Assessment of Homologous Internalization of Constitutively Active N111G Mutant of AT1 Receptor

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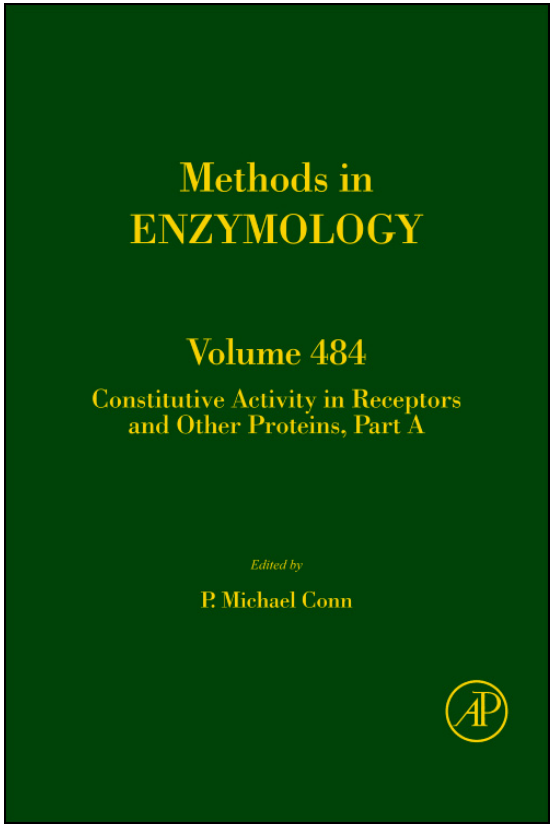

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CHAPTER NINE

Assessment of Homologous Internalization of Constitutively Active N111G Mutant of AT_1 Receptor

Mohiuddin Ahmed Bhuiyan[*,†] *and* Takafumi Nagatomo[†]

Contents

Abstract

Constitutively active mutants (CAMs) of G-protein-coupled receptors mimic the active conformation of the receptor in their ability to activate second messenger systems in the absence of agonist. They have revealed novel properties of drugs that reverse the basal levels of constitutive activity, indicating that the drugs have the inverse agonist activity. Internalization plays an important role in receptor endocytosis and signal transduction. The present chapter provides the investigation of the internalization behavior of CAM N111G of Angiotensin II type 1 (AT_1) receptor and correlates the result with the mechanism of constitutive activity of the mutant. Both wild-type (WT) and N111G mutant receptors were transiently expressed in COS-7 cells and total inositol phosphate production was measured in presence and absence of the angiotensin II receptor blockers (ARBs). The binding affinities toward agonist and ARBs were also determined. We found that the ARBs have the inverse agonist activity in CAM N111G of AT_1

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receptor. The internalization of the mutant, which was much lower than WT receptor, was significantly increased in presence of the ARBs. The results indicate that internalization of CAM N111G of AT_1 receptor is induced by the ARBs, which may be an important characteristic of inverse agonist activities of the ARBs in N111G.

1. Introduction

G-protein-coupled receptors (GPCRs) form one of the largest protein families, with several hundred members in humans (Venter *et al.*, 2001). 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 receptors are the members of the GPCR superfamily. Two subtypes of angiotensin II receptors have been identified (Chiu *et al.*, 1989; Whitebread *et al.*, 1989) and pharmacologically characterized, designated as angiotensin II type 1 and type 2 (AT_1 and AT_2) receptors (Bumpus *et al.*, 1999). Interaction of angiotensin II (Ang II) with the AT_1 receptor induces vasoconstriction, sodium reabsorption, and stimulation of aldosterone release (Matsusaka and Ichikawa, 1997).

Mutagenesis studies of the AT_1 receptor have recently identified amino acid residues important in the binding of the natural ligand, Ang II and nonpeptide 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 (Noda *et al.*, 1995) and nonpeptide (Bhuiyan *et al.*, 2009) ligands. Ang II contains two residues, Tyr^4 and Phe^8, which are essential for agonism (Noda *et al.*, 1996). 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 (Feng *et al.*, 1998). This 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 (Noda *et al.*, 1996). 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 inactive (R) to active state (R^*) and allow receptor activation. Small side chain (glycine) substitution 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 (Feng *et al.*, 2005).

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 (Ferguson, 2001; Kohout and Lefkowitz, 2003; Lodowski *et al.*, 2003). Homologous internalization of GPCRs can also take place through β-arrestin-independent pathway. Initially, internalization of the GPCRs was viewed as a mean to uncouple the receptor from its signaling components, thereby dampening the overall response (Gagnon *et al.*, 1998; Hertel *et al.*, 1985; Tsao *et al.*, 2001; Waldo *et al.*, 1983). The results of many studies indicate that the itinerary of the internalized GPCR is receptor- and cell-specific (Zhang *et al.*, 1997). At least four pathways of agonist-induced internalization of GPCRs exist (Claing *et al.*, 2000, 2002), 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 (Claing *et al.*, 2000, 2002; Ferguson, 2001; Ferguson *et al.*, 1996; Krupnick and Benovic, 1998; Lefkowitz, 1998; Pitcher *et al.*, 1998). Thus in this chapter, we described the investigation about the binding profiles of both WT and N111G mutant of AT_1 receptors with AT_1 receptor agonist and ARBs, such as 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 in N111G mutant. Finally, we examined the internalization of the specified receptors and correlated the result with the mechanism of constitutive activity of N111G mutant.

2. Preparation of Receptor Plasmid and Protein

2.1. Site-directed mutagenesis and plasmid preparation

The synthetic rat AT_1 receptor gene, cloned in the shuttle expression vector pMT-2, was used for expression. Site-directed mutagenesis was performed on the WT AT_1 receptor by polymerase chain reaction (PCR) method with the QuichChange Site-Directed Mutagenesis Kit (Stratagene, CA, USA) according to the protocol of the manufacturer as described in earlier studies (Jongejan *et al.*, 2005). Briefly, forward and reverse oligonucleotides were constructed to introduce desired mutation. PCR products were purified and finally DNA sequence analysis was done to confirm the site-directed mutation. Receptor plasmid was prepared using Midiprep kit (BIO-RAD, CA, USA) after transformation in XL1-Blue supercompetent cells through heat pulse according to manufacturer's protocol.

2.2. 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 transiently 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 buffer saline (PBS), pH 7.4; following two times wash by PBS. The cells were centrifuged at 3000 rpm for 10 min at 4 °C and the cell pellets were washed by Hank's buffered salt solution (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 (Kinematica, Switzerland) for 10 s. The mass was centrifuged at 4 °C for 5 min at 1260 × *g* and the supernatant containing the membrane fraction was ultra centrifuged at 4 °C for 20 min at 30,000 × *g*. The resulting pellets were suspended in binding assay buffer containing 20 m*M* phosphate buffer, 100 m*M* sodium chloride, 20 m*M* magnesium chloride, 1 m*M* EGTA, and 0.2% BSA, pH 7.4 and used for binding experiments. The protein contents of the membranes were measured by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

3. Radioligand Binding Assay

Binding assays for WT and N111G mutant of AT_1 receptors were carried out in incubation tube that contained 10 μg of membrane protein, [^{125}I]-Sar^1-Ile^8-Ang II (Perkin Elmer, Inc., Boston, USA), unlabeled drug as required, and binding buffer in a final volume of 125 μl. Both saturation and competition binding assays were carried out as described previously (John *et al.*, 2001; Miura *et al.*, 2006). Briefly, for saturation binding studies, six to seven concentrations (5–800 p*M*) of [^{125}I]-Sar^1-Ile^8-Ang II were tested in duplicate. Nonspecific binding was defined as the amount of radioligand binding remaining in presence of 10 μM Ang II (Peptide Institute Inc. Japan). For competition binding studies, membranes were incubated with 250 p*M* of [^{125}I]-Sar^1-Ile^8-Ang II and different concentrations (10^{-4} to 10^{-11} *M*) of unlabelled drugs, such as candesartan (Takeda Chemical Industries Ltd., Japan), losartan (Merck Research Laboratories, NJ, USA), telmisartan (Nippon Boehringer Ingelheim, Japan), and valsartan (Novartis Institutes for BioMedical Research, Inc., Cambridge, MA, USA)

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 m*M* Tris–HCl (pH 8.0). The bound ligand fraction was determined from the counts/min remaining on the membrane in γ-counter.

[^{125}I]-Sar^1-Ile^8-Ang 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 n*M*, respectively (Table 9.1). B_{max} values for the WT receptor and N111G mutant were calculated from the maximal specific binding of [^{125}I]-Sar^1-Ile^8-Ang II as 1.52 ± 0.07 and 0.68 ± 0.12 pmol/mg of protein, respectively (Table 9.1). The receptor expression for the mutant was decreased significantly compared to WT AT_1 receptors ($P < 0.001$). Table 9.2 shows the binding affinities (pK_i) for agonist and ARBs, valsartan, losartan, candesartan, and telmisartan, toward WT and N111G mutant of AT_1 receptors. The binding affinity of Ang II to the mutant N111G was markedly increased compared to WT receptor ($P < 0.05$) (Table 9.2, Fig. 9.1). Binding affinities of ARBs were two- to threefold decreased to the mutant N111G compared to AT_1 WT receptor

Table 9.1 Dissociation constant (K_d) and maximum binding sites (B_{max}) of [^{125}I]-Sar^1-Ilu^8-angiotensin II for wild-type and mutant N111G of AT_1 receptors

	AT_1 wild-type receptor	N111G mutant receptor
K_d value (n*M*)	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-Ile-Angiotensin II was used to label AT_1 wild-type and N111G mutant receptors transiently expressed in COS-7 cells. Data represent the mean ± SEM of four independent experiments, each performed in duplicate. **$P < 0.001$ versus wild-type.

Table 9.2 Binding affinities (K_i) in n*M* of agonists and antagonists to wild-type and N111G mutant of AT_1 receptors

	Wild type	N111G Mutant
Agonist		
Angiotensin 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-Ile^8-Angiotensin II (250 p*M*) was used to label AT_1 wild-type and N111G mutant receptors transiently expressed in COS-7 cells. Data represent the mean ± SEM of four independent experiments, each performed duplicate. *$P < 0.05$; **$P < 0.001$ versus wild type.

($P < 0.05$; Table 9.2) although candesartan showed almost no change in binding affinity to the mutant.

4. Inositol Phosphate Accumulation Assay

COS-7 cells at about 90% confluent in 10-cm dishes were seeded into 24-well plates taking about 10^5 cells per well. After 24 h the cells were transfected transiently using Lipofectamine™ 2000 with plasmid DNA of

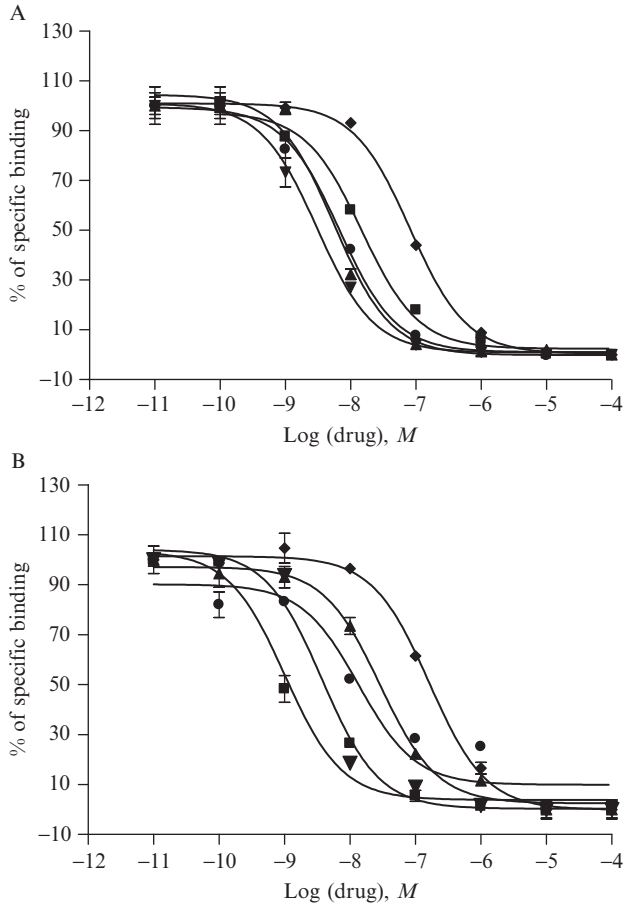

Figure 9.1 Determination of binding affinities (pK_i) of Ang II (■), candesartan (▼), losartan (◆), valsartan (▲), and telmisartan (●) to the (A) wild-type and (B) N111G mutant of AT_1 receptors as assessed by [^{125}I]-Sar^1-Ile^8-Angiotensin II radioligand.

both WT and N111G mutant of AT_1 receptors. Twenty-four hours after transfection the cells were labeled with 1 μCi/ml [^{3}H]myo-inositol (Amersham Biosciences, NJ, USA) in DMEM and incubated for 20 h at 37 °C in 5% CO_2. The cells were washed with HBSS and exposed with HBSS containing 20 m*M* phosphate buffer and 20 m*M* LiCl, pH 7.4 for 30 min at 37 °C. Agonist and four ARBs were added to each well and the incubation was continued for an additional 1 h at 37 °C. At the end of the incubation, the medium was removed, and the reaction was stopped by adding 1 ml of 10 m*M* formic acid (previously stored at 4 °C) to each well. The cells were then neutralized by 1 ml 500 m*M* KOH and 9 m*M* sodium tetraborate per well. The contents of each well were extracted and centrifuged for 5 min at 1400× *g* and the upper layer was transferred to a 1 ml AG1-X8 resin (100–200 mesh; BIO-RAD Laboratories, Inc., CA, USA) loaded column. The columns were washed two times with 5 ml 60 m*M* sodium formate and 5 m*M* borax. Total soluble IP was eluted with 5 ml 1 *M* ammonium formate and 0.1 *M* formic acid. Radioactivity was measured by liquid scintillation counter.

The cells expressing N111G mutant exhibited higher levels of agonist-independent (basal) IP production (Fig. 9.2A) showing constitutive activity. A decrease in the size of Asn^{111} side chain induces an intermediate activated receptor conformation, which may be responsible for the constitutive activity of N111G mutant (Noda *et al.*, 1996). IP production by the cells expressing N111G mutant of AT_1 receptor was markedly decreased when incubated in presence of the ARBs used in the study (Fig. 9.2B) confirming the inverse agonist activity of the ARBs.

5. Internalization Assay

Internalization assay was performed as described previously (Modrall *et al.*, 2001). Briefly, COS-7 cells in 12-well plates that had been transiently transfected were incubated separately at 37 °C in serum-free DMEM with 1 μ*M* Ang II or 1 μ*M* ARBs for 0, 2, 5, 10, 15, 30, and 45 min. The cells were washed twice by ice-cold PBS and incubated for 3 h at 4 °C with 0.1 n*M* [^{125}I]-Sar^1-Ile^8-Ang II in binding buffer. The cells were then washed with ice-cold PBS and surface bound [^{125}I]-Sar^1-Ile^8-Ang II was removed using the acid wash technique of Crozat *et al.* (1986) in which cells were exposed to 150 m*M* NaCl, 50 m*M* glycine, pH 3, for 10 min at 4 °C. For cell lysis, 300 μl of 1 *M* NaOH solution was added and the content was neutralized by 300 μl of 1 *M* HCl. The cell-associated radioactivity was measured by δ-counting. Percent of internalization was calculated from the difference in cell surface binding values at different time interval considering the cell surface binding value at zero as 100%.

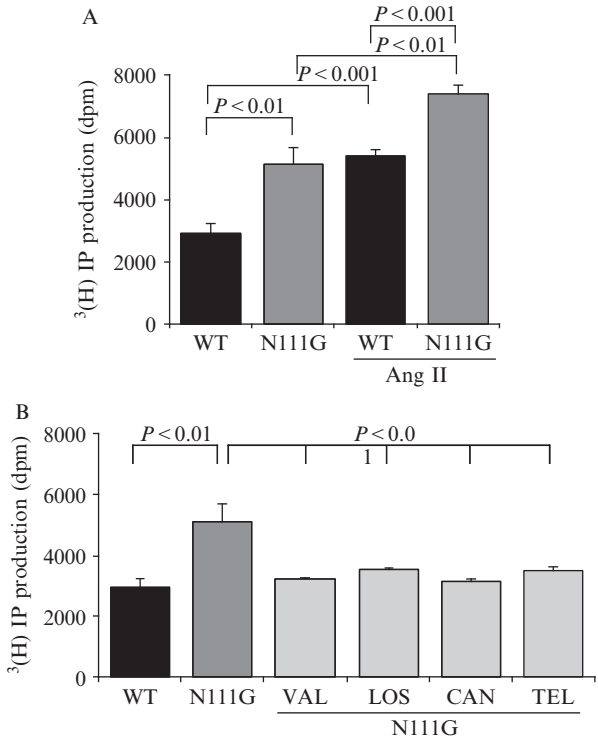

Figure 9.2 Effect of AT_1 receptor agonist and antagonists on inositol phosphate (IP) production by wild-type and N111G mutant of AT_1 receptors. (A) IP production in the absence (solid bar) and presence of 1 μM Ang II (gray bar) by COS-7 cells expressing wild-type and N111G mutant of AT_1 receptors. (B) Inhibition of IP production by 1 μM valsartan, losartan, candesartan, and telmisartan, respectively (light gray bars). Data are the mean ± SEM of 4–6 independent experiments, each performed in duplicate. Student's *t*-test was performed for statistical analyses.

Treatment of the WT and N111G mutant of AT_1 receptors with 1 μM Ang II for 45 min induced 74.63 ± 1.00% and 19.56 ± 2.87% receptor internalization, respectively (Table 9.3, Fig. 9.3). The mutant N111G showed very low internalization (only 26%) compared to WT AT_1 receptor after 45 min (Table 9.3, Fig. 9.3) and is unable to bind with adaptor protein that may cause the mutant receptor not to be internalized and increase in the receptor protein in the cell surface. This in turn promotes higher IP production without agonist stimulation. However, it is very interesting that the internalization of N111G mutant significantly increased in presence of the ARBs of this study. The ARBs may cause the change in the conformation of CAM N111G of AT_1 receptor from active to inactive state to promote internalization, which is consistent with the mechanism of inverse agonists of constitutive active GPCRs. On the other hand, there was

Table 9.3 Internalization of AT_1 wild-type and N111G mutant of AT_1 receptor without and with different AT_1 receptor antagonists

Receptor	Treatment	% of internalization (after 45 min)	K_e (h^{-1})
AT_1 WT	Ang II	74.63 ± 1.00	0.87 ± 0.37
	Valsartan	70.97 ± 1.35	0.65 ± 0.23
N111G	Ang II	19.56 ± 2.87*	0.41 ± 0.26
	Valsartan	63.22 ± 0.38*	0.24 ± 0.13
	Candesartan	62.43 ± 0.68*	0.29 ± 0.14
	Losartan	54.78 ± 0.61*	0.43 ± 0.21
	Telmisartan	60.85 ± 0.66*	0.25 ± 0.10

K_e is the rate constant of internalization. The data are the mean ± SEM of four independent experiments, each performed in duplicate. Student's *t*-test was performed for statistical analyses. *A *P* value of less than 0.01 compared to AT_1 wild-type receptor was taken as significant.

no significant change in the internalization of the WT receptor after the use of valsartan with the receptor indicating that the internalization of WT receptor is not induced by the ARBs. Some GPCRs, such as vasopressin V2, AT_1, and bradykinin B_2 receptor subtypes, were reported to internalize upon antagonist binding (Houle *et al.*, 2000; Hunyady 1999; Pfeiffer *et al.*, 1998). Pheng *et al.* (2003) also reported that the binding of 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.

6. Western Blot Analysis

Equal amounts (20 μg) of whole cell lysates of the WT and mutant receptors were resolved by SDS–polyacrylamide gel electrophoresis and transferred onto Hybond ECL nitrocellulose membranes (Amersham Biosciences, DE, USA) using a semidry system (Trans-Blot SD Semi-dry Transfer Cell, BIO-RAD, USA) in immunotransfer buffer. The membranes were blocked with 10% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) by slow shaking 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, CA, USA) at 1:1000 dilutions in TBS-T with 1% milk for 1 h at room temperature, followed by incubation with HRP-conjugated anti-rabbit IgG (Promega, WI, USA) at 1:2000 dilutions in TBS-T with 1% milk overnight at 4 °C. The membranes were washed three times with TBS-T in each step and finally the blots on the membranes were visualized by adding Amersham ECL Western blotting detection reagent (GE Healthcare, UK). Western blot probed with anti-AT_1 antibody detected

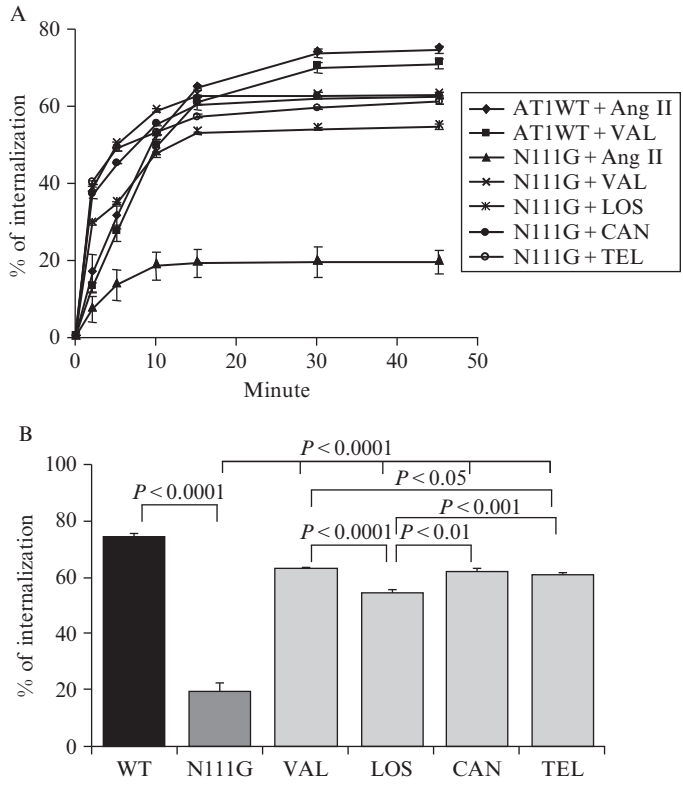

Figure 9.3 Internalization assay of wild-type and N111G mutant of AT_1 receptors. (A) % of internalization of wild-type AT_1 receptors treated with 1 μM Ang II and valsartan, and N111G mutant of AT_1 receptors treated with 1 μM Ang II and 1 μM ARBs. (B) % of internalization of wild-type (solid bar) and N111G mutant of AT_1 receptors in absence (gray bar) and presence of 1 μM ARBs (light gray bar) after 45 min of incubation. Internalization of ^{125}I-labeled Sar^1-Ile^8-angiotensin II at 37 °C by wild-type and N111G mutant of AT_1 receptors was determined as described in Materials and Methods. Data are the mean ± SEM of four independent experiments, each performed in duplicate. Student's *t*-test was performed for statistical analyses.

the specified protein of the receptors and immunoreactive bands were observed at 43 kDa both in WT and N111G mutant of AT_1 receptors (Fig. 9.4).

7. Data Analysis

Nonlinear regression analyses of saturation and competition binding assay were performed using GraphPad Prism software (San Diego, CA, USA). The results of the experiments were expressed as the mean ± SEM.

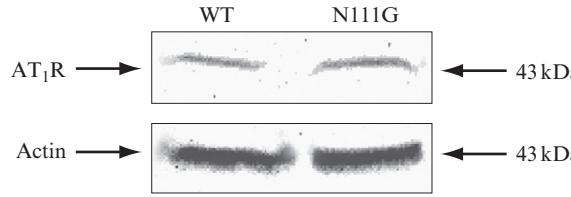

Figure 9.4 Western blot analysis showing the band of AT_1 receptor and actin for both wild-type and N111G mutant of AT_1 receptors.

In competition binding experiments, the values of inhibition constants (K_i) were calculated by the following equation (Cheng and Prusoff, 1973):

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

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

8. Concluding Remark

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. 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. The results demonstrate that asparagine at position 111 of TMD III of AT_1 receptor is very important site for both agonist and antagonist binding. N111G mutant of AT_1 receptor can undergo ligand-induced internalization following stimulation of ARBs. If these results are applicable *in vivo*, the study can suggest that chronic treatment with the ARBs may induce cell surface receptor losses, leading to apparent conditional knock out of receptor activity, this possibly being of clinical significance of the present study.

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