Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

Title Page

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

Gyöngyi Szakadáti, András D. Tóth, Ilona Oláh, László Sándor Erdélyi, Tamas Balla, Péter Várnai, László Hunyady, András Balla

Department of Physiology (G.S., A.D.T., I.O., L.S.E., P.V., L.H. A.B.), Semmelweis University, Faculty of Medicine, H-1444 Budapest, Hungary

MTA-SE Laboratory of Molecular Physiology (L.S.E., P.V., L.H. A.B.), Hungarian Academy of Sciences and Semmelweis University, H-1094 Budapest, H-1094 Budapest, Hungary

Section on Molecular Signal Transduction (T.B.), Program for Developmental Neuroscience, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Fate of biased activated AT_1-receptor

Address correspondence to: Dr. László Hunyady, H-1444 Budapest, P. O. Box 259, Hungary, Fax: 36-1-266-6504, Phone: 36-1-459-1500/60401, E-mail: Hunyady@eok.sote.hu

Number of text pages: 32

Number of tables: 0

Number of figures: 7

Number of supplemental figures: 8

Number of references: 51

Number of words in the Abstract: 233

Number of words in the Introduction: 746

Number of words in the Discussion: 1383

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), [Sar^1,Ile^4,Ile^8]-AngII (SII-AngII), super *Renilla* luciferase (Sluc), type 1 angiotensin receptor (AT_1-R), yellow fluorescent protein (YFP), YFP-labeled Rab protein (Rab-YFP), YFP-tagged phospholipase C delta 1 PH domain (PLCδ1-PH-YFP).

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Biased agonism on the type I angiotensin receptor (AT_1-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 AT_1-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 AT_1-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 AT_1-R differed from those stimulated by the biased agonists. Similarly, AngII-stimulated wild type AT_1-R showed differences compared to a biased mutant AT_1-R (DRY/AAY AT_1-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)P_2 depletion. Moreover, the stability of the β-arrestin binding is a major determinant of the later fate of the internalized AT_1-R receptor.

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

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 (AT_1-R) angiotensin receptor. After binding to AT_1-R, the $G_{q/11}$ protein mediates the activation of phosphoinositide-specific phospholipase Cβ (PLCβ) and hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) (Hunyady and Catt, 2006). Binding of AngII also triggers events leading to regulation of AT_1-R, such as desensitization, internalization, degradation, and recycling to the cell surface (Ferguson, 2001; Hunyady et al., 2000). Desensitization and internalization of AT_1-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

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

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 β-arrestin-mediated

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

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 (AT_1-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 AT_1-R is dependent on the type of activation and the affinity of ligand binding to AT_1-R. Bias ligand bound receptors are unable to couple to G_q proteins and activate PLCβ show accelerated internalization due to lack of PtdIns(4,5)P_2 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.

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

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 AT_1-Rs (AT_1-R-Rluc, AT_1-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

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

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% CO_2 at 37 ^{o}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 $1x10^5$ 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 Ca^{2+} 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 $CaCl_2$, 0.7 mM $MgSO_4$, 10 mM glucose, and Na-HEPES 10 mM, pH 7.4; and the BRET measurements were performed at 37 ^{o}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

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

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 AT_1-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 $CaCl_2$, 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. AT_1-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

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

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 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.

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

Results

BRET assay for the detection of agonist induced internalization of AT_1-R. Our previous results raised the possibility that biased stimulation of the AT_1-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 AT_1-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 AT_1-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 AT_1-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 AT_1-R. In contrast to the biased agonists, AngIV stimulation evoked very similar

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

BRET interaction of the receptor with Rab5 containing endosomes as the AngII initiated response (Fig. 1C).

It is well established that AT_1-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 AT_1-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 AT_1-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

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

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 AT_1-Rs.

Characterization of the biased agonist induced internalization of AT_1-R. We also investigated the agonist-induced internalization of the receptor with confocal microscopy using HEK293 cells stably expressing AT_1-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 AT_1-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 AT_1-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

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

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 AT_1-R with β-arrestin2. Next we investigated the possible involvement of β-arrestin2 in the altered internalization of the AT_1-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 AT_1-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)P_2 hydrolysis (Supplemental Figure 5A) and β-arrestin2 binding (Supplemental Figure 5B). The AngIV was maximally effective in 10 μM concentration in PtdIns(4,5)P_2 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)P_2 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-AT_1-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 AT_1-R-DRY/AAY-Rluc with SII-AngII and AngII. Stimulation of AT_1-R-DRY/AAY-Rluc with SII-

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

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 AT_1-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 AT_1-R endocytosis. Next we investigated the role of PtdIns(4,5)P_2 in the regulation of AT_1-R internalization. PtdIns(4,5)P_2 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)P_2 (Balla and Balla, 2006; Balla, 2013). Among those phosphatidylinositol 4-kinases (PI4Ks) control the first step in PtdIns(4,5)P_2 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 AT_1-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, A1 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

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

biased agonist induced response (Fig. 5), while the PI4KIIIβ was found not to be responsible for the maintenance of PtdIns(4,5)P_2 involved in the regulation of endocytosis.

Plasma membrane PtdIns(4,5)P_2 depletion effects AT_1-R internalization upon unbiased or biased activation. We also investigated the role of PtdIns(4,5)P_2 in the stimulation evoked Rab5 recruitment of AT_1-R. First, we utilized a plasma membrane PtdIns(4,5)P_2 depletion system, as earlier described (Toth et al., 2012). The appearance of AT_1-R in early endosomes was followed by BRET-measurements between AT_1-R-Rluc and fluorescently-tagged Rab5 in cells coexpressing the rapamycin-inducible PtdIns(4,5)P_2 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)P_2. 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)P_2 completely blocked both unbiased or biased activation induced AT_1-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)P_2 in the regulation of AT_1-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 AT_1-R stimulation induced plasma membrane PtdIns(4,5)P_2 hydrolysis in HEK293 cells (Supplemental Figure 8A). The Fig. 6 shows that overexpression of DN-GRK2 accelerated the AngII evoked AT_1-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

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

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 AT_1-R. Our results suggested that biased stimulation of the AT_1-R can lead to altered intracellular processing of the internalized receptor. To investigate this possibility, we performed BRET experiment to follow the AT_1-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 AT_1-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 AT_1-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 AT_1-R is partially responsible for the intracellular processing of the receptor. However, BAPTA-AM pretreatment did not modify the association of the AT_1-R with Rab4, Rab7 and Rab11 (data not shown) arguing against the role of Ca^{2+} signal generation in this process.

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

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 chlathrin-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 chlathrin-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

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

et al., 2003; Violin et al., 2010; Wei et al., 2003), differences were expected in the fate of AT_1-R after biased activation. All biased agonists (SII-AngII, TRV120023 and TRV120027) induced co-localization of AT_1-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 AT_1-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 G_q activation initiated calcium signal (Supplemental Figure 4) in the background of the dissimilar rate of endocytosis after bias activation of AT_1-R. We were also not able to demonstrate correlation between the β-arrestin binding and the accelerated internalization of the AT_1-R after stimulation with biased agonists (Fig. 3). Moreover, it seems that the course of AT_1-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 AT_1-R agonist AngIV caused similarly reduced and less stable AT_1-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)P_2 breakdown in our system, while the biased agonists not (Supplemental Figure 1), we investigated the role of PtdIns(4,5)P_2 in the regulation of the AT_1-R internalization. Pharmacological approach revealed that PI4KIIIα is responsible for the maintenance of the PtdIns(4,5)P_2 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 AT_1-R (Fig. 4 and 5). The inhibitory effect

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

of the inhibition of PtdIns(4,5)P_2 resynthesis by the blockade of PI4KIIIα 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

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

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 AT_1-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 AT_1-R with Rab11 is a latter event after stimulation. The AngII stimulus caused a drop in the BRET ratio between Rab11-YFP and AT_1-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 AT_1-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

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

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.

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgements

The excellent technical assistance of Mártonné Schultz is greatly appreciated.

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

Authorship Contributions

Participated in research design: Szakadáti, Tóth, Erdélyi, Várnai, Hunyady, and A. Balla.

Conducted experiments: Szakadáti, Tóth, Oláh, Erdélyi, and A. Balla.

Contributed new reagents or analytic tools: T. Balla.

Performed data analysis: Szakadáti, Tóth, Erdélyi, Várnai, Hunyady, and A. Balla.

Wrote or contributed to the writing of the manuscript: Szakadáti, Tóth, Erdélyi, T.Balla, Várnai, Hunyady, and A. Balla.

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

References

Anborgh PH, Seachrist JL, Dale LB and Ferguson SS (2000) Receptor/beta-arrestin complex formation and the differential trafficking and resensitization of beta2-adrenergic and angiotensin II type 1A receptors. *Molecular endocrinology (Baltimore, Md* **14**(12): 2040-2053.

Balla A and Balla T (2006) Phosphatidylinositol 4-kinases: old enzymes with emerging functions. *Trends Cell Biol* **16**(7): 351-361.

Balla A, Kim YJ, Varnai P, Szentpetery Z, Knight Z, Shokat KM and Balla T (2008) Maintenance of hormone-sensitive phosphoinositide pools in the plasma membrane requires phosphatidylinositol 4-kinase IIIalpha. *Molecular biology of the cell* **19**(2): 711-721.

Balla A, Toth DJ, Soltesz-Katona E, Szakadati G, Erdelyi LS, Varnai P and Hunyady L (2012) Mapping of the localization of type 1 angiotensin receptor in membrane microdomains using bioluminescence resonance energy transfer-based sensors. *The Journal of biological chemistry* **287**(12): 9090-9099.

Balla A, Tuymetova G, Tsiomenko A, Varnai P and Balla T (2005) A plasma membrane pool of phosphatidylinositol 4-phosphate is generated by phosphatidylinositol 4-kinase type-III alpha: Studies with the PH domains of the oxysterol binding protein and FAPP1. *Molecular biology of the cell* **16**(3): 1282-1295.

Balla T (2013) Phosphoinositides: tiny lipids with giant impact on cell regulation. *Physiol Rev* **93**(3): 1019-1137.

Bojjireddy N, Botyanszki J, Hammond G, Creech D, Peterson R, Kemp DC, Snead M, Brown R, Morrison A, Wilson S, Harrison S, Moore C and Balla T (2014) Pharmacological and genetic targeting of the PI4KA enzyme reveals its important role in maintaining plasma membrane phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate levels. *The Journal of biological chemistry* **289**(9): 6120-6132.

Bonde MM, Hansen JT, Sanni SJ, Haunso S, Gammeltoft S, Lyngso C and Hansen JL (2010) Biased signaling of the angiotensin II type 1 receptor can be mediated through distinct mechanisms. *PloS one* **5**(11): e14135.

Bucci C, Thomsen P, Nicoziani P, McCarthy J and van Deurs B (2000) Rab7: a key to lysosome biogenesis. *Molecular biology of the cell* **11**(2): 467-480.

Carman CV, Parent JL, Day PW, Pronin AN, Sternweis PM, Wedegaertner PB, Gilman AG, Benovic JL and Kozasa T (1999) Selective regulation of Galpha(q/11) by an RGS domain in the G protein-coupled receptor kinase, GRK2. *The Journal of biological chemistry* **274**(48): 34483-34492.

Dale LB, Seachrist JL, Babwah AV and Ferguson SS (2004) Regulation of angiotensin II type 1A receptor intracellular retention, degradation, and recycling by Rab5, Rab7, and Rab11 GTPases. *The Journal of biological chemistry* **279**(13): 13110-13118.

Danser AH (2003) Local renin-angiotensin systems: the unanswered questions. *Int J Biochem Cell Biol* **35**(6): 759-768.

Ferguson SS (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacological reviews* **53**(1): 1-24.

Ferguson SS, Menard L, Barak LS, Koch WJ, Colapietro AM and Caron MG (1995) Role of phosphorylation in agonist-promoted beta 2-adrenergic receptor sequestration. Rescue of

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

a sequestration-defective mutant receptor by beta ARK1. *The Journal of biological chemistry* **270**(42): 24782-24789.

Gaborik Z, Jagadeesh G, Zhang M, Spat A, Catt KJ and Hunyady L (2003) The role of a conserved region of the second intracellular loop in AT1 angiotensin receptor activation and signaling. *Endocrinology* **144**(6): 2220-2228.

Gaborik Z, Szaszak M, Szidonya L, Balla B, Paku S, Catt KJ, Clark AJ and Hunyady L (2001) Beta-arrestin- and dynamin-dependent endocytosis of the AT1 angiotensin receptor. *Molecular pharmacology* **59**(2): 239-247.

Godin CM and Ferguson SS (2012) Biased Agonism of the Angiotensin II Type 1 Receptor. *Mini reviews in medicinal chemistry* **12**(9): 812-816.

Hansen JL, Aplin M, Hansen JT, Christensen GL, Bonde MM, Schneider M, Haunso S, Schiffer HH, Burstein ES, Weiner DM and Sheikh SP (2008) The human angiotensin AT(1) receptor supports G protein-independent extracellular signal-regulated kinase 1/2 activation and cellular proliferation. *Eur J Pharmacol* **590**(1-3): 255-263.

Heuser JE and Anderson RG (1989) Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. *The Journal of cell biology* **108**(2): 389-400.

Holloway AC, Qian H, Pipolo L, Ziogas J, Miura S, Karnik S, Southwell BR, Lew MJ and Thomas WG (2002) Side-chain substitutions within angiotensin II reveal different requirements for signaling, internalization, and phosphorylation of type 1A angiotensin receptors. *Molecular pharmacology* **61**(4): 768-777.

Hunyady L, Baukal AJ, Gaborik Z, Olivares-Reyes JA, Bor M, Szaszak M, Lodge R, Catt KJ and Balla T (2002) Differential PI 3-kinase dependence of early and late phases of recycling of the internalized AT1 angiotensin receptor. *The Journal of cell biology* **157**(7): 1211-1222.

Hunyady L and Catt KJ (2006) Pleiotropic AT1 receptor signaling pathways mediating physiological and pathogenic actions of angiotensin II. *Molecular endocrinology* **20**(5): 953-970.

Hunyady L, Catt KJ, Clark AJ and Gaborik Z (2000) Mechanisms and functions of AT(1) angiotensin receptor internalization. *Regulatory peptides* **91**(1-3): 29-44.

Kim J, Ahn S, Ren XR, Whalen EJ, Reiter E, Wei H and Lefkowitz RJ (2005) Functional antagonism of different G protein-coupled receptor kinases for beta-arrestin-mediated angiotensin II receptor signaling. *Proceedings of the National Academy of Sciences of the United States of America* **102**(5): 1442-1447.

Kim KS, Abraham D, Williams B, Violin JD, Mao L and Rockman HA (2012) beta-Arrestin-biased AT1R stimulation promotes cell survival during acute cardiac injury. *American journal of physiology Heart and circulatory physiology* **303**(8): H1001-1010.

Le MT, Vanderheyden PM, 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. *The Journal of biological chemistry* **277**(26): 23107-23110.

Lefkowitz RJ (2007) Seven transmembrane receptors: something old, something new. *Acta Physiol (Oxf)* **190**(1): 9-19.

Li H, Li HF, Felder RA, Periasamy A and Jose PA (2008) Rab4 and Rab11 coordinately regulate the recycling of angiotensin II type I receptor as demonstrated by fluorescence resonance energy transfer microscopy. *Journal of biomedical optics* **13**(3): 031206.

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL and Lefkowitz RJ (2001) Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. *Proceedings of the National Academy of Sciences of the United States of America* **98**(5): 2449-2454.

Maxfield FR and McGraw TE (2004) Endocytic recycling. *Nature reviews* **5**(2): 121-132.

Orlandi PA and Fishman PH (1998) Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *The Journal of cell biology* **141**(4): 905-915.

Qian H, Pipolo L and Thomas WG (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. *Molecular endocrinology (Baltimore, Md* **15**(10): 1706-1719.

Reiter E, Ahn S, Shukla AK and Lefkowitz RJ (2012) Molecular mechanism of beta-arrestin-biased agonism at seven-transmembrane receptors. *Annual review of pharmacology and toxicology* **52**: 179-197.

Sallese M, Mariggio S, D'Urbano E, Iacovelli L and De Blasi A (2000) Selective regulation of Gq signaling by G protein-coupled receptor kinase 2: direct interaction of kinase N terminus with activated galphaq. *Molecular pharmacology* **57**(4): 826-831.

Saraste J and Goud B (2007) Functional symmetry of endomembranes. *Molecular biology of the cell* **18**(4): 1430-1436.

Seachrist JL and Ferguson SS (2003) Regulation of G protein-coupled receptor endocytosis and trafficking by Rab GTPases. *Life sciences* **74**(2-3): 225-235.

Seachrist JL, Laporte SA, Dale LB, Babwah AV, Caron MG, Anborgh PH and Ferguson SS (2002) Rab5 association with the angiotensin II type 1A receptor promotes Rab5 GTP binding and vesicular fusion. *The Journal of biological chemistry* **277**(1): 679-685.

Soergel DG, Subach RA, Cowan CL, Violin JD and Lark MW (2013) First clinical experience with TRV027: pharmacokinetics and pharmacodynamics in healthy volunteers. *Journal of clinical pharmacology* **53**(9): 892-899.

Sonnichsen B, De Renzis S, Nielsen E, Rietdorf J and Zerial M (2000) Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. *The Journal of cell biology* **149**(4): 901-914.

Szidonya L, Supeki K, Karip E, Turu G, Varnai P, Clark AJ and Hunyady L (2007) AT1 receptor blocker-insensitive mutant AT1A angiotensin receptors reveal the presence of G protein-independent signaling in C9 cells. *Biochem Pharmacol* **73**(10): 1582-1592.

Takahashi S, Kubo K, Waguri S, Yabashi A, Shin HW, Katoh Y and Nakayama K (2012) Rab11 regulates exocytosis of recycling vesicles at the plasma membrane. *Journal of cell science* **125**(Pt 17): 4049-4057.

Toth DJ, Toth JT, Gulyas G, Balla A, Balla T, Hunyady L and Varnai P (2012) Acute depletion of plasma membrane phosphatidylinositol 4,5-bisphosphate impairs specific steps in endocytosis of the G-protein-coupled receptor. *Journal of cell science* **125**(Pt 9): 2185-2197.

Turu G, Szidonya L, Gaborik Z, Buday L, Spat A, Clark AJ and Hunyady L (2006) Differential beta-arrestin binding of AT1 and AT2 angiotensin receptors. *FEBS Lett* **580**(1): 41-45.

Varnai P and Balla T (1998) Visualization of phosphoinositides that bind pleckstrin homology domains: calcium- and agonist-induced dynamic changes and relationship to myo-[3H]inositol-labeled phosphoinositide pools. *The Journal of cell biology* **143**(2): 501-510.

Violin JD, Crombie AL, Soergel DG and Lark MW (2014) Biased ligands at G-protein-coupled receptors: promise and progress. *Trends in pharmacological sciences* **35**(7): 308-316.

Violin JD, DeWire SM, Yamashita D, Rominger DH, Nguyen L, Schiller K, Whalen EJ, Gowen M and Lark MW (2010) Selectively engaging beta-arrestins at the angiotensin II type 1 receptor reduces blood pressure and increases cardiac performance. *The Journal of pharmacology and experimental therapeutics* **335**(3): 572-579.

Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM and Lefkowitz RJ (2003) Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proceedings of the National Academy of Sciences of the United States of America* **100**(19): 10782-10787.

Whalen EJ, Rajagopal S and Lefkowitz RJ (2011) Therapeutic potential of beta-arrestin- and G protein-biased agonists. *Trends in molecular medicine* **17**(3): 126-139.

Woo J and von Arnim AG (2008) Mutational optimization of the coelenterazine-dependent luciferase from Renilla. *Plant methods* **4**: 23.

Zerial M and McBride H (2001) Rab proteins as membrane organizers. *Nature reviews* **2**(2): 107-117.

Zhang J, Ferguson SS, Barak LS, Menard L and Caron MG (1996) Dynamin and beta-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. *The Journal of biological chemistry* **271**(31): 18302-18305.

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

This work was supported by the Hungarian Scientific Research Fund OTKA [NK100883 and K105006], National Development Agency NFU TAMOP [4.2.2-08/1/KMR and 4-2.1/B-09/1/KMR-2010-0001]. A.B. was also supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences.

This research was supported in part by the Intramural Research Program of the National Institutes of Health (Section on Molecular Signal Transduction, Program for Developmental Neuroscience, Eunice Kennedy Shriver National Institute of Child Health and Human Development).

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

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).

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3. BRET assay between AT_1-R and β-arrestin2 upon agonist stimulation in HEK293 cells. HEK293 cells were transfected with the AT_1-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 AT_1-R in HEK293 cells. HEK293 cells were transfected with the plasmids of the AT_1-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 AT_1-R in HEK293 cells. HEK293 cells were transfected with the plasmids of the AT_1-R-Rluc and with Rab5-YFP

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

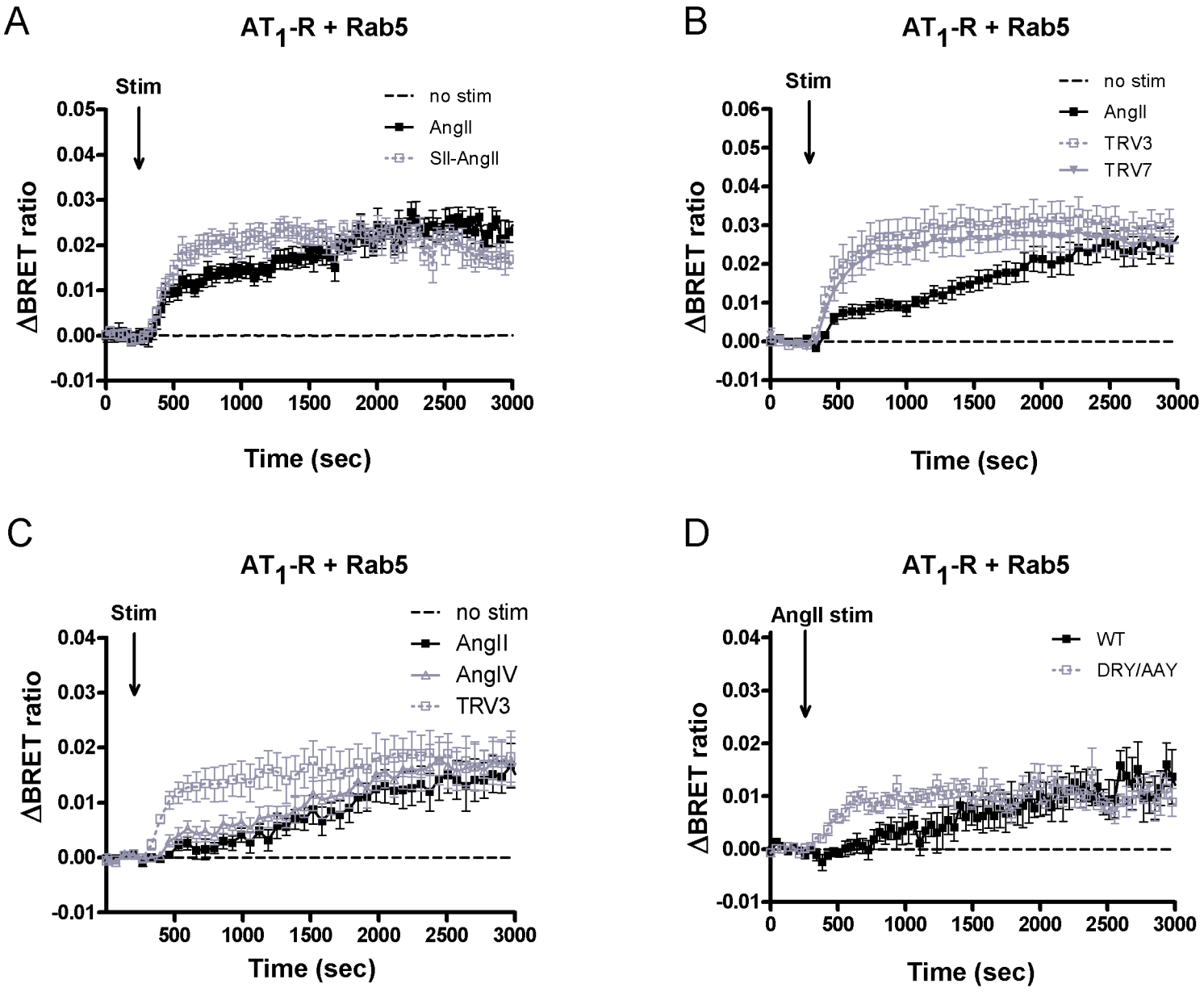
proteins, and after 24 hours the experiments were carried out. Cells were pretreated for 10 min with vehicle BRET medium (black filled symbols), BRET medium supplemented with 10 nM A1 (black open squares), or with 250 nM PIK-93 (grey open triangles) and exposed to either vehicle (dashed line) or 100 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, AT_1-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 AT_1-R and different Rab proteins upon agonist stimulation in HEK293 cells. HEK293 cells were transfected with the plasmids of the AT_1-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 AT_1-R-Rluc; (B, D, F) Rab11-YFP and AT_1-R-Rluc. The BRET records are average of at least 3 independent experiments. Mean values ± SEM are shown (n = 3).

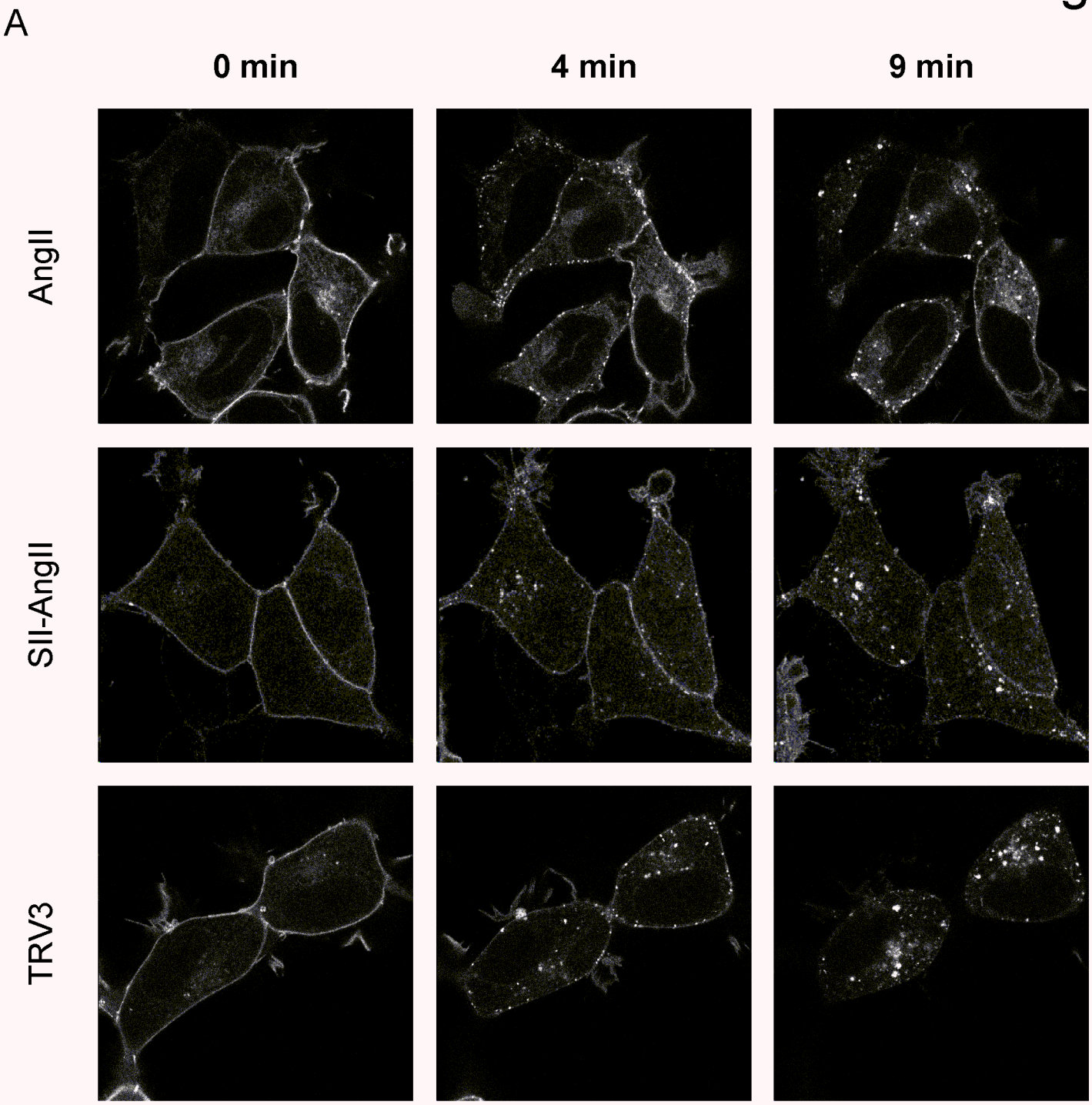
Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 1



Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2



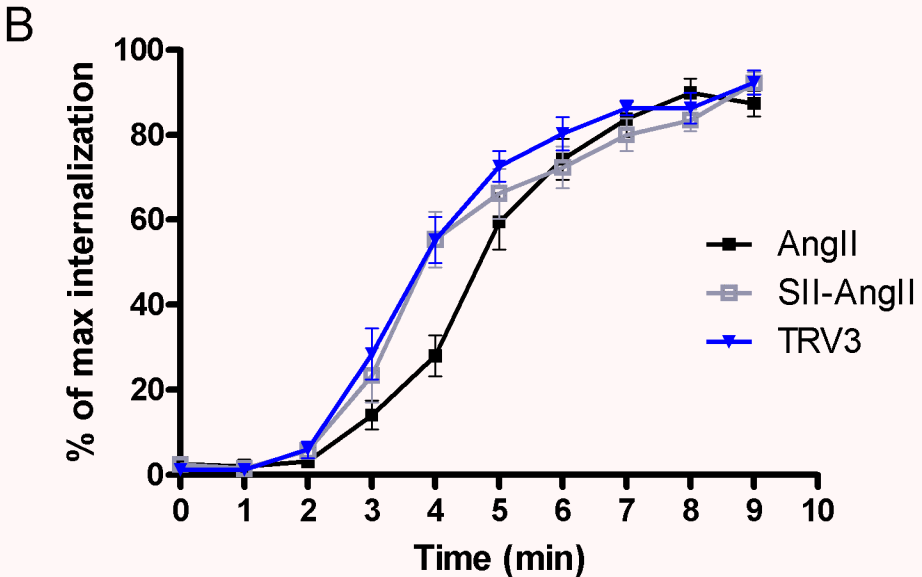
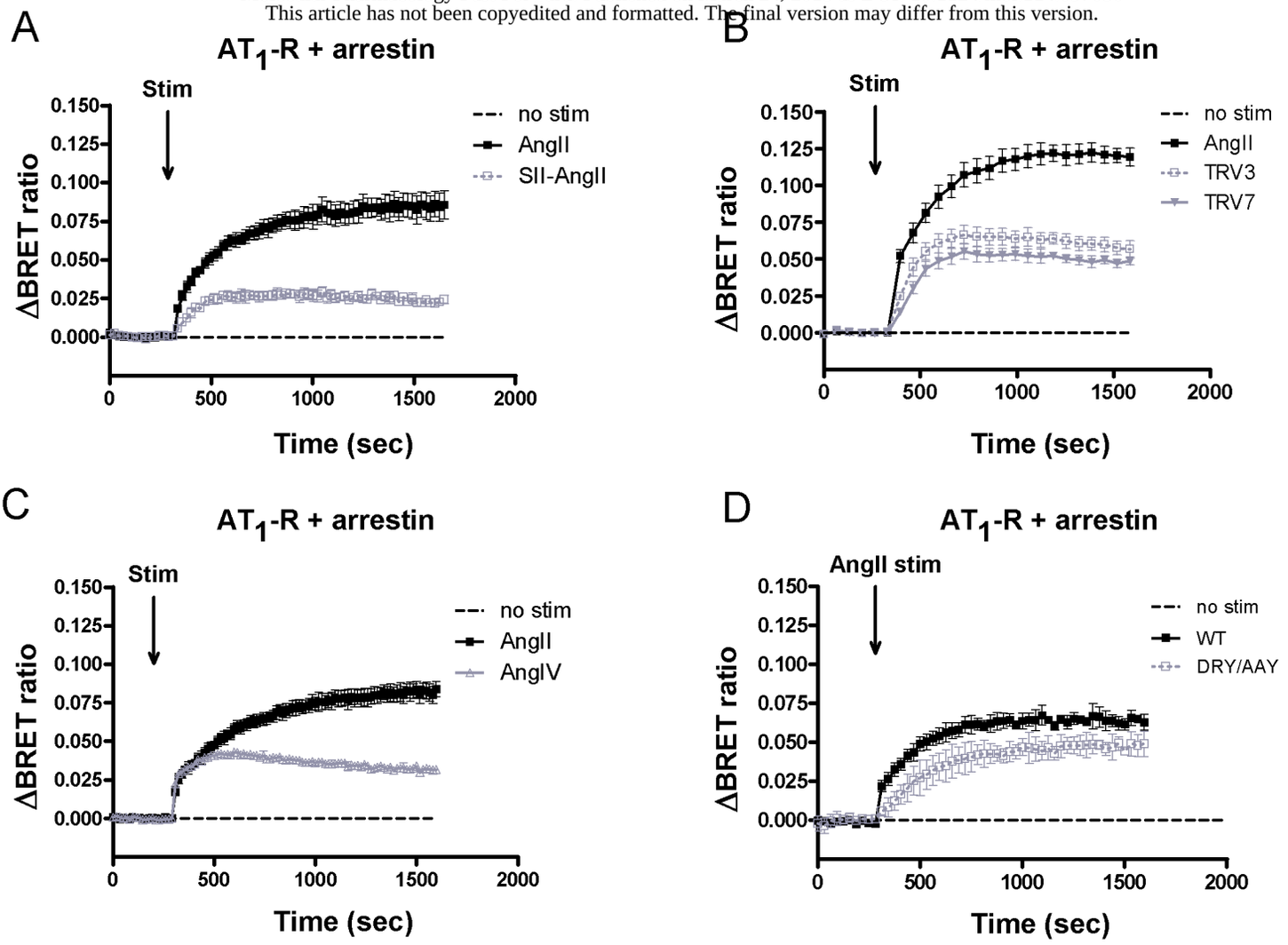


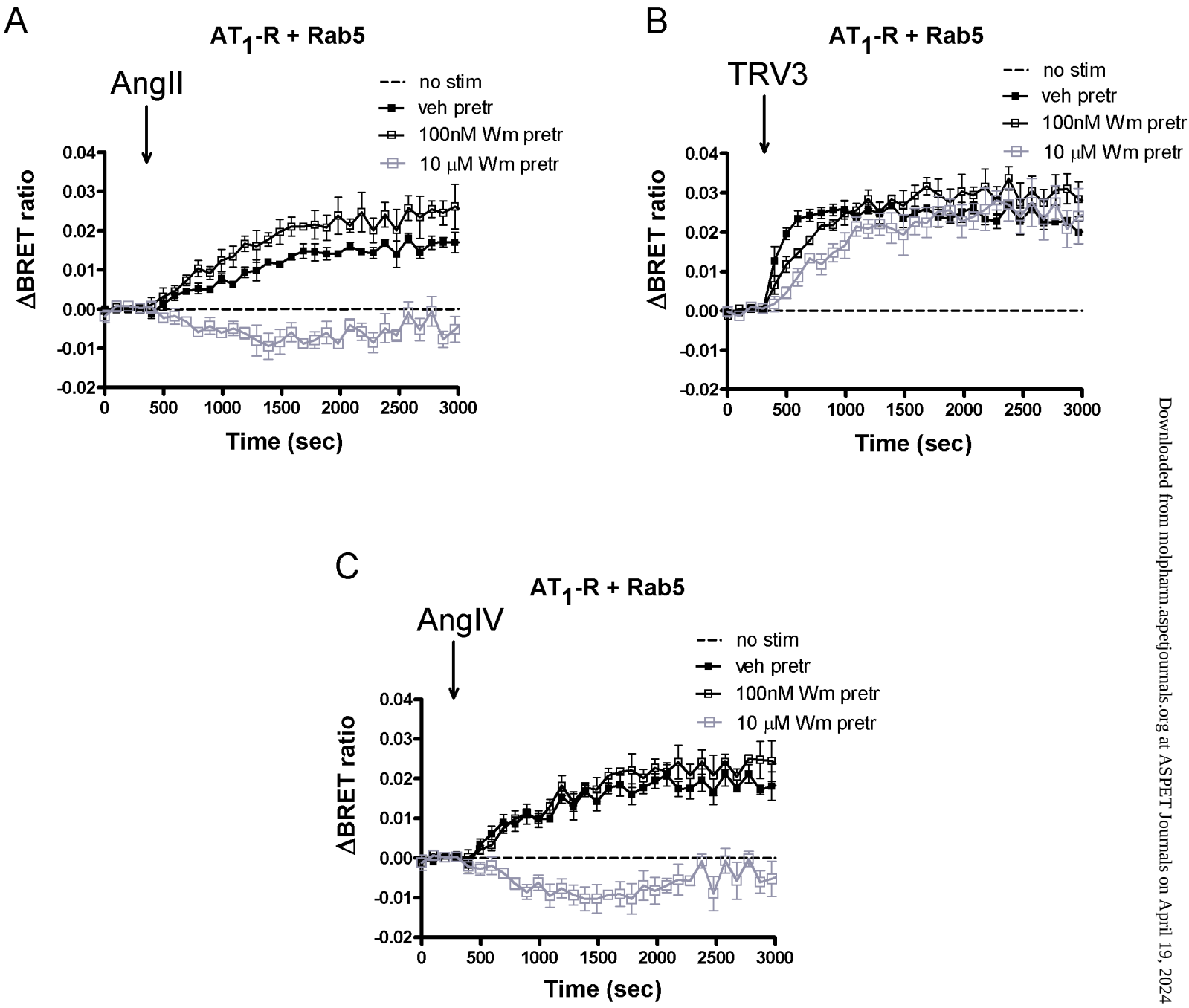
Figure 3

Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.



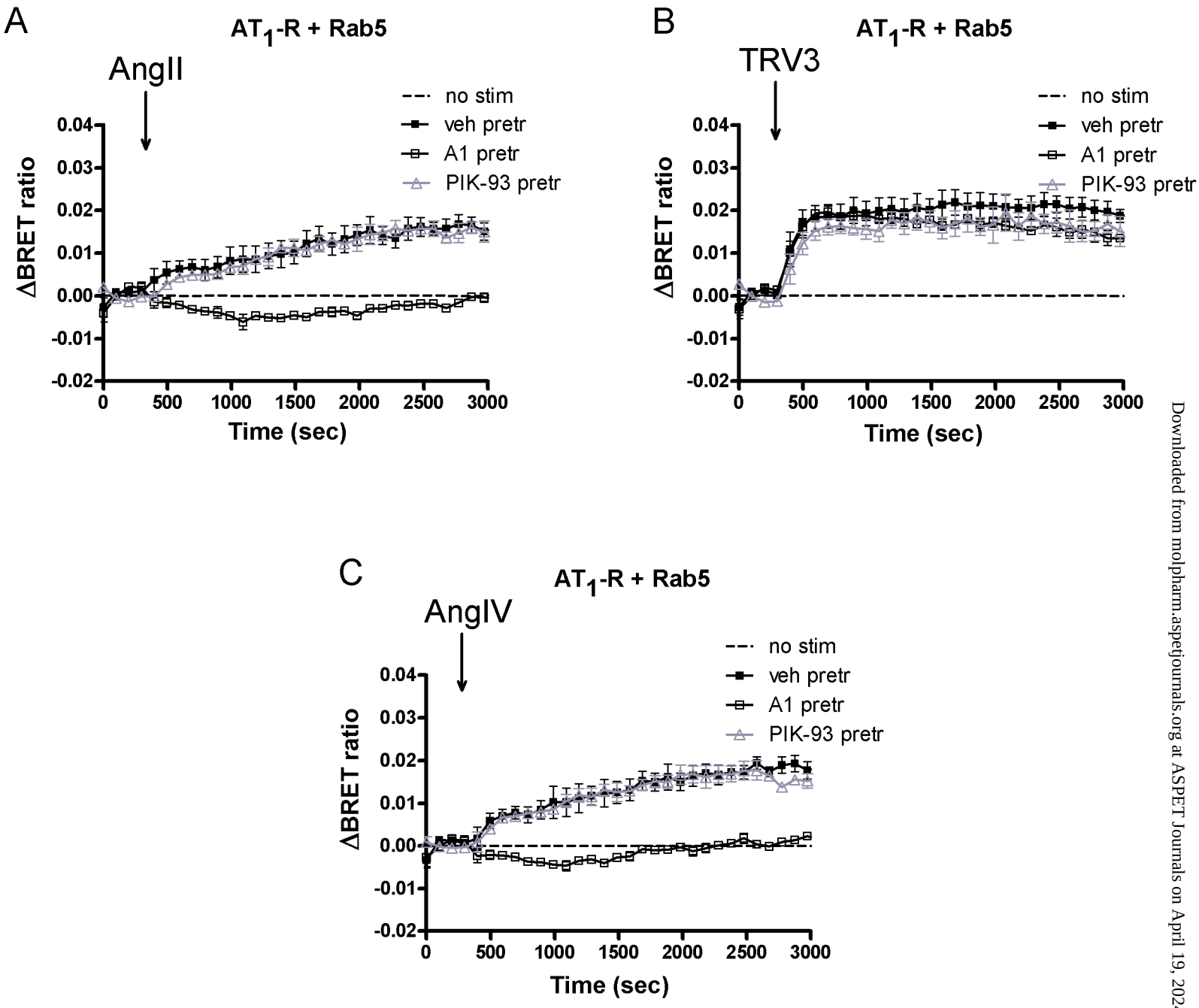
Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 4



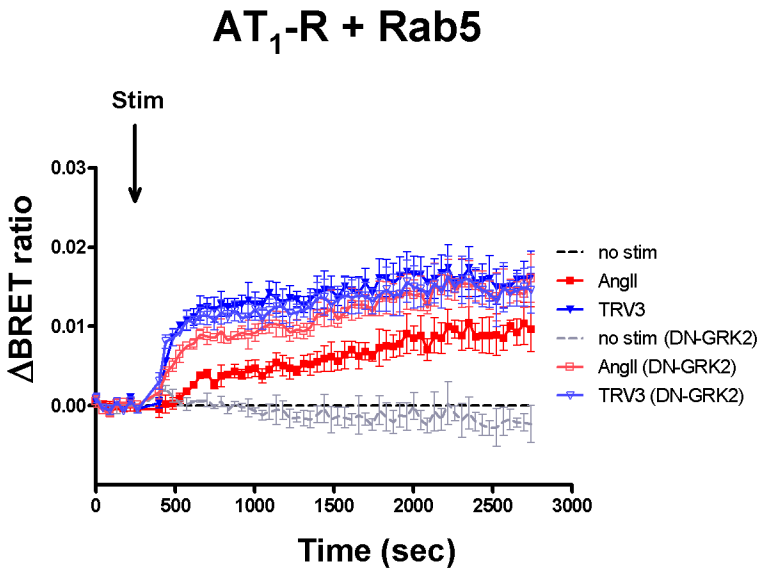
Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5



Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 6



Molecular Pharmacology Fast Forward. Published on March 24, 2015 as DOI: 10.1124/mol.114.097030
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 7