Interruption of the ionic lock in the bradykinin B_2 receptor results in constitutive internalization and turns several antagonists into strong agonists

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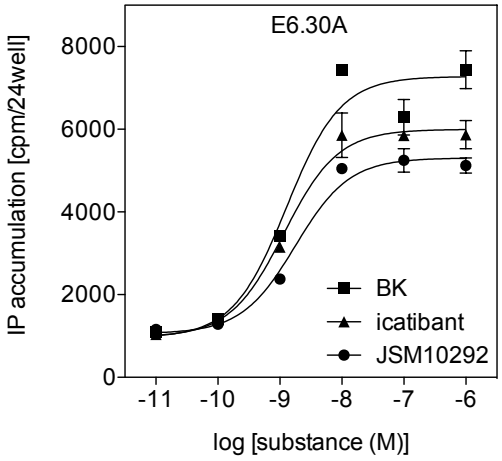
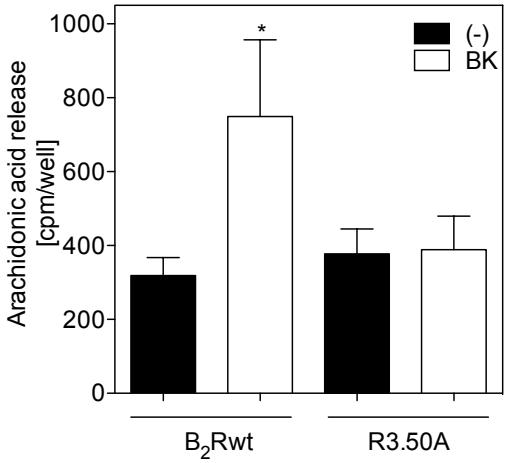
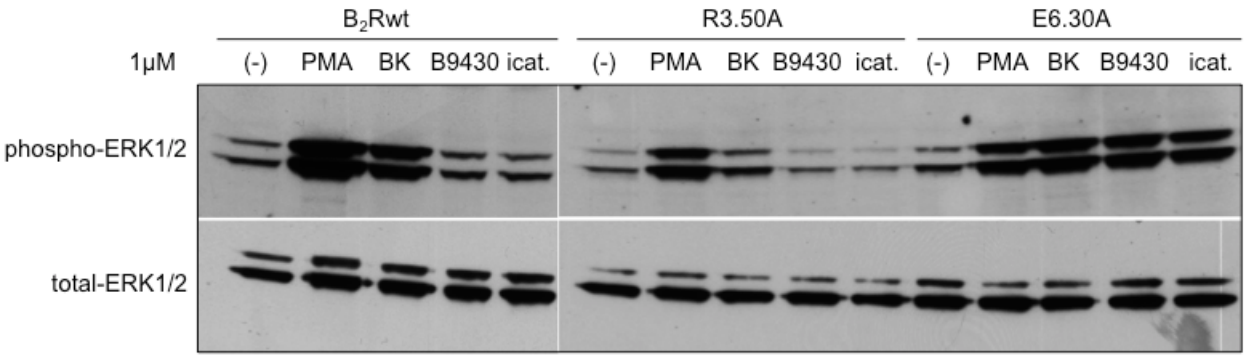


Supplemental Figure 1. Dose-dependent IP accumulation of E6.30A mutant expressing cells in response to B_2R agonist and antagonists. HEK293 cells stably expressing mutant E6.30A and pre-incubated overnight with [^{3}H]inositol, were stimulated with the indicated concentrations of BK, icatibant or JSM10292 for 30 min at 37°C as described in "Material and Methods". Shown is a representative experiment that was repeated three times with similar results.

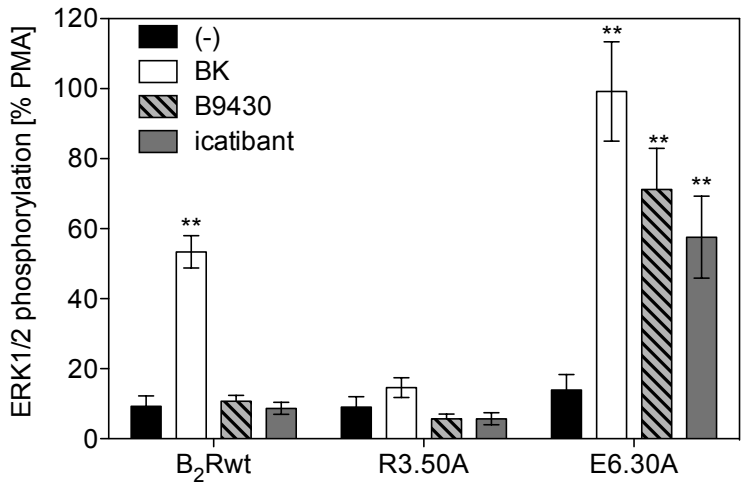


Supplemental Figure 2. Arachidonic acid release. HEK293 cells stably expressing B_2Rwt and R3.50A were cultured in 24-wells. To each well 0.5 μCi [^{3}H]arachidonic acid was added and cultivation was continued for 18 h. Thereafter, cells were rinsed with 37°C warm medium containing 2 mg/ml fatty acid free BSA (bovine serum albumin) and further incubated for 1 h at 37°C in the same medium. Subsequently, cells were washed once with BSA-containing medium and incubated in 300 μl of the same medium in the absence or presence of 1 μM BK for 20 min at 37°C. The release of [^{3}H]arachidonic acid and its metabolites was determined by measuring 200 μl of the cell supernatant in a β-counter after addition of scintillation liquid. Data represent means ± SEM of three independent experiments performed in triplicate. (Comparison of non-stimulated (-) and BK-stimulated arachidonic acid release: $^*P < 0.05$).

A



B



Supplemental Figure 3. B_2R-stimulated activation of ERK1/2. (A) Western blot of B_2R-induced phospho-ERK1/2 and total-ERK1/2. Cells stably expressing B_2Rwt, mutant R3.50A, or mutant E6.30A were serum-starved in Opti-MEM overnight. They were either left unstimulated (-) or treated with 1 µM BK, B9430 or icatibant (icat.) for 10 min at 37°C as indicated. Treatment with 1µM PMA (phorbol-12-myristate-13-acetate, Calbiochem), a potent protein kinase C activator, served as a reference for maximal response (=100%). Protein extraction was performed as described in "Material and Methods (Receptor phosphorylation)" and detection of phospho- and total-ERK1/2 levels (loading control) was carried out using the appropriate antibodies (monoclonal, Cell Signaling, dilution 1/2000). The blot shown is representative of three experiments. **(B) Quantification.** PMA treatment served as positive control and reference for maximal response (=100%). Phospho-ERK1/2 levels were normalized to PMA stimulation and total-ERK1/2 levels in each cell line. Values represent means ± SEM of three independent experiments. (One-way ANOVA with Dunnett's Multiple Comparison Test; Comparison of non-stimulated (-) versus stimulated: $^{**}P < 0.01$).