BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2010.00875.x
www.brjpharmacol.org

RESEARCH PAPER

β-Arrestin 1 and 2 stabilize the angiotensin II type I receptor in distinct high-affinity conformations

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Keywords
AT_1 receptor; β-arrestins; fusion proteins; ternary complex; high-affinity conformation

Received
23 November 2009
Revised
23 March 2010
Accepted
11 April 2010

BACKGROUND AND PURPOSE

The angiotensin II type 1 (AT_1) receptor belongs to family A of 7 transmembrane (7TM) receptors. The receptor has important roles in the cardiovascular system and is commonly used as a drug target in cardiovascular diseases. Interaction of 7TM receptors with G proteins or β-arrestins often induces higher binding affinity for agonists. Here, we examined interactions between AT_{1A} receptors and β-arrestins to look for differences between the AT_{1A} receptor interaction with β-arrestin1 and β-arrestin2.

EXPERIMENTAL APPROACH

Ligand-induced interaction between AT_{1A} receptors and β-arrestins was measured by Bioluminescence Resonance Energy Transfer 2. AT_{1A}-β-arrestin1 and AT_{1A}-β-arrestin2 fusion proteins were cloned and tested for differences using immunocytochemistry, inositol phosphate hydrolysis and competition radioligand binding.

KEY RESULTS

Bioluminescence Resonance Energy Transfer 2 analysis showed that β-arrestin1 and 2 were recruited to AT_{1A} receptors with similar ligand potencies and efficacies. The AT_{1A}-β-arrestin fusion proteins showed attenuated G protein signalling and increased agonist binding affinity, while antagonist affinity was unchanged. Importantly, larger agonist affinity shifts were observed for AT_{1A}-β-arrestin2 than for AT_{1A}-β-arrestin1.

CONCLUSION AND IMPLICATIONS

β-Arrestin1 and 2 are recruited to AT_{1A} receptors with similar ligand pharmacology and stabilize AT_{1A} receptors in distinct high-affinity conformations. However, β-arrestin2 induces a receptor conformation with a higher agonist-binding affinity than β-arrestin1. Thus, this study demonstrates that β-arrestins interact with AT_{1A} receptors in different ways and suggest that AT_1 receptor biased agonists with the ability to recruit either of the β-arrestins selectively, would be possible to design.

Abbreviations

AT_1, angiotensin II type 1; ERK, extracellular signal-regulated kinase; IP, inositol phosphate; 7TM, 7 transmembrane

Introduction

The angiotensin II type 1 (AT_1) receptor (nomenclature follows Alexander *et al.*, 2009) belongs to family A of 7 transmembrane (7TM) receptors and plays a central role in blood pressure regulation and fluid homeostasis (Hunyady and Catt, 2006). The pathophysiological importance of AT_1 receptors is emphasized by the use of AT_1 receptor blockers and angiotensin converting enzyme inhibitors in

treatment of cardiovascular diseases such hypertension and cardiac failure (Burnier and Brunner, 2000; Weir, 2007).

The AT_1 receptor signals through two major pathways, namely the G_q protein-dependent signalling pathway, leading to inositol phosphate (IP) accumulation and calcium release and the G protein-independent signalling, which involves recruitment of the scaffolding proteins, β-arrestin1 and β-arrestin2 (Aplin *et al.*, 2008; 2009; Hunyady and Catt, 2006).

The two β-arrestin isoforms share 78% sequence identity and regulate the majority of 7TM receptors (Gurevich and Gurevich, 2006). The binding of β-arrestins to AT_1 receptors hinders these receptors from further interaction with G proteins. This process is termed desensitization. Besides terminating G protein-dependent signalling, β-arrestin1 and β-arrestin2 also function as scaffolds, as they mediate internalization and initiate a second 'wave' of signalling involving many kinases and signalling molecules (Lefkowitz and Shenoy, 2005; Zhai *et al.*, 2005; Aplin *et al.*, 2007; Xiao *et al.*, 2007; Hansen *et al.*, 2008).

The two β-arrestin isoforms may have different, and in some cases opposite, properties in terms of kinase activation and internalization (Ahn *et al.*, 2004; Kuo *et al.*, 2006; Fan *et al.*, 2007; Kumar *et al.*, 2007; Sneddon and Friedman, 2007). It has been shown that down regulation of β-arrestin2 leads to a decrease in AT_1 receptor-mediated activation of the extracellular signal-regulated kinase (ERK1/2), while down regulation of β-arrestin1 markedly increases ERK1/2 activation (Ahn *et al.*, 2004). Recently it has also been demonstrated that β-arrestin2 increases smooth muscle cell proliferation and migration, thereby aggravating atherosclerosis, while β-arrestin1 was shown to have the opposite effect (Kim *et al.*, 2008). These findings clearly suggest that it could be beneficial to design drugs working as biased agonists that selectively recruit either β-arrestin1 or -2. Furthermore, the different properties of β-arrestin1 and β-arrestin2 in modulating receptor signalling may to some extent reflect different modes of interaction between the receptor and the two β-arrestin isoforms.

Based on their interaction with β-arrestins, the 7TM receptors are divided into two classes. Class A receptors, which include receptors such as the β_2 adrenoceptor and vasopressin 1a (V_{1a}) receptor, interact transiently with β-arrestins. In contrast, class B receptors, such as the AT_1 and the vasopressin 2 (V_2) receptor, interact more stably with β-arrestins (Oakley *et al.*, 2000). Furthermore, class A and B receptors differ in their preference towards the two β-arrestin isoforms. Class A receptors have been shown to bind β-arrestin2 more tightly than β-arrestin1, whereas class B receptors bind β-arrestin1 and 2 equally well (Oakley *et al.*, 2000). However, these studies do not address how β-arrestins affect the receptor conformation or how they affect ligand binding properties.

It has been observed that both G proteins and β-arrestins are able to increase receptor affinity for agonists, as described in the ternary complex model (Samama *et al.*, 1993; Gurevich *et al.*, 1997; Martini *et al.*, 2002; Jorgensen *et al.*, 2005). The interaction between 7TM receptors and β-arrestin has been studied by using fusion proteins, where the C terminus tail of the 7TM receptor was fused directly to the N terminus end of β-arrestin.

In order to study the interactions between AT_{1A} receptors and β-arrestins quantitatively, we used two strategies: β-arrestin recruitment-based Bioluminescence Resonance Energy Transfer (BRET) assay and studies of fusion proteins between AT_{1A} receptors and β-arrestin1 or 2. The results showed that β-arrestin1 and 2 were both recruited to AT_{1A} receptor with similar ligand potencies and efficacies. Furthermore, we found that the AT_{1A}-β-arrestin2 fusion protein displayed a higher affinity for agonists than the AT_{1a}-β-arrestin1 protein, a difference most likely to reflect the induction of different receptor conformations.

Methods

Molecular biology

All constructs are expressed in pcDNA3.1. The wild-type (WT) HA-tagged rat AT_{1A} receptor was made by inserting cDNA encoding the peptide sequence of the HA tag in the N-terminus of the rat AT_{1A} receptor. To create the AT_{1A}-β-arrestin fusion proteins, the coding sequence of HA-tagged rat AT_{1A} receptor lacking the stop codon was digested with NheI and BamHI and inserted into the N terminus end of cDNA sequence of β-arrestin1 and β-arrestin2 respectively. All constructs were verified by sequencing. The AT_{1A} receptor fused to Rluc is described in (Hansen *et al.*, 2004b).

In our experiment we used two different cell types: COS-7 cells and HEK293T cells. These cell lines provide good models for studying binding characteristics and protein recruitment, because they are easily transfected and easy to handle. The assay of IP accumulation and radioligand binding assays include many washing steps, and COS-7 cells were used in these assays, as they adhere more tightly to the wells. For the BRET experiments the HEK293 cells were preferred, because they present

the receptor better on the cell surface than COS-7 cells

β-arrestin recruitment assay

HEK293T cells (4.5×10^6) were seeded into a p10 dish and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, Gentamycin (0.5 mg·mL^{-1}) and L-glutamine (2 mM). After 24 h, the cells were transfected using Polyethylenimine (PEI). We used 1 µg of AT_{1A}-Rluc and 3ug of either GFP2-β-arrestin1 or GFP2-β-arrestin2.

Forty-eight hours post transfection, HEK293T cells were washed with PBS, detached with PBS/trypsin-EDTA (0.25% trypsin; 1 mM EDTA, Invitrogen), harvested by centrifugation (5 min, 1000× g), resuspended in PBS supplemented with 0.5 mM Ca^{2+} and 0.5 mM Mg^{2+} and incubated at room temperature on a shaker (app. 250 r.p.m.) until the time of the experiments. The resuspended cells were distributed in 96-well microplates (black/white optiplate, PerkinElmer) and incubated in the presence or absence of different ligands for 15 min after agonist addition for dose–response curves. This time was chosen after preliminary time-dependent β-arrestin recruitment experiments showed that the BRET response reached a plateau after approximately 10–15 min (data not shown).

DeepBlueC coelenterazine (Coelenterazine 400a, Biosynth) was added 2 s before reading, using an injector at a final concentration of 5 µM. Measurement of Rluc-mediated luminescence and GFP^2-mediated emission from each well was made using a Tecan Infinite F500 microplate reader (Tecan Group Ltd, Männedorf, Switzerland). The BRET ratio was determined by calculating the ratio of the light emitted by GFP2 (515 nm) over the light emitted by the Rluc (410 nm). The background BRET signal from Rluc was determined by coexpressing the Rluc construct with empty vector, and the BRET2 ratio generated from this transfection was subtracted from all other BRET2 ratios. The maximum BRET2 response generated by angiotensin II (Ang II) was set to 100% and the response mediated by all the other ligands was normalized to this value.

Immunocytochemistry and confocal microscopy

HEK293 cells were grown in DMEM supplemented with L-glutamine and 10% FBS. On day 1, cells were seeded in 10 cm dishes, 4×10^6 cells per dish in 10 mL medium. On day 2, cells were transfected with 5 µg HA-tagged WT AT_{1A} receptor or AT_{1A}-β-arrestin1 or AT_{1A}-β-arrestin2 using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. For transfection, DNA and Lipofectamine2000 were both diluted in Optimem (Invitrogen) and medium was changed on cells 4 h after transfection. On day 3, cells were seeded on poly-L-lysine coated coverslips (Corning) in 2 mL medium. Cells were grown until about 50% confluency and immunocytochemistry was performed.

To visualize receptors expressed on the cell surface, an antibody feeding assay was performed. Cells were incubated for 30 min at 37°C with anti-HA11 antibody (1:1000, Covance), stimulated with Ang II or left untreated for 30 min, washed with PBS and fixed with 4% paraformaldehyde in PBS. Cells were subsequently blocked and permeabilized in blotting solution (50 mM Tris-Cl pH 7.5, 1 mM $CaCl_2$, 3% milk, 0.1% Triton X-100) for 20 min and stained with fluorescently conjugated Alexa anti-IgG_1 488 (green) antibody (1:500 in blotting solution, Invitrogen) for 45 min. Coverslips were mounted onto glass slides using mounting media from Vectashield containing 4′,6′-diamidino-2-phenylidole (DAPI).

To visualize total amount of receptor protein, cells were initially washed with PBS, fixed and permeabilized as above, and then incubated with anti-HA-11 antibody (1:1000 in blotting solution) for 1 h. Cells were washed three times in Tris-buffered saline with 1 mM $CaCl_2$, incubated with secondary antibody and mounted as described above. Mounted slides were analysed on an LSM510 laser confocal microscope (Zeiss) using the 63x oil objective.

PI hydrolysis assay

COS-7 cells (2.5×10^6) were seeded in a p10 dish and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (50 units·mL^{-1}), streptomycin (50 units·mL^{-1}) and L-glutamine (2 mM). After 24 h, the cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The day after transfection, cells were split into poly-D-lysine coated 96-well plates (50 000 cells per well) in inositol-free DMEM supplemented with non-essential amino acids, 10% fetal calf serum and myo-[2-^{3}H]inositol (2 µCi·mL^{-1} medium). The following day, cells were washed with Hanks balanced salt solution (HBSS), supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM LiCl (HBSS++) and incubated with HBSS++ for 20 min at 37°C. The buffer was aspirated and cells were incubated with increasing concentrations of ligands for 20 min at 37°C. Ligands were removed, and cells were incubated on ice with formic acid (10 mM) for at least 45 min. 20 µL of the lysis solution was transferred to a solid white 96-well plate, and 80 µL of freshly diluted SPA YSi beads (12 mg·mL^{-1}, GE Healthcare) were added. The plates were shaken vigorously on a

shaker for half an hour, and incubated for at least 8 h at room temperature prior to measurement. The IP response was normalized to maximum Ang II response observed for the WT AT_{1A} receptor.

Competition radioligand binding assay

This assay was performed as described previously (Hansen *et al.*, 2004a). Briefly, COS-7 cells were transfected and seeded into 48-well plates as described above for the IP hydrolysis assay. Following day, cells were washed once with cold HBSS, supplemented with $CaCl_2$ (0.9 mM) and $MgCl_2$ (1.05 mM) and cooled at 4°C for 30 min. The HBSS was aspirated and cells were incubated on ice for 4 h with 1 nM [tyrosyl-3,5-^{3}H]Ang II (5-isoleucine) or 10^{-11} M of [^{125}I]-SI-Ang II and increasing amounts of unlabelled ligand. Each concentration point was performed in duplicate. After incubation, cells were washed twice in cold HBSS, before the addition of 0.5 mL lysis buffer (1.0% Triton X-100, 50 mM Tris/HCl, pH 7.5, 100 mM NaCl and 5 mM EDTA) for 30–60 min at room temperature. The lysis buffer was transferred to scintillation tubes containing 8 mL of Ultima Gold (PerkinElmer).

Data analysis

Data are shown as means ± SEM. Results were analysed by non-linear regression using Prism GraphPad Software. Values for inhibition and dissociation constant (K_i and K_d) were estimated from competition binding by using the equations: $K_d = IC_{50}\text{-}L$ and $K_i = IC_{50}/(1 + L/K_d)$, where L is the concentration of radioligand. Values of $P < 0.05$ were considered to show significant differences between means.

Materials

Ang II, Ang III (Val^4)Ang III and telmisartan were all from Sigma-Aldrich (St. Louis, MO, USA). SII-Ang II (Sar-Arg-Val-Ile-Ile-His-Pro-Ile-COOH) was synthesized at Cleveland Clinic, Lerner Research Institute (Cleveland, OH, USA). [^{3}H]-Ang II (tyrosyl-3,5-[^{3}H]Ang II 5-isoleucine) was from GE Healthcare while [^{125}I]-SI-Ang II (Sar-Arg-Val-Tyr-Val-His-Pro-Ile-COOH) was from PerkinElmer.

Results

Recruitment of β-arrestins 1 and 2 and G protein coupling to AT_{1A} receptors

To characterize the interaction between β-arrestin1 and 2 and AT_{1A} receptors, we co-expressed the C terminally Rluc-tagged AT_{1A} receptor (AT_{1A}-Rluc) with N terminally GFP2-tagged β-arrestin1 and 2 (GFP2-β-arrestin1 and GFP2-β-arrestin2), respectively, and measured the ligand-induced recruitment of β-arrestins using a BRET2 assay (Figure 1A and B). The expression of GFP2-β-arrestin1 and GFP2-β-arrestin2 was comparable in these experiments when measured by GFP2 as was the AT_1-Luc expression when measured by coelenterazine h luminescence as described previously (data not shown and Jensen *et al.*, 2002). We chose to study agonists with different potencies and efficacies with respect to G_q protein activation, measured by IP hydrolysis assay (Figure 1C). Accordingly, we used the following ligands: Ang II (full agonist with high potency); Ang III and (Val^4) Ang III (full agonists with low potency), SII-Ang II (biased agonist with low binding affinity and no agonist ability on G protein activation) (Holloway *et al.*, 2002; Wei *et al.*, 2003; Hansen *et al.*, 2008) and finally the high-affinity antagonist, telmisartan.

As depicted in Figure 1A and B, β-arrestin1 and 2 were recruited equally well to the AT_{1A} receptor in response to the tested ligands; the corresponding pEC_{50} values and efficacies are shown in Table 1. As observed for the G_q protein activation, both Ang III and (Val^4)Ang III were low-potency full agonists with respect to β-arrestin1 and 2 recruitment (Figure 1). In our experiments, the biased agonist SII-Ang II showed only weak ability to recruit β-arrestin1 and 2, and was also unable to induce G protein activation. Several studies show that SII-Ang II can act as both a full or a partial agonist with respect to β-arrestin recruitment (Holloway *et al.*, 2002; Wei *et al.*, 2003; Rajagopal *et al.*, 2006). This discrepancy remains to be fully elucidated but it has been shown that the expression of specific G protein kinases may influence the result (Kim *et al.*, 2005). Telmisartan produced no response in any of the assays, which was consistent with previous observations (Hansen *et al.*, 2008).

Subcellular localization of fusion constructs

To further analyse the interaction between AT_{1A} receptors and β-arrestins, we constructed fusion proteins between AT_{1A} receptors and β-arrestin1 and 2 respectively (Figure 2). The localization of the AT_{1A} receptor fusion proteins was visualized using immunocytochemistry and confocal microscopy on HEK293 cells transiently expressing HA-tagged WT AT_{1A} receptors, AT_{1A}-β-arrestin1 or AT_{1A}-β-arrestin2 constructs. Two different approaches were applied to visualize cell surface expressed proteins and total proteins respectively. To visualize receptors expressed on the cell surface, the antibody feeding assay was performed (Whistler *et al.*, 2002). In this assay, live cells were incubated with anti-HA11 antibody. As the cells are not permeabilized, this antibody incubation will only label receptors that are on the cell surface during the time of incubation. As

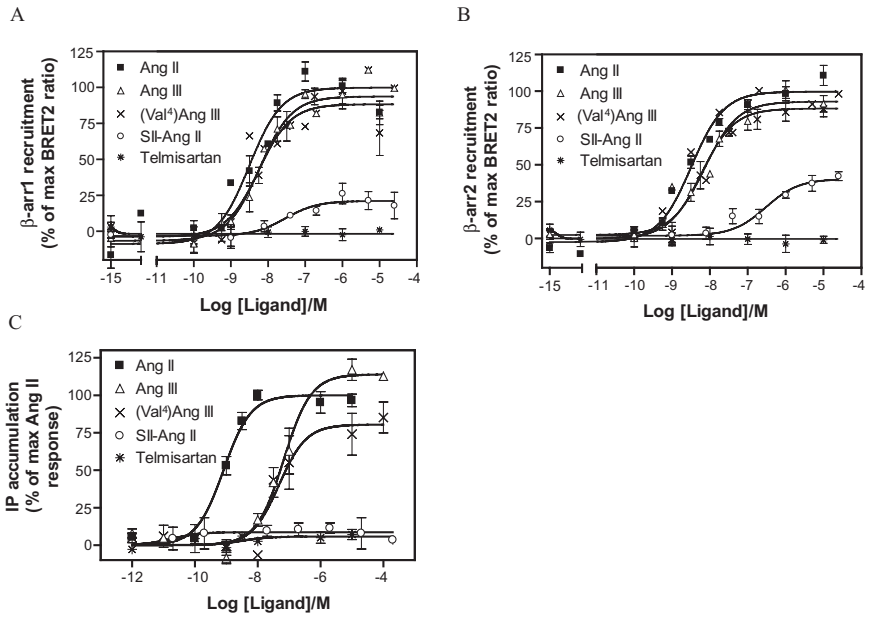

Figure 1

Ligand-induced β-arrestin1 and 2 recruitment and G protein coupling to WT AT_{1A} receptor. (A) and (B) The BRET2 response measured between the AT_{1A}-luc and GFP2-β-arrestin1 (β–arr1) and GFP2-β-arrestin2 (β-arr2), as a function of ligand concentration. The following ligands were used: Ang II, Ang III (Val^4)Ang III, SII-Ang II and telmisartan. (C) The AT_{1A} receptor-mediated G_q protein coupling was measured using a IP hydrolysis assay in response to ligand stimulation. Values are reported as a percentage of the maximum response to Ang II. Potencies and relative efficacies are reported in Table 1 and experimental details are described in the *Methods* section. AT_1, angiotensin II type 1; BRET2, Bioluminescence Resonance Energy Transfer 2; IP, inositol phospholipid; WT, wild type.

Table 1

AT_{1A} receptor-mediated β-arrestin recruitment and G protein coupling

Ligands	β-arrestin1 recruitment pEC_{50}	β-arrestin1 recruitment Relative efficacy	*n*	β-arrestin2 recruitment pEC_{50}	β-arrestin2 recruitment Relative efficacy	*n*	G_q coupling pEC_{50}	G_q coupling Relative efficacy	*n*
Ang II	8.4 ± 0.3	100 ± 0.0	7	8.5 ± 0.2	100 ± 0.0	7	9.0 ± 0.1	100 ± 0.0	5
Ang III	8.3 ± 0.2	90.5 ± 12.7	7	8.2 ± 0.4	86.1 ± 14.4	7	7.0 ± 0.1	122 ± 6.8	3
(Val^4)Ang III	8.3 ± 0.3	86.6 ± 18.2	7	8.3 ± 0.4	89.9 ± 12.4	7	7.5 ± 0.2	64.2 ± 4.4	3
SII-Ang II	7.5 ± 1.2	27.4 ± 19.4	7	6.8 ± 0.7*	38.2 ± 14.2	7		NR	3
Telmisartan		NR	7		NR	7		NR	3

The average pEC_{50} ± SEM and relative efficacy ± SEM values are reported. Relative efficacies are calculated as a percentage of maximum Ang II response. Dose-response curves are shown in Figure 1. Statistical analysis was performed by one-tailed paired *t*-test to determine significant differences between GFP2-β-arrestin1 and GFP2-β-arrestin2 recruitment (one-tailed, paired Student's *t*-test). *$P < 0.05$.
AT_1, angiotensin II type 1; NR, no response.

depicted in Figure 2A, WT AT_{1A} receptors were predominantly localized on the cell surface in the basal state, indicating low levels of internalization. In contrast, the HA-tagged AT_{1A}-β-arrestin1 and AT_{1A}-β-arrestin2 showed a cytoplasmic distribution under the same conditions (Figure 2A), indicating that these receptors constitutively internalize and/or recycle. This observation is in good agreement with the established role of β-arrestins as scaffolding molecules for clathrin-coated pit components, whereby the receptor is internalized (Krueger and Dipalma, 1997). Treatment with saturating amounts of Ang II for 30 min. resulted in primarily cytoplasmic staining, suggesting significant internalization of the WT AT_{1A} receptor (Figure 2B). The fusion proteins were also localized intracellularly in the Ang II-treated cells (Figure 2B).

To visualize the total amount of receptor protein, cells were fixed, permeabilized and then treated with anti-HA11 antibody. As shown in Figure 2C,

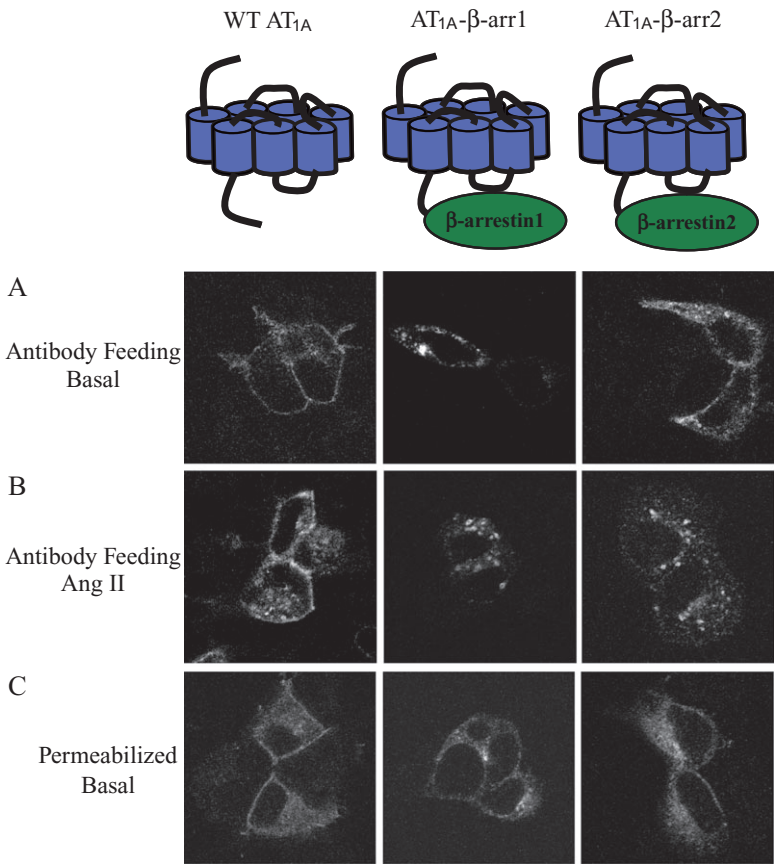

Figure 2

Subcellular localization of HA-tagged WT AT_{1A} receptor, AT_{1A}-β-arrestin1 and AT_{1A}-β-arrestin2 in transiently transfected HEK-293 cells. (A) Antibody feeding assay of unstimulated cells. This procedure selectively stains receptors that are located on the cell surface during incubation with the anti-HA antibody. (B) Antibody feeding assay stains showing cells treated with 10^{-7} M Ang II for 30 min. (C) Preparations showing whole cell localization of WT AT_{1A} receptor and the fusion constructs in permeabilized cells. AT_1, angiotensin II type 1; WT, wild type.

WT AT_{1A} receptors along with the AT_{1A}-β-arrestin fusion proteins were distributed both in the membrane and in the cytoplasm.

G protein-mediated signal transduction by AT_{1A}-β-arrestin1 and AT_{1A}-β-arrestin2 is attenuated

To determine if β-arrestin fusion to AT_{1A} receptor resulted in complete attenuation of G_q signalling, we compared the ability of WT AT_{1A} receptors and fusion proteins to activate IP hydrolysis. As illustrated in Figure 3A, AT_{1A}-β-arrestin1 and AT_{1A}-β-arrestin2 fusion proteins almost abolished IP hydrolysis, compared with WT AT_{1A} receptors. Efficacies and pEC_{50} values are shown in Table 2. Similarly, the IP hydrolysis in response to the low-affinity agonists, Ang III and (Val^4)Ang III, was totally abolished by AT_{1A}-β-arrestin1 and AT_{1A}-β-arrestin2 (Figure 3B and C) and neither the biased agonist SII-Ang II nor the antagonist telmisartan induced G protein signalling of the fusion proteins (Figure 3D and E). Although, we cannot completely exclude the possibility that part of the uncoupling results from a lower cell surface expression, these results are in agreement with previous observations where the ability of β-arrestin fusion proteins to activate G proteins was abolished (Martini *et al.*, 2002; Jorgensen *et al.*, 2005), suggesting that the coupling between the AT_{1A} receptors and β-arrestins is functional.

The AT_{1A}-β-arrestin fusion proteins show increased affinity for agonists

Previous studies have shown that 7TM receptor-β-arrestin fusion proteins induced significant affinity shifts in agonist binding, indicating that β-arrestins stabilize receptors in a high-affinity conformation (Martini *et al.*, 2002; Jorgensen *et al.*, 2005). Here, we used the same approach to analyse if β-arrestins induce high-affinity conformations of the AT_{1A} receptor, and to analyse whether β-arrestin1 and 2 are equally potent in this regard. In order to

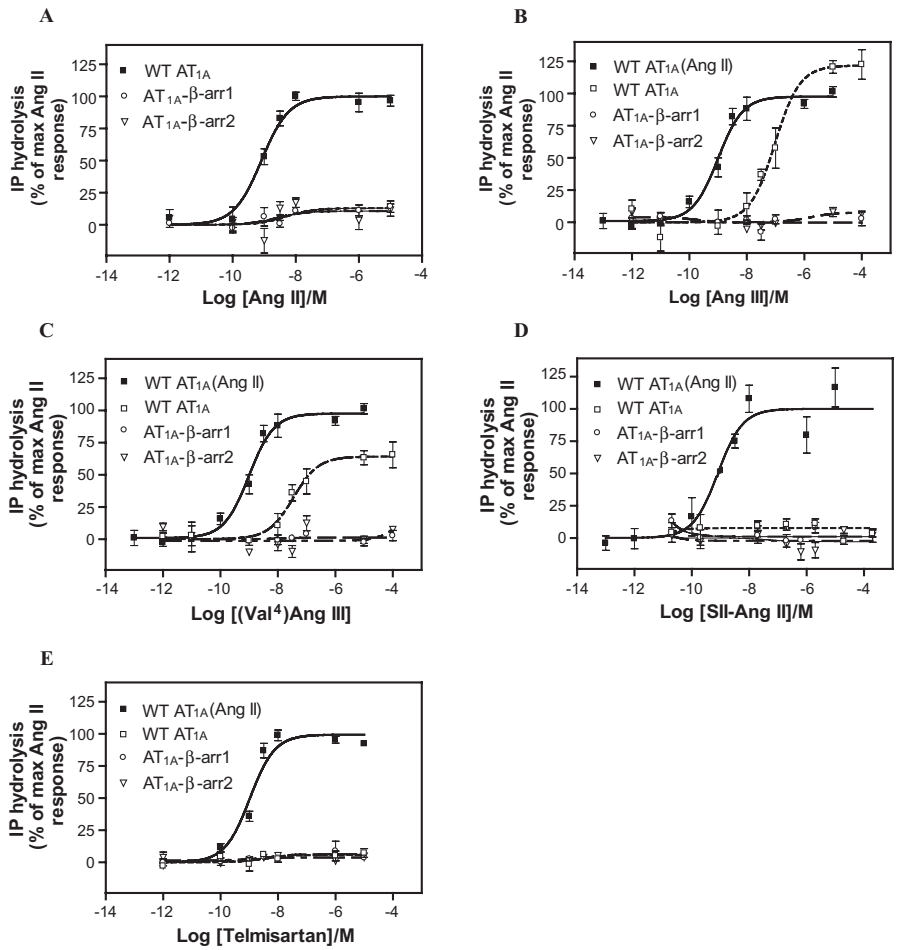

Figure 3

Ligand-induced IP hydrolysis of the AT_{1A}-β-arrestin fusion proteins. Dose–response curves for Ang II (A) Ang III (B) (Val^4)Ang III (C) SII-Ang II (D) and telmisartan (E) induced IP hydrolysis by WT AT_{1A} receptors, AT_{1A}-β-arrestin1 and AT_{1A}-β-arrestin2. The relative efficacies of fusion proteins are calculated as a percentage of the maximal Ang II response observed for the WT AT_{1A} receptor from experiments performed in parallel. Potencies and relative efficacies are reported in Table 2. AT_1, angiotensin II type 1; IP, inositol phospholipid; WT, wild type.

Table 2

Ligand-induced IP hydrolysis

Assay IP3 hydrolysis	WT AT_{1A} pEC_{50}	Relative efficacy	*n*	AT_{1A}-β-arr1 pEC_{50}	Relative efficacy	*n*	AT_{1A}-β-arr2 pEC_{50}	Relative efficacy	*n*
Ang II	9.0 ± 0.1	100 ± 0.0	5	7.9 ± 0.5	9.5 ± 2.2*	4	8.6 ± 1.0	5.2 ± 2.9*	4
Ang III	7.0 ± 0.1	122 ± 6.8*	3		NR	3		NR	3
(Val^4)Ang III	7.5 ± 0.2	64.2 ± 4.4*	3		NR	3		NR	3
SII-Ang II		NR	3		NR	3		NR	3
Telmisartan		NR	3		NR	3		NR	3

Average pEC_{50} and relative efficacy values are reported. Data are presented as mean ± SEM from n experiments. Relative efficacies were calculated as a percentage of maximal Ang II response observed for the WT AT_{1A} receptors. Dose-response curves are shown in Figure 3.
*$P < 0.05$; significantly different from Ang II responses with WT AT_{1A} receptors (one-tailed, paired Student's *t*-test).
AT_1, angiotensin II type 1; IP, inositol phospholipid; NR, no response; WT, wild type.

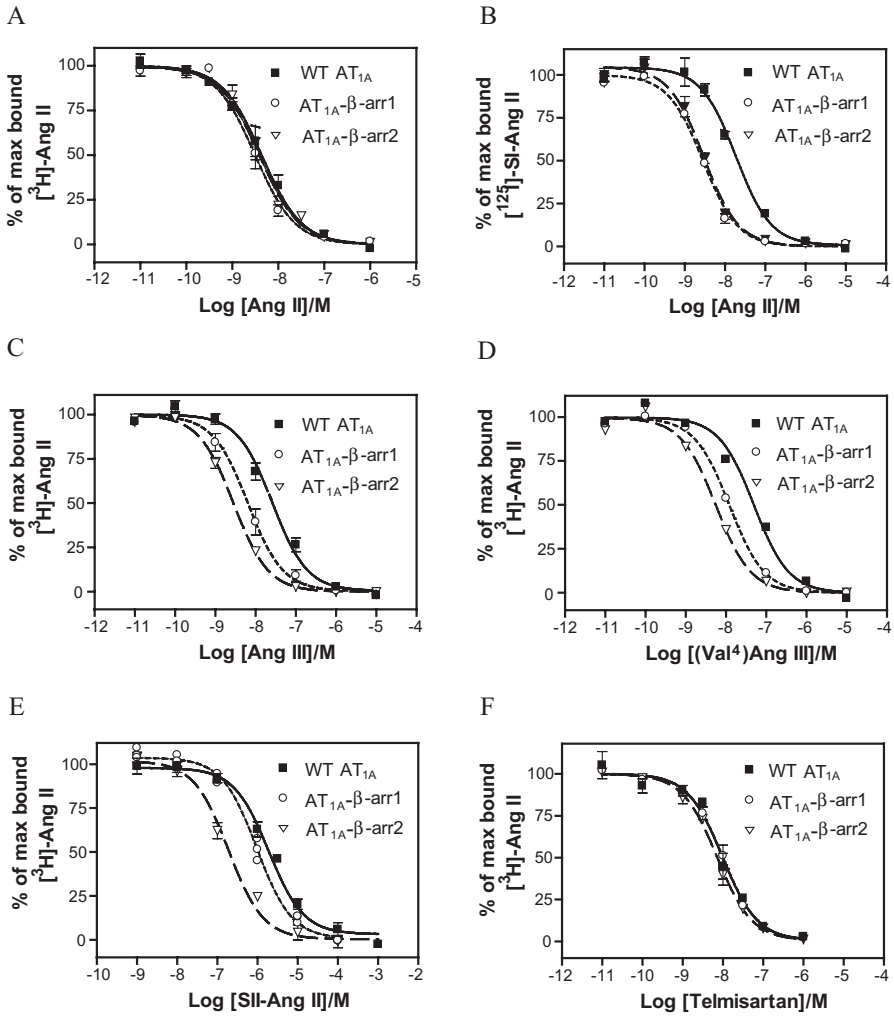

Figure 4

Competition radioligand binding for the WT AT_{1A} receptor, AT_{1A}-β-arrestin1 and AT_{1A}-β-arrestin2. AT_{1A} receptors were transiently expressed in COS-7 cells and competitive radioligand binding assays performed as described. [^{3}H]-Ang II (10^{-9} M) (A, C–E) or [^{125}I]-SI-ang II (10^{-11} M) (B) was used as radioligand tracers with the following unlabelled ligands: Ang II (A and B), Ang III (C) (Val^4)Ang III (D) SII-Ang II (E) and telmisartan (F). Average pK_i values are reported in Table 3. AT_1, angiotensin II type 1; WT, wild type.

investigate whether β-arrestins induce high-affinity conformations of the AT_1 receptor, we performed competition binding assays on the AT_{1A}-β-arrestin1 and AT_{1A}-β-arrestin2 fusion proteins in parallel with the WT AT_{1A} receptor, by using the radioligand [^{3}H]-Ang II and increasing amounts of unlabelled Ang II. Surprisingly, we did not observe any apparent affinity shifts with respect to Ang II binding when comparing to the WT AT_{1A} (Figure 4A); pK_i values are shown in Table 3. To further analyse if β-arrestins induced high-affinity receptor conformations, we used a different approach, which was inspired by previous studies demonstrating that more significant shifts in K_i values are observed in heterologous competition binding assays than in homologous competition binding assays (Martini *et al.*, 2002; Jorgensen *et al.*, 2005). Therefore, we tested the affinities for Ang II in a heterologous competition assay, with the radioligand [^{125}I]-SI-Ang II, and indeed seven- to eightfold shifts were observed for AT_{1A}-β-arrestin1 and AT_{1A}-β-arrestin2, when compared with WT AT_{1A} receptors (WT AT_{1A}, $K_i = 2.22 \times 10^{-8}$; AT_{1A}-β-arrestin1, $K_i = 2.84 \times 10^{-9}$; AT_{1A}-β-arrestin2, $K_i = 3.10 \times 10^{-9}$) (Figure 4B). The K_i values estimated from this experiment can be affected partially (or completely) by a change in the affinity for SI-Ang II. We have performed a homologous competition assay using labelled SI-Ang II and unlabelled SI-Ang II. Here, the K_i of the fusion constructs was identical to that of the WT AT_1 receptors (data not shown). However, this does not completely exclude an affinity shift of the tracer, but it

Table 3

Radioligand competition binding of the WT AT_{1A} receptor (WT $AT_{1A}R$) and the fusion constructs with β-arrestins 1 or 2 (β-arr1, β-arr2)

Assay Competition binding	WT $AT_{1A}R$ pK_i	*n*	$AT_{1A}R$-β-arr1 pK_i	Fold shift	*n*	$AT_{1A}R$-β-arr2 pK_i	Fold shift	*n*
Ang II	7.7 ± 0.1	3	8.5 ± 0.0	8	3	8.5 ± 0.1	8	4
Ang III	7.6 ± 0.1	5	8.4 ± 0.1*,†	6	4	8.6 ± 0.1*	10	5
(Val[4])Ang III	7.4 ± 0.2	4	8.0 ± 0.2*,†	4	3	8.3 ± 0.3*	9	4
SII-Ang II	5.8 ± 0.1	4	6.0 ± 0.1†	NS	3	6.8 ± 0.1*	9	3
Telmisartan	8.1 ± 0.1	5	8.1 ± 0.1	NS	3	8.3 ± 0.0	NS	5

Data represent mean pK_i ± SEM from n experiments. Fold shift denotes the ratio of K_i of the WT AT_{1A} receptor to fusion constructs. Curves are depicted in Figure 4.
*$P < 0.05$; significantly different from K_i values of WT AT_{1A} receptor.
†$P < 0.05$, significant differences between K_i values of AT_{1A}-β-arrestin1 and 2 (one-tailed, paired Student's *t*-test).
AT_1, angiotensin II type 1; NS, not significant ($P > 0.05$); WT, wild type.

clearly demonstrates that the relationship between SI-Ang II and Ang II binding is affected by fusing the receptor to β-arrestins.

When analysing the agonists Ang III (Val[4])Ang III, and SII-Ang II and using [^{3}H]-Ang II as the tracer ligand, we also observed that β-arrestin fusion proteins increased the ligand binding affinity compared with WT AT_{1A} receptors. These results indicate that β-arrestins stabilize the receptor in a conformation that has a high affinity for the agonists investigated, which is most likely to be caused by a specific interaction between the two molecules. On the other hand, we cannot completely exclude the possibility that it is caused by 'non-specific' interactions or as a consequence of tagging the receptor on the C-terminal tail. However, tagging the AT_1 receptor or other 7TM receptors in the C-terminal tail with GFP2 does not affect binding affinity of the receptor, which makes this explanation less likely (Jensen *et al.*, 2002; Jorgensen *et al.*, 2005).

Importantly, we also observed significant differences in how β-arrestin1 and 2 affect ligand binding abilities. When competing with the low-affinity agonists Ang III and (Val[4])Ang III, larger affinity shifts were observed for AT_{1A}-βarrestin2 (nine- to 10-fold affinity shift) than for AT_{1A}-βarrestin1 (four- to sixfold affinity shift). This is illustrated in Figure 4C–D and summarized in Table 3. As illustrated in Figure 4E and Table 3, the interaction of AT_{1A} receptors with β-arrestin2 also increased the affinity for the biased agonist SII-Ang II up to ninefold, in contrast to AT_{1A}-βarrestin1 where no affinity shift was observed. This observation shows that β-arrestin1 and β-arrestin2 induce different conformations in the AT_1 receptor and may reflect that β-arrestin2 interacts more stably with the AT_{1A} receptor, than β-arrestin1.

As expected no change in the binding affinity for the antagonist telmisartan was observed for either fusion proteins (Figure 4F). Thus, our data showed that both β-arrestins stabilized the AT_{1A} receptor in a conformation with higher agonist binding affinity and, furthermore, that β-arrestin2 induce larger affinity shifts than β-arrestin1.

Discussion and conclusions

Increasing evidence suggest that the functions of β-arrestin1 and 2 in 7TM receptor signalling may differ. Therefore, we wanted to test whether the two β-arrestin isoforms interacted differently with the receptor. A comparative study on AT_{1A} receptor interaction with β-arrestin1 and 2 was carried out with ligands of different binding affinities and signalling properties, such as agonists, biased agonists and antagonists with varying affinities for the receptor. In this study, we demonstrated that β-arrestin1 and 2 were recruited to the AT_{1A} receptor in response to agonists with comparable potencies and efficacies. Thus, both β-arrestin isoforms interact equally well with the AT_{1A} receptors in the BRET2 assay, which is consistent with previous studies, demonstrating that Class B receptors do not show any preference towards a specific β-arrestin isoform (Oakley *et al.*, 2000).

As there were no apparent differences in β-arrestin1 and 2 recruitment to AT_{1A} receptors, we constructed fusion proteins between these receptors and β-arrestin1 and 2 respectively. Fusion proteins have been useful tools to study the interaction between β-arrestins (or G proteins) and other 7TM receptors (Seifert *et al.*, 1999; Milligan, 2000; Martini *et al.*, 2002; Jorgensen *et al.*, 2005). Fusing two interacting proteins offer the obvious advantage

that it brings two proteins of interest in close proximity. Furthermore, fusing two proteins of interest will, ideally, abolish the interaction with other signalling molecules and create a stoichiometry of 1:1 between the fused proteins. With the fusion receptor strategy, we demonstrate that the fusion of AT_{1A} to β-arrestins mimics the natural AT_{1A}/β-arrestin interaction and that fusion proteins can be used to disclose small differences in protein-protein interactions. First we showed that the subcellular localization of fusion proteins was predominantly cytoplasmic even in the absence of agonist stimulation, suggesting constitutive coupling between AT_{1A} receptors and β-arrestins (Figure 2). Second, we showed that fusion of β-arrestin1 and β-arrestin2 to AT_{1A} receptors abolished coupling to G_q. Although, we cannot completely exclude that part of the uncoupling results from a lower cell surface expression, similar findings have been made for other 7TM/β-arrestin fusion receptors, such as the NK_1 or the GLP-1 receptor (Martini *et al.*, 2002; Jorgensen *et al.*, 2005). Third, both β-arrestin1 and β-arrestin2 constrain the AT_{1A} receptor in a high-affinity conformation. The results show that β-arrestins increase AT_{1A} receptor affinity for all agonists, compared with WT AT_{1A} receptors (Figure 4B–E, and Table 3), while no affinity shift was evident in response to the antagonist telmisartan (Figure 4F, and Table 3). Thus, the high-affinity AT_{1A} receptor conformation stabilized by β-arrestins is only formed in presence of agonists which is in good agreement with the alternative ternary complex model (Samama *et al.*, 1993; Gurevich *et al.*, 1997).

Ang II, Ang III and (Val^4)Ang III were shown to be full agonists on β-arrestin recruitment (Figure 1A and B), and both β-arrestin1 and 2 were able to increase the AT_{1A} receptor affinity for these agonists up to 10-fold, compared with WT receptors. Interestingly, β-arrestin2 induced higher affinity shifts than β-arrestin1, which could reflect the fact that β-arrestin2 formed a more stable complex with AT_{1A} receptors than β-arrestin1. On the other hand, we were not able to detect these differences in the BRET2 assay, where β-arrestin1 and 2 were both recruited to AT_{1A} receptors with similar potencies and efficacies for the tested ligands. Nevertheless, this observation still demonstrates that β-arrestin1 and 2 interact with the AT_{1A} receptor in different ways, most likely reflecting a stabilization of different conformations within the fusion proteins.

The discrepancy between BRET2 results and radioligand binding experiments may be surprising but can be explained by differences between the two assays. In the binding assay, steady-state or equilibrium is almost reached, whereas the BRET assay is a functional assay where equilibrium is not reached, due to the constant dynamics inside the cell. Furthermore, the temperature at which the experiments are performed is different. The BRET assay is carried out at room temperature whereas binding is performed at 4°C. The differences could also arise because the affinity shifts we observe in the binding experiments are not caused by a tighter interaction between the two proteins, but instead by the fact that a different state is generated. As equilibrium is not reached in the BRET assay, this conformational change does not necessarily transform into an actual shift in EC_{50} values.

As mentioned previously, the AT_1 receptor signals through two major pathways: the G protein-dependent signalling pathway and the G protein-independent signalling pathway, which is mediated by β-arrestins. The two signalling pathways of the AT_1 receptor have been linked to different biological phenotypes. The G protein-dependent signalling pathway has been associated with effects such as cell death and fibrosis, while the G protein-independent pathway has been associated with cell survival and cell renewal (Zhai *et al.*, 2005; Aplin *et al.*, 2008). Thus, differential activation of the AT_1 receptor by a biased agonist may be favourable in certain pathological states. Our present finding demonstrates that it even may be possible to develop drugs that selectively recruit β-arrestin1 or 2, which could be an advantage in certain pathological states. A recent study has demonstrated that β-arrestin1 and 2 have opposing roles in the development of atherosclerosis (Kim *et al.*, 2008); β-arrestin2 was found to aggravate atherosclerosis by inducing smooth muscle cell proliferation and migration, while β-arrestin1 had the opposite effect. Thus, selective inhibition of β-arrestin2 could be a novel therapeutic target in treatment of atherosclerosis.

In conclusion, the present study shows that fusion proteins are valuable tools to detect the differences between β-arrestin1 and 2 interactions with 7TM receptors. Both β-arrestin1 and β-arrestin2 fused to AT_{1A} receptors stabilized the receptor in a high-affinity conformation. Interestingly, β-arrestin2 induced a receptor conformation with a higher agonist-binding affinity than β-arrestin1. Thus, in our study we demonstrate that β-arrestin1 and 2 interact with the AT_{1A} receptor in different ways, which suggest that it could be possible to design AT_1 receptor biased agonists with the ability to recruit either of the β-arrestins selectively.

Acknowledgements

We thank Birte Kofoed for excellent technical assistance. This work was sponsored by The Danish

National Research Foundation, The Koebmand i Odense Johan og Hanne Weimann f. Seedorffs legat, Aase og Ejnar Danielsens Foundation, The Novo Nordisk Foundation, The Danish Heart Foundation and the Augustinus Foundation.

Conflict of interest

None.

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