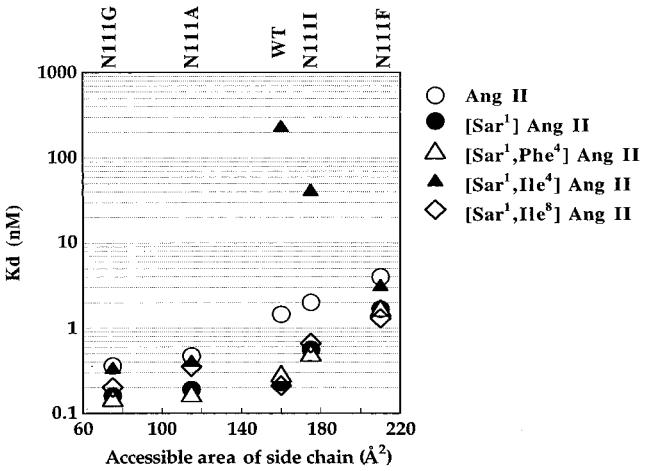

FIGURE 3: Influence of residue at position 111 on Ang II binding affinity. Shown are changes in the K_d of various Ang II analogues when the residue at position 111 of the AT_1 receptor is altered by the different amino acid substitutions indicated. The values for accessible surface area of the side chains of Gly (75 Å^2), Ala (115 Å^2), Asn (160 Å^2), Ile (175 Å^2), and Phe (210 Å^2) are from Creighton (1984). The values represent the mean ± SEM of three to five independent determinations carried out in duplicate. The standard error was within 5% and therefore is not represented in the figure.

built from the structural coordinates of constrained Ang II analogues obtained from nuclear magnetic resonance studies (Nikiforovich et al., 1994). Ang II was docked to the AT_1 receptor with experimentally determined salt-bridge interactions (Noda et al., 1995a,b; Feng et al., 1995) as constraints, and minimal energy interaction was simulated. This model indicates that the Tyr^4 side chain of Ang II interacts with transmembrane helix III and involves, more specifically, residues Ser^{107}, Asn^{111}, and Leu^{112} (Figures 1 and 2). The AT_1 receptor model described by Joseph et al. (1995) also suggests that Asn^{111} is a candidate for a hydrogen-bonding interaction with the hydroxyl group of Tyr^4. Previous mutagenesis studies indicate a role for Asn^{111} in Ang II binding but have excluded a significant role for Ser^{107} (Ji et al., 1995; Monnot et al., 1996). The Asn^{111} residue is conserved in all cloned Ang II receptors. The evidence for a role for Asn^{111} is significant because contact between small peptide hormones and transmembrane helix III of their receptors is thought to be not as common as the contact of the ligands with transmembrane helix III of monoamine receptors and opsins (Strader et al., 1994).

Binding Affinity of [Ile^4]Ang II Is Specifically Affected by the Size of the Residue at Position 111 of the AT_1 Receptor. A size constraint in the interaction between the Ang II Tyr^4 side chain and the AT_1 receptor is thought to be operational because several position 4 analogues of Ang II bind with reduced affinity to the AT_1 receptor (Bumpus & Khosla, 1977). Ang II and [Sar^1]Ang II, a full agonist analogue of Ang II, bind to the AT_1 receptor with a K_d of ~1 nM and ~0.2 nM, respectively. Figure 3 shows that the Tyr^4 → Phe^4 substitution in [Sar^1]Ang II has no effect on binding affinity but that a Tyr^4 → Ile^4 substitution produces an ~1100-fold reduction of binding affinity. On the basis of this observation, we predicted that if Asn^{111} of the AT_1 receptor interacts directly with Tyr^4 of Ang II, the interaction does not involve a conventional hydrogen bonding between the hydroxyl group of Tyr^4 and the $-CONH_2$ group of Asn^{111}. In fact, it may involve interaction between the bulky aromatic ring of Tyr^4 and the Asn^{111} side chain. Therefore, substituting bulkier residues for Asn^{111} should result in a loss of affinity toward Ang II, [Sar^1]Ang II, and [Sar^1,Ile^8]Ang II but increase the binding affinity of [Sar^1,Ile^4]Ang II. Asn^{111} was independently replaced with two smaller residues, Gly and Ala, and two larger residues, Ile and Phe, to calibrate the effect of modifying the side chain size at position 111 of the AT_1 receptor. The mutant receptors were expressed in COS-1 cells by transient transfection. The expression of receptor protein in each instance was assessed by Western blot analyses. As control, the effect of reduction of Ser^{107} and Ser^{115} side chain size was also examined. We expected these serine residues to be located on the same phase of the transmembrane α-helix III but one helical turn proximal and distal to Asn^{111}, respectively. S107G and S115G mutants displayed wild-type-like phenotypes, indicating that reduction in size of these helix III side chains does not disrupt either the α-helix structure or the interhelical packing interactions necessary for correct folding of the receptor (Table 1).

The difference in Ang II, [Sar^1]Ang II, [Sar^1,Phe^4]Ang II, and [Sar^1,Ile^8]Ang II binding affinity for N111G, N111A, N111I, and N111F mutants relative to the wild-type receptor was similar in level and direction (Figure 3). The binding affinity of the mutant receptors for [Sar^1]Ang II, [Sar^1,Phe^4]-Ang II, and [Sar^1,Ile^8]Ang II was N111G > N111A > N111I > N111F. This gradation of effect is consistent with a change of the accessible side chain surface area at position 111. These findings show that position 1 and 8 substitutions of Ang II are not recognized by position 111 mutants of the AT_1 receptor. Furthermore, the structural difference between Tyr^4 and Phe^4 side chains is not relevant for this interaction. In stark contrast, [Sar^1,Ile^4]Ang II binding affinity for the wild-type receptor was reduced 10 μg 1100-fold in comparison to [Sar^1]Ang II binding affinity; substitution of Ile^4 for Tyr^4 causes an ~27 Å^2 decrease in side chain surface area. Figure 3 shows that the loss in binding affinity due to the introduction of Ile at position 4 of [Sar^1]Ang II is regained upon increasing the side chain size at position 111 by substitution with Phe (~50 Å^2 increase). An intermediate effect on binding affinity was seen when the surface area of the position 111 side chain was increased by ~15 Å^2 through the introduction of Ile. The affinity changes observed with the position 4 substituted analogue of [Sar^1]Ang II and the position 111 mutants of the receptor correlate well with values for the volume and surface area of substituted side chains at these sites but not with values for hydrogen-bonding potential and hydrophobicity. These affinity changes are consistent with complementation and support our modeling studies that predict a direct interaction between Asn^{111} and the Tyr^4 side chain of Ang II through van der Waals contacts.

Gly^{111} and Ala^{111} Mutants of the AT_1 Receptor Are Constitutively Active. We observed that AT_1 receptor agonists (e.g., Ang II and [Sar^1]Ang II) and partial agonists (e.g., [Sar^1,Ile^8]Ang II) showed a small to moderate (<5-fold) increase in binding affinity for the Gly^{111} and Ala^{111} receptor mutants compared to wild type, whereas AT_1 receptor antagonists (e.g., losartan) exhibited a markedly (23−34-fold) lower binding affinity (Table 1 and Figure 3). Perlman et al. (1995) and Monnot et al. (1996) have also reported that the Ala^{111} mutant has a higher affinity for peptide agonists but a lower binding affinity for the antagonists losartan and L158.809.

An increase in binding affinity for agonists in the absence of G-protein coupling suggests that the mutation induced an "active state" conformation of the receptor, which in turn

Table 1: Ligand Binding and Activation Properties of Wild-Type and Helix III Mutant AT_1 Receptors[a]

	K_d (nM)		total IP production (cpm × 10^{-3})			
receptor[a]	[Sar^1]Ang II	Losartan	basal[b]	maximal[c]	B_{max} (pmol/mg)	AS/cell × 10^{-3}
mock			2.9 ± 0.2	2.1 ± 0.1		
wild type	0.23 ± 0.02	10 ± 1.5	5.3 ± 0.15	47.8 ± 3.4	5.4 ± 0.10	160 ± 4.0
S107G	0.34 ± 0.03	14 ± 4	6.7 ± 0.15	41.5 ± 1.0	4.8 ± 0.14	141 ± 4.1
S115G	0.29 ± 0.09	39 ± 12	5.0 ± 0.1	47.1 ± 2.6	5.1 ± 0.11	162 ± 3.5
N111G	0.16 ± 0.05	229 ± 31	28.2 ± 0.8	47.4 ± 4.7	3.6 ± 0.07	112 ± 3.7
N111A	0.19 ± 0.07	342 ± 17	14.0 ± 0.9	42.6 ± 3.1	6.8 ± 0.14	165 ± 4.2
N111I	0.56 ± 0.02	3150 ± 484	3.4 ± 0.2	23.9 ± 2.4	3.1 ± 0.13	130 ± 3.3
N111F	1.67 ± 0.20	918 ± 115	2.8 ± 0.25	11.3 ± 1.6	3.8 ± 0.77	134 ± 4.2

[a] The K_d and B_{max} values represent the mean ± SEM obtained from three to five independent transfection experiments performed in duplicate. The B_{max} values represent the total receptor present in intracellular membranes and plasma membrane. The cell surface receptor expression represented as average sites per cell (AS/cell) was determined from three transfection experiments in which the total receptor expression was monitored by immunoblotting and saturation binding, and the plasma membrane expression was monitored by total binding of ^{125}I-[Sar^1,Ile^8]Ang II to 10^7 intact transfected cells. [b] In the absence of ligand. [c] In the presence of 10 μM [Sar^1]Ang II.

promotes ligand-independent activation of intracellular signal transduction (Costa & Herz, 1989; Samama et al., 1993; Black & Shankley, 1995; Bond et al., 1995; Perez et al., 1996). Therefore, to examine the influence of Asn^{111} mutations on signal transduction properties of the receptor, we measured IP production in COS-1 cells transfected with the wild type and N111A and N111G mutants of the AT_1 receptor. Table 1 shows that, at comparable levels of cell surface receptor expression, [Sar^1]Ang II-dependent maximal IP formation with Gly^{111} and Ala^{111} receptor mutants was identical to the level seen in wild-type receptor transfected cells. Basal IP production in the Gly^{111} and Ala^{111} receptor mutants was 57% and 28%, respectively, of maximal activity (Table 1). Under identical conditions, basal IP production in wild-type, Ile^{111}, and Phe^{111} transfected COS-1 cells was 5.3%, 2.3%, and ~0% of [Sar^1]Ang II-dependent maximal IP production (in these comparisons, zero was the level of IP production in mock-transfected COS-1 cells). These results indicate that a reduction of the Asn^{111} side chain leads to a partial constitutively activated form of the receptor. In contrast, increased side chain size leads to forms of the receptor that have reduced basal and maximal activity.

N111G Mutation Induces a Conformational Change in the Ang II Binding Pocket. N111G, N111A, N111I, and N111F mutant receptors have a 23−310-fold lower binding affinity than the wild-type receptor for the nonpeptide antagonist losartan (Table 1). However, the decrease in affinity is unlikely to be due to the loss of a direct interaction between Asn^{111} and losartan because the increase in K_d does not correlate with change in the size or hydrophobicity/hydrophilicity of the substituted side chains. Previous studies from this laboratory and others have led to the view that losartan binds to a pocket in the AT_1 receptor that overlaps with the Ang II binding pocket. This binding involves interactions between specific agonist and antagonist groups and the AT_1 receptor that are common, such as those involving residues Lys^{199} and His^{256} (Noda et al., 1995a,b), as well as several interactions that are distinct (Feng et al., 1995; Ji et al., 1995; Perlman et al., 1995; Monnot et al., 1996). Therefore, it appears that the N111G mutation induces a conformational change (R′) in the Ang II binding pocket that causes misalignment of the residues required for losartan binding. The same conformational change favors the binding of agonists and partial agonist analogues of Ang II (Figure 3). Thus, [Sar^1,Ile^4]Ang II, which binds poorly to the wild-type receptor, binds to the Gly^{111} mutant receptor with an affinity comparable to that of [Sar^1]Ang II. The ~1000-fold increase in binding affinity for [Sar^1,Ile^4]Ang II following the N111G mutation is unexpected from the creation of a simple cavity because the N111A mutant also exhibited the same property. The increase in binding affinity must be an intrinsic property of this mutation because the high-affinity state was not affected in the presence of GTPγS or EDTA-washed membrane—both are conditions that promote dissociation of endogenous G-protein precoupling to the receptor. These findings suggest that all groups that participate in stabilizing the bound Ang II need to be properly aligned within the receptor pocket in the conformation R′ induced by the N111G mutation. Because Asn^{111} is a receptor residue that is also involved in docking Tyr^4, an Ang II residue that contains an important agonism-specifying side chain, the Tyr^4−Asn^{111} interaction is likely to be the switch that controls a conformation-dependent reconfiguration of the ground state Ang II binding pocket that concomitantly stabilizes Ang II binding and induces the active state of the receptor (R*). The finding that the N111G mutation produces only a partial, constitutively activated receptor would suggest that further stabilization of the R′ conformation is needed for full activation.

Agonism-Specifying Side Chains of Ang II Are Not Needed for Stabilizing the R′ State of the N111G Mutant. [Sar^1,Ile^4]Ang II and [Sar^1,Ile^8]Ang II are partial agonists of the AT_1 receptor (Figure 4). These Ang II analogues display higher (40−100 nM) EC_{50} values than [Sar^1]Ang II (3.2 nM) at the AT_1 receptor, and maximal IP stimulation by [Sar^1,Ile^4]Ang II and [Sar^1,Ile^8]Ang II is 70% and 20% of that produced by [Sar^1]Ang II, respectively. [Sar^1,Ile^4]Ang II has higher partial agonist activity compared to [Sar^1,Ile^8]Ang II despite the fact that the Ile^4 modification of [Sar^1]Ang II has a larger effect on AT_1 receptor binding affinity than the Ile^8 modification. We designed and tested an analogue of Ang II, [Sar^1,Ile^4,Ile^8]Ang II, in which both agonism-specifying aromatic side chains were replaced with an aliphatic isoleucine side chain. The affinity of [Sar^1,Ile^4,Ile^8]Ang II for the AT_1 receptor was 2350 ± 163 nM; this Ang II analogue did not stimulate IP production in the wild-type AT_1 receptor at concentrations 5-fold (Figure 4) and 500-fold (i.e., at 1 mM; data not shown) greater than its K_d. Because concomitant replacements of Tyr^4 and Phe^8 side chains with the Ile side chain are required for total loss of agonism at the AT_1 receptor, agonism-producing interactions between Tyr^4 and Phe^8 and the receptor are likely to be additive. In addition, we found that replacement (with Ala) of His^{256}—a residue that forms an important interaction with the Ang II Phe^8 side chain—produces a partial activation-defective mutant of the AT_1 receptor that can be rescued by

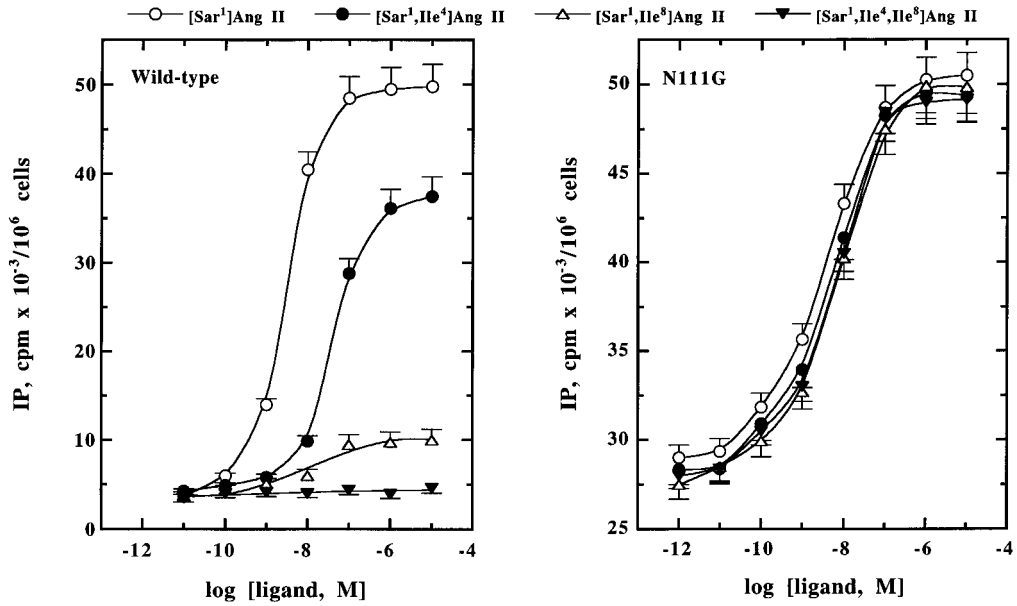

FIGURE 4: Concentration−response curves for various analogues of Ang II at comparable levels of the wild-type and the N111G mutant receptors expressed in COS-1 cells. The IP response produced roughly correlated with the GTPγS-sensitive high-affinity state (R·G complex) induced by various analogues. However, GTPγS has no significant effect on the binding affinity of various analogues to the N111G mutant, indicating that R·G complex formation does not modulate the ligand affinity in this mutant receptor.

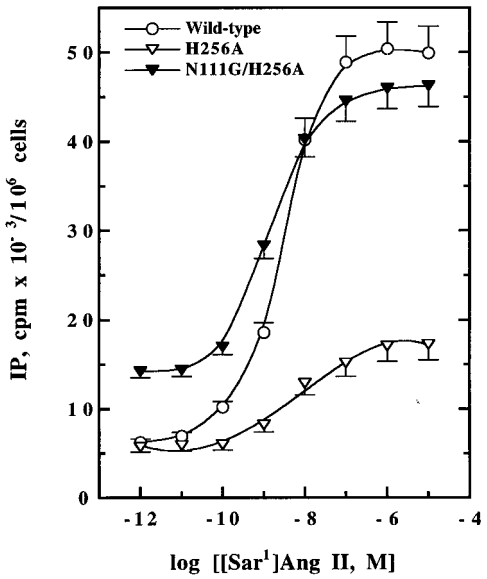

FIGURE 5: Activation of the N111G, H256A double mutant AT_1 receptor by [Sar^1]Ang II. Its relationship to the H256A single mutant and the wild-type AT_1 receptor expressed at comparable levels is indicated.

the additional Asn^{111} → Gly change. This finding suggests that His^{256} is not required when the Asn^{111} side chain is deleted and that the Phe^8−His^{256} interaction enables the Tyr^4−Asn^{111} switch to engage the wild-type AT_1 receptor in the activation process (Figure 5).

The affinity of [Sar^1,Ile^4,Ile^8]Ang II for the N111G mutant was 7.1 ± 1 nM. When the activation of the N111G mutant was examined using [Sar^1,Ile^4]Ang II, [Sar^1,Ile^8]Ang II, and [Sar^1,Ile^4,Ile^8]Ang II, the maximal stimulation was identical to that produced by [Sar^1]Ang II (Figure 4). This equivalency of stimulation is consistent with the observation that the N111G mutant binds [Sar^1]Ang II, [Sar^1,Ile^4]Ang II, and [Sar^1,Ile^8]Ang II with similar affinities and binds [Sar^1,Ile^4,-Ile^8]Ang II with an ~15-fold lower affinity (K_d = 3.1 ± 0.1 nM). These observations demonstrate that (1) a full activation of the N111G mutant receptor does not require aromatic side chains at positions 4 and 8 of Ang II and (2) the remainder of the Ang II structure plays a crucial role in stabilizing and fully activating the receptor. The role of these regions of Ang II has previously been thought to be important only in the correct positioning of the aromatic Tyr^4 and Phe^8 side chains within the AT_1 receptor Ang II-binding pocket.

In light of this explanation, the reasons for an anomalous decrease in binding affinity toward the wild-type receptor produced by the Tyr^4 → Ile^4 change in [Sar^1]Ang II become clearer. The >1000-fold loss of binding affinity cannot be explained by size and hydrophobicity changes resulting from the Tyr^4 → Ile^4 change. Previously, it has been suggested that Tyr^4 → nonaromatic transitions alter the Ang II secondary structure and render the analogue a poor AT_1 receptor ligand. However, the high binding affinity of [Ile^4]-Ang II for the N111G receptor mutant does not support the view that this position 4 change causes a major distortion in the Ang II secondary structure. We therefore conclude that the contribution of Ang II interactions, other than those of Tyr^4 and Phe^8, with the AT_1 receptor becomes critically important after the R′ state is achieved. It remains to be established whether the progression of R′ to the fully activated R* state in the N111G mutant by [Sar^1,Ile^4,Ile^8]-Ang II occurs via an active (induced-fit) or a passive (stabilization of an equilibrium-driven R* state, i.e., best-fit) process.

Antagonist Decreases the Basal Activity of the Wild-Type and N111G Mutant Receptor. In the experiments described above, we considered the association of a conformational change in the Ang II-binding pocket with the process of receptor activation. Differing levels of basal activity can readily be observed in COS-1 cells transfected with the wild-type (5.3% of maximal) or the N111G mutant (57% of maximal) receptor (Table 1). The following series of experiments were designed to address whether the wild-type and the N111G mutant receptors can be driven to the fully inactive state, i.e., the "true" R state, with an antagonist. The nonpeptide antagonist EXP3174 (a structural analogue of losartan that has higher binding affinity, K_i = ~1 nM for the AT_1 receptor) was used in these studies.

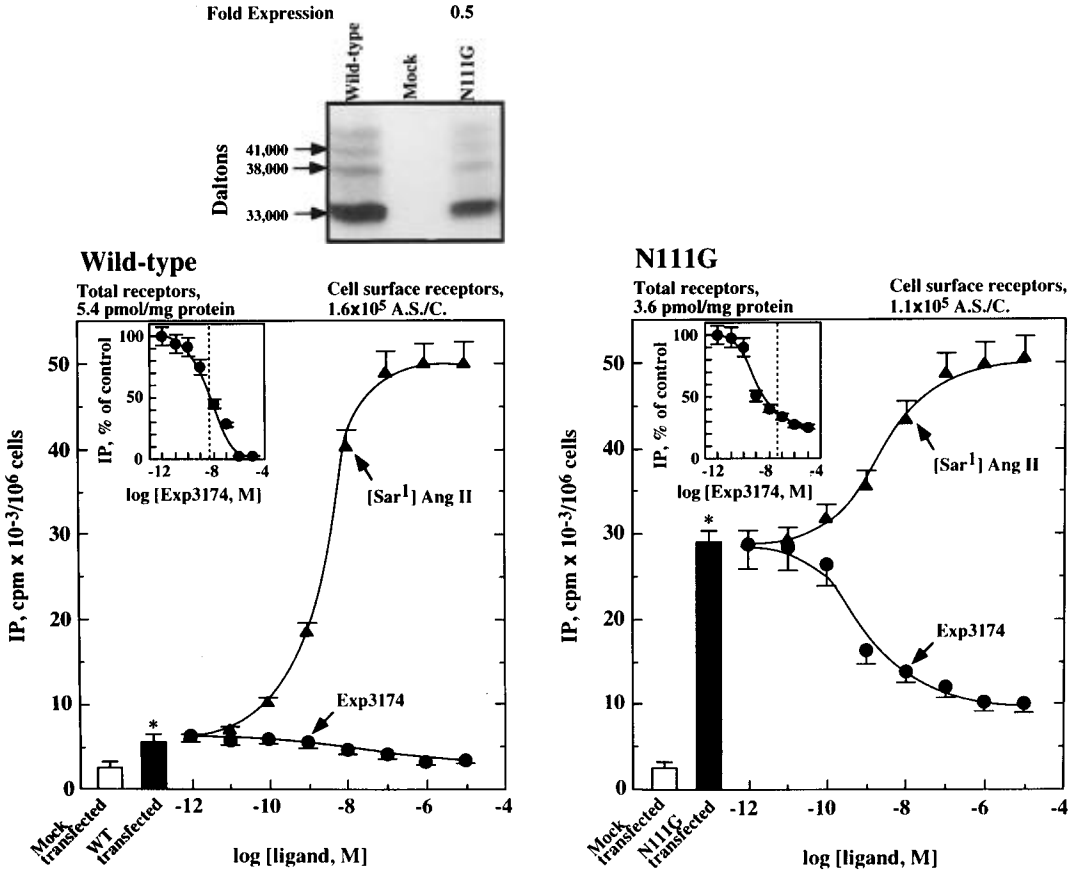

FIGURE 6: Modulation of activity of the wild-type and Gly^{111} mutant AT_1 receptors by [Sar^1]Ang II and the nonpeptide antagonist EXP3174. The values represent the mean of three independent measurements. The asterisk (*) represents a significant increase of IP production in transfected COS-1 cells in the absence of the ligand compared to mock-transfected COS-1 cells. The total number and number of cell surface receptors were estimated from ^{125}I-[Sar^1,Ile^8]Ang II binding studies carried out on total membranes and whole cells, respectively. The average sites per cell (AS/C) values were estimated by saturation binding using intact transfected cells from triplicate samples in three independent transfections. The inset in each case represents suppression of the basal activity; the vertical dashed line indicates the K_d of EXP3174 for the wild-type (1.3 ± 0.08 nM) and the Gly^{111} (46 ± 4.1 nM) receptor. In the top panel, a representative immunoblot of the wild-type and the N111G mutant receptors expressed in COS-1 cells is shown. The band of ~33 kDa corresponds to the unglycosylated AT_1 receptor because deglycosylation does not alter the mobility of this band and the remainder of the bands collapse to this band upon treatment with protein *N*-glycosidase F. The relative levels of expression shown at the top were estimated by phosphorimage analysis, using a calibration curve obtained from the immunoblot analysis of the purified preparation of bovine opsin, which also carries the epitope for the monoclonal antibody 1D4 (Noda et al., 1995a,b).

Basal IP production was not affected when COS-1 cells transfected with the wild-type and the N111G mutant receptor were preincubated with EXP3174 for 1 h at concentrations 100-fold greater than K_d (data not shown). In similar experiments, 18 h preincubations with EXP3174 resulted in complete suppression of receptor-dependent basal IP production in COS-1 cells transfected with wild-type receptor and ~70% suppression of basal IP production in COS-1 cells transfected with the N111G mutant receptor (Figure 6). The protein expression, the cell surface receptor number and the Ang II-dependent stimulation are not altered by prolonged preincubation with EXP3174. These findings illustrate that EXP3174 is a true inverse agonist of the AT_1 receptor. The inability of concentrations of EXP3174 that exceed K_d by >100-fold to fully suppress the basal activity of the N111G mutant, even after an 18-h preincubation, indicates that the rate of interconversion between R′ and R is much slower in the N111G mutant receptor than in the wild-type receptor. A substantially higher constitutive activity in the N111G mutant receptor compared with the wild-type receptor further indicates that, in the absence of an agonist, the population of receptors that exist in the R state is considerably reduced by the N111G mutation.

AT_1 Receptor Activation by Ang II: Selection versus Induction. Constitutive activation caused by several mutations, some naturally occurring and some generated through mutagenesis, indicates a basal constrained state of the G-protein-coupled receptors (Kjelsberg et al., 1992; Clapham, 1993; Lefkowitz, 1993). Intramolecular constraint is proposed to control the equilibrium between the R and R* states. In current models of receptor activation, conversion of R to the fully active state (R*) does not require the hormone; rather, the hormone binds preferentially to the activated receptor to shift the equilibrium toward R* (Costa & Herz, 1989; Black & Shankley, 1995; Samama et al., 1993; Robinson et al., 1992; Bond et al., 1995). In the absence of agonists, the receptor is thought to be in a state of flux between R and R*, and the agonists and antagonists stabilize, respectively, active and inactive states (Black & Shankley, 1995). This hypothesis is supported predominantly by the mode of action of inverse agonists; proof against agonist induction of the active state is scant at this time. The results presented here for the AT_1 receptor argue against the "conformational selection" route for the generation of the R* state. For example, if we assume that [Sar^1,Ile^4,Ile^8]Ang II (L_A)-dependent activation of the N111G mutant receptor proceeds from an intermediate conformational state (R′) (that is, $R' \leftrightarrow R^* \rightarrow R^*{\cdot}L_A$), conformational selection theory predicts that the spontaneously generated R* state of the wild-type receptor will also be stabilized by L_A (that is, R

↔ R* → R*·L_A). This was not observed under conditions where >99% of the receptors is occupied by L_A, implying that an accumulation of R*·L_A does not occur by selection.

The observation that the basal conformation of the N111G mutation is a facsimile of a transient intermediate in the activation of the AT_1 receptor provides an important insight into the mechanism of the agonist (L) mediated activation of the receptor. That constitutively active mutants of the AT_1 receptor can be created suggests that the wild-type protein is normally constrained to an inactive state (R). What is the mechanism for relaxing the constraint? Evidence presented here demonstrates that [Sar^1,Ile^4,Ile^8]Ang II (L_A), at a concentration ≈500-fold over K_d, does not generate the R*·L_A complex starting from the R state (i.e., the wild-type receptor), whereas, at a concentration ≈100-fold over K_d, [Sar^1,Ile^4,Ile^8]Ang II generates R*·L_A starting from R′ (i.e., the N111G mutant). The simplest way to explain these data is that under equilibrium conditions the fraction of the wild-type receptor that exists as R* must be rare at best and the R→R′ transition is a determinative step that occurs after Ang II binds but does not occur when [Sar^1,Ile^4,Ile^8]Ang II binds to the wild-type receptor. Ang II, present in the binding pocket of the receptor to generate R′, also promotes the overall transition of R ↔ R*; i.e., the receptor activation occurs by agonist induction. Thus, our findings suggest that the stabilization of the R* state of the AT_1 receptor requires two distinct Ang II-dependent steps. The initial rate-limiting step (R → R′) is achieved through thermodynamically linked interactions between Ang II−[Tyr^4] and the [Asn^{111}]−AT_1 receptor and between Ang II−[Phe^8] and the [His^{256}]−AT_1 receptor. When the ligand-binding pocket is not occupied, the receptor is stabilized by a complex set of intramolecular interactions that chiefly constrain Asn^{111}. Release of Asn^{111} from this interaction produces a conformational change in the ligand-binding pocket such that this pocket now favors agonists and partial agonists of the AT_1 receptor but not antagonists. In this state (R′), all groups that participate in the binding of Ang II still need to be properly aligned and stabilized from conformational entropy. In the second step (R′ → R*), unspecified structural features in Ang II other than the agonism-specifying Tyr^4 and Phe^8 side chains are needed to stabilize the fully activated state (R*) of the receptor. Whether the agonist induction process is unique to this subfamily of receptors or is a more general phenomenon that has not been fully explored in previous studies of G-protein-coupled hormone receptors remains to be established. In this regard, our observations of the AT_1 receptor provide a novel paradigm for the study of the receptor activation process and establish a framework against which other models can be tested.

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