Biochemical and Biophysical Research Communications 409 (2011) 544–549

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

A cleavable signal peptide enhances cell surface delivery and heterodimerization of Cerulean-tagged angiotensin II AT1 and bradykinin B2 receptor

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ARTICLE INFO

Article history:
Received 4 May 2011
Available online 11 May 2011

Keywords:
AGTR1
BDKRB2
Receptor heterodimer
Fluorescence resonance energy transfer (FRET)
Cerulean
G protein-coupled receptor

ABSTRACT

Heterodimerization of the angiotensin II AT1 receptor with the receptor for the vasodepressor bradykinin, B2R, is known to sensitize the AT1-stimulated response of hypertensive individuals in vivo. To analyze features of that prototypic receptor heterodimer in vitro, we established a new method that uses fluorescence resonance energy transfer (FRET) and applies for the first time AT1-Cerulean as a FRET donor. The Cerulean variant of the green fluorescent protein as donor fluorophore was fused to the C-terminus of AT1, and the enhanced yellow fluorescent protein (EYFP) as acceptor fluorophore was fused to B2R. In contrast to AT1–EGFP, the AT1-Cerulean fusion protein was retained intracellularly. To facilitate cell surface delivery of AT1-Cerulean, a cleavable signal sequence was fused to the receptor's amino terminus. The plasma membrane-localized AT1-Cerulean resembled the native AT1 receptor regarding ligand binding and receptor activation. A high FRET efficiency of 24.7% between membrane-localized AT1-Cerulean and B2R-EYFP was observed with intact, non-stimulated cells. Confocal FRET microscopy further revealed that the AT1/B2 receptor heterodimer was functionally coupled to receptor desensitization mechanisms because activation of the AT1-Cerulean/B2R-EYFP heterodimer with a single agonist triggered the co-internalization of AT1/B2R. Receptor co-internalization was sensitive to inhibition of G protein-coupled receptor kinases, GRKs, as evidenced by a GRK-specific peptide inhibitor. In agreement with efficient AT1/B2R heterodimerization, confocal FRET imaging of co-enriched receptor proteins immobilized on agarose beads also detected a high FRET efficiency of 24.0%. Taken together confocal FRET imaging revealed efficient heterodimerization of co-enriched and cellular AT1/B2R, and GRK-dependent co-internalization of the AT1/B2R heterodimer.

1. Introduction

The angiotensin II type 1 receptor (AT1) is the major receptor for the vasopressor angiotensin II. By regulating vascular tone, cardiovascular function, salt and water homeostasis the AT1 receptor exerts an indispensable physiological role. In addition to its important physiological functions, the AT1 receptor has become one of the major drug targets for the treatment of cardiovascular disorders, which are characterized by an exaggerated AT1-stimulated response [1,2]. Mechanisms underlying AT1 sensitization are therefore of great importance to elucidate pathomechanisms of diseases such as hypertension, atherosclerosis or heart failure.

Different mechanisms may contribute to an exaggerated angiotensin II response under pathophysiological conditions. In recent years, receptor homo- and heterodimerization emerged as a major mechanism controlling angiotensin II responsiveness in vivo [3–6]. An altered dimerization of the AT1 receptor may contribute to the angiotensin II hyperresponsiveness during the pathogenesis of cardiovascular diseases [7–9]. For instance, covalently linked AT1 receptor dimers may contribute to the exaggerated angiotensin II response at the onset of atherosclerosis because the covalent stabilization of AT1 dimers may create a signaling platform, which is kinetically favored to interact with the downstream signal transduction cascade [7]. Covalently stabilized AT1 receptor dimers are also a characteristic feature of preeclampsia hypertension [8]. In preeclamptic individuals it is the covalent interaction with the bradykinin B2 receptor (B2R), which accounts for the formation of a hyperresponsive AT1 receptor platform [4,8]. A similar observation linked AT1/B2 receptor heterodimers to the enhanced AT1 response in renal mesangial cells of spontaneously hypertensive rats [9]. Those examples suggest that the covalent stabilization of AT1 receptors by homo- or hetero- dimerization may facilitate receptor signaling by creating a non-dissociable signaling platform.

Previous studies mainly focused on AT1 dimerization under in vivo conditions. In view of the pathophysiological role, transfected cell systems are of increasing interest to facilitate the study of mechanisms underlying the formation of AT1

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0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.05.041

homo- and heterodimers [10,11]. Fluorescence resonance energy transfer (FRET) is a versatile technology for the analysis of protein interactions such as receptor homo- and heterodimerization [12,13]. FRET is capable to detect interactions between donor and acceptor fluorophores in close proximity occurring in the range of 1–10 nm in distance [12,13]. Therefore we applied FRET for the analysis of AT1/B2R heterodimerization. As FRET donor, the Cerulean variant of the enhanced green fluorescent protein was chosen and fused to the carboxyl terminus of AT1 (AT1-Cerulean) because Cerulean is 2.5-fold brighter than the cognate ECFP and yields a substantially improved signal-to-noise-ratio in FRET measurements [14]. As FRET acceptor, the enhanced yellow fluorescent protein was fused to the carboxyl terminus of the bradykinin B2 receptor (B2R-EYFP). With a high FRET efficiency of more than 24%, confocal FRET imaging revealed the close interaction between membrane-localized AT1-Cerulean and B2R-EYFP indicative of efficient heterodimerization of AT1/B2 receptors.

2. Materials and methods

2.1. Cell culture and cell transfection

Human embryonic kidney cells (HEK293) were cultivated and transfected with plasmids encoding AT1-Cerulean and B2R-EYFP as described [4]. Overlapping PCR was performed to create cDNAs encoding AT1-Cerulean and B2R-EYFP, i.e. the cDNA of Cerulean [14] and EYFP was fused with a linker (GlyGlyGlyGlyGly) in frame to the C-terminus of AGTR1 (amino acid 359) and BDKRB2 (amino acid 391), respectively. To enhance the membrane localization of AT1-Cerulean, the cleavable signal sequence of influenza hemagglutinin (MKTIIALSYIFCLVFA; h-sp; [15]) was added by PCR to the N-terminus of AT1-Cerulean to create (h-sp)AT1-Cerulean. Analogously, the signal sequence of the secreted protein resistin (MKALCLLLLPVLGLLVSS; r-sp) was fused to the N-terminus of AT1-Cerulean to create (r-sp)AT1-Cerulean. The identity of all DNA constructs was verified by DNA sequencing. Cellular inositol phosphate levels were quantified as described [4]. For receptor internalization, cells co-expressing (h-sp)AT1-Cerulean/B2R-EYFP were stimulated with angiotensin II (2 μM) for 40 min at 37 °C. To inhibit G-protein-coupled receptor kinases (GRKs), a GRK-specific peptide inhibitor was expressed [16]. Radioligand binding affinities were determined with membranes of transiently transfected HEK293 cells in the presence of protease inhibitors with 50 μg protein/point for 1 h at 22 °C. Saturation isotherms were determined by incubating membranes with varying concentrations of Sar^1,[^{125}I]Tyr^4,Ile^8-angiotensin II (2200 Ci/mmol; Perkin Elmer) or [2,3,-*prolyl*-3,4-^{3}H]bradykinin (96 Ci/mmol; Perkin Elmer). Non-specific binding was determined by the addition of 1 μM unlabeled angiotensin II or bradykinin for AT1 or B2 receptors, respectively [8].

2.2. Co-immunoenrichment studies

Co-immunoenrichment of B2R-EYFP and (h-sp)AT1-Cerulean was performed with solubilized membranes of HEK293 cells expressing B2R-EYFP and/or (h-sp)AT1-Cerulean, similarly as described [10,11,17]. For visualization of (co-)enriched receptor proteins by confocal FRET imaging, membranes (prepared from 3×10^7 cells) were solubilized with PBS containing 1% sodium deoxycholate, 0.01% Tween 20, and protease inhibitors. The solubilisate was diluted 1:10 (with PBS supplemented with 1% with bovine serum albumin and 0.01% Tween 20), and applied to ProteinA sepharose loaded with B2R-specific antibodies or control antibodies. After overnight incubation at 4 °C and three washing steps (with PBS containing 0.01% Tween 20), ProteinA sepharose beads were imaged by confocal FRET microscopy. The following antibodies were used for immunoblotting and/or immuno-enrichment: affinity-purified B2R-specific antibodies raised in rabbit/rat against an antigen encompassing amino acids 356–391 of the human B2R sequence, and affinity-purified AT1-specific antibodies raised in rabbit/rat against an antigen encompassing amino acids 306–359 of the human AT1R sequence [10,11].

2.3. Confocal FRET Imaging

Confocal FRET imaging of HEK cells co-expressing membrane-localized AT1-Cerulean and B2R-EYFP was performed with a confocal laser-scanning microscope (SP5-CLSM, Leica) similarly as described [13]. Cerulean was excited using the 458 nm line of the argon laser at moderate laser power, and emission was detected at 470–485 nm. EYFP was excited at 495 nm, and emission was detected at 520–535 nm. After image collection, EYFP was bleached at 514 nm at 100% laser power. Quantification of donor and acceptor fluorescence emission intensities at 470–485 nm and 520–535 nm before and after photobleaching of a selected region of interest was used to calculate FRET efficiencies. Calculation of the FRET efficiency (E_F) was performed with the FRET-AB Wizard (Leica Application Suite Advanced Fluorescence Version 2.3.6) according to the formula $E_F=(I_{after} - I_{before}) \times 100/I_{after}$, where I_{after} is the Cerulean intensity after the EYFP bleach and I_{before} is the Cerulean intensity before the EYFP bleach. The formula yields the increase in Cerulean fluorescence induced by the EYFP bleach normalized by the Cerulean fluorescence after the bleach [13]. Confocal FRET imaging of enriched receptors immobilized on sepharose beads was performed essentially as described for cellular receptors expressed in HEK cells.

2.4. Statistical analyses

Unless otherwise stated, data are expressed as mean ± S.D. To determine significance between two groups, comparisons were made using the unpaired two-tailed Student's *t* test, *p* values of <0.05 were considered significant.

3. Results

3.1. A cleavable signal peptide facilitates cell surface delivery of AT1-Cerulean

In view of the physiological importance of AT1/B2R heterodimers in vivo [8,9], we sought to establish a FRET-based method to analyze features of AT1/B2R heterodimers in vitro. For FRET studies we chose the Cerulean variant of the enhanced green fluorescent protein, which is a highly efficient FRET donor [14]. In contrast to AT1–EGFP, fusion of Cerulean to the C-terminus of AT1 induced the predominant intracellular retention of AT1-Cerulean (Fig. 1A).

To increase the cell surface delivery of AT1-Cerulean, the cleavable signal peptide of the influenza hemagglutinin (h-sp) was fused to the N-terminus of the receptor. The cleavable signal peptide significantly enhanced the cell surface delivery of (h-sp)AT1-Cerulean as determined by radioligand binding (Fig. 1B). Fluorescence microscopy revealed the plasma membrane localization of (h-sp)AT1-Cerulean and the co-localization with B2R-EYFP (Fig. 1C). A similar enhancement of plasma membrane localization of AT1-Cerulean was achieved with the signal peptide of the mammalian secreted protein, resistin, as visualized with (r-sp)AT1-Cerulean-expressing cells (Fig. 1D). As a control, ligand affinity and activation of membrane-localized AT1-Cerulean and B2R-EYFP receptors were comparable to the wild-type AT1 and B2 receptor, respec-