Supporting Information

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SI Materials and Methods

Cell Lines, Chemicals, and Plasmids. HEK-293 cells stably expressing AT1aR (1) and β2AR (2) and plasmids for DRY/AAY mutant of AT1aR (1) and β2AR$^{GRK-/PKA-}$ mutant (2) were described previously. The cells were maintained in designated culture media at 37°C in a humidified 5% CO_2 incubator and transfected by using FuGENE 6 (Roche Applied Biosciences). The Luc–β-arr–YFP and Luc–β-arr (169)–YFP constructs were kindly provided by Michel Bouvier (Université de Montréal, Montréal), and HEK-293 cells stably expressing PTH1R were a kind gift from Robert Nissenson (University of California, San Francisco). Coelenterazine h was purchased from Promega, 96-well microplates for BRET assay were from Corning, and H-89 and GFX were obtained from Calbiochem.

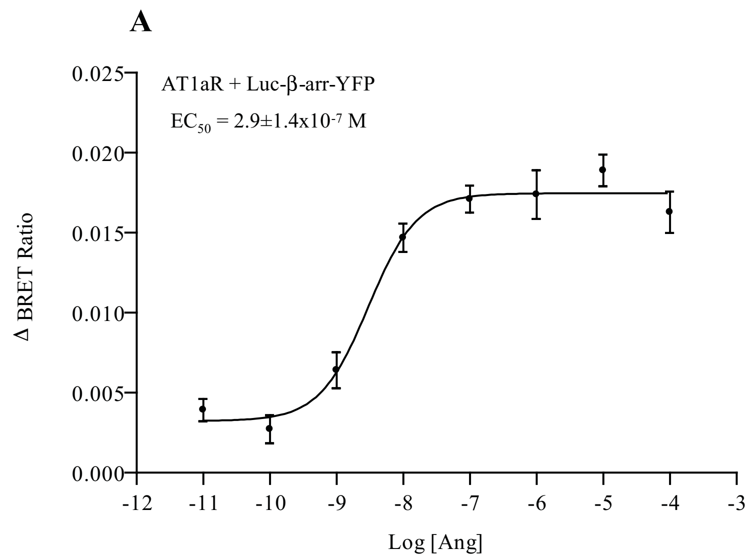
Confocal Microscopy. HEK-293 cells coexpressing the receptor and β-arr biosensor were plated on fibronectin-coated, 35-mm, glass-bottom plates. Cells were stimulated with appropriate ligand, and images of YFP fluorescence were collected by using single-line excitation (488 nm) on a Zeiss laser scanning microscope (LSM-510).

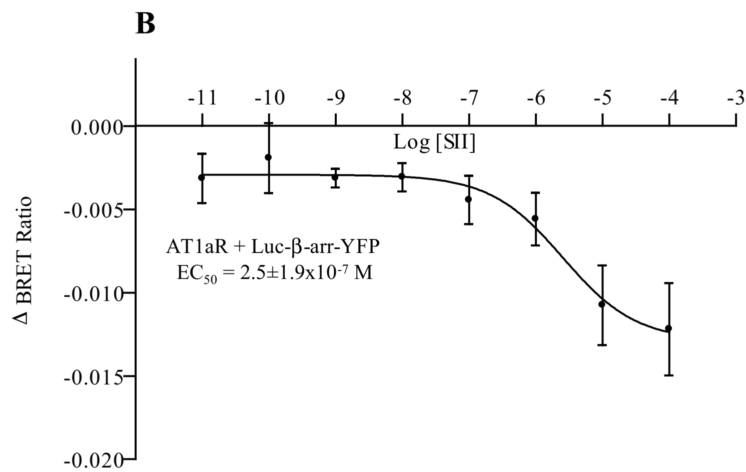
BRET Assay. BRET assays were performed as described in ref. 3. Briefly, 24 h after transfection, HEK-293 cells coexpressing the receptor and the biosensor were distributed in fibronectin coated 96-well microplates (white wall, clear bottom). Before the assay, cells were washed twice with PBS, the transparent bottom of the plate was covered with a white back-tape adhesive, and then cells were incubated with coelenterazine h (final concentration of 5 μM) for 10 min. Subsequently, the cells were stimulated with appropriate ligand for the indicated time period and light emission was detected (460–500 nm for Luc and 510–550 nm for YFP) by using a multilabel reader (Mithras LB 940; Berthold Technologies). The BRET signal was determined as the ratio of the light emitted by YFP and the light emitted by Luc. For dose–response curves, different concentrations (as indicated in the figures) of ligands were used and the BRET ratio was monitored at 5–10 min after ligand stimulation. The values were corrected by subtracting the background BRET signals detected when Luc–β-arr was expressed alone.

For the *in vitro* BRET assay, the Luc–β-arr–YFP was purified from HEK-293 cells using GFP antibody-coupled agarose (Santa Cruz Biotechnology). The β2AR was expressed as 6-histidine fusion protein in Sf9 cells using the baculovirus system and purified using Ni-NTA resin. The purified receptor was reconstituted in phosphatidyl choline vesicles as described in ref. 4. To monitor the changes in BRET ratio, the purified Luc–β-arr–YFP was incubated with β2AR containing vesicles for 10–15 min at room temperature in presence or absence of indicated ligands. Subsequently, coelenterazine h was added to the reaction, and the changes in the BRET ratio were recorded.

To compare the effect of different ligands with varying efficacies on the conformational changes in β-arrestin, seven additional ligands for the β2AR were used at receptor-saturating concentrations as described in ref. 2.

1. Wei H, *et al.* (2003) Independent β-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc Natl Acad Sci USA* 100:10782–10787.
2. Drake M, *et al.* (2007) β-arrestin-biased agonism at the β2-adrenergic receptor. *J Biol Chem* 283:5669–5676.
3. Charest PG, Terrillon S, Bouvier M (2005) Monitoring agonist-promoted conformational changes of β-arrestin in living cells by intramolecular BRET. *EMBO Rep* 6:334–340.
4. Cerione RA, *et al.* (1984) Reconstitution of a hormone-sensitive adenylate cyclase system. The pure β-adrenergic receptor and guanine nucleotide regulatory protein confer hormone responsiveness on the resolved catalytic unit. *J Biol Chem* 259:9979–9982.





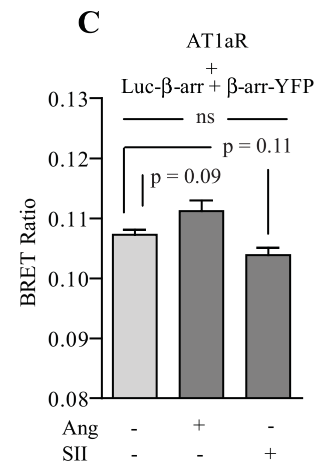
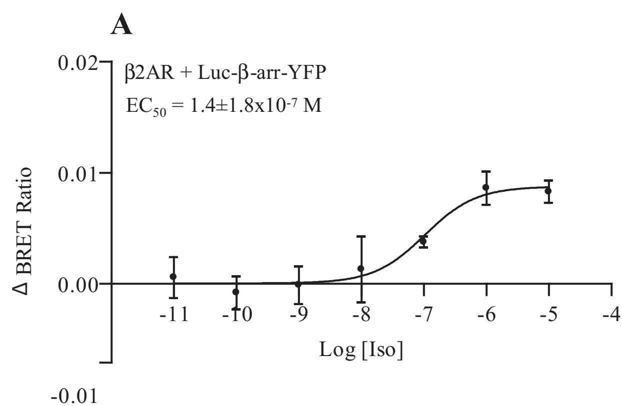
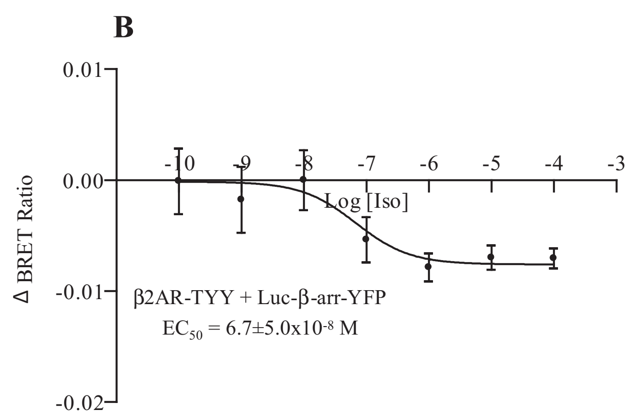


Fig. S1. Dose dependency of the conformational changes in β-arrestin 2 upon stimulation of AT1aR by Ang (*A*) and SII (*B*). Ang II (100 nM, 10 min) and SII (10 μM, 5 min) did not induce changes in intermolecular BRET ratio upon coexpression of Luc–β-arr and β-arr–YFP (*C*). Data are mean ± SD of four independent experiments.





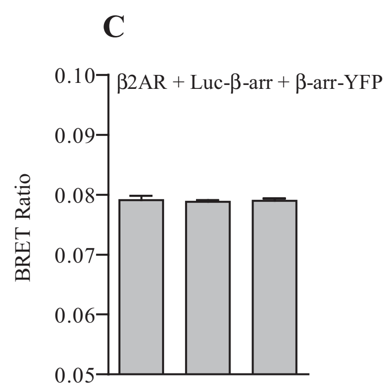


Fig. S2. Dose dependency of the conformational changes in β-arrestin 2 upon stimulation of β2AR (*A*) or β2AR^{TYY} (*B*) by Isoproterenol. Isoproterenol (1 μM, 10 min) and propranolol (10 μM, 10 min) did not induce changes in intermolecular BRET ratio upon coexpression of Luc–β-arr and β-arr–YFP (*C*). Data are mean ± SD of four independent experiments.

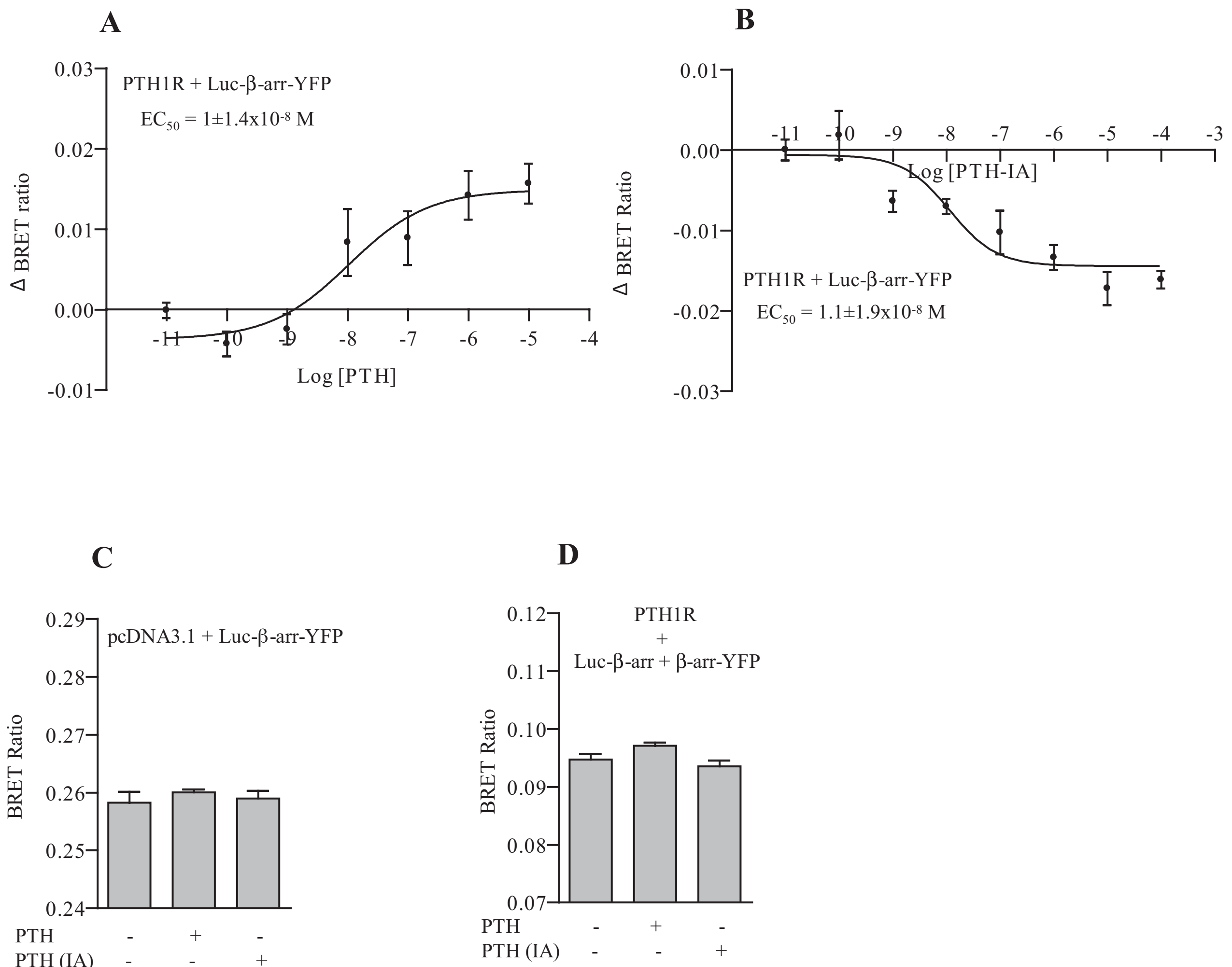


Fig. S3. Dose dependency of the conformational changes in β-arrestin 2 upon stimulation of PTH1R by PTH (*A*) and PTH-IA (*B*). PTH (1–34) (100 nM, 10 min) and PTH-IA (1 μM, 10 min) did not induce changes in intermolecular BRET ratio in either mock transfected cells (*C*) or upon coexpression of Luc–β-arr and β-arr–YFP (*D*). Data are mean ± SD of four independent experiments.

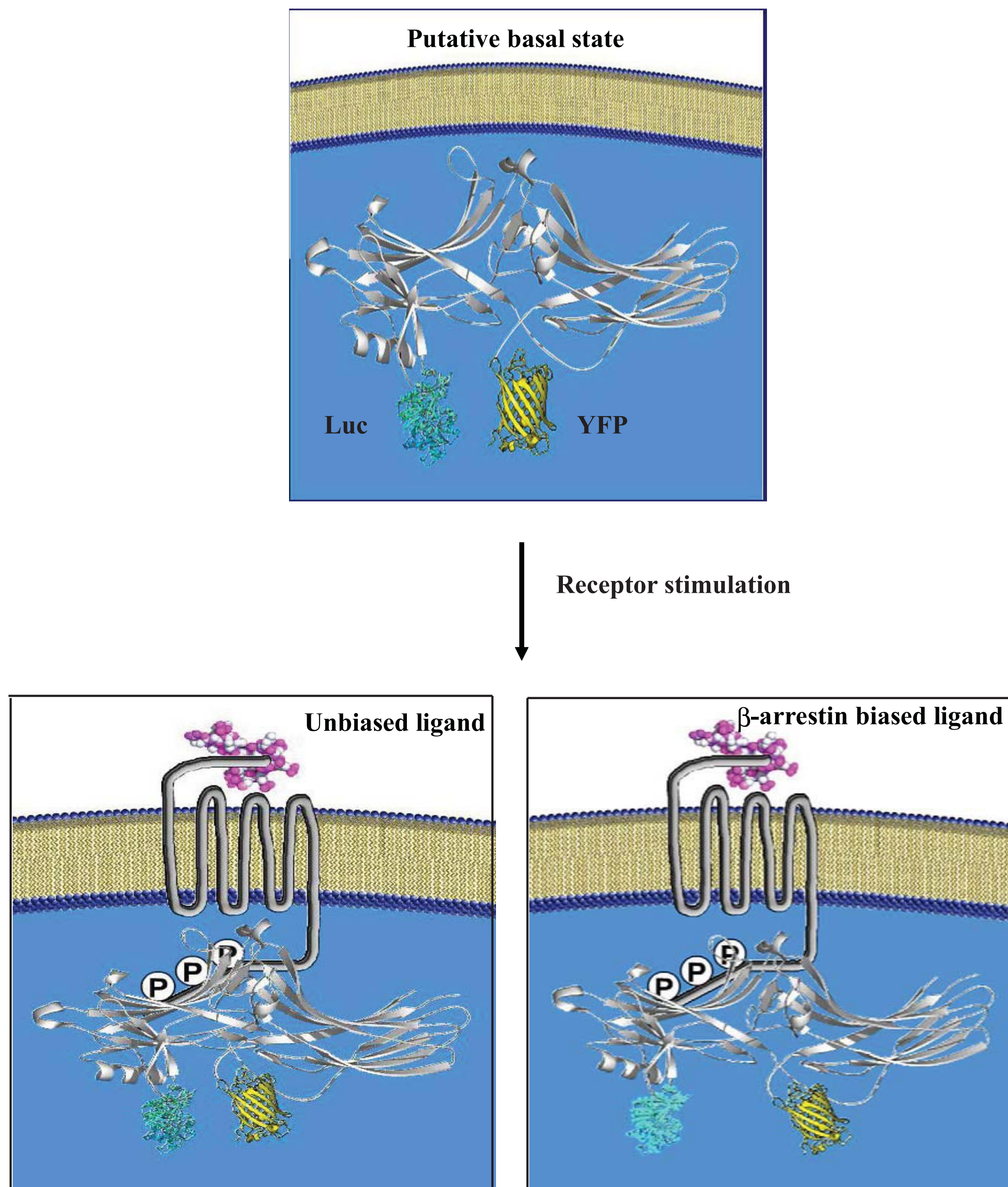


Fig. S4. A schematic representation of putative basal and active states of Luc–β-arr–YFP biosensor. While an increase versus a decrease in the BRET ratio reflects different conformations, it does not necessarily indicate the movement of the N and the C termini closer to or away from each other. Such a scenario, which represents one possibility, is shown in the diagram just for illustrative purposes.

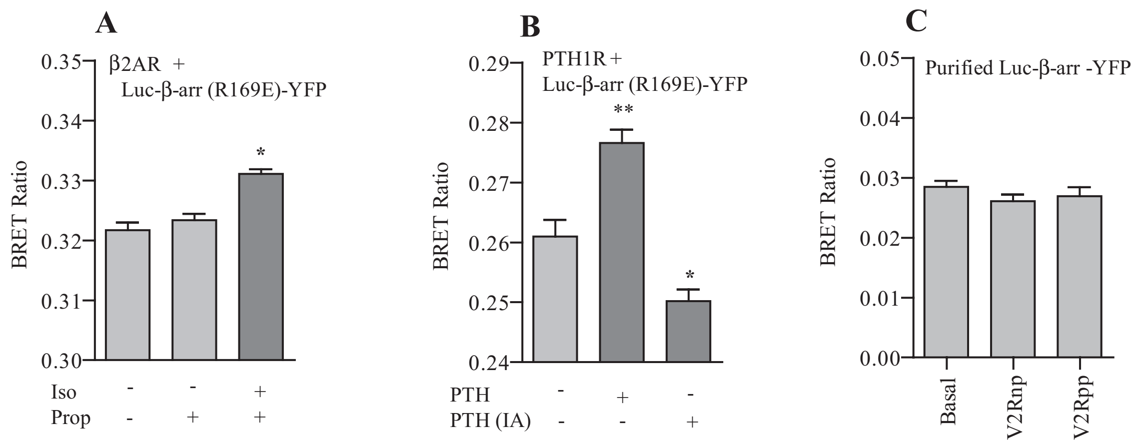


Fig. S5. Phosphorylation independent conformational change in β-arrestin. The phosphorylation independent β-arrestin mutant Luc–β-arr (R169E)–YFP was coexpressed with either the β2AR (*A*) or the PTH1R (*B*), and the changes in the BRET ratio were measured as described earlier. Luc–β-arr–YFP does not undergo a conformational change upon incubation with V_2Rpp or V_2Rnp (*C*). Purified Luc–β-arr–YFP fusion protein was incubated for 15 min on ice with 1:10 molar ratio of either V_2Rpp or V_2Rnp (phosphorylated and nonphosphorylated versions of a peptide corresponding to the C terminus of the vasopressin receptor) [Xiao K, Shenoy SK, Nobles K, Lefkowitz RJ (2004) Activation-dependent conformational changes in β-arrestin 2. *J Biol Chem* 279:55744–55753]. Subsequently, coelenterazine h was added to the reaction, and BRET changes were recorded ($n = 4$, two independent purifications of Luc–β-arr–YFP).