European Journal of Pharmacology 590 (2008) 255–263

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

The human angiotensin AT_1 receptor supports G protein-independent extracellular signal-regulated kinase 1/2 activation and cellular proliferation

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ARTICLE INFO

Article history:
Received 16 October 2007
Received in revised form 7 April 2008
Accepted 13 May 2008
Available online 20 May 2008

Keywords:
GPCR
7TM receptor
Differential activation
Angiotensin
Arrestin
AT_1 receptor

ABSTRACT

The angiotensin AT_1 receptor is a key regulator of blood pressure and body fluid homeostasis, and it plays a key role in the pathophysiology of several cardiovascular diseases such as hypertension, cardiac hypertrophy, congestive heart failure, and arrhythmia. The importance of human angiotensin AT_1 receptor signalling is illustrated by the common use of angiotensin AT_1 receptor-inverse agonists in clinical practice. It is well established that rodent orthologues of the angiotensin AT_1 receptor can selectively signal through G protein-dependent and -independent mechanisms in recombinant expression systems, primary cells and *in vivo*. The *in vivo* work clearly demonstrates profoundly different cellular consequences of angiotensin AT_1 receptor signalling in the cardiovascular system, suggesting pharmacological potential for drugs which specifically affect a subset of angiotensin AT_1 receptor actions. However, it is currently unknown whether the human angiotensin AT_1 receptor can signal through G protein-independent mechanisms — and if so, what the physiological impact of such signalling is. We have performed a detailed pharmacological analysis of the human angiotensin AT_1 receptor using a battery of angiotensin analogues and registered drugs targeting this receptor. We show that the human angiotensin AT_1 receptor signals directly through G protein-independent pathways and supports NIH3T3 cellular proliferation. The realization of G protein-independent signalling by the human angiotensin AT_1 receptor has clear pharmacological implications for development of drugs with pathway-specific actions and defined biological outcomes.

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1. Introduction

The angiotensin AT_1 receptor is a key regulator of blood pressure and body fluid homeostasis, and its importance in renal and cardiovascular pathophysiology is underscored by the widespread use of receptor blockers and inhibitors of the angiotensin converting enzyme (ACE) in clinical practice (Healey et al., 2005; Zaman et al., 2002). The development of these drugs occurred prior to the growing appreciation of G protein coupled receptors (also seven transmembrane spanning domain receptors, henceforth 7TM receptors) as regulators of multiple signal transduction pathways rather than as binary "on/off" switches for G proteins. There is mounting evidence that the angiotensin AT_1 receptor along with many other 7TM receptors can attain conformations that do not couple to heterotrimeric G proteins, yet activate G protein-independent signal transduction pathways (Kenakin, 2005; Lefkowitz and Shenoy, 2005; Urban et al., 2006). In this respect, β-arrestins are particularly interesting modulatory proteins, acting as scaffolds for "second wave" — or alternative signal transduction. β-arrestin-dependent signalling from various 7TM receptors has recently been linked to cell motility (Ge et al., 2004), developmental regulation (Wilbanks et al., 2004), and behavioural phenotypes (Beaulieu et al., 2005).

For the angiotensin AT_1 receptor, the rodent orthologues have served as models for studying differential signal transduction and accordingly have been studied in great detail at the cellular level (Ahn

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doi:10.1016/j.ejphar.2008.05.010

et al., 2004a,b; Hines et al., 2003; Holloway et al., 2002; Seta et al., 2002; Szidonya et al., 2007; Yee et al., 2006). In addition, several important cellular phenotypes have been ascribed to the G protein-independent signalling from the rodent angiotensin AT_1 receptors suggesting that differential activation of the angiotensin AT_1 receptor may be pharmacologically possible and favourable to target in certain pathological settings (Aplin et al., 2007b; Hunton et al., 2005; Rajagopal et al., 2006; Zhai et al., 2005). Probing the rat angiotensin AT_1 receptor endogenously present in cardiac myocytes, we have recently demonstrated that G protein-independent signalling supports non-hypertrophic proliferation of cardiac myocytes (Aplin et al., 2007a,b). Transgenic and knock-out studies of the mouse angiotensin AT_{1a} receptor have also associated G protein-independent signalling with beneficial contractile regulation of atrial myocytes (Rajagopal et al., 2006) and non-fibrotic compensated hypertrophic cardiac phenotypes (Zhai et al., 2005). Additionally, cell-based studies have implicated G protein-independent signalling by rodent angiotensin AT_1 receptor in activation of pathways that govern cell migration and cellular survival (Howes et al., 2006; Hunton et al., 2005).

Despite its status as a drug target, it is not known whether the human angiotensin AT_1 receptor supports G protein-independent signalling and which cellular events this might regulate. To this end, we have performed a detailed pharmacological analysis of the human angiotensin AT_1 receptor in several signal transduction assays, using a battery of Angiotensin analogues and registered drugs targeting the receptor. We show that the human angiotensin AT_1 receptor can signal through both G protein-dependent and -independent pathways.

2. Materials and methods

2.1. Recombinant DNA plasmids

Using PCR-based methods, the angiotensin AT_1 receptor was cloned from human liver cDNA, fully sequence verified, and subcloned into the pSI expression vector (Promega, Madison, WI) for functional expression in mammalian cells. The consensus wild type angiotensin AT_1 receptor sequence was defined from GenBank accession number M93394, identical to that reported previously (Takayanagi et al., 1992). To generate the receptor variants (Constitutively active N111A and L245T), point mutations were introduced using the QuikChange mutagenesis protocol (Stratagene, La Jolla, CA). Each variant was subcloned back into the original plasmid vector and sequenced to eliminate potential PCR-generated mutations in non-coding regions of the plasmids. The EGFP-tagged bovine-β-arrestin 2, was described previously (Penn et al., 2001) and angiotensin AT_1 receptor-Rluc was created by PCR using the angiotensin AT_1 receptor as template and subsequently sub cloned into pRluc-N3 vector (Packard) using HindIII and BamHI (both from New England Biolabs; Ipswich, MA) as restriction enzymes.

2.2. Receptor Selection and Amplification Technology (RSAT)

The RSAT assay is based on the fact that NIH3T3 cells become contact inhibited upon reaching confluency but that transiently expressed oncogenes, proto-oncogenes, and many 7TM receptors confer partial or total transformation of these cells, causing a loss of contact inhibition and allowing them to continue to proliferate when they would otherwise stop (Brauner-Osborne and Brann, 1996; Burstein et al., 2006). In RSAT, a reporter gene (in this case β-galactosidase) is co-transfected into the cells with the 7TM receptor of interest to quantify this proliferative response. The β-galactosidase reporter is constitutively expressed in this system and does not participate in driving the biological response but rather works as an indirect measure of proliferation (Brauner-Osborne and Brann, 1996). The RSAT assay was performed as described previously (Hansen et al., 2004a; Weiner et al., 2001). Briefly, NIH3T3 cells at 70 to 80% confluence were transfected with human angiotensin AT_1 receptor cDNA (25 ng of receptor and 20 ng of β-galactosidase reporter/well of a 96-well plate) using the PolyFect Reagent (QIAGEN, Valencia, CA) as described in the manufacturer's protocol. To measure constitutive activity 5 ng of Gαq was added pr. well in addition to receptor and reporter DNA. One day after transfection, ligands were added in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (100 g/ml), and 2% Cyto-SF3. After 6 days, the media was aspirated off, cells were lysed, *O*-nitrophenyl-β-D-galactopyranoside was added, and the resulting absorbance was measured spectrophotometrically. All concentration–response curves were performed in duplicate.

2.3. Inositol hydrolysis assay

This assay was performed as described previously (Hansen et al., 2004a,b). Briefly, COS-7 cells ($2.0 \cdot 10^6$) were seeded in a 10-cm tissue culture plate and transfected the following day, using Lipofectamine 2000 ™. The day after transfection, the cells were split into 24 wells of a poly-L-lysine coated 48-well tissue culture plate in inositol-free DMEM (Dulbecco's modified Eagle's medium) with reduced concentrations of $CaCl_2$ (0.9 mM) and $MgCl_2$ (0.8 mM), supplemented with Gentamycin, non-essential amino acids, 10% dialyzed fetal calf serum and 1lCi/mL myo-[2–^{3}H]inositol (BD Biosciences, Franklin Lakes, NJ). Sixteen to twenty-four hours after application of the radioligand, the cells were assayed as described (Nanevicz et al., 1996).

2.4. β-arrestin recruitment Bioluminescence Resonance Energy Transfer 2 ($BRET^2$) assay

The β-arrestin recruitment assay was performed as described previously (Hansen et al., 2004b). $BRET^2$ is a specific patented assay from Packard Biosciences that uses bioluminescence from luciferase to GFP^2, a second generation modification of GFP. However, as described in (Jensen et al., 2002), EGFP-tagged proteins are generally applicable in this assay as well. Briefly, 2 million COS-7 cells were seeded into a 10 cm plate and grown in 10% fetal calf serum/Dulbecco's modified Eagle's medium (DMEM) overnight. After 24 h, the cells were transfected using Lipofectamine 2000™ (Invitrogen) according to the manufacturer's protocol with 1 μg of Angiotensin AT_1 receptor-Rluc and 3 μg of The EGFP-tagged bovine-β-arrestin 2. After 2 days, the cells were washed twice with PBS to remove the indicator dye before detachment in PBS. Cells were then submitted to either basal or agonist treatment for 20 min at room temperature before measuring the $BRET^2$ ratios. The $BRET^2$ ratio equals ((emission (515/30)−(emission(410/80)**Cf*))/(emission (410/80), where *Cf* denotes the cross-Rluc luminescence cross-talk ratio into the 515/30 filter defined as emission (515/30)/(emission (410/80) when Rluc expressed alone is excited.

2.5. Extracellular signal-regulated kinase phosphorylation assay

ERK1/2 phosphorylation assay —Except for minor modifications, this assay was performed as described previously (Hansen et al., 2000; Theilade et al., 2002). Briefly, 2 million COS-7 cells were seeded into a 10 cm dish and grown in DMEM with 10% fetal calf serum overnight. After 24 h, the cells were transfected using Lipofectamine ™ 2000 according to the manufacturer's protocol. On the day after transfection, the cells were seeded onto 6-well plates (500 000 cells pr. well). Next, 45 h after transfection, the cells were serum-starved for 3 h, incubated with inverse agonists for 30 min and/or agonist for 12 min at 37 °C, quickly rinsed in ice-cold phosphate-buffered saline, and then lysed. SDS-PAGE and immunoblotting were performed as described previously (Theilade et al., 2002), and the bands were visualized using the enhanced chemiluminescence system (BD Biosciences). To assess the relative efficacies of agonists, a densitometric gel quantification of the P-ERK and total ERK band intensities was performed, and the ratios of P-ERK to total ERK were normalized with reference to the receptor maximum angiotensin II response (100%).

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