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Investigation of the fate of type I angiotensin receptor after biased activation

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The abbreviations used are: Angiotensin II (AngII), bioluminescence resonance energy transfer (BRET), G protein-coupled receptors (GPCRs), GPCR kinases (GRK), phosphatidylinositol 4-kinase (PI4K), phospholipase C (PLC), [Sar1,Ile4,Ile8]-AngII (SII-AngII), super Renilla luciferase (Sluc), type 1 angiotensin receptor (AT1-R), yellow fluorescent protein (YFP), YFP-labeled Rab protein (Rab-YFP), YFP-tagged phospholipase C delta 1 PH domain (PLCδ1-PH-YFP).
Abstract

Biased agonism on the type I angiotensin receptor (AT₁-R) can achieve different outcomes, via activation of G protein-dependent and -independent cellular responses. In this study, we investigated whether the biased activation of the AT₁-R can lead to different regulation and intracellular processing of the receptor. We analyzed β-arrestin binding, endocytosis and subsequent trafficking steps such as early and late phases of recycling of the AT₁-R in HEK293 cells expressing wild type or biased mutant receptors in response to different ligands. We used Renilla luciferase tagged receptors and yellow fluorescent protein (YFP) tagged β-arrestin2, Rab5, Rab7 and Rab11 proteins in bioluminescence resonance energy transfer (BRET) measurements to follow the fate of the receptor after stimulation. We found that not only is the signaling of the receptor different upon using selective ligands, but the fate within the cells is also determined by the type of the stimulation. β-arrestin binding and the internalization kinetics of the angiotensin II (AngII)-stimulated AT₁-R differed from those stimulated by the biased agonists. Similarly, AngII-stimulated wild type AT₁-R showed differences compared to a biased mutant AT₁-R (DRY/AAY AT₁-R) regarding β-arrestin binding and endocytosis. We found that the differences in the internalization kinetics of the receptor in response to biased agonist stimulation are due to the differences in plasma membrane PtdIns(4,5)P₂ depletion. Moreover, the stability of the β-arrestin binding is a major determinant of the later fate of the internalized AT₁-R receptor.
Introduction

Ligand binding to receptors can initiate several parallel signal transduction pathways leading to various final outcomes in the same cell. It has been recognized that several ligands are capable of selectively initiating distinct signal transduction pathways from the same receptor leading to the concept of biased agonism. It is well-accepted that biased agonism is an important feature of G protein-coupled receptors (GPCR), and it is proposed that development of biased agonists can serve new therapeutic approach (Whalen et al., 2011). The binding of agonists to GPCRs initiates G protein-mediated pathway, which results production of second messengers. The down-regulation of activated receptors involves several consecutive or parallel processes, such as desensitization, followed by internalization into vesicles and recycling or degradation. Receptor internalization is regulated by GPCR kinases (GRKs), with subsequent binding of β-arrestin (Lefkowitz, 2007). The β-arrestin binding not only mediates desensitization and internalization, but it also triggers additional signal transduction pathways, which are often called as G protein-independent signaling routes.

The angiotensin II (AngII) is the main effector of the renin-angiotensin system, can activate type 1 (AT1-R) angiotensin receptor. After binding to AT1-R, the Gq/11 protein mediates the activation of phosphoinositide-specific phospholipase Cβ (PLCβ) and hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) (Hunyady and Catt, 2006). Binding of AngII also triggers events leading to regulation of AT1-R, such as desensitization, internalization, degradation, and recycling to the cell surface (Ferguson, 2001; Hunyady et al., 2000). Desensitization and internalization of AT1-R are regulated by phosphorylation by GRKs, which promote β-arrestin binding and additional dynamin-dependent steps (Anborgh et al., 2000; Qian et al., 2001). Although considerable amount of knowledge has been gained in
understanding the mechanisms involved in the biased agonism of GPCRs (Godin and Ferguson, 2012; Reiter et al., 2012), less information is available about the fate (intracellular processing) of the receptors after biased activation.

The Rab proteins are key players in vesicular transport mechanisms, with the individual isoforms marking distinct vesicles in the endocytic and exocytic compartments (Zerial and McBride, 2001). Internalization, recycling and degradation of receptors involve different Rab proteins such as Rab5, Rab7 and Rab11 (Seachrist and Ferguson, 2003). It was shown that Rab5, Rab7 and Rab11 work together to regulate the vesicular trafficking of AT$_1$-R (Dale et al., 2004). We have shown in confocal microscopy studies using GFP tagged AT$_1$-R that AngII stimulates rapid translocation of the receptor to Rab5 containing early endosomes and subsequently to juxtanuclear Rab11 containing vesicles (Hunyady et al., 2002).

Studies of the G protein-independent signaling of AT$_1$-R have been accelerated by several approaches. Mutations within the highly conserved Asp$^{125}$Arg$^{126}$Tyr$^{127}$ sequence of AT$_1$-R (DRY/AAY mutation), abrogated G protein coupling, yet such receptors can initiate ERK1/2 activation (Hansen et al., 2008; Szidonya et al., 2007; Wei et al., 2003). The development and utilization of a biased AT$_1$-R agonist, [Sar$^1$,Ile$^4$,Ile$^8$]-AngII (SII-AngII), further widened the possibilities to investigate G protein-independent mechanisms (Holloway et al., 2002). Stimulation of AT$_1$-R by SII-AngII does not activate G proteins, but stimulates G protein-independent mechanisms, and is also able to induce internalization of the receptor (Luttrell et al., 2001; Wei et al., 2003). SII-AngII is widely used tool to study the biased agonism of the AT$_1$-R in spite of its relatively low affinity to AT$_1$-R. Recently, new biased peptide ligands of AT$_1$-R were discovered (TRV120023, TRV120027), which have higher receptor binding affinities than SII-AngII (Violin et al., 2010). These TRV peptides selectively activate the $\beta$-arrestin-mediated
signaling pathway and possess beneficial effects on cardiac contractility and performance (Kim et al., 2012; Violin et al., 2010). The favorable properties and potential clinical applications of TRV120027 are further investigated in trials for treatment of acute heart failure (Soergel et al., 2013; Violin et al., 2014).

We used BRET-based approach to investigate the fate of the receptor in response to stimuli. We used fluorescently-labeled constructs marking the molecular steps during internalization and luciferase-fused receptors in BRET measurements. Our results demonstrate that biased agonists or stimulation of biased receptor with the native AngII (AT₁-R-DRY/AAY) yields different fates of the activated receptors in terms of β-arrestin binding, internalization and appearance in various intracellular compartments. Our findings also suggest that the intracellular processing of the AT₁-R is dependent on the type of activation and the affinity of ligand binding to AT₁-R. Bias ligand bound receptors are unable to couple to G₉ proteins and activate PLCβ show accelerated internalization due to lack of PtdIns(4,5)P₂ hydrolysis in the plasma membrane. In contrast, the degradation and recycling of the internalized receptor is mainly determined by the strength of β-arrestin binding.
Material and Methods

Materials. Cell culture dishes and plates for BRET measurements were purchased from Greiner (Kremsmunster, Austria). Coelenterazine h were from either Invitrogen (Carlsbad, CA) or Regis Technologies (Morton Grove, IL). The SII-AngII was purchased from Bachem AG (Bubendorf, Switzerland). The TRV120023 (Sar-Arg-Val-Tyr-Lys-His-Pro-Ala-OH) and TRV120027 (Sar-Arg-Val-Tyr-Ile-His-Pro-D-Ala-OH) peptides were synthesized by Proteogenix (Schiltigheim, France) to more than 98% purity. The wortmannin was purchased from Calbiochem (San Diego, CA), the PIK93 was obtained from Sigma (St. Louis, MO), and the A1 inhibitor was synthesized as described (Bojjireddy et al., 2014). Rapamycin was obtained from Merck (Darmstadt, Germany). Unless otherwise stated, all other chemicals and reagents were purchased from Sigma (St. Louis, MO). The human embryonic kidney (HEK293) cells were from ATCC (American Type Culture Collection, Manassas, VA).

DNA constructs. For the construction of yellow fluorescent protein (YFP) labeled constructs the plasmid backbones of eYFP-C1 or eYFP-N1 (Clontech, Mountain View, CA) were used. The cDNA of the eYFP-tagged β-arrestin2 (β-arrestin2-YFP) and Renilla luciferase-tagged AT1-Rs (AT1-R-Rluc, AT1-R-DRY/AAY-Rluc) were constructed as described previously (Balla et al., 2012; Turu et al., 2006). The eYFP-labeled full length Rab5, Rab7 and Rab11 were constructed by replacing the eGFP coding region with eYFP in the GFP tagged constructs, as described previously (Hunyady et al., 2002). The eYFP-tagged PLC delta 1 PH domain (PLCδ1-PH-YFP) was constructed as described previously (Varnai and Balla, 1998), whereas the PLCδ1-PH-Sluc was constructed by replacing the eYFP coding region with sequence of super Renilla luciferase (Woo and von Arnim, 2008). The constructs of the rapamycin inducible heterodimerization system (PM-FRB-mRFP and mRFP-FKBP-5ptase) were constructed as
described previously (Toth et al., 2012). The expression vector of dominant negative mutant GRK2(K220M) was kindly provided by Dr. S. S. G. Ferguson (Ferguson et al., 1995).

**Cell culture and transfection.** The experiments were performed on HEK293 cell line. The cells were cultured in DMEM with Pen/Strep (Invitrogen) and 10% heat-inactivated fetal bovine serum (FBS) in 5% CO₂ at 37 °C. For BRET experiments, the cells were cultured in plastic dishes and were trypsinized prior to transfection and were transiently transfected by using Lipofectamine 2000 (Invitrogen) and plated on poly-lysine pretreated white 96-well plates in 1x10⁵ cells/well density for BRET measurements. The DNA amounts were 0.25 μg Rluc containing construct/well and 0.0625 – 0.125 μg YFP containing construct/well; the amount of Lipofectamine 2000 was 0.5 μl/well. For suspension Ca²⁺ measurements and for MAPK assay the HEK293 cells were cultured in 10-cm dishes and in 6-well plates, respectively. The cells were transiently transfected with Lipofectamine 2000 according to the manufacturer's protocol.

**BRET measurement.** We used Renilla luciferase fused receptor as the energy donor and an eYFP tagged protein as acceptor. The BRET measurements were performed after 24 h of the transfection on white 96-well plates. The medium of the cells were changed prior to measurements to a modified Krebs-Ringer buffer containing 120 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl₂, 0.7 mM MgSO₄, 10 mM glucose, and Na-HEPES 10 mM, pH 7.4; and the BRET measurements were performed at 37 °C. The BRET measurements were started after addition of the cell permeable substrate, coelenterazine h (Invitrogen or Regis Technologies) at a final concentration of 5 μM, and the counts were recorded by using either Berthold Mithras LB 940 or Varioskan Flash readers that allow the detection of signals using filters at 485 and 530 nm wavelengths, the detection time was 0.25-0.5 sec. The BRET ratios were calculated as 530 nm/485 nm ratio. Measurements were done in triplicates. The plotted BRET curves are the
average of at least 3 independent experiments. BRET ratios were baseline-corrected to the vehicle curve using GraphPad Prism software. The approximate BRET ratio using the cytosolic eYFP and AT1-R-Rluc pair is ~0.8 (data not shown).

**Cytoplasmic Ca\(^{2+}\) measurements in cell suspensions.** HEK293 cells were transfected with the various constructs (6 μg DNA/ 10 cm dish) by using Lipofectamine 2000. After 24 hours, the cells grown on 10-cm culture plates were removed by mild trypsinization and aliquots of cells (~10^6 cells) were loaded with 2 μM Fura-2/AM in DMEM medium containing 1.2 mM CaCl₂, 3.6 mM KCl, 25 mM HEPES containing 1 mg/ml bovine serum albumin, 0.06% Pluronic acid, and 200 μM sulfinpyrazone, for 45 min at room temperature. Cells were then washed with the same medium without Fura-2/AM and stored at room temperature in the dark. The cells were centrifuged rapidly before the measurements and dispersed in 3 ml of the modified Krebs-Ringer buffer used for all other analysis. Calcium measurements were performed at room temperature in a PTI Deltascan spectrofluorometer (Photon Technology International, Princeton, NJ).

**ERK1/2 MAPK assay.** Twenty-four hours after transfection, the HEK293 cells were serum-starved for 4 h, and stimulated for 5 min with 100 nM AngII or 10 μM SII-AngII. Cells were scraped into SDS sample buffer containing protease and phosphatase inhibitors, briefly sonicated, boiled, and separated on SDS-polyacrilamide gels. The proteins were transferred to PVDF membranes and incubated with the appropriate primary (Santa Cruz Biotechnology, Santa Cruz, CA) and secondary (Cell Signaling, Danvers, MA) antibodies. The antibodies were visualized by enhanced chemiluminescence, using Immobilion Western HRP substrate reagents (Millipore, Billerica, MA).

**Confocal microscopy.** AT1-R-GFP stably expressing HEK293 cells (Hunyady et al., 2002) were plated on polylysine-pretreated glass coverslips (3×10^5 cells/35-mm dish). After 24 h
the localization and distribution of the receptor were analyzed using a Zeiss LSM 710 confocal laser-scanning microscope. Postacquisition data analysis of the confocal images was performed with either the ZEN (Carl Zeiss) or the MetaMorph (Molecular Devices) software. Quantification of the internalization of \( \text{AT}_1 \)-R-GFP (location in intracellular vesicles, which clearly are separated from the plasma membrane) as function of time calculated from morphometric analysis by MetaMorph software obtained in seven separate experiments.
Results

BRET assay for the detection of agonist induced internalization of AT₁-R. Our previous results raised the possibility that biased stimulation of the AT₁-R can lead to different fates of the internalized receptor (Balla et al., 2012). In order to investigate this question in more detail, we analyzed the proximity of the receptor with different Rab proteins, used as markers of the steps along the endocytotic/recycling/degradation pathways of the internalized receptors. Rab5-YFP is a marker for early endosomes, Rab7-YFP is that for the multivesicular body/late endosome, while the Rab11-YFP marks the late recycling route (Zerial and McBride, 2001). As shown in Fig. 1A, we detected an elevated BRET ratio between AT₁-R-Rluc and Rab5-YFP after both AngII and SII-AngII stimulation (black filled and gray open symbols, respectively), which indicates receptor mediated endocytosis. In our previous studies (Balla et al., 2012), we noted that at the early phase after stimulation SII-AngII induced a more robust co-localization of the receptor with Rab5 endosomes compared to AngII stimulation (Fig. 1A, grey open symbols, also in Supplemental Figure 6C). Therefore, we investigated the effects of newly developed biased agonists on the internalization and sequential intracellular trafficking steps of the AT₁-R. Fig. 1B shows that stimulation with both 1 μM TRV120023 (labeled as TRV3) and 1 μM TRV120027 (labeled as TRV7) yielded BRET curves between the receptor and Rab5 very reminiscent of those evoked by SII-AngII. Taken together the results shown in Fig. 1A and B suggest that biased stimulation apparently accelerated the internalization of the AT₁-R. Since both SII-AngII and the TRV compounds have lower affinity than AngII, we also tested a low affinity, but unbiased agonist, the hexapeptide angiotensin IV (AngIV) (Le et al., 2002) on the internalization rate of the AT₁-R. In contrast to the biased agonists, AngIV stimulation evoked very similar
BRET interaction of the receptor with Rab5 containing endosomes as the AngII initiated response (Fig. 1C).

It is well established that AT1-R induced G_q activation causes PLC-mediated hydrolysis of the membrane PtdIns(4,5)P_2, which can be monitored by following the translocation of PLCδ1-PH-GFP from the plasma membrane to the cytosol (Varnai and Balla, 1998). We confirmed that AngIV at the concentration used (10 μM) is able to initiate G_q activation and PtdIns(4,5)P_2 hydrolysis in BRET measurements: AngIV, similarly to AngII, decreased the BRET ratio between the PLCδ1-PH domains fused with YFP and Sluc. When the PH domains are bound to plasma membrane they are within BRET distance, whereas being in the cytoplasm they are not therefore the decreased BRET signal reflects the dissociation of the PtdIns(4,5)P_2 sensor domains from the plasma membrane due to the breakdown of the PtdIns(4,5)P_2. The lipid is slowly resynthesized causing the reappearance of the probes in the plasma membrane (BRET ratio increase) (Supplemental Figure 1). It has been well documented, that AngII stimulation of the DRY/AAY mutant receptor leads to biased activation, similar to those seen during SII-AngII stimulation of wild type receptors (Wei et al., 2003). Fig. 1D shows that AngII stimulation of the AT1-R-DRY/AAY-Rluc caused similar BRET interaction with Rab5-YFP as the biased ligand stimulation of the wild type receptor (Fig. 1A and B). This result suggested that the AngII-induced internalization of the mutant receptor occurs faster than with the wild type AT1-R. To demonstrate that the properties of the luciferase-tagged receptors are similar to those of the untagged receptors, we analyzed calcium signaling and ERK activation. The results showed that both of these responses were comparable with the untagged and Rluc-tagged receptors and that neither SII-AngII nor the DRY/AAY receptor stimulated by AngII showed Ca^{2+} responses, yet
they displayed a reduced but still detectable ERK1/2 activation (Supplemental Figure 2). These data together suggested that the tagged receptors function similarly to the non-tagged AT1-Rs.

**Characterization of the biased agonist induced internalization of AT1-R.** We also investigated the agonist-induced internalization of the receptor with confocal microscopy using HEK293 cells stably expressing AT1-R-GFP. Fig. 2 demonstrates the slightly faster internalization of a biased-activated receptor; more receptors were detectable in intracellular vesicles after 4 min stimulation with either SII-AngII or TRV120023 (labeled as TRV3) compared to the AngII stimulation.

We then investigated the possibility that the more rapid internalization of the SII-AngII stimulated receptor is the result of the use of an alternative endocytic pathway(s). We used 300 mM sucrose treatment to inhibit clathrin-mediated endocytosis (Heuser and Anderson, 1989). At this concentration, (300 mM) sucrose significantly diminished the BRET ratio increase between the receptor and Rab5 after stimulation with either AngII or SII-AngII, which suggested that both ligands initiated primarily a clathrin-mediated endocytic mechanism (Supplemental Figure 3A and B). We also studied the effect of filipin, which is widely used for the inhibition of clathrin-independent caveolae-mediated pathway (Orlandi and Fishman, 1998). During stimulation of the cells with 100 nM AngII or 10 μM SII-AngII, the preincubation of cells with filipin did not change the BRET ratio kinetic between Rab5 and AT1-R (Supplemental Figure 3C).

Next, we analyzed whether the faster rate of the Rab5 recruitment is the consequence of the absence of classical (G protein-dependent) calcium signaling in biased-stimulated AT1-R. We used BAPTA-AM pretreatment to blunt the evoked calcium signal. As shown in Supplemental Figure 4A, BAPTA-AM pretreatment did not alter the kinetics of the Rab5 recruitment. We
confirmed that the BAPTA-AM pretreatment was sufficient to inhibit the calcium signal in HEK293 cells (Supplemental Figure 4B).

**Effect of biased stimulation on the association of AT₁-R with β-arrestin2.** Next we investigated the possible involvement of β-arrestin2 in the altered internalization of the AT₁-R when the receptor is activated by biased agonists. Both SII-AngII and the TRV compounds were able to cause β-arrestin2 recruitment, although the biased agonists evoked smaller responses than AngII stimulation (Fig. 3A and B). It is noteworthy, that the AngII induced binding of β-arrestin2 appeared more sustained than that evoked by the biased agonists, (Fig 3A and B, grey symbols, also in Supplemental Figure 6A, grey symbols). Here, AngIV caused similar type of AT₁-R - β-arrestin2 BRET interaction as the low affinity biased agonists (Fig. 3C). We also measured complete concentration-response curves in order to check that the used agonist concentrations are maximally effective. The Supplemental Figure 5 demonstrates that 100 nM AngII induced maximal PtdIns(4,5)P₂ hydrolysis (Supplemental Figure 5A) and β-arrestin2 binding (Supplemental Figure 5B). The AngIV was maximally effective in 10 μM concentration in PtdIns(4,5)P₂ breakdown and β-arrestin2 recruitment assays (Supplemental Figure 5A and 5B). 10 μM SII-AngII and 1 μM concentrations of TRV compounds evoked maximal β-arrestin binding (Supplemental Figure 5B), but did not cause detectable PtdIns(4,5)P₂ hydrolysis (Supplemental Figure 1) or vasoconstriction in wire myography experiments using mouse aortic rings (data not shown).

The BRET curve of the AngII-induced β–arrestin binding of the DRY/AAY-AT₁-R is very reminiscent of that of the wild type receptor and the amplitude is ~75% of the wild type counterpart (Fig. 3D, black filled symbols). We also compared the effects of stimulation of AT₁-R-DRY/AAY-Rluc with SII-AngII and AngII. Stimulation of AT₁-R-DRY/AAY-Rluc with SII-
AngII resulted in slightly weaker and less stable β-arrestin binding than the AngII induced response (Supplemental Figure 6). Yet, the translocations to Rab5 endosomes of the AT₁-R-DRY/AAY-Rluc in response to either SII-AngII or AngII stimulation were almost identical (Supplemental Figure 6).

Effect of inhibitors of phosphatidylinositol kinases on the agonist induced AT₁-R endocytosis. Next we investigated the role of PtdIns(4,5)P₂ in the regulation of AT₁-R internalization. PtdIns(4,5)P₂ is located on the inner leaflet of the plasma membrane and is important both in signal transduction processes and in the endocytosis of cell surface receptors, including GPCRs (Toth et al., 2012). Several phosphatidylinositol kinases are involved in the maintenance of the plasma membrane PtdIns(4,5)P₂ (Balla and Balla, 2006; Balla, 2013). Among those phosphatidylinositol 4-kinases (PI4Ks) control the first step in PtdIns(4,5)P₂ synthesis (Balla et al., 2008; Balla et al., 2005). First, we examined the effect of low and high concentrations of wortmannin (Wm) (the former selectively inhibits most phosphatidylinositol 3-kinases, the latter also inhibits type III PI4Ks). Fig 4. shows that 100 nM Wm did not alter the agonist induced endocytosis of the AT₁-R. In contrast, 10 μM Wm was able to diminish the AngII- and AngIV-induced endocytosis (Fig. 4A and C) but not the internalization evoked by the biased agonists (Fig. 4B). This result suggested the involvement of PI4Ks rather than PI3Ks and therefore, we tested more selective inhibitors of the individual PI4K isoforms. PIK-93 inhibits PI4KIIIβ/PI4KB but to a much lesser degree PI4KA (Balla et al., 2008), while a compound, Al inhibits PI4KIIIα/PI4KA more potently than PI4KB (Bojjireddy et al., 2014). Using these inhibitors and following the BRET signal between the receptor and Rab5, the activity of PI4KIIIα was found necessary for the AngII and AngIV induced endocytosis but not for the
biased agonist induced response (Fig. 5), while the PI4KIIIβ was found not to be responsible for the maintenance of PtdIns(4,5)P2 involved in the regulation of endocytosis.

**Plasma membrane PtdIns(4,5)P2 depletion effects AT1-R internalization upon unbiased or biased activation.** We also investigated the role of PtdIns(4,5)P2 in the stimulation evoked Rab5 recruitment of AT1-R. First, we utilized a plasma membrane PtdIns(4,5)P2 depletion system, as earlier described (Toth et al., 2012). The appearance of AT1-R in early endosomes was followed by BRET-measurements between AT1-R-Rluc and fluorescently-tagged Rab5 in cells coexpressing the rapamycin-inducible PtdIns(4,5)P2 depletion system. Briefly, addition of rapamycin induces heterodimerization between the plasma membrane targeted FRB and cytoplasmic FKBP, thus 5ptase domain is translocated to the plasma membrane, where it degrades PtdIns(4,5)P2. We confirmed the efficiency of this method in BRET measurements using PLCδ1-PH-YFP and PLCδ1-PH-Sluc sensors (data not shown). The depletion of plasma membrane PtdIns(4,5)P2 completely blocked both unbiased or biased activation induced AT1-R Rab5 recruitment (Supplemental Figure 7A and 6B).

We also performed experiments using a dominant-negative GRK2 (DN-GRK2) construct to show the role of PtdIns(4,5)P2 in the regulation of AT1-R internalization. It was demonstrated that DN-GRK2 significantly blunts the receptor activation induced PLCβ activity (Carman et al., 1999; Sallese et al., 2000); and we confirmed that overexpression of the DN-GRK2 (GRK2-K220M) decreased the AT1-R stimulation induced plasma membrane PtdIns(4,5)P2 hydrolysis in HEK293 cells (Supplemental Figure 8A). The Fig. 6 shows that overexpression of DN-GRK2 accelerated the AngII evoked AT1-R internalization, but had no effect on the biased-agonist (TRV120023) induced response. The overexpression of DN-GRK2 did not effect the β-arrestin2 binding properties (Supplemental Figure 8B), which supports that not only GRK2 but other GRK
isoforms such as GRK3/5/6 could be involved in receptor phosphorylation and β–arrestin2 recruitment in HEK293 cells (Kim et al., 2005).

**BRET assay for detection of intracellular processing of AT1-R.** Our results suggested that biased stimulation of the AT1-R can lead to altered intracellular processing of the internalized receptor. To investigate this possibility, we performed BRET experiment to follow the AT1-R inside the cell using various markers of endomembranes, such as Rab7 and Rab11, as the markers of degradation and recycling pathways, respectively. As discussed earlier, Rab7-YFP is used to detect multivesicular body/late endosome pathway and the Rab11-YFP is marker of the late recycling route. As shown in Fig. 7, we detected elevated BRET ratio with Rab7-YFP following both AngII and biased agonist (SII-AngII, TRV120023 and TRV120027) stimulation. Enhanced initial BRET interactions were detected after stimulation with biased ligands between AT1-R and Rab5 (Fig. 1), and both the Rab7 and Rab11 containing compartments (Fig. 7). The amplitudes of the association of the Rab7 and the receptor were in good correlation with the initiated β–arrestin binding upon stimulation with various ligands (Fig. 7A, 7C, 7E and Fig. 3). In case of Rab11-YFP and AT1-R-Rluc the AngII stimulus caused an initial decrease in the BRET ratio, but after ~15 min of stimulation the ratio started to elevate (Fig. 7B, 7D, 7F; black filled symbols). The low affinity agonists caused increased co-localization with Rab11 endosomes after ~5-10 min of the stimulation, which is strikingly different from the AngII evoked response, where the increased BRET ratio was preceded by a period of ~15 min BRET decrease (Fig. 7B, 7D, 7F). We also analyzed whether the calcium signal of the AT1-R is partially responsible for the intracellular processing of the receptor. However, BAPTA-AM pretreatment did not modify the association of the AT1-R with Rab4, Rab7 and Rab11 (data not shown) arguing against the role of Ca^{2+} signal generation in this process.
Discussion

In the present study we investigated and analyzed the consequences of biased activation of AT$_1$-R, focusing on β-arrestin binding and intracellular processing of wild-type as well as DRY/AAY mutant AT$_1$-Rs. Agonist activation of most GPCRs, such as AT$_1$-R, initiates phosphorylation of the receptor by G protein-coupled receptor kinases. The phosphorylated carboxyl-terminal tail of the receptor recruits the cytosolic adaptor protein, β–arrestin and uncouples the receptor from the corresponding heterotrimeric G protein. The receptor bound to β-arrestin is also sorted from the plasma membrane into endocytic vesicles. Receptor internalization can occur by several mechanisms, such as via clathrin-coated vesicles, or by other vesicles, including caveolae (Maxfield and McGraw, 2004). GPCRs, which internalize via clathrin-mediated pathway, require agonist binding and subsequent β–arrestin binding to become endocytosed. The adaptor between the phosphorylated receptor and the endocytotic machinery is the β–arrestin molecule itself (Ferguson, 2001). AT$_1$-Rs are internalized predominantly via the clathrin-mediated pathway at physiological hormone concentrations, but β–arrestin-independent internalization was also reported at higher AngII concentrations (Gaborik et al., 2001; Zhang et al., 1996).

We used a BRET-based approach to investigate the distribution of the receptor in response to ligands that possess biased agonist properties. We used several YFP-labeled fusion constructs and Renilla luciferase fused receptors as intermolecular probe pairs in BRET measurements. After addition of AngII, the BRET ratios are increased between AT$_1$-R and Rab5, Rab7, which are signs of traveling receptor through endosomes decorated with those proteins (Fig. 1 and Fig. 7). Since the DRY/AAY mutation of the receptor or the use of biased agonists (such as SII-AngII, TRV120023 and TRV120027) fail to achieve G protein activation (Gaborik
et al., 2003; Violin et al., 2010; Wei et al., 2003), differences were expected in the fate of AT1-R after biased activation. All biased agonists (SII-AngII, TRV120023 and TRV120027) induced co-localization of AT1-R with Rab5 endosomes (indicating internalization of the receptor), which was apparently more robust than the AngII evoked responses (Fig. 1). The low affinity unbiased AT1-R agonist AngIV caused very similar internalization as AngII (Fig. 1C). Our results did not prove the role of either fundamentally different endocytic routes (Supplemental Figure 3) or Gq activation initiated calcium signal (Supplemental Figure 4) in the background of the dissimilar rate of endocytosis after bias activation of AT1-R. We were also not able to demonstrate correlation between the β-arrestin binding and the accelerated internalization of the AT1-R after stimulation with biased agonists (Fig. 3). Moreover, it seems that the course of AT1-R - β-arrestin2 recruitment may correlate with the affinity of the ligand, since it is known that the SII-AngII, TRV120023, and TRV120027 have significantly lower affinity than AngII (Bonde et al., 2010; Violin et al., 2010), and also the low affinity unbiased AT1-R agonist AngIV caused similarly reduced and less stable AT1-R - β-arrestin2 recruitment as the biased agonists (Fig. 3C). We used various concentrations of agonists to check their effects (Supplemental Figure 5), since locally produced AngII levels in many target tissues are at least one order magnitude higher than the concentration of circulating AngII (Danser, 2003).

Since both AngII and AngIV initiated PtdIns(4,5)P2 breakdown in our system, while the biased agonists not (Supplemental Figure 1), we investigated the role of PtdIns(4,5)P2 in the regulation of the AT1-R internalization. Pharmacological approach revealed that PI4KIIIα is responsible for the maintenance of the PtdIns(4,5)P2 pool, which is involved in the receptor mediated endocytosis, since both 10 μM wortmannin and 10 nM A1 were able to suspend the endocytosis after AngII or AngIV stimulation of the AT1-R (Fig. 4 and 5). The inhibitory effect
of the inhibition of PtdIns(4,5)P$_2$ resynthesis by the blockade of PI4KIII$\alpha$ activity on the AT$_1$-R endocytosis is consistent with the observation that PtdIns(4,5)P$_2$ depletion by a rapamycin-inducible heterodimerization system also interfered with GPCR internalization Supplemental Figure 7) (Toth et al., 2012).

Several studies have investigated the roles of different Rab proteins, such as Rab4, Rab5, Rab7 and Rab11, in the AT$_1$-R intracellular processing (Dale et al., 2004; Hunyady et al., 2002; Seachrist and Ferguson, 2003; Seachrist et al., 2002). We extended those studies using biased agonists in living cell experiments. It is important to emphasize in the interpretation of results of the previous and our recent studies that a given intracellular vesicle may contain more than one isoforms of Rab proteins (Sonnichsen et al., 2000).

The Rab4 shows overlapping distribution in early endosomes with Rab5 and in recycling endosomes with Rab11. In our studies, the BRET curves of Rab4 and Rab5 with AT$_1$-R were very similar, which suggests that the Rab4 is partly located in Rab4/Rab5 positive endosomes (data not shown) in HEK293 cells. As mentioned above, the Rab7 is localized to both late endosomal and lysosomal compartments, as well (Bucci et al., 2000). The Rab7 is important in the regulation of in the intracellular processing of GPCRs routing the receptors from early endosomes to late endosomal and to lysosomal compartments. It seems that the biased agonists and the AngIV-induced receptor trafficking did not prefer the association with Rab7 compartments as much as the AngII-induced mechanism (Fig. 7). Our data raise the possibility that the association with Rab7 is mainly determined by the affinity of the ligands toward the receptor (Fig. 7A, 7C and 7E). The Rab11 is also located in several compartments, such as perinuclear recycling endosomes and the trans-Golgi network, where it influences the slow endosomal recycling and the endosome to trans-Golgi network trafficking, respectively. It was
demonstrated earlier by confocal (Hunyady et al., 2002), and by FRET microscopy (Li et al., 2008) that the Rab4 and Rab11 coordinates together the recycling of the AT1-R; during the early recycling the receptor associated to mostly to Rab4 and during the late-stage recycling to mostly Rab11. That is in concert with our results, which show that the association of the AT1-R with Rab11 is a latter event after stimulation. The AngII stimulus caused a drop in the BRET ratio between Rab11-YFP and AT1-R-Rluc, but after ~15 min of the stimulation the ratio started to increase (Fig. 7B, 7D, 7F; black filled symbols). This phenomenon could raise the possibility that some fraction of the overexpressed receptor is already located in Rab11 intracellular compartments in HEK293, and after stimulus these receptors shift to other compartments, which results the drop in the BRET ratio. It is known that the Rab11 is very important player not just in the regulation of endocytic recycling compartment but also in the regulation of biosynthetic recycling compartment (Saraste and Goud, 2007), moreover Rab11 regulates exocytosis of vesicles at the plasma membrane (Takahashi et al., 2012). It is possible that the AngII stimulation initiates a mechanism, which translocate the preformed or previously endocytosed receptors to the cell surface regulating the responsiveness of the cells.

Interestingly, the association with Rab11 is very dissimilar in case of stimulation with the other agonists (biased agonists and AngIV), where the association starts at earlier timepoint after the stimulation (Fig. 7B, 7D and 7F). The dissimilarity between the AngII and the other ligands suggest that the ligand affinity and the strength of arrestin binding can determine the later fate of the stimulated receptor.

Taken together, the wild type and the DRY/AAY mutant AT1-Rs, and also the AngII or biased agonist stimulated receptors differ in their sorting between intracellular compartments. Our data suggest that neither the reduced arrestin binding nor the fundamentally different
internalization routes nor the calcium signal (Supplemental Figure 3 and 4), but rather the transient depletion of the plasma membrane PtdIns(4,5)P$_2$ pool is responsible for the reduced rate of the AngII-induced AT$_1$-R endocytosis compared to the biased agonists induced responses (Fig. 4 and 5; Supplemental Figure 7 and 8). Even though the main determinant of the endocytic rate is the presence or absence of PLC activation, the later fate of the AT$_1$-R within the cells seems mainly dependent on the course of the β-arrestin binding to the stimulated receptor. The hormonal responsiveness of tissues and cells is dynamic and determined by the delicate balance between externalization (delivery mechanisms, which transports the receptors from intracellular compartments to the plasma membrane) and internalization pathways of the receptors. It is very promising that the delicate balance between receptor resensitization/externalization and desensitization/internalization can be modified by biased agonists, which raise the possibility to apply biased ligands in diseases where the intracellular receptor processing should be changed.
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Authorship Contributions

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References


Footnotes

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Figure Legends

Figure 1. BRET assay between wild type AT$_1$-R and Rab5 upon stimulation in HEK293 cells. (A-C) HEK293 cells were transfected with the plasmids of the AT$_1$-R-Rluc and with YFP fused Rab5 protein, and after 24 hours the cells were exposed to either 100 nM AngII (black filled squares), (A) 10 μM SII-AngII (grey open symbols); (B) 1 μM TRV120023 (labeled as TRV3, grey open squares), 1 μM TRV120027 (labeled as TRV7, grey filled triangles); (C) 10 μM AngIV (grey open triangles), 1 μM TRV3 (grey open squares) or vehicle (dashed lines) at the indicated time points. (D) HEK293 cells were transfected with the plasmids of the indicated receptor-Rluc (black filled symbols: wild type; grey open symbols: DRY/AAY mutant) and with Rab5-YFP, and after 24 hours the cells were exposed to either 100 nM AngII or vehicle (dashed line) at the indicated time points. The BRET records are average of at least 3 independent experiments. Mean values ± SEM are shown (n = 3).

Figure 2. Effect of agonist stimulation on the distribution of AT$_1$-R-GFP in HEK293 cells. (A) AT$_1$-R-GFP stably expressing HEK293 cells were exposed to either 100 nM AngII (upper row of micrographs) or 10 μM SII-AngII (middle row of micrographs) or 1 μM TRV120023 (labeled as TRV3, lower row of micrographs) and the GFP fluorescence was detected by a Zeiss LSM 710 confocal laser-scanning microscope. The representative confocal micrographs show the localization and cellular distribution of the AT$_1$-R before (0 min) and 4 min, or 9 min after the agonist treatment. (B) Quantification of the internalization of AT$_1$-R-GFP as function of time calculated from morphometric analysis by MetaMorph software obtained in seven separate experiments (means ± SEM).
Figure 3. BRET assay between AT1-R and β-arrestin2 upon agonist stimulation in HEK293 cells. HEK293 cells were transfected with the AT1-R-luciferase and β-arrestin2-YFP, and after 24 hours the cells were exposed to 100 nM AngII (black filled squares), (A) 10 μM SII-AngII (grey open symbols); (B) 1 μM TRV120023 (labeled as TRV3, grey open squares), 1 μM TRV120027 (labeled as TRV7, grey filled triangles); (C) 10 μM AngIV (grey open triangles), or vehicle (dashed lines) at the indicated time points. (D) HEK293 cells were transfected with the plasmids of the indicated receptor-Rluc (black filled symbols: wild type; grey open symbols: DRY/AAY mutant) and with β-arrestin2-YFP, and after 24 hours the cells were exposed to either 100 nM AngII or vehicle (dashed line) at the indicated time points. The BRET records are average of at least 3 independent experiments. Mean values ± SEM are shown (n = 3).

Figure 4. Effect of wortmannin on the agonist induced internalization of AT1-R in HEK293 cells. HEK293 cells were transfected with the plasmids of the AT1-R-Rluc and with Rab5-YFP proteins, and after 24 hours the experiments were carried out. Cells were pretreated for 10 min with vehicle BRET medium (black filled symbols), BRET medium supplemented with 100 nM wortmannin (black open symbols), or with 10 μM wortmannin (grey open symbols) and exposed to either vehicle (dashed line) or 100 nM AngII (A) or 1 μM TRV120023 (labeled as TRV3) (B) or 10 μM AngIV (C) at the indicated time points. The BRET curves are average of 3 independent experiments, each performed in triplicates. Mean values ± SEM are shown (n = 3).

Figure 5. Effect of A1 and PIK-93 on the agonist induced internalization of AT1-R in HEK293 cells. HEK293 cells were transfected with the plasmids of the AT1-R-Rluc and with Rab5-YFP
proteins, and after 24 hours the experiments were carried out. Cells were pretreated for 10 min with vehicle BRET medium (black filled symbols), BRET medium supplemented with 10 nM A1 (black open squares), or with 250 nM PIK-93 (grey open triangles) and exposed to either vehicle (dashed line) or 100 nM AngII (A) or 1 μM TRV120023 (labeled as TRV3) (B) or 10 μM AngIV (C) at the indicated time points. The BRET curves are average of 3 independent experiments, each performed in triplicates. Mean values ± SEM are shown (n = 3).

Figure 6. Effects of attenuation of plasma membrane PtdIns(4,5)P2 hydrolysis on BRET curves between AT1-R and Rab5 or β-arrestin2. HEK293 cells were transfected with plasmids encoding DN-GRK2, AT1-R-Rluc and with Rab5-YFP. After 24 hours the cells were exposed to either 100 nM AngII (red trace), 1 μM TRV120023 (labeled as TRV3, blue trace), or vehicle (dashed lines) at the indicated time point. The BRET records are average of 3 independent experiments. Mean values ± SEM are shown (n = 3).

Figure 7. BRET assay between AT1-R and different Rab proteins upon agonist stimulation in HEK293 cells. HEK293 cells were transfected with the plasmids of the AT1-R-Rluc and with the indicated YFP fused Rab proteins, and after 24 hours the cells were exposed to either 100 nM AngII (black filled symbols), (A, B) 10 μM SII-AngII (grey open symbols); (C, D) 1 μM TRV120023 (labeled as TRV3, blue traces), 1 μM TRV120027 (labeled as TRV7, purple traces); (E, F) 10 μM AngIV (grey open squares), 1 μM TRV3 (blue traces) or vehicle (dashed lines) at the indicated time points. BRET pairs: (A, C, E) Rab7-YFP and AT1-R-Rluc; (B, D, F) Rab11-YFP and AT1-R-Rluc. The BRET records are average of at least 3 independent experiments. Mean values ± SEM are shown (n = 3).
Figure 2

(A) Images showing the internalization of AngII, SII-AngII, and TRV3 over time.

(B) Graph depicting the percentage of maximum internalization over time for AngII, SII-AngII, and TRV3.
Figure 6

AT$_1$-R + Rab5

$\Delta$BRET ratio vs Time (sec)

- no stim
- AngII
- TRV3
- no stim (DN-GRK2)
- AngII (DN-GRK2)
- TRV3 (DN-GRK2)