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Dependence of AT_1 Angiotensin Receptor Function on Adjacent Asparagine Residues in the Seventh Transmembrane Helix

LÁSZLÓ HUNYADY, HONG JI, GOWRAGANAHALLI JAGADEESH, MENG ZHANG, ZSUZSANNA GÁBORIK, BALÁZS MIHALIK, and KEVIN J. CATT

Department of Physiology, Semmelweis University of Medicine, H-1088 Budapest, Hungary (L.H., Z.G., B.M.), Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892-4510 (H.J., M.Z., K.J.C.), and Division of Cardio-Renal Drug Products, Center for Drug Evaluation and Research, Food and Drug Administration, Rockville, Maryland 20857 (G.J.)

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ABSTRACT

For several G protein-coupled receptors, amino acids in the seventh transmembrane helix have been implicated in ligand binding and receptor activation. The function of this region in the AT_1 angiotensin receptor was further investigated by mutation of two conserved polar residues (Asn294 and Asn295) and the adjacent Phe293 residue. Analysis of the properties of the mutant receptors expressed in COS-7 cells revealed that alanine replacement of Phe293 had no major effect on AT_1 receptor function. Substitution of the adjacent Asn294 residue with alanine (N294A) reduced receptor binding affinities for angiotensin II, two nonpeptide agonists (L-162,313 and L-163,491), and the AT_1-selective nonpeptide antagonist losartan but not that for the peptide antagonist [Sar^1,Ile^8]angiotensin II. The N294A receptor also showed impaired G protein coupling and severely attenuated inositol phosphate generation. In contrast, alanine replacement of Asn295 decreased receptor binding affinities for all angiotensin II ligands but did not impair signal transduction. Additional substitutions of Asn295 with a variety of amino acids did not identify specific structural elements for ligand binding. These findings indicate that Asn295 is required for the integrity of the intramembrane binding pocket of the AT_{1a} receptor but is not essential for signal generation. They also demonstrate the importance of transmembrane helices in the formation of the binding site for nonpeptide AT_1 receptor agonists. We conclude that the Asn294 residue of the AT_1 receptor is an essential determinant of receptor activation and that the adjacent Asn295 residue is required for normal ligand binding.

The AT_1 angiotensin receptor is a GPCR that mediates the physiological actions of the octapeptide pressor hormone Ang II (Spät *et al.,* 1991; Ganguly and Davis, 1994; Griendling *et al.,* 1996). GPCRs share a common basic structure of seven transmembrane helices connected by alternating intracellular and extracellular loops. All members of this receptor family couple to specific G proteins that mediate the activation of several plasma membrane effector systems (Probst *et al.,* 1992; Strader *et al.,* 1994). AT_1 receptors, which occur as highly homologous and functionally similar AT_{1a} and AT_{1b} subtypes in rodents, are primarily coupled through the $G_{q/11}$ group of G proteins to stimulation of phospholipase C activity and Ins(1, 4, 5)P_3-induced Ca^{2+} signal generation. This is usually accompanied by activation of protein kinase C, mediated by increased diacylglycerol production in concert with the agonist-evoked intracellular Ca^{2+} signal (Spät *et al.,* 1991; Ganguly and Davis, 1994; Griendling *et al.,* 1996). The molecular mechanisms by which activated GPCRs are coupled to signal generation are currently the focus of intensive studies investigating their structure-function relationships.

In many peptide hormone receptors, the cognate physiological ligands bind to a site formed by the extracellular loops and the outermost regions of the transmembrane helices (Strader *et al.,* 1995; Hunyady *et al.,* 1996). The conformational changes evoked by agonist binding are transmitted by the transmembrane helices to the intracellular loops, which are believed to comprise the regions that couple the receptor to its intracellular signaling systems. The current structural information about GPCRs is based on low-resolution maps of bovine and frog rhodopsins (Schertler *et al.,* 1993; Schertler and Hargrave, 1995). These studies, together with recent data obtained by electron cryomicroscopy of crystals of frog

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ABBREVIATIONS: GPCR, G protein-coupled receptor; Ang II, angiotensin II; $InsP_2$, inositol bisphosphate; $InsP_3$, inositol trisphosphate; GTPγS, guanosine 5′-(γ-thio)triphosphate; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

rhodopsin (Unger *et al.,* 1997), have confirmed the seven-transmembrane helix nature of GPCRs and have provided information about the positioning of the helices but have not elucidated the structural changes that are involved in the mechanism of activation of these receptors. For this reason, several theoretical models of the three-dimensional conformation of the AT_1 receptor and other GPCRs have been constructed (Baldwin, 1993; Donnelly *et al.,* 1994; Joseph *et al.,* 1995; Underwood *et al.,* 1995; Yamano *et al.,* 1995; Inoue *et al.,* 1997). Although predictions based on such studies have been helpful in defining the binding sites of several GPCRs, the manner in which agonist binding changes the conformation of the receptor molecule has not been clarified.

Pharmacological studies have identified two major classes of nonpeptide compounds that bind selectively to angiotensin AT_1 and AT_2 receptors (Griendling *et al.,* 1996). Structure-function studies have shown that the binding site of such nonpeptide antagonists is located within the intramembrane binding pocket, with contributions from several residues located in the transmembrane helices (Ji *et al.,* 1994; Hunyady *et al.,* 1996; Inoue *et al.,* 1997; Karnik *et al.,* 1997). More recent studies on nonpeptide analogs have led to the identification of compounds that act as partial agonists of the AT_1 receptor, causing increased blood pressure *in vivo* and stimulation of signal transduction *in vitro* (Perlman *et al.,* 1995; Kivlighn *et al.,* 1996). Mutational studies focusing primarily on the extracellular regions have failed to identify residues that contribute to the binding of nonpeptide agonists (Perlman *et al.,* 1995). However, the nonpeptide agonists and antagonists are closely related chemically, and it is likely that some of the residues that participate in the binding of nonpeptide antagonists also contribute to the binding of nonpeptide agonists.

The seventh transmembrane helix has been suggested to be an important area for both ligand binding and activation of the AT_1 receptor and several other GPCRs (Luo *et al.,* 1994; Marie *et al.,* 1994; Hunyady *et al.,* 1995, 1996; Strader *et al.,* 1995; Laporte *et al.,* 1996; Inoue *et al.,* 1997). Mutations of amino acid residues in the seventh helix have been reported to interfere with the binding of nonpeptide Ang II antagonists. These residues include Tyr292 (the amino acid located in the position at which the retinal chromophore binds covalently to the rhodopsin molecule), Leu300 and Phe301 (two apolar amino acids located in the center of the conserved NPXXY sequence), and Asn294 and Asn295 (two polar residues located between Tyr292 and the NPXXY sequence) (Marie *et al.,* 1994; Schambye *et al.,* 1994b; Ji *et al.,* 1995; Hunyady *et al.,* 1996; Inoue *et al.,* 1997; Karnik *et al.,* 1997). In addition, mutations in the seventh transmembrane helix have been found to interfere with the binding of peptide ligands and with signal transduction from the receptor (Marie *et al.,* 1994; Hunyady *et al.,* 1995; Ji *et al.,* 1995; Laporte *et al.,* 1996).

Recent studies have suggested that an interaction between Asn295 and Asn111 in the third helix stabilizes the inactive conformation of the receptor, based on the finding that mutation of Asn295 or Asn111 caused constitutive activation of the AT_1 receptor (Noda *et al.,* 1996; Balmforth *et al.,* 1997; Groblewski *et al.,* 1997). The Asn111 residue has also been reported to interact with the side chain of Tyr4 of Ang II, and this has been suggested to initiate the process of receptor activation (Noda *et al.,* 1996). Because the available structure-function data suggest that the seventh transmembrane helix of the AT_1 angiotensin receptor is a region in which the binding sites of peptide and nonpeptide ligands overlap (Hunyady *et al.,* 1996), the role of this region in the binding of nonpeptide agonists was analyzed. Receptors bearing single amino acid substitutions of two polar residues (Asn294 and Asn295) and an adjacent apolar residue (Phe293) were created and expressed in COS-7 cells to study the role of this region in the ligand binding and activation of the AT_1 receptor.

Materials and Methods

Mutagenesis of rat smooth muscle AT_{1a} receptor cDNA. The rat AT_{1a} receptor cDNA was subcloned into the mammalian expression vector pcDNAI/Amp (Invitrogen, San Diego, CA), as described earlier (Hunyady *et al.,* 1994). The AT_{1a} receptors used in this study also contained an octapeptide tag (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) inserted after the starting methionine of mutant and wild-type rat AT_{1a} receptors, to permit the immunodetection of binding-deficient mutant receptors. In accordance with a recent report (Hein *et al.,* 1997), the octapeptide tag had no major effect on the binding and inositol phosphate signaling properties of the wild-type rat AT_{1a} receptor. The amino acid numbers used in this study refer to the positions in the published rat AT_{1a} receptor sequence (Murphy *et al.,* 1991); the presence of the octapeptide tag does not affect the numbering. Mutant rat AT_{1a} receptors were created using the Mutagene kit (Bio-Rad, Hercules, CA). Each mutant contained a silent restriction site to facilitate the screening of colonies. Oligonucletides were from Midland Certified Reagent Co. (Midland, TX). All mutations were verified by dideoxy sequencing using Sequenase II (Amersham-USB, Arlington Heights, IL).

Transient expression of AT_{1a} receptor mutants. COS-7 cells were cultured in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. For measurements of inositol phosphate responses or [Sar^1,Ile^8]Ang II binding to intact cells, the cells were seeded in 24-well plates (50,000 cells/well) 3 days before transfection. Transient transfection was performed by replacing the culture medium with 0.4-ml aliquots of Opti-MEM I containing 8 μg of Lipofectamine and 1 μg of plasmid DNA for each sample. The cells were incubated for 5–6 hr in this solution, which was then replaced with culture medium. All experiments were performed 48 hr after the initiation of the transfection procedure.

[Sar^1,Ile^8]Ang II binding to intact cells. To determine the surface expression level and structural integrity of the mutant receptors, the number of Ang II binding sites was determined by incubating the transfected cells with ^{125}I-[Sar^1,Ile^8]Ang II (0.05–0.1 μCi/sample) and increasing concentrations of unlabeled [Sar^1,Ile^8]Ang II in medium 199 (with HEPES) for 6 hr at 4°. The cells were washed twice with ice-cold Dulbecco's phosphate-buffered saline, and the radioactivity associated with the cells was measured by γ-counting after solubilization with 0.5 M NaOH/0.05% SDS. The displacement curves were analyzed with the Ligand computer program, using a one-site model, as described earlier (Hunyady *et al.,* 1995).

Binding to COS-7 cell membranes. COS-7 cells were seeded in 15-cm tissue culture dishes 72 hr before the transfection, which was performed by the calcium phosphate precipitation method (Life Technologies, Gaithersburg, MD), using 50 μg of DNA. After 48 hr, the cells were washed, scraped into 1.5 ml of ice-cold 10 mM Tris·HCl, pH 7.4, 1 mM EDTA, and then lysed by freezing. Crude membranes were prepared by centrifuging the samples at 16,000 $\times$ *g*. The pellet was resuspended in binding buffer (containing 100 mM NaCl, 5 mM $MgCl_2$, and 20 mM Tris·HCl, pH 7.4), and the protein content was determined. Binding assays were performed at 25° in 0.2 ml of binding buffer supplemented with 2 g/liter BSA, except when nonpeptide agonists were tested. In these experiments, BSA was re-

placed with 0.1% lysozyme. Saturation binding experiments with ^{125}I-Ang II and ^{125}I-[Sar^1,Ile^8]Ang II were performed by adding increasing concentrations of the respective radioligand (up to 2 nM) to 15–30 μg of crude membranes. In displacement studies, each sample contained 0.05–0.1 μCi of ^{125}I-[Sar^1,Ile^8]Ang II, 15–30 μg of crude membranes, and the indicated concentrations of unlabeled ligand. G protein coupling was evaluated by measuring the binding of ^{125}I-Ang II (0.05–0.1 μCi) under similar conditions in the presence of the indicated concentrations of GTPγS. After 90-min incubations at 25°, the unbound tracer was removed by rapid filtration, and the bound radioactivity was measured by γ-counting.

Inositol phosphate measurements. In these experiments, the culture medium was replaced, 24 hr after transfection, with 0.5 ml of inositol-free Dulbecco's modified Eagle's medium containing 1 g/liter BSA, 20 μCi/ml [^{3}H]inositol, 2.5% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin, as described earlier (Hunyady *et al.,* 1994). After a 24-hr labeling period, the cells were washed twice and incubated in inositol-free modified medium 199 (with HEPES), in the presence of 10 mM LiCl, for 30 min at 37° and were then stimulated with the indicated concentration of Ang II for 20 min. Incubations were stopped by the addition of perchloric acid (5%, v/v, final), and the inositol phosphates were extracted as described earlier (Hunyady *et al.,* 1995). After neutralization, the samples were applied to a Bio-Rad AG1X8 column. The columns were washed four times with 3 ml of water and twice with 3 ml of 0.2 M ammonium formate in 0.1 M formic acid, to remove inositol and inositol monophosphates. After these washing steps, the combined $InsP_2$/$InsP_3$ fractions were eluted with two 3-ml aliquots of 1 M ammonium formate in 0.1 M formic acid, and radioactivities were determined by liquid scintillation counting.

Materials. The cDNA clone (pCa18b) of the rat smooth muscle AT_{1a} receptor (Murphy *et al.,* 1991) subcloned into the mammalian expression vector pCDM8 (Invitrogen) was kindly provided by Dr. Kenneth E. Bernstein (Department of Pathology, Emory University, Atlanta, GA). Restriction enzymes were obtained from Boehringer Mannheim (Indianapolis, IN) or New England Biolabs (Beverly, MA). Culture media were from Biofluids (Rockville, MD). The medium 199 used in these experiments was modified to contain 3.6 mM K^+, 1.2 mM Ca^{2+}, 1 g/liter BSA, and 20 mM HEPES. Lipofectamine and Opti-MEM I were from Life Technologies. Losartan was a gift from Dr. P. C. Wong (DuPont, Wilmington, DE). L-162,313 and L-163,491 were kindly provided by Dr. W. J. Greenlee (Merck Research Laboratories, Rahway, NJ). ^{125}I-Ang II and ^{125}I-[Sar^1,Ile^8]Ang II were obtained from Hazleton Laboratories (Vienna, VA) or DuPont New England Nuclear Research (Boston, MA), and [^{3}H]inositol was from Amersham (Arlington Heights, IL).

Statistical analysis. The significance of changes in the binding affinities and inositol phosphate responses of the mutant AT_1 receptors were determined by analysis of variance combined with Scheffe's range test.

Results

Binding properties of mutant and wild-type AT_{1a} receptors. The wild-type and F293A, N294A, and N295A mutant AT_{1a} receptors were expressed in COS-7 cells, to study their radioligand binding and signaling characteristics. The positions of the mutated amino acids in the seventh transmembrane helix of the rat AT_{1a} receptor are shown in Fig. 1. Binding affinities were determined by measuring the inhibition of binding of the radioiodinated peptide antagonist ^{125}I-[Sar^1,Ile^8]Ang II in the presence of increasing concentrations of the unlabeled nonpeptide antagonist (losartan), the peptide antagonist (Fig. 2), or two nonpeptide agonists (L-162,313 and L-163,491) (Fig. 3). The K_d values are shown in Table 1. The binding affinities of the F293A receptor for [Sar^1,Ile^8]Ang II and losartan were not significantly different from those of the wild-type receptor. The N294A receptor had normal affinity for [Sar^1,Ile^8]Ang II, indicating that the structure of this mutant receptor was intact. However, its affinity for losartan was markedly reduced, with a 62-fold increase in K_d. The N295A mutant receptor exhibited diminished binding affinity for both [Sar^1,Ile^8]Ang II and losartan, with K_d values 16 $\pm$ 2 and 104 $\pm$ 6 times those of the wild-type receptor, respectively.

Mutations of the adjacent asparagine residues in the seventh transmembrane helix also affected the binding affinities for the two nonpeptide agonists (Fig. 3, Table 1). The F293A mutation caused no significant change in the binding of these compounds. The K_d values of the N294A receptor for L-162,313 and L-163,491 were 3.9- and 2.8-fold higher, respectively, than those of the wild-type receptor. However, the affinities of the N295A mutant receptor for these nonpeptide agonists were more severely impaired, with K_d values 26- and 25-fold higher, respectively, than those of the wild-type receptor.

The structural requirements at position 295 for ligand binding to the AT_{1a} receptor were further analyzed by measurement of the binding affinities of a series of substitution mutants for [Sar^1,Ile^8]Ang II, losartan, and the AT_1-specific nonpeptide agonist L-163,491 (Table 1). The binding affinity for [Sar^1,Ile^8]Ang II was most severely impaired with the above-

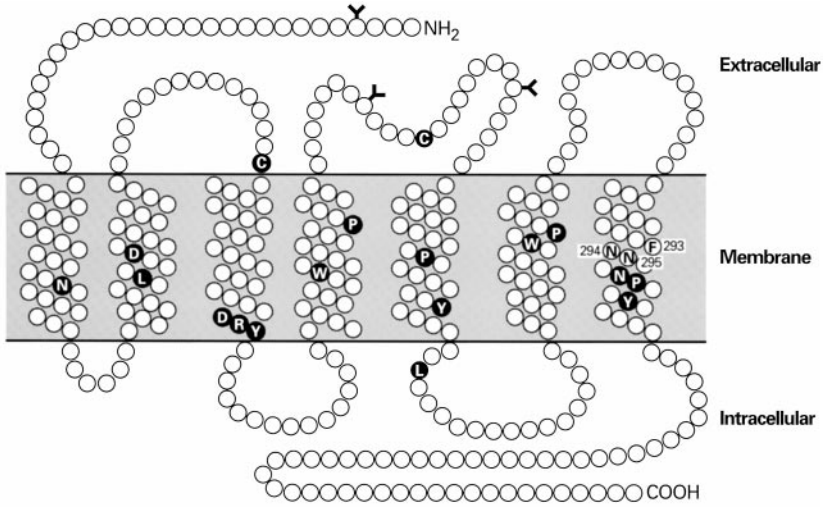

Fig. 1. Two-dimensional representation of the AT_{1a} receptor. The locations of the intramembrane helices are based on the model of Baldwin (1993). The most conserved residues in the GPCRs are shown (*white letters on black background*). The amino acid residues analyzed in this article and the putative glycosylation sites of the receptor are also shown.

described alanine-substituted (N295A) mutant receptor. Substitution with amino acids with larger side chains had less effect on [Sar^1,Ile^8]Ang II binding to the receptor, regardless of the nature of the side chain introduced (Table 1). For example, the inhibitory effects of three apolar substitutions for Asn295 (N295A, N295V, and N295L) decreased with increasing side-chain length. Substitution with leucine, which has the largest apolar side chain, caused the least impairment (2.4-fold) of [Sar^1,Ile^8]Ang II binding, similar to the effect of the conserved substitution N295Q (2.0-fold). Replacement by valine, with its smaller side chain, further impaired [Sar^1,Ile^8]Ang II binding affinity (6.5-fold), similar to the effects of the N295S (6.3-fold) and N295T (5.9-fold) mutations, but to a lesser extent than replacement with alanine (16-fold). Substitution of Asn295 with a negatively charged residue (N295D) did not significantly impair receptor affinity for [Sar^1,Ile^8]Ang II, and substitution with lysine caused a relatively minor (3.7-fold) decrease in binding affinity.

The structural requirements for the nonpeptide compounds losartan and L-163,491 were different from those for the peptide antagonist at position 295 of the AT_{1a} receptor. Losartan binding affinity was reduced >3-fold by substitution with all tested amino acids. The most pronounced impairments were observed after substitution with alanine (70-fold), serine (72-fold), or glutamine (74-fold). Losartan binding was much less affected by substitution with threonine (4.5-fold) or valine (3.6-fold). Alanine substitution had the largest inhibitory effect on the binding of nonpeptide ligands, similar to the results obtained with [Sar^1,Ile^8]Ang II. The structural requirements for receptor binding of the nonpeptide agonist L-163,491 (Kaye *et al.,* 1995) differed from those for losartan in several respects; the conserved substitution with glutamine in the N295Q receptor did not affect agonist binding, in contrast to its major effect on losartan binding. The binding of L-163,491 was substantially reduced by substitution with serine (9.2-fold reduction) and was less affected by replacement with threonine, aspartic acid, lysine, leucine, or valine (2–4-fold) (Table 1).

Saturation binding studies. The affinities of the alanine mutant AT_{1a} receptors for the physiological agonist Ang II and the peptide antagonist [Sar^1,Ile^8]Ang II were also evaluated in saturation binding experiments with ^{125}I-labeled radioligands (Table 2). Under these conditions, Scatchard analysis of ^{125}I-Ang II binding to the wild-type receptor detected only the high-affinity agonist binding site, with a K_d of 0.12 nM. For this reason, the number of Ang II binding sites measured in COS-7 cells expressing the wild-type receptor is approximately 16% of the sites calculated from binding analysis with [Sar^1,Ile^8]Ang II, which binds with a single affinity (K_d = 0.23 nM) to the entire AT_1 receptor population (Table 2).

In accordance with the findings of the binding-inhibition studies, the F293A mutation had no major effect on Ang II binding, and the N295A mutation decreased the binding affinities for the two ligands to similar extents (7.5-fold). The affinity of the N294A receptor for Ang II showed a moderate decrease (3.2-fold), whereas its affinity for [Sar^1,Ile^8]Ang II was similar to that of the wild-type receptor. It is interesting to note that the reduction in the binding affinity of the N294A mutant AT_{1a} receptor for Ang II was similar to the above-described impairment of its affinity for nonpeptide agonists.

Inositol phosphate responses and G protein coupling of alanine mutant AT_{1a} receptors. The signal transduction properties of the alanine mutant receptors were

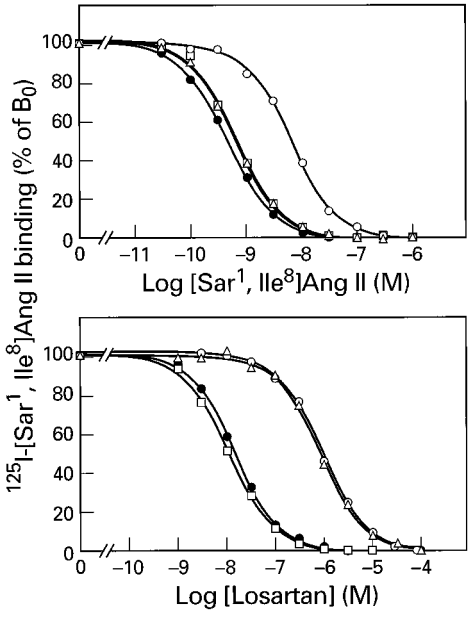

Fig. 2. Inhibition of ^{125}I-[Sar^1,Ile^8]Ang II binding by [Sar^1,Ile^8]Ang II (*upper*) or losartan (*lower*). The binding of ^{125}I-[Sar^1,Ile^8]Ang II to the membranes of COS-7 cells expressing wild-type (●) or F293A (□), N294A (△), or N295A (○) mutant AT_{1a} receptors was measured in the presence or absence of the respective antagonists. All values are expressed as percentages of the B_0 values determined in the absence of unlabeled ligand. Data are shown as means of duplicate determinations from a representative example of three to five experiments with similar results. K_d values are shown in Table 1.

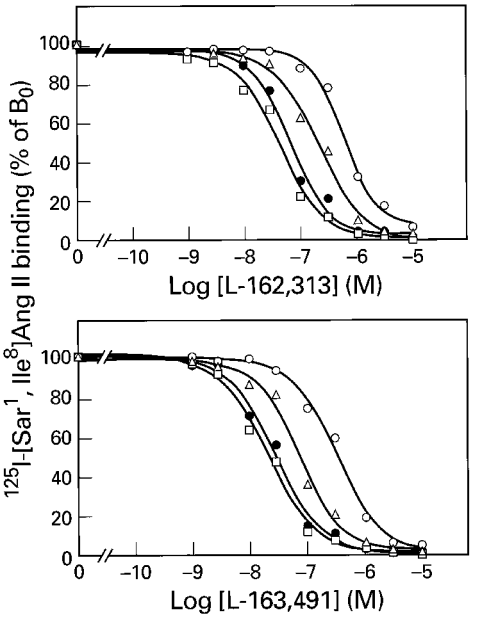

Fig. 3. Inhibition of ^{125}I-[Sar^1,Ile^8]Ang II binding by the nonpeptide agonist L-162,313 (*upper*) or L-163,491 (*lower*). The binding of ^{125}I-[Sar^1,Ile^8]Ang II to the membranes of wild-type (●) or F293A (□), N294A (△), or N295A (○) mutant AT_{1a} receptor-transfected COS-7 cells was measured in the presence or absence of the respective nonpeptide agonists. All values are expressed as percentages of the B_0 values measured in the absence of unlabeled ligand. Data are shown as means of duplicate determinations from a typical experiment representing four experiments with similar results. K_d values are shown in Table 1.

studied by measuring the inositol phosphate ($InsP_2$ plus $InsP_3$) responses of the expressed mutant receptors in [^{3}H]inositol-prelabeled COS-7 cells in the presence of lithium. The data were normalized to the measured receptor levels, because under these conditions the combined $InsP_2$ plus $InsP_3$ response is directly proportional to the receptor number (Hunyady *et al.,* 1995). Representative curves for the inositol phosphate responses of alanine mutant AT_{1a} receptors are shown in Fig. 4. The amplitudes of the inositol phosphate responses for the F293A, N294A, and N295A mutant receptors were 107 ± 27%, 16.3 ± 0.7% ($p < 0.01$), and 73.0 ± 11.3%, respectively, compared with those of the wild-type AT_{1a} receptor (three experiments). The EC_{50} values for the wild-type and F293A, N294A, and N295A mutant AT_{1a} receptors for inositol phosphate responses were 0.91 ± 0.36 nM, 1.1 ± 0.3 nM, 7.3 ± 1.6 nM ($p < 0.01$), and 5.0 ± 1.1 nM ($p < 0.05$), respectively (three experiments). These data demonstrate that the reduced efficacy of the N294A mutant receptor for inositol phosphate signal generation was accompanied by decreased potency of Ang II to activate this receptor. The capacity of the F293A mutant receptor for inositol phosphate generation was similar to that of the wild-type receptor, whereas the decreased potency of the N295A mutant receptor is in accordance with its reduced affinity for Ang II. The maximal inositol phosphate response of the N295A mutant receptor did not differ significantly from that of the wild-type receptor. Thus, although the affinity of this mutant receptor was reduced, the peptide agonist could induce receptor activation when present at sufficiently high concentrations.

The G protein coupling of the N294A receptor was also investigated, by measuring the effect of GTPγS on the binding of the physiological agonist Ang II. As reported earlier (Hunyady *et al.,* 1994), GTPγS substantially reduced the binding of ^{125}I-Ang II to the wild-type AT_{1a} receptor in membranes of transiently transfected COS-7 cells (Fig. 5). However, the inhibitory effect of GTPγS on Ang II binding was significantly decreased with the N294A mutant AT_{1a} receptor (four experiments) ($p < 0.001$), in accordance with its impaired coupling to inositol phosphate generation.

Discussion

Alanine substitution of Asn294 affects agonist binding and signal transduction. In this study, mutational analysis has defined the functional properties of three adjacent amino acids located in the seventh transmembrane helix of the AT_1 receptor. According to predictions based on modeling studies of GPCRs, the two consecutive polar residues (Asn294 and Asn295) are likely to face the interior of the AT_1 angiotensin receptor, whereas Phe293 is expected to face the lipid environment of the plasma membrane (Baldwin, 1993; Donnelly *et al.,* 1994). In accordance with these proposals, we found that alanine replacement of Phe293 had no major effect on angiotensin receptor function, whereas similar mutations of Asn294 and Asn295 significantly affected the binding and signaling functions of the receptor.

The N294A receptor exhibited markedly reduced binding affinity for losartan and decreased affinities for peptide and nonpeptide agonists, but its affinity for [Sar^1,Ile^8]Ang II was completely normal. These data differ from those of earlier reports, in which mutations of several extracellular residues and alanine replacement of Asn294 did not affect the binding

TABLE 1

Binding affinities of wild-type and mutant AT_{1a} receptors for antagonist and nonpeptide agonist ligands

Data are means ± standard errors of K_d values derived from inhibition of ^{125}I-[Sar^1,Ile^8]Ang II binding to AT_1 receptors by each ligand, determined as described in Materials and Methods. The numbers of independent experiments, each performed in duplicate, are shown in parentheses.

Receptor	K_d			
	[Sar1,Ile8]Ang II	Losartan	L-163,491	L-162,313
	nM			
Wild-type	0.24 ± 0.03 (7)	12.5 ± 1.3 (9)	13.0 ± 0.9 (8)	23.5 ± 3.6 (4)
F293A	0.48 ± 0.04[a] (3)	7.81 ± 1.02[a] (4)	12.5 ± 1.1[a] (4)	21.3 ± 2.7[a] (4)
N294A	0.43 ± 0.02 (5)	523 ± 33 (4)	35.3 ± 2.6 (4)	89.2 ± 10.5 (4)
N295A	5.10 ± 0.38 (5)	870 ± 24 (4)	299 ± 37 (4)	568 ± 19 (4)
N295S[b]	1.50 ± 0.06 (4)	898 ± 18 (3)	119 ± 5 (4)	
N295T	1.41 ± 0.14 (4)	56.2 ± 4.4 (4)	30.8 ± 2.2 (4)	
N295D	0.30 ± 0.05[a] (3)	208 ± 15 (3)	48.9 ± 7.0 (3)	
N295K	0.88 ± 0.04 (4)	363 ± 42 (4)	39.8 ± 4.7 (4)	
N295Q	0.49 ± 0.04 (4)	929 ± 27 (4)	11.0 ± 0.8[a] (4)	
N295L	0.58 ± 0.05 (4)	277 ± 13 (3)	25.6 ± 1.7 (4)	
N295V	1.57 ± 0.10 (4)	44.7 ± 2.9 (4)	34.6 ± 2.9 (4)	

[a] Not significant. All other differences from the K_d values of the wild-type receptor are significant at the $p < 0.01$ level.
[b] The N295S mutant receptor did not contain the FLAG epitope.

TABLE 2

Binding parameters for wild-type and mutant AT_{1a} receptors with ^{125}I-Ang II and ^{125}I-[Sar^1,Ile^8]Ang II

Saturation binding curves were determined using membranes from COS-7 cells transfected with wild-type or F293A, N294A, or N295A mutant AT_{1a} receptors, as described in Materials and Methods. K_d values, calculated using a one-site model, are shown as means ± standard errors. The numbers of experiments are shown in parentheses. Each determination was performed in duplicate.

Receptor	^{125}I-Ang II		^{125}I-[Sar1,Ile8]Ang II	
	K_d	Sites	K_d	Sites
	nM	*pmol/mg*	*nM*	*pmol/mg*
Wild-type	0.12 ± 0.01	0.68 ± 0.11 (8)	0.23 ± 0.02	3.99 ± 0.53 (7)
F293A	0.15 ± 0.01	0.67 ± 0.03 (6)	0.30 ± 0.04	4.29 ± 1.62 (7)
N294A	0.34 ± 0.06	0.33 ± 0.07 (5)	0.27 ± 0.03	2.80 ± 0.52 (9)
N295A	0.91 ± 0.11	0.59 ± 0.04 (7)	2.02 ± 0.24	3.27 ± 0.80 (8)

affinities of the human AT_1 receptor for Ang II and two nonpeptide agonists, L-162,313 and L-162,782 (Perlman *et al.,* 1995, 1997). The most prominent feature of the N294A receptor was its markedly attenuated capacity to engender inositol phosphate responses, even in the presence of high concentrations of Ang II. These data suggest that Asn294 is an important determinant of agonist-induced receptor activation and signal transduction. The moderately impaired agonist binding of the N294A receptor may reflect its inability to achieve the optimal conformation required for agonist-induced activation (Karnik *et al.,* 1997). This conclusion is supported by the finding that the effect of GTPγS on Ang II binding was reduced with the N294A mutant AT_{1a} receptor.

Replacements of Asn295 affect ligand binding of the AT_{1a} receptor without interfering with its activation. Alanine replacement of Asn295 caused a substantial decrease in receptor binding affinity for all AT_1 receptor ligands tested, without significant impairment of the maximal inositol phosphate response to Ang II stimulation. The role of Asn295 in the binding function of the AT_{1a} receptor was evaluated with a series of mutant AT_{1a} receptors bearing other replacements of this residue. In general, substitution with amino acids with polar, apolar, or charged side chains had less effect on the binding affinities for the tested ligands than did alanine substitution. In the case of [Sar^1,Ile^8]Ang II, substitution with amino acids with smaller side chains (e.g., serine and threonine) caused more marked impairment of binding than did substitution with residues with larger side chains (e.g., aspartic acid, glutamine, leucine, or lysine). Among the substituents with apolar side chains, leucine, with the largest side chain, caused the least impairment of [Sar^1,Ile^8]Ang II binding. Conversely, substitution with alanine, which has the smallest side chain, caused the most marked reduction of binding affinity. There was no clear requirement regarding the nature of the amino acid substitution in position 295. Substitution with polar (glutamine), charged (aspartic acid or lysine), or apolar (leucine) residues caused moderate impairment of binding. These data suggest that the Asn295 residue has a structural effect on [Sar^1,Ile^8]Ang II binding, and they argue against a direct contact between the bound ligand and this residue.

Losartan binding to the AT_{1a} receptor was reduced by all tested substitutions of the Asn295 residue. These findings are in accordance with previous studies on the affinity of the N295S mutant AT_1 receptor for losartan (Schambye *et al.,* 1994a,b; Balmforth *et al.,* 1997). The unique requirement for asparagine in position 295 for normal losartan binding may reflect a direct interaction between this ligand and Asn295 of the receptor. The intramembrane location of Asn295 and the effects of its replacement on the binding of peptide and nonpeptide antagonists suggest that this residue is essential for the integrity of the intramembrane binding pocket of the AT_1 receptor.

Another interesting feature of the N295A mutation was its deleterious effect on the binding of the nonpeptide agonists L-162,313 and L-163,491. This finding differs from those of previous studies, in which replacement of Asn295 by aspartic acid had no effect on Ang II and nonpeptide agonist binding (Perlman *et al.,* 1995, 1997). Although aspartic acid was found to substitute for Asn295 without detectable loss of binding affinity for L-162,313, the efficacy with which

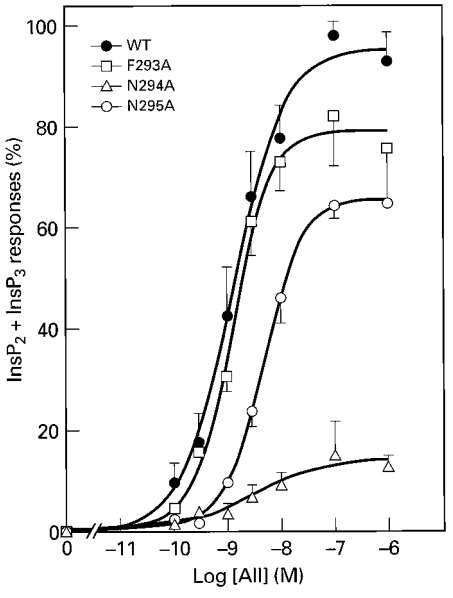

Fig. 4. Inositol phosphate dose-response curves for AT_{1a} receptors. The combined accumulation of radioactivity in the $InsP_2$ plus $InsP_3$ fraction of wild-type (●) or F293A (□), N294A (△), or N295A (○) mutant AT_{1a} receptor-transfected COS-7 cells was measured after [^{3}H]inositol prelabeling, as described in Materials and Methods. Data were normalized to the number of expressed extracellular binding sites for each mutant, and stimulation is expressed as a percentage of the response mediated by the wild-type receptor with 1 μM Ang II (*AII*). Values are shown as means and ranges of duplicates from one of three similar experiments. In these experiments, basal versus stimulated (1 μM Ang II) values for $InsP_2$ plus $InsP_3$ labeling for wild-type, F293A, N294A, and N295A AT_{1a} receptors were 932 ± 72 versus 8127 ± 1236, 935 ± 84 versus 7386 ± 1196, 1081 ± 21 versus 932 ± 117, and 804 ± 88 versus 7551 ± 2414 cpm, respectively (three experiments).

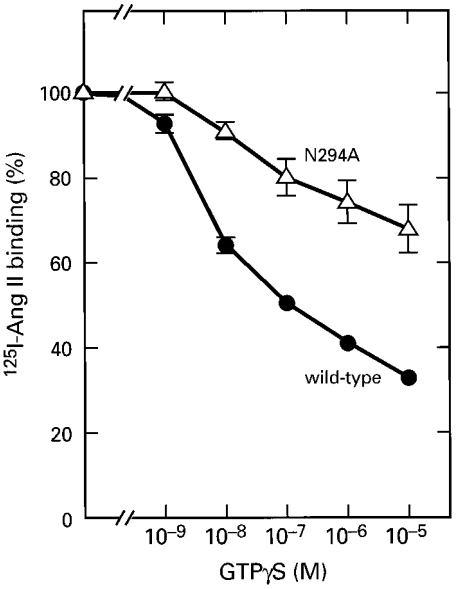

Fig. 5. Effects of GTPγS on the ^{125}I-Ang II binding of wild-type (●) and N294A (△) AT_{1a} receptors. Membranes were prepared from COS-7 cells transfected with wild-type or N294A AT_{1a} receptors, and the binding of ^{125}I-Ang II was measured in the presence or absence of the indicated concentrations of GTPγS, as described in Materials and Methods. Data are expressed as a percentage of the binding measured in the absence of GTPγS and are shown as means ± standard errors of values obtained in four independent experiments, each performed in duplicate. Errors not shown are within the data symbols. The reduced effect of GTPγS on agonist binding to the N294A mutant receptor was significant at the $p <$ 0.01 level.

L-162,313 and other nonpeptide agonists stimulated inositol phosphate responses was greatly reduced with the N295D mutant (Perlman *et al.,* 1997). In our study, the affinity of the N295D mutant receptor for the AT_1-selective nonpeptide agonist L-163,491 was moderately impaired. All tested substitutions of Asn295 caused less impairment of L-163,491 binding, compared with that of losartan. In contrast to its marked effect on losartan binding, the conserved substitution of glutamine for Asn295 had no effect on the binding affinity for L-163,491. These data suggest that the structural requirements in position 295 for the binding of L-163,491 differ from those for losartan. Although these data do not provide evidence for a role of Asn295 in nonpeptide agonist binding, the marked inhibition of binding to the N295A and N295S mutant receptors suggests that the intact intramembrane binding pocket is an important requirement for the binding of nonpeptide agonists.

Our data also showed that alanine substitution of Asn295 reduced the high-affinity binding of Ang II to the receptor. In accordance with this finding, the potency with which Ang II stimulated inositol phosphate responses in cells expressing the N295A receptor was also reduced. Although earlier studies suggested that mutation of Asn295 to serine causes a relatively minor change in the Ang II binding affinity of the receptor (Schambye *et al.,* 1994a,b; Balmforth *et al.,* 1997), the present data, in accordance with previous studies on the rat AT_{1b} receptor (Ji *et al.,* 1995), demonstrate that the N295A mutation also affects the binding of peptide ligands.

In a recent report, the finding that mutation of Asn295 to serine caused constitutive activation of the AT_1 receptor led to the proposal that an interaction between Asn295 and Asn111 (in the third intramembrane helix) stabilizes the inactive conformation of the AT_1 receptor (Balmforth *et al.,* 1997). However, constitutive activation was not observed when Asn295 was replaced by aspartic acid (Perlman *et al.,* 1997), and it was not detectable with the N295A mutant AT_{1a} receptor in this study, despite its reasonably high levels of expression.

Mutations in the seventh transmembrane helix affect the binding of nonpeptide agonists. Initial studies on agonist binding to the AT_1 receptor demonstrated that several extracellular residues are essential determinants of Ang II binding (Hjorth *et al.,* 1994). Based on these data, it has been proposed that the peptide binding site of the AT_1 receptor is located in the extracellular region (Hjorth *et al.,* 1994). However, more recent studies have emphasized the role of the intramembrane helices in the agonist-induced conformational change (Noda *et al.,* 1995, 1996; Hunyady *et al.,* 1996; Inoue *et al.,* 1997; Karnik *et al.,* 1997). His256, in the sixth transmembrane helix, has been reported to interact with the side chain of the carboxyl terminal Phe8 of Ang II (Noda *et al.,* 1995), and Tyr111 of the AT_1 receptor has been proposed to interact with the side chain of Tyr4 of Ang II (Noda *et al.,* 1996). These data, as well as recent modeling studies (Underwood *et al.,* 1995; Inoue *et al.,* 1997), point to the importance of the intramembrane binding pocket in the agonist-induced conformational change of the AT_1 receptor.

Asn294 has a role in the agonist-induced conformational change. The seventh transmembrane helix has been implicated in several recent studies as the site of initiation of agonist-induced conformational changes in a variety of GPCRs. The retinal chromophore of rhodopsin binds covalently to the seventh transmembrane helix, which has also been implicated in agonist binding to several different GPCRs (Probst *et al.,* 1992; Baldwin, 1993; Strader *et al.,* 1995; Hunyady *et al.,* 1996). More recently, conserved aromatic residues in the seventh transmembrane domain of the same receptor were found to be essential for agonist binding and stimulation of inositol phosphate production (Roth *et al.*, 1997). Rotation of transmembrane helices, which results in a new hydrogen bond between Asp74 of the second transmembrane helix and Tyr292 of the seventh transmembrane helix, has also been proposed as the initial event of AT_1 receptor activation (Inoue *et al.,* 1997).

The polar nature of the amino acid located in the position of Asn294 is a conserved feature of most GPCRs (Probst *et al.,* 1992; Baldwin, 1993). In a published alignment of GPCR sequences, >60% of the listed sequences contain asparagine in the position corresponding to Asn294 of the AT_1 receptor, and >90% of the GPCRs have a polar amino acid that can participate in hydrogen bond formation (e.g., serine, threonine, histidine, or arginine) (Probst *et al.,* 1992). In view of the location of Asn294 in the seventh intramembrane helix (Baldwin, 1993; Liu *et al.,* 1995; Mizobe *et al.,* 1996), this residue is unlikely to directly mediate receptor coupling to G_q or other G proteins. It is more probable that this residue is required for an intramolecular interaction that in turn stabilizes the active conformation of the receptor. In recent reports, an interaction between Asn111 and Tyr292 (Groblewski *et al.,* 1997) and/or Asn295 (Balmforth *et al.,* 1997) was proposed to be a major determinant of the inactive conformation of the AT_1 receptor. It is possible that an interaction between Asn294 and other amino acids is an additional structural requirement for AT_1 receptor activation, similar to the proposed role for the interaction between Tyr292 of the seventh transmembrane helix and Asp74 of the second transmembrane helix (Bihoreau *et al.,* 1993; Marie *et al.,* 1994; Joseph *et al.,* 1995).

In summary, the present data provide evidence that the binding of nonpeptide agonists to the AT_1 receptor depends on residues located in the intramembrane binding pocket. In addition, Asn294 has a crucial role in receptor activation, and Asn295 is a major determinant of the binding of all tested Ang II ligands to the AT_1 angiotensin receptor. The complementary functions of these adjacent asparagine residues could provide a molecular basis for the proposed role of the seventh transmembrane helix in transforming agonist binding into the rearrangement of intramembrane helices that is believed to result in receptor activation.

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Send reprint requests to: Kevin J. Catt M.D., Ph.D., Endocrinology and Reproduction Research Branch, NICHD, NIH, Building 49, Room 6A-36, Bethesda, MD 20892-4510. E-mail: catt@helix.nih.gov