Application of G Protein-Coupled Receptor-Heteromer Identification Technology to Monitor β -Arrestin Recruitment to G Protein-Coupled Receptor Heteromers

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ABSTRACT

Understanding the role of G protein-coupled receptor (GPCR; also known as a 7 transmembrane receptor) heteromerization in the physiology and pathophysiology of cellular function has now become a major research focus. However, there is currently a lack of cell-based assays capable of profiling the specific functional consequences of heteromerization in a ligand-dependent manner. Understanding the pharmacology specifically associated with heteromer function in contrast to monomer or homomer function enables the so-called biochemical fingerprints of the receptor heteromer to be ascertained. This is the first step in establishing the physiological relevance of heteromerization, the goal of everyone in the field, as these fingerprints can then be utilized in future endeavors to elucidate heteromer function in native tissues. The simple, robust, ligand-dependent methodology described in this study utilizes a novel configuration of components of a proximity-based reporter system. This is exemplified by the use of bioluminescence resonance energy transfer due to the advantages of real-time live cell monitoring of proximity specifically between the heteromer complex and a protein that is recruited in a ligand-dependent manner, in this case, β -arrestin 2. Further, the demonstration of Z'-factor values in excess of 0.6 shows the potential of the method for screening compounds for heteromer-selective or biased activity. Three previously characterized GPCR heteromers, the

chemokine receptor heteromers CCR2-CCR5 and CCR2-CXCR4, as well as the angiotensin II receptor type 1-bradykinin receptor type 2 heteromer, have been used to illustrate the profiling capability and specificity of the GPCR heteromer identification technology.

INTRODUCTION

There is now considerable evidence for G protein-coupled receptors (GPCRs; also known as 7 transmembrane receptors) forming dimers or oligomers with different receptor subtypes, termed heteromers.^{1–6} Milligan and Smith recently highlighted the need for developing cell-based screening approaches that incorporate the concept of GPCR heteromerization.⁷ They also asserted that GPCR heteromers should be considered as “individual molecular species” and therefore represent drug targets distinct from those observed when screening a single GPCR in isolation. Importantly, a GPCR heteromer complex is also likely to include a range of other proteins that contribute to GPCR function, including structure, localization, trafficking, scaffolding, signaling, desensitization (largely involving phosphorylation), internalization, resensitization/recycling, and/or degradation. Therefore, two GPCRs within such a complex can influence each other's function without physically touching and, consequently, still fall under the recently agreed definition of a “receptor heteromer” 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.”⁵

Having recognized the potential importance of GPCR heteromerization, the challenge now is to find ways to effectively interrogate the pharmacology associated selectively with the heteromer complex. This is not a trivial exercise as a cell expressing two different GPCRs is likely to contain a mixture of receptor populations, with monomers or homomers of each constituent GPCR being present in addition to heteromers. This confounds analysis using downstream signaling outputs as it is usually not clear which particular receptor population(s) contributes to signal mediation and therefore the biochemical fingerprint.⁵ Technology configurations that enable

ABBREVIATIONS: AT₁R, angiotensin II receptor type 1; BRET, bioluminescence resonance energy transfer; B2R, bradykinin receptor type 2; eBRET, extended BRET; FCS, fetal calf serum; GPCR, G protein-coupled receptor; HIT, heteromer identification technology; RC, reporter component; SD, standard deviation; s.e.m., standard error of the mean.

specific assessment of the heteromer are very limited in number and have been reviewed recently.^{6,8} Other elegant studies published previously in this area include observations of differential receptor trafficking via enzyme-linked immunosorbent assay and confocal microscopy,⁹ the use of the synthetic bivalent dimerizing ligand AP21967 or a nonselective agonist to provide insights into β -arrestin recruitment to the vasopressin receptors (V1aR and V2R),¹⁰ and the study of yellow fluorescent protein (YFP)/ β -arrestin recruitment to melatonin receptor MT₁/Rluc, either melatonin-induced or constitutively, in the presence and absence of GPR50 co-expression.¹¹

The current study highlights the utility of the recently developed GPCR heteromer identification technology (GPCR-HIT) configuration (Fig. 1) using bioluminescence resonance energy transfer (BRET) (see Ref.¹² for review) as the preferred proximity-based reporter system. Importantly, the method is equally adaptable to other reporter systems such as fluorescence resonance energy transfer, bimolecular fluorescence complementation, bimolecular luminescence complementation, enzyme fragment complementation, and the TangoTM protease-based system (see Ref.⁶ for review), each of which may have certain benefits in certain applications.

The GPCR-HIT configuration enables monitoring of constitutive or dynamic interactions between receptors that, crucially, are both ligand-dependent in terms of reporter output and specifically monitor the receptor heteromer population in the cell (Fig. 1). The first GPCR (GPCR A) is linked to the first component of a proximity-based reporter system (Reporter Component 1 [RC1]), the second GPCR (GPCR B) is untagged with respect to the reporter system, and a GPCR-interacting protein (Protein C) is linked to the second (complementary) component of the reporter system (RC2). These three partners are co-expressed in cells and the signal resulting from increased proximity of RC1 and RC2 is measured after addition of ligand selective for GPCR B, generally resulting in Protein C (*e.g.*, β -arrestin) binding to that receptor. In a situation where the GPCRs are not proximal (Fig. 1A), RC1 and RC2 are themselves not sufficiently proximal to enable a signal from the reporter system to be detected. In contrast when the GPCRs are in proximity (Fig. 1B), a signal from the reporter system can be detected indicative of the two GPCRs forming a heteromer (being part of the same macromolecular complex, shown in Figure 1B in its simplest terms). Importantly, the inclusion of Protein C (*e.g.*, β -arrestin) in this configuration means that the reporter signal is ligand dependent regardless of whether the heteromer itself is formed in a constitutive or ligand-dependent manner. With certain receptor heteromer combinations, activation of GPCR B may result in allosteric modulation of GPCR A such that Protein C binds to GPCR A even though it is not ligand bound. In this situation, the signal is still heteromer-specific as the GPCR A-Protein C interaction is dependent upon both heteromerization and allosteric activation by GPCR B. The use of a ligand selective for GPCR B specifically enables profiling of the heteromer as no signal is detected from GPCR A monomers/homomers or GPCR B monomers/homomers (Fig. 1C). In contrast, addition of a ligand selective for GPCR A is likely to result in a signal from both GPCR A monomers/homomers and the heteromers and is therefore not heteromer specific

(Fig. 1D). Clearly, addition of a nonselective ligand or a combination of selective ligands for both receptors will also result in a signal from both of these receptor populations (GPCR A-GPCR A and GPCR A-GPCR B). When screening, compounds may bind to GPCR A, GPCR B, or the heteromer. Therefore, by using the hits from the primary screen to carry out a secondary screen of cells expressing Protein C/RC2 with either GPCR A/RC1 or GPCR B/RC1, compounds not displaying heteromer-selective or biased efficacy can be discounted.

We have profiled the CCR2-CCR5 and CCR2-CXCR4 chemokine receptor heteromers in terms of their ability to recruit the trafficking, scaffolding, and signaling protein β -arrestin 2 specifically to the heteromer complex. These heteromers have been well characterized previously,^{13–19} but not with respect to β -arrestin interactions. Chemokine receptors play important roles in both inflammation and human immunodeficiency virus-1 infectivity.^{13–19} Consequently, understanding the intricacies of their function has considerable relevance to treatment of major human diseases. We have also investigated the more controversial angiotensin II receptor type 1 (AT₁R)-bradykinin receptor type 2 (B2R) heteromer.^{20–26} Evidence has been presented for a role of this heteromer in vascular smooth muscle contraction,²¹ pre-eclampsia,²² and experimental hypertension,²⁴ although others have recently questioned these findings, at least in COS-7, HEK293, and NIH3T3 cells.²⁶

We have used BRET in this study to assess the real-time kinetic profiles resulting from any consequent increase in proximity, building upon the previously validated monitoring of GPCR/ β -arrestin interactions utilizing BRET.^{27–30} BRET occurs between a complementary *Renilla* luciferase (Rluc) variant as donor and green fluorescent protein variant as acceptor.^{31,32} Upon Rluc-catalyzed oxidation of the coelenterazine substrate (such as coelenterazine *h* for first generation BRET (BRET¹) or EnduRenTM for extended BRET (eBRET),²⁸ energy is transferred to the acceptor if within 10 nm, resulting in acceptor light emission peaking at a characteristic wavelength. Indeed, recent characterization of BRET efficiency indicated that distances beyond 5 nm were very difficult to detect, at least for the Rluc-YFP combination.³³ Acceptor emission is therefore indicative of very close donor-acceptor proximity and consequently the existence of a complex containing proteins fused to the donor or acceptor. The advantages of the GPCR/Rluc8 and β -arrestin 2/Venus combination to generate BRET dose-response, kinetic profiles, and Z'-factor data have been shown recently.²⁹

MATERIALS AND METHODS

cDNA Constructs and Ligands

CCR2 and B2R cDNAs were obtained from the Missouri S&T cDNA Resource Center (www.cdna.org). CCR5/Rluc8 and CXCR4/Rluc8 cDNA constructs were generated from plasmids containing the respective receptor cDNA tagged with Rluc kindly provided by Aron Chakera (Oxford University, United Kingdom). Similarly, the B2R/Rluc8 cDNA construct was generated from B2R/Rluc (formerly produced by PCR amplification of B2R cDNA to remove the stop codon and ligation into pcDNA3 containing Rluc). With all of these

Fig. 1. Illustration of the GPCR-HIT configuration. GPCR A is linked to the first component of a proximity-based reporter system (RC1). GPCR B is untagged with respect to the reporter system. Protein C is a GPCR-interacting protein linked to the second (complementary) component of the reporter system (RC2). Addition of ligand selective for GPCR B results in Protein C (e.g., β -arrestin) binding to that receptor or the heteromer. If the GPCRs are not proximal (**A**), RC1 and RC2 are themselves not sufficiently proximal to enable a signal from the reporter system to be detected. However, proximity of the GPCRs (**B**) enables detection of a signal from the reporter system indicative of heteromerization. Likely, consequences of treating cells with ligand selective for GPCR B (**C**) or GPCR A (**D**) are illustrated in terms of signals from the various receptor populations. GPCR, G protein-coupled receptor; HIT, heteromer identification technology; RC, reporter component. Color images available online at www.liebertonline.com/adt.

constructs, the Rluc coding region was replaced with Rluc8 cDNA from pcDNA3.1-Rluc8 kindly provided by Andreas Loening and Sanjiv Gambhir (Stanford University, CA)³⁴ as described previously for other GPCR constructs.²⁹ The use of chemokine receptors tagged with Rluc has been published previously,³⁵ as has the use of Rluc8 in place of Rluc.²⁹ AT₁R cDNA was kindly provided by Walter Thomas (University of Queensland, Australia) and has been validated previously.³⁶ The β -arrestin 2/Venus cDNA construct was prepared previously from pCS2-Venus kindly provided by Atsushi Miyawaki (RIKEN Brain Science Institute, Wako-city, Japan).²⁹ Agonists used were CCL2 (MCP-1), CCL4 (MIP-1 β), CXCL12 (SDF-1 α), bradykinin, and Ang II (Sigma).

Cell Culture and Transfections

HEK293FT cells were maintained at 37°C, 5% CO₂ in Complete Media (Dulbecco's modified Eagle's medium containing 0.3 mg/mL glutamine, 100 IU/mL penicillin, and 100 μ g/mL streptomycin; Gibco) supplemented with 10% fetal calf serum (FCS) and 400 μ g/mL Geneticin (Gibco). Transfections were carried out 24 h after seeding using Genejuice (Novagen) according to manufacturer's instructions. Cells were harvested with 0.05% Trypsin-EDTA (Gibco).

Dose-Response and Real-Time Kinetic BRET Assays

HEK293FT cells were transfected with cDNA as described in the figure legends. When assessing GPCR A/ β -arrestin 2 proximity in the

absence of GPCR B, empty pcDNA3 vector was transfected instead of GPCR B cDNA. Cells were harvested 24 h post-transfection in HEPES-buffered phenol red-free Complete Medium containing 5% FCS and added to a poly-L-lysine-coated white 96-well plate (Nunc). Dose-response curves were generated using BRET¹, with medium in the plate being replaced with PBS containing 5 μ M coelenterazine *h* (Molecular Probes) and assays carried out immediately. Kinetic BRET assays were carried out using eBRET as described previously.^{28,29} For these assays, 48 h post-transfection, the plate was incubated at 37°C, 5% CO₂ for 2 h with 30 μ M EnduRen (Promega) to ensure substrate equilibrium was reached. All BRET measurements were taken at 37°C using the VICTOR Light plate reader with Wallac 1420 software (PerkinElmer). Filtered light emissions were sequentially measured at 400–475 and 520–540 nm. The BRET signal was calculated by subtracting the ratio of 520–540 nm emission over 400–475 nm emission for a vehicle-treated cell sample from the same ratio for a second aliquot of the same cells treated with agonist, as described previously.^{28,31} In this calculation, the vehicle-treated cell sample represents the background, eliminating the requirement for measuring a donor-only control sample.^{28,31} For BRET kinetic assays, the final pretreatment reading is presented at the zero time point (time of ligand/vehicle addition).

BRET Z'-Factor Assays

HEK293FT cells were transfected as for the kinetic and dose-response BRET assays. Twenty-four hours post-transfection, cells were harvested in HEPES-buffered phenol red-free Complete Media containing 5% FCS and added to an entire poly-L-lysine-coated white 96-well plate (Nunc). Forty-eight hours post-transfection, the plate was incubated at 37°C, 5% CO₂ for 2 h with 30 μ M EnduRen (Promega) to ensure that substrate equilibrium was reached. Forty-eight agonist-treated wells (positive control) and 48 vehicle-treated wells (negative control) were assayed at 37°C using the VICTOR Light plate reader with Wallac 1420 software (PerkinElmer). Filtered light emissions were sequentially measured at 400–475 and 520–540 nm. The BRET data were expressed as fluorescence/luminescence, meaning the 520–540 nm emission over the 400–475 nm emission, as described previously.²⁹ These are not BRET ratios as normally defined because the background is not subtracted. Instead, the background (negative control) data are presented along with the treated (positive control) data so that the variance associated with each can be compared.²⁹ As a measure of assay performance, the Z'-factor was calculated with respect to the mean and standard deviation (SD) of control data using the equation $Z' = 1 - [(3 \text{ SD of positive control} + 3 \text{ SD of negative control}) / (\text{mean of positive control} - \text{mean of negative control})]$.³⁷

Measurement of Total Inositol Phosphate Production

HEK293FT cells were seeded in six-well plates and transiently transfected the next day. 24 h post-transfection cells were split into 24-well plates, in inositol-free Complete Medium (MP Biomedicals) containing 1% dialyzed FCS. Six to eight hours later, the medium was replaced with inositol-free medium containing 1% dialyzed FCS and

1 μ Ci/mL [³H]myo-inositol (Amersham Pharmacia Biotech), followed by overnight incubation. The medium was removed and cells washed twice with Assay Buffer (1 mg/mL fatty acid-free BSA, 140 mM NaCl, 20 mM HEPES, 4 mM KCl, 8 mM D-glucose, 1 mM MgCl₂, 1 mM CaCl₂, and 10 mM LiCl) before incubating at 37°C for 40 min in Assay Buffer with or without agonist. Postincubation, the Assay Buffer was replaced with 10 mM formic acid for 40 min at 4°C. The acid was subsequently transferred to 5 mL tubes and the inositol phosphates bound to AG1-X8 anion-exchange resin (Bio-Rad Laboratories). After washes with water and 60 mM ammonium formate/5 mM sodium tetraborate, inositol phosphates were eluted with 1 M ammonium formate/0.1 M formic acid. Samples (0.9 mL) were mixed with 4 mL Optiphase hisafe 2 scintillant (Wallac) and radioactivity measured using a 1209 Rackbeta liquid scintillation counter (Wallac).

Data Presentation and Statistical Analysis

Data are presented and analyzed using Prism graphing software (GraphPad). Sigmoidal curves with variable slope were fitted to the dose-response data using nonlinear regression. Statistical analysis of logEC₅₀ and Hill slope data was carried out using two-tailed unpaired Student's *t*-tests.

RESULTS

Dose-Response Curves for Chemokine Receptor Heteromers

We investigated both the CCR2-CCR5 and CCR2-CXCR4 heteromers. Addition of the CCR5-selective agonist CCL4 to cells expressing CCR5/Rluc8, β -arrestin 2/Venus, and CCR2 resulted in a dose-dependent increase in BRET signal indicative of β -arrestin 2/Venus being recruited to activated CCR5/Rluc8 (Fig. 2A). In this situation, CCR5/Rluc8 could be existing as a monomer, homomer, or heteromer with other receptors. Addition of the CCR2-selective agonist CCL2 to a second aliquot of the same transfected cells also resulted in a dose-dependent increase in BRET signal (Fig. 2A). This is consistent with β -arrestin 2/Venus being recruited to CCR2 (GPCR B in Fig. 1) in a ligand-dependent manner and CCR5/Rluc8 (GPCR A/RC1 in Fig. 1) being sufficiently proximal to CCR2 to allow detectable energy transfer to β -arrestin 2/Venus (Protein C/RC2 in Fig. 1), even though β -arrestin is binding to CCR2 (GPCR B). Alternatively, allosteric modulation of CCR5/Rluc8 by the activated CCR2 could enable binding of β -arrestin 2/Venus directly to CCR5/Rluc8. Either way, a BRET signal in this scenario implies that CCR2 and CCR5 are in proximity in a heteromer complex. The mean concentrations of CCL4 and CCL2 eliciting half-maximal BRET responses (mean BRET EC₅₀ values $\pm$ standard error of the mean [s.e.m.]) were not significantly different (35.5 ± 6.3 and 21.5 ± 2.1 nM respectively). However, the Hill slopes differed significantly ($P < 0.0001$), the mean values for CCL4 and CCL2 $\pm$ s.e.m. being 1.3 ± 0.05 and 3.4 ± 0.06 , respectively.

Similar dose-response data were generated with addition of CXCR4-selective agonist CXCL12 or CCL2 to cells co-expressing CXCR4/Rluc8, β -arrestin 2/Venus, and CCR2 (Fig. 2B). The mean BRET EC₅₀ values $\pm$ s.e.m. were not significantly different (34.3 ± 10.1 and 16.8 ± 1.6 nM, respectively). Further and in contrast to that

Fig. 2. Dose-response BRET data for GPCR-HIT profiling chemokine receptor heteromerization for CCR2-CCR5 and CCR2-CXCR4. HEK293FT cells transiently co-expressing CCR5/Rluc8, β -arrestin 2/Venus, and CCR2 were treated with increasing doses of CCL4 (black triangles) or CCL2 (white squares) (**A**). HEK293FT cells transiently co-expressing CXCR4/Rluc8, β -arrestin 2/Venus, and CCR2 were treated with increasing doses of CXCL12 (black triangles) or CCL2 (white squares) (**B**). Cells were monitored at 37°C using BRET¹ to generate dose-response curves after about 30 min of agonist treatment, except for CXCL12-treated cells in (**B**) for which data were generated after about 10 min of treatment due to the more transient nature of the signal. Sigmoidal dose-response curves with variable slope were fitted using Prism graphing software (GraphPad). Data shown are representative of three independent experiments. BRET, bioluminescence resonance energy transfer.

observed with the CCR2-CCR5 heteromer, the Hill slopes did not differ significantly.

Real-Time Kinetic Profiles for Chemokine Receptor Heteromers

Addition of CCL4 (100 nM) to cells expressing CCR5/Rluc8 and β -arrestin 2/Venus resulted in a robust and continually increasing ligand-induced BRET signal indicative of β -arrestin 2/Venus being recruited to activated CCR5/Rluc8 (Fig. 3A). Addition of CCL2 (100 nM) to a second aliquot of the same transfected cells did not result in any increase in the BRET signal and co-treatment with CCL2 and CCL4 (both 100 nM) resulted in a similar kinetic profile to that observed with CCL4 alone (Fig. 3A). Similar treatments of cells transfected with untagged CCR2 in addition to CCR5/Rluc8 and β -arrestin 2/Venus gave different results. CCL4 treatment again elicited an increase in BRET signal with a similar kinetic profile, although of reduced magnitude. However, following co-expression of CCR2 with CCR5, CCL2 also induced an increase in BRET signal that, as described above, indicates the proximity of CCR2 and CCR5 in a heteromer complex that recruits β -arrestin 2. Further, and in contrast to that observed in the absence of CCR2, combined treatment with CCL2 and CCL4 induced a substantially higher BRET signal than observed with CCL2 or CCL4 alone.

Addition of CXCL12 (100 nM) to cells expressing CXCR4/Rluc8 and β -arrestin 2/Venus resulted in a ligand-induced BRET signal indicative of β -arrestin 2/Venus being recruited to activated CXCR4/Rluc8 (Fig. 3C). Notably, the kinetic profile of this recruitment differed considerably from that observed with CCR5/Rluc8 and β -arrestin 2/Venus (Fig. 3A), the BRET signal peaking after 10–15 min and decreasing thereafter (Fig. 3C). Addition of CCL2 (100 nM) to a second aliquot of the same transfected cells did not result in any increase in BRET signal and co-treatment with CCL2 and CXCL12 (both 100 nM) resulted in a similar kinetic profile to that observed with CXCL12 alone (Fig. 3C). Similar treatments of cells transfected with untagged CCR2 in addition to CXCR4/Rluc8 and β -arrestin 2/Venus again gave different results. CXCL12 treatment elicited an increase in BRET signal with a similar kinetic profile, but as observed with CCL4 treatment previously, of reduced magnitude in the presence of CCR2. Indeed, the signal returned to baseline before 40 min (Fig. 3D). In contrast, CCL2 again induced an increase in

BRET signal, this time indicating the proximity of CCR2 and CXCR4 in a heteromer complex that recruits β -arrestin 2. Indeed, this signal was remarkably similar to that observed for CCL2 in Figure 3B in both profile and magnitude. Further, and in contrast to that observed in the absence of CCR2, combined treatment with CCL2 and CXCL12 induced a substantially higher BRET signal than observed with CCL2 or CXCL12 alone. The increase in magnitude is particularly remarkable considering that after 40 min, no ligand-induced BRET signal was observed as a consequence of CXCL12 treatment alone (Fig. 3D).

Demonstration of Potential for Screening Chemokine Receptor Heteromers

Z' values in excess of 0.5 indicate high assay performance³⁷ and, in the present study, values of 0.68 were generated for eBRET assays detecting CCL2-induced signals from cells expressing CCR2 and β -arrestin 2/Venus with either CCR5/Rluc8 (Fig. 4A, B) or CXCR4/Rluc8 (Fig. 4C, D). With both of these combinations, extensive assay windows were observed. These started after ~30 min of agonist incubation and extended for at least 30 min thereafter (Fig. 4A, C). The signal dynamic range and data variation across a 96-well plate are illustrated after ~45 min (Fig. 4B, D).

Fig. 3. Kinetic BRET data for GPCR-HIT profiling chemokine receptor heteromerization for CCR2-CCR5 and CCR2-CXCR4. HEK293FT cells transiently co-expressed CCR5/Rluc8 and β -arrestin 2/Venus in the absence (A) or presence (B) of CCR2. Alternatively, HEK293FT cells transiently co-expressed CXCR4/Rluc8 and β -arrestin 2/Venus in the absence (C) or presence (D) of CCR2. The cells were monitored at 37°C using eBRET to generate kinetic profiles. Selective agonists (alone or in combination; 100 nM) or vehicle were added after 10 min and measurements recommenced for a further 50 min. Data shown are representative of three independent experiments. eBRET, extended BRET.

Investigation of the AT₁R-B2R Heteromer in Terms of β -Arrestin Recruitment

AT₁R-B2R is an interesting heteromer to profile as it has caused controversy in the scientific literature to date. Addition of bradykinin (1 μ M) to cells expressing B2R/Rluc8, β -arrestin 2/Venus, and AT₁R resulted in a ligand-induced BRET signal indicative of β -arrestin 2/Venus being recruited to activated B2R/Rluc8 (Fig. 5A). In this situation, B2R/Rluc8 could be existing as a monomer, homomer, or in a heteromer. In contrast to observations with the chemokine receptors, addition of angiotensin II (Ang II; 1 μ M) to a second aliquot of the same cells did not result in a ligand-induced BRET signal (Fig. 5A), indicating that β -arrestin 2/Venus (Protein C/RC2 in Fig. 1) was not brought into proximity with B2R/Rluc8 (GPCR A/RC1 in Fig. 1) despite activation of the co-expressed AT₁R (GPCR B in Fig. 1). To ensure this negative result was not due to AT₁R not being expressed or functional, parallel secondary signaling assays were carried out using the same protein expression profile as for the BRET kinetic assays, resulting in >4-fold increase in total inositol phosphate

production with Ang II activation compared with vehicle control (Fig. 5B). Further, a wide range of B2R/Rluc8: β -arrestin 2/Venus:AT₁R cDNA ratios were evaluated and none of them resulted in a reproducible Ang II-induced BRET signal (data not shown). The lack of signal is illustrated by the highly negative Z' value generated for eBRET assays showing the inability of Ang II to induce BRET signals from cells co-expressing B2R/Rluc8, β -arrestin 2/Venus, and AT₁R (Fig. 5C, D). Signal dynamic range and data variation across a 96-well plate are illustrated after ~45 min. As a Z' value of -1 is the lower detection limit of an assay system,³⁷ a Z' value of -3.99 indicates our inability to detect a BRET signal indicative of β -arrestin 2 recruitment to the AT₁R-B2R heteromer. This is in contrast to the Z' value of 0.68 for CCR2-CCR5 and CCR2-CXCR4 (Fig. 4).

DISCUSSION

There are very few cell-based assays available with the capability to consider GPCR heteromerization,^{4,6-8} and a distinct lack of assays capable of specifically assessing heteromerization without altering constituent receptor function as the basis of the assay.^{6,8} The GPCR-HIT assay configuration described in this study has the capacity to

profile heteromers specifically, by placing an RC that does not discernibly alter receptor function on only one of the two receptor types. Consequently, insights into the biochemical characteristics specifically of the receptor heteromer can be ascertained and used for its identification in a native tissue, the so-called biochemical fingerprint of the receptor heteromer.⁵

The ligand-dependent nature of our methodology enables specific signals to be observed in clear comparison to negative controls, without needing complex saturation or competition assays.^{32,38} The advantages of utilizing β -arrestin-dependent assays for GPCR drug profiling and screening have already been described,²⁷ including the observations that most GPCRs interact with β -arrestins and that assays exploiting this interaction are not dependent on GPCR G protein preference. This is a particular advantage for the current application as heteromers may not couple to the same G protein as constituent protomers.^{1-4,7} Of course β -arrestin recruitment may also differ with the heteromer (a biochemical fingerprint of the receptor heteromer in itself), but Protein C does not need to be β -arrestin as it could be another protein that is recruited in a ligand-dependent manner, such as a G protein or

Fig. 4. BRET Z' -factor data illustrating the potential for GPCR-HIT screening of chemokine receptor heteromerization for CCR2-CCR5 and CCR2-CXCR4. Entire 96-well plates of HEK293FT cells transiently co-expressing CCR2 and β -arrestin 2/Venus with CCR5/Rluc8 (**A**, **B**) or CXCR4/Rluc8 (**C**, **D**) were monitored at 37°C using eBRET assaying 48 CCL2 (100 nM)-treated wells and 48 vehicle-treated wells. Fluorescence/luminescence values are presented against time (**A**, **C**) or well number at ~45 min (**B**, **D**). Solid lines in **B** and **D** show the means of the positive control (CCL2) and negative control (vehicle). Broken lines display three standard deviations from the mean of each data set. Z' values are calculated as described in the Materials and Methods section. Data shown are representative of three independent experiments.

Protein Kinase C. The ligand dependency of the interaction with Protein C/RC2, assuming that ligands are not membrane permeable, enables assessment of receptors that have successfully trafficked to the plasma membrane and are sufficiently functional to bind agonist, in this case becoming phosphorylated and recruiting β -arrestin 2.³⁹ This selects against assessment of tagged proteins clumped in the endoplasmic reticulum or degradative compartments due to incorrect or excessive expression. Further, the dose dependency of these combined events can also be evaluated. Significantly steeper Hill slopes have been observed for dose-response curves of certain heteromers (exemplified by CCR2-CCR5), but not others (exemplified by CCR2-CXCR4). The reason for this difference is currently unclear; however, as the protein expression profile is identical in both cases and the only difference is the agonist treatment, this observation may help to shed light on the mechanism of GPCR heteromerization and/or allosterism across the complex in the future.

There is good evidence for functionally relevant heteromerization for CCR2-CCR5^{13–17} and CCR2-CXCR4.^{14,17–19} We have therefore

used these receptor combinations to illustrate GPCR-HIT and its ability to generate dose-response, kinetic, and Z' data. Receptor expression levels in the range used in this study were previously published as being similar to those observed in primary lymphocytes.¹⁸

The distinct kinetic profiles observed for the different chemokine receptors recruiting β -arrestin 2 illustrate the utility of real-time live cell monitoring with BRET to provide this level of output texture. With both CCR2-CCR5 and CCR2-CXCR4, CCL2-induced β -arrestin 2 recruitment to the heteromer complex is clearly demonstrated and is similar in both cases (Fig. 3). However, expression of CCR2 appears to reduce the BRET signal induced by CCL4 increasing CCR5/Rluc8- β -arrestin 2/Venus proximity or CXCL12 increasing CXCR4/Rluc8- β -arrestin 2/Venus proximity (Fig. 3), a reduction that is not reflected in the luminescence observed from Rluc8 (data not shown). One explanation for this is that the CCL4- or CXCL12-induced recruitment of β -arrestin 2 is lower with the heteromers than with the monomers/homomers despite potency being unaffected (Fig. 2). This would be consistent with previous data indicating that MT₁/Rluc-GPR50 heteromers exhibit reduced melatonin-induced recruitment of YFP/ β -arrestin compared with MT₁/Rluc monomers/homomers.¹¹

Alternatively, heteromerization may affect trafficking to or from the plasma membrane. However, treatment with both ligands appears to overcome the effect, perhaps implying that β -arrestin 2 recruitment is facilitated by both types of receptor in the complex being in active conformations. These findings might be considered to be contrary to the negative binding cooperativity observed for both CCR2-CCR5^{15,16} and CCR2-CXCR4.¹⁹ One suggestion we have to reconcile these data is that the significant conformational changes believed to result in negative binding cooperativity may also influence the ability of the heteromer to recruit β -arrestin 2. Indeed, investigation of such a putative coordinated response to costimulation has the potential to reveal interesting insights into the physiological role of chemokine heteromerization. Another consideration is the BRET technology itself, which is highly sensitive to donor-acceptor distance and relative orientation.^{32,33} Therefore, it is possible that the proximity of the donor and acceptor in these complexes is sufficiently close to enable detection of changes in

Fig. 5. Lack of GPCR-HIT signal from cells co-expressing B2R and AT₁R. eBRET at 37°C was used to generate kinetic profiles with HEK293FT cells transiently expressing B2R/Rluc8 and β -arrestin 2/Venus with or without AT₁R (**A**). Agonist (bradykinin or Ang II; 1 μ M) or vehicle were added after 10 min and measurements recommenced for a further 50 min. No ligand-induced increase in BRET signal was observed upon addition of Ang II (1 μ M) to B2R/Rluc8 and β -arrestin 2/Venus with or without AT₁R (**A**). Data representative of three independent experiments. To demonstrate plasma membrane expression and functionality of AT₁R in this context, the total inositol phosphate production resulting from activation of AT₁R with Ang II (1 μ M) when the cells were co-expressing B2R/Rluc8, β -arrestin 2/Venus, and AT₁R was assessed (**B**). Data shown as fold over basal (total inositol phosphate production from vehicle-treated samples) and are mean $\pm$ standard error of the mean of three independent experiments. Z'-factor data were generated using entire 96-well plates of HEK293FT cells transiently co-expressing B2R/Rluc8, β -arrestin 2/Venus, and AT₁R (**C**, **D**). The plates were monitored at 37°C using eBRET assaying 48 Ang II (1 μ M)-treated wells compared with 48 vehicle-treated wells. Fluorescence/luminescence values are presented against time (**C**) or well number at ~45 min (**D**). Solid lines show the means of the positive control (Ang II) and negative control (vehicle). Broken lines display three standard deviations from the mean of each data set. Data representative of three independent experiments. AT₁R, angiotensin II receptor type 1; B2R, bradykinin receptor type 2.

these parameters resulting from both receptors being stabilized in active conformations instead of just one. For BRET¹ with Rluc-YFP this would imply a donor-acceptor distance of just 5 nm.³³ Either way, this provides good evidence for specific reporting of β -arrestin 2 recruitment to the heteromer complex.

To further illustrate the specificity of the assay, we have also provided data for another well characterized, but controversial heteromer involving AT₁R and B2R^{20–26} that did not result in a signal under the conditions tested. As we have demonstrated the ability of BRET to monitor Ang II-induced interactions between AT₁R and

β -arrestins previously,²⁸ the lack of Ang II-induced BRET signal observed with B2R/Rluc8, β -arrestin 2/Venus, and AT₁R is therefore consistent with any of three scenarios: (1) AT₁R and B2R do not interact as a heteromer in HEK293FT cells under these experimental conditions (consistent with suggestions by Hansen and co-workers);²⁶ (2) the AT₁R-B2R heteromer exhibits pharmacology distinct from that observed at either constituent monomer/homomer, namely, that this heteromer does not interact with β -arrestin 2 in a ligand-dependent manner to a detectable level; (3) formation of the B2R/Rluc8-AT₁R- β -arrestin 2/Venus complex is not detected due to the high sensitivity of BRET to relative donor-acceptor distance and orientation, despite receptor expression and functionality being demonstrated. Importantly, all of these explanations support the specificity of the assay. Were the assay not specific, recruitment of β -arrestin 2/Venus to the plasma membrane by activation of untagged AT₁R would be expected to result in a BRET signal with the B2R/Rluc8 already at the plasma membrane.

Finally, in addition to profiling heteromers, our methodology has the potential for screening of heteromers in a cell-based assay system as demonstrated by the Z' data. This is not native tissue; however, it is far better than screening GPCRs in isolation as is the current norm.^{4,7} Screening of the heteromer complex will provide primary hits for drugs acting on one or both of the protomers. Secondary screening of the constituent GPCR protomers (as monomers or homomers), interacting with

β -arrestin in the absence of the other protomer, will then allow subtraction of compounds interacting with the protomers without being affected by heteromerization. This will leave a pool of heteromer-selective or heteromer-biased hits for further development and validation. The complexity resulting from GPCR heteromerization provides exciting opportunities for developing new classes of improved pharmaceuticals. Consequently, we believe that the GPCR-HIT approach outlined in this study has the potential to contribute to this new frontier in the development of GPCR therapeutics.

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DISCLOSURE STATEMENT

In addition to being Head of the Laboratory for Molecular Endocrinology-GPCRs, Western Australian Institute for Medical Research and Centre for Medical Research, University of Western Australia, K.D.G.P. is Chief Scientific Officer of Dimerix Bioscience Pty Ltd, a spin-out company of the University of Western Australia that has been assigned the rights to the "GPCR-HIT" technology. K.A.E. and K.D.G.P. have minor shareholdings in Dimerix.

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