# The Effect of Hormone-induced PtdIns(4,5)$P_2$-depletion on Endocytosis Suggests the Importance of Local Regulation of Inositol Lipid Signalling

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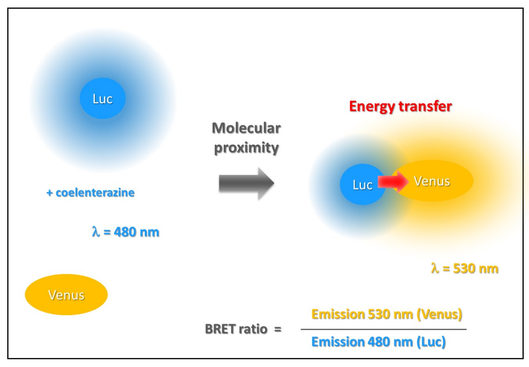
## Introduction

Receptor endocytosis plays an important role in regulating the responsiveness of cells to specific hormones. Phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)$P_2$] has been shown to be critical for many endocytic processes including the internalization of G protein-coupled receptors (GPCRs). Our aim in this study was to compare the effect of two distinct plasma membrane PtdIns(4,5)$P_2$ depletion methods on GPCR internalization in HEK 293 cells.

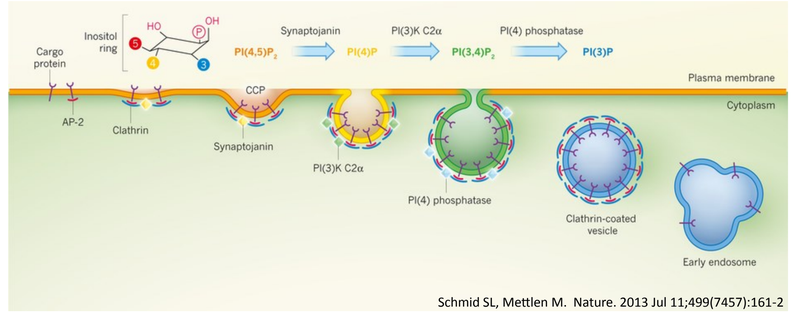
We used bioluminescence resonance energy transfer (BRET) and confocal microscopy to determine the effectiveness of our depletion methods and to follow the endocytosis of $\beta_2$ adrenergic receptor ($\beta_2$AR).

## Bioluminescence Resonance Energy Transfer




Bioluminescence resonance energy transfer (BRET) is a highly sensitive method for detection of molecular interactions. The energy acceptor Venus emits light only when the donor luciferase is within a distance of 10 nm. By genetic fusion of each molecule to our proteins of interest, we could investigate their interactions in live cells, reflected best in the changes of the BRET ratio.

## Phosphoinositides in clathrin-mediated endocytois

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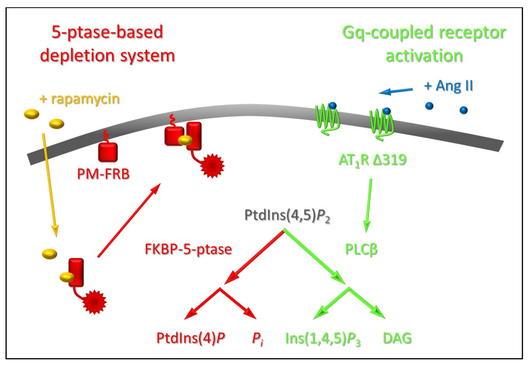
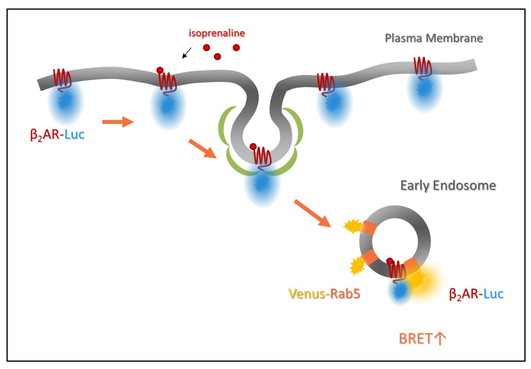
One of the latest models for the role of phophoinositides and their metabolizing enzymes in clathrin-mediated endocytosis. Although it is now well established that PtdIns(4,5)$P_2$ and also other phosphoinositides are essential players in this process, their exact function and point of action, as well as the relative contribution of each lipid to the process still remain unclear.

## Two distinct PtdIns$P_2$ depletion methods



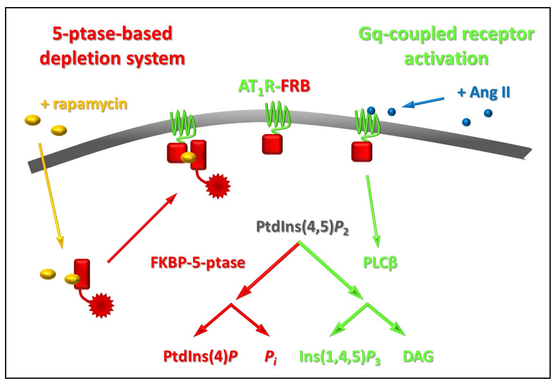

Previously we confirmed the PtdIns(4,5)$P_2$-dependence of GPCR endocytosis using the 5-phosphatase (5-ptase) based lipid depletion system depicted on the left (in red). The initially cytoplasmic FKBP-5-ptase translocates to the plasma membrane as rapamycin induces heterodimerization of FKBP with FRB anchored to the plasma membrane, and PtdIns(4,5)$P_2$ is dephosphorylated as the red arrows show. Here we tested the effects of the green arrows where the same lipid is degraded by activation of PLCβ through an internalization-incompetent mutant of $AT_1R$ (Δ319).

## Monitoring receptor endocytosis with BRET




In our previous studies we used several interactions as indicators for various stages of the endocytic process. Here we examined the interaction of $\beta_2$ adrenergic receptor ($\beta_2$AR) fused to luciferase with the endosomal marker Rab5 labeled with Venus. An increase in the BRET ratio between these molecules is indicative of the receptor arriving at the early endosomal compartment.

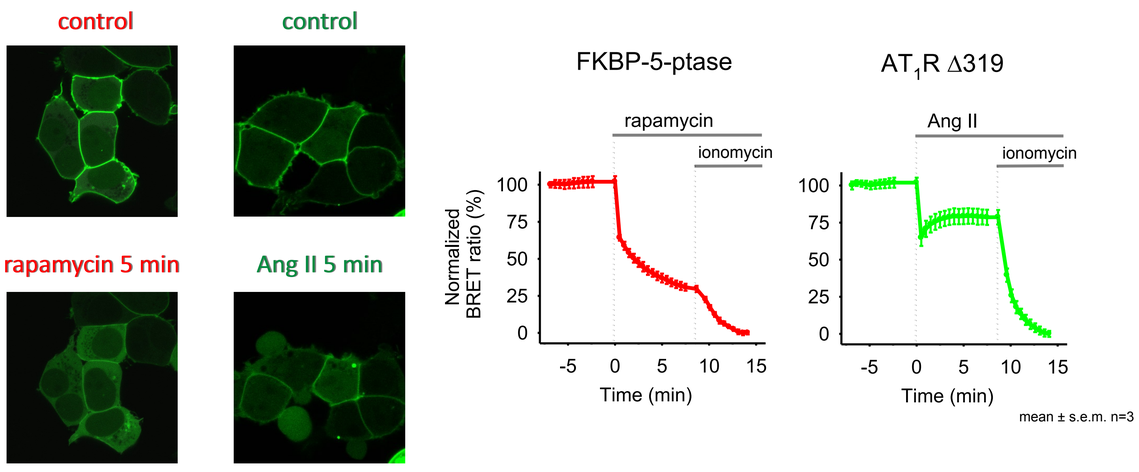
## Combining both depletion methods in one system




In order to combine both depletion methods into one system, we fused the FRB domain to $AT_1R$. Here we used either the wild type receptor, or another internalization-incompetent mutant (TSTS-AAAA). When coexpressed with FKBP-5-ptase, this $AT_1R$-FRB construct is able to degrade PtdIns(4,5)$P_2$ through both ways, depending on stimulation. This way we could compare the two methods more directly and also achieve similar rates of depletion.

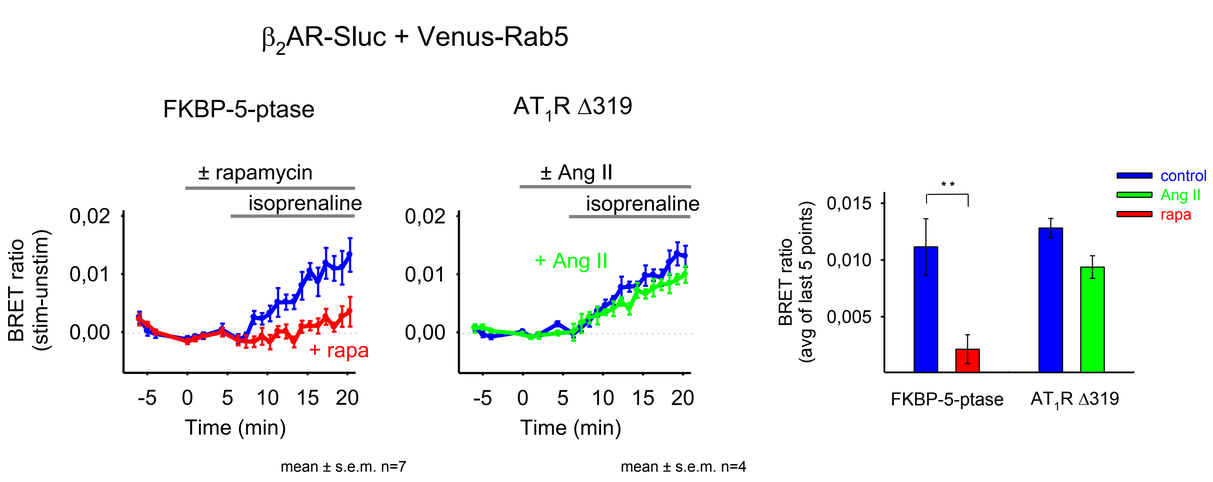
## Results

### Detection of PtdIns$P_2$ degradation by confocal microscopy and BRET



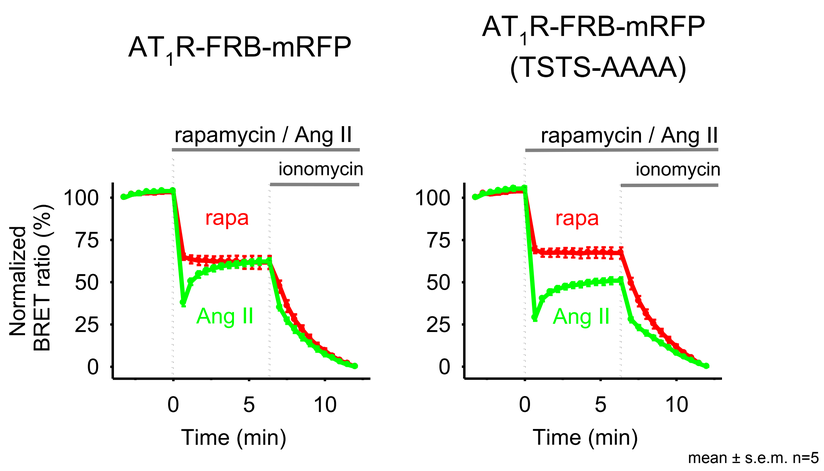

For confocal detection of PtdIns(4,5)$P_2$-depletion we transfected HEK 293 cells with either our 5-ptase based system (left side) or $AT_1R$ Δ319 (right side), and the specific PtdIns(4,5)$P_2$-binding PH domain of PLCδ1 tagged with Venus (PLCδ1 PH-Venus). For BRET measurements, we used the luciferase-tagged version of this domain instead (PLCδ1 PH-Sluc) and coexpressed it with a plasma membrane targeted Venus (PM-Venus). The BRET ratio decreased upon lipid depletion (300 nM rapamycin or 100 nM Ang II) as the PH domain relocated to the cytoplasm (visible on the confocal images). We used ionomycin (10 μM) to induce total depletion and normalized our data to this baseline.

### The effect of PtdIns$P_2$ depletion on $\beta_2$AR endocytosis



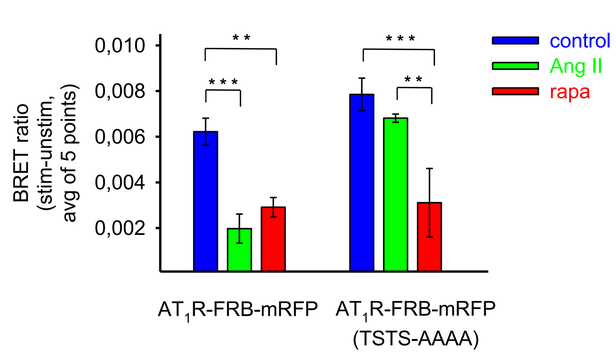

To compare the effect of the two PtdIns(4,5)$P_2$-depletion methods, we transfected HEK 293 cells with either of them as indicated, along with $\beta_2$AR-Sluc and Venus-Rab5, and after addition of rapamycin (300 nM) or Ang II (100 nM) cells were stimulated with isoprenaline (1 μM). The inhibitory effect was quantified as the average of the last 5 points in each experiment. The data were tested by two-way ANOVA with Bonferroni post-hoc test. **p<0,01

### Comparison of PtdIns$P_2$ depletion in the combined system




We compared the PtdIns(4,5)$P_2$ depletion rate in the combined system by BRET as described for the separate systems (to the left). We tested both the wild type $AT_1R$-FRB and the TSTS-AAAA desensitization- and internalization-incompetent mutant, and lipid depletion reached a higher degree for the mutant version after Ang II stimulation.

### Comparison of endocytosis inhibition in the combined system




We tested the ability of the combined system to inhibit endocytosis as described for the separate systems (to the left). Despite the higher degree of PtdIns(4,5)$P_2$-depletion, TSTS-AAAA mutant $AT_1R$-FRB was unable to inhibit $\beta_2$AR endocytosis when stimulated by Ang II, whereas the wild type showed potent inhibition. Rapamycin was equally effective in both cases. ** p<0,01; *** p<0,001

## Conclusion

Our data suggest that stimulation of wild type $AT_1R$ inhibits $\beta_2$AR internalization by competition for the endocytic machinery. Using internalization-incompetent $AT_1R$ mutants we found that the effect of plasma membrane PtdIns(4,5)$P_2$ depletion on $\beta_2$AR internalization depends on the method of lipid degradation, suggesting the importance of local phosphoinositide pools in the regulation of receptor endocytosis.

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