SUPPLEMENTARY MATERIAL

Receptor activity-independent recruitment of βarrestin2 reveals specific signalling modes

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MATERIALS AND METHODS

Materials

Mouse anti-Myc (9E10) and anti-HA (12CA5) monoclonal antibodies were produced by our core facility as ascite fluids. Rabbit anti-Myc (A14), mouse anti-PERK1/2 (E4), rabbit anti-ERK1/2 (K23), goat anti-rabbit and anti-mouse coupled to Texas Red as well as goat anti-mouse coupled to Alexa 633 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies were from Amersham Pharmacia Biotech (Little Chalfont, United Kingdom). The renaissance chemiluminescence kit was from PerkinElmer Life Sciences (Boston, MA). Coelenterazine h was from Molecular Probes (Eugene, OR). The noncommercially available ligand AP21967 was graciously provided by Ariad Pharmaceuticals (Cambridge, MA). All other reagents were of analytical grade and obtained from various commercial suppliers.

Expression plasmids

The original constructs of HA-V1aR and Myc-V2R expressed in pRK5 (Terrillon *et al.*, 2003), Myc-δOR expressed in pcDNA3 (Invitrogen) (Petaja-Repo *et al.*, 2002) and βarrestin2-YFP expressed in pGFP-N1-Topaz (PerkinElmer, Life Sciences) (Angers *et al.*, 2000) were previously described. The pC4EN-F_1 (FKBP), pC4M-F_2E (MyrFKBP2) and pC4-

R_HE (FRB) vectors were a generous gift of Dr. V. Rivera (Ariad Pharmaceuticals, Cambridge, MA) (http: //www.ariad.com/regulationkits).

HA-V1aR-FKBP: The HA-V1aR coding sequence was amplified by PCR using sense and antisense primers harbouring unique *SgrAI* and *XbaI* sites so as to introduce an *XbaI* site at the 3' end of V1aR coding sequence and to remove the receptor's stop codon. The FKBP coding sequence was amplified out of its original vector between *XbaI* and *AccI* site-containing primers to generate an *AccI* site downstream of the FKBP's stop codon. Both fragments were then subcloned in frame into the *SgrAI*/*AccI* sites of the pRK5 vector, so as to generate the HA-V1aR-FKBP construct in which the carboxyl terminal of V1aR was separated from the first codon of FKBP by a 3 amino acid linker (CSR).

Myc-V2R-FKBP: The Myc-V2R coding sequence was amplified without its stop codon using sense and antisense primers harbouring unique *EcoRI* and *XbaI* sites in order to create a *XbaI* site at the 3' end of Myc-V2R. The Myc-V2R and FKBP fragments were then subcloned together into the *EcoRI*/*AccI* sites of the pRK5 vector, so as to generate the Myc-V2R-FKBP construct in which the carboxyl terminal of V2R was separated from the first codon of FKBP by a 2 amino acid linker (SR).

FRB-βarrestin2-YFP: Sense and antisense primers were made to introduce a *SpeI* site just before the coding sequence of the βarrestin2-YFP expressed in pGFP-N1-Topaz vector. The FRB coding sequence amplified without its stop codon between *SnaBI* and *SpeI*, was ligated in frame in 5' of the βarrestin2-YFP, so that a 5 amino acid long linker (TSSAT) separated the last codon of FKBP and the initiator methionine of βarrestin2.

V1aR-Rluc: sense and anti-sense primers were designed to introduce an *NheI* site at the 3' end of the receptor coding sequence and to remove the V1aR stop codon. pRK5-V1aR-Rluc was constructed using the NheI/XbaI sites to excise Rluc from the pRL-CMV vector (Promega, Madison, WI) and insert it in frame in pRK5-V1aR vector so that a 3 amino acid long linker (LAT) separated the carboxyl tail of V1aR and the initiator methionine of Rluc, as previously described (Terrillon *et al.*, 2003).

V2R-RLuc: sense and antisense primers were made to both introduce an *AgeI* site at the 3' end of V2R coding sequence expressed in pRK5 and to remove the receptor's stop codon. An *AgeI* site was also created at the beginning of the Rluc coding sequence expressed in pRL-CMV vector. The pRL-CMV vector was then cut with *AgeI*/*XbaI* to excise the Rluc coding region and insert it in frame into the pRK5-V2R vector to yield the pRK5-V2R-Rluc construct

(Terrillon *et al.*, 2003) with a 3 amino acid linker (PVT) separating the carboxyl terminal of V2R and the initiator methionine of Rluc.
All constructs were verified by DNA sequencing.

Cell culture and Transfection

HEK 293T cells were grown in DMEM supplemented with 10 % foetal bovine serum, 100 U/mL penicillin/streptomycin and 2 mM L-glutamine, at 37°C in a humidified atmosphere with 95 % air and 5 % CO_2. For immunofluorescence and BRET experiments, $3x10^5$ and $2x10^6$ cells were seeded in each well of a 6 well-plate and 100-mm Petri dishes, respectively, and transient transfections performed using the calcium phosphate precipitation method (Mellon *et al.*, 1981). Twenty-four hours after transfection, cells dedicated to BRET assay were transferred into 6 well-plates. In all other cases, to insure the highest efficiency of transfection, Fugene 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) was utilized as described in the manufacturer's protocol. For ELISA, inositol phosphate and cyclic AMP assays, $2x10^6$ cells plated per 100-mm Petri dishes, were transfected 24-hours before being transferred into poly-lysine coated 12 well-plates. For ERK1/2 phosphorylation experiments, $2x10^6$ cells plated per 100-mm Petri dishes were transfected 8-hours before being transferred into poly-lysine coated 6 well-plates. The number of receptors co-expressed with FRB-βarrestin2-YFP determined by [^{3}H]-AVP binding carried out in membrane preparations (Terrillon *et al.*, 2003) was as follow: 2.2 ± 0.4 pmol/mg and 1.7 ± 0.6 pmol/mg for V2R-FKBP and V1aR-FKBP respectively.

Cyclic AMP assays

HEK 293T cells transfected with Myc-V2R-FKBP + FRB-βarrestin2-YFP were divided into 12-well polylysine-coated plates and incubated with 2 μCi/mL of [^{3}H]-adenine (NEN Life sciences products). Sixteen-hours post-labelling, cells were washed twice with PBS and stimulated for 15 min with increasing concentration of AVP (from 10^{-12}M to 10^{-6}M) in PBS/1mM isobutylmethylxantine. The reaction was stopped by the addition of 5% trichloroacetic acid and 1 mM of unlabeled cAMP to decrease the enzymatic degradation of [^{3}H]-cAMP. Purification of total cAMP was then performed over Dowex/alumina sequential chromatography as previously described (Salomon *et al.*, 1974). .

Inositol phosphate assays

HEK 293T cells transfected with HA-V1aR-FKBP + FRB-βarrestin2-YFP were divided into 12-well polylysine-coated plates and incubated with 2 μCi/mL of myo-[2-^{3}H]inositol (PerkinElmer, Boston, MA). After a 24 h labelling period, cells were washed once with DMEM and incubated for 30 min at 37°C with DMEM/200 mM Hepes pH 7.4/20 mM LiCl. Then, cells were washed once with IP buffer (DMEM/200 mM Hepes pH 7.4/10 mM LiCl) and stimulated for 20 min with increasing concentration of AVP (from 10^{-12}M to 10^{-6}M) in IP buffer. The reaction was stopped with 1 ml of methanol/HCl and the content was transferred to an AG 1-X8 (formate) column (BioRad Laboratories, Hercules, CA). Each column was washed twice with 3 ml of water followed by 2 ml of 5 mM sodium tetraborate/60 mM sodium formate. Total inositol phosphates were then eluted from the columns with 2 ml of 1 M ammonium formate/100 mM formic acid. Myo-[2-^{3}H]inositol phosphate β-radioactivity was detected in a liquid scintillation counter. .

SUPPLEMENTARY REFERENCES

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LEGENDS TO SUPPLEMENTARY FIGURE 1

Internalisation of the V2R/V1aR heterodimer following the translocation of FRB-βarrestin2-YFP to V1aR-FKBP. HEK 293T cells transfected with FRB-βarrestin2-YFP in the presence of HA-V1aR-FKBP + Myc-V2R were incubated with rabbit polyclonal anti-Myc antibody A14 and mouse monoclonal anti-HA antibody 12CA5 for 1h at 4°C. After treatment with 500 nM AP21967 for 30 min at 37°C, cells were fixed, permeabilized and labelled with Texas Red-conjugated goat anti rabbit and Alexa 633-conjugated goat anti mouse to visualize V2R and V1aR, respectively. The samples were analysed by confocal laser-scanning microscopy as described in *Material and Methods*.

Supplementary Figure 1