Manifold active-state conformations in GPCRs: Agonist-activated constitutively active mutant AT_1 receptor preferentially couples to Gq compared to the wild-type AT_1 receptor

ChangWoo Lee[a,c,*], Si Ae Hwang[c], Sei-Heon Jang[c], Hye-Shin Chung[d], Manjunatha B. Bhat[b], Sadashiva S. Karnik[a,*]

[a] Department of Molecular Cardiology, The Cleveland Clinic Foundation, Cleveland, OH 44195, USA
[b] Center for Anesthesiology Research, The Cleveland Clinic Foundation, Cleveland, OH 44195, USA
[c] Department of Genetic Engineering, Daegu University, Gyeongsan 712-714, Republic of Korea
[d] Department of Biotechnology, Hannam University, Daejon 305-811, Republic of Korea

Abstract The angiotensin II type I (AT_1) receptor mediates regulation of blood pressure and water-electrolyte balance by Ang II. Substitution of Gly for Asn^{111} of the AT_1 receptor constitutively activates the receptor leading to Gq-coupled IP_3 production independent of Ang II binding. The Ang II-activated conformation of the $AT1_{N111G}$ receptor was proposed to be similar to that of the wild-type AT_1 receptor, although, various aspects of the Ang II-induced conformation of this constitutively active mutant receptor have not been systematically studied. Here, we provide evidence that the conformation of the active state of the wild-type and the constitutively active AT_1 receptors are different. Upon Ang II binding an activated conformation of the wild-type AT_1 receptor activates G protein and recruits β-arrestin. In contrast, the agonist-bound $AT1_{N111G}$ mutant receptor preferentially couples to Gq and is inadequate in β-arrestin recruitment.
© 2007 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Angiotensin II receptor; G protein; β-Arrestin

1. Introduction

The angiotensin II type I (AT_1) receptor belongs to the G protein-coupled receptor (GPCR) family and plays an important role in blood pressure regulation and cardiomyocyte growth [1,2]. The octapeptide hormone angiotensin II (Ang II) binds to the AT_1 receptor and generates diverse signals such as intracellular production of inositol-1,4,5-triphosphates (IP_3) and activation of cytoplasmic kinases, mitogen-activated protein kinase, Src, and Rho [2]. Mutations of GPCRs, either found in humans or artificially generated, provide important clues to the understanding of structure/function relationship of the receptor [3]. Many of those mutations cause constitutive activation of the receptor. Since the first description of the constitutive activity of $α_1$-adrenergic receptor [4], constitutively active mutations were found in several GPCRs from clinical studies and provided valuable insights into the mechanisms of GPCR activation [5]. Constitutively active mutants (CAM) of human AT_1 receptor are not yet reported presumably due to its importance in regulating blood vessel constriction during fetal growth. However, by site-directed mutagenesis, a mutation of the residue Asn^{111} located in the TM3 of the AT_1 receptor, when substituted with a Gly was shown to cause ≈40% increase of IP_3 level in the absence of the receptor agonist, Ang II [6]. The shorter the side chain of amino acid residue substituted at the position of Asn^{111}, the greater the degree of constitutive activation [7].

The CAM receptors including the $AT1_{N111G}$ receptor are considered to be mimicking the active conformations of the wild-type receptors in that the mutations disrupt the constraining interactions between the TM domains. Activation of the AT_1 receptor requires loss of constraining interactions between TM2 and TM7 [8,9]. Additional constraints have been suggested, for instance between TM3 and TM7 [10–12]. These conclusions are largely based upon experiments measuring one or the other secondary messenger accumulation in the cell. Such a comparison may be inadequate and may mask the potential difference between agonist-activated conformation of wild-type and CAM receptors. For instance, study on the luteinizing hormone (LH) receptor showed that coupling of the constitutively active mutant LHR_{D578G} to cAMP production is different from that of the wild-type receptor when the 76 amino acid-long C-terminal peptide of the G_{i2} protein was co-expressed, suggesting that the two types of LH receptors have distinct mechanisms of cAMP induction [13]. Unfortunately, the agonist-induced active conformations of the constitutively active GPCRs in terms of coupling to signaling molecules other than heterotrimeric G proteins have not been explored.

In the current study, we sought to elucidate the Ang II-induced active conformations of the wild-type and the CAM AT_1 receptors with regards to coupling preferences to Gq and β-arrestin 1 in COS-1 cells. We show that the Ang

*Corresponding authors. Address: Department of Molecular Cardiology, The Cleveland Clinic Foundation, Cleveland, OH 44195, USA. Fax: +1 216 444 9263 (S.S. Karnik).
E-mail addresses: karniks@ccf.org (S.S. Karnik), leec@daegu.ac.kr (C. Lee).

II-AT1$_{WT}$ and Ang II-AT1$_{N111G}$ differ in this assay with consequence on function, for instance calcium homeostasis.

2. Materials and methods

2.1. Materials

The cDNA clone for human Gq-alpha dominant-negative mutant (Q209L/D277N) was obtained from the UMR cDNA Resource Center (www.cdna.org). [Sar^1]Ang II was purchased from Bachem, Torrance, CA. Monoclonal antibodies to HA and Gq were purchased from Roche and Cell Signaling Technology, respectively. Monoclonal antibody to β-arrestin 2 (H-9) was purchased from Santa Cruz. Since this anti-β-arrestin 2 antibody recognized both β-arrestin 1 and 2, we used this antibody for the detection of transfected β-arrestin 1 in our experiment. COS-1 cells are from American Type Culture Collection, Rockville, MD. All other reagents unless stated otherwise were from Sigma.

2.2. Cell culture and expression of the AT$_1$ receptor

The synthetic rat AT$_1$ receptor gene, cloned in the shuttle expression vector pMT3, was used for expression. Creation of the constitutively active AT$_1$ receptor mutant AT1$_{N111G}$ by site-directed mutagenesis was described in a previous study [6]. To express the AT$_1$ receptor protein with Gq or β-arrestin 1, 60–65% confluent COS-1 cells grown in 10-cm petri dish were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were transfected with 10 μg of purified plasmid DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.3. Immunoprecipitations and Western blotting

Transfected cells were cultured for 48 h, treated with 1 μM [Sar^1]AngII for 1 h, and then washed with cold PBS. Cells were scraped and suspended in lysis buffer (25 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 1 mM PMSF, 2 mM AEBSF, 130 μM bestatin, 14 μM E-64, 1 μM leupeptin, 0.3 μM aprotinin, 25 mM β-glycerophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 10% glycerol) for 30 min. Cell lysates were centrifuged at 13 000 rpm for 10 min at 4 °C to remove cell debris. Anti-HA antibody was added to the supernatant with 20 μl of Protein G-Sepharose (GE Healthcare) to immunoprecipitate the HA-AT$_1$ receptors. After overnight incubation at 4 °C on a rocker platform, the immunoprecipitates were collected by centrifugation at 10 000 rpm for 1 min at 4 °C. Pellets were dissolved in Laemmli's sample buffer, boiled for 5 min at 95 °C, and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis with a 10% separation gel. Following electrophoresis the proteins were transferred to nitrocellulose membranes and then blocked for 1 h at room temperature in 5% non-fat dry milk and 0.1% Tween-20 in PBS, pH 7.4. Incubation with anti-Gq antibody or anti-β-arrestin antibody was carried out overnight at 4 °C. Following washes with PBS, incubation with horseradish peroxidase-conjugated secondary antibody was carried out for 1 h at room temperature. The detection was made with enhanced chemiluminescence (GE Healthcare) and the films were scanned for densitometry analysis. Student's t-test was used for statistical analysis in Figs. 2 and 3.

2.4. Intracellular Ca^{2+} measurement

Changes in the cytoplasmic Ca^{2+} were measured using the fluorescent calcium indicator dye Fura-2 (excitation max ∼340 nm and 380 nm, emission max 510 nm, Invitrogen). Transfected COS-1 cells growing on cover glasses in a 6-well culture plate were loaded with 2 μM Fura-2 AM in BSS containing 0.1% Pluronic 127 and 2 mM Ca^{2+} at 37 °C for 30 min, followed by washing with BSS to remove the extracellular dye. Calcium measurements were done in single cells using an inverted microscope (Zeiss Axiovert 135) connected to a CCD camera (Photon Technology International). The data were collected at every 1.5 s interval and analyzed using the Image Master software (Photon Technology International). The release of intracellular Ca^{2+} in individual cells was measured after exposure to 1 μM [Sar^1]AngII in a Ca^{2+}-free balanced salt solution (BSS, 140 mM NaCl, 2.8 mM KCl, 2 mM MgCl$_2$, 0.5 mM EGTA, and 10 mM HEPES, pH 7.2) by rapid solution exchange. Results are presented as average changes in

the ratio of Fura-2 fluorescence upon excitation at 340 nm and 380 nm. Data from 6 to 8 cells were collected and plotted using Graph-Pad Prism software.

3. Results

3.1. Functional evidence for difference in Ang II-AT1$_{WT}$ and Ang II-AT1$_{N111G}$

To characterize the functional interaction of G-protein, Gq, and β-arrestin 1 with AT1$_{WT}$ and AT1$_{N111G}$ receptors, we examined Ang II-stimulated calcium release from intracellular calcium store. The HA-tagged AT1$_{WT}$ and AT1$_{N111G}$ receptors were expressed in COS-1 cells by transient transfection of expression plasmids. The Ca^{2+}-flux was measured by single cell imaging and the data were plotted (Fig. 1). The Ca^{2+}-flux signal of AT1$_{WT}$ treated with Ang II was 1.4-fold compared to baseline. The Ca^{2+}-flux signal of AT1$_{N111G}$ treated with Ang II was 1.3-fold compared to its baseline. A simple explanation for the lower Ca^{2+} response of the AT1$_{N111G}$ receptor could be that the constitutive IP$_3$ production causes constitutive decrease of the Ca^{2+} stores. However, similar to our finding, reduced Ca^{2+} response from Ang II-treated AT1$_{N111G}$ receptor was reported previously by Auger-Messier et al. [14]. They attributed the reduction to downregulation of the IP$_3$ receptors in HEK 293 cells and showed that the intracellular Ca^{2+} store is intact [14].

In the next set of experiments, the HA-tagged AT1$_{WT}$ and AT1$_{N111G}$ receptors were co-expressed with the dominant-negative mutant Gq (Q209L/D277N) and treated with 1 μM Ang II. The amplitude of Ca^{2+}-flux signals was smaller in the presence of the dominant-negative Gq mutant than respective controls. Interestingly, the onset of Ca^{2+}-release upon Ang II treatment was delayed (54.9 ± 0.6 s from the time of addition of Ang II) for COS-1 cells expressing the AT1$_{WT}$ receptor compared to the COS-1 cells expressing the AT1$_{N111G}$ mutant receptor (24.3 ± 1.6 s) when dominant-negative mutant Gq was expressed. This suggests that the dominant-negative Gq was more efficient in inhibiting the dynamic coupling between Gq and AT1$_{WT}$ receptor than Gq and AT1$_{N111G}$ mutant receptor.

It is well established that β-arrestin 1 is also a negative regulator of AT$_1$ receptor coupling to Gq [15,16]. Therefore, to test whether β-arrestin 1 over-expression inhibited Gq-mediated Ca^{2+}-signaling from the AT$_1$ receptor, the AT1$_{WT}$ receptor and AT1$_{N111G}$ was co-expressed with β-arrestin 1 in COS-1 cells. The mobilization of Ca^{2+} in cells with AT1$_{WT}$ receptor co-expressed with β-arrestin 1 was completely blocked. Compared to that the Ca^{2+} flux in the AT1$_{N111G}$ co-expressed with β-arrestin 1 was not completely abolished and furthermore the onset of Ca^{2+} release was delayed. This observation suggested that β-arrestin 1 prefers AT1$_{WT}$ rather than AT1$_{N111G}$ in the presence of Ang II.

3.2. Biochemical evidence for difference in Ang II-AT1$_{WT}$ and Ang II-AT1$_{N111G}$

To characterize the Ang II-induced conformational differences between AT1$_{WT}$ and AT1$_{N111G}$ receptors in binding the G protein and β-arrestin 1, we used receptor-Gq and receptor-β-arrestin 1 pull-down assays. The HA-tagged AT1$_{WT}$ and AT1$_{N111G}$ receptors were expressed in COS-1 cells with either
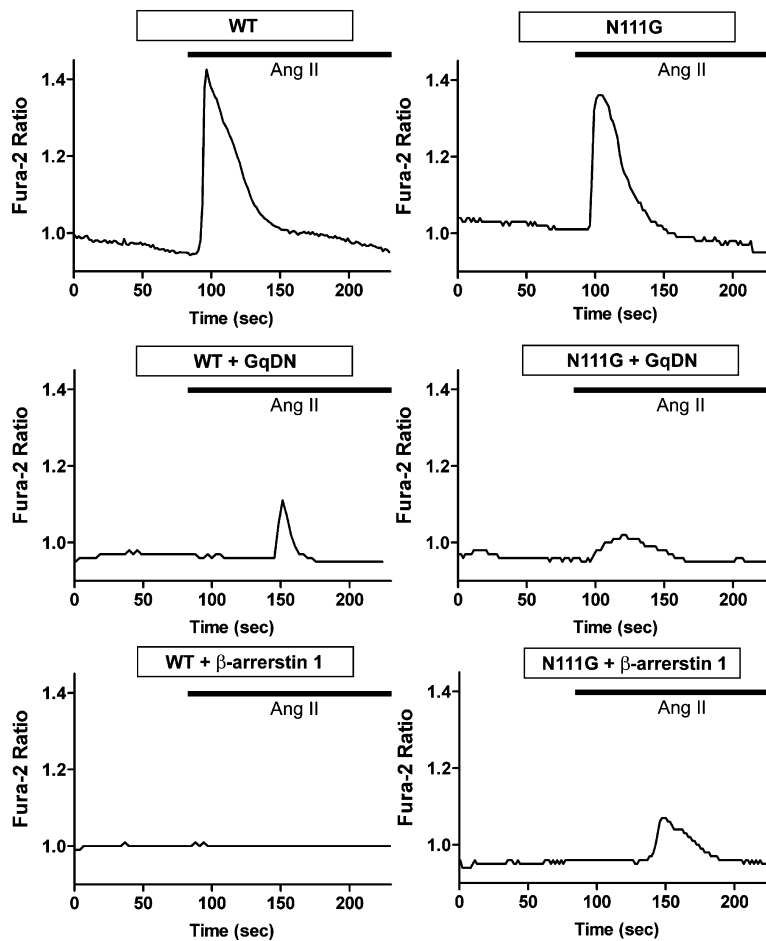
Fig. 1. Ca^{2+}-mobilization from the $AT1_{WT}$ receptor and the constitutively active $AT1_{N111G}$ receptor when β-arrestin 1 and a dominant-negative Gq (Q209L/D277N) was co-expressed, respectively. Delay of Ca^{2+}-response from the AT_1 receptor with GqDN or β-arrestin 1 was measured from the time point Ang II was added to the medium to the highest peak of each Ca^{2+}-response curve. Values are averages of 6–8 cells. Experiments were repeated three times.

Gq or β-arrestin 1, respectively. After cell lysis, receptor-Gq complexes were precipitated by anti-HA antibody, separated by SDS–PAGE, and transferred onto nitrocellulose membrane. The amount of Gq protein coupled to the $AT1_{WT}$ or $AT1_{N111G}$ mutant receptor were determined using anti-Gq antibody (Fig. 2A). The result shows that equivalent amounts of Gq are pre-coupled to the $AT1_{WT}$ receptor and the $AT1_{N111G}$ mutant receptor. Although coupling to Gq is similar under basal condition, upon ligand binding there is a 1.4-fold (1.39 ± 0.23, $P < 0.05$) increase of Gq coupling to the $AT1_{N111G}$ receptor whereas the amount of Gq coupled to the $AT1_{WT}$ receptor remained constant. These results suggest that the conformation of the $AT1_{N111G}$ is different from that of the $AT1_{WT}$ receptor in Gq-coupling. This observation is consistent with a previous study by Auger-Messier et al. that stimulation with Ang II stabilized the complex between the CAM $AT1_{N111G}$ and Gq [17]. To test the expression level of AT_1 receptors in transfection, the nitrocellulose membrane was stripped and reprobed with anti-HA antibody. The AT_1 receptor expression level was similar as shown in Fig. 2B.

To study the coupling of β-arrestin 1 to the $AT1_{WT}$ or the $AT1_{N111G}$ receptor, HA-tagged receptors were co-expressed with β-arrestin 1 in COS-1 cells. Upon Ang II binding, contrary to what was observed for Gq coupling to the receptor,

the amount of total β-arrestin 1 coupled to the $AT1_{WT}$ receptor were increased 2.2-fold (2.23 ± 0.75, $P < 0.05$) whereas there was 1.4-fold (1.41 ± 0.35, $P < 0.05$) increase of total β-arrestin 1-coupling to the $AT1_{N111G}$ receptor (Fig. 3A). A lower molecular weight band that reacting with anti-β-arrestin antibody was found in the pull-down assay. This band seems to be enhanced in cells expressing the $AT1_{WT}$ receptor treated with Ang II. We interpret this finding as a potential proteolysis event of β-arrestin 1 as has been described in the visual system [18]. Next, the nitrocellulose membrane was stripped and reprobed with anti-HA antibody to see the expression level of the AT_1 receptors in transfection. The overall expression level of the AT_1 receptors was similar among samples as shown in Fig. 3B. The amount of receptor monomers increased upon Ang II treatment in both $AT1_{WT}$ and $AT1_{N111G}$ receptors. The coupling difference between the wild-type and CAM AT_1 receptors is unlikely to be due to differences in monomer concentration. Increase in monomers upon receptor activation has been shown for other GPCRs [19–21] and this may be the case in AT_1 receptor system also. These results clearly support the idea that Ang II-activated conformation of the $AT1_{WT}$ receptor preferentially recruits β-arrestin 1 but the agonist-activated conformation of the $AT1_{N111G}$ receptor is deficient in this.
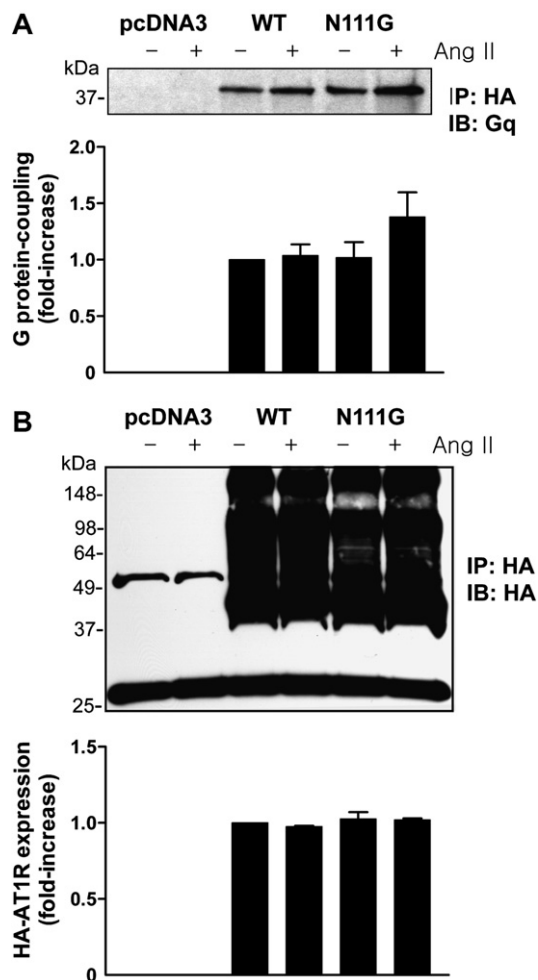
Fig. 2. Differences in Ang II-AT1$_{WT}$ and Ang II-AT1$_{N111G}$ for Gq-coupling. (A) Wild-type or mutant HA-AT1 receptors were co-expressed with Gq in COS-1 cells grown in 10-cm petri dish and stimulated for 1 h with 1 μM [Sar^1]AngII. Cells were lysed, immuno-precipitated with 10 μg of anti-HA antibody followed by SDS–PAGE and transferred onto nitrocellulose membrane. Gq-coupling to the AT$_1$ receptors was determined by immunoblotting with anti-Gq antibody (1:1000). Fold-increases were calculated compared to the wild-type AT$_1$ receptor without Ang II treatment. Data correspond to the means ± S.D. from three independent experiments. A representative blot is shown. (B) The nitrocellulose membrane was stripped with western blot stripping buffer (Pierce) and re-blotted with anti-HA antibody (1:1000) to see the amount of AT$_1$ receptor expression.

4. Discussion

The experiments presented above elucidate a previously un-known difference between Ang II-induced conformations of the wild-type and the CAM AT$_1$ receptors. Specifically, the agonist-bound AT1$_{N111G}$ mutant differs in recruiting β-arrestin due to preferential association with the G protein, Gq. In pre-vious studies these receptors were shown to efficiently activate the Gq-phospholipase C pathway leading to nearly similar lev-els of intracellular IP$_3$ production [6–10,14]. However, Thomas et al. demonstrated functional distinction in phosphorylation between the active-state conformations of the AT1$_{WT}$ and AT1$_{N111G}$ receptors [22]. Furthermore, Feng et al. demon-strated functional distinction in desensitization and EGFR trans-activation by AT1$_{WT}$ and AT1$_{N111G}$ receptors [23]. The
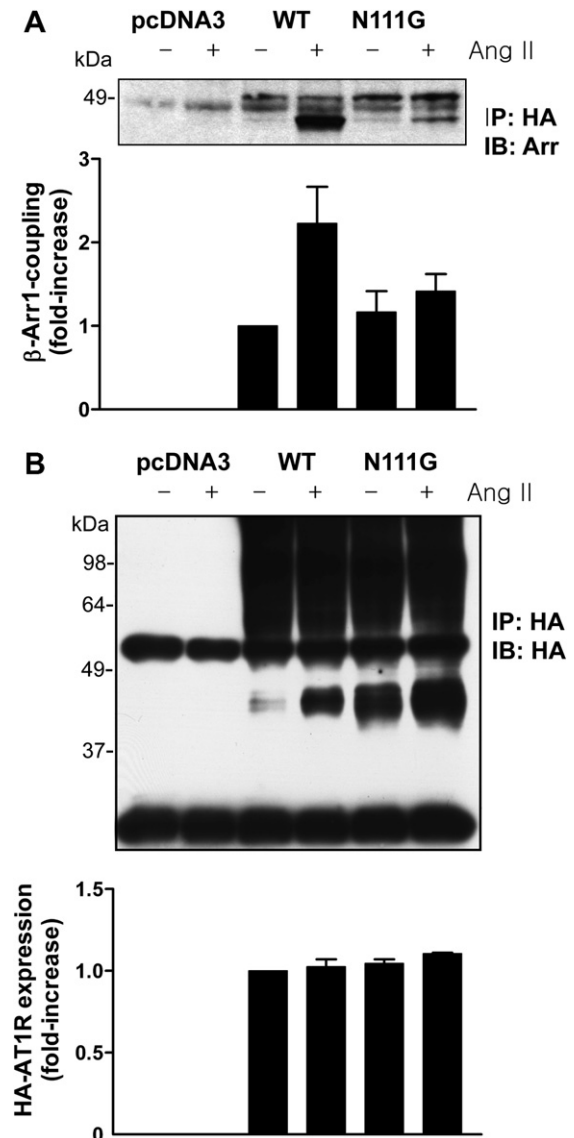

Fig. 3. Differences in Ang II-AT1$_{WT}$ and Ang II-AT1$_{N111G}$ for β-arrestin 1-coupling. (A) To see whether the coupling of β-arrestin 1 changes upon AT$_1$ receptor activation, wild-type or mutant HA-AT$_1$ receptors were co-expressed with β-arrestin 1 in COS-1 cells in 10-cm petri dish and stimulated for 1 h with 1 μM [Sar^1]AngII. Cell lysates were immunoprecipitated with 10 μg of anti-HA antibody followed by SDS–PAGE and transferred onto nitrocellulose membrane. β-Arrestin 1-coupling to the AT$_1$ receptors were determined by immunoblotting with anti-β-arrestin antibody (1:1000). Fold-increases were calculated compared to wild-type AT$_1$ receptor without Ang II treatment. Data correspond to the means ± S.D. from three independent experiments. A representative blot is shown. (B) The nitrocellulose membrane was stripped with western blot stripping buffer (Pierce) and re-blotted with anti-HA antibody (1:1000) to see the amount of AT$_1$ receptor expression.

data presented in this study clearly suggests that deactivation of their agonist-activated states differ. Hence the Ang II-in-duced conformation of the AT1$_{WT}$ and the AT1$_{N111G}$ recep-tors are different and in each instance resulting in difference in subsequent binding of intracellular proteins: the activated state in the wild-type receptor traverses to interact with β-arr-estin and in the AT1$_{N111G}$ receptor this transition is inept resulting in its preferential coupling to Gq.

It was observed that the CAM receptors exhibit increased basal activity and upon agonist binding the maximal activity of the CAM receptors reach 80–100% of the wild-type receptors. It was elucidated that the interactions between transmembrane domains are crucial in keeping the receptor in its inactive R conformation and the CAM receptors achieve the active R^* conformation with higher probability in the absence of agonists. Several models were suggested to explain the activation mechanism of the CAM AT_1 receptors [7,11,24]. Feng et al. have shown that the substitution of Asn^{111} appears to release the constraining intramolecular bonds between Asn^{111} and residues in TM7 [7]. Le et al. have shown that Ang II binding to the $AT1_{WT}$ receptor induces a pre-activated R' state of the receptor and further conformational changes affecting both Ang II and the receptor shift the conformation to fully active R^* conformation [11]. Nikiforovich et al. have suggested that the release of transmembrane interactions by substitutions of Asn^{111} for other residues causes steric hindrance in other parts of the AT_1 receptor, and further releasing the constraints becomes a driving force for the activation of the AT_1 receptor [24]. However, upon agonist binding, the R^* conformation achieved in the wild-type and CAM receptors are considered to be functionally identical (Fig. 4A). The results reported here question adherence to this model in the case of agonist-bound $AT1_{WT}$ and $AT1_{N111G}$ receptors (Fig. 4B).

A potential defect in the ability of the R^* state of $AT1_{N111G}$ mutant in transition to a phosphorylation-competent state was suggested previously [22]. Our findings are consistent with this suggestion, assuming that β-arrestin interaction with the AT1 receptor requires prior phosphorylation. Considering that G proteins, GRKs, β-arrestins and other signaling molecules compete for the overlapping binding sites on GPCRs, we envisage a working model consisting of manifold R^* states that each favors recruitment of distinct signaling molecules. Hence, it is worthwhile to investigate the active conformations of the $AT1_{N111G}$ receptor with regards to coupling preferences to signaling molecules other than heterotrimeric G proteins. Our findings expand current understanding of the active-state conformation (R^*) of the AT_1 receptors not only to IP$_3$ induction via Gq coupling but also to the realm of β-arrestin coupling (Fig. 4B). The findings reported by Feng et al. with regards to unconventional desensitization could also be accounted for by the model we suggest [23].

Our understanding of how different types of ligands bind and subsequently activate receptors is becoming more complex. An increase in scientific sophistication has enabled us to detect many different types of receptor conformations resulting from the binding of various ligands or sometimes the same ligand. In the rhodopsin system, receptor activation causes movements of TM3 and TM6 relative to each other and may impart the proper conformation of the intracellular loops for G protein activation [25–28]. This paradigm of TM domain motion seems conserved in other GPCRs, especially the AT_1 receptors [8,9,29] although some details may differ. Whether differences in these different conformational states translate into physiological reality is yet to be determined.

There are reasons to believe that the cellular consequence and signaling pathways activated by the wild-type and the CAM AT_1 receptors may actually differ. For instance, it has been shown that β-arrestin recruitment by the activated wild-type receptor is responsible for a distinct cytoplasmic phase of ERK 1/2 activation, which is responsible for phosphorylation of cytoplasmic targets [30]. Although the significance of potential proteolysis of β-arrestin 1 reported in this study is unclear, it is evident that the phenomenon is substantially reduced in the CAM AT_1 receptor. A recent study shows that there are morphological differences and Rho signaling important for cytoskeletal change between HEK 293 cells expressing the wild-type and the CAM $AT1_{N111G}$ receptor [31]. Thus, the $AT1_{N111G}$ mutant may serve as a useful research tool for the understanding of exaggerated G protein signaling [32,33]. In this regard, different CAM receptors may actually differ, and well characterized CAM receptors may serve as important research tools to understand beneficial and/or harmful *in vivo* consequence of select signals of the AT_1 receptor. It is important to characterize such differences between distinct signals arising from receptors such as the AT_1 receptor, where apart from mutation induced effects, the fragments of Ang II [34] or Ang II-analogs used in therapy, could lead to manifold activated-states of the receptor in vivo.
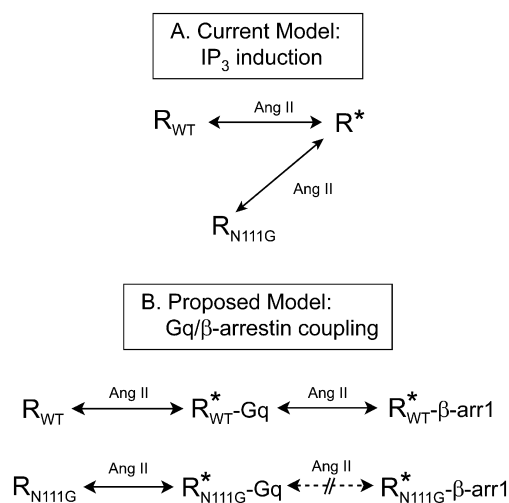
Fig. 4. A model for ligand-induced conformations of the wild-type and the constitutively active AT_1 receptors. (A) Based upon IP$_3$ accumulation, the active conformation of the constitutively active $AT1_{N111G}$ is considered to be similar to that of the agonist-induced wild-type AT_1 receptor. (B) Results from this study suggest that agonist-induced conformations of the $AT1_{WT}$ and the constitutively active $AT1_{N111G}$ receptors are different in that the $AT1_{WT}$ receptor traverses from the coupling of Gq to β-arrestin 1 upon agonist binding whereas the constitutively active $AT1_{N111G}$ is inept in this transition thus resulting to predominant Gq coupling.

References

[1] Peach, M.J. (1977) Renin–angiotensin system: biochemistry and mechanisms of action. Physiol. Rev. 57, 313–370.
[2] Hunyady, L. and Catt, K.J. (2006) Pleiotropic AT1 receptor signaling pathways mediating physiological and pathogenic actions of angiotensin II. Mol. Endocrinol. 20, 953–970.

[3] Karnik, S.S., Gogonea, C., Patil, S., Saad, Y. and Takezako, T. (2003) Activation of G-protein-coupled receptors: a common molecular mechanism. Trends Endocrinol. Metab. 14, 431–437.

[4] Cotecchia, S., Exum, S., Caron, M.G. and Lefkowitz, R.J. (1990) Regions of the alpha 1-adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function. Proc. Natl. Acad. Sci. USA 87, 2896–2900.

[5] Parnot, C., Miserey-Lenkei, S., Bardin, S., Corvol, P. and Clauser, E. (2002) Lessons from constitutively active mutants of G protein-coupled receptors. Trends Endocrinol. Metab. 13, 336–343.

[6] Noda, K., Feng, Y.H., Liu, X.P., Saad, Y., Husain, A. and Karnik, S.S. (1996) The active state of the AT1 angiotensin receptor is generated by angiotensin II induction. Biochemistry 35, 16435–16442.

[7] Feng, Y.H., Miura, S., Husain, A. and Karnik, S.S. (1998) Mechanism of constitutive activation of the AT1 receptor: influence of the size of the agonist switch binding residue Asn(111). Biochemistry 37, 15791–15798.

[8] Groblewski, T., Maigret, B., Larguier, R., Lombard, C., Bonnafous, J.C. and Marie, J. (1997) Mutation of Asn111 in the third transmembrane domain of the AT1A angiotensin II receptor induces its constitutive activation. J. Biol. Chem. 272, 1822–1826.

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

[10] Balmforth, A.J., Lee, A.J., Warburton, P., Donnelly, D. and Ball, S.G. (1997) The conformational change responsible for AT1 receptor activation is dependent upon two juxtaposed asparagine residues on transmembrane helices III and VII. J. Biol. Chem. 272, 4245–4251.

[11] Le, M.T., Vanderheyden, P.M., Szaszak, M., Hunyady, L. and Vauquelin, G. (2002) Angiotensin IV is a potent agonist for constitutive active human AT1 receptors. Distinct roles of the N- and C-terminal residues of angiotensin II during AT1 receptor activation. J. Biol. Chem. 277, 23107–23110.

[12] Baleanu-Gogonea, C. and Karnik, S. (2006) Model of the whole rat AT1 receptor and the ligand-binding site. J. Mol. Model. 12, 325–337.

[13] Lee, C., Ji, I. and Ji, T.H. (2004) Distinct mechanisms of cAMP induction by constitutively activating LH receptor and wild-type LH receptor activated by hCG. Endocrine 25, 111–115.

[14] Auger-Messier, M., Arguin, G., Chaloux, B., Leduc, R., Escher, E. and Guillemette, G. (2004) Down-regulation of inositol 1,4,5-trisphosphate receptor in cells stably expressing the constitutively active angiotensin II N111G-AT(1) receptor. Mol. Endocrinol. 18, 2967–2980.

[15] Qian, H., Pipolo, L. and Thomas, W.G. (2001) Association of beta-Arrestin 1 with the type 1A angiotensin II receptor involves phosphorylation of the receptor carboxyl terminus and correlates with receptor internalization. Mol. Endocrinol. 15, 1706–1719.

[16] Barnes, W.G., Reiter, E., Violin, J.D., Ren, X.R., Milligan, G. and Lefkowitz, R.J. (2005) beta-Arrestin 1 and Galphaq/11 coordinately activate RhoA and stress fiber formation following receptor stimulation. J. Biol. Chem. 280, 8041–8050.

[17] Auger-Messier, M., Clement, M., Lanctot, P.M., Leclerc, P.C., Leduc, R., Escher, E. and Guillemette, G. (2003) The constitutively active N111G-AT1 receptor for angiotensin II maintains a high affinity conformation despite being uncoupled from its cognate G protein Gq/11alpha. Endocrinology 144, 5277–5284.

[18] Azarian, S.M., King, A.J., Hallett, M.A. and Williams, D.S. (1995) Selective proteolysis of arrestin by calpain. Molecular characteristics and its effect on rhodopsin dephosphorylation. J. Biol. Chem. 270, 24375–24384.

[19] Cvejic, S. and Devi, L.A. (1997) Dimerization of the delta opioid receptor: implication for a role in receptor internalization. J. Biol. Chem. 272, 26959–26964.

[20] Latif, R., Graves, P. and Davies, T.F. (2002) Ligand-dependent inhibition of oligomerization at the human thyrotropin receptor. J. Biol. Chem. 277, 45059–45067.

[21] Grant, M., Collier, B. and Kumar, U. (2004) Agonist-dependent dissociation of human somatostatin receptor 2 dimers: a role in receptor trafficking. J. Biol. Chem. 279, 36179–36183.

[22] Thomas, W.G., Qian, H., Chang, C.S. and Karnik, S. (2000) Agonist-induced phosphorylation of the angiotensin II (AT(1A)) receptor requires generation of a conformation that is distinct from the inositol phosphate-signaling state. J. Biol. Chem. 275, 2893–2900.

[23] Feng, Y.H., Ding, Y., Ren, S., Zhou, L., Xu, C. and Karnik, S.S. (2005) Unconventional homologous internalization of the angiotensin II type-1 receptor induced by G-protein-independent signals. Hypertension 46, 419–425.

[24] Nikiforovich, G.V., Mihalik, B., Catt, K.J. and Marshall, G.R. (2005) Molecular mechanisms of constitutive activity: mutations at position 111 of the angiotensin AT1 receptor. J. Pept. Res. 66, 236–248.

[25] Farahbakhsh, Z.T., Ridge, K.D., Khorana, H.G. and Hubbell, W.L. (1995) Mapping light-dependent structural changes in the cytoplasmic loop connecting helices C and D in rhodopsin: a site-directed spin labeling study. Biochemistry 34, 8812–8819.

[26] Altenbach, C., Yang, K., Farrens, D.L., Farahbakhsh, Z.T., Khorana, H.G. and Hubbell, W.L. (1996) Structural features and light-dependent changes in the cytoplasmic interhelical E-F loop region of rhodopsin: a site-directed spin-labeling study. Biochemistry 35, 12470–12478.

[27] Farrens, D.L., Altenbach, C., Yang, K., Hubbell, W.L. and Khorana, H.G. (1996) Requirement of rigid-body motion of transmembrane helices for light activation of rhodopsin. Science 274, 768–770.

[28] Han, M., Lin, S.W., Smith, S.O. and Sakmar, T.P. (1996) The effects of amino acid replacements of glycine 121 on transmembrane helix 3 of rhodopsin. J. Biol. Chem. 271, 32330–32336.

[29] Boucard, A.A., Roy, M., Beaulieu, M.E., Lavigne, P., Escher, E., Guillemette, G. and Leduc, R. (2003) Constitutive activation of the angiotensin II type 1 receptor alters the spatial proximity of transmembrane 7 to the ligand-binding pocket. J. Biol. Chem. 278, 36628–36636.

[30] Wei, H., Ahn, S., Shenoy, S.K., Karnik, S.S., Hunyady, L., Luttrell, L.M. and Lefkowitz, R.J. (2003) Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. Proc. Natl. Acad. Sci. USA 100, 10782–10787.

[31] Auger-Messier, M., Turgeon, E.S., Leduc, R., Escher, E. and Guillemette, G. (2005) The constitutively active N111G-AT1 receptor for angiotensin II modifies the morphology and cytoskeletal organization of HEK-293 cells. Exp. Cell Res. 308, 188–195.

[32] Ramchandran, R. et al. (2006) Angiotensinergic stimulation of vascular endothelium in mice causes hypotension, bradycardia, and attenuated angiotensin response. Proc. Natl. Acad. Sci. USA 103, 19087–19092.

[33] Allen, A.M., Dosanjh, J.K., Erac, M., Dassanayake, S., Hannan, R.D. and Thomas, W.G. (2006) Expression of constitutively active angiotensin receptors in the rostral ventrolateral medulla increases blood pressure. Hypertension 47, 1054–1061.

[34] Reudelhuber, T.L. (2005) The renin-angiotensin system: peptides and enzymes beyond angiotensin II. Curr. Opin. Nephrol. Hypertens. 14, 155–159.