Single Mutations at Asn[295] and Leu[305] in the Cytoplasmic Half of Transmembrane α-Helix Domain 7 of the AT$_1$ Receptor Induce Promiscuous Agonist Specificity for Angiotensin II Fragments: A Pseudo-Constitutive Activity

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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). In this study, we showed 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 inositol 1,4,5-phosphates 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 because of the lack of endogenous Ang II fragments produced by the cells—a pseudo-constitutive activity. It is surprising that the Ang II analog [Sar¹,Ile⁴,Ile⁸]Ang II and the native angiotensin II fragments Ang 1–7, Ang IV, and Ang 5–8, which 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 (R^N). These data allow us to identify 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.

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 stimuli 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. How-

ever, 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 Gα$_{q/11}$-mediated activation of phospholipase C$_{β1}$, generating diacylglycerol and inositol 1,4,5-triphosphate. Although naturally occurring constitutively active AT$_1$ mutants are not known, engineered

ABBREVIATIONS: GPCR, G protein-coupled receptor; TM, transmembrane α-helix domain; HEK, human embryonic kidney; IP, inositol 1,4,5-triphosphate; RT-PCR, reverse transcription-polymerase chain reaction; EXP3174, 2-butyl-4-chloro-1-((2′-(1H-tetrazol-5-yl)(1,1′-biphenyl)-4-yl)methyl)-1H-imidazole-5-carboxylic acid; Ang I, angiotensin I (Asp¹-Arg²-Val³-Tyr⁴-Ile⁵-His⁶-Pro⁷-Phe⁸-His⁹-Leu¹⁰); Ang II, angiotensin II (Asp¹-Arg²-Val³-Tyr⁴-Ile⁵-His⁶-Pro⁷-Phe⁸); Ang IV, angiotensin IV (Val³-Tyr⁴-Ile⁵-His⁶-Pro⁷-Phe⁸); Ang 1–7, angiotensin 1–7 (Asp¹-Arg²-Val³-Tyr⁴-Ile⁵-His⁶-Pro⁷); Ang 5–8, angiotensin 5–8 (Ile⁵-His⁶-Pro⁷-Phe⁸); WIN64338, [4-[[2[[bis(cyclohexylamino) methylene]amino]-3-(2-naphthalenyl)-1-oxopropyl]amino]phenyl]methyl]tributyl phosphonium chloride HCl; NPC18325, D-Arg¹-Arg²-[aminotridecanoyl]3-Ser⁴-D-Tic⁵-Oic⁶+++-Arg⁷.

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; Balmforth et al., 1997; Groblewski 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 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). It seems that 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 because of 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 used 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 caused by the presence of endogenous Ang II fragments produced by the cells—a pseudo-constitutive activity. It is surprising that these mutations induced promiscuous activation of the mutant AT_1 receptors by Ang II and its native fragments.

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 analogs 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). [myo-^{3}H]Inositol (22 mCi/ml) was purchased from Amersham Biosciences (Piscataway, NJ). The monoclonal antibody 1D4 was purchased from the National Cell Culture Center (Biovest International, Coon Rapids, MN). The enzyme immunoassay kit for angiotensin peptides was purchased from SPI-BIO (Montigny le Bretonneux, 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 previously (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 described previously (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 plasmid DNA/10^7 cells was column-purified and used in transfection (columns from QIAGEN, Valencia, CA). COS-7 and HEK293 cells (American Type Culture Collection) were transfected with the Gene-PORTER transfection reagents (Genlantis, San Diego, CA). Transfected cells cultured for 48 h were harvested and Mg^{2+}-free cell membranes were prepared by the nitrogen Parr cell disruption bomb method (Feng et al., 1998). In brief, after centrifugation of the cell membrane homogenate at 30,000g [14,400 rpm with an SA-600 rotor (Sorvall, Newton, CT) for 15 min], the pellet was resuspended in 5 ml of HEPES/EDTA buffer (50 mM HEPES, pH 7.4, and 1.5 mM EGTA, containing protease inhibitors) and re-centrifuged at 30,000g for 15 min. Finally, the pellet (crude membrane fraction) was resuspended in 1.0 ml of HEPES/EDTA containing 10% glycerol (per 10^8 cells). The membrane fraction was aliquoted into 1.5-ml Eppendorf tubes and stored at $-70°$C. The receptor expression was assessed in each case by immunoblot analysis (data 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 h 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. Nonspecific 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°C for 1 h 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., Clifton, NJ). The filters were extensively washed further with ice-cold binding buffer to wash away the free radioligand. The bound ligand fraction was determined from the counts per minute remaining on the membrane. Equilibrium-binding kinetics were determined using the computer program Ligand. The K_d values represent the mean $\pm$ S.E. of three to five independent determinations.

Production of Total Inositol (1,4,5)Phosphates. COS-7 and HEK293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% bovine calf serum. The medium for HEK293 cells contains 1% nonessential amino acids. Twenty-four hours after transfection, COS-7 and HEK293 cells cultured in 60-mm Petri dishes were labeled for 24 h with [myo-^{3}H]inositol (1μCi/ml) at 37°C in inositol-free Dulbecco's modified Eagle's medium containing 10% bovine calf serum. On the day of IP assay (i.e., 48 h 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°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 before the experiment for 15 min in incubation buffer (138 mM NaCl, 5 mM KCl, 10 mM LiCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 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, St. Louis, MO). This

treatment has been shown to greatly decrease the intracellular Na$^+$ content because of the presence of Na$^+$/K$^+$-ATPases in all nucleated mammalian cells (Glossmann et al., 1974; Tsai and Lefkowitz, 1978; Ceresa and Limbird, 1994; Quitterer et al., 1996).

Measurement of AT$_1$ Receptor Internalization. Internalization of the AT$_1$ receptors was measured by a method described earlier (Thomas et al., 2000). In brief, 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 (1 μM for N111G and 30 μM for other receptors) for 10 min at 37°C. Surface-bound ligands were removed by gentle acid wash (50 mM sodium citrate, 0.2 mM sodium phosphate, 90 mM NaCl, and 0.1% bovine serum albumin, pH 5.0; 10 min at 4°C), which does not affect subsequent receptor binding. Then, a radioligand binding assay was performed (5 h at 4°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, Carlsbad, CA) and used for routine reverse transcriptase and polymerase chain reaction (RT-PCR) with an RT-PCR kit (Invitrogen). The primers used for amplification of angiotensinogen transcripts were: forward, gctgatccagcctcactatg; and reverse, 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 base pairs for angiotensinogen.

Enzyme Immunoassay of Ang II and Its Fragments. All cells at approximately 90% confluence in 60-mm dishes were washed carefully three times and then cultured with 2 ml of serum-free media containing cocktail protease inhibitors in a cell culture incubator. Six hours after culture, the 2 ml of 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) following the company's protocol. The antibody of this kit cross-reacts with Ang I, Ang II, Ang III, and Ang IV but not with Ang 1–7 and other nonangiotensin 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.

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^1,Ile^4,Ile^8]Ang II, and AT$_1$-specific inverse agonist EXP3174 (Fig. 1). Mutant N295S and L305Q show significantly increased binding affinities for Ang II ($\sim$4-fold) and [Sar^1,Ile^4,Ile^8]Ang II ($\sim$3-fold) and significantly decreased binding affinities for nonpeptide inverse agonist EXP3174 (24-fold) compared with the wild-type AT$_1$ receptor. The patterns of increase or decrease of binding affinities are similar to those of 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 compared with the wild-type AT$_1$ receptor.

When membrane proteins prepared from transfected HEK293 cells were used in ligand binding assays, patterns and magnitudes of binding affinities for these receptors similar to those shown in Fig. 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 compared with the wild-type AT$_1$ and N111G. For basal IP productions, no significant differences were observed for N295S and L305Q compared with 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, respectively, than that of the wild-type receptor. It is surprising that 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 versus 31.2%). Wild-type AT$_1$ also induced higher basal IP in HEK293 cells than in the COS-7 cells (Table 1), similarly to N111G.

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
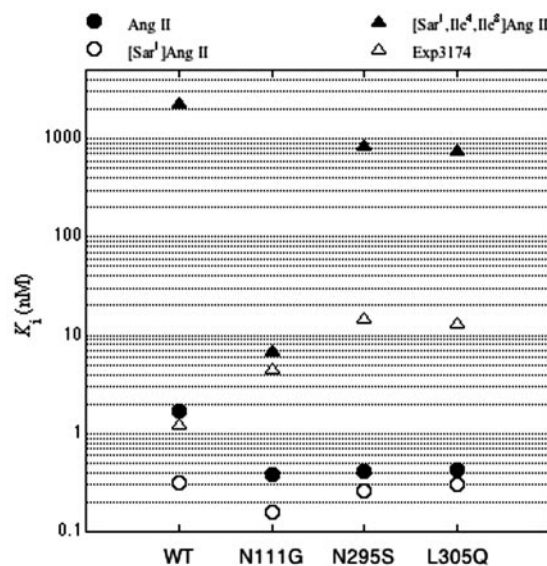

Fig. 1. Influence of mutations of AT$_1$ receptor on ligand binding affinity. Shown are changes in the K_i of various ligands when the residues at positions 111, 295, and 305 of the AT$_1$ receptor are altered by the amino acid substitution indicated. The values represent the mean ± S.E.M. of at least three independent determinations carried out in duplicate. The S.E. 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.

1), we thought that HEK293 cells might produce a 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. It is interesting that the conditioned media induced 16.3, 7.4, and 4.5% basal IP productions for N295S, L305Q, and the wild type. These IP productions were significantly greater compared with 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 base pairs was amplified on RNAs isolated from HEK293 cells. RT-PCR failed to show any specific amplification on RNAs prepared from the COS-7 cells (Fig. 3, inset).

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. Figure 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 Fig. 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 [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 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 compared with 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 Fig. 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 concentrations of ≥ 5 μM (data not shown). Activations of the mutant receptors by these Ang II fragments were independent of cell types.
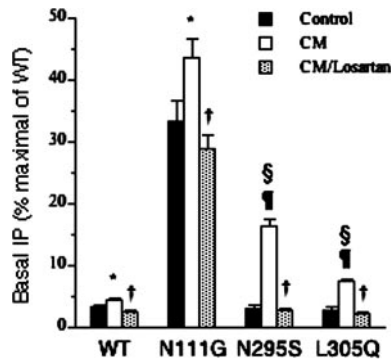

Fig. 2. Basal IP production of the AT$_1$ receptors in COS-7 cells in the presence and absence of conditioned media and Losartan (1 μM). Basal activity expressed as percentage of maximal activity of wild type in the presence of 5 μM Ang II. *, $p < 0.05$; ¶, $P < 0.01$, significant changes between conditioned media and control media. †, significant changes ($p < 0.05$ or $P < 0.01$) between conditioned media and conditioned media plus Losartan. §, significant changes ($p < 0.01$) between N295S and N111G and between L305Q and N111G. CM, conditioned medium.
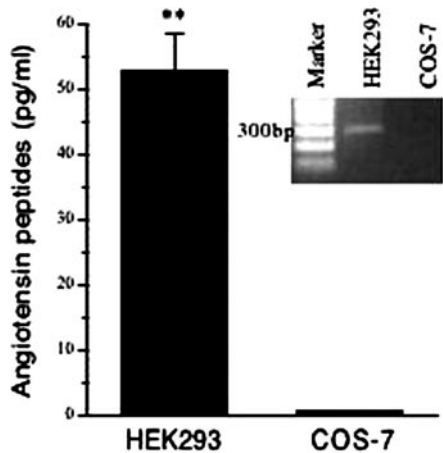

Fig. 3. Production of Angiotensin peptides detected by enzyme immunoassay. Inset, RT-PCR of angiotensinogen mRNA, as described under *Materials and Methods*.

TABLE 1

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

Data represent mean $\pm$ S.E.M. for three to five separate experiments. The basal IP (cpm $\times 10^{-3}/10^6$ cells) was assayed in the absence of ligand and the maximal IP (cpm $\times 10^{-3}/10^6$ cells) was induced by 5 μM [Sar^1]Ang II. The B_{max} values (pmol/mg) represent the expressed receptors present in the total membrane preparations. Mock, vector only.

	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 $\pm$ 0.08	1.16 $\pm$ 0.08	2.23 $\pm$ 0.23	1.21 $\pm$ 0.11	18.7 $\pm$ 1.61	12.1 $\pm$ 0.87	7.8 $\pm$ 0.76	1.05 $\pm$ 0.05	3.3 $\pm$ 0.21	1.15 $\pm$ 0.05
Maximal IP	1.13 $\pm$ 0.09	1.25 $\pm$ 0.08	37.2 $\pm$ 1.62	36.5 $\pm$ 1.54	37.1 $\pm$ 2.15	36.2 $\pm$ 1.57	36.3 $\pm$ 1.55	36.2 $\pm$ 1.63	36.2 $\pm$ 1.48	36.4 $\pm$ 1.33
B_{max}	N/A	N/A	3.97 $\pm$ 0.07	4.03 $\pm$ 0.08	4.04 $\pm$ 0.11	3.95 $\pm$ 0.10	3.96 $\pm$ 0.09	4.32 $\pm$ 0.12	4.01 $\pm$ 0.11	4.22 $\pm$ 0.13

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 under a low-sodium condition (10 mM) as described under *Materials and Methods*. 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 near that of the N111G (48.8%) (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, compared with that under the 148 mM normal sodium condition. The -fold changes in basal IP induced by N295S and L305Q were significantly greater (>5-fold) than the -fold change by the wild type and N111G. The low-sodium 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 D74G, the corresponding mutant of the AT$_1$ receptor, 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. Because 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 under low-Na$^+$ conditions. To test this hypothesis, EXP3174 and candesartan, the specific

inverse agonists of AT$_1$ receptor, were added to the cells at the same time as the low-Na$^+$ condition was applied. It is striking that EXP3174 and candesartan inhibited the increment in basal IP production to approximately 90 and 85%, respectively, for N295S and 68 and 70%, respectively, for
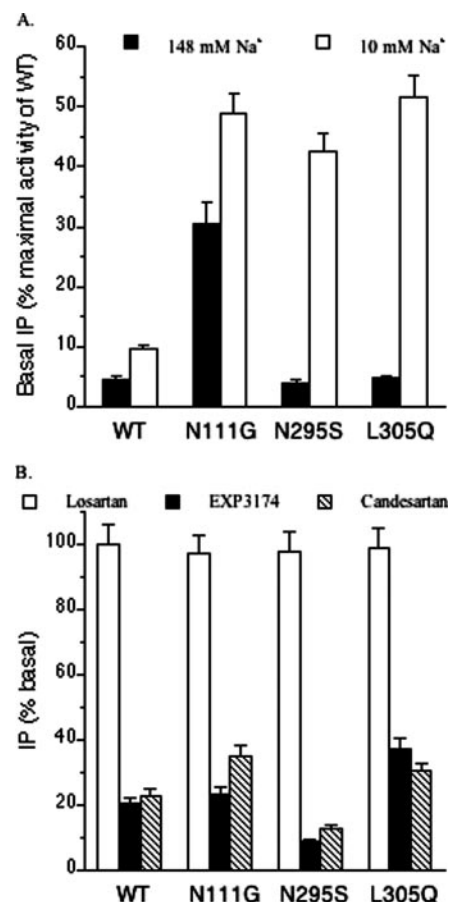

Fig. 5. Basal IP production of the AT$_1$ receptors in COS-7 cells at low (10 mM) Na$^+$ concentration (A). Basal activities expressed as percentage of maximal activity of the wild type. The maximal IP production of wild-type AT$_1$ receptor induced by 5 μM [Sar^1]Ang II at normal Na$^+$ concentration was used as 100%. B, inhibition of basal activity of the AT$_1$ receptors at 10 mM Na$^+$ concentration by AT$_1$ receptor-specific antagonists losartan, EXP3174, and candesartan.
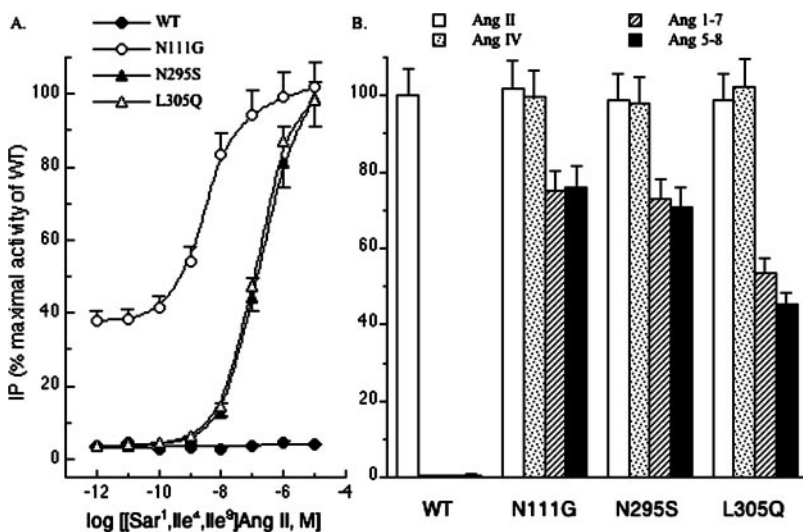

Fig. 4. Activation of AT$_1$ receptors by Ang II analogs. A, dose-response curve of AT$_1$ receptors to inactive analog [Sar^1,Ile^4,Ile^8]Ang II. B, activation of AT$_1$ receptors by endogenous Ang II fragments Ang IV (5 μM), Ang 1–7 and Ang 5–8 (50 μM). The mutant receptors expressed in COS-7 cells are at levels comparable with those of the wild type. The maximum activation of WT represents the 100% activation (approximately 39,000 cpm/10^6 cells) of the wild-type AT$_1$ receptor when stimulated by 0.1 μM [Sar^1]Ang II.

L305Q, whereas the specific neutral antagonist Losartan failed to inhibit the basal activity as shown in Fig. 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.

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. Because 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 probably caused by 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 used in this study are naturally occurring short peptides. Ang 1–7 and Ang IV are native agonists for their own specific receptors (Albiston et al., 2001; Santos et al., 2003). 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 greater -fold-increase in basal activity than N111G and the wild-type receptor under low-Na$^+$ conditions. 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 if a similar mutation were to occur 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 that this type of basal activity should be termed *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; Miura et al., 1999; Feng and Douglas, 2000; Boucard et al., 2003; Hunyady et al., 2003; Karnik et al., 2003; Martin et al., 2004). In light of recent discovery of constitutively active mutations, the extended ternary complex model (Kjelsberg et al., 1992; Lefkowitz et al., 1993; Gether and Kobilka, 1998), the two-state model (Leff, 1995), and the cubic ternary model (Weiss et al., 1996; Gether and Kobilka, 1998) have been proposed to describe GPCR activation. These models suggest that GPCRs exist in an equilibrium of two functionally distinct states, inactive (R) and active (R*).

Karnik's laboratory (Noda et al., 1996; Feng et al., 1998) 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, [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 (R^N) or a state proximal to R^N states (Fig. 6). The R^N state is relatively stable so that no
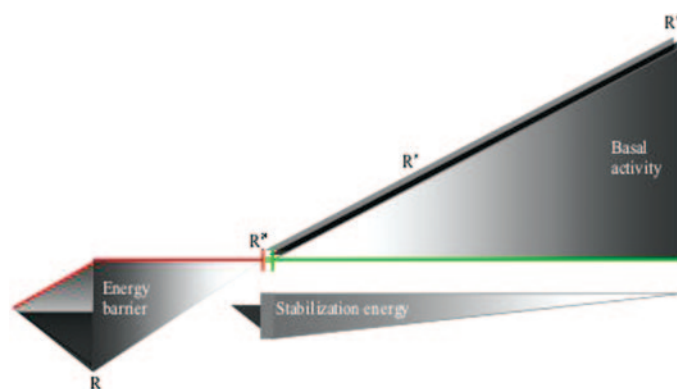

Fig. 6. Four-states model for receptor activation: R, inactive basal states; R^N, neutral states; R′, intermediate partial active states; and R*, active states. Red line indicates structural constraints that require agonism-specifying groups of agonist to overcome the energy barrier and induce conformational 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 R^N and R′ to R* states. Here R, 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, R^N, R′, and R* state appear randomly as a result of 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 receptors. This model does not explain superactivity that may partially result from impaired desensitization and internalization.

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 R^N to the R state, whereas an agonist or a partial agonist would drive the R^N to the R* or R′ state. The N295S and L305Q receptors in the R^N state no longer require the agonism-specifying side chains of Ang II that are normally required for the wild type to overcome the energy barrier 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} are located 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. (2003) reported that TM7 goes through a pattern of helical movements upon the receptor activation, suggesting an interhelical network interaction involving Asn^{111}-Asp^{74}-Asn^{298} among TM3, TM2, and TM7. 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 (Boucard et al., 2003; Miura et al., 2003; Martin et al., 2004). N295S and L305Q may have resulted in conformational changes similar to those in N111G, leading to pseudo-constitutive activity or 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.
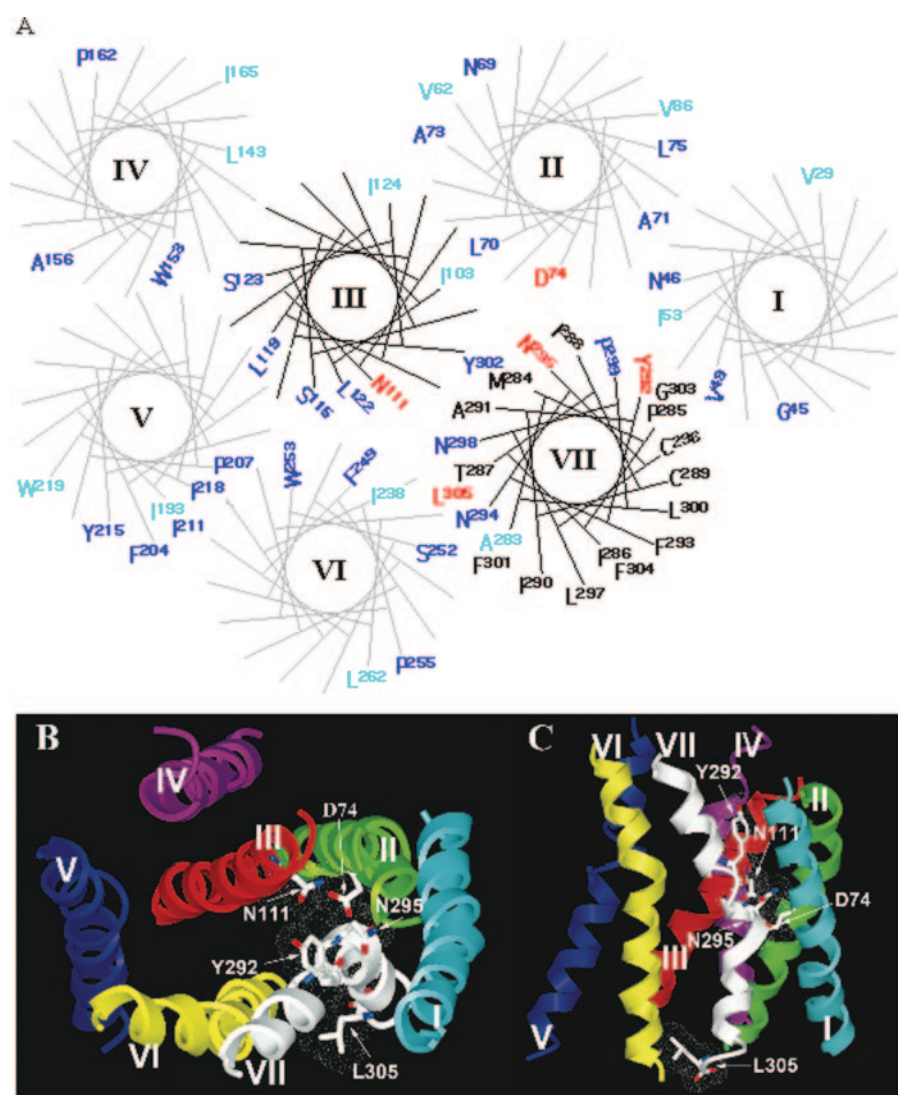

Fig. 7. Interhelical network interactions of the rat AT$_1$ receptor. Using MacVector 7.2 (Accelrys, San Diego, CA) for ClustalW alignment of primary sequences, the seven helices of the rat AT$_1$ receptor were determined based on three references: bovine rhodopsin structure (Palczewski et al., 2000; Protein Data Bank code 1F88); Baldwin model of GPCR receptors (Baldwin, 1993); and computational prediction of TM topology (hidden Markov model-based methods 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 Leu^{305} at the end of TM7, which 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 (C) of the AT$_1$ receptor at ground state. The models were drawn with software ICM-Browser (MolSoft LLC, San Diego, CA). The ribbon representation of 7TMs is color-coded and also identified with a Roman numeral. Stick and ball representation and dot envelope representation by accessibility are shown for residues Asp^{74}, Asn^{111}, Tyr^{292}, Asn^{295}, and Leu^{305}.

The small but significant basal activity (8.73%) of D74G at low Na$^+$ concentrations indicates that the highly conserved Asp^{74} is not the only factor that mediates the Na$^+$ effects. Lowering the Na$^+$ concentration could overcome a weak 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 used 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,b). Although this Ang II analog induces no IP production, it 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 with our observation concerning activation of N295S and L305Q, [Sar^1,Ile^4,Ile^8]Ang II should be considered a partial agonist for the wild type. It is 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 (Bond et al., 1995; Noda et al., 1996; Groblewski et al., 1997; Leurs et al., 1998). 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 pseudoconstitutive activity, EXP3174 exhibited similar inhibitory actions at low Na$^+$ concentrations. 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 R^N state with a remarkably extended spectrum of agonist specificity. Although an agonist-mediated conformational selection model for GPCR activation has been proposed (Kjelsberg et al., 1992; Lefkowitz et al., 1993; 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). In fact, 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.

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