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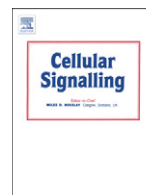

Heteromerization of angiotensin receptors changes trafficking and arrestin recruitment profiles

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ABSTRACT

The cardiovascular hormone angiotensin II (AngII) exerts its actions via two G protein-coupled receptor (GPCR) subtypes, AT_1 and AT_2, which often display antagonistic functions. Methodological constraints have so far precluded detailed analyses of the ligand-dependency, cellular localization, and functional relevance of AngII receptor interactions in live cells. In this study, we utilize a protein-fragment complementation assay (PCA) and GPCR-Heteromer Identification Technology (GPCR-HIT) to provide the first detailed investigation of the ligand-dependency and cellular localization of AngII receptor interactions in human embryonic kidney 293 cells. Fluorescent-tagged receptor constructs for PCA and GPCR-HIT displayed normal affinity and selectivity for AngII (AT_1: IC_{50} = 1.0–1.6 nM; AT_2: IC_{50} = 2.0–3.0 nM). Well-characterized angiotensin receptor interactions were used as positive and negative controls to demonstrate the sensitivity and specificity of these fluorescence-based assays. We report that AT_1–AT_2 receptor heteromers form constitutively, are localized to the plasma membrane and perinuclear compartments, and do not internalize following AngII stimulation despite arrestin being recruited specifically to the heteromer. Our findings using novel fluorescence-based technologies reveal a previously unrecognized mechanism of angiotensin receptor cross-talk involving cross-inhibition of AT_1 receptor internalization through heteromerization with the AT_2 receptor subtype.

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1. Introduction

G protein-coupled receptors (GPCRs) are the largest group of membrane receptors and transduce signals from extracellular stimuli involved in diverse biological processes ranging from phototransduction to cell growth and apoptosis [1,2]. Traditionally, GPCRs were thought to act as monomers, with a specific ligand binding a single GPCR and initiating a downstream signaling cascade via a single heterotrimeric G protein subunit [3]. However, numerous studies suggest that GPCRs can exist and function as dimers or higher order oligomers [3]. GPCRs can exist as either heteromers or homomers: a heteromer being defined as a macromolecular complex composed of at least two (functional) receptor units with biochemical properties that are demonstrably different from those of its individual components, and a homomer being similar, but combining two or more identical (functional) receptor units [4]. Although the concept of receptor–receptor interactions was introduced almost 30 years ago [5], many fundamental facets of GPCR heteromerization remain controversial and unresolved.

The cardiovascular hormone angiotensin II (AngII) exerts its actions via two receptor subtypes, AT_1 and AT_2, both of which belong to the GPCR superfamily [6]. Most of the classic biological actions attributed to AngII have been assigned to the AT_1 receptor subtype, which has also served as a prototypical model for studying GPCR signaling [7]. In contrast, the AT_2 receptor is one of the most controversial and poorly understood aspects of renin–angiotensin system biology [8]. Unlike AT_1 receptors, AT_2 receptors do not couple with arrestins and fail to internalize following AngII stimulation [9–11]. AT_2 receptor function and signaling often, but not always, oppose the actions of AT_1 [8], suggesting that important interplay exists between these two receptor

Abbreviations: PCA, Protein-fragment Complementation Assay; GPCR-HIT, G protein-coupled receptor Heteromer Identification Technology.

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subtypes. However, the nature of this interplay has not been fully elucidated. Given emerging evidence that many effects of the AT_2 receptor are ligand-independent [12–15], heteromerization of AT_1–AT_2 receptors may significantly alter AT_1 receptor function and regulation by AngII. Indeed, the existence of AT_1–AT_2 heteromers has been previously reported [16,17], but methodological constraints associated with co-immunoprecipitation have precluded detailed studies of the ligand-dependency, cellular localization and functional relevance of AngII receptor heteromerization in live cells. Whether AT_1–AT_2 receptor heteromerization is an important regulatory aspect of AngII signaling and function remains an open question.

Several different approaches have been used to detect GPCR complexes in live cells. The most commonly used techniques have been those based on resonance energy transfer (RET) between donor and acceptor molecules [18]. Both fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) have been used to study GPCR homo- and heteromerization [19]. More recently, novel assays such as the GPCR-Heteromer Identification Technology (GPCR-HIT), have been developed that allow for ligand-dependent monitoring of GPCR heteromers in real-time. GPCR-HIT utilizes specific ligand-induced GPCR interactions to detect GPCR heteromerization and provides information about specific ligand-dependent recruitment of proteins to heteromers [20–22].

An alternative approach for studying protein interactions in viable cells, which relies on fluorescent molecular reconstitution rather than on energy transfer, is the protein-fragment complementation assay (PCA; also known as bimolecular fluorescence complementation or BiFC) [23]. For example, if yellow fluorescent protein (YFP) is split into two fragments, neither of these molecules will fluoresce when expressed alone. However, if the two non-fluorescing fragments are fused to two interacting proteins, the fragments will be brought into close proximity by the protein-protein interaction such that refolding occurs and the YFP molecule is reconstituted (Fig. 1A). Because PCA is based on the formation of a fluorescent molecule rather than energy transfer between existing fluorophores, it is advantageous as it avoids interference from changes in fluorescence intensity and lifetime that are caused by conditions unrelated to the protein interactions and is therefore potentially more sensitive than either FRET or BRET in this regard. The other contrast with energy transfer based technologies is that the complementation involves physical interaction of the two fragments as well as a time delay for refolding to occur, meaning that the fluorescence does not reflect the interaction in real-time [24]. Thus, given the different strengths of the GPCR-HIT and the PCA approaches, the use of both assays in parallel provides a particularly powerful strategy for studying the temporal and spatial dynamics of GPCR interactions in live cells.

The aim of this study was to combine the different attributes of PCA and GPCR-HIT to identify and monitor the formation of AngII receptor complexes in live cells. In parallel, these novel assays are used to demonstrate the existence of AT_1–AT_2 receptor heteromers. This study provides the first detailed investigation of complex ligand-dependency and cellular localization, as well as ligand-induced β-arrestin recruitment and internalization specifically of AT_1–AT_2 heteromers. We establish that these heteromers occur constitutively, are localized to the plasma membrane and perinuclear compartments, and do not internalize following AngII stimulation despite recruiting β-arrestin. These findings reveal a previously unrecognized mechanism of angiotensin receptor 'cross-talk' involving cross-inhibition of AT_1 receptor internalization through heteromerization with the AT_2 receptor subtype. Establishing the occurrence of this receptor interaction provides new insight into the biology of the renin–angiotensin system as it operates at a subcellular level.

2. Materials and methods

2.1. Materials

Primers were purchased from Proligo (Sigma-Aldrich, MO, USA). Restriction enzymes were purchased from Promega (NSW, Australia)

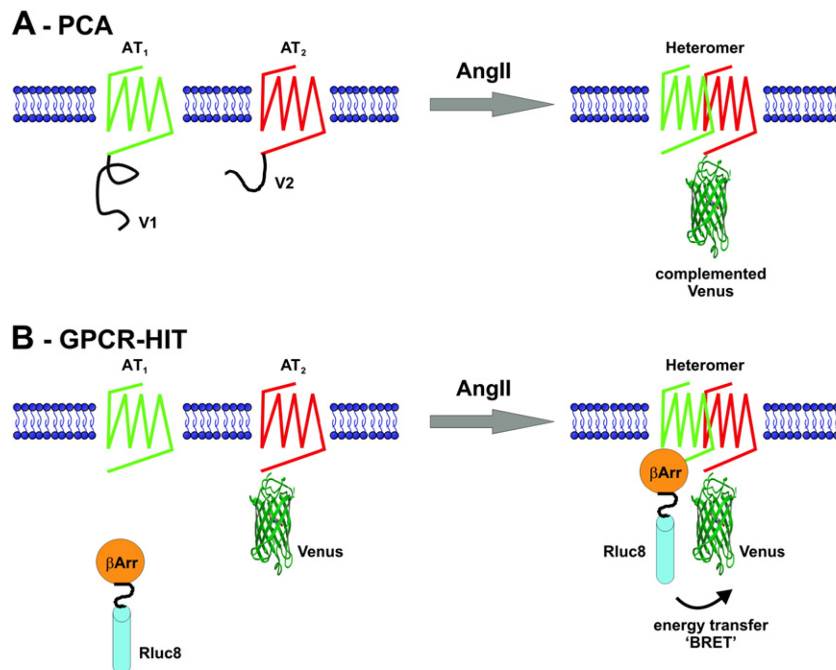

Fig. 1. Development of a protein-fragment complementation assay (PCA) and GPCR-Heteromer Identification Technology (GPCR-HIT) for studying AngII receptor heteromerization. A) PCA for studying AngII receptor heteromerization. When the Venus fluorescent protein is split into N-terminal (V1) and C-terminal (V2) amino acid fragments, neither displays inherent autofluorescence. However, when V1 and V2 are linked to two interacting proteins that come into close enough proximity to allow reformation of the Venus fluorophore, fluorescence is generated. Therefore, if AT_1 and AT_2 receptors that bear C-terminal V1 and V2 tags form heteromers, fluorescence will be generated. The ligand-dependency of such an interaction can be tested in the presence and absence of AngII. B) GPCR-HIT assay for studying AngII receptor heteromerization. In contrast to the AT_1 receptor, the AT_2 receptor does not normally interact with arrestins in a ligand-dependent manner. However, if AT_1 and AT_2 receptors exist as heteromers, AngII stimulation will cause translocation of βArr2–Rluc8 to the activated AT_1 receptor, enabling BRET to occur between βArr2–Rluc8 and AT_2–Venus.

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