The Arrestin-selective Angiotensin AT$_1$ Receptor Agonist [Sar$^1$,Ile$^4$,Ile$^8$]-AngII Negatively Regulates Bradykinin B$_2$ Receptor Signaling via AT$_1$-B$_2$ Receptor Heterodimers*

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Background: Hemodynamic regulation involves extensive cross-talk between the renin-angiotensin and kallikrein-kinin systems.

Results: In vascular smooth muscle, "biased" AT$_1$ agonists inhibit both AT$_1$ and B$_2$ signaling by internalizing AT$_1$-B$_2$ heterodimers.

Conclusion: AT$_1$ antagonists and arrestin-selective biased AT$_1$ agonists have opposing effects on B$_2$ signaling.

Significance: Negative allosteric modulation of B$_2$ signaling by biased AT$_1$ agonists may impact their clinical utility.

The renin-angiotensin and kallikrein-kinin systems are key regulators of vascular tone and inflammation. Angiotensin II, the principal effector of the renin-angiotensin system, promotes vasoconstriction by activating angiotensin AT$_1$ receptors. The opposing effects of the kallikrein-kinin system are mediated by bradykinin acting on B$_1$ and B$_2$ bradykinin receptors. The renin-angiotensin and kallikrein-kinin systems engage in cross-talk at multiple levels, including the formation of AT$_1$-B$_2$ receptor heterodimers. In primary vascular smooth muscle cells, we find that the arrestin pathway-selective AT$_1$ agonist, [Sar$^1$,Ile$^4$,Ile$^8$]-AngII, but not the neutral AT$_1$ antagonist, losartan, inhibits endogenous B$_2$ receptor signaling. In a transfected HEK293 cell model that recapitulates this effect, we find that the actions of [Sar$^1$,Ile$^4$,Ile$^8$]-AngII require the AT$_1$ receptor and result from arrestin-dependent co-internalization of AT$_1$-B$_2$ heterodimers. BRET$_{50}$ measurements indicate that AT$_1$ and B$_2$ receptors efficiently heterodimerize. In cells expressing both receptors, pretreatment with [Sar$^1$,Ile$^4$,Ile$^8$]-AngII blunts B$_2$ receptor activation of G$_{q/11}$-dependent intracellular calcium influx and G$_{i/o}$-dependent inhibition of adenyl cyclase. In contrast, [Sar$^1$,Ile$^4$,Ile$^8$]-AngII has no effect on B$_2$ receptor ligand affinity or bradykinin-induced arrestin3 recruitment. Both radioligand binding assays and quantitative microscopy-based analysis demonstrate that [Sar$^1$,Ile$^4$,Ile$^8$]-AngII promotes internalization of AT$_1$-B$_2$ heterodimers. Thus, [Sar$^1$,Ile$^4$,Ile$^8$]-AngII exerts lateral allosteric modulation of B$_2$ receptor signaling by binding to the orthosteric ligand binding site of the AT$_1$ receptor and promoting co-sequestration of AT$_1$-B$_2$ heterodimers. Given the opposing roles of the renin-angiotensin and kallikrein-kinin systems in vivo, the distinct properties of arrestin pathway-selective and neutral AT$_1$ receptor ligands may translate into different pharmacologic actions.

The renin-angiotensin system (RAS)$^2$ is a critical regulator of vascular tone and volume homeostasis and an important therapeutic target in hypertension and congestive heart failure (1). In response to a drop in systemic blood pressure, renin, a pro tease produced in the juxtaglomerular apparatus of the kidney, converts circulating angiotensinogen to angiotensin I, which is in turn cleaved by angiotensin converting enzyme (ACE) to generate the biologically active peptide, AngI[1–8] or angiotensin II (AngII). Most of the physiological effects of AngII are mediated by G protein-coupled type 1 angiotensin II (AT$_1$) receptors (2–4). AT$_1$ receptors couple primarily to G$_{q/11}$-phospholipase C$_{B}$-protein kinase C (PKC) and G$_{12/13}$-Rho-GEF (guanine nucleotide exchange factor) signaling pathways. In vascular smooth muscle cells (VSMC), AT$_1$ receptors increase vascular tone by triggering phospholipase C$_{B}$-inositol-trisphosphate-dependent intracellular calcium release and calcium-dependent contraction. In the adrenal cortex, AngII stimulates production of aldosterone, a potent mineralocorticoid that promotes renal sodium retention to expand blood volume. Clinically, prolonged activation of the AT$_1$ receptor has been asso-

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2 The abbreviations used are: RAS, renin-angiotensin system; ACE, angiotensin converting enzyme; AngII, angiotensin II; AT$_1$, type 1 angiotensin II receptor; B$_2$, type 2 bradykinin receptor; BK, bradykinin; BRET, bioluminescence resonance energy transfer; GPCR, G protein-coupled receptor; HA, influenza virus hemagglutinin; KKS, kallikrein-kinin system; RFU, relative fluorescence unit; RLUC, relative lucinescence units; RLuc, Renilla luciferase; SI, [Sar$^1$,Ile$^4$,Ile$^8$]-AngII; YFP, yellow fluorescent protein; VSMC, vascular smooth muscle cells; Sar, sarcosine.
associated with hypertension, arrhythmia, and progression of diabetic nephropathy (5, 6).

The parallel kallikrein-kinin system (KKS) also plays diverse roles in the regulation of vascular tone, tissue inflammation, coagulation, and pain (7). Two forms of the KKS exist in humans, the tissue KKS and plasma KKS. Tissue kallikrein is primarily expressed in the kidney, vascular system, brain, and pancreas, where it acts on low molecular weight kininogen to release bradykinin (BK). The plasma KKS consists of factor XII, prekallikrein, and high molecular weight kininogen. It is responsible for the activation of the intrinsic blood-clotting pathway (8). The cellular effects of KKS are thought to reflect primarily the action of BK, a peptide hormone that produces proinflammatory and vasodilatory effects by activating two cell surface G protein-coupled receptors (GPCRs), the B1 and B2 bradykinin receptors (9). B2 receptors are constitutively expressed in a majority of tissues and are particularly abundant in endothelium and VSMC (10). The B1 receptor is generally not expressed under physiological conditions but can be induced by inflammation, diabetes mellitus, and genetic deletion of the B2 receptor (11–13). B2 receptors couple to the Gα11/12-phospholipase Cβ-PKC signaling pathway to promote calcium entry and to Gβγproteins leading to inhibition of adenylyl cyclase-cAMP signaling (9). Like the majority of GPCRs, desensitization and sequestration of AT1 and B2 receptors is mediated by arrestins, which bind to ligand activated receptors and target them to clathrin-coated pits for endocytosis (14, 15).

The KKS interfaces with the RAS at multiple levels (1, 16). Plasma and tissue kallikrein can convert prorenin to renin. Conversely, ACE1, the protease responsible for processing angiotensin I to AngII also cleaves BK to its inactive fragment, BK[1–5]. Thus, factors that enhance the generation of AngII tend to dampen BK signaling. Not surprisingly, given this reciprocal regulation, the KKS and RAS exert opposing effects in the regulation of vascular tone. AngII causes VSMC constriction and promotes hypertension, whereas under normal physiological conditions, BK stimulates endothelial nitric oxide synthase, leading to relaxation of the underlying VSMC and vasodilation (17, 18). Nitric oxide from endothelial cells also inhibits VSMC and renal mesangial cell proliferation (19, 20). However, in the setting of vascular injury and endothelial denudation, BK can act directly on VSMC to induce their contraction and activate multiple signaling pathways in a manner similar to vasoconstrictors like AngII (21).

Another potential mechanism for cross-regulation of the RAS and KKS is through the formation of GPCR hetero-oligomers (22, 23). Although their existence has been questioned (24, 25), substantial evidence has emerged supporting the hypothesis that AT1 and B2 receptors form both homo- and heterodimers (26, 27). Although it is clear that many, if not most, GPCRs can signal as monomeric entities (28, 29), it is also clear that the assembly of GPCR homo- and heterodimers can modulate many aspects of GPCR signaling through lateral allosteric effects generated by contact between the transmembrane and intracellular domains of GPCRs that imposes steric constraints on the dimer partner (30). Trafficking of nascent receptors to the plasma membrane, ligand binding affinity, G protein-coupling efficiency, downstream signaling, and endocytosis of activated receptors are all influenced by the formation of receptor multimers (31–34). In the case of the AT1 and B2 receptors, heterodimer formation reportedly sensitizes vascular cells to AngII signaling (35), leading to exaggerated pressor responses in experimental hypertension and human pre-eclampsia (26, 36).

Targeting the RAS is a mainstay of treatment for hypertension, congestive heart failure, and diabetic nephropathy. Several classes of drug are in clinical use, including direct renin inhibitors, ACE inhibitors, and small molecule AT1 receptor antagonists, each of which would be expected to have different effects on the complex interplay that occurs between the RAS and KKS (25). Recently, a novel class of biased AT1 receptor ligand that antagonizes AT1 receptor-G protein signaling while simultaneously acting as an agonist for arrestin recruitment and receptor internalization has been advanced as a potential therapeutic for congestive heart failure (37, 38). The prototypic arrestin pathway-selective AT1 receptor agonist is [Sar1,Ile4,Ile8]-AngII (SII (39, 40)). In vitro, SII acts as a weak partial agonist for AT1 receptor coupling to its G protein effectors while retaining near native ability to recruit arrestins (41). This “bias” toward arrestin coupling permits SII to induce AT1 receptor internalization and activate arrestin-dependent signaling pathways independent of detectable G protein signaling (40).

In this study we examined the effects of SII on AngII and BK signaling in primary aortic VSMC and in engineered HEK293 cell systems designed to permit us to dissect effects on ligand binding, G protein signaling, and receptor internalization. We find that unlike the neutral AT1 receptor antagonist losartan, SII antagonizes B2 signaling. This effect requires the AT1 receptor and results from co-internalization of AT1–B2 heterodimers in response to SII. Thus, by binding to the orthosteric ligand binding site of the AT1 receptor, SII exerts lateral allosteric effects on B2 receptors leading to a reduction in BK responsiveness. These results demonstrate that arrestin pathway-selective biased AT1 receptor agonists represent a unique pharmacological entity that can produce effects not observed using conventional antagonists.

**EXPERIMENTAL PROCEDURES**

**Primary VSMC Isolation and Culture**—Primary rat aortic VSMC were isolated from 75–100-g Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) as previously described (42). VSMC were maintained in minimum essential medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Sigma). Cells were fed every 2 days and subcultured upon reaching 90% confluence. Before each experiment, cells were seeded into multiwell plates as appropriate and incubated for 24–48 h in serum-free growth medium supplemented with 0.1% bovine serum albumin and 1% antibiotic/antimycotic solution. All experiments on primary VSMC were performed between passages four and nine.

**cDNA Expression Constructs**—pcDNA3.1 expression plasmids encoding untagged angiotensin AT1 receptor (AGTR1), angiotensin AT2 receptor (AGTR2), bradykinin B1 receptor...
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(BDKRB1), and bradykinin B2 receptor (BDKRB2) were obtained from the Missouri Science and Technology cDNA Resource Center (Rolla, MO). C-terminal yellow fluorescent protein (YFP)-tagged receptor expression plasmids were constructed by cloning untagged receptor cDNAs into pYFP-N1 (Clontech Laboratories, Inc., Mountain View, CA). Receptor expression plasmids tagged at the C terminus with the Venus-enhanced YFP (43) were constructed by cloning untagged receptor cDNAs into a modified pIRES-puro3 vector containing the Venus tag in position 2098–2817. The vector, which contains a polypeptide linker (PVN5GGGGS) between the C terminus of the receptor and the Venus protein to improve receptor expression, was a gift from Michel Bouvier (University of Montreal, Montreal, Quebec, Canada). The pcDNA3.1 plasmid encoding arrestin3 tagged at the C terminus with Renilla luciferase (RLuc) was also a gift from Michel Bouvier. The pcDNA3.1 plasmid encoding hemagglutinin (HA) epitope-tagged rat AT1A receptor was a gift from Marc G. Caron (Duke University, Durham, NC).

Generation of HEK293 FRT/TO Cell Lines—HA epitope-tagged AT1A receptor expression plasmids were reconstructed by cloning the receptor cDNA into pcDNA5/FRT/TO (Invitrogen). A cell line carrying TET-inducible cDNA encoding HA-tagged AT1A receptors was generated by co-transfection of pcDNA5/FRT/TO-AT1A and the pOG44 plasmid encoding Flp-recombinase (1:9 pcDNA5:POG44) into the HEK293 Flp-InTM host cell line (Invitrogen) using FuGENE 6 (Roche Diagnostics). Doxycline-inducible (1 µg/ml × 48 h) expression of HA-tagged AT1A receptors was documented by real-time polymerase chain reaction and anti-HA immunoblotting. A HEK293 line carrying tetracycline-inducible shRNA targeting the arrestin2 and 3 isoforms (CGTCCACGTCACCAACAC) was generated as previously described (44). Doxycycline-inducible down-regulation of arrestin 2/3 expression was documented by immunoblotting.

HEK293 Cell Culture and Transfection—HEK293 cells were obtained from the American Type Culture Collection. HEK293 cells, HEK293 FRT/TO arrestin2/3 shRNA cells, HEK293 FRT/TO AT1A cells, and GloSensorTM cAMP HEK293 cells (Promega, Inc., Madison, WI) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution. To maintain selection, hygromycin (200 µg/ml) and blasticidin (50 µg/ml) were added to the medium of HEK293 FRT/TO AT1A cells, and zeocin (50 µg/ml), blasticidin (50 µg/ml), and puromycin (50 µg/ml) were added to the medium of HEK293 FRT/TO arrestin2/3 shRNA cells. GloSensorTM cells were maintained in medium containing hygromycin (200 µg/ml). Transient transfection of HEK293 cells for intracellular calcium, GloSensorTM cAMP, receptor-arrestin bioluminescence resonance energy transfer (BRET), and microscopy-based receptor trafficking studies was performed in 10-cm dishes (8 million cells/dish) using FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions with 6 µg of plasmid DNA per dish and 3 µl of FuGENE 6 per µg of DNA. Transient transfection of HEK293 cells for static BRET and radioligand binding studies was performed in 35-mm multiwell plates (1 million cells/well) in phenol red-free DMEM supplemented with 5% fetal bovine serum and 1% antibiotic/antimycotic solution. Plasmid DNA (1.5 µg total per well) was diluted in 100 µl of serum-free DMEM and mixed with 100 µl DMEM containing 8 µg of polyethyleneimine. The DNA-polyethyleneimine mixture was allowed to stand for 15 min before adding to the cells, which were then incubated for 16 h before refeeding.

FLIPRTETRA Assay of Calcium Influx—VSMC were plated into black-wall clear-bottom 96-well plates (BD Biosciences) at a density of 20,000 cells/well and starved in serum-free DMEM supplemented with 0.1% bovine serum albumin, 1% antibiotic/antimycotic solution for 48 h before assay. Twenty-four hours after transfection, HEK293 cells were passed at 70–80% confluence onto collagen-coated black well/ clear-bottom 96-well plates, allowed to grow for 24 h, then placed in serum-free medium for 5–6 h before assay. Fresh FLIPR Calcium 5 assay reagent (100 µl/well; Molecular Devices, Sunnyvale, CA) was added, and plates were incubated for an additional hour before stimulation. Stimulations were carried out on a FLIPR[TETRA] screening system (Molecular Devices) with 470–495-nm excitation and 515–575-nm emission filters per the manufacturer’s operating instructions (45).

FLIPRTETRA Assay of cAMP Production—Assays were performed using HEK293 GloSensorTM cAMP cells that stably express a genetically encoded biosensor composed of a cAMP binding domain fused to a mutated form of Photinus pyralis luciferase (46). Twenty-four hours after transient transfection with the receptors of interest, HEK293 GloSensorTM cAMP cells were seeded into poly-d-lysine-coated white-wall clear-bottom 96-well plates (BD Biosciences) at a density of 50,000 cells/well. At 48 h, the growth medium was removed and replaced with 100 µl of CO2-independent medium (Invitrogen) containing 2% w/v of the GloSensor cAMP reagent. After a 2 h preincubation, stimulations were performed at room temperature in the FLIPRTETRA system with luminescence recorded every 2 s for 600 reads as previously described (45). To assay B2 receptor-mediated inhibition of cAMP production, cells were pretreated with AT1 and B2 receptor ligands as appropriate, then exposed to forskolin as described in the figure legends.

Receptor Dimerization and Estimation of BRET50—HEK293 cells were transiently transfected with a constant amount (200 pg) of B2 receptor tagged at the C terminus with Renilla luciferase (B2-RLuc) and varying amounts (10–1500 ng) of AT1, AT2, AT1, or B2 receptor tagged at the C terminus with Venus fluorescent protein using the polyethyleneimine method. For competition experiments, cells were additionally transfected with 500 ng of untagged AT1 or B2 receptor. Forty-eight hours post transfection, cells were resuspended in BRET buffer (1 mM CaCl2, 140 mM NaCl, 2.7 mM KCl, 900 µM MgCl2, 370 µM NaH2PO4, 5.5 mM d-glucose, 12 mM NaHCO3, 25 mM HEPES, pH 7.4) and placed in white-wall clear-bottom 96-well plates. Background and total Venus fluorescence were read on an OptiplateTM microplate reader (PerkinElmer Life Sciences) with 485-nm excitation and 525–585 emission filters. Coelenterazine (NanoLight Technology, Pinetop, AZ) was then added to a final concentration of 5 µM, and the cells were incubated at room temperature for 2 min, after which luciferase (440–480) and Venus (525–585) emissions were read to calculate the...
BRET ratio (emission eYFP/emission RLuc). Net BRET ratio was calculated by background-subtracting the BRET ratio measured for the B2-RLuc construct expressed alone in the same experiment. BRET saturation curves were plotted as the net BRET ratio relative to the ratio of acceptor (total Venus fluorescence) to donor (total RLuc luminescence). The curves were modeled using GraphPad Prism (San Diego, CA), and the calculated B2 receptor number (B2max) and binding affinity (Kd) were determined by fitting competition binding curves using GraphPad Prism. To determine the effect of SII on cell surface B2 receptor density, cells were exposed to SII or vehicle for 20 min at 37 °C then placed on ice. B2 receptor B2max was then determined as specific binding of 200 pm [3H]bradykinin for 2 h at 4 °C.

**FLIPRTETRA Assay of B2 Receptor-Arrestin3 Recruitment**—HEK 293 cells in phenol red-free DMEM supplemented with 5% fetal bovine serum and 1% antibiotic/antimycotic solution were transiently transfected with plasmids encoding B2-YFP, Arr3-RLuc, and HA-tagged AT1A receptor. Forty-eight hours after transfection, cells were resuspended in BRET buffer and aliquoted into white-wall clear-bottom 96-well plates at a density of 100,000 cells per well. Cells were pretreated with SII (100 μM) or vehicle at room temperature for 20 min. Coelenterazine (5 μM) was added to each well 2 min before stimulation with 10 μM BK. After stimulation, luciferase donor (440–480 nm) and YFP acceptor (526–586 nm) emission was read in the FLIPRTETRA every 4 s for 20 min. Net BRET ratio was determined by calculating the mean of emission YFP/emission RLuc determined for each time point after stimulation minus the BRET ratio of the first three reads before stimulation. Plots of Net BRET versus time were corrected for the background signal drift observed in unstimu-
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**RESULTS**

[Sar¹,Ile⁴,Ile⁸]-AngII and Losartan Differentially Affect B₂ Receptor Signaling in Primary VSMC—Conventional GPCR agonists, like AngII for the AT₁ receptor, are generally considered to promote receptor coupling to its full complement of potential downstream effectors. True neutral antagonists, like the small molecule AT₁ receptor blocker losartan, are thought to have no net effect on receptor confirmation and act only through competitive inhibition of orthosteric agonist binding. In contrast, biased agonists like SII are pharmacologically dis-
distinct entities in that they stabilize receptor conformation(s) that productively engage only a subset of downstream effectors (30, 48). In the case of SII, it promotes arrestin-dependent internalization of AT$_1$ receptors and activates downstream arrestin-mediated signaling while generating little to no measureable heterotrimeric G protein signaling (39–41).

Fig. 1 depicts the effects of SII and losartan on BK-stimulated calcium influx in primary rat aortic VSMC, which natively express both AT$_1$ and B$_2$ receptors (42). In these cells BK produces a dose-dependent increase in calcium entry with an EC$_{50}$ of 23.0 ± 1.4 nM (Fig. 1A). Preincubation with SII for 20 min before stimulation led to a marked reduction in BK-induced calcium entry across a range of SII concentrations at or above its K$_D$ for the rat AT$_{1_A}$ receptor of 0.4–1.0 μM (Fig. 1B). As shown in Fig. 1C, this property was not shared with losartan, which had no significant effect on the BK response even at concentrations >10-fold above its K$_D$ of 12 nM. The inhibitory effect of SII was apparent across a range of physiologically relevant BK concentrations (Fig. 1D).

Recapitulation of the SII Effect in Transfected HEK293 Cell Models—Any number of mechanisms might account for the inhibitory effect of SII on BK signaling in VSMC. Although structurally distinct, SII might exert direct effects on B$_2$ receptors, as has been reported for losartan (49). SII might impact B$_2$ receptor signaling by acting on AT$_1$-B$_2$ receptor heterodimers (26, 27, 35, 36), potentially changing B$_2$ receptor ligand affinity, effector coupling, or trafficking. Alternatively, SII acting via AT$_1$ receptors might generate G protein-independent signals that antagonize B$_2$ effector coupling. To dissect these potential mechanisms, we employed HEK293 cell systems in which the levels of expression of B$_2$ and AT$_1$ receptors could be experimentally manipulated.

To confirm that the inhibitory effect of SII on BK signaling requires co-expression of the AT$_1$ receptor, we utilized the tetrcycline-inducible HEK293 FRT/TO AT$_{1_A}$ cell line to alter expression of the AT$_{1_A}$ receptor. As shown in Fig. 2A, 48-h induction with doxycycline led to a marked up-regulation of AT$_{1_A}$ mRNA. After induction, Ang II produced a robust calcium signal that was sensitive to losartan, confirming doxycycline-inducible expression of functional AT$_1$ receptors (Fig. 2B). To test whether the inhibitory effect of SII on B$_2$ signaling required the AT$_1$ receptor, HEK293 FRT/TO AT$_{1_A}$ cells were transiently transfected with B$_2$ receptors, and the effects of SII and losartan were determined with and without doxycycline treatment (Fig. 2C). Without doxycycline, AngII provoked a small rise in intracellular calcium that was sensitive to both SII and losartan. Under these conditions, BK-stimulated calcium influx was insensitive to both AT$_1$ receptor ligands. With doxycycline induction, AngII produced a robust SII- and losartan-sensitive calcium signal. In contrast, BK-stimulated calcium entry was significantly inhibited only by SII pretreatment. As shown in Fig. 2D, SII pretreatment attenuated BK-induced intracellular calcium entry by ~30% across a range of BK concentrations (10–200 nM) and modestly shifted the EC$_{50}$ from 2.4 to 5.3 nM. As in primary VSMC, losartan had no effect on BK calcium signaling in doxycycline induced HEK293 FRT/TO AT$_{1_A}$ cells (Fig. 2E). Moreover, the SII effect was absent from HEK293 FRT/TO AT$_{1_A}$ cells that were not doxycycline-inducible, demonstrating that its actions were mediated via the AT$_1$ receptor (Fig. 2F).

B$_2$ receptors also inhibit adenylyl cyclase by activating G$_{i/o}$ family heterotrimeric G proteins (9). We tested whether SII affected B$_2$ receptor coupling to G$_{i/o}$ proteins using GloSensor$^TM$ cAMP HEK293 cells, which are engineered to express a luciferase complementation cAMP reporter (46). In these assays, forskolin was used to stimulate cAMP production, and
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SII was tested for the ability to reverse the inhibitory effect of BK. Fig. 3A depicts representative forskolin concentration-response curves in GloSensor™CAMP HEK293 cells transiently transfected with B₂ and AT₁ receptor expression plasmids. As shown in Fig. 3B, exposure to a saturating concentration of BK produced a modest but significant 10% reduction in maximum forskolin-stimulated CAMP luminescence (12.2 ± 0.7-fold versus 13.6 ± 0.5-fold change in CAMP relative luminescence units (RLU)). Preincubation with SII for 20 min before stimulation reversed the BK effect (13.3 ± 0.2-fold versus 13.6 ± 0.5-fold change in CAMP RLUs) consistent with SII-dependent inhibition of B₂ receptor-Gi/o protein coupling. The SII effect required the AT₁ receptor, as it was absent from GloSensor™CAMP HEK293 cells expressing B₂ receptor alone (Fig. 3C).

**BRET Analysis of AT₁-B₂ Receptor Heterodimerization** — Because it is sensitive to very small differences in distance and orientation between donor and acceptor fluorophores, BRET has proven to be a valuable tool for studying protein-protein interactions including GPCR dimerization (50). Although it does not specifically discriminate between receptor dimers and higher order multimers, an intermolecular BRET signal that is saturable and susceptible to competition by untagged receptor provides strong evidence of direct and stoichiometric protein-protein interaction.

Fig. 4A depicts the increase in net BRET signal observed when HEK293 cells were transfected with a fixed amount of plasmid DNA encoding the BRET donor, B₂-RLuc, and increasing amounts of the BRET acceptor, AT₁-Venus. As shown, the BRET signal between the B₂-RLuc:AT₁-Venus donor:acceptor pair could be competed away by untagged AT₁-Venus. No change in BRET signal was observed upon treatment with AngII, SII, or BK. Fig. 4B shows similar results obtained using the B₂-RLuc:B₂-Venus donor:acceptor pair, suggesting that B₂ receptors are capable of forming both B₂-B₂ homo- and AT₁-B₂ heterodimeric complexes.

As shown in Fig. 4C, the B₂-RLuc:AT₁-Venus BRET signal was saturable, another hallmark of a stoichiometric protein-protein interaction. Because the magnitude of the BRET signal (B_max) is influenced by both proximity and orientation of the BRET donor:acceptor fluorophores, it does not provide information about the relative avidity of the interaction. However, BRET_{50}, defined as the donor:acceptor (YFP/RLuc) fluores-
cence ratio that produces a half-maximal BRET signal, can be used to compare the relative affinity of different potential dimer partners. The BRET$_{50}$ value obtained by nonlinear regression fitting of the B$_2$-RLuc:AT$_1$-Venus saturation curve was 0.31 ± 0.25 eYFP/RLuc. Fig. 4D shows BRET saturation curves obtained using B$_2$-RLuc and Venus-tagged B$_1$, B$_2$, and AT$_2$ receptors. The BRET$_{50}$ values for these three donor-acceptor pairs ranged from 8.99 to 12.65. To estimate the relationship between BRET$_{50}$ and receptor expression ratio, we empirically determined the relationship between receptor number, assayed by saturation binding, and the maximal RLuc or YFP fluorescence produced by that number of receptors when excited directly. Although approximate, these conversion factors indicate that BRET$_{50}$ for the AT$_1$-B$_2$ receptor heterodimer occurred at about a 1:1 receptor expression ratio, whereas that for the B$_2$-B$_2$ homodimer occurred at about a 3:1 ratio (Fig. 4, C and D). This suggests AT$_1$-B$_2$ receptor heterodimers are at least as likely to assemble as B$_2$-B$_2$ receptor homodimers when the receptors are co-expressed.

[Sar$^1$,Ile$^4$,Ile$^8$]-AngII Does Not Affect B$_2$ Receptor Ligand Binding Affinity or Arrestin3 Recruitment—Because SII pre-treatment significantly inhibited B$_2$ receptor signaling via the G$_q$, phospholipase C and G$_i/o$-adenyl cyclase pathways, we next tested whether SII had an effect on B$_2$ receptor ligand binding affinity or arrestin recruitment. Because we wanted to look for SII effects on B$_2$ receptor affinity, not on subcellular distribution, competition binding assays were performed on intact cells, and all ligand treatments, including preincubation with SII, were performed at 4 °C to prevent receptor internalization. As shown in Fig. 5, neither co-expression of AT$_1$ receptors nor pretreatment with SII had an effect on B$_2$ receptor affinity for BK. In HEK293 cells expressing the B$_2$ receptor alone, SII did not change the $K_d$ or $B_{max}$ of BK binding, suggesting the hypothesis that this AngII-derivative peptide has no direct effect on BK binding to B$_2$ receptors (Fig. 5A). Similarly, co-expression of AT$_1$ and B$_2$ receptors had no effect on BK binding affinity whether or not cells were pretreated with SII (Fig. 5B). Thus, under conditions favoring the formation of AT$_1$-B$_2$ receptor dimers, neither the presence of the AT$_1$ receptor nor SII binding to the AT$_1$ receptor had a significant effect on B$_2$ receptor binding affinity.

Like G heterotrimeric protein activation and ligand binding affinity, arrestin recruitment is a sensitive indicator of ligand-induced changes in receptor conformation. To determine whether SII affected arrestin recruitment to the B$_2$ receptor we employed a BRET based assay using arrestin3-RLuc and B$_2$ receptor-YFP as the donor-acceptor pair (51). Assays were run using a FLIPRTETRA instrument that collects real-time net BRET data, enabling comparison of the effect of SII on the kinetics of arrestin3 recruitment. A representative assay of change in BRET signal intensity as a function of time after the addition of BK to HEK293 cells transiently expressing both AT$_1$ and B$_2$ receptors is shown in Fig. 6A. Fig. 6B shows the kinetics of arrestin3 recruitment plotted after subtraction of the baseline drift in net BRET observed in unstimulated wells assayed simultaneously on the plate. Nonlinear regression modeling of these curves provided a measure of the extent of arrestin-B$_2$ receptor binding ($\Delta$Net BRET) and rate of arrestin recruitment ($T_{\%}$ to maximum $\Delta$Net BRET). The $\Delta$Net BRET values for B$_2$ receptor-YFP-arrestin3-2-RLuc association were 0.27 ± 0.01 and 0.29 ± 0.01 with and without SII pretreatment, respectively, and the $T_{\%}$ values were 350 ± 65 s and 355 ± 62 s, respectively, indicating that SII binding did not alter the capacity of BK to promote arrestin3 binding to B$_2$ receptors (Fig. 6, C and D).

[Sar$^1$,Ile$^4$,Ile$^8$]-AngII Promotes AT$_1$ Receptor-dependent Internalization of B$_2$ Receptors—Because it is well established that SII promotes internalization of AT$_1$ receptors (39, 40, 52), we hypothesized that SII binding to the AT$_1$ receptor might inhibit B$_2$ receptor signaling by promoting the sequestration of AT$_1$-B$_2$ heterodimers. We initially performed BK saturation binding to look for loss of cell surface B$_2$ receptors in response to SII. As shown in Fig. 7, exposing HEK293 cells co-expressing B$_2$ and AT$_1$ receptors to SII for 20 min at 37 °C before initiating the saturation binding assay reduced BK binding by ~60%. SII had no effect in HEK293 cells expressing B$_2$ receptors alone. Because SII did not affect B$_2$ receptor number or affinity when...
allowed to bind at 4 °C (Fig. 5), these data were consistent with AT1 receptor-dependent sequestration of B2 receptors in response to SII.

To directly visualize the effect of SII on B2 receptor trafficking, we performed quantitative high-content image analysis of cells expressing YFP-tagged AT1 or B2 receptors (47). In this assay, fluorescence images are captured in real time, and analytical software is used to identify and count the number of receptor “objects” in each image for all time points. Receptor objects are defined by exceeding a pre-specified brightness and size threshold and counted on a per cell basis. Typically, the number of GPCR objects increases with stimulation, as receptors traffic into endosomes, and then either remains constant or declines as endosomal vesicles coalesce or disperse (47). As shown in Fig. 8A, SII promoted efficient internalization of AT1-YFP receptors expressed in HEK293 cells. The number of receptor objects increased rapidly to a maximum within 5–10 min of stimulation, then slowly declined. Likewise, BK promoted internalization of B2-YFP receptors (Fig. 8B). The kinetics of internalization were different, with slower and more sustained accumulation of receptor objects. B2-YFP receptor internalization also occurred when cells transfected with both B2-YFP and untagged AT1 receptors were exposed to SII (Fig. 8C). The SII effect was absent in HEK293 cells expressing B2-YFP alone, consistent with SII induced internalization of AT1-B2 receptor heterodimers. Interestingly, the kinetics of SII-induced B2-YFP receptor internalization resembled those produced by BK, with slower and more sustained formation of receptor objects, suggesting that the presence of the B2 receptor in the complex dictates the kinetics of AT1-B2 receptor heterodimer trafficking.

AT1 receptor internalization is arrestin-dependent (14). Because our data indicated that SII-mediated inhibition of B2 receptor signaling correlated with AT1 receptor-dependent internalization of a substantial fraction of the cell sur-
face B<sub>2</sub> receptor pool (Fig. 7), we tested whether arrestins were required for the SII effect on BK signaling. These experiments employed HEK293 FRT/TO arrestin2/3 shRNA cells, which incorporate a tetracycline-inducible shRNA targeting both the arrestin2 and arrestin3 isoforms (Fig. 9A; Ref. 44).

As in transfected HEK293 and HEK293 FRT/TO AT<sub>1A</sub> cells, SII inhibited BK-induced calcium influx in non-induced HEK293 FRT/TO arrestin 2/3 shRNA cells transiently coexpressing AT<sub>1</sub> and B<sub>2</sub> receptors (Fig. 9B). Down-regulating arrestin2/3 expression with doxycycline eliminated the SII effect (Fig. 9C), indicating that arrestin-dependent sequestration of AT<sub>1</sub>-B<sub>2</sub> receptor heterodimers is the principal mechanism whereby the biased AT<sub>1</sub> receptor agonist negatively regulates BK signaling.

**DISCUSSION**

The concept that GPCR heterodimers can exhibit altered ligand-dependent trafficking compared with their monomeric or homodimeric counterparts has been previously established. For example, the non-selective opioid agonist etorphine, which causes internalization of δ-opioid but not κ-opioid receptors, does not cause δ-opioid receptor internalization when it is coexpressed with κ-opioid receptor (32). In our study we found that the arrestin pathway-selective AT<sub>1</sub> receptor agonist SII promotes arrestin-dependent internalization of B<sub>2</sub> bradykinin receptors. The effect is dependent upon coexpression of AT<sub>1</sub> receptors, as SII has no effect on B<sub>2</sub> receptor ligand affinity, G-protein-dependent signaling, or arrestin recruitment in the absence of the AT<sub>1</sub> receptor. BRET analysis, which offers stronger evidence of protein-protein interaction at atomic-level distances than co-immunoprecipitation or conventional confocal microscopy, indicates that the AT<sub>1</sub> and B<sub>2</sub> receptors efficiently oligomerize, suggesting that modulation of B<sub>2</sub> receptor signaling by SII occurs in the context of AT<sub>1</sub>-B<sub>2</sub> heterodimers or higher order stoichiometric complexes.

Allosteric modulation of GPCR signaling occurs when a molecule that interacts with a receptor at a site distant from its orthosteric ligand binding site imposes conformational restraints that measurably alter the binding, signaling, or trafficking properties of the receptor (30, 48). For example, the small molecule calcimimetic compound, cinacalcet, acts as a positive allosteric modulator of the G protein-coupled calcium-sensing receptor, increasing its affinity for calcium by binding to the receptor outside of the ligand binding site (53). Anything that affects the conformation of a GPCR has the potential to modulate signaling, whether an orthosteric ligand, allosteric small molecule, membrane lipid, or a cytosolic or intrinsic membrane protein. In our case, SII behaves as a lateral allosteric modulator of the B<sub>2</sub> receptor in that it binds to the orthosteric site of the AT<sub>1</sub> receptor component of AT<sub>1</sub>-B<sub>2</sub> receptor heterodimers and modulates BK signaling by promoting the removal of B<sub>2</sub> receptors from the plasma membrane.

**FIGURE 8.** [Sar<sup>1</sup>,Ile<sup>4</sup>,Ile<sup>8</sup>]-AngII promotes AT<sub>1</sub> receptor-dependent internalization of B<sub>2</sub> receptors. HEK293 cells were transiently transfected with cDNA encoding AT<sub>1</sub>-YFP, B<sub>2</sub>-YFP, or B<sub>2</sub>-YFP plus untagged AT<sub>1</sub> receptor as indicated. Stimulus-dependent trafficking of YFP-tagged receptors was monitored by high content fluorescence microscopy using an InCell 2000<sup>TM</sup> Analyzer with image acquisition every 30 s for 30 min after agonist application. Trafficking quantitation was performed using endosome segmentation with GE InCell Investigator<sup>TM</sup> software as previously described (47). A, shown is SII-induced internalization of AT<sub>1</sub>-YFP receptors. Representative images obtained before (0 min) and 10 min after stimulation are shown above a plot of the mean count of receptor objects per cell over time. B, shown is BK-induced internalization of B<sub>2</sub>-YFP receptors. Representative images obtained before and 20 min after stimulation are shown above a plot of the mean count of receptor objects per cell over time. C, shown is SII-induced internalization of B<sub>2</sub>-YFP receptors in HEK293 cells co-expressing untagged AT<sub>1</sub> receptors. Representative images obtained before and 20 min after stimulation are shown above a plot of the mean count of receptor objects per cell over time. In each panel, error bars represent the S.E. of at least three independent image analyses.
Modulation of AT$_1$-B$_2$ Receptor Dimers

The magnitude of the SII effect on B$_2$ signaling would be expected to vary depending on the abundance of AT$_1$-B$_2$ heterodimers relative to B$_2$-B$_2$ homodimers. Our BRET$_{50}$ data suggest that AT$_1$-B$_2$ heterodimers are at least as favored as B$_2$-B$_2$ homodimers when both receptors are coexpressed, and our saturation binding data from cotransfected HEK293 cells suggest that as much as 60% of the B$_2$ receptor pool is complexed with AT$_1$ receptors under these conditions. In primary VSMC, we find that SII pretreatment blunts BK-stimulated calcium signaling by 30–50% over a range of physiologically relevant BK concentrations. Because G protein-mediated signaling is often amplified, it is difficult to infer from the signaling data what proportion of endogenous B$_2$ receptors in VSMC exist as AT$_1$-B$_2$ heterodimers. However, co-immunoprecipitation studies performed using primary VSMC have shown that the majority of endogenous B$_2$ receptors exist in high molecular weight complexes that contain AT$_1$ receptor immunoreactivity (35). If AT$_1$-B$_2$ receptor heterodimers in vivo are hyperresponsive to AngII, as has been reported (26, 36), then changes in the relative expression of the two receptors in disease may promote “unbalanced” signaling by changing the ratio of homodimeric and heterodimeric receptors. For example, AT$_1$ receptor expression and RAS signaling activity are both up-regulated in experimental diabetes (54, 55), which may pull a larger fraction of B$_2$ receptors into AT$_1$-B$_2$ heterodimers.

The RAS and KKS constitute an interlocking signaling network involved in the regulation of vascular function (1, 16). Coordinate regulation is achieved through shared pathway components. Plasma and tissue kallikrein, which generate BK from kininogens, also convert prorenin to renin, an action that would simultaneously enhance RAS activity. Meanwhile ACE1, which generates AngII from angiotensin I, converts BK into its inactive fragment BK[1–5], an action that would likewise tip the balance toward RAS activation. Such interdependence means that drugs acting on the RAS would be expected to have different effects on KKS depending on which RAS pathway component is targeted. Direct renin inhibitors like aliskiren would preserve KKS integrity while inhibiting not only generation of AngII by ACE1 but also generation of Ang[1–7] by ACE2 (56). Ang[1–7], produced by cleavage of AngII, is a ligand for the AT$_2$ receptor/Mas protooncogene that is thought to mediate cardiovascular and renal protective effects. Moving downstream, clinical ACE inhibitors not only inhibit AngII production but they enhance KKS activity by blocking BK degradation. Finally, clinical angiotensin receptor blockers like losartan inhibit AT$_1$ receptor signaling without blocking BK degradation. These seemingly subtle differences may underlie differences in the clinical efficacy of these drugs. Randomized controlled trials to date indicate that although both ACE inhibitors and angiotensin receptor blockers are effective in reducing the progression of diabetic and hypertensive renal disease, only ACE inhibitors significantly reduce the risk of non-fatal myocardial infarction (57).

In this context, arrestin pathway-selective AT$_1$ agonists like SII, and potentially TRV120027 (37, 38), may constitute another pharmacodynamically distinct entity. Unlike ACE inhibitors, which simultaneously inhibit RAS and enhance KKS signaling, or angiotensin receptor blockers, which target RAS alone, our data suggest that SII-like drugs may inhibit both pathways by promoting B$_2$ receptor internalization. Given the generally salutary effects of BK in promoting vasodilation via endothelial nitric oxide signaling, one might suppose that dual RAS-KKS inhibition would be disadvantageous. On the other hand, dysregulation of the KKS is associated with progression of the vascular and renal complications of diabetes mellitus (58). In the setting of endothelial denudation, BK can act directly on B$_2$ and B$_3$ bradykinin receptors expressed by VSMC to promote vasoconstriction in a manner similar to AngII (21). Moreover, genetic deletion of B$_2$ receptors protects against nephropathy progression in experimental diabetes mellitus (59) and is associated with genomic changes in endothelial injury, oxidative stress, and inflammatory pathways (60). Although it is thus unclear whether dual RAK-KKS inhibition
by SII-like drugs would prove beneficial or harmful in vivo, our data indicate that arrestin pathway-selective AT1 receptor agonism represents a novel approach to RAS targeting with unique functional properties.

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