Supplementary data file

Article's title: Investigation of the fate of type I angiotensin receptor after biased activation

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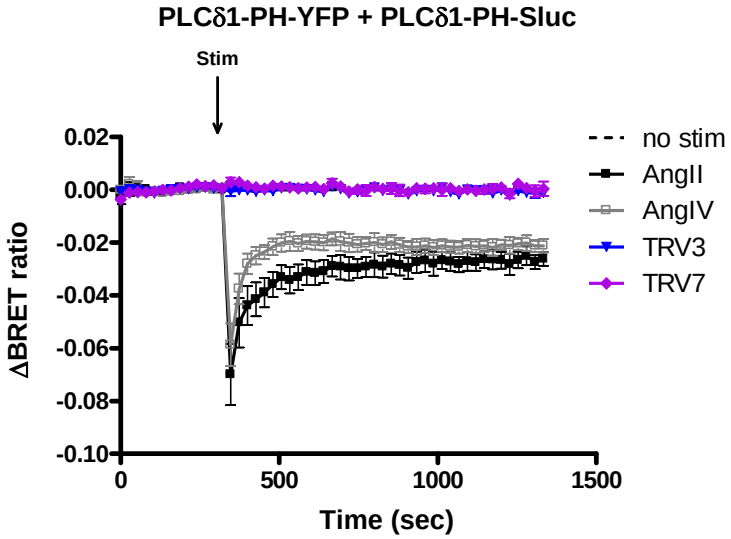
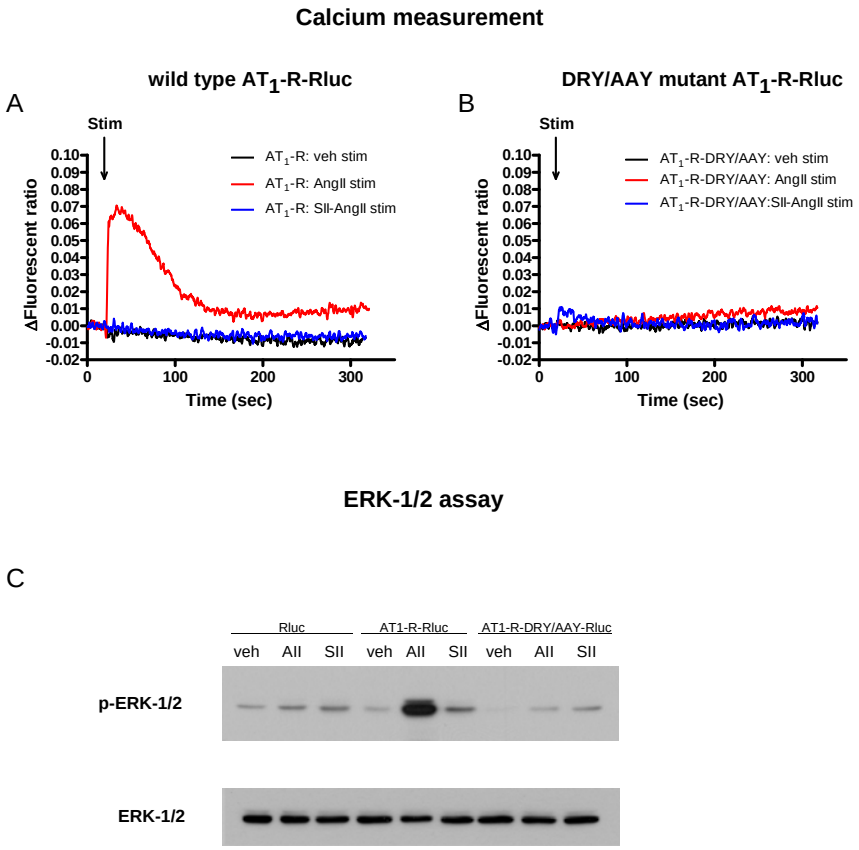
Running title: Fate of biased activated AT_1-receptor

Supplemental Figure 1.



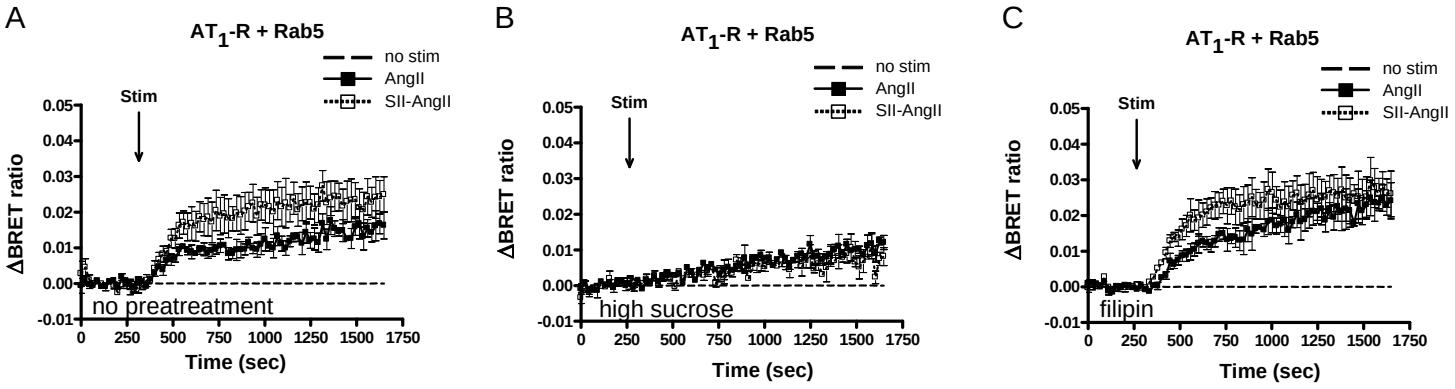
Supplemental Figure 1. Effects of AT_1-R agonist stimulation on PtdIns(4,5)P_2 breakdown in HEK293 cells. HEK293 cells were transfected with the plasmids of the AT_1-R, PLCδ1-PH-YFP and PLCδ1-PH-Sluc, and after 24 hours the cells were exposed to either 100 nM AngII (black trace), 1 μM TRV120023 (labeled as TRV3, blue trace), 1 μM TRV120027 (labeled as TRV7, purple trace), 10 μM AngIV (grey trace), or vehicle (dashed lines) at the indicated time point. The BRET records are average of 3 independent experiments. Mean values ± SEM are shown (n = 3).

Supplemental Figure 2.



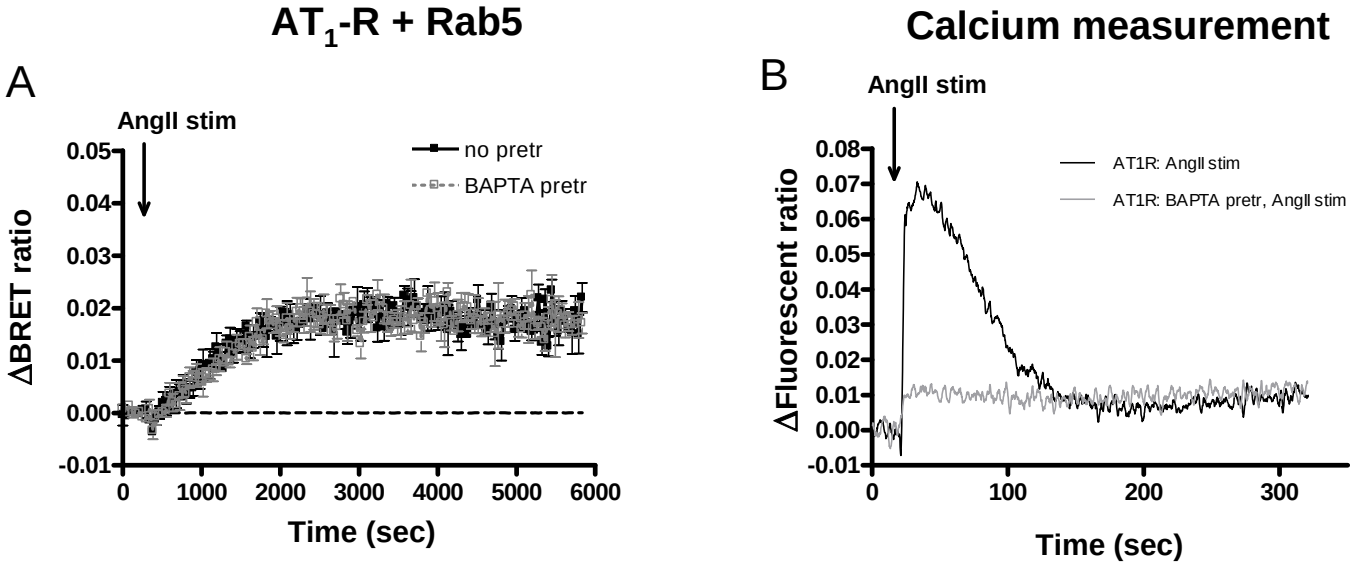
Supplemental Figure 2. Functional analysis of the AT_1-R-Rluc and AT_1-R-DRY/AAY-Rluc. HEK293 cells were transfected with the plasmids of the indicated AT_1-R-luciferase (wild type or DRY/AAY mutant), and after 24 hours the cells were used for studies. (A-B) Cytoplasmic Ca^{2+} measurement. The Fura-2-loaded HEK293 cells were exposed to either 100 nM AngII (red trace), 10 μM SII-AngII (blue trace), or vehicle (black trace) at the indicated time points. The curves are representative of 3 independent experiments. (C) ERK-1/2 MAPK activation. HEK293 cells were exposed to vehicle, 100 nM AngII or 10 μM SII-AngII for 5 min. The western blot is a representative of 3 independent experiments.

Supplemental Figure 3.



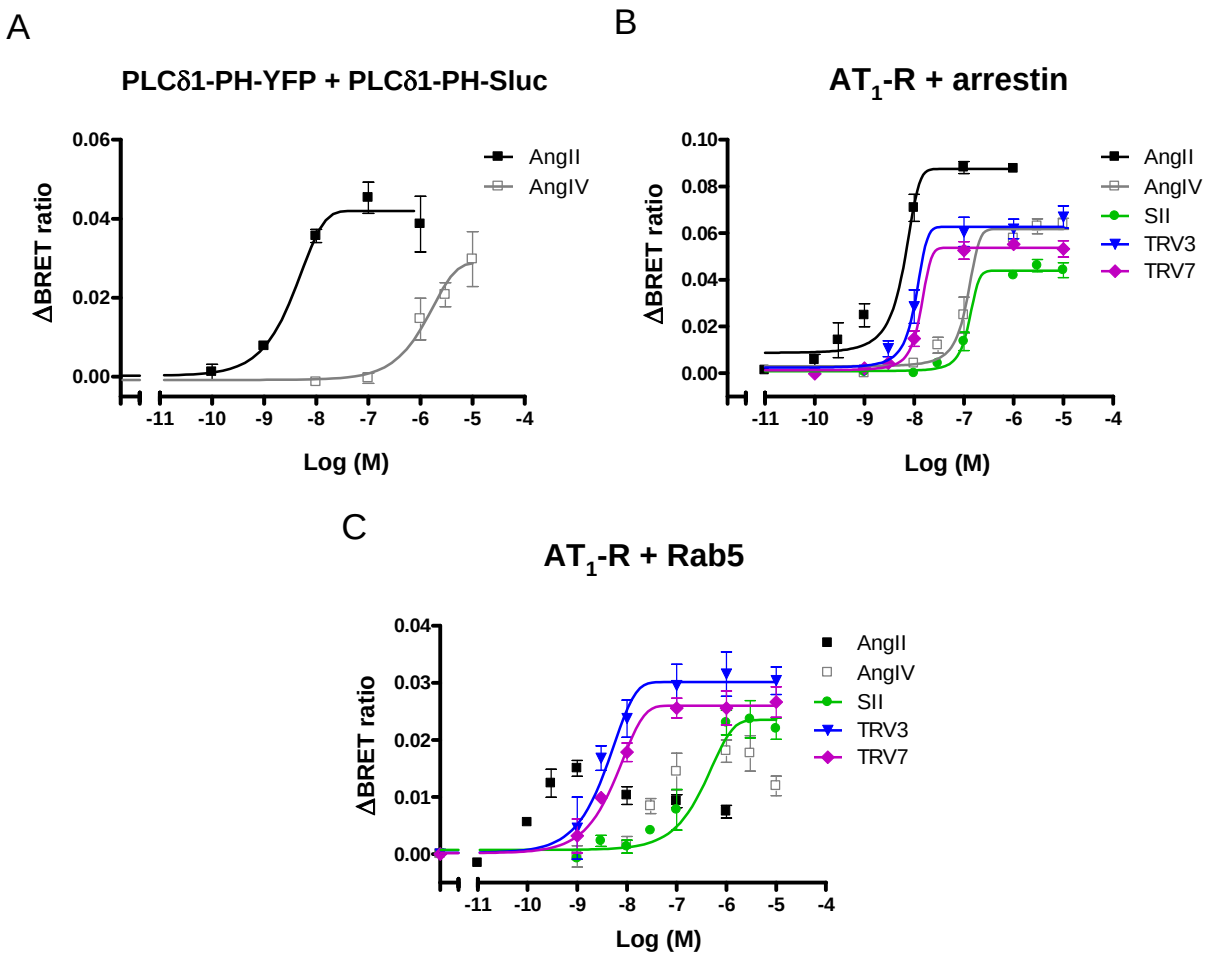
Supplemental Figure 3. BRET assay between AT_1-R and Rab5 upon either AngII or SII-AngII stimulation in HEK293 cells. HEK293 cells were transfected with the plasmids of the AT_1-R-Rluc and with Rab5-YFP proteins, and after 24 hours the experiments were carried out. Cells were pretreated for 30 min with vehicle BRET medium (**A**), BRET medium supplemented with 300 mM sucrose (**B**), or 5 μg/ml filipin (**C**) and exposed to either vehicle (dashed line) or 100 nM AngII (black filled symbols) or 10 μM SII-AngII (grey open symbols) at the indicated time points. The BRET curves are average of 3 independent experiments, each performed in triplicates. Mean values ± SEM are shown (n = 3).

Supplemental Figure 4.



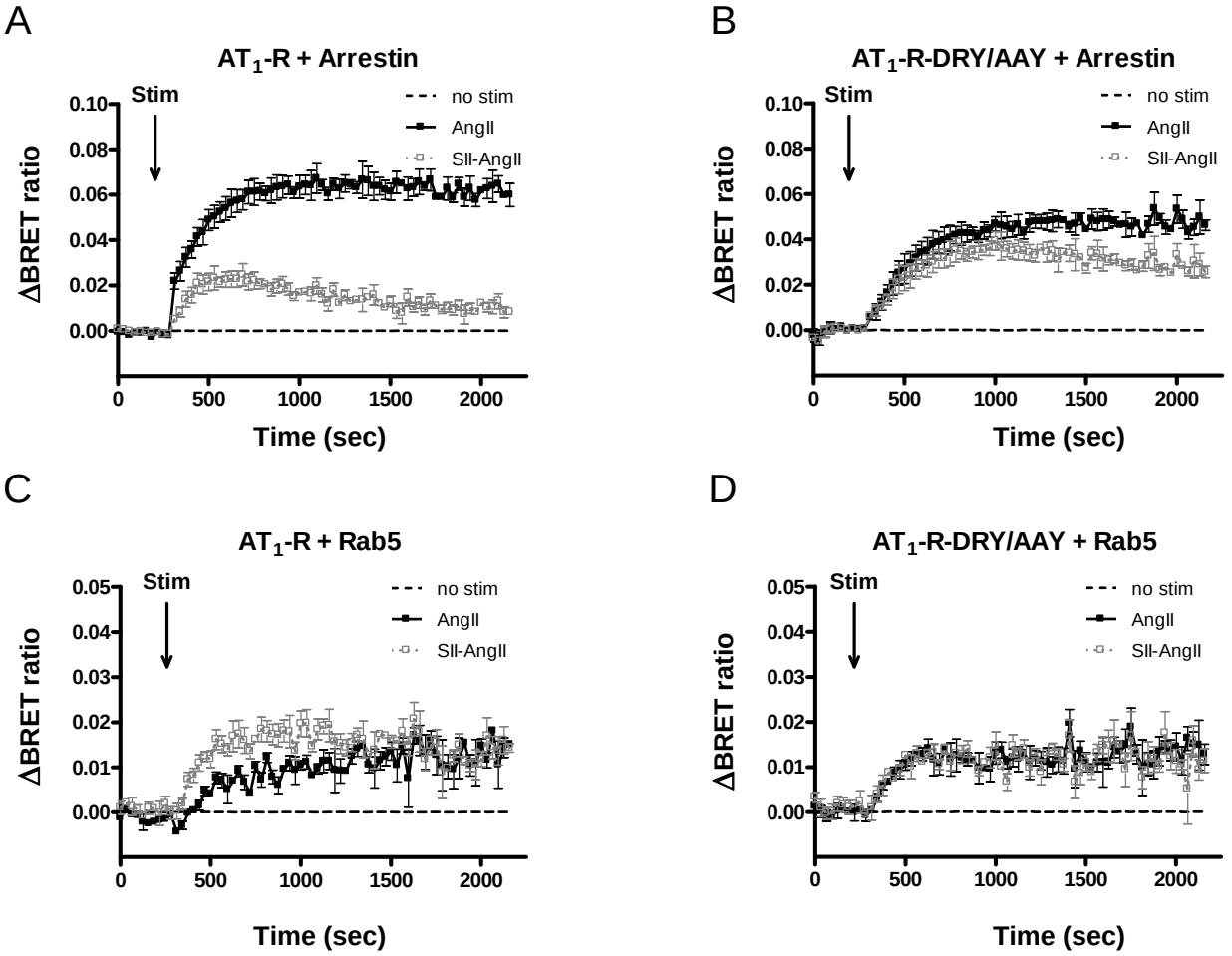
Supplemental Figure 4. Effect of intracellular Ca^{2+} chelation on AT_1-R internalization upon AngII stimulation in HEK293 cells. HEK293 cells were transfected with the plasmids of the AT_1-R-Rluc and with either Rab5-YFP (A) or PLCδ1-PH-YFP (C), and after 24 hours the cells were pretreated for 30 min with either vehicle BRET medium (black traces) or BRET medium supplemented with 10 μM BAPTA-AM for 30-45 min (grey traces) and exposed to either vehicle (dashed line, A and B) or 100 nM AngII (A-C) or 10 μM ionomycin (C) at the indicated time points. The BRET curves (A and C) are average of 3 independent experiments, each performed in triplicates, mean values ± SEM are shown (n = 3); the fluorescent ratio curves for the cytoplasmic Ca^{2+} measurements (B) are representative of 3 independent experiments.

Supplemental Figure 5.



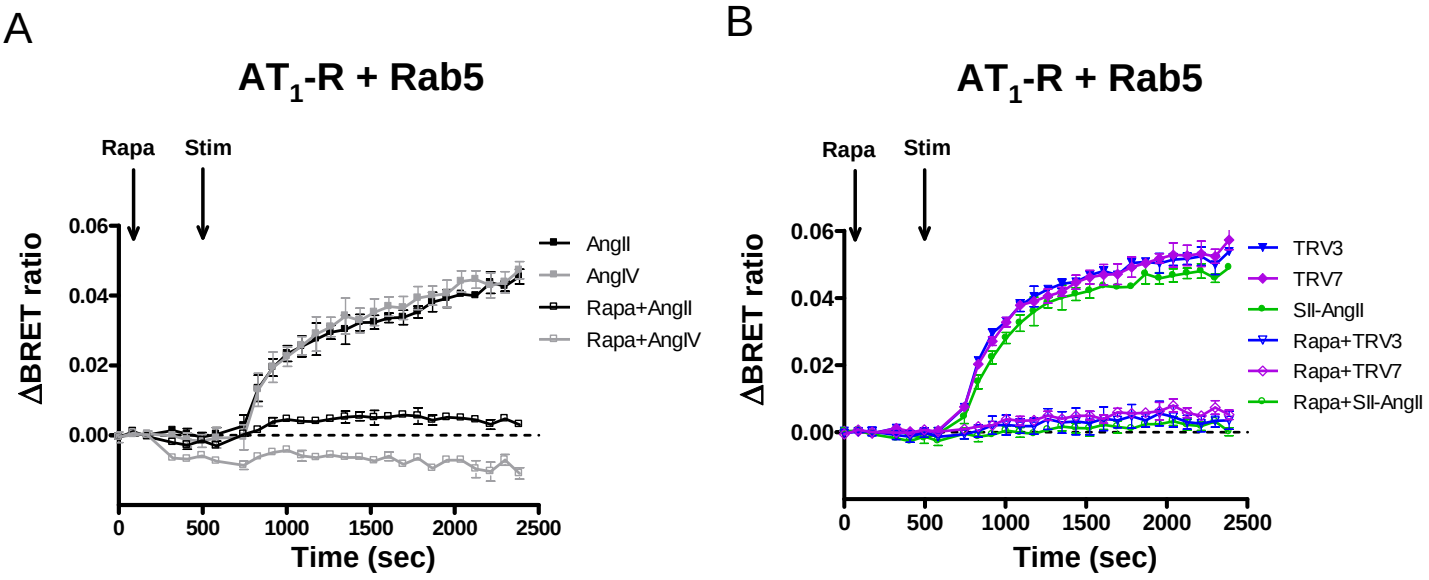
Supplemental Figure 5. Dose-response curves of AT_1-R induced PtdIns(4,5)P_2 hydrolysis (A), β-arrestin2 binding (B), and Rab5 recruitment (C) in HEK293 cells. HEK293 cells were transfected with the plasmids of the AT_1-R, PLCδ1-PH-YFP and PLCδ1-PH-Sluc (A), AT_1-R-Rluc and β-arrestin2-YFP (B), AT_1-R-Rluc and Rab5-YFP (C) and after 24 hours the cells were exposed to various concentrations of AngII, AngIV, SII-AngII, TRV120023 (labeled as TRV3), TRV120027 (labeled as TRV7). The BRET records are average of 3 independent experiments, and the following data points were used for the calculations: (A) BRET values in the first minute after stimulation, (B) BRET values between 5-6 minutes after stimulation, (C) BRET values between 9-11 minutes after stimulation. Mean values ± SEM are shown (n = 3).

Supplemental Figure 6.



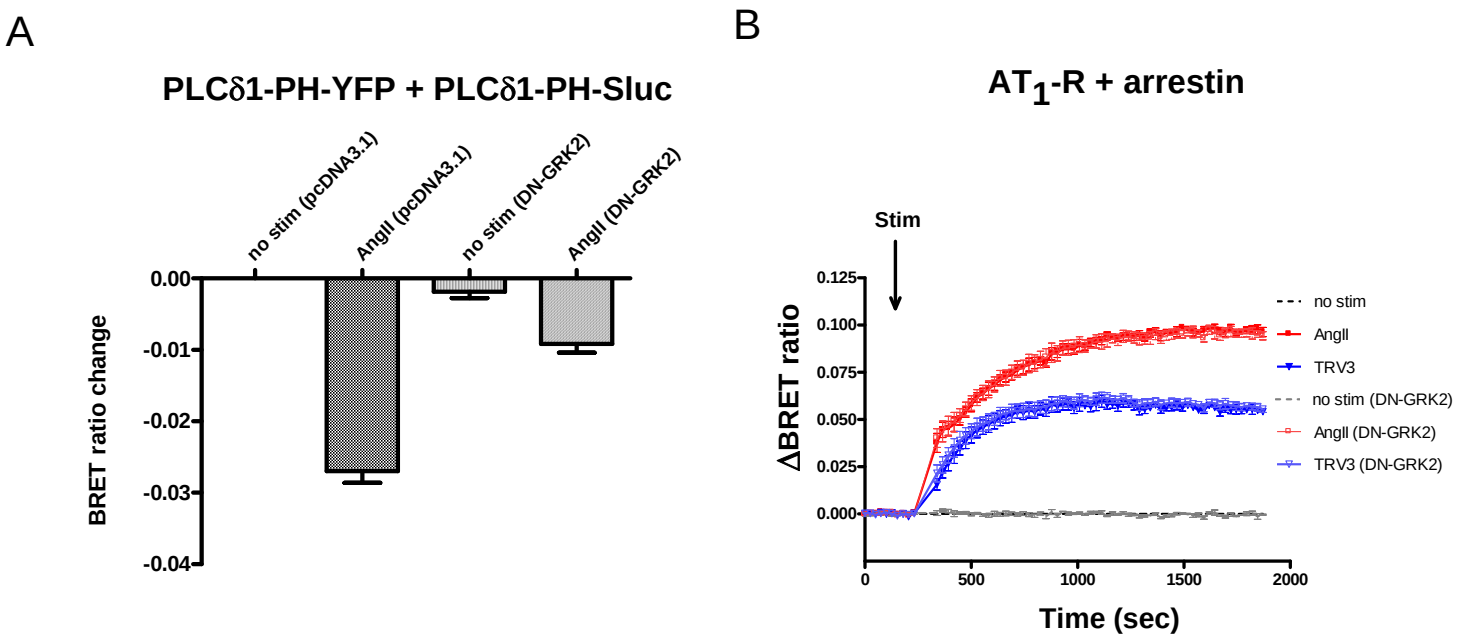
Supplemental Figure 6. BRET assay between wild type or mutant AT_1-Rs and β-arrestin2 or Rab5 upon either AngII or SII-AngII stimulation in HEK293 cells. HEK293 cells were transfected with the plasmids of the indicated receptor-Rluc and with either β-arrestin2-YFP (A-B) or Rab5-YFP (C-D), and after 24 hours the cells were exposed to either vehicle (dashed line) or 100 nM AngII (black filled symbols) or 10 μM SII-AngII (grey open symbols) at the indicated time points. BRET pairs: (A) AT_1-R-Rluc and β-arrestin2-YFP; (B) AT_1-R-DRY/AAY-Rluc and β-arrestin2-YFP; (C) AT_1-R-Rluc and Rab5-YFP; (D) AT_1-R-DRY/AAY-Rluc and Rab5-YFP. The BRET records are average of at least 3 independent experiments. Mean values ± SEM are shown (n = 3).

Supplemental Figure 7.



Supplemental Figure 7. Plasma membrane PtdIns(4,5)P_2 depletion effects AT_1-R internalization. HEK293T cells were transfected with plasmids encoding AT1-R-Rluc, Venus-Rab5, PM-FRB-mRFP and mRFP-FKBP-5ptase. After 24 hours the cells were pretreated with either rapamycin (300 nM) or vehicle (DMSO) for 5 minutes, followed by stimulation with the indicated agonists (100 nM AngII, 1 μM TRV120023, 1 μM TRV120027, 10 μM SII-AngII or 10 μM AngIV) and (A) Rab5 recruitment and (B) β-arrestin2 binding were detected in BRET measurements.

Supplemental Figure 8.



Supplemental Figure 8. Effects of DN-GRK overexpression on plasma membrane PtdIns(4,5)P_2 hydrolysis and β-arrestin2 binding of AT_1-R. HEK293 cells were transfected with plasmids encoding (A) DN-GRK2, AT_1-R, PLCδ1-PH-YFP and PLCδ1-PH-Sluc, or (B) DN-GRK2, AT_1-R-Rluc and β-arrestin2-YFP. (A) The cells were exposed to 100 nM AngII and the PtdIns(4,5)P_2 hydrolysis caused BRET ratio change was detected for three minutes. (B) The cells were exposed to either 100 nM AngII (red trace), 1 μM TRV120023 (labeled as TRV3, blue trace), or vehicle (dashed lines) at the indicated time point. The BRET records are average of 3 independent experiments. Mean values ± SEM are shown (n = 3).