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Constitutively Active Mutant N111G of Angiotensin II Type 1 (AT_1) Receptor Induces Homologous Internalization Through Mediation of AT_1-Receptor Antagonist

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Abstract. The present study investigated the internalization behavior of the constitutively active mutant (CAM) N111G of angiotensin II type 1 (AT_1) receptor and correlated the result with the mechanism of the constitutive activity of the mutant. The inverse agonist activity of valsartan, losartan, candesartan, and telmisartan was also examined by inositol phosphate (IP) accumulation study as well as receptor-internalization assay. Both wild-type (WT) and N111G mutant receptors were transiently expressed in COS-7 cells and the binding affinities towards the agonist and these four AT_1 antagonists were determined. Production of total IP was measured in the presence and absence of the compounds. The agonist-induced receptor internalization of both WT and N111G mutant receptors was also investigated. Although the mutant showed similar binding characteristics with agonist and the antagonists used as WT, the internalization of the mutant was much lower ($19.56 \pm 2.87\%$) than that of the WT receptor ($74.63 \pm 1.00\%$). Internalization of the mutant significantly increased ($63.22 \pm 0.03\%$) in the presence of valsartan, which also showed significant inverse agonist activity in the N111G mutant. The results indicate that internalization of CAM N111G of the AT_1 receptor is induced by the use of valsartan, which may be an important characteristic of inverse agonist activities of AT_1 antagonists in N111G.

Keywords: angiotensin II type 1 (AT_1) receptor, internalization, constitutive activity, inverse agonist activity, binding affinity

Introduction

G-protein coupled receptors (GPCRs) form one of the largest protein families, with several hundred members in humans (1). Despite the wide variety of ligands and physiological roles, all these receptors are structurally characterized by seven transmembrane domains and most of them are thought to share common activation and desensitization mechanisms. Angiotensin II (Ang II) receptors are members of the GPCR superfamily. Two subtypes of Ang II receptors have been identified (2, 3) and pharmacologically characterized: Ang II type 1 (AT_1) and type 2 (AT_2) receptors (4). Interaction of Ang II with the AT_1 receptor induces vasoconstriction, sodium reabsorption, and stimulation of aldosterone release (5).

Mutagenesis studies of the AT_1 receptor have recently identified amino acid residues important in the binding of the natural ligand Ang II and non-peptide antagonists. A conserved residue, Lys^{199} in the fifth transmembrane domain (TMD V) of the AT_1 receptor, has recently been reported to be crucial for the binding of both peptide (6) and non-peptide (7) ligands. Ang II contains two residues, Tyr^4 and Phe^8, which are essential for agonism (8). The activation of AT_1 receptor from the basal state requires an interaction between Asn^{111} in TMD III of the AT_1 receptor and the Tyr^4 residue of Ang II (9). This

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shows the importance of Asn^{111} residue in TMD III of the AT_1 receptor. No naturally occurring, constitutively active mutant (CAM) of AT_1 receptors have been reported, but engineered mutation of the Asn^{111} residue to glycine (N111G) results in constitutive activation of the AT_1 receptor (8). In the wild-type (WT) AT_1 receptor, interaction of Tyr^4 of Ang II with Asn^{111} in the receptor appears to act as the trigger to convert the inactive state (R) to the active state (R*) and allow receptor activation. Small side chain substitution (glycine) of Asn^{111} in the AT_1 receptor presumably releases this conformational switch, allowing constitutive activity and removing the requirement of Tyr^4 in Ang II for maximal receptor activation.

Agonist binding to a GPCR induces conformational changes in the receptor, leading to activation of G$\alpha\beta\gamma$ heterotrimers (10). One function of the activated G-proteins is to activate GPCR kinases (GRKs) that in turn phosphorylate the specific receptor for desensitization. Subsequently, β-arrestins bind to the GRK-phosphorylated motifs of the receptor and induce the receptor internalization. This homologous GPCR internalization is agonist-specific and GRK-dependent. This type of feedback regulation is conventional because it requires activation of classic G-proteins (11 – 13). Homologous internalization of GPCRs can also take place through a β-arrestin–independent pathway. Initially, internalization of the GPCRs was viewed as a means to uncouple the receptor from its signaling components, thereby dampening the overall response (14 – 17). The results of many studies indicate that the itinerary of the internalized GPCR is receptor- and cell-specific (18). At least four pathways of agonist-induced internalization of GPCRs exist (19, 20), and they may be cell-type specific. The classical GPCR internalization pathway involves GRKs, β-arrestin, clathrin-coated pits, and the GTPase dynamin and is exemplified by the β_2-adrenergic receptor (11, 19 – 24).

Thus the present study was designed to examine the binding profiles of both WT and the N111G mutant of AT_1 receptors with the AT_1-receptor agonist Ang II and AT_1-receptor blockers (ARBs), valsartan, losartan, candesartan, and telmisartan. We also determined total inositol phosphate (IP) accumulation by the cells expressing specified receptors and showed the inverse agonist activity of the ARBs used in this study in the N111G mutant. Finally, we examined the internalization of the specified receptors and correlated the result with the mechanism of constitutive activity of the N111G mutant.

Materials and Methods

Drugs

Ang II used in the study was purchased from Peptide Institute, Inc., Osaka. Candesartan was obtained as gift from Takeda Chemical Industries, Ltd., Osaka. Losartan was obtained from Merck Research Laboratories, Rahway, NJ, USA. Valsartan was gift from Novartis Institutes for BioMedical Research, Inc., Cambridge, MA, USA. Telmisartan was obtained from Nippon Boehringer Ingelheim Co., Ltd., Tokyo. [^{125}I]-Sar^1-Ilu^8-angiotensin II (2200 Ci/mmol) was purchased from Perkin Elmer, Inc., Boston, MA, USA. [^{3}H]-Myo-inositol (83 Ci/mmol) was purchased from Amersham Biosciences, Piscataway, NJ, USA.

Site-directed mutagenesis

The synthetic rat AT_1-receptor gene, cloned in the shuttle expression vector pMT-2, was used for expression and mutagenesis, as described in earlier studies (25). The mutant was prepared by the polymerase chain reaction (PCR) method with the QuichChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). DNA sequence analysis was done to confirm the mutation.

Cell culture, transfection, and membrane preparation

COS-7 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 37°C. The WT and mutant AT_1 receptors were transfected into COS-7 cells using Lipofectamine™ 2000 according to the manufacturer's protocol (Invitrogen Life Technologies, Rockville, MD, USA). To express the AT_1 receptor protein, 12 μg of purified plasmid DNA/10^7 cells was used in the transfection. Transfected COS-7 cells that had been cultured for 48 h were harvested with ice-cold phosphate-buffered saline (PBS), pH 7.4; washed by HBSS with 1.5% 0.5 M EDTA; 0.15% 50 mg/ml PMSF, and 0.15% 2 mg/ml aprotinin; and finally suspended in 0.25 M sucrose solution containing 1.5% 0.5 M EDTA, 0.15% 50 mg/ml PMSF, and 0.15% 2 mg/ml aprotinin. The cells were then disrupted by Polytron Homogenizer for 10 s. The mass was centrifuged at 4°C for 5 min at $1260 \times g$ and the supernatant was ultra centrifuged at 4°C for 20 minutes at $30{,}000 \times g$. The resulting pellets were suspended in binding assay buffer and used for the binding experiments. The protein contents of the membranes were measured by the method of Lowry et al. (26) using bovine serum albumin as the standard.

Radioligand binding assay

Binding assays for WT and N111G mutant of AT_1

receptors were carried out in an incubation tube that contained 10 μg of membrane protein, [^{125}I]-Sar^1-Ilu^8-angiotensin II, unlabeled drug as required, and binding buffer containing 20 mM phosphate buffer, 100 mM sodium chloride, 20 mM magnesium chloride, 1 mM EGTA, and 0.2% BSA, pH 7.4 in a final volume of 125 μl. Both saturation and competition binding assays were carried out as described previously (27, 28). Briefly, for saturation binding studies, six to seven concentrations (5 – 800 pM) of [^{125}I]-Sar^1-Ilu^8-angiotensin II were tested in duplicate. Nonspecific binding was defined as the amount of radioligand binding remaining in the presence of 10 μM Ang II. For competition binding studies, membranes were incubated with 250 pM of [^{125}I]-Sar^1-Ilu^8-angiotensin II and unlabelled drugs for 1 h at 25°C. The incubation was terminated by rapid filtration under vacuum through Whatman GF/C filters that had been presoaked in 0.5% polyethyleneimine followed by three times washing with ice-cold 50 mM Tris HCl (pH 8.0). The bound ligand fraction was determined from the counts/min remaining on the membrane.

IP accumulation assay

COS-7 cells at about 90% confluence in 10-cm dishes were seeded into 24-well plates. The cells were then transfected with plasmid DNA of both the WT and N111G mutant AT_1 receptors. At 24 h after transfection, the cells were labeled with 1 μCi/ml [^{3}H]myo-inositol in DMEM and incubated for 20 h at 37°C. The cells were washed with Hank's buffered salt solution (HBSS) and exposed with HBSS containing 20 mM phosphate buffer and 20 mM LiCl, pH 7.4. The cells were incubated for 30 min at 37°C. Agonist and four ARBs were added to each well and incubation continued for an additional 1 h at 37°C. At the end of the incubation, the medium was removed, and the total soluble IP was extracted from the cells by the perchloric acid extraction method as described previously (29, 30).

Internalization assay

Internalization assay was performed as described previously (31). Briefly, COS-7 cells in 12-well plates that had been transiently transfected were incubated at 37°C in serum-free DMEM with or without 1 μM Ang II for the indicated time. The cells were washed twice by ice-cold PBS and incubated for 3 h at 4°C with 0.1 nM [^{125}I]-Sar^1-Ilu^8-angiotensin II in binding buffer. The cells were then washed with ice-cold PBS and surface-bound [^{125}I]-Sar^1-Ilu^8-angiotensin II was removed using the acid wash technique of Crozat et al. (32) in which cells were exposed to 150 mM NaCl – 50 mM glycine, pH 3, for 10 min at 4°C. The cell-associated radioactivity was measured by gamma counting.

Western blot analysis

Equal amounts of whole cell lysates were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto Hybond ECL nitrocellulose membranes (Amersham Biosciences) using a semi dry system in immunotransfer buffer. The membranes were blocked in blocking buffer (Tris-buffered saline, TBS with 10% nonfat dried milk) and incubated for 1 h at room temperature. After blocking, membranes were exposed to AT_1 rabbit polyclonal IgG and actin rabbit polyclonal IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1:1000 dilutions in blocking buffer with 1% milk for 1 h and kept at 4°C for a whole night in a rotating shaker. The membranes were washed three times with TBS containing 0.1% Tween-20 and incubated with HRP-conjugated anti-rabbit IgG (Promega, Madison, WI, USA) for 1 h at room temperature at 1:2000 dilutions in blocking buffer with 1% milk. The membranes were washed and then the blots on the membranes were visualized by adding Amersham ECL Western blotting detection reagent (GE Healthcare, Piscataway, NJ, USA).

Data analyses

Nonlinear regression analyses of saturation and competition binding assay were performed using GraphPad Prism software (San Diego, CA, USA). The results of experiments were expressed as the mean ± S.E.M. In competition binding experiments, the values of inhibition constants (K_i) were calculated by the following equation (33):

$$K_i = IC_{50} / \{1 + ([L] / K_d)\}$$

, where the inhibition concentrations (IC_{50}) were determined as the concentrations of ligands that inhibited [^{125}I]-Sar^1-Ilu^8-angiotensin II binding by 50%; [L] = the concentration of [^{125}I]-Sar^1-Ilu^8-angiotensin II used, and K_d = the dissociation constant of [^{125}I]-Sar^1-Ilu^8-angiotensin II for the receptor. Statistical analyses were performed by the Student's unpaired *t*-test (two tailed).

Results

Radioligand binding characteristics for WT and N111G mutant of AT_1 receptors

WT and N111G mutant AT_1-receptor genes were transiently expressed in COS-7 cells. [^{125}I]-Sar^1-Ilu^8-angiotensin II radioligand binding assay showed that the WT receptor and N111G mutant were bound as expected, with a dissociation constant (K_d) of 0.55 ± 0.02 and 0.78 ± 0.21 nM, respectively (Table 1). On the other hand, B_{max} values for the WT receptor and N111G mutant were calculated from the maximal specific

Table 1. Dissociation constant (K_d) and maximum binding sites (B_{max}) of [^{125}I]-Sar^1-Ilu^8-angiotensin II for wild-type (WT) and N111G mutant AT_1 receptors

	AT_1 WT receptor	N111G mutant receptor
K_d value (nM)	0.55 ± 0.02	0.78 ± 0.21
B_{max} value (pmol/mg protein)	1.52 ± 0.07	0.68 ± 0.12**

[^{125}I]-Sar^1-Ilu^8-Angiotensin II was used to label AT_1 WT and N111G mutant receptors transiently expressed in COS-7 cells. Data represent the mean ± S.E.M. of 4 independent experiments, each performed in duplicate. ***P*<0.001 vs. WT.

Table 2. Binding affinities (K_i) of agonists and antagonists to wild-type (WT) and N111G mutant AT_1 receptors

	AT_1 WT receptor	N111G mutant receptor
	K_i (nM)	K_i (nM)
Agonist		
Ang II	30.58 ± 11.47	0.72 ± 0.09*
Antagonist		
Valsartan	8.21 ± 3.19	20.31 ± 3.75*
Losartan	59.41 ± 2.65	119.50 ± 5.01**
Candesartan	3.37 ± 0.27	3.23 ± 0.12
Telmisartan	2.96 ± 1.11	7.38 ± 0.82*

[^{125}I]-Sar^1-Ilu^8-Angiotensin II (250 pM) was used to label AT_1 WT and N111G mutant receptors transiently expressed in COS-7 cells. Data represent the mean ± S.E.M. of 4 independent experiments, each performed in duplicate. **P*<0.05, ***P*<0.001 vs. WT.

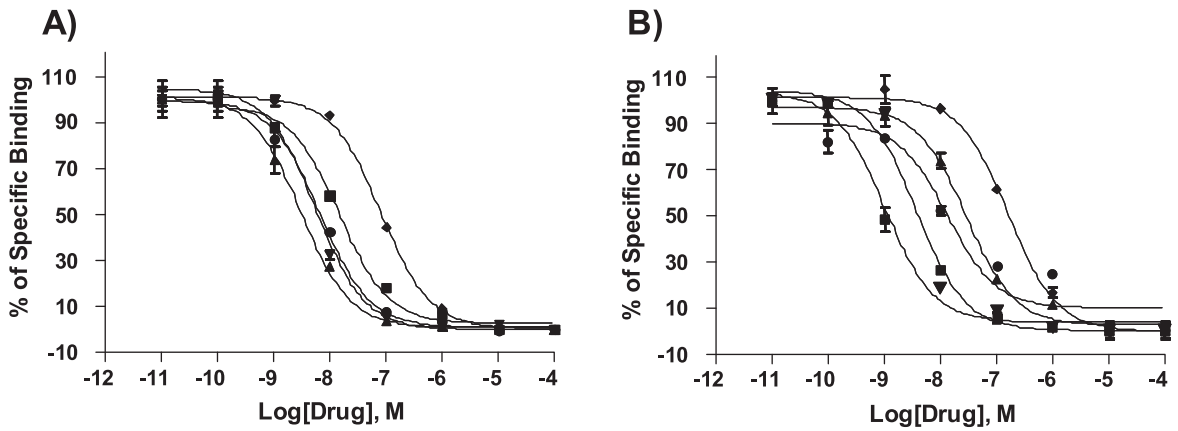

Fig. 1. Determination of binding affinities (pK_i) of Ang II (square), candesartan (inverted triangle), losartan (diamond), valsartan (triangle), and telmisartan (circle) to the wild-type (WT) (A) and N111G mutant (B) AT_1 receptors as assessed by [^{125}I]-Sar^1-Ilu^8-angiotensin II radioligand.

binding of [^{125}I]-Sar^1-Ilu^8-angiotensin II as 1.52 ± 0.07 and 0.68 ± 0.12 pmol/mg of protein, respectively (Table 1). The result demonstrated that the receptor expression for the mutant was decreased compared to WT AT_1 receptors (*P*<0.001).

Table 2 shows the binding affinities (pK_i) for agonist and ARBs, valsartan, losartan, candesartan, and telmisartan, towards WT and N111G mutant of AT_1 receptors. The binding affinity of Ang II to the mutant N111G was markedly increased compared to WT receptors (*P*<0.05) (Table 2, Fig. 1). ARBs showed 2 – 3-fold decreased binding affinities to the mutant N111G compared to AT_1 WT receptor (*P*<0.05) (Table 2), although candesartan showed almost no change in binding affinity to the mutant.

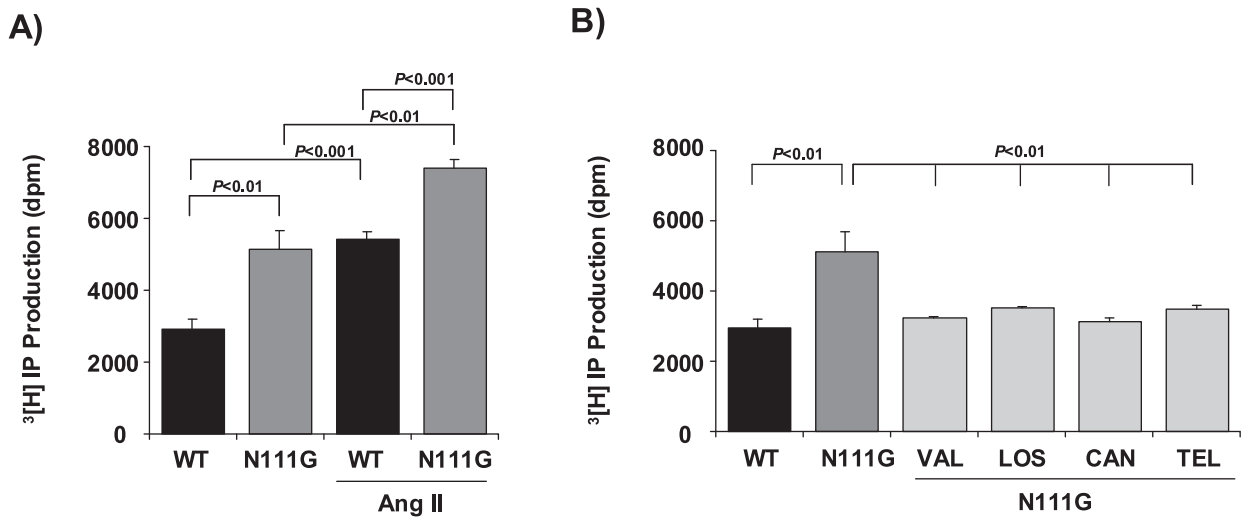

Fig. 2. Effect of AT_1-receptor agonist and antagonists on IP production by wild-type (WT) and N111G mutant AT_1 receptors. A) IP production in the absence (solid bar) and presence of 1 μM Ang II (gray bar) by COS-7 cells expressing WT and N111G mutant AT_1 receptors. B) Inhibition of IP production by 1 μM valsartan, losartan, candesartan, and telmisartan, respectively (light gray bars). Data are the mean ± S.E.M. of 4 – 6 independent experiments, each performed in duplicate.

IP accumulation assay in transfected COS-7 cells

IP production was measured in COS-7 cells expressing both WT AT_1 and N111G mutant AT_1 receptors (Fig. 2). Even though the level of expression of N111G mutant of AT_1 receptors (0.68 ± 0.12 pmol/mg of protein) was less than that of the WT (1.52 ± 0.07 pmol/mg of protein), the cells expressing the N111G mutant exhibited higher levels of agonist-independent (basal) IP production (Fig. 2A), showing constitutive activity. IP production by the cells expressing the N111G mutant AT_1 receptor was markedly decreased when incubated in the presence of the ARBs used in the study (Fig. 2B).

Internalization of WT and N111G mutant of AT_1 receptors

In view of the apparent difference in G protein coupling and activation between WT AT_1 and N111G mutant AT_1 receptors, we examined the capacity of the mutant to undergo homologous internalization. Treatment of the WT and N111G mutant AT_1 receptors with 100 nM Ang II for 45 min induced 74.63 ± 1.00% and 19.56 ± 2.87% receptor internalization, respectively (Fig. 3). Internalization of the N111G mutant AT_1 receptor was much lower than that of the WT AT_1 receptor (only 26% compared to the WT AT_1 receptor). However, when the ARB valsartan was used, internalization of the N111G mutant AT_1 receptor was significantly increased to 63.22 ± 0.38%, which is 3-fold higher than that of the N111G mutant in the absence of valsartan (Fig. 3).

Western blot analysis

To investigate the expression of both WT and N111G mutant AT_1 receptors, we performed Western blot analysis. The Western blot probed with anti-AT_1 antibody detected the specified protein of the receptors, and immunoreactive bands were observed at 43 kDa in both WT and N111G mutant AT_1 receptors (Fig. 4).

Discussion

Internalization plays an important role in receptor endocytosis and signal transduction. The present study demonstrated the correlation between endocytosis and signal transduction of AT_1 receptors due to its site directed mutagenesis. Homologous internalization of GPCRs is an active process that requires specific ligand binding, conformational changes of the receptor, and signal transduction initiated by the activated receptor (10). Earlier, it was reported that site directed mutagenesis of Asn^{111} of TMD III of the AT_1 receptor to glycine (N111G) results in constitutive activation of the receptor (8), and N111G showed higher basal IP production without agonist-induced stimulation compared to the WT AT_1 receptor. Thereby the present investigation has been performed to find out the correlation between the constitutive activity and internalization behavior of the N111G mutant receptor.

In saturation binding experiments, the data represent the different level of cell expression of N111G mutant receptor compared to WT AT_1 receptor. The mutant N111G showed a 2-fold lower B_{max} value assessed by

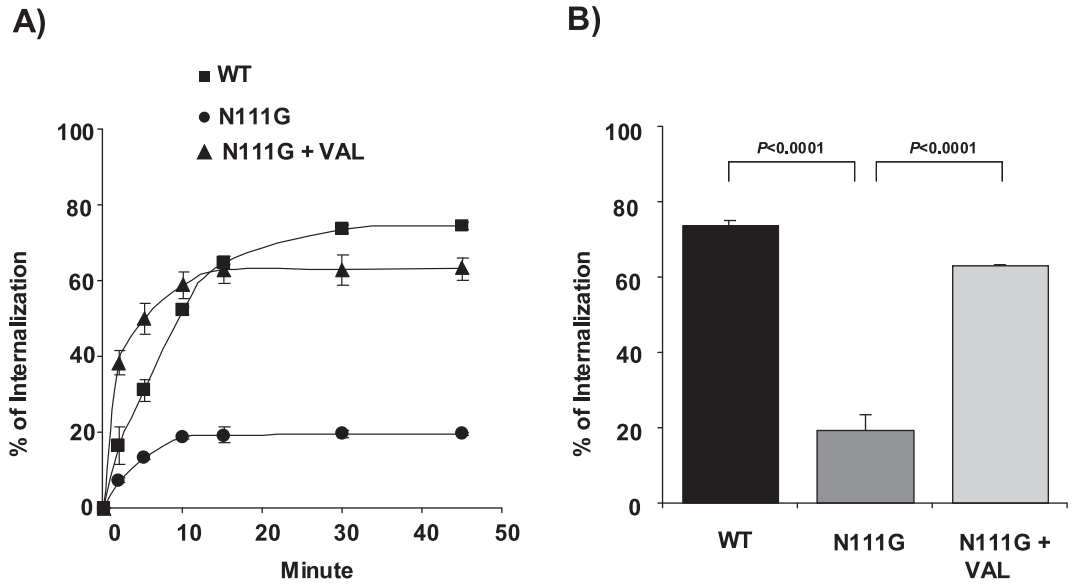

Fig. 3. Internalization assay of wild-type (WT) and N111G mutant AT_1 receptors. A) % of internalization of WT (square) and N111G mutant (circle) AT_1 receptors and internalization of N111G mutant receptors in the presence of 1 μM valsartan (triangle). B) % of internalization of WT (solid bar) and N111G mutant AT_1 receptors in the absence (gray bar) and presence (light gray bar) of 1 μM valsartan after 45 min of incubation. Internalization of ^{125}I-labeled Sar^1-Ilu^8-angiotensin II at 37°C by WT and N111G mutant AT_1 receptors was determined as described in Materials and Methods. Data are the mean ± S.E.M. of 4 independent experiments, each performed in duplicate.

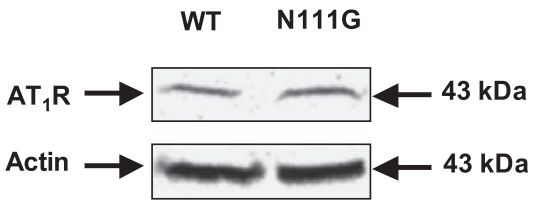

Fig. 4. Western blot analysis showing the band of AT_1 receptor and actin for both wild-type (WT) and N111G mutant AT_1 receptors.

radioligand [^{125}I]-Sar^1-Ilu^8-angiotensin II binding compared to the WT AT_1 receptor, although there was no significant changes in the values of the dissociation constant (K_d) of [^{125}I]-Sar^1-Ilu^8-angiotensin II for the mutant and WT AT_1 receptors (Table 1). The competition binding experiment demonstrated that the binding affinity of the agonist, Ang II, was significantly increased for the N111G mutant compared to the WT AT_1 receptor, whereas the binding affinities of ARBs, valsartan, losartan, and telmisartan, were 2 – 3-fold decreased towards the N111G mutant compared to the WT AT_1 receptor (Table 2, Fig. 1), although candesartan showed almost no change in binding affinity to the mutant. The results demonstrate that asparagine at position 111 of TMD III of the AT_1 receptor is a very important site for both agonist and antagonist binding.

IP production by the COS-7 cells expressing the WT and N111G mutant AT_1 receptor is shown in Fig. 2. The results of IP production experiments show that N111G is a CAM (Fig. 2A) as described in the earlier study (8). A decrease in the size of the Asn^{111} side-chain induces an intermediate activated receptor conformation, which may be responsible for the constitutive activity of the N111G mutant (8). Valsartan, losartan, candesartan, and telmisartan significantly suppressed the basal IP production by the cells expressing the constitutively active N111G mutant of AT_1 receptor (Fig. 2B), which shows the inverse agonist activity of these compounds in the N111G mutant, although in a previous study, losartan was found to have weak inverse agonist activity in the same mutant (34). Earlier studies suggest that the binding of inverse agonists not only blocks potential entry of Ang II into the receptor pocket but also induces the inactive conformation of the receptor (35).

To determine the possible reason for the behavior of the N111G mutant AT_1 receptor in signaling transduction, the present study investigated internalization for cell localization of the receptor protein. The mutant N111G showed very low internalization behavior compared to WT AT_1 receptor after 45 min (Fig. 3). However, it was very interesting that the internalization of N111G mutant significantly increased in the presence of valsartan. The AT_1 antagonist valsartan may cause a change in the conformation of the CAM N111G from the active to inactive state to promote internalization, which is consistent with the mechanism of inverse

agonists of constitutive active GPCRs. Some GPCRs, such as vasopressin V2–, AT_1-, and bradykinin B_2–receptor subtypes, were reported to internalize upon antagonist binding (36 – 38). Pheng et al. (39) also reported that the binding of the Y_1-receptor antagonist, GR231118 induced time-dependent internalization of Y_1 receptors in HEK293 cells, and this process was mediated in part by clathrin-dependent and G-protein independent mechanisms.

In the present study, the N111G mutant of AT_1 receptor showed higher basal IP production without agonist-induced stimulation, which indicates that it might act like a CAM receptor. More interestingly, N111G showed very low internalization from the cell surface compared to WT AT_1 receptor. On the other hand, this mutant showed binding characteristics with Ang II and ARBs similar to those of the WT. The possible reason of these differential findings may be decreased size of the side chain due to the mutation of asparagine to glycine, which makes the receptor unable to bind with adaptor protein, resulting in the inability of the mutant receptor to undergo internalization and thus increased the amount of receptor protein on the cell surface. This in turn promotes higher IP production without agonist stimulation. Thus, our present study suggests that higher signal transduction by the mutant may be due to lack of sufficient internalization of the receptor from the cell surface. An earlier study (9) suggested that the conformation induced in the N111G mutant AT_1 receptor mimics the partially activated state (R') and transition to the fully activated conformation no longer requires Tyr^4 of Ang II. In the native AT_1 receptor, the R' conformation is generated by replacement with residues smaller than Asn^{111}. Thus, when valsartan is used, the active conformation of the N111G mutant is again changed to the basal state of the receptor by the mechanism of inverse agonist activity of ARBs, which may in turn increase internalization of the receptor into the cell.

In summary, the N111G mutant AT_1 receptor can undergo ligand-induced internalization following stimulation of the ARB valsartan in COS-7 cells. If these results are applicable in vivo, the study may suggest that chronic treatment with valsartan may induce cell surface receptor losses, leading to apparent conditional knockout of receptor activity, this possibly being of clinical significance.

References

1 Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, et al. The sequence of the human genome. Science. 2001;291:1304–1351.

2 Whitebread S, Mele M, Kamber B, DeGasparo M. Preliminary biochemical characterization of two angiotensin II receptor subtypes. Biochem Biophys Res Commun. 1989;163:284–291.

3 Chiu AT, Herblin WF, McDall DE, Ardecky RJ, Carini DJ, Duncia JV, et al. Identification of angiotensin receptor subtypes. Biochem Biophys Res Commun. 1989;165:196–203.

4 Bumpus FM, Catt KJ, Chiu AT, DeGasparo M, Godfraind T, Husain A, et al. Nomenclature for angiotensin receptors. A report of Nomenclature Committee of the Council for High Blood Pressure Research. Hypertension. 1999;17:720–721.

5 Matsusaka T, Ichikawa I. Biological functions of angiotensin and its receptors. Annu Rev Physiol. 1997;59:395–412.

6 Noda K, Saad Y, Kinoshita A, Boyle TP, Graham RM, Husain A, et al. Tetrazole and carboxylate groups of angiotensin receptor antagonists bind to the same subsite by different mechanisms. J Biol Chem. 1995;270:2284–2289.

7 Bhuiyan MA, Ishiguro M, Hossain M, Nakamura T, Ozaki M, Miura S, et al. Binding sites of valsartan, candesartan and losartan with angiotensin II receptor 1 subtype by molecular modeling. Life Sci. 2009;85:136–140.

8 Noda K, Feng YH, Liu XP, Saad Y, Husain A, Karnik SS. The active state of the AT_1 angiotensin receptor is generated by angiotensin II induction. Biochemistry. 1996;35:16435–16442.

9 Feng YH, Miura S, Hussain A, Karnik SS. Mechanism of constitutive activation of the AT_1 receptor: influence of the size of the agonist switch binding residue Asn(111). Biochemistry. 1998;37:15791–15798.

10 Feng YH, Ding Y, Ren S, Zhou L, Xu C, Karnik SS. Unconventional homologous internalization of the angiotensin II type-1 receptor induced by G-protein-independent signals. Hypertension. 2005;46:419–425.

11 Ferguson SS. Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. Pharmacol Rev. 2001;53:1–24.

12 Kohout TA, Leftkowitz RJ. Regulation of G-protein couple receptor kinases and arrestins during receptor desensitization. J Mol Pharmacol. 2003;63:9–18.

13 Lodowski DT, Pitcher JA, Capel WD, Leftkowitz RJ, Tesmer JJ. Keeping G-proteins at bay: a complex between G-protein couple receptor kinase 2 and Gbetagamma. Science. 2003;300:1256–1262.

14 Hertel C, Coulter S, Perkins JP. A comparison of catecholamine-induced internalization of beta-adrenergic receptors and receptor-mediated endocytosis of epidermal growth factor in human astrocytoma cells. Inhibition by phenylarsine oxide. J Biol Chem. 1985;260:12547–12553.

15 Waldo GL, Northup JK, Perkins JP, Harden TK. Characterization of an altered membrane form of the beta-adrenergic receptor produced during agonist-induced desensitization. J Biol Chem. 1983;258:13900–13908.

16 Gagnon AE, Kallal L, Benovic JL. Role of clathrin-mediated endocytosis in agonist-induced down-regulation of the beta2-adrenergic receptor. J Biol Chem. 1998;273:6976–6981.

17 Tsao P, Cao T, Von Zastrow M. Role of endocytosis in mediating downregulation of G-protein-coupled receptors. Trends Pharmacol Sci. 2001;22:91–96.

18 Zhang J, Barak LS, Winkler KE, Caron MG, Ferguson SS. A central role for beta-arrestins and clathrin-coated vesicle-mediated endocytosis in beta2-adrenergic receptor resensitization. Differential regulation of receptor resensitization in two

distinct cell types. J Biol Chem. 1997;272:27005–27014.
19 Claing A, Perry SJ, Achiriloaie M, Walker JK, Albanesi JP, Lefkowitz RJ, et al. Multiple endocytic pathways of G protein-coupled receptors delineated by GIT1 sensitivity. Proc Natl Acad Sci U S A. 2000;97:1119–1124.
20 Claing A, Laporte SA, Caron MG, Lefkowitz RJ. Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and β-arrestin proteins. Prog Neurobiol. 2002;66:61–79.
21 Ferguson SS, Barak LS, Zhang J, Caron MG. G-protein-coupled receptor regulation: role of G-protein-coupled receptor kinases and arrestins. Can J Physiol Pharmacol. 1996;74:1095–1110.
22 Krupnick JG, Benovic JL. The role of receptor kinases and arrestins in G protein-coupled receptor regulation. Annu Rev Pharmacol Toxicol. 1998;38:289–319.
23 Lefkowitz RJ. G protein-coupled receptors. III. New roles for receptor kinases and β-arrestins in receptor signaling and desensitization. J Biol Chem. 1998;273:18677–18680.
24 Pitcher JA, Freedman NJ, Lefkowitz RJ. G protein-coupled receptor kinases. Annu Rev Biochem. 1998;67:653–669.
25 Jongejan A, Bruysters M, Ballesteros JA, Haaksma E, Bakker RA, Pardo L, et al. Linking agonist-binding to histamine H_1 receptor activation. Nat Chem Biol. 2005;1:98–103.
26 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193:265–275.
27 Miura S, Fujino M, Hanzawa H, Kiya Y, Imaizumi S, Matsuo Y, et al. Molecular mechanism underlying inverse agonist of angiotensin II type I receptor. J Biol Chem. 2006;281:19288–19295.
28 John H, Jennifer NH, Steven JF, Daniel KY. Identification of angiotensin II type 2 receptor domains mediating high-affinity CGP 42112A binding and receptor activation. J Pharmacol Exp Ther. 2001;298:665–673.
29 Miura S, Karnik SS. Constitutive activation of angiotensin II type 1 receptor alters the orientation of transmembrane helix-2. J Biol Chem. 2002;277:24299–24305.
30 Miura S, Feng YH, Hussain A, Karnik SS. Role of aromaticity of agonist switches of angiotensin II in the activation of the AT_1 receptor. J Biol Chem. 1999;274:7103–7110.
31 Modrall JG, Nanamori M, Sadoshima J, Barnhart CB, Stanley JC, Neubig RR. ANG II type 1 receptor downregulation does not require receptor endocytosis or G protein coupling. Am J Physiol Cell Physiol. 2001;281:C801–C809.
32 Crozat A, Penhoat A, Saez JM. Processing of angiotensin II (A-II) and (Sar^1, Ala^8) A-II by cultured bovine adrenocortical cells. Endocrinology. 1986;118:2312–2318.
33 Cheng Y-C, Prusoff WH. Relationship between the inhibition constant (Ki) and the concentration of inhibition, which causes 50% inhibition (IC_{50}) of an enzymatic reaction. Biochem Pharmacol. 1973;22:3099–3108.
34 Le MT, Vanderheyden PM, Szaszak M, Hunyady L, Kersemans V, Vauquelin G. Peptide and nonpeptide antagonist interaction with constitutively active human AT_1 receptors. Biochem Pharmacol. 2003;65:1329–1338.
35 Miura S, Kiya Y, Kanazawa T, Imaizumi S, Fujino M, Matsuo Y, et al. Differential bonding interactions of inverse agonists of angiotensin II type 1 receptor in stabilizing the inactive state. Mol Endocrinol. 2008;22:139–146.
36 Pfeiffer R, Kirsch J, Fahrenholz F. Agonist and antagonist-dependent internalization of the human vasopressin V2 receptor. Exp Cell Res. 1998;244:327–339.
37 Hunyady L. Molecular mechanisms of angiotensin II receptor internalization. J Am Soc Nephrol. 1999;10:S47–S56.
38 Houle S, Larrivee JF, Bachvarova M, Bouthillier J, Bachvarov DR, Marceau F. Antagonist-induced intracellular sequestration of rabbit bradykinin B(2) receptor. Hypertension. 2000;35:1319–1325.
39 Pheng LH, Dumont Y, Fournier A, Chabot JG, Beaudet A, Quirion R. Agonist- and antagonist-induced sequestration /internalization of neuropeptide Y Y1 receptors in HEK293 cells. Br J Pharmacol. 2003;139:695–704.