



Novel Pharmacology Following Heteromerization of the Angiotensin II Type 2 Receptor and the Bradykinin Type 2 Receptor

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The angiotensin type 2 (AT₂) receptor and the bradykinin type 2 (B₂) receptor are G protein-coupled receptors (GPCRs) that have major roles in the cardiovascular system. The two receptors are known to functionally interact at various levels, and there is some evidence that the observed crosstalk may occur as a result of heteromerization. We investigated evidence for heteromerization of the AT₂ receptor and the B₂ receptor in HEK293FT cells using various bioluminescence resonance energy transfer (BRET)-proximity based assays, including the Receptor Heteromer Investigation Technology (Receptor-HIT) and the NanoBRET ligand-binding assay. The Receptor-HIT assay showed that G α_q , GRK2 and β -arrestin2 recruitment proximal to AT₂ receptors only occurred upon B₂ receptor coexpression and activation, all of which is indicative of AT₂-B₂ receptor heteromerization. Additionally, we also observed specific coupling of the B₂ receptor with the G α_z protein, and this was found only in cells coexpressing both receptors and stimulated with bradykinin. The recruitment of G α_z , G α_q , GRK2 and β -arrestin2 was inhibited by B₂ receptor but not AT₂ receptor antagonism, indicating the importance of B₂ receptor activation within AT₂-B₂ heteromers. The close proximity between the AT₂ receptor and B₂ receptor at the cell surface was also demonstrated with the NanoBRET ligand-binding assay. Together, our data demonstrate functional interaction between the AT₂ receptor and B₂ receptor in HEK293FT cells, resulting in novel pharmacology for both receptors with regard to G α_q /GRK2/ β -arrestin2 recruitment (AT₂ receptor) and G α_z protein coupling (B₂ receptor). Our study has revealed a new mechanism for the enigmatic and poorly characterized AT₂ receptor to be functionally active within cells, further illustrating the role of heteromerization in the diversity of GPCR pharmacology and signaling.

Keywords: angiotensin receptor, bradykinin receptor, GPCR, BRET, receptor-HIT, heteromer, NanoBRET

INTRODUCTION

Angiotensin II (AngII) and bradykinin (BK) are two peptide hormones that have major regulatory roles in the cardiovascular system. AngII exerts its effects through two G protein-coupled receptors (GPCRs), the AngII type 1 (AT₁) and the AngII type 2 (AT₂) receptors, while BK exerts most of its cardiovascular effects through the BK type 2 (B₂) GPCR. While the AT₁ receptor mediates most of the classical actions of AngII, such as vasoconstriction, antinatriuresis, cell proliferation and hypertrophy (1), the effects of the AT₂ receptor are less well characterized, and its molecular pharmacology and physiological functions remain to be fully elucidated (2, 3). Through the B₂ receptor, BK mediates vasodilation that antagonizes the classical AngII vasoconstriction.

Although GPCRs are able to act as single, monomeric units, it is also believed that they can form homomeric or heteromeric complexes that may result in altered signaling. In particular, GPCR heteromers have been a major focus of research in GPCR pharmacology over the past decade. This has led to the characterization of numerous GPCR heteromers, including the AT₁-AT₂ heteromer (4–11), and also the controversial AT₁-B₂ heteromer (12–19). As yet, a functional heteromer between the AT₂ and the B₂ receptor has not been categorically demonstrated, however there are numerous examples of crosstalk between the two receptors. One of the least contentious aspects of AT₂ receptor functioning is its action as a vasodilator. AT₂ receptor-mediated vasodilation has been shown to occur *via* several signaling pathways, including the same nitric oxide (NO)/cyclic 3'-5' guanosine

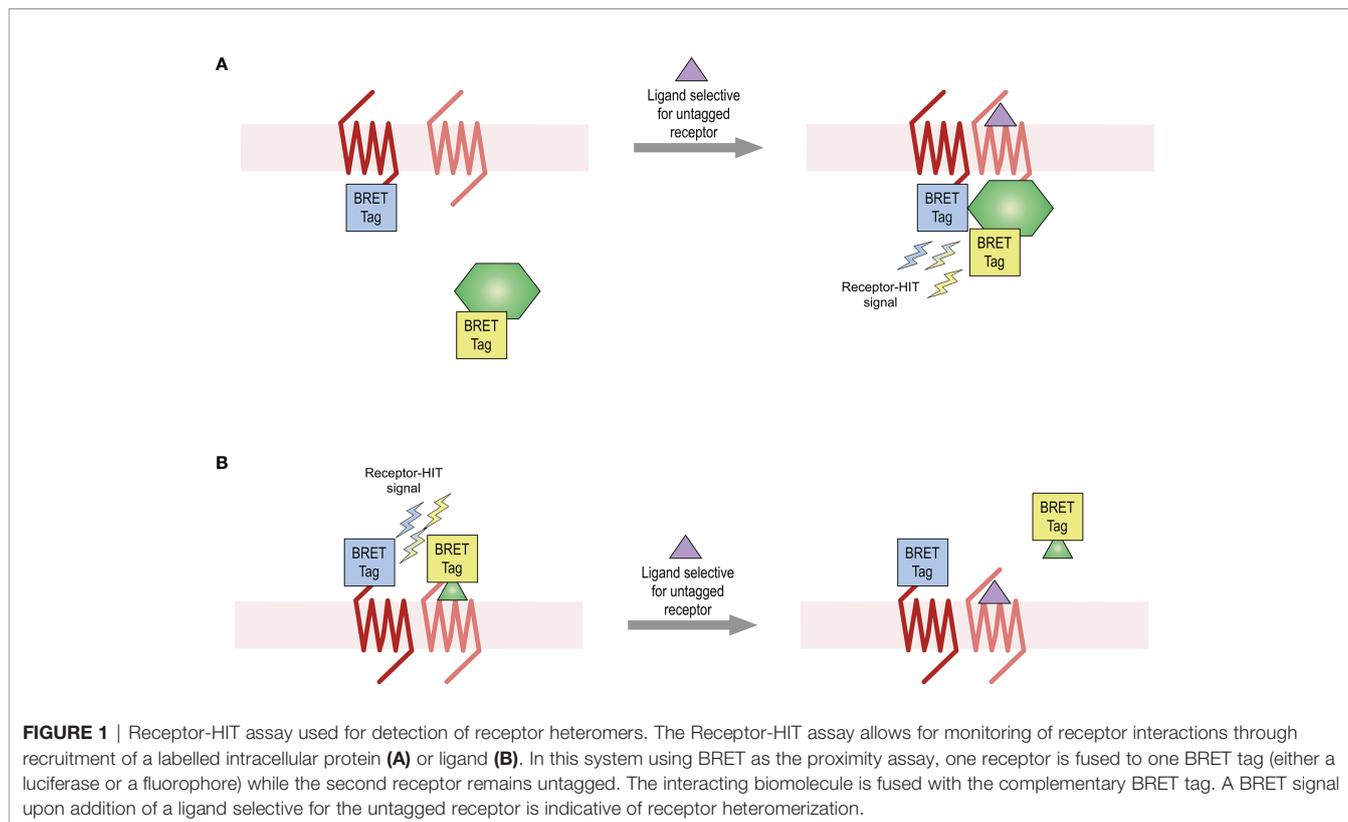
monophosphate (cGMP) pathway involved in B₂ receptor-mediated vasodilation (20). Furthermore, numerous studies have shown that BK is involved in AT₂ receptor-mediated NO/cGMP vasodilation (21–23). Confocal fluorescence resonance energy transfer studies have shown the distance between the two receptors in PC12W cell membranes to be 50 ± 5 Å, suggesting that the observed functional interactions may be a result of heteromerization between the AT₂ receptor and the B₂ receptor (24).

This study aimed to provide further evidence for the existence of the AT₂-B₂ heteromer in HEK293FT cells, using various bioluminescence resonance energy transfer (BRET)-based proximity assays including the Receptor-Heteromer Investigation Technology (Receptor-HIT) (25, 26) and the NanoBRET ligand binding assay (27, 28). Receptor-HIT, which has most commonly been applied to GPCRs (GPCR-HIT) (5, 25, 27, 29–32), is an assay that enables detection and characterization of heteromers through ligand-dependent interaction with biomolecules (**Figure 1**). Using various BRET assays, this study provided evidence for the existence of the AT₂-B₂ heteromer in our system and also revealed novel pharmacology obtained by the receptors upon heteromerization.

MATERIALS AND METHODS

cDNA Constructs and Ligands

All receptor constructs are human unless otherwise specified. AT₂-Rluc8 (rat) and B₂-Rluc8 cDNA constructs were generated from plasmids containing the respective receptor cDNA tagged



with Rluc. The Rluc coding region was replaced with Rluc8 cDNA from pcDNA3.1-Rluc8 kindly provided by Andreas Loening and Sanjiv Gambhir (Stanford University, CA) (33), as described previously for other constructs (34). AT₂-Rluc (rat), AT₂-Venus (rat) (5) and HA-AT₂ (rat; referred to as AT₂ in the BRET¹ and eBRET assays) were kindly provided by Walter Thomas (University of Queensland). B₂ and HA-B₂ (referred to from here-on-in as B₂) and EP₃ cDNA was obtained from the Missouri S&T cDNA Resource Center (www.cdna.org). B₂-Rluc was previously produced by PCR amplification of B₂ cDNA to remove the stop codon and ligation into pcDNA₃ containing Rluc. B₂-Venus was generated by replacing the Rluc8 coding region from B₂-Rluc8 with Venus cDNA. NES-Venus-mGsq was kindly provided by Nevin Lambert (Augusta University, Augusta, Georgia). G α_q -Rluc8, G α_{i3} -Rluc8 and G β_3 were from the TRUPATH kit, which was a gift from Bryan Roth (Addgene kit #1000000163), with Venus-G γ_6 being generated from GFP2-G γ_6 , also from the TRUPATH kit (35). G α_z -Rluc8 was kindly provided by Martina Kocan (The Florey Institute of Neuroscience and Mental Health). GRK2-Rluc8 was synthesized by GeneArt (ThermoFisher Scientific, Regensburg, Germany). The β -arrestin2-Venus cDNA construct was prepared previously from pCS2-Venus kindly provided by Atsushi Miyawaki (RIKEN Brain Science Institute, Wako-city, Japan) (34). Signal peptide and flag-tagged AT₂ (referred to as AT₂ in the NanoBRET ligand binding assays) and Nluc-AT₂ were generated previously (27). Nluc-B₂ was generated by replacing the AT₂ coding region from Nluc-AT₂ with B₂ cDNA. Ligands used were AngII, BK and PGE₂ (Sigma), icatibant and PD 123319 (Tocris Bioscience) and TAMRA-AngII (AnaSpec).

Cell Culture and Transfection

HEK293FT cells were maintained at 37°C, 5% CO₂ in complete medium (Dulbecco's modified Eagle's medium (DMEM) containing 0.3 mg/ml glutamine, 100 IU/ml penicillin, and 100 μ g/ml streptomycin) supplemented with 10% fetal calf serum (FCS) (GIBCO BRL, Carlsbad, CA). Transient transfections were carried using either GeneJuice (Merck, Kilsyth, Australia) or FuGENE (Promega) according to manufacturer's instructions. All assays were carried out 48 hours post transfection.

Receptor-HIT

Receptor-HIT is an assay that enables the identification and pharmacological profiling of receptor heteromers in live cell systems. The assay uses a proximity-based reporter system such as BRET to enable detection of heteromers through their ligand-dependent interaction with proteins or ligands (5, 25, 27, 29–32). The Receptor-HIT assay comprises three elements (**Figure 1**), which in these studies on the BRET platform are a BRET-tagged receptor, an untagged receptor, and a BRET-tagged interacting protein (**Figure 1A**) or a BRET-tagged interacting ligand [**Figure 1B** (27)]. If a change in BRET signal occurs upon addition of a ligand that is selective for the untagged receptor, this indicates proximity between the tagged receptor and the tagged interacting biomolecule. This Receptor-HIT signal signifies the close proximity of the two receptors, and is indicative of receptor heteromerization.

BRET¹ and eBRET Assays

HEK293FT cells were transfected with cDNA as described in figure legends. BRET¹ and eBRET assays used rat AT₂ constructs. For all BRET¹ assays (with the exception of **Figures 6E, F**), 5 μ M coelenterazine *h* (Promega) was added and basal BRET was measured for 10–20 mins before adding agonist or vehicle and then continuing to measure BRET. Antagonist assays had a pretreatment of antagonist or vehicle (30 min) prior to addition of coelenterazine *h*. For the BRET¹ assays in **Figures 6E, F**, cells were pretreated for 30 min with agonist or vehicle, with cells in **Figure 6F** having an additional pretreatment of antagonist or vehicle (30 min) prior to treatment with agonist. Following pretreatment, coelenterazine *h* was added to a final concentration of 5 μ M and BRET was measured immediately. For eBRET assays, cells were incubated at 37°C, 5% CO₂ for 2 hours with 30 μ M EnduRen (Promega) to ensure substrate equilibrium was reached. Basal BRET was measured for 10–20 mins before adding agonist or vehicle and then continuing to measure BRET. Antagonist assays had a pretreatment of antagonist or vehicle (30 min) prior to addition of coelenterazine *h*. All BRET¹ and eBRET measurements were taken at 37°C using either a LUMIstar Omega plate reader (BMG Labtech, Mornington, Victoria, Australia) with 460–490 nm and 520–550 nm filters; a CLARIOstar plate reader (BMG Labtech) with 420–480 nm and 520–620 nm filters; or a VICTOR Light plate reader (Perkin Elmer) with 400–475 nm and 520–540 nm filters. The ligand-induced BRET signal was calculated by subtracting the ratio of the long wavelength emission over the short wavelength 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 (34, 36). In this calculation, the vehicle-treated cell sample represents the background, eliminating the requirement for measuring a donor-only control sample (34, 36). For BRET kinetic assays, the final pretreatment reading is presented at the zero time point (time of agonist/vehicle addition).

NanoBRET Assays

HEK293FT cells were transfected with cDNA as described in figure legends. NanoBRET assays used human AT₂ constructs. For NanoBRET assays, cells were pretreated for 30 min with PD 123319 and then TAMRA-AngII was added (final concentration of 1 μ M). Following another 30 min incubation, furimazine was added and BRET measured immediately at 37°C using a PHERAstar FS plate reader (BMG Labtech) with 420–500 nm and 610-LP filters or a LUMIstar Omega plate reader (BMG Labtech) with 410–490 nm and 610-LP filters. The BRET signal was calculated by subtracting the ratio of the long wavelength emission over the short wavelength emission and the data were normalized as percentage of TAMRA-AngII binding.

IP₁ Accumulation Assays

Measurement of IP₁ accumulation was performed using the IP-One Tb kit (Cisbio Bioassays) according to manufacturer's instructions. Cells were treated for 30 minutes at 37°C with

agonists or vehicle. Antagonist assays had an additional pretreatment with antagonist or vehicle for 30 mins at 37°C, which was removed prior to treatment with agonist. The cells were then lysed by adding the supplied assay reagents, and the assay was incubated for 1 hour at room temperature. Fluorescence was measured at 620 nm and 665 nm 50 μs after excitation at 340 nm using the EnVision 2102 multilabel plate reader (PerkinElmer).

Data Presentation and Statistical Analysis

Data were presented and analyzed using Prism 9 software (GraphPad). Competition binding data and concentration-response data were fitted using logarithmic nonlinear regression (three parameter). Unpaired *t*-tests, one-way ANOVAs and two-way ANOVAs were used to determine statistical significance where appropriate (**p* < 0.05).

RESULTS

Gα_q Coupling to the AT₂-B₂ Heteromer

Following activation by an agonist, GPCRs typically interact with and activate heterotrimeric G proteins to initiate intracellular signaling cascades. The B₂ receptor primarily couples to the Gα_q class of G proteins (37) while the AT₂ receptor is an unusual GPCR in that it does not readily couple to any G proteins (38). To investigate Gα_q coupling by the receptors, we used a Venus-tagged mini G (mG) protein construct that comprises an engineered GTPase domain of the Gα_s protein that has been modified to confer Gα_q specificity (NES-Venus-mG_{sq}). As expected, no ligand-induced recruitment of NES-Venus-mG_{sq} to the Rluc8-tagged AT₂ receptor (AT₂-Rluc8) was observed (Figure 2A). In contrast, and also as expected, coexpression of NES-Venus-mG_{sq} with the B₂ receptor tagged with Rluc8 (B₂-Rluc8) resulted in a BK-induced BRET signal (Figure 2B) indicative of recruitment of Gα_q to the receptor.

We then investigated Gα_q coupling using the Receptor-HIT assay, again using NES-Venus-mG_{sq}. Receptor-HIT uses a proximity-based reporter system such as BRET to enable detection and characterization of heteromers through their ligand-dependent interactions with labelled proteins or ligands (5, 25, 29–32) (Figure 1). Upon coexpression of the unlabeled B₂ receptor in cells expressing AT₂-Rluc8 and NES-Venus-mG_{sq} we now observed a BK-induced BRET signal (Figure 2C), indicating recruitment of NES-Venus-mG_{sq} proximal to AT₂-Rluc8. This Receptor-HIT signal indicates the close proximity of the AT₂ receptor and the B₂ receptor, and suggests their interaction within a heteromeric complex. Coexpression of untagged AT₂ receptor to cells expressing B₂-Rluc8 and NES-Venus-mG_{sq} did not alter the BRET signal (Figure 2D) from that seen without AT₂ expression (Figure 2B).

We investigated the mG_{sq} Receptor-HIT signal further by conducting concentration-response analysis. Figure 2E shows that there is no change in potency of mG_{sq} coupling to AT₂-B₂ heteromers compared to B₂ receptors (pEC₅₀ ± SEM = 7.94 ± 0.29 vs. 7.73 ± 0.19, respectively; *p* > 0.05, unpaired *t*-test). When we conducted the mG_{sq} Receptor-HIT assay in the presence of

selective antagonists (Figure 2F), we saw that the AT₂ receptor antagonist PD 123319 did not inhibit coupling of mG_{sq} to AT₂-B₂ heteromers. In contrast, the putative B₂ receptor antagonist icatibant was able to significantly reduce the level of mG_{sq} recruitment, indicating the requirement of B₂ receptor activation for Gα_q coupling.

Finally, we investigated the specificity of the Receptor-HIT signal by conducting a similar experiment but instead using a GPCR not known to heteromerize with the AT₂ receptor, the prostaglandin E receptor 3 (EP₃ receptor). Here we found that only coexpression and activation of the B₂ receptor resulted in a Receptor-HIT signal between AT₂-Rluc8 and NES-Venus-mG_{sq} (Figure 2G). No signal was observed when EP₃ was coexpressed with AT₂-Rluc8 and NES-Venus-mG_{sq} and treated with PGE₂ (Figure 2G), despite both B₂ and EP₃ being expressed within the cells, as shown by their activation of G protein (Figure 2H; Gα_q for B₂, and Gα_{i3} for EP₃).

Activation of the IP₁ Signaling Pathway

Gα_q activation initiates a signaling cascade that leads to inositol phosphate signaling, which can be monitored by measuring the accumulation of the metabolite IP₁. Using an IP₁ assay and aliquots of transfected cells also used in the β-arrestin2 assays described below, we next investigated downstream Gα_q signaling mediated by the receptors. As expected, we found that AngII did not induce IP₁ production in cells expressing AT₂/Rluc8 (Figure 3A). However, coexpression of the B₂ receptor resulted in robust BK-induced IP₁ production (Figure 3A). When we conducted concentration-response analysis, we found that there was no significant difference in the potency of IP₁ production between cells expressing just the B₂ receptor and cells expressing the B₂ receptor and the AT₂ receptor (Figure 3B; pEC₅₀ ± SEM = 8.55 ± 0.20 vs. 8.45 ± 0.04, respectively; *p* > 0.05, unpaired *t*-test), just as we saw no difference in potency of mG_{sq} recruitment in the BRET assay. When we conducted these IP₁ assays with an antagonist pretreatment, we found that 10 μM of the AT₂ receptor antagonist PD 123319 had no inhibitory effect on 0.1 μM BK-induced IP₁ production (Figure 3C). Interestingly, in this assay 10 μM of the putative B₂ selective antagonist icatibant was also unable to inhibit 0.1 μM BK-induced IP₁ production. Indeed, it acted as a partial agonist in this assay, as can be seen by the substantial IP₁ production in cells treated only with icatibant and no BK. Further analysis illustrated the concentration-dependent effect of IP₁ production mediated by icatibant (Figure 3D). This concentration-response analysis also showed that high concentrations of icatibant were in fact able to inhibit BK-induced IP₁ production. However, the potency of this effect was shifted substantially to the right of its inhibitory actions on BK-induced β-arrestin2 recruitment. These findings support reports of the partial agonism of icatibant, which has previously been observed mediating IP₁ production through the B₂ receptor (39).

Gα_z Recruitment to the AT₂-B₂ Heteromer

We next investigated Gα_z protein recruitment to the receptors, using Gα_z tagged with Rluc8 (Gα_z-Rluc8). As Gα_z is not a known signaling partner for either the AT₂ receptor or the B₂

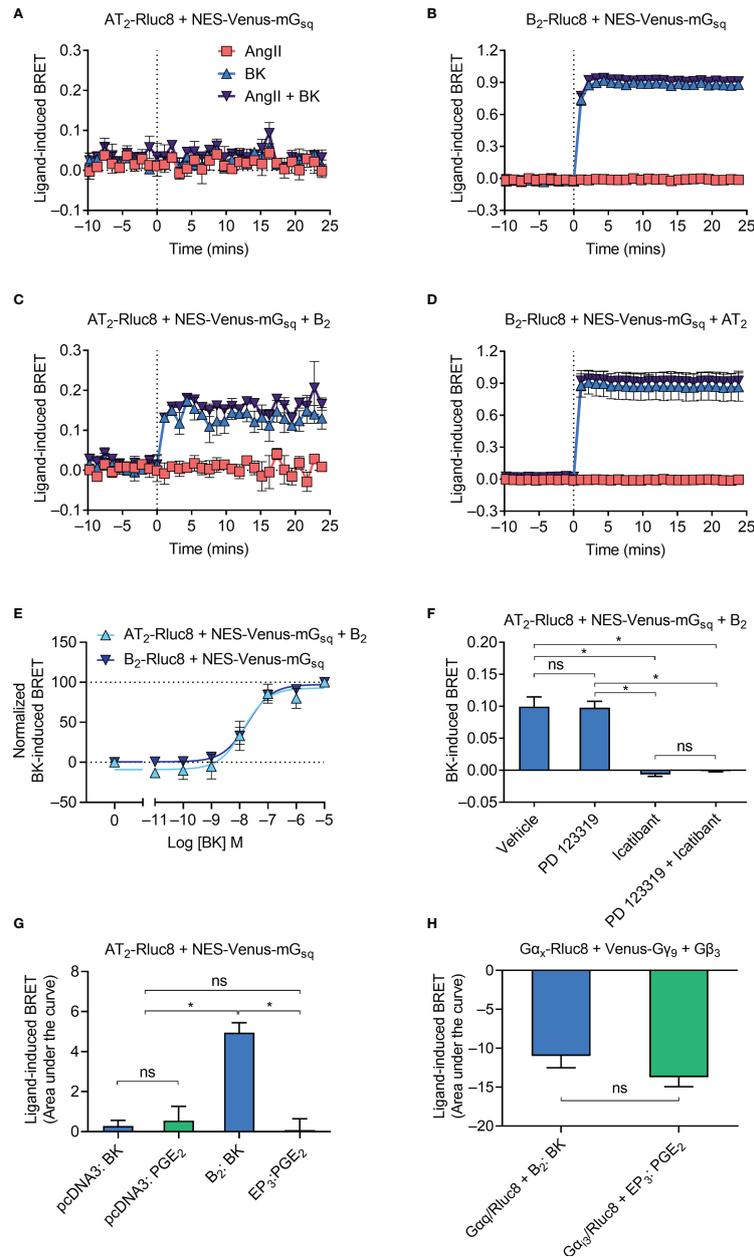


FIGURE 2 | $G\alpha_q$ recruitment to the AT₂-B₂ heteromer. HEK293FT cells were transfected with plasmid cDNA as described on graphs. **(A–D)** Time course analysis showing recruitment of NES-Venus-mG_{sq} to receptors following addition of ligands at 0 mins. **(E)** BK concentration-response analysis showing recruitment of NES-Venus-mG_{sq} to receptors. Normalized data taken from BRET assays at 17 min after agonist addition. **(F)** NES-Venus-mG_{sq} Receptor-HIT assay in the presence of 50 μ M antagonists (or vehicle) and 0.1 μ M BK. Data are from BRET assays at 17 min after agonist addition. * $p < 0.05$; ns, not significant (one-way ANOVA with Tukey's multiple comparisons test). **(G)** Area under the curve ligand-induced BRET data. * $p < 0.05$; ns, not significant (one-way ANOVA with Tukey's multiple comparisons test). **(H)** Area under the curve ligand-induced BRET data. * $p < 0.05$; ns, not significant (unpaired t -test). All data are presented as mean \pm SEM of \geq three independent experiments performed in triplicate.

receptor, we did not expect to observe any recruitment, and this was confirmed in our BRET assay expressing either Venus-tagged receptor and $G\alpha_z$ -Rluc8 (**Figures 4A, B**). Coexpression of the untagged B₂ receptor did not alter the BRET signal between AT₂-Venus and $G\alpha_z$ -Rluc8 (**Figure 4C**), however, coexpression of the untagged AT₂ receptor interestingly

resulted in a marked decrease in the BRET signal between B₂-Venus and $G\alpha_z$ -Rluc8 upon treatment with BK (**Figure 4D**). A decrease in the BRET signal suggests that there is a preformed complex between B₂-Venus and $G\alpha_z$ -Rluc8, which either disassociates or undergoes conformational rearrangement that increases the distance between the two BRET tags (40–42).

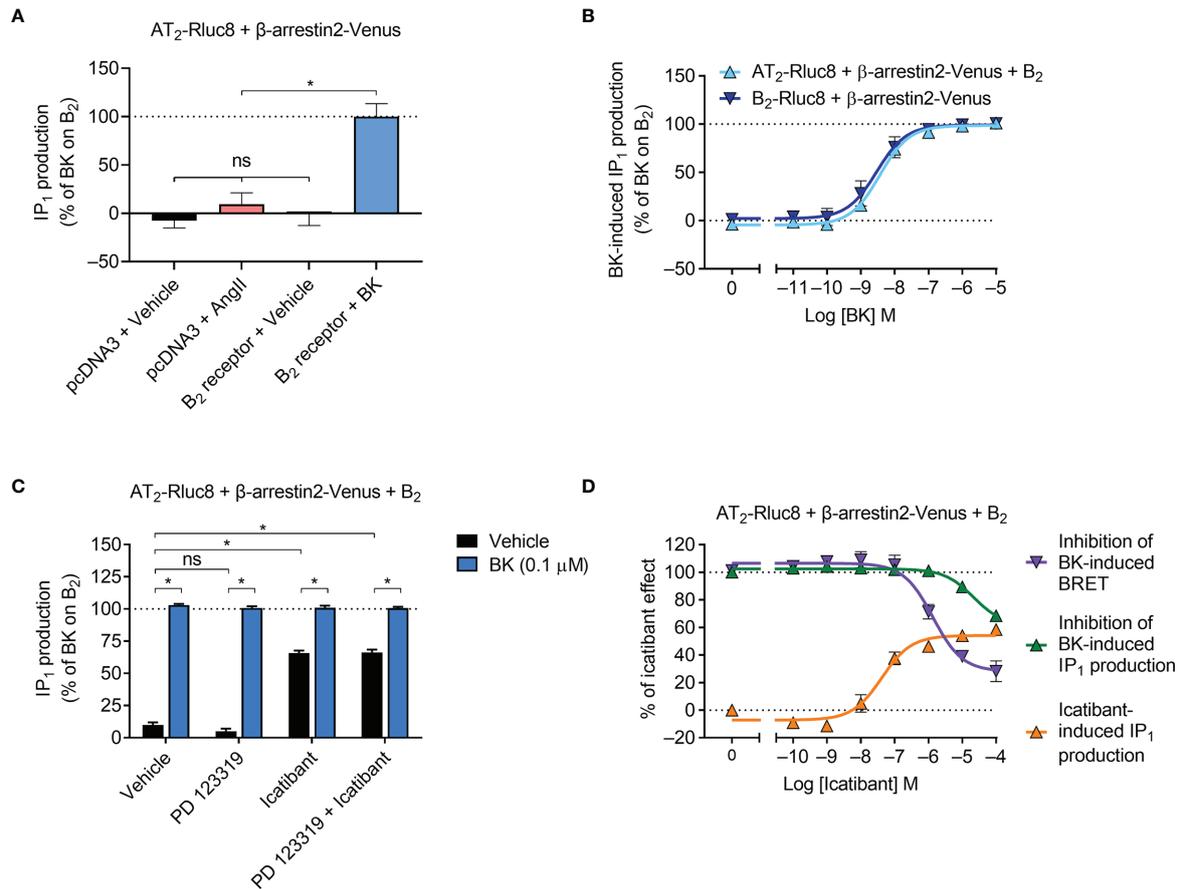


FIGURE 3 | IP₁ signaling by the AT₂-B₂ heteromer. HEK293FT cells were transfected with plasmid cDNA as described on graphs. **(A)** Ligand-induced IP₁ signaling in cells expressing AT₂-Rluc8, with or without the B₂ receptor. **p* < 0.05; ns, not significant (one-way ANOVA with Tukey's multiple comparisons test). **(B)** Concentration-response analysis showing BK-induced IP₁ signaling. **(C)** IP₁ assay in the presence of 10 μ M antagonists (or vehicle) and 0.1 μ M BK. **p* < 0.05 (two-way ANOVA with Sidak's multiple comparisons test). **(D)** Concentration-response analysis comparing agonistic and antagonistic actions of icatibant, the latter antagonizing 0.1 μ M BK. All data are presented as mean \pm SEM of \geq three independent experiments performed in duplicate (IP₁ assay) or triplicate (BRET).

In either case, this BRET signal provides further evidence in support of the existence of a functional AT₂-B₂ heteromer, and illustrates completely novel pharmacology it has adopted.

We also investigated the concentration-dependence of the G α_z BRET signal (**Figure 4E**) and found a similar potency of BK-induced concentration-dependence as observed for mG_{sq} coupling (pEC₅₀ \pm SEM = 7.94 \pm 0.29, unpaired *t*-test). Likewise, when we conducted the assay in the presence of selective antagonists, we again found that the G α_z BRET signal could be blocked by B₂ receptor inhibition (icatibant), but not AT₂ inhibition (PD 123319) (**Figure 4F**).

GPCR Kinase 2 Recruitment to the AT₂-B₂ Heteromer

Following agonist stimulation, GPCR kinases (GRKs) are rapidly recruited to GPCRs, where they phosphorylate the receptor's C terminal tail. This initiates receptor desensitization and interaction with β -arrestin proteins. We investigated GRK recruitment using BRET with Rluc8-tagged GRK2 (GRK2-

Rluc8) and Venus-tagged receptors. There was no ligand-induced recruitment of GRK2-Rluc8 to the Venus-tagged AT₂ receptor (AT₂-Venus; **Figure 5A**). This lack of GRK2 recruitment is expected, as it is well known that the AT₂ receptor does not recruit β -arrestin or internalize upon stimulation with AngII (43–45), and therefore it is unlikely it would recruit GRKs. In contrast, but also as expected, when cells expressing GRK2-Rluc8 and Venus-tagged B₂ receptor (B₂-Venus) were treated with BK (but not AngII) we saw an immediate increase in the BRET signal, indicating rapid recruitment of GRK2 to the B₂ receptor (**Figure 5B**).

When we coexpressed untagged B₂ receptor in cells expressing AT₂-Venus and GRK2-Rluc8, we saw BK-induced recruitment of GRK2 proximal to the AT₂ receptor (**Figure 5C**). Interestingly, this BRET signal had a much more sustained signal than that observed between GRK2-Rluc8 and B₂-Venus (**Figure 5B**), which declined steadily over time. As with the mG_{sq} Receptor-HIT assay, this Receptor-HIT signal indicates the close proximity of the AT₂ receptor and the B₂ receptor, and

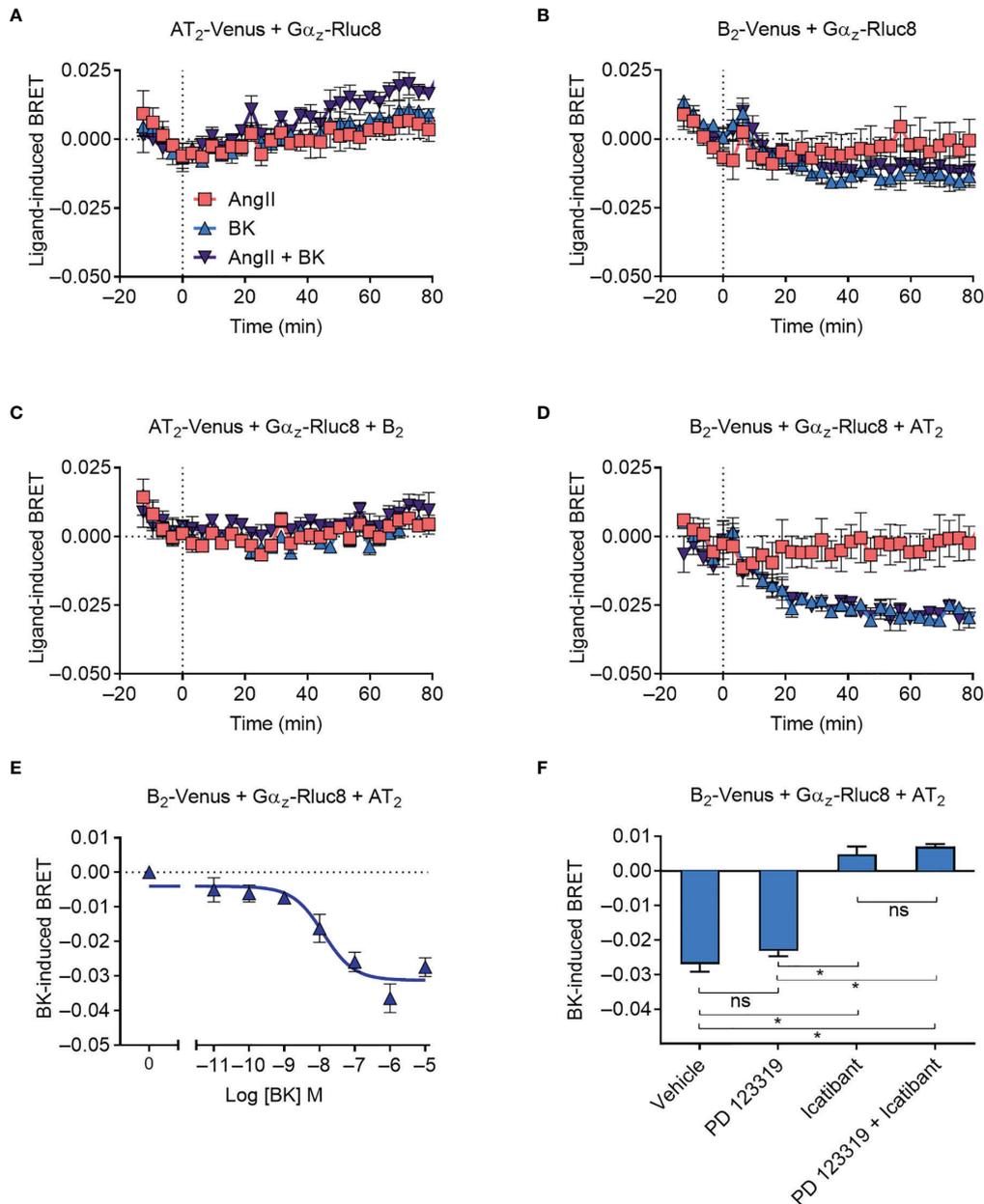


FIGURE 4 | Gα₂ recruitment to the AT₂-B₂ heteromer. HEK293FT cells were transfected with plasmid cDNA as described on graphs. **(A–D)** Time course analysis showing interaction of Gα₂-Rluc8 with receptors following addition of ligands at 0 mins. **(E)** BK concentration-response analysis showing recruitment of Gα₂-Rluc8 to B₂ receptors. Data taken from BRET assays at 60 min after agonist addition. **(F)** Gα₂-Rluc8 Receptor-HIT assay in the presence of 10 μM antagonists and 0.1 μM BK. Data taken from BRET assays at 30 min after agonist addition. *p < 0.05; ns, not significant (one-way ANOVA with Tukey's multiple comparisons test). All data are presented as mean ± SEM of ≥ three independent experiments performed in triplicate.

suggests their interaction within a heteromeric complex. Coexpression of untagged AT₂ receptor did not alter the BRET signal between B₂-Venus and GRK2-Rluc8 (**Figure 5D**) from that seen without AT₂ expression (**Figure 5B**).

We also investigated the GRK2 Receptor-HIT signal further by conducting concentration-response analysis. **Figure 5E** shows that there is a significant leftward shift in the potency of GRK2 recruitment to AT₂-B₂ heteromers compared to B₂ receptors

(pEC₅₀ ± SEM = 8.07 ± 008 vs. 7.35 ± 0.06, respectively; p < 0.05, unpaired *t*-test). When we conducted the GRK2 Receptor-HIT assay in the presence of selective antagonists (**Figure 5F**) we saw, as in the mG_{sq} and Gα₂ Receptor-HIT assays, that the AT₂ receptor antagonism did not inhibit the recruitment of GRK2 to AT₂-B₂ heteromers, while B₂ receptor antagonism significantly reduced the level of GRK2 recruitment, indicating the specificity of the BRET signals.

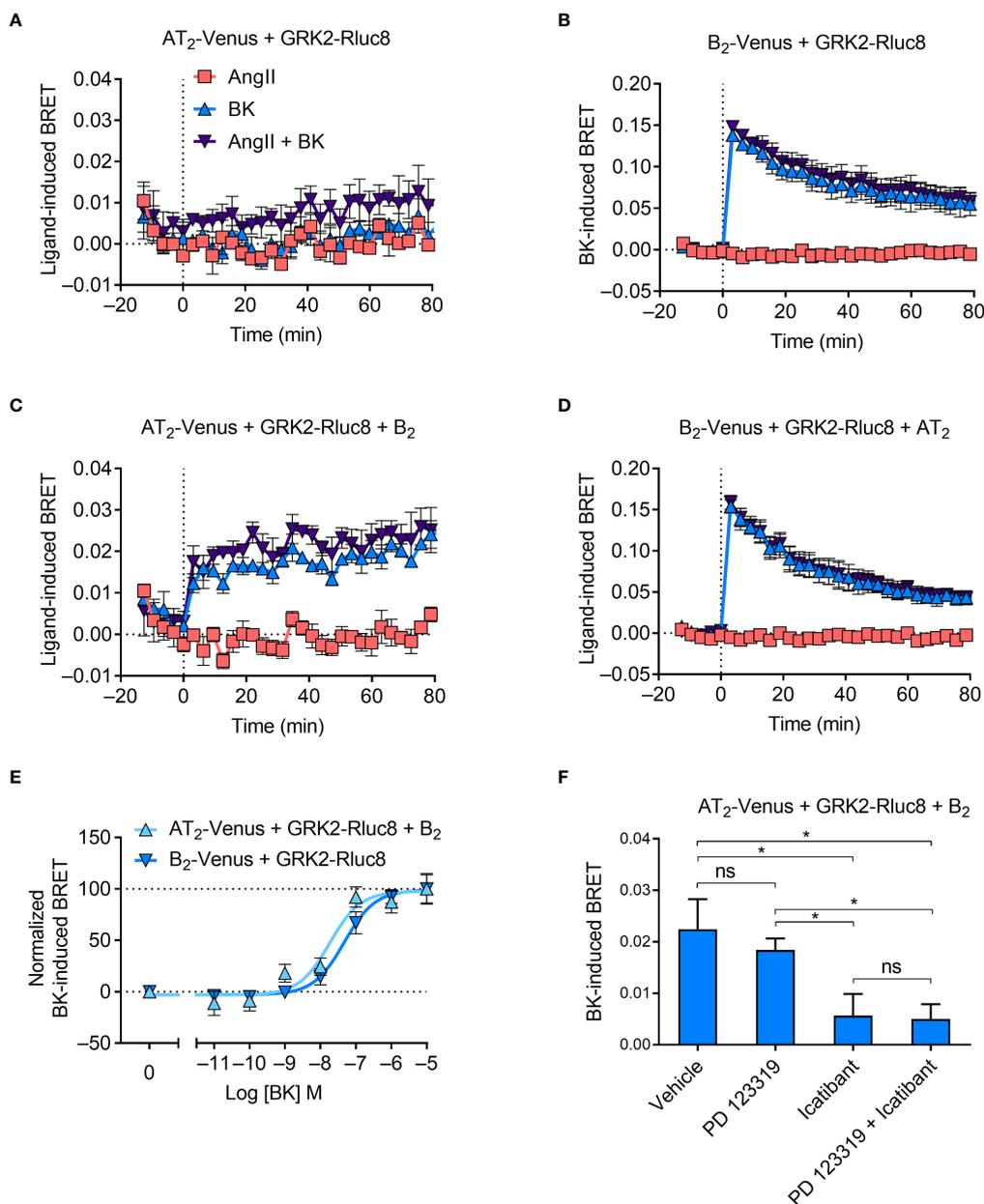


FIGURE 5 | GRK2 recruitment to the AT₂-B₂ heteromer. HEK293FT cells were transfected with plasmid cDNA as described on graphs. **(A–D)** Time course analysis showing recruitment of GRK2-Rluc8 to receptors following addition of ligands at 0 mins. **(E)** Concentration-response analysis showing recruitment of GRK2-Rluc8 to receptors. Normalized data taken from BRET assays at 10 min after agonist addition. **(F)** GRK2-Rluc8 Receptor-HIT assay in the presence of 50 μ M antagonists and 1 μ M BK. Data are from BRET assays at 20 min after agonist addition. * $p < 0.05$; ns, not significant (one-way ANOVA with Tukey's multiple comparisons test). All data are presented as mean \pm SEM of \geq three independent experiments performed in triplicate.

β -arrestin2 Recruitment to the AT₂-B₂ Heteromer

Following GRK recruitment and subsequent receptor phosphorylation, GPCRs recruit the scaffold protein β -arrestin, which desensitizes the receptor from classical cell surface G protein signaling and initiates internalization (46). Individual GPCRs have different β -arrestin recruitment profiles resulting in unique

desensitization and internalization characteristics. Upon treatment with BK, the B₂ receptor rapidly recruits β -arrestin leading to swift desensitization and extensive internalization (47, 48). In contrast and as already mentioned, the AT₂ receptor does not recruit β -arrestin or internalize upon stimulation with AngII (43–45).

As expected, there was no ligand-induced recruitment of β -arrestin2-Venus to AT₂-Rluc8 (**Figure 6A**), whereas when we

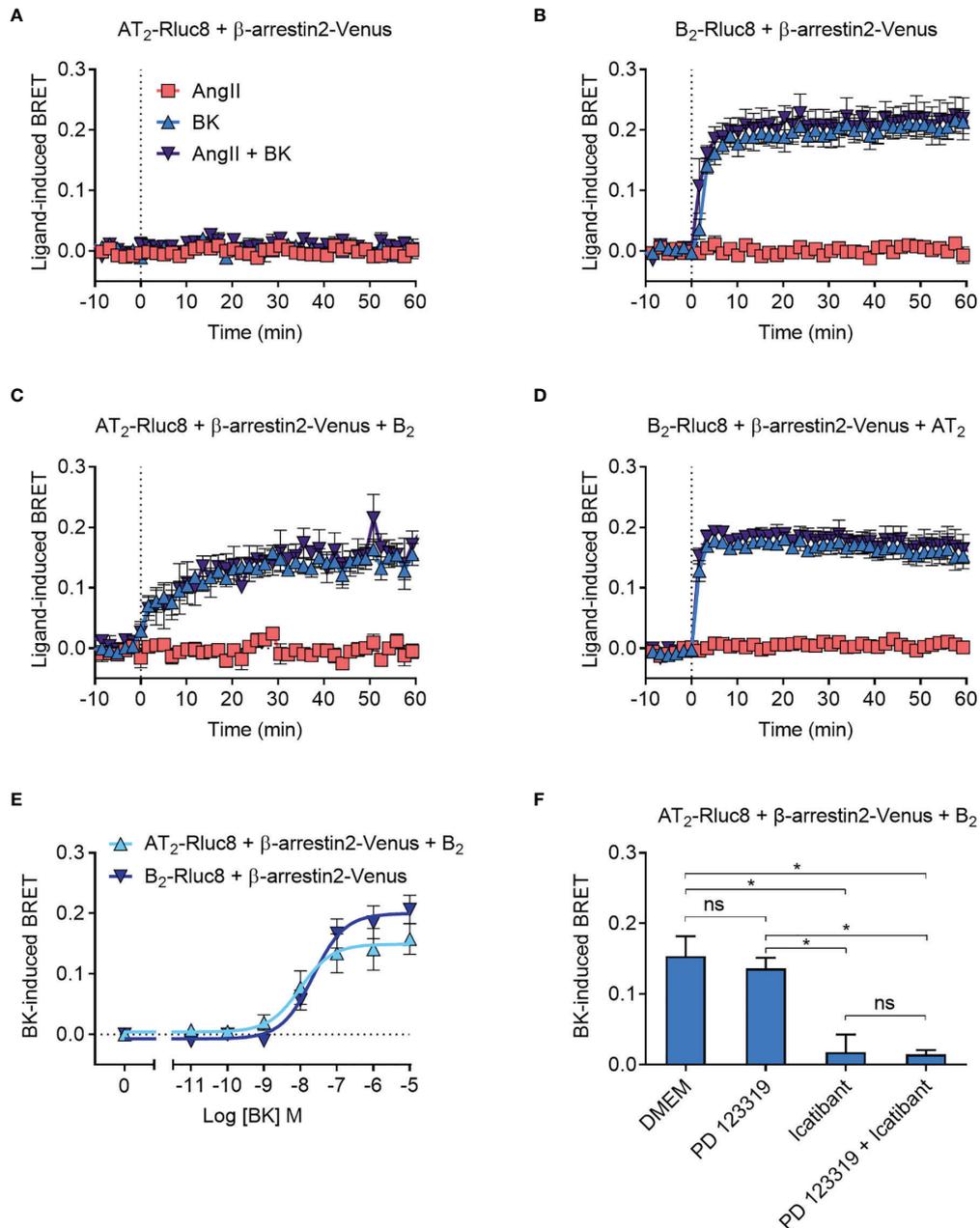


FIGURE 6 | β-arrestin2-Venus recruitment to the AT₂-B₂ heteromer. HEK293FT cells were transfected with plasmid cDNA as described on graphs. **(A–D)** Time course analysis showing recruitment of β-arrestin2-Venus to receptors following addition of ligands at 0 mins. **(E)** Concentration-response analysis showing recruitment of β-arrestin2-Venus to receptors. Data taken from BRET assays at 40 min after agonist addition. **(F)** β-arrestin2-Venus Receptor-HIT assay in the presence of 10 μM antagonists and 0.1 μM BK. Data taken from BRET assays at 40 min after agonist addition. *p < 0.05; ns, not significant (one-way ANOVA with Tukey's multiple comparisons test). All data are presented as mean ± SEM of ≥ three independent experiments performed in triplicate.

coexpressed B₂-Rluc8 with β-arrestin2 tagged with Venus (β-arrestin2-Venus) we observed strong and rapid BK-induced recruitment of β-arrestin2-Venus to B₂-Rluc8 (**Figure 6B**). When AT₂-Rluc8 was co-expressed with the untagged B₂ receptor in the Receptor-HIT configuration, there was a marked increase in ligand-induced BRET when the cells were treated with BK but not AngII (**Figure 6C**), indicating BK-

dependent translocation of β-arrestin2-Venus proximal to the B₂ receptor. This BRET signal between AT₂-Rluc8 and β-arrestin2-Venus confirms the close proximity of AT₂-Rluc8 and the B₂/β-arrestin2-Venus complex and is indicative of AT₂-B₂ heteromerization. Additionally, and similar to what was seen with GRK2, β-arrestin2-Venus recruitment to AT₂-B₂ heteromers had an altered kinetic profile to what was seen

with B₂ monomers/homomers. When we conducted the Receptor-HIT assay in the reverse configuration by coexpressing the untagged AT₂ receptor with B₂-Rluc8 and β -arrestin2-Venus there was no change in BRET signal (**Figure 6D**) from that seen without AT₂ expression (**Figure 6B**).

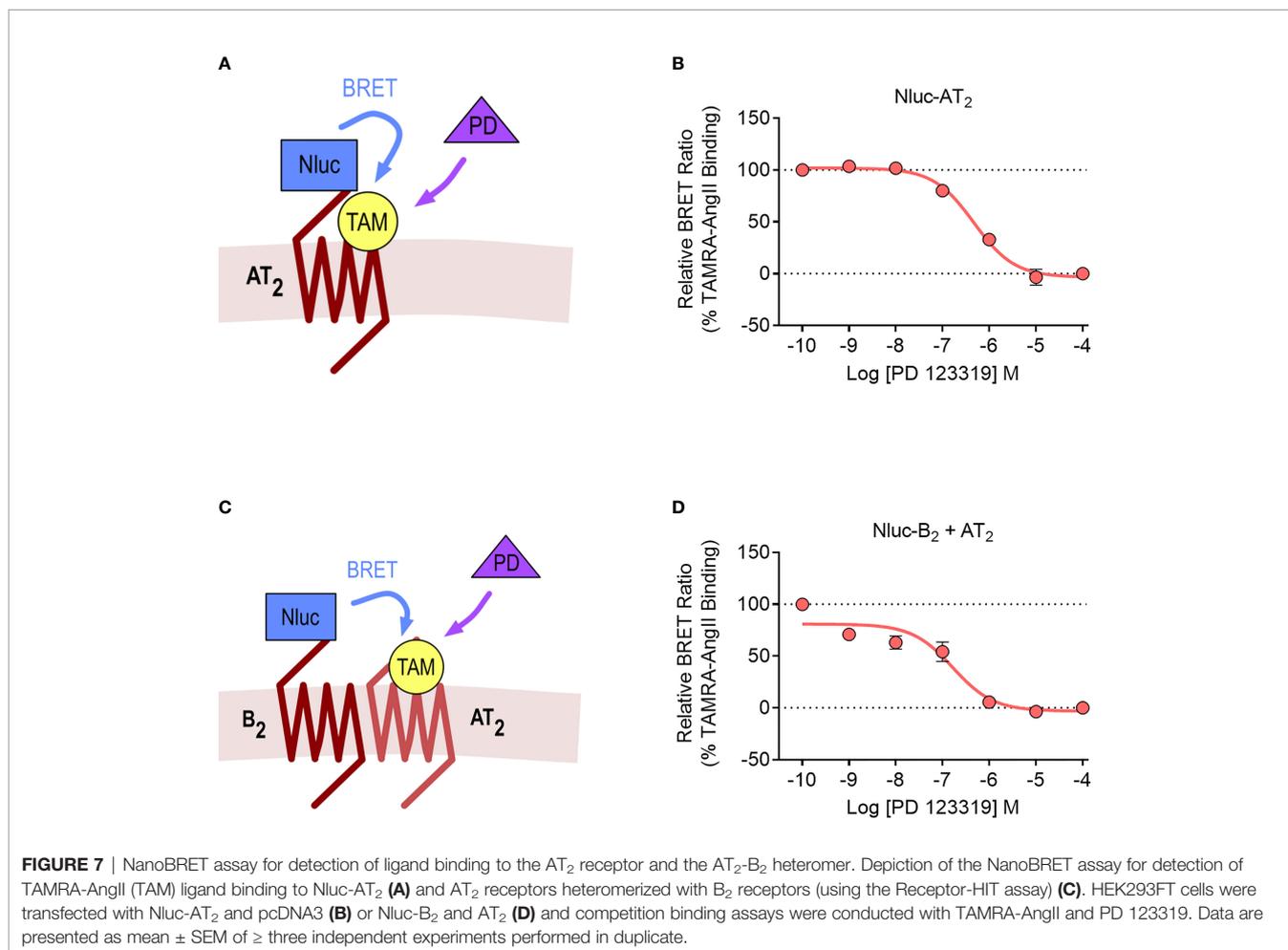
To further investigate the β -arrestin2 Receptor-HIT signal we again conducted concentration-response analysis (**Figure 6E**). This showed that there was no significant difference in potency between BK-induced β -arrestin2-Venus recruitment to B₂ receptors and AT₂-B₂ heteromers ($pEC_{50} \pm SEM = 7.64 \pm 0.06$ vs. 7.94 ± 0.19 , respectively; $p > 0.05$, unpaired *t*-test). We then conducted the β -arrestin2 Receptor-HIT assay in the presence of selective antagonists. Similar to the previous Receptor-HIT assays, we saw that the BK-induced recruitment of β -arrestin2 to the AT₂-B₂ heteromer could be blocked by B₂ receptor inhibition but not AT₂ inhibition (**Figure 6F**), which demonstrates the importance of B₂ receptor coexpression and activation.

NanoBRET Ligand Binding to the AT₂-B₂ Heteromer

We lastly investigated the AT₂-B₂ heteromer using the NanoBRET ligand binding assay (28). In this assay, the

NanoLuc (Nluc) luciferase (49) is fused to the N-terminus of a GPCR, and binding of fluorescent ligands can be detected with BRET. In our study, we fused Nluc to the N-terminus of the AT₂ receptor (Nluc-AT₂) and treated cells with an AngII analogue tagged with the TAMRA fluorophore (TAMRA-AngII) (**Figures 7A, B**). When we treated cells with increasing concentrations of the AT₂ receptor antagonist PD 123319 in a competition binding assay, we were able to see a reduction in the BRET signal, indicating displacement of TAMRA-AngII binding to Nluc-AT₂.

We then conducted the ligand binding assay in the Receptor-HIT configuration, as recently published (27). Here, Nluc was fused to the N-terminus of the B₂ receptor (Nluc-B₂) and was coexpressed with the untagged AT₂ receptor. A BRET signal upon addition of TAMRA-AngII would indicate both the binding of TAMRA-AngII to the untagged AT₂ receptor and also its close proximity to Nluc-B₂, and this specific binding of TAMRA-AngII to AT₂ receptors would be confirmed by displacement with PD 123319 (**Figure 7C**). When we conducted the assay, this was precisely what we observed, a TAMRA-AngII-induced BRET signal that could be displaced by increasing concentrations of PD 123319 (**Figure 7D**). When we compared the pIC₅₀ values between cells expressing Nluc-AT₂



and cells expressing Nluc-B₂ and AT₂ we found no significant differences ($pIC_{50} \pm SEM = 6.33 \pm 0.10$ vs. 6.84 ± 0.20 , respectively; $p > 0.05$, unpaired *t*-test).

DISCUSSION

This study provides evidence for the existence of the AT₂-B₂ receptor heteromer in transfected HEK293FT cells. This is illustrated by the Receptor-HIT signals that show the requirement of B₂ receptor coexpression and activation for recruitment of mG_{sq}, GRK2 and β -arrestin2 proximal to the AT₂ receptor. Evidence also came from the G α_z assay that demonstrated BK-induced modulation of B₂ receptor/G α_z coupling, which was not present without AT₂ receptor coexpression. Finally, the results of the heteromer ligand binding assay confirmed the close proximity of the two receptors at the cell surface, showing a Receptor-HIT signal between Nluc-B₂ and TAMRA-AngII bound to AT₂ receptors.

Perhaps the most interesting finding of this study was the novel G protein signaling pharmacology observed in the form of BK-induced modulation of B₂ receptor/G α_z coupling that was not present without AT₂ receptor coexpression. Following a search of the literature, we were unable to find any evidence that either the AT₂ or the B₂ receptor individually couple to G α_z , and this fits with the lack of ligand-induced interaction we observed in our BRET assays expressing only the single receptor. It is therefore particularly interesting that heteromerization may lead to new G protein coupling for both receptors. The G α_z protein is in the G $\alpha_{i/o}$ class of G proteins and therefore its canonical effect is inhibition of adenylyl cyclase and cAMP signaling (50). Gene and protein expression studies show that it is expressed at particularly high levels in the nervous system, and also at detectable levels in the gastrointestinal and reproductive systems as well as the adrenal gland and smooth muscle tissue (50, 51). There is therefore some overlap in expression profiles with the AT₂ receptor and the B₂ receptor, both of which are expressed in the brain, vasculature, adrenal gland and reproductive tissues (37, 38, 52). This suggests that the AT₂-B₂ heteromer could have physiological roles outside of the cardiovascular system, which is where most of the research into functional interactions between the two receptors has primarily been focused. In particular, the coexpression of G α_z and the two receptors in the nervous system is especially interesting, due to the growing appreciation of the functional role of the AT₂ receptor in mediating neurological processes (53, 54). Indeed, an AT₂ receptor antagonist progressed to Phase II clinical trials for the treatment of neuropathic pain (54, 55), although the trial had to be terminated due to toxicological concerns arising from pre-clinical data that only became available after the start of the trial (56). In addition, it is well established that the B₂ receptor is also involved in the mediation of various types of pain, including neuropathic pain (57), and it is interesting to speculate on a possible involvement of the AT₂-B₂ heteromer in mediating pain or other neurological processes.

The AT₂ receptor is an unusual GPCR in that it does not readily signal through G proteins, and nor does it undergo agonist-induced desensitization or internalization. In addition,

despite decades of research, even the physiological effects mediated by the AT₂ receptor are still not well understood. It is most commonly believed to antagonize many of the characteristic AngII/AT₁-mediated actions, such as vasoconstriction, anti-natriuresis, growth and cell proliferation. However, numerous studies describe opposing effects of the AT₂ receptor, reporting its mediation, rather than opposition of these effects (3). Despite these conflicting studies, one of the least controversial aspects of AT₂ receptor pharmacology is its action as a vasodilator, *via* stimulation of the NO/cGMP signaling pathway (58). This is the same pathway used by the B₂ receptor to mediate vasodilation following BK-induced activation of G α_q signaling (59). It is well known that BK can be involved in AT₂ receptor-mediated NO signaling (22, 23), and the results of our study may provide further insight into this signaling cascade, demonstrating that G α_q can be recruited proximal to the AT₂ receptor when it is heteromerized with the B₂ receptor. Furthermore, a previous study has suggested that functional heteromerization of these two receptors leads to enhanced NO signaling (24). The recruitment of mG_{sq} proximal to the AT₂ receptor within the AT₂-B₂ heteromer in our study, potentially provides a mechanism for this enhanced NO signal, as BK-mediated NO signaling could be mediated not only by B₂ receptors but also by AT₂-B₂ heteromers. It is important to note that not all AT₂ receptor-mediated NO signaling requires the presence of B₂ receptors, as B₂ receptor knockout mice can produce NO directly from AT₂ receptors (21). This therefore indicates an alternate pathway used by the AT₂ receptor to mediate NO signaling, which does not require the presence of B₂ receptors, and may therefore not necessarily be directly impacted by AT₂-B₂ heteromerization.

An interesting recent study has revealed that β -arrestin2 is an integral component of an endothelial NO synthase (eNOS) signaling pathway (60). Here, β -arrestin2 was found to colocalize in sinusoidal endothelial cells with GPCR kinase interactor 1 and eNOS, stimulating eNOS activity in a ERK1/2- and Src-dependent manner. The study revealed that endothelin-1-mediated eNOS activity required β -arrestin2, and therefore it is likely that it is also involved in NO signaling by both the AT₂ and the B₂ receptor, as well as the AT₂-B₂ heteromer.

It is now well established that endocytosed GPCR-bound β -arrestins are able to aid in the initiation of signaling cascades through their function as scaffold proteins. Numerous signaling molecules are regulated through this property of β -arrestins, such as the MAPKs ERK, JNK, and p38. The AT₂ receptor most commonly exerts inhibitory effects on MAPK cascades through activation of phosphatases (61–64). The potential for additional signaling through β -arrestin scaffolds adds another level of complexity to AT₂ receptor signaling. Furthermore, this heteromerization-mediated recruitment of β -arrestin could explain some of the contradictory studies that report AT₂-mediated activation of MAPKs (65, 66).

Coexpression of the AT₂ receptor and the B₂ receptor in the same cell is of course a primary requisite for formation of a heteromer. Expression of both receptors in endothelial cells is well documented (37, 67), and as both receptors initiate endothelium-

mediated vasodilation *via* the NO/cGMP pathway (59), this is a probable location that we may expect functional AT₂-B₂ heteromers to be present. This is further supported by the previous heteromer study, which found that heteromerization of these two receptors resulted in enhanced NO signaling (24). Both receptors are also found in smooth muscle cells of the vasculature and in the heart, indicating the potential for heteromer formation in these cells, and further allowing for a role for the heteromer in the cardiovascular system. Beyond the cardiovascular system, the two receptors are also coexpressed in uterine smooth muscle cells, epithelial cells and fibroblasts (59, 68, 69), suggesting a broad range of cells the heteromer may be present in.

Although the AT₂ receptor does not interact with the traditional GPCR interacting proteins, it is, however, known to interact with other signaling and regulatory proteins at its intracellular face. Interactions with the ErbB3 epidermal growth factor receptor (70), the scaffold protein connector enhancer of Ksr (71) and tissue inhibitor of metalloproteinases-3 (72) are implicated in AT₂ receptor-mediated antigrowth effects, while interactions with the transcription factor promyelocytic zinc finger protein are involved in the mediation of cardiac hypertrophy (73). Interactions with the Na⁺/H⁺ exchanger NHE6 are important for AT₂ receptor regulation of sodium levels (74), and interactions with AT₂ receptor-interacting protein 1 result in antigrowth effects (75, 76) and neural differentiation (77). It is possible that when the AT₂ receptor is heteromerized with the B₂ receptor, recruitment and interaction with the proteins investigated in this study (Gα_q, Gα_z, GRK2 and β-arrestin2) could modulate the above AT₂ receptor interactions, leading to alterations in signaling. In addition, many studies reveal that the AT₂ receptor is constitutively active (65, 78–81). If recruitment and interaction of the proteins in this study to the AT₂-B₂ heteromer were able to block the interaction between the AT₂ receptor and its various signaling partners, this would be a mechanism of reducing the constitutive activity observed for this receptor.

Despite decades of research, the AT₂ receptor remains incompletely characterized in terms of its molecular pharmacology and its physiological functions (2, 3). Its lack of canonical GPCR pharmacology, such as agonist-induced G protein coupling, desensitization and internalization, make it a unique and enigmatic receptor within the field. The antagonist assays conducted in this study initially suggested that it has a somewhat silent role within the heteromer, as in every functional assay we investigated, B₂ activation but not AT₂ activation was required for the heteromer response. However, the Gα_z results demonstrate an important functional role of the AT₂ receptor

within the heteromer, as it confers novel Gα_z coupling to the B₂ receptor. This is indicative of bi-directional modulation within the heteromer, as both the presence of the AT₂ receptor and activation of the B₂ receptor is required for modulation of Gα_z proximity.

In summary, we have provided evidence for the existence of the AT₂-B₂ heteromer and have demonstrated some of its apparent novel pharmacology. Extension of these findings beyond HEK293FT cells to more physiologically relevant systems will enable further characterization of the pharmacology mediated by the heteromer. Heteromerization of the AT₂ and B₂ receptors likely underpins some of the functional crosstalk observed between the receptors in the cardiovascular system, and it is possible that the heteromer may also have physiological roles in other areas of the body, such as the nervous system. AT₂-B₂ heteromerization is a newly identified mechanism for the enigmatic and poorly characterized AT₂ receptor to be functionally active within cells.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

EKMJ, MAA, RJH, HBS and RSA conducted the experiments and analyzed the results. EKMJ, MAA, RMS and KDGP conceived the experiments. EKMJ and KDGP wrote the paper. All authors reviewed the manuscript.

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Chief Scientific Advisor to Dimerix. KP, ES and RS are named inventors on patents covering the Receptor-HIT technology (WO/2008/055313 Detection System and Uses Therefor).

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