Molecular Pharmacology Fast Forward. Published on May 18, 2005 as doi: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

MOL 11601

Single Mutations at Asn^{295} and Leu^{305} in the Cytoplasmic Half of TM7 of the AT_1 Receptor Induce Promiscuous Agonist Specificity for Angiotensin II Fragments – A PSEUDO-CONSTITUTIVE ACTIVITY

Ying-Hong Feng, Lingyin Zhou, Rongde Qiu, and Robin Zeng

Dept. of Pharmacology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814, Y.H., and R.D.; Department of Medicine, CWRU School of Medicine, Cleveland, Ohio 44106, L.Y. and R.

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

MOL 11601

Running Title: Promiscuous Activation of AT_1 mutants

Corresponding author: Ying-Hong Feng, M.D., Ph.D.
Department of Pharmacology, C2021
Uniformed Services University of the Health Sciences
4301 Jones Bridge Road
Bethesda, MD 20814
Phone: (301) 295-3232
FAX: (301) 295-3220
e-mail: yhfeng@usuhs.mil

Statistics:		
	Number of text pages:	32
	Tables:	1
	Figures:	7
	References:	36
	Abstract:	210 words
	Introduction:	583 words
	Discussion:	1498

Abbreviations: GPCR, G protein-coupled receptor; Ang I, angiotensin I (Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8-His^9-leu^{10}); Ang II, angiotensin II (Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8); Ang IV, angiotensin IV (Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8); Ang 1-7, angiotensin 1-7 (Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7); and Ang 5-8, angiotensin 5-8 (Ile^5-His^6-Pro^7-Phe^8); IP, inositol (1,4,5)triphosphate; TM, transmembrane α-helix domain.

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

The most striking feature of a G protein-coupled receptor (GPCR) is its highly exclusive agonist specificity. This feature guarantees that a GPCR recognizes only its specific native agonist(s). Herein we show that two point mutations of N295S and L305Q enabled the AT_1 receptors to recognize multiple Ang II fragments. Similar to the well-established constitutively active AT_1 mutant receptor N111G, the mutations of N295S and L305Q induced an increased production of basal IP in the absence of exogenous Ang II when expressed in HEK293 cells. Distinct from the N111G, however, is the fact that the increased basal activity disappeared in COS-7 cells due to lack of endogenous Ang II fragments produced by the cells – a pseudo-constitutive activity. Surprisingly, the Ang II analog, [Sar^1,Ile^4,Ile^8]Ang II, and the native angiotensin II fragments Ang 1-7, Ang IV, and Ang 5-8 that are inactive in activating the wild-type receptor activated N295S and L305Q. Results generated by lowering the Na^+ concentration suggest that the mutant N295S and L305Q may be trapped in neutral conformational states ($\mathbf{R}^{\mathbf{N}}$). These data have identified for the first time a novel pattern of GPCR mutations with a broad spectrum of agonist specificity, suggesting possible existence of functional GPCRs in nature that are activated through conformational “selection” rather than “induction” mechanisms.

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

The highly exclusive agonist specificity of a GPCR guarantees that the receptor only recognizes its specific native agonist(s) for binding and activation. This is an essential property for a GPCR to convey discrete extracellular stimulus to intracellular signals. The N-terminus, three extracellular loops, and the extracellular halves of the seven TM core of a GPCR are often involved in direct interaction with the native agonist, particularly, for peptide agonists of less than 40 amino acids (Ji et al., 1998; Karnik et al. 2003). Mutations in these regions often alter the binding affinity and potency of the agonist, but rarely change its agonist specificity. However, mutations in a GPCR that induce its constitutive activation may result in altered agonist specificity, i.e. N111G mutation of the AT_1 receptor (Le et al., 2002). This N111G mutation-induced constitutive activation, a transitional intermediate partial active state (**R′**), is mimicry of native agonist Ang II-induced activation. Most receptors with this mode of constitutive activity also show increased binding affinity for native agonists (Noda et al., 1996; Feng et al., 1998). This mimicry property could be essential to the induction of the altered agonist specificity.

AT_1 receptors are GPCRs that mediate the actions of Ang II, an octapeptide hormone that regulates blood pressure, homeostasis, and cell growth (Feng and Douglas, 2000). Stimulation of AT_1 receptors leads to $G\alpha_{q/11}$-mediated activation of phospholipase $C_{\beta 1}$, generating diacylglycerol and inositol (1,4,5)triphosphate. Although naturally occurring constitutively active AT_1 mutants are not known, engineered mutations of Asn^{111} to Gly^{111}, Ala^{111}, and Ser^{111} have displayed constitutive activities through a mechanism that mimics the Ang II-induced activation (Noda et al., 1996; Groblewski e al., 1997; Balmforth et al., 1997; Feng et al., 1998). Other engineered mutations of N295S and L305Q were also reported to be constitutively active in HEK293 cells (Balmforth et al., 1997; Parnot et al., 2000). However, the constitutive activities

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

of N295S and L305Q observed in HEK293 cells surprisingly disappeared in COS-1 and COS-7 cells (Feng et al., 1998; Hunyady et al., 1998). Apparently, cell type does not explain the complete loss of constitutive activities because the constitutive activity of N111G did not disappear in COS cells. This suggests that N295S and L305Q may differ from N111G in constitutive activity due to unidentified intrinsic properties. If so, would N295S and L305Q also differ from N111G in agonist specificity?

Ang II consists of eight residues (Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8). The residues Tyr^4 and Phe^8 are considered agonist switches because they are essential for agonism of the wild-type AT_1 receptors (Noda et al., 1996; Feng et al., 1998; Miura et al., 1999). The other residues of Ang II are necessary for stabilization of AT_1 receptor conformation. Thus, [Sar^1,Ile^4,Ile^8]Ang II, an analog of the potent full agonist [Sar^1]Ang II, is incapable of activation of wild-type AT_1 receptors whereas [Sar^1,Ile^4]Ang II and [Sar^1,Ile^8]Ang II are partial agonists (Noda et al., 1996; Feng et al., 1998; Miura et al., 1999).

In this study, various measures, i.e. Ang II analogs, and low Na^+ concentrations were utilized to further investigate the reason why the increased basal activities of N295S and L305Q in HEK293 cells disappear in COS cells and to examine whether these mutations alter agonist specificity for Ang II and its native fragments. This work shows that the increased basal activity of N295S and L305Q in HEK293 cells was due to the presence of endogenous Ang II fragments produced by the cells – a pseudo-constitutive activity. Surprisingly, these mutations induced promiscuous activation of the mutant AT_1 receptors by Ang II and its native fragments.

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Materials. Oligonucleotides were synthesized by Sigma-Genosys (Woodlands, TX) or MWG Biotech (High Point, NC). Ang II, Ang I, Ang 1-7, Ang IV, Ang 5-8, and [Sar^1]Ang II were purchased from Bachem (King of Prussia, PA). Other peptide analogues of [Sar^1]Ang II were synthesized by GeneMed Synthesis (South San Francisco, CA). Losartan, EXP3174, and Candesartan were gifts from DuPont Merck Co. (Wilmington, DE). ^{125}I-[Sar^1,Ile^8]Ang II (2200 Ci/mmol) was purchased from The Peptide Radioiodination Center of Washington State University (Pulman, WA). [^{3}H]myo-inositol (22 mCi/ml) was purchased from Amersham (Piscataway, NJ). The monoclonal antibody 1D4 was purchased from the Cell Culture Center (Endotronics Inc.). The enzyme immunoassay kit for angiotensin peptides was purchased from SPI-BIO (www.spibio.com, France).

Mutagenesis and expression of the AT_1 receptors. The synthetic rat AT_1 receptor gene with a consensus Kozak sequence at the N-terminus and an 1D4 epitope tag (TETSQVAPA) at the C-terminus, cloned in the shuttle expression vector pMT-3, was used for expression and mutagenesis, as described earlier (Noda et al., 1996; Feng et al., 1998; Miura et al., 1999). AT_1 mutants were prepared with the restriction fragment-replacement method and the polymerase chain reaction method as previously described (Noda et al., 1996; Feng et al., 1998; Miura et al., 1999). The DNA sequence analysis was performed by Cleveland Genomics Inc. to confirm the mutations. To express the AT_1 receptor protein, 3 µg of column (Qiagen) purified plasmid DNA per 10^7 cells was used in transfection. COS-7 and HEK293 cells (ATCC) were transfected with the GenePORTER^{TM} transfection reagents (GTS Inc). Transfected cells cultured for 48 hours were harvested and Mg^{2+}-free cell membranes were prepared by the nitrogen PARR bomb

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

disruption method (Feng et al., 1998). Briefly, after centrifugation of the cell membrane homogenate at 30,000 g (14,400 rpm with a SA-600 rotor) for 15 min, the pellet was re-suspended in 5 ml of HE buffer *(50 mM HEPES, pH 7.4 and 1.5 mM EGTA, containing protease inhibitors)* and re-centrifuged at 30,000 g for 15 min. Finally, the pellet (crude membrane fraction) was re-suspended in 1.0 ml of HE containing 10% glycerol (per 10^8 cells). The membrane fraction was aliquoted into 1.5 ml Eppendorf tubes and stored at –70^{o}C. The receptor expression was assessed in each case by immunoblot analysis (not shown) and by ^{125}I-[Sar^1,Ile^8]Ang II saturation binding analysis.

Ligand Binding Study. ^{125}I-[Sar^1,Ile^8]Ang II binding experiments were carried out under equilibrium conditions as described earlier (Noda et al., 1996; Feng et al., 1998; Miura et al., 1999). For competition binding studies, membranes expressing the wild-type (WT) or mutant receptors were incubated for 1 hour with 0.3 nM ^{125}I-[Sar^1,Ile^8]Ang II and various concentrations of the ligands in assay buffer. The K_d value of ^{125}I-[Sar^1,Ile^8]Ang II was determined by competition binding study in the presence of various concentrations (0.03 to 3 nM) of ^{127}I-[Sar^1,Ile^8]Ang II. For the saturation binding study, increasing concentrations of ^{125}I-[Sar^1,Ile^8]Ang II that was 10-fold higher than the K_d value of the receptor were used to obtain >90% bound form of the receptor. Non-specific binding (which was <5% of total binding in our experiments) of the 0.3 nM ^{125}I-[Sar^1,Ile^8]Ang II radioligand to the membrane was measured in the presence of 50 µM ^{127}I-[Sar^1,Ile^8]Ang II. All binding experiments were carried out at 22^{o}C for 1 hour in a 250 µl volume unless otherwise indicated. After equilibrium was reached, the binding reactions were stopped by filtering the binding mixture under vacuum (Brandel Type M-24R) through FP-200 GF/C glass fiber filters (Whatman Inc.). The filters were extensively

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

washed further with ice-cold binding buffer to wash away 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®. The K_d values represent the mean ± S.E. of three to five independent determinations.

Production of Total Inositol (1,4,5)Phosphates (IP). COS-7 and HEK293 cells were cultured in DMEM containing 10% bovine calf serum. The medium for HEK293 cells contains 1% non-essential amino acids. Twenty-four hours after transfection, COS-7 and HEK293 Cells cultured in 60 mm Petri dishes were labeled for 24 hours with [^{3}H]*myo*-inositol (1μCi/ml) at 37^{o}C in inositol-free DMEM containing 10% bovine calf serum. On the day of IP assay (i.e. 48 hours after transfection), the labeled cells were washed three times with serum-free medium and incubated with their specific media containing 10 mM LiCl for 20 min. Then medium alone or ligands were added to the cells. After incubation for 45 min at 37^{o}C, the medium was removed, and total soluble IP was extracted from the cells by the perchloric acid extraction method as described previously (Noda et al., 1996; Feng et al., 1998; Miura et al., 1999). The amount of [^{3}H]-IP eluted from the column was counted and a concentration-response curve was generated using iterative nonlinear regression analysis (Noda et al., 1996; Feng et al., 1998; Miura et al., 1999). To determine the total IP under low Na^+ conditions, cells were incubated prior to the experiment for 15 min in incubation buffer (138 mM NaCl, 5 mM KCl, 10 mM LiCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Na^+-HEPES, pH 7.2) or in incubation buffer where 138 mM Na^+ had been substituted by 138 mM *N*-methyl-D-glucamine (Sigma). This treatment has been shown to greatly decrease the intracellular Na^+ content due to the presence of Na^+/K^+-ATPases in all

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

nucleated mammalian cells (Glossmann et al., 1974; Tsai and Lefkowitz, 1978; Quitterer et al., 1996; Ceresa and Limbird, 1994).

Measurement of AT_1 receptor Internalization. Internalization of the AT_1 receptors was measured by a method described earlier (Thomas et al., 2000). Briefly, cells transiently transfected with AT_1 receptors in 12-well plates were stimulated with and without Ang II (100 nM) or [Sar^1,Ile^4,Ile^8]Ang II (1µM for N111G and 30 µM for other receptors) for 10 minutes at 37^{o}C. Surface-bound ligands were removed by gentle acid wash (50 mM sodium citrate, 0.2 mM sodium phosphate, 90 mM NaCl, 0.1% bovine serum albumin, pH 5.0; 10 minutes at 4^{o}C), which does not affect subsequent receptor binding. Then a radio-ligand binding assay was performed (5 hours at 4^{o}C) to measure receptors remaining at the cell surface. Internalized receptors were expressed as a percentage loss of cell surface binding compared with cells not exposed to Ang II or [Sar^1,Ile^4,Ile^8]Ang II.

RT-PCR of angiotensinogen. Total RNA from HEK293 and COS-7 cells were prepared with an RNAeasy kit (Invitrogen) and used for routine reverse transcriptase and polymerase chain reaction (RT-PCR) with an RT-PCR kit (Life Technologies). The primer pair used for amplification of angiotensinogen transcripts was forward primer (gctgatccagcctcactatg) and reverse primer (gctgttgggtagactctgtg). Sequence analysis indicated that these primers should recognize the transcripts of humans (HEK293 cells) and monkeys (COS-7 cells). The expected sizes of PCR products are 308 bp for angiotensinogen.

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

Enzyme immunoassay of Ang II and its fragments. All cells at about 90% confluence in 60 mm dishes were washed carefully three times and then cultured with 2 ml serum-free media containing cocktail protease inhibitors in a cell culture incubator. Six hours after culture, the 2 ml media were collected and microcentrifuged at full speed to remove cell debris. The levels of endogenous angiotensin peptides in the media were measured with the enzyme immunoassay kit (SPI-BIO, France) following the company's protocol. The antibody of this kit cross-reacts with Ang I, Ang II, Ang III, and Ang IV, but doesn't cross-react with Ang 1-7 and other non-angiotensin peptides. Thus, it measures a mixed level of Ang II and its fragments.

Statistical Analysis. The results are expressed as the mean ± S.E.M. of three to five independent determinations. The significance of measured values was evaluated with an unpaired Student's *t*-test.

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Binding profiles of Ang II receptors in various cell types: Membrane proteins were prepared from COS-7 cells expressing AT_1 receptors. All mutant receptors bind Ang II, [Sar^1]Ang II, [$Sar^1Ile^4Ile^8$]Ang II and AT_1-specific inverse agonist EXP3174 (Fig 1). Mutant N295S and L305Q show significantly increased binding affinities for Ang II (~4-fold) and [$Sar^1Ile^4Ile^8$]Ang II (~3-fold) and significantly decreased binding affinities for non-peptide inverse agonist EXP3174 (24-fold) as compared to the wild-type AT_1 receptor. The patterns of increase or decrease of binding affinities are similar to constitutively active mutant N111G although the magnitude varies (Fig 1). The binding affinities of N295S and L305Q for [Sar^1]Ang II are not significantly increased as compared to the wild-type AT_1 receptor.

When membrane proteins prepared from transfected HEK293 cells were used in ligand binding assays, similar patterns and magnitudes of binding affinities for these receptors as shown in figure 1 were found. No significant difference in binding affinities between COS-7 cells and HEK293 cells was observed in our binding experiments (data not shown).

The elusive constitutive activities of N295S and L305Q: To compare the basal activities of the receptors in HEK293 cells and COS-7 cells, we optimized the transfection protocol by varying the amount of plasmid DNAs. With the amount of DNA shown in Table 1, comparable expression levels (similar B_{max} values for all receptors in the two type of cells) of the receptors on the cell surface were achieved and used for the IP production assay. Table 1 also shows the absolute amount of IP production. The maximal IP productions induced by 5 μM [Sar^1]Ang II demonstrate no significant differences for N295S and L305Q as compared to the

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

wild-type AT_1 and N111G. For basal IP productions, no significant differences were observed for N295S and L305Q as compared to the wild type, except in HEK293 cells.

The basal IP activities of all four receptors were significantly higher in HEK293 cells than in the COS-7 cells. When basal IP productions were expressed as percentage of maximal activities of the wild-type AT_1 receptors in the presence of 5 μM [Sar^1]Ang II for a specific cell type, the constitutive activities of the wild-type, N111G, N295S, and L305Q in HEK293 cells were 3%, 48.7%, 18.4%, and 6%, respectively. These constitutive activities are 16.3, 6.1, and 2-fold greater than the wild-type receptor. Surprisingly, the 18.4% and 6% basal IP produced by N295S and L305Q disappeared in COS-7 cells. Their basal IP productions in the COS-7 cells fell into the range of the wild type and mock (Table 1). However, the basal IP of N111G remained significantly higher than the wild type (around 31.2% in COS-7 cells). The N111G also induced significantly higher production of basal IP in HEK293 cells than in the COS-7 cells (48.7% vs 31.2%). Similar to N111G, wild-type AT_1 also induced higher basal IP in HEK293 cells than in the COS-7 cells (Table 1).

Increased IP activities of N295S and L305Q in COS-7 cells in the presence of conditioned media of HEK293 cells: Because HEK293 cells also displayed higher basal IP than the other types of cells for the wild-type receptor (Table 1), we thought that HEK293 cells might produce trace amount of endogenous angiotensin II attributable to the increased basal IP activities of the wild-type, N111G, N295S, and L305Q receptors. To test this hypothesis, the COS-7 cells expressing N295S, L305Q, and wild-type receptors were stimulated with the conditioned media of HEK293 cells and the basal IP productions were measured for the receptors. Interestingly, the conditioned media induced 16.3%, 7.4%, and 4.5% basal IP

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

productions for N295S, L305Q, and the wild type. These IP productions were significantly greater when compared to the control media (Fig 2). These results suggest that HEK293 cells may produce native angiotensin peptides to activate the receptors.

Production of Angiotensin peptides by HEK293 cells: To examine whether HEK293 cells produce angiotensin peptides at the mRNA level, RT-PCR experiments were performed with primers designed for angiotensinogen. As expected, a specific band at 308 bp was amplified on RNAs isolated from HEK293 cells. RT-PCR failed to show any specific amplification on RNAs prepared from the COS-7 cells (inset of Fig 3).

To determine whether and to what degree the HEK293 cells produce angiotensin peptides at the peptide level, an enzyme immunoassay was employed to assay the angiotensin concentration in the culture media of HEK293 cells. Fig 3 shows that the conditioned media of HEK293 cells contained 53 pg/ml of angiotensin peptides whereas the conditioned media of COS-7 cells contained a negligible amount of angiotensin peptides (less than 3 pg/ml).

Promiscuous activation of N295S and L305Q by Ang II analog and endogenous Ang II fragments: As shown in Figure 4A, an inactive Ang II analog [Sar^1,Ile^4,Ile^8]Ang II that is completely incapable of activating the wild-type receptor activated mutant N295S and L305Q. Stimulation of N295S and L305Q using 50 μM of [Sar^1,Ile^4,Ile^8]Ang II produced maximal IP productions that were equivalent to the maximal activity of the wild-type receptor in the presence of 0.1 μM [Sar^1]Ang II. This observation was highly reproducible and consistent regardless of the cell types tested in the study. However, [Sar^1,Ile^4,Ile^8]Ang II at such a high concentration failed to activate the wild-type receptor. When the maximal IP production of the wild type in the

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

presence of 0.1 μM [Sar^1]Ang II was taken as a reference for 100%, [Sar^1,Ile^4,Ile^8]Ang II exerted an equivalent efficacy but reduced potency in inducing IP production by N295S and L305Q when compared to the N111G mutant. This was demonstrated by the shift of the curves to the right-hand side (Fig. 4A).

To examine whether native Ang II fragments also activate the mutants N295S and L305Q, we measured IP productions for these mutants in the presence of Ang IV, Ang 1-7, and Ang 5-8 peptide fragments that are completely incapable of activating wild-type AT_1 receptors. As shown in Figure 4B, peptides Ang IV, Ang 1-7, and Ang 5-8 became either full agonists (Ang IV) or partial agonists (Ang 1-7 and Ang 5-8) for these mutant receptors. Ang I, a partial agonist of wild-type AT_1, became a full agonist for all three mutants (data not shown). A peptide (HDIFPVYR) with randomized sequence of Ang II failed to activate any mutant at 5 μM or greater concentrations (data not shown). Activations of the mutant receptors by these Ang II fragments were independent of cell types.

Differential increases in basal activity of AT_1 receptors by lowering Na^+ concentration: Lowering the intracellular Na^+ concentration has been shown to increase the basal activity of the bradykinin B_2 receptor independently of agonist binding (Glossmann et al., 1974; Tsai and Lefkowitz, 1978; Quitterer et al., 1996). Na^+ has also been shown to alter Ang II binding affinity as well (Glossmann et al., 1974). To test whether mutant N295S and L305Q are more similar to the wild type or constitutively active N111G in the absence of any ligand, the COS-7 cells were cultured with low Na^+ condition (10 mM) as described in the method section. The absolute constitutive activities of N295S (42.6%) and L305Q (51.5%) at 10 mM Na^+ condition in COS-1 cells increased substantially to levels close to that of the N111G (48.8%)

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

(Fig 5A). The basal IP productions under 10 mM Na^+ condition increased 11-fold for N295S and L305Q, but only 2- and 1.6-fold for the wild type and N11G, respectively, as compared to that under the 148 mM normal Na^+ condition. The fold change in basal IP induced by N295S and L305Q were significantly greater (>5-fold) than the fold change by the wild type and N111G. Low Na^+ condition (10 mM) also increased efficacy of [Sar^1]Ang II in maximal IP for N111G (16.5%), N295S (16.3%), and L305Q (15.9%), but not for the wild-type receptor in COS-7 cells.

It was thought that interaction of Na^+ ions with a highly conserved aspartate in the second transmembrane domain of GPCR (Asn^{74} for AT_1 receptors) prevents the residue from contributing to spontaneous isomerization (Quitterer et al., 1996). Removal of the negatively charged carboxyl group of the Aspartate by mutagenesis has been shown to abolish the Na^+ effect on α_2-adrenergic receptors (Ceresa and Limbird, 1994). Although the corresponding mutant of the AT_1 receptor, D74G, failed to produce any activity at basal or upon activation by [Sar^1]Ang II in the presence of 148 mM Na^+, the inactive mutant D74G showed 8.73% basal IP production but no further activation upon [Sar^1]Ang II stimulation at low Na^+ condition (data not shown).

Inverse agonist and internalization of N295S and L305Q: Since inverse agonists block the constitutive activity of N111G (Noda et al., 1996; Feng et al., 1998), they may also inhibit the increased basal activities of mutants N295S and L305Q observed at low Na^+ condition. To test this hypothesis, EXP3174 and Candesartan, the specific inverse agonists of AT_1 receptor, were added to the cells at the same time when the low Na^+ condition was applied. Strikingly, EXP3174 and Candesartan inhibited the increment in basal IP production to about 90% and 85%

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

for N295S, and 68% and 70% for L305Q whereas the specific neutral antagonist Losartan failed to inhibit the basal activity as shown in Figure 5B.

It was reported that Ang II but not [Sar^1,Ile^4,Ile^8]Ang II induced internalization of the wild type and the constitutively active N111G (Thomas et al., 2000). To test whether N295S and L305Q are different from the wild type or N111G in internalization, Ang II and [Sar^1,Ile^4,Ile^8]Ang II were used. The result showed that Ang II induced a wild-type-like internalization profile for N295S and L305Q receptors in COS-7 cells. Similar to the wild type and N111G, the two mutant receptors also failed to respond to [Sar^1,Ile^4,Ile^8]Ang II in internalization. Thus, N295S and L305Q was not different from the wild type in internalization in COS-7 cells when stimulated with Ang II and [Sar^1,Ile^4,Ile^8]Ang II.

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

The aim of this study was to understand why the constitutive activity of N295S and L305Q, which appeared in HEK293 cells, disappears in COS-7 cells and to assess the mechanisms underlying AT_1 receptor activation. Changes in IP production under a variety of conditions were monitored in the mutant N295S and L305Q receptors and compared with the wild type (ground state, **R**) and a constitutively active mutant N111G receptor (an intermediate active state, **R′**).

In this study we have found that the previously reported constitutive activity of N295S and L305Q results from the endogenous Ang II and its fragments produced by the HEK293 cells. Since the enzyme immunoassay kit used in the study also detects other Ang II fragments including Ang I, Ang III, Ang, IV, and Ang 5-8, the exact amount of Ang II produced in the cell medium remains unknown. It is possible that Ang II, the active hormone, is not the major form of angiotensin peptide produced by the HEK293 cells because the wild-type AT_1 receptor produced only minimal amounts of basal activity in HEK293 cells. The basal activities increased in N295S and L305Q over the wild-type in HEK293 cells (6.2- and 2-fold, respectively, Table 1) are likely due to increased Ang II affinity and the presence of other Ang II fragments. This prompted us to further examine whether inactive Ang II fragments activate N295S and L305Q.

The inactive angiotensin II fragments utilized in this study are naturally occurring short peptides. Ang 1-7 and Ang IV are native agonists for their own specific receptors (Santos et al, 2003; Albiston et al, 2001). Ang 5-8 has no known function. These inactive Ang II fragments plus active hormone Ang II, Ang I, and Ang III all activate N295S and L305Q whereas only Ang I, Ang II, and Ang III activate the wild-type AT_1 receptor. In addition, [Sar^1,Ile^4,Ile^8]Ang II activates N295S and L305Q as it activates N111G. Moreover, N295S and L305Q induce a much

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

greater fold-increase in basal activity than N111G and the wild-type receptor at a low Na^+ condition. Thus, N295S and L305Q are distinct from the wild-type receptor and similar to the constitutively active mutant N111G receptors in properties of promiscuous activation by structurally similar peptides. However, N295S and L305Q are not constitutively active by definition because they show no detectable basal activity in the absence of ligand.

The promiscuous agonist specificity displayed by N295S and L305Q in this study could be disastrous in the case that a similar mutation occurs in AT_1 receptors in nature. A receptor with this unique property could become either constitutively active as in the case of HEK293 cells or wild type as in the case of COS-7 cells. Thus, we propose to term this type of basal activity as pseudo-constitutive activity. Thus, mutant N295S and L305Q represent a novel pattern of mutations for GPCRs with pseudo-constitutive activity.

Activation of GPCRs can occur through agonist-dependent as well as agonist-independent spontaneous mechanisms. Like other GPCRs, the molecular mechanism involved in AT_1 receptor activation remains unclear (Noda et al., 1996; Feng et al., 1998; Feng and Douglas, 2000; Miura et al., 1999; Karnik , 2003; Hunyady et al, 2003; Boucard et al, 2003; Martin et al, 2004). In light of recent discovery of constitutively active mutations, the extended ternary complex model (Lefkowitz et al., 1993; Kjelsberg et al., 1992; Gether and Kobilka, 1998), the two-state model (Leff, 1995), and the cubic ternary model (Gether and Kobilka, 1998; Weiss et al., 1996) have been proposed to describe GPCR activation. These models suggest that GPCRs exist in an equilibrium of two functionally distinct states: the inactive (**R**) and active state (**R***).

Karnik's laboratory (Noda et al., 1996; Feng et al., 1998) has proposed that constitutively active mutant receptors may exist in partial active states (**R′**). This model explains the constitutive activity for N111G. It also explains why the inactive Ang II analog,

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

[Sar^{1},Ile^{4},Ile^{8}]Ang II, drives N111G to its fully active **R*** state. Our data suggest that mutant N295S and L305Q receptors may have overcome most energy barriers and exist in a neutral state ($\mathbf{R^N}$) or a state proximal to $\mathbf{R^N}$ states (Fig 6). The $\mathbf{R^N}$ state is relatively stable so that no basal activity will be produced because additional stabilization energy is required to drive the conformation to either the **R** or **R*** direction. An inverse agonist would drive the $\mathbf{R^N}$ to the **R** state whereas an agonist or a partial agonist would drive the $\mathbf{R^N}$ to the **R*** or **R′** state. The N295S and L305Q receptors in the $\mathbf{R^N}$ state no longer require agonism-specifying side chains of Ang II that are normally required for the wild type to overcome the energy barrier in order to remove the structural constraints. However, they do require certain stabilization energy to 'trap' the conformation to **R′** or **R*** for effector activation. The naturally occurring inactive Ang II fragments and synthetic inactive Ang II analog, [Sar^{1},Ile^{4},Ile^{8}]Ang II, are actually agonists or partial agonists to N295S and L305Q because they activate the receptors.

Asn^{295} and Leu^{305} locate in a region of TM7 that is not thought to interact directly with Ang II (Fig 7). Mutations at these positions resulted in new selectivity or promiscuous agonist specificity for Ang II fragments, similar to constitutively active N111G (Le et al, 2002). These mutations barely affect the receptor internalization as shown in the internalization study, suggesting little possibility of a modified basal activity through alteration of the internalization. TM7 of the AT_1 receptor plays a pivotal role in the receptor activation. An interaction between TM2 Asp^{74} and TM7 Tyr^{292} was proposed in an earlier model (Marie et al, 1994). Most recently, Karnik's laboratory reported interactions involving an extensive hydrogen bonding network (Asn^{69}-Tyr^{302}, Asp^{74}-backbone carbonyl of Asn^{294} and Asn^{295}, Asp^{74}-water-Asn^{298}, and Thr^{80}-Tyr^{292}) between TM2 and TM7 (Miura et al, 2003). Boucard et al reported that TM7 goes through a pattern of helical movements upon the receptor activation, suggesting an interhelical

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

network interaction involving Asn^{111}-Asp^{74}-Asn^{298} among TM3, TM2, and TM7 (Boucard et al, 2003). Removal of constraints through agonist or mutation by breaking the interhelical interaction network and inducing TM movements may explain receptor activation and the constitutive activity of N111G (Miura et al, 2003; Boncard et al 2003; Martin et al, 2004). N295S and L305Q may have resulted in conformational changes similar to N111G, leading to pseudo-constitutive activity or $\mathbf{R^N}$ state with promiscuous agonist specificity. Mutations of N295S and L305Q must have generated new interhelical interaction networks with little constraints preventing the receptors from full activation. However, the exact new interhelical interaction network generated by point mutations in N295S and L305Q is not known at this time.

The small but significant basal activity (8.73%) of D74G at low Na^+ concentration indicates that the highly conserved Asp^{74} is not the only factor that mediates the Na^+ effects. Lowering the Na^+ concentration could overcome a week constraint in D74G, leading to 8.73% basal activity. It is also possible that N295S and L305Q still possess a weak constraint and that a certain degree of energy is needed to overcome this barrier. Likewise, lowering the Na^+ concentration could overcome this weak constraint in N295S and L305Q, leading to much greater basal activities on the basis of mutations.

Since its first use in Dr. Karnik's laboratory, the inactive Ang II analog, [Sar^1,Ile^4,Ile^8]Ang II has been utilized in many studies to characterize AT_1 receptors (Noda et al., 1996; Thomas et al, 2000; Holloway et al, 2002; Wei et al, 2003; Ahn et al, 2004a; Ahn et al, 2004b). This Ang II analog, while inducing no IP production, has been shown to induce phosphorylation (Thomas et al, 2000; Holloway et al, 2002) and Erk1/2 activation through binding to the wild-type AT_1. Consistent to our observation concerning activation of N295S and L305Q, [Sar^1,Ile^4,Ile^8]Ang II should be considered as a partial agonist for the wild type. It is

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

quite possible that this analog still contains an agonism-specifying side-chain or group required for AT_1 activation. The power of this agonism-specifying side-chain or group may be very weak but clearly critical and sufficient for induction of a distinct conformation required for Erk1/2 activation and the receptor phosphorylation. It may also be sufficient to overcome the minimal energy barrier in N295S and L305Q as revealed at low Na^+ concentration.

Inhibition of basal activity by receptor-specific inverse agonist is a hallmark of constitutively active receptors (Leurs et al., 1998; Bond et al., 1995; Noda et al., 1996; Groblewski et al., 1997). EXP3174 is an inverse agonist that has been shown to inhibit basal activity of the wild-type AT_1 and constitutively active mutant N111G (Noda et al., 1996). For the mutants with pseudo-constitutive activity, EXP3174 exhibited similar inhibitory actions at low Na^+ concentration. This is different from the observations employing three non-peptide antagonists (WIN64338, WIN51556, and NPC18325) of bradykinin B_2 receptors (Quitterer et al., 1996). However, it is not clear whether these antagonists are inverse or partial agonists or neutral antagonists.

In summary, this study identified a novel pattern of mutation that traps the receptor at the $\mathbf{R^N}$ state with a remarkably extended spectrum of agonist specificity. Although an agonist-mediated conformational selection model for GPCR activation has been proposed (Lefkowitz et al., 1993; Kjelsberg et al., 1992; Leff, 1995; Noda et al 1996; Gether and Kobilka, 1998), most GPCRs i.e. rhodopsin and β_2-adrenergic receptor, are found to be activated through an agonist-mediated conformational induction model (Karnik et al, 2003). Actually, GPCRs activated through the conformational selection model are very scarce at this time. Our data may suggest the possible existence of GPCRs in nature that can be activated by agonists through a conformational selection mechanism.

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgments

We thank Dr. Sadashiva Karnik for providing the wild-type AT_1 and mutant N111G receptor genes. We thank Ms. Justine Cowan and Dr. Shiv Srivastava for critical reading of this manuscript.

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

References

Ahn S, Wei H, Garrison TR, Lefkowitz RJ (2004a) Reciprocal regulation of angiotensin receptor-activated extracellular signal-regulated kinases by beta-arrestins 1 and 2. *J Biol Chem* **279:**7807-7811.

Ahn S, Shenoy SK, Wei H, Lefkowitz RJ (2004b) Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. *J Biol Chem* **279:**35518-35525

Albiston AL, McDowall SG, Matsacos D, Sim P, Clune E, Mustafa T, Lee J, Mendelsohn FA, Simpson RJ, Connolly LM, Chai SY (2001) Evidence that the angiotensin IV (AT(4)) receptor is the enzyme insulin-regulated aminopeptidase. *J Biol Chem* **276:**48623-48626.

Baldwin, J. M. (1993) The probable arrangement of the helices in G protein-coupled receptors. *EMBO J.* **12,** 1693–1703.

Balmforth AJ, Lee LJ, Warburton P, Donnelly D and Ball SG (1997) The conformational change responsible for AT_1 receptor activation is dependent upon two juxtaposed asparagine residues on transmembrane helices III and VII. *J Biol Chem* **272:**4245–4251.

Bond RA, Leff P, Johnson TD, Milano CA, Rockman HA, McMinn TR, Apparsundaram S, Hyek MF, Kenakin TP, Allen LF and Lefkowitz RJ (1995) Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the β_2-adrenoreceptor. *Nature* **374:**272-276.

Boucard AA, Roy M, ve Beaulieu ME, Lavigne P, Escher E, Guillemette G, and Richard Leduc R (2003) Constitutive Activation of the Angiotensin II Type 1 Receptor Alters the Spatial Proximity of Transmembrane 7 to the Ligand-binding Pocket. *J Biol Chem* **278**: 36628-36636.

Ceresa BP and Limbird LE (1994) Mutation of an Aspartate residue highly conserved among G-protein-coupled receptors results in nonreciprocal disruption of α_2-adrenergic receptor-G-protein interactions. *J Biol Chem* **269:**29557-29564.

Chen G, Way J, Armour S, Watson C, Queen K, Jayawickreme CK, Chen WJ and Kenakin T (2000) Use of constitutive G protein-coupled receptor activity for drug discovery. *Mol Pharmacol* **57:**125-134.

Feng YH and Douglas JG (2000) Angiotensin receptors: an overview, in *Angiotensin II receptor antagonists* (Epstein M and Brunner H eds) pp 29-48, Hanley & Belfus, Philadelphia.

Feng YH, Miura SI, Husain A and Karnik SS (1998) Mechanism of constitutive activation of the AT_1 receptor: Influence of the size of the agonist switch binding residue Asn^{111}. *Biochemistry* **37:**15791-15798.

Gether U and Kobilka BK (1998) G protein-coupled receptors. II. Mechanism of agonist activation. *J Biol Chem* **273:**17979-17982.

Glossmann H, Baukal A and Catt KJ (1974) Cation dependence of high-affinity angiotensin II binding to adrenal cortex receptors. *Science* **185:**281-283.

Groblewski T, Maigret B, Languier R, Lombard C, Bonnafous JC and Marie J (1997) Mutation of Asn^{111} in the third transmembrane domain of the AT_{1A} angiotensin II receptor induces its constitutive activation. *J Biol Chem* **292:**1822–1826.

Holloway AC, Qian H, Pipolo L, Ziogas J, Miura S, Karnik S, Southwell BR, Lew MJ, Thomas WG (2002) Side-chain substitutions within angiotensin II reveal different requirements for signaling, internalization, and phosphorylation of type 1A angiotensin receptors. *Mol Pharmacol* **61:**768-77.

Hunyady L, Ji H, Jagadeesh G, Zhang M, Gaborik Z, Mihalik B and Catt KJ (1998) Dependence of AT_1 angiotensin receptor function on adjacent asparagine residues in the seventh transmembrane helix. *Mol Pharmacol* **54:**427-434.

Hunyady L, Vauquelin G, and Vanderheyden P (2003) Agonist induction and conformational selection during activation of a G-protein-coupled receptor. Trends in Pharmacol Sci **24:** 81-86

Ji TH, Grossmann M, and Ji I. (1998) G Protein-coupled Receptors I. diversity of receptor-ligand interactions. *J Biol Chem* **273**:17299–17302

Kjelsberg MA, Cotecchia S, Ostrowski J, Caron MG and Lefkowitz RJ (1992) Constitutive activation of the α_{1B} adrenergic receptor by all amino acid substitutions at a single site. *J Biol Chem* **267:**1430-1433.

Karnik SS, Gogonea C, Patil S, Saad Y, and Takezako T. (2003) Activation of G-protein-coupled receptors: a common molecular mechanism. *Trends Endo Metabol* **14**: 431-437

Le MT, Vanderheyden PML, Szaszák M, Hunyady L, Vauquelin G. (2002) Angiotensin IV is a potent agonist for constitutive active human AT_1 receptors. *J Biol Chem* **277:**23107-23110

Leff P (1995) The two-state model of receptor activation. *Trends Pharmacol Sci* **16:**89-97.

Lefkowitz RJ, Cotecchia S, Samama P and Costa T (1993) Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol Sci* **14:**303-307.

Leurs R, Smit MJ, Alewijnse AE and Timmerman H (1998) Agonist-independent regulation of constitutively active G-protein-coupled receptors. *TIBS* **23:**418-422.

Martin SS, Boucard AA, Clément M, Escher E, Leduc R, and Guillemette G (2004) Analysis of the third transmembrane domain of the human type 1 angiotensin II receptor by cysteine-scanning mutagenesis. *J Biol Chem* **280** (in press).

Miura SI, Feng YH, Husain A and Karnik SS (1999) Role of aromaticity of agonist switches of angiotensin II in the activation of the AT_1 receptor. *J Biol Chem* **274:**7103-7110

Miura S, Zhang J, Boros J, Karnik SS (2003) TM2-TM7 interaction in coupling movement of transmembrane helices to activation of the angiotensin II type-1 receptor. *J Biol Chem* **278:**3720-3725.

Noda K, Feng YH, Liu X, Saad Y, Husain A and Karnik SS (1996) The active state of the AT_1 angiotensin receptor generated by angiotensin II induction. *Biochemistry* **35:**16435-16442.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **289:**739-45.

Parnot C, Bardin S, Miserey-Lenker S, Guedin D, Corvol P and Clauser E. (2000) Systematic identification of mutations that constitutively activate the angiotensin II type 1A receptor by screening a randomly mutated cDNA library with an original pharmacological bioassay. *Proc Natl Acad Sci USA* **97:**7615-7620.

Quitterer U, AbdAlla S, Jarnagin K and Wuller-Esterl W (1996) Na^+ ions binding to the bradykinin B_2 receptor suppress agonist-independent receptor activation. *Biochemistry* **35:**13368-13377.

Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, Mendes EP, Lemos VS, Campagnole-Santos MJ, Schultheiss HP, Speth R, Walther T (2003) Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci U S A* **100:**8258-63.

Thomas WG, Qian H, Chang CS and Karnik SS (2000) Agonist-induced phosphorylation of the angiotensin II (AT_{1A}) receptor requires generation of a conformation that is distinct from the inositol phosphate-signaling state. *J Biol Chem* **275:**2893-2900.

Tsai BS and Lefkowitz RJ (1978) Agonist-specific effects of monovalent and divalent cations on adenylate cyclase-coupled □-adrenergic receptors in rabbit platelets. *Mol Pharmacol* **14:**540-549.

Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM, Lefkowitz RJ (2003) Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc Natl Acad Sci U S A* **100:**10782-10787.

Weiss JM, Morgan PH, Lutz MW and Kenakin TP (1996) The cubic ternary complex receptor-occupancy model. I. Model description. *J Theor Biol* **178:**151-167.

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

This work was supported by an SDG grant 0030019N from the American Heart Association and an RO1 grant HL65492 from the National Heart, Lung, and Blood Institute to YHF.

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

Legend

Fig. 1. Influence of mutations of AT_1 receptor on ligand binding affinity. Shown are changes in the K_i of various ligands when the residues at position 111, 295, and 305 of the AT_1 receptor are altered by the amino acid substitution indicated. The values represent the mean ± SEM of at least three independent determinations carried out in duplicate. The standard error was within 5% and therefore is not represented in the figure. The Mg^{2+}-membrane preparations used for the binding assays were prepared from COS-7 cells expressing the AT1 receptors.

Fig. 2. Basal IP production of the AT_1 receptors in COS-7 cells in the presence and absence of conditioned media and Losartan (1 µM). Basal activity expressed as percentage of maximal activity of wild type in the presence of 5 µM Ang II. Asterisks and ¶ indicate significant changes ($p < 0.05$ and $P < 0.01$) between conditioned media and control media. † indicates significant changes ($p < 0.05$ or $P < 0.01$) between conditioned media and conditioned media plus Losartan. § indicates significant changes ($p < 0.01$) between N295S and N111G, and between L305Q and N111G. CM, conditioned medium.

Fig. 3. Production of Angiotensin peptides detected by enzyme immunoassay. Inset: RT-PCR of angiotensinogen mRNA, as described in materials and methods.

Fig. 4. Activation of AT_1 receptors by Ang II analogs. (*A*) Dose response curve of AT_1 receptors to inactive analog [Sar^1,Ile^4,Ile^8]Ang II. (*B*) Activation of AT_1 receptors by endogenous Ang II fragments Ang IV (5 µM), Ang 1-7 and Ang 5-8 (50 µM). The mutant receptors expressed in COS-7 cells are at comparable levels to the wild type. The maximum activation of WT

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

represents the 100% activation (about 39,000 cpm/10^6 cells) of the wild-type AT_1 receptor when stimulated by 0.1 μM [Sar^1]Ang II.

Fig. 5. Basal IP production of the AT_1 receptors in COS-7 cells at low (10 mM) Na^+ concentration (A). Basal activities expressed as percentage of maximal activity of the wild type. The maximal IP production of wild-type AT_1 receptor induced by 5 μM [Sar^1]Ang II at normal Na^+ concentration was used as 100%. (B) Inhibition of basal activity of the AT_1 receptors at 10 mM Na^+ concentration by AT_1 receptor-specific antagonist Losartan, EXP3174, and Candesartan.

Fig. 6. Four-states model for receptor activation. **R**, inactive basal states. $\mathbf{R^N}$, neutral states. **R′**, intermediate partial active states. **R***, active states. Red line indicates structural constraints that require agonism-specifying groups of agonist to overcome the energy barrier and induce conformational change through further isomerization. Green lines indicate that no structural constraint exists and no agonism-specifying groups of agonist are required for overcoming the energy barrier but require only stabilization energy to trap the $\mathbf{R^N}$ and **R′** to **R*** states. Here **R**, $\mathbf{R^N}$, **R′**, and **R*** represent four distinct conformational states. For any single molecule of wild type or mutant receptors, it can only exist as one state without equilibrium between different states if absolutely identical conditions are maintained. For more molecules of an identical wild type or mutant receptor, multiple forms of each **R**, $\mathbf{R^N}$, **R′**, and **R*** state appear randomly due to various folding paths of each individual receptor molecule. However, the predominant form or the predominant states of the receptors are determined by the intrinsic structural property of the

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

receptors. This model does not explain superactivity that may be partially resulted from impaired desensitization and internalization.

Fig. 7. Interhelical network interactions of the rat AT_1 receptor. Using MacVector 7.2 (Acceryles) for CLUSTALW alignment of primary sequences, the seven helices of the rat AT_1 receptor were determined based on three references: bovine rhodopsin structure (Palczewski et al, 2000; Protein Data Bank code: 1F88); Baldwin model of GPCR receptors (Baldwin, 1993); and computational prediction of TM topology (HMMTOP and TMHMM). Helical wheel representation of the seven TMs of the rat AT_1 receptor (A). The residues at the beginning and end of each TM helix are highlighted in cyan except for the L^{305} at the end of TM7 that is in red. Red shows the critical residues involved in activation and agonist binding. The charged residues and residues that are highly conserved in more than 90% GPCRs are in blue. The helical wheels of TM1, TM3, TM5, and TM7 are illustrated clockwise. The helical wheels of TM2, TM4, and TM6 are shown counterclockwise to reflect the opposite orientations. Extracellular view (B) and side-view of the AT_1 receptor at ground state. The models were drawn with software ICM-browser (MolSoft L.L.C.). The ribbon representation of 7TMs was color-coded and also identified with a roman numeral. Stick and ball representation and dot envelope representation by accessibility were shown for residues Asp^{74}, Asn^{111}, Tyr^{292}, Asn^{295} and Leu^{305}.

Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011601
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1

Expression profile of Ang II receptors in HEK293 cells and COS-7 cells

Data represent mean ± SEM for three to five separate experiments. The basal IP (cpm $x10^{-3}/10^6$ cells) was assayed in the absence of ligand and the maximal IP (cpm $x10^{-3}/10^6$ cells) was induced by 5 μM [Sar^1]Ang II. The B_{max} values represent the expressed receptors present in the total membrane preparations.

	Mock		WT		N111G		N295S		L305Q	
	HEK293	COS-7	HEK293	COS-7	HEK293	COS-7	HEK293	COS-7	HEK293	COS-7
DNA (μg/ml)	2.5	3	2.5	3	2.5	3	2.5	3	2.5	3
Basal IP	1.15±0.08	1.16±0.08	2.23±0.23	1.21±0.11	18.7±1.61	12.1±0.87	7.8±0.76	1.05±0.05	3.3±0.21	1.15±0.05
Maximal IP	1.13±0.09	1.25±0.08	37.2±1.62	36.5±1.54	37.1±2.15	36.2±1.57	36.3±1.55	36.2±1.63	36.2±1.48	36.4±1.33
B_{max} (pmol/mg)	N/A	N/A	3.97±0.07	4.03±0.08	4.04±0.11	3.95±0.10	3.96±0.09	4.32±0.12	4.01±0.11	4.22±0.13

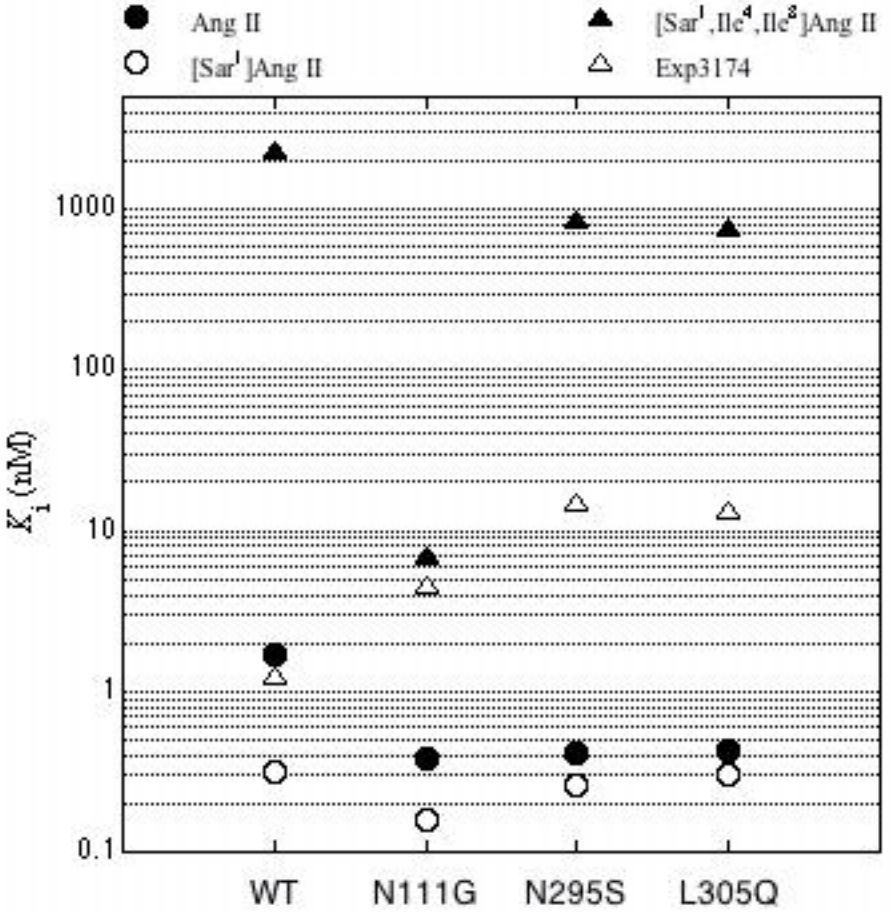
Ang II
[Sar1]Ang II
[Sar1,Ile4,Ile8]Ang II
Exp3174
1000
100
10
1
0.1
Ki (nM)
WT
N111G
N295S
L305Q

Figure 1

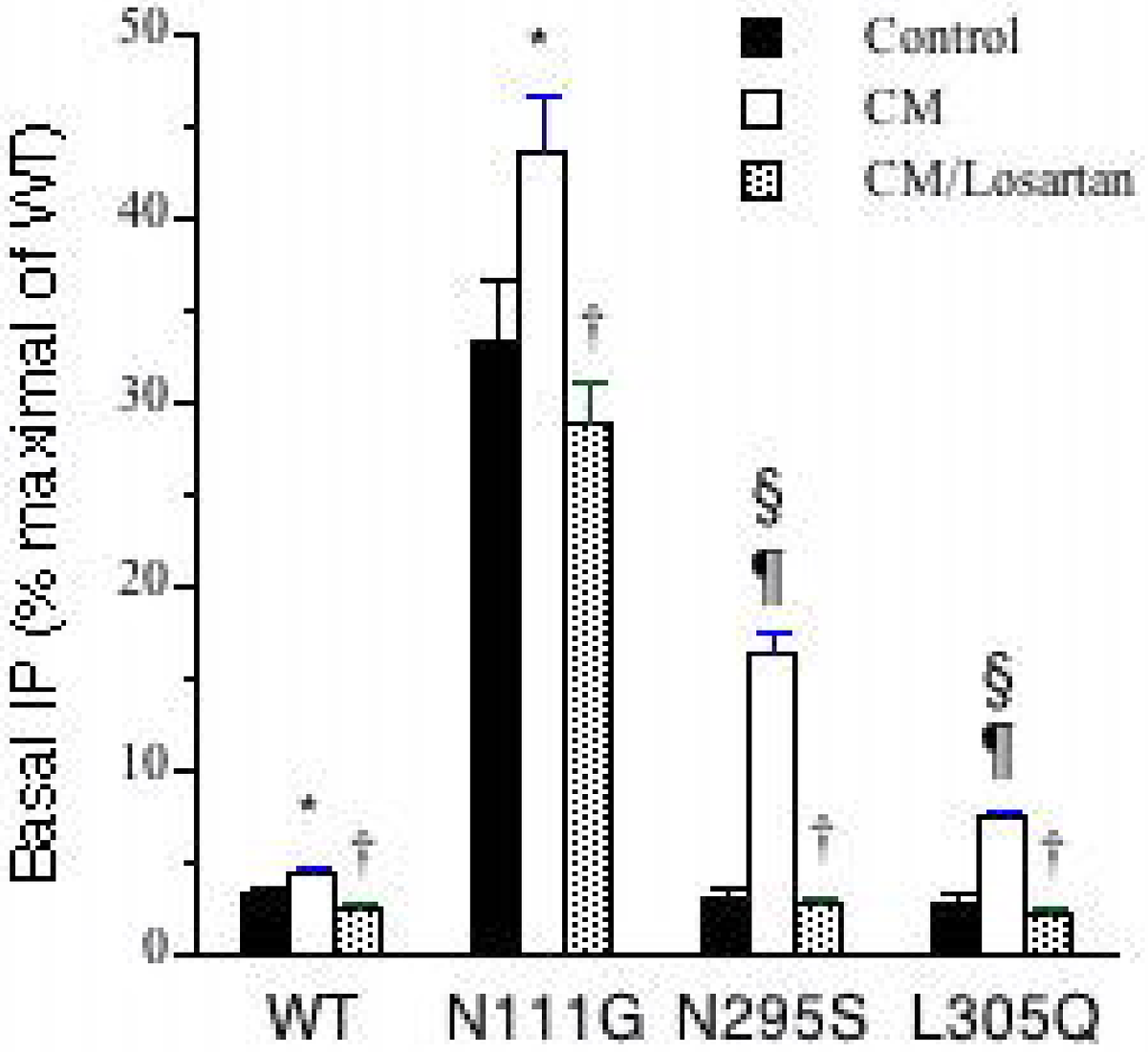
Basal IP (% maximal of WT)
50
40
30
20
10
0
Control
CM
CM/Losartan
*
†
§
¶
WT
N111G
N295S
L305Q

Figure 2

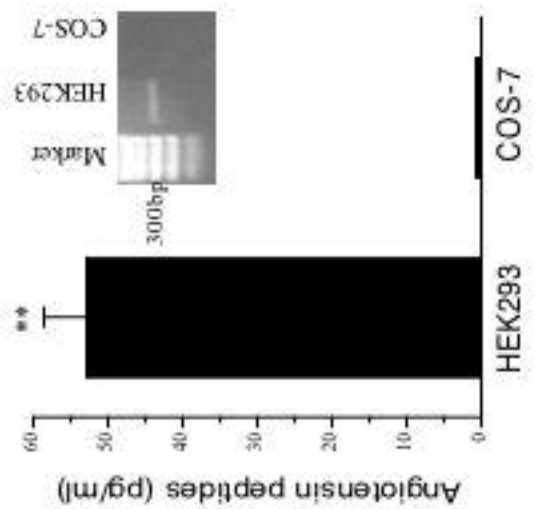

Figure 3

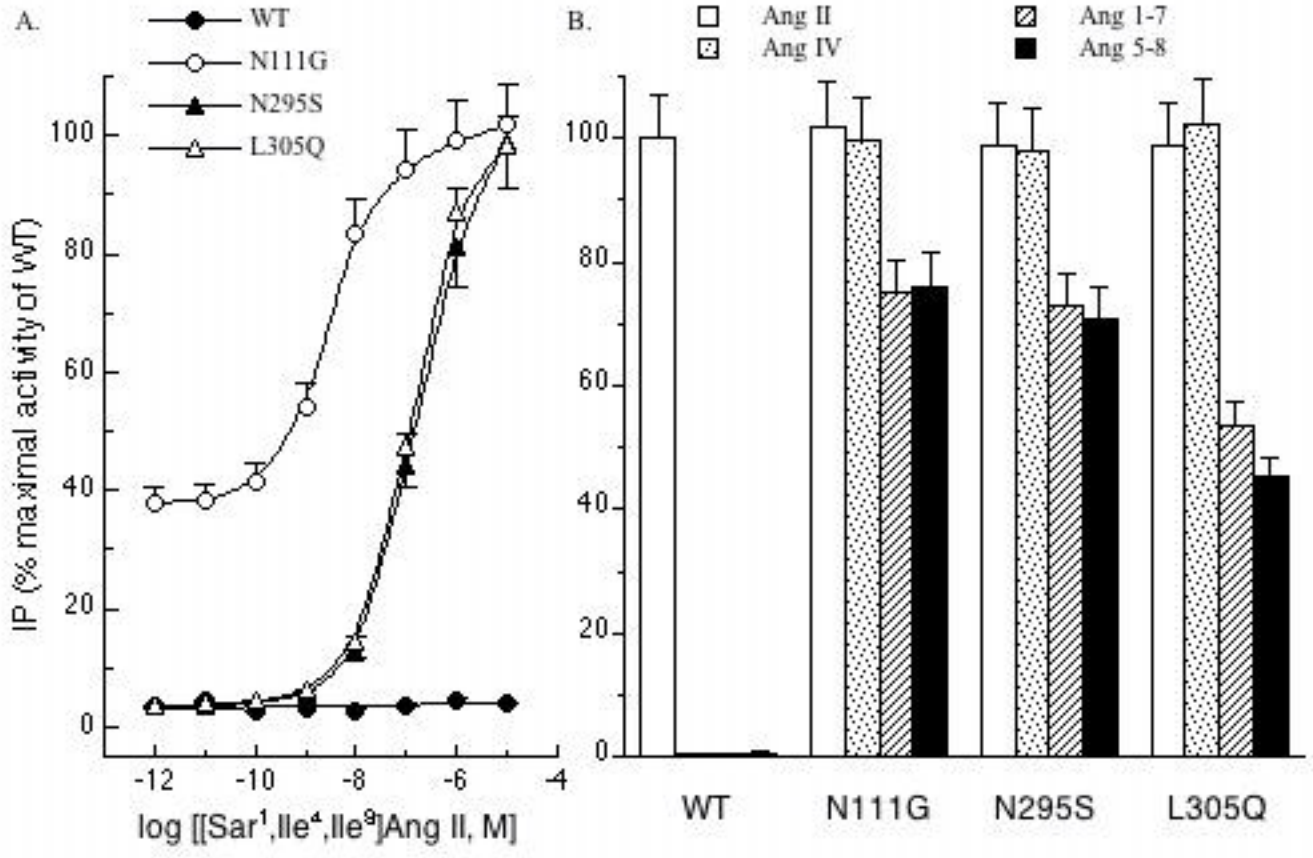
A.
WT
N111G
N295S
L305Q
IP (% maximal activity of WT)
100
80
60
40
20
0
-12
-10
-8
-6
-4
log [[Sar1,Ile4,Ile8]Ang II, M]
B.
Ang II
Ang IV
Ang 1-7
Ang 5-8
100
80
60
40
20
0
WT
N111G
N295S
L305Q

Figure 4

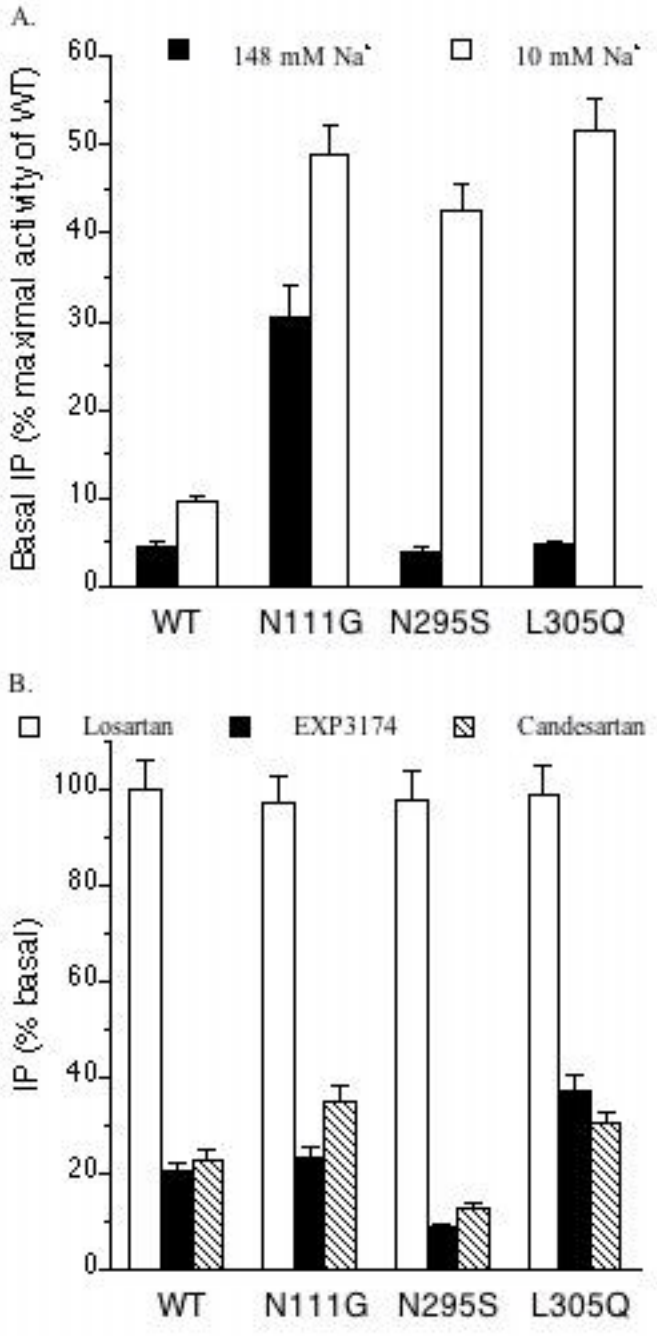
A.
148 mM Na+
10 mM Na+
Basal IP (% maximal activity of WT)
60
50
40
30
20
10
0
WT
N111G
N295S
L305Q
B.
Losartan
EXP3174
Candesartan
IP (% basal)
100
80
60
40
20
0
WT
N111G
N295S
L305Q

Figure 5

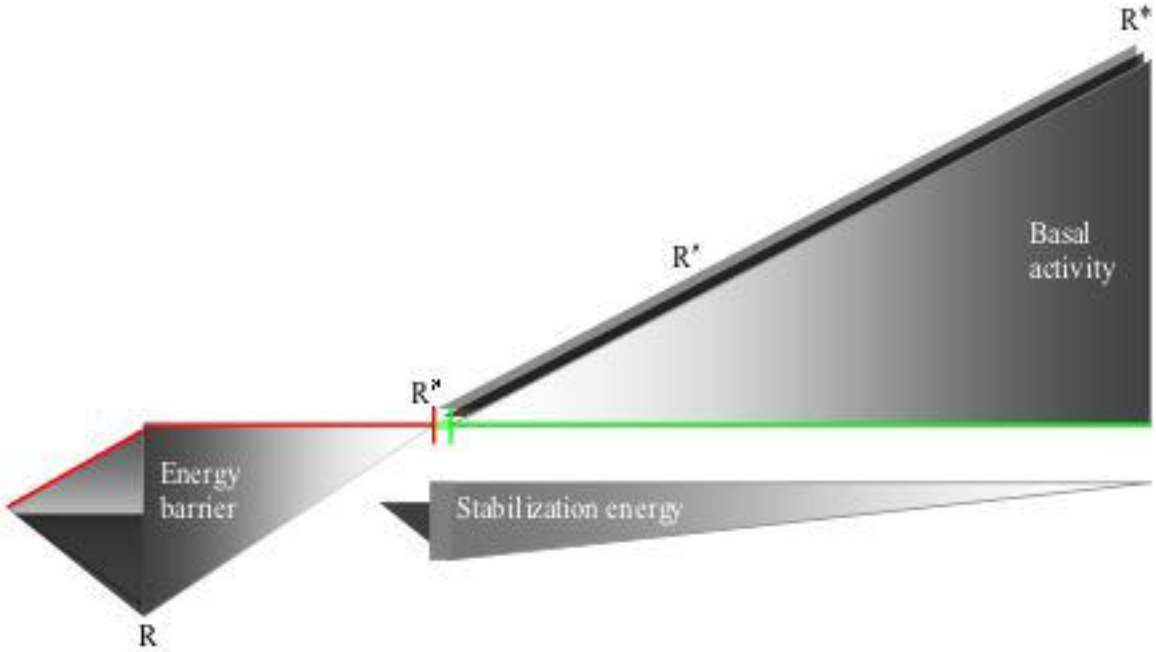

Figure 6

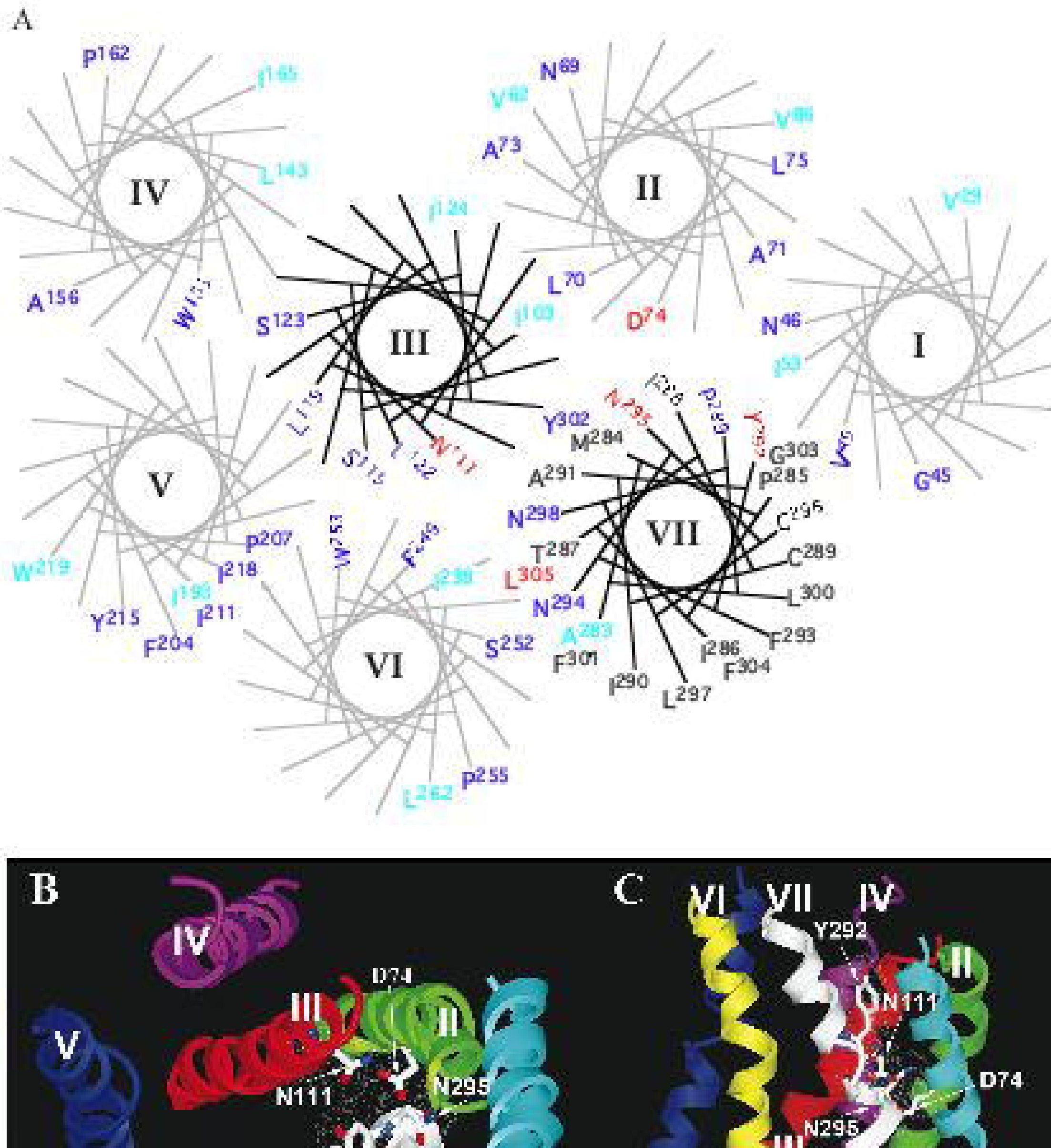
A
IV
III
II
I
V
VI
VII
P162
A156
S123
D74
A73
L75
L70
A71
N69
N46
G45
Y302
M284
A291
N298
T287
L305
N294
P285
G303
C295
C289
L300
F293
I286
F304
L297
I290
F301
S252
P255
P207
I218
I211
Y215
F204
W219
B
C
D74
N111
N295
Y292
L305

Figure 7