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Quantifying biased signaling in GPCRs using BRET-based biosensors

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ABSTRACT

There has been a growing appreciation that G protein-coupled receptor (GPCR) functional selectivity (viz. biased signaling), in particular between G protein- and β-arrestin-dependent signaling, can be achieved with specific ligands, and that such directed signaling represents a promising avenue for improving drug efficacy and therapy. Thus, for any given GPCRs it is important to define means to pharmacologically characterize and classify drugs for their propensity to bias signaling. Here we describe an experimental protocol and step-by-step approach to assess functional selectivity between Gαq and β-arrestin-dependent responses using the prototypical angiotensin II (AngII) type 1 receptor (AT1R) expressed in HEK 293 cells. The protocol describes the expression of Bioluminescence Resonance Energy Transfer (BRET) sensors for either Gαq or β-arrestin with AT1R, and the use of the operational model of pharmacological agonism to quantify ligand bias. Such methods are equally applicable to other GPCRs and their downstream signaling effectors.

1. Introduction

G protein-coupled receptors (GPCR) represent the most common drug targets. The analysis of known modes of action of commercial drugs reveals that over 30% of all drug targets are GPCRs [1]. Given their biological and clinical importance, signaling by GPCRs has been intensely studied over the past decade which has led to the identification of new intracellular signaling cascades independent of canonical heterotrimeric G protein activation. Namely, β-arrestin signaling pathways have since been recognized as key events in GPCRs responses and as potential new therapeutic targets [2,3]. Indeed, studies of β-arrestin signaling have shown that it can be selectively activated by ligands independently of G protein activation, leading to the concept of ligand-directed or biased signaling [3]. From its initial recognition, biased signaling has since been expanded as a classical view for many GPCRs and as a new mean for improving therapeutics. Several discovery programs are currently underway that capitalize on the therapeutic potential of biased ligands. Preclinical data suggest that biased ligands may limit some adverse effects associated with GPCRs by specifically engaging beneficial signaling pathways. Examples of biased ligands currently in clinical development include JNJ7777120 being developed as an anti-inflammatory agent targeting histamine H4 receptors [4], that acts both as an antagonist of G protein-dependent responses and as an agonist for β-arrestin signaling. The G protein-biased μ opioid agonist TRV130 [5] and the β-arrestin-biased AT1R agonist TRV027 [6] are two other ligands undergoing clinical validation.

Drug discovery and studies of biased signaling mechanisms require the use of highly sensitive assays and a standardized methodology to quantify responses related to the signaling pathways. Bioluminescence Resonance Energy Transfer (BRET) is a sensitive and non-destructive method now commonly used in live cells to vet protein–protein interactions [7,8]. It has successfully been applied to study GPCR dimerization [9], and expanded lately to study other proximal GPCRs interactors, such as for different signaling effectors, including G proteins and β-arrestin [10–12]. BRET results from the transfer of energy between a donor (luciferases such as the one from *Renilla reniformis*: RLuc) and an acceptor (fluorescent proteins such as green fluorescent proteins: GFP). BRET donor and acceptor are either tagged individually on two proteins

Abbreviations: BRET, Bioluminescence Resonance Energy Transfer; AngII, angiotensin II; AT1R, angiotensin II type 1 receptor.

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of interest or onto the same protein in an intramolecular setting. The BRET response depends on the distance and the relative orientation of the donor and acceptor pair. Because of its fast reaction kinetics, BRET allows for real time detection of complexes or conformational changes that may be transient. In many studies, two proteins that potentially assemble in signaling complexes are fused separately to a donor and an acceptor. In other cases, subtle changes in protein conformation upon complex assembly or disassembly can be studied using modified proteins that are labeled with both BRET donor and acceptor moieties engineered into the same protein [8,11]. In terms of analytical methods, the operational model of agonism derived from the Black–Leff operational model (see Kenakin et al. [18], for more details), which incorporates into a single parameter the propensity of a ligand to transduce signals (e.g., the pharmacological efficacy and potency) under a "transduction ratio", has become the reference for quantifying biased signaling for different pathways. This method involves comparing the full concentration–responses of different ligands obtained for distinct signaling pathways with that of a reference ligand, usually the endogenous ligand, in order to identify whether unknown ligands have distinct biases toward one pathway. Here, we describe the use of such methods to quantify the properties of different AngII ligands to engage either Gαq or β-arrestin2, two downstream effectors of AT1R. To do so, we use both inter- and intra-molecular BRET sensors to assess respectively Gq (by measuring the interaction between Gαq and Gβγ) and β-arrestin2 (as an intramolecular conformational sensor) responses.

2. Materials and methods

2.1. BRET biosensor constructs and reagents

For the cloning of polycistronic Gq BRET sensor (Gq-poly), we first modified the multi-cloning site (MCS) of pLVX-IRES-Hyg vector (Clontech) between SpeI and BamHI sites by using linker ligation, which ablated the MfeI site by Klenow ligation. The resulting vector named pLVXi2H has a new MCS, which reads as follows: 5′-ACTAGTTCGAACCCGGGCGCGCCTCCGGACTCGAGTCAATTGTTTAAACCAGTGTGCTGGTGTACATGCATGTTAACCGGTCTAGAGCGGCCGCGGATCC-3′. IVS–IRES (^{944}BstBI–^{1560}HindIII) of pIRES–Hyg3 vector (Clontech, CA) was amplified by PCR using a 3′ reverse primer with overlapping sequences (underlined) with Gαq-RlucII (5′-CATCAATTGTATCAGTTGTGGCAAGCTTATCATCGTG-3′) and Gαq-RlucII was PCR-amplified by using 5′ forward primer with overlapping sequences (underlined) with IRES (5′-TGCCACAACTGATACAATTGATGACTCTGGAGTCCATCATG-3′). Overlapping PCR amplification was performed by using these two PCR products as templates. The final PCR product (IVS–IRES–GαqRlucII) was subcloned into SpeI/AgeI sites of pLVXi2H vector by infusion technology (Clontech, CA). GFP10-Gγ1 was amplified by PCR and subcloned into SpeI/BstBI sites of PLVxi2H vector containing IVS–IRE–GαqRlucII. IRES was amplified by using the 5′ primer (5′-TCTGGTCTAACTCGAGAACTGCAG GTCGAGCATGCAT-3′) and 3′ primer (5′-CGTCCTTATAATCCATTATCAGTTGTGGCAAGCTTATCATCGT-3′) with overlapping sequences with the Flag-Gβ1 (underlined). Flag-Gβ1 was amplified by PCR using 5′ primer (5′-GCTTGCCACAACTGATAATGGATTATAAGGACGATGACGA-3′) with overlapping sequences with IRES (underlined) and 3′ primer (5′-GCCGCTCTAGACCGGTT AGTTCCAGATCTTGAGGAAGCTAT-3′). Overlapping PCR amplification was performed by using these two PCR products as templates and using the 5′ primer of IRES and 3′ primer of Flag-Gβ1 as primers. The final PCR product (IRES–Flag-Gβ1) was subcloned into XhoI/AgeI sites of pLVXi2H vector containing GFP10-Gγ1–IVS–IRES–Gαq-RlucII. Gαq-RlucII [13], GFP10-Gγ1 [14] and signal peptide-Flag tagged human AT1R (sp-Flag AT1R) [15] were described elsewhere. Flag-Gβ1 was obtained from Guthrie cDNA resource center (www.cdna.org). The GFP10–β-arrestin2–RlucII "double brilliance" (GFP10–β-arr2–RlucII) construct was designed similarly to the GFP10–β-arr1–RlucII, previously described by our group [12] using PCR amplification and cloned into Acc65I and HindIII sites of GFP10–EPAC–RlucII [16]. Angiotensin ligands used were described elsewhere [12] and consisted of the octapeptide AngII: (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), SVdF: (Sar^1-Val^5-D-Phe^8) AngII, SBpa: (Sar^1-Val^5-Bpa^8 (*p*-benzoyl-L-phenylalanine)) AngII, DVG: (Asp^1-Val^5-Gly^8) AngII, and SII: (Sar^1-Ile^4-Val^5-Ile^8) AngII. UBO-QIC, was obtained from Institute for Pharmaceutical Biology of the University of Bonn (Germany). Coelenterazine 400a was from Nanolight® technology, AZ. Phusion DNA polymerase was purchased from Thermo Scientific.

2.2. Cell transfection and BRET assays

HEK 293 cells were cultured in DMEM supplemented with 6% fetal bovine serum (FBS) and 20 μg/ml gentamycin. Cells were grown at 37 °C in 5% CO_2 and 90% humidity. Cells were seeded at a density of 7.5×10^5 cells per 100 mm dish and were transiently transfected the next day with AT1R (0.5–6 μg) along with either Gq-polycistronic BRET sensor (4.5 μg) or GFP10–βarr2–RlucII (0.075 μg) sensor using calcium phosphate method as described previously [15]. After 24 h, cells were detached and seeded onto poly-ornithine-coated 96-well white plates at a density of 25,000 cells per well in complete media. The next day, cells were washed once with Tyrode's buffer (140 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 12 mM $NaHCO_3$, 5.6 mM D-glucose, 0.5 mM $MgCl_2$, 0.37 mM NaH_2PO_4, 25 mM HEPES, pH 7.4) and left in 80 μl (Gq sensor) or 90 μl (β-arr2 sensor) of Tyrode's buffer. For kinetic measurements, BRET signals were monitored every 2 s after addition of coelenterazine 400a to a final concentration of 5 μM using a Synergy2 (BioTek®) microplate reader. Filter set was 410 ± 80 nm and 515 ± 30 nm for detecting the RlucII *Renilla* luciferase (donor) and GFP10 (acceptor) light emission. AngII (to a final concentration of 100 nM) or Tyrode's buffer was injected after 11 measurements (20 s), and BRET was recorded over a period of 4 min. For concentration–response curves of the Gq-poly sensor, BRET signals were monitored after adding coelenterazine 400a for 90 s, followed by the addition of various concentrations of ligands for either 2 or 10 min. For the β-arr2 sensor, BRET signals were measured 20 min after addition of various concentrations of ligands and coelenterazine 400a was added 10 min before BRET measurements. BRET ratio was determined by calculating the ratio of the light emitted by GFP10 over the light emitted by the RlucII.

2.3. Data analysis and calculation of bias

Recently, a simple mathematical method was introduced to quantify ligand bias [17], which still uses the transduction ratio (τ/K_A). Such ratios can be extrapolated from full concentration–response curves in functional assays without the need to perform additional experiments to determine the K_D of a ligand, or the maximal responses of the system, or the τ and K_A of the reference ligand from receptor depletion analysis [17–19]. Equations for such analysis can be embedded in GraphPad Prism software v6, and has been explained in [17]. Here, we provide an example file as supplementary material, where the equation can be found under the rubric "Nonlinear regression (curve fit)"/ "User-defined equations"/"Operational Model (Tau/K_A ratios)", where the transduction ratio, τ/K_A, is defined as "*R*" (Appendix A).