Supplementary Information - **EMBO *reports* online**

β_1-adrenergic receptor antagonists signal via PDE4 translocation

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SUPPLEMENTARY METHODS

Materials.

βAR ligands Isoproterenol (ISO), CGP-20712A (CGP), Propranolol (PRO), Carvedilol (CAR), Metoprolol (MET), CGP-12177, Alprenolol (ALP), ICI-118551 (ICI) as well as IBMX (3-Isobutyl-1-methylxanthine) and Rolipram were from Sigma-Aldrich. Forskolin (FSK) was from Tocris and cAMP-AM (8-Bromo-2'-O-methyladenosine-3',5'-cyclic monophosphate . acetoxymethyl ester) from Biolog. The following antibodies were used in this study: PKA substrate antibody and phospho-ERK1/2 antibody (Cell Signaling Technology, Danvers, MA), GFP and ERK1 antibodies (BD Transduction Laboratories, Franklin Lakes, NJ), α-Flag(M1) resin and antibodies against α-Flag(M2) and β-tubulin (Sigma-Aldrich), and antibodies against Myc-tag (Roche Applied Sciences).

Design of expression vectors.

Generation of adenoviruses encoding C-terminally Myc-tagged PDE4D variants or N-terminally Flag-tagged β_1AR [1] as well as generation of the EPAC2-Cyt and EPAC2-PM sensors [2, 3] have been described previously. A pcDNA3 expression vector encoding β_1AR-EPAC2 was created by fusing the cDNA encoding the cAMP-EPAC2 sensor to the 3' end of the human β_1AR open reading frame. A pcDNA3 expression vector encoding mCherry-β_1AR was kindly provided by David Zuckerman and Carolyn Machamer (Johns Hopkins University). This construct was generated by inserting the coding sequence for mCherry behind a cleavable signal sequence followed by human β_1AR.

Cell culture and adenovirus infection.

Ventricular cardiac myocytes were isolated from the excised hearts of 1–2 day old neonatal mice as described previously [1]. They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Nu Serum IV (BD Falcon), 5% fetal bovine serum, 1 mM glutamine, 30 µg/ml penicillin, 100 µg/ml streptomycin, 3 mM HEPES, and 1 x ITS media supplement (Sigma-Aldrich) on plates precoated with 10 mg/ml laminin. Experiments were carried out on day 3 of culture. All animal studies were performed according to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of the University of California San Francisco. Hek293

cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1 mM glutamine, 30 µg/ml penicillin, and 100 µg/ml streptomycin. All cells were cultured at 37°C and under a 5% CO_2 atmosphere. For expression of exogenous βARs, PDE4D constructs or the EPAC2-Cyt/PM sensors, cells were infected with the respective adenoviruses at an MOI of 10 (Hek293) or 500 (cardiac myocytes). As "mock" controls, cells were infected with comparable titers of an adenovirus encoding for green fluorescent protein (GFP).

Immunoprecipitation of Flag-tagged β_1ARs from cell lysates.

Flag-tagged β_1ARs were imunoprecipitated as described previously [1]. In brief, after the respective cell treatment, cells were rinsed once with ice-cold PBS and then lysed in 500 µl buffer containing 20 mM HEPES, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% n-Dodecyl-β-D-maltopyranoside (DDM, Anatrace), 1 µM microcystin-LR (Calbiochem) and Complete protease inhibitor cocktail (Roche Applied Sciences). Lysates were rotated at 4˚C for 30 min followed by a 10-min centrifugation at 20,000 x g and 4°C. Soluble extracts were precleared by a 30-min incubation with 30 µl of ProteinG Sepharose. Flag-tagged receptors were then immunoprecipitated using M1-affinity resin (α-Flag antibody resin; Sigma Aldrich). After incubation for 3 h at 4°C, the resin was washed three times and proteins were eluted in 40 µl of elution buffer (200 µg/ml Flag peptide, 20 mM HEPES, 50 mM NaCl, 0.1 % cholesterol, 8 mM EDTA).

PDE assay.

PDE activity was measured according to the method of Thompson and Appleman [4] as described in detail previously [5]. In brief, samples were assayed in a reaction mixture of 200 µl containing 40 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 1.4 mM β-mercaptoethanol, 1 µM cAMP, 0.75 mg/ml bovine serum albumin, and 0.1 µCi of [^{3}H]cAMP for 10 min at 33°C. The reaction was terminated by heat inactivation in a boiling water bath for 1 min. The PDE reaction product 5'-AMP was then hydrolyzed by incubation of the assay mixture with 50 µg of *Crotalus atrox* snake venom (Sigma-Aldrich) for 20 min at 33°C, and the resulting adenosine was separated by anion exchange chromatography using 1 ml of AG1-X8 resin (BioRad, Hercules, CA) and quantitated by scintillation counting. PDE4 activity was defined as the PDE activity inhibited by the PDE4-selective inhibitor, Rolipram (10 µM).

Measurement of cellular cAMP levels.

After the respective cell treatment, the medium was removed and the cell layer washed with ice-cold PBS. 0.8 ml of 95% ice-cold ethanol containing 0.1% trichloroacetic acid (TCA) was then added to each well (6-well plates). After a 30-min incubation of the plates on ice, the TCA solution containing the cAMP was collected and cAMP concentration in these samples was then determined by radioimmunoassay (RIA) as previously described [6] or by enzyme immunoassay (EIA) using a kit from Cayman Chemicals. The cell protein, which remains on the cell culture plates, was dissolved in

300 µl of 1 N NaOH per well, and this solution was used for determination of protein content.

Measurement of local cAMP levels using the β_1AR-EPAC2, EPAC2-Cyt and EPAC2-PM sensors.

Cells grown on collagen-coated glass coverslips were transfected with the vector encoding the β_1AR-EPAC2 sensor or infected with adenoviruses encoding the EPAC2-Cyt or EPAC2-PM sensors. After overnight culture, cells were serum-starved for 2 h. For FRET microscopy, coverslips were placed in a modified Sykes-Moore Chamber and kept in 500 µl Locke's medium (5 mM HEPES (pH 7.4), 154 mM NaCl, 5.6 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 5 mM glucose, 0.05% BSA) at 37°C. Images were acquired with a Nikon TE2000 inverted fluorescence microscope using a 60x fluorescence objective. CFP (donor) fluorescence was viewed by exciting at 430-455 nm and measuring emission at 470-490 nm. YFP (acceptor) fluorescence was viewed by exciting at 500-520 nm and measuring emission at 535-565 nm. FRET was viewed by exciting at 430-455 nm (donor excitation) and measuring fluorescence at 535-565 nm (acceptor emission). Background and bleedthrough were subtracted from FRET images to obtain corrected FRET images using MetaMorph software (Molecular Devices) and average FRET intensity was measured directly in the corrected FRET images.

Adenylyl cyclase activity assay.

Hek293 cells were homogenized in ice-cold buffer containing 25 mM HEPES (pH 7.4), 20% w/v sucrose, 150 mM NaCl, 2 mM EDTA, 0.2 mM EGTA, 1 µM microcystin-LR and Complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) using a glass homogenizer. The cell extracts were then spun for 20 min at 20,000 x *g* and 4°C. The resulting membrane pellets were resuspended in lysis buffer and subjected to adenylyl cyclase activity assays according to the method of Alvarez and Daniels [7] with minor variations. In brief, samples were assayed in a reaction mixture of 100 µl containing 40 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.2 mM cAMP, 10 mM phosphoenol pyruvate, 3 units of pyruvate kinase, 10 µM GTP, 1 mM ATP, and 2 µCi of [α^{32}P]-ATP for 15 min at 37°C. The reaction was terminated with the addition of 20 µl of 2.2 N HCl containing 0.01 µCi [^{3}H]-cAMP (added to determine the recovery of cAMP from subsequent column chromatography) followed by boiling for 2 min. Cyclic AMP was then separated from the substrate ATP by column chromatography using 2.5 cm^3 Alumina WN-6. The column was eluted into scintillation vials with 5 ml of 0.1 M ammonium acetate (pH 6.5), the eluate was mixed with 12 ml of Aquasol-2 scintillation fluid (PerkinElmer, Waltham, MA) and the eluted cAMP quantified by scintillation counting.

Measurement of βAR ligand binding *in vitro*.

Hek293 cells were harvested in lysis buffer containing 20 mM HEPES, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1 µM microcystin-LR (Calbiochem) and Complete protease

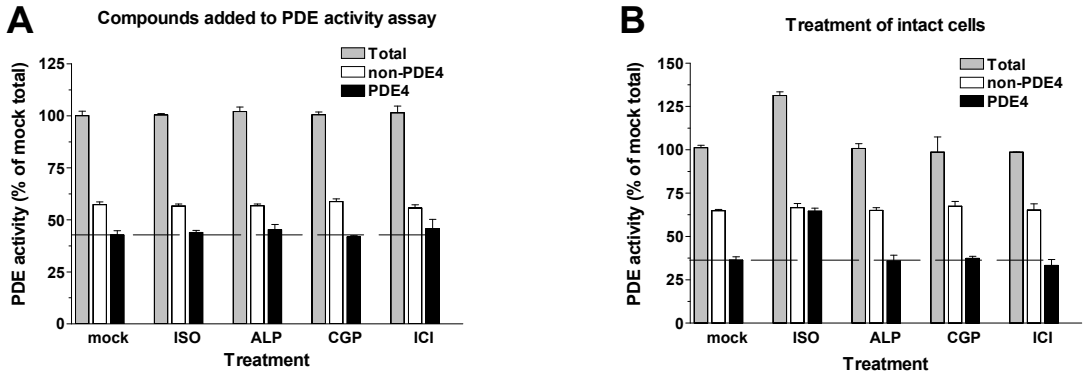
inhibitor cocktail (Roche Diagnostics) using a glass homogenizer. The extracts were spun at 20,000 x g for 20 min and the resulting membrane pellets were resuspended in lysis buffer. To measure βAR ligand binding, membrane extracts (~10 µg protein) were incubated in a 200 µl reaction mix of binding buffer (20 mM Tris-HCl (pH 7.4), 7 mM $MgCl_2$, 0.1 mM DTT) and 10 nM [^{3}H]-CGP-12177 (Perkin-Elmer) for 30 min at 30 °C followed by a 30-min incubation on ice. The samples were then filtered over GF/B-Whatman filters (Whatman, Clifton, NJ) that had been presoaked in 0.3% (v/v) polyethyleneimine diluted in water and placed onto a 3025 sampling manifold from Millipore (Bedford, MA). After filtration of the samples, the filters were washed twice with 1 ml ice-cold binding buffer, dried and subjected to scintillation counting. The resulting values for [^{3}H]-CGP-12177 binding were corrected for unspecific binding of the tracer to the filters as determined in reactions containing no protein extract. Membrane preparation from mock-transfected Hek293 cells did not show specific binding of tracer suggesting that Hek293 cells do not express significant levels of endogenous β_1AR.

Measurement of βAR ligand binding to live cells.

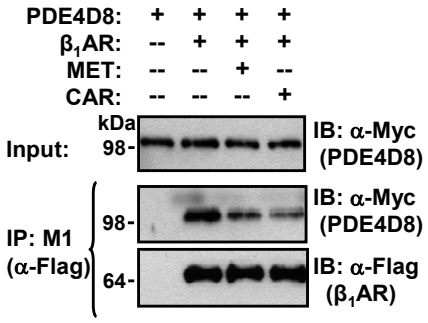
Cells, grown on plates coated with Matrigel (BD Biosciences), were treated for the indicated times with βAR agonists or antagonists. The cells were then washed three times with medium and subsequently incubated with medium containing [^{3}H]-CGP-12177 (10 nM) for 3 min at RT. After removal of the medium, cells were lysed and [^{3}H]-CGP-12177 in the cell lysate was quantified by scintillation counting.

Data analysis.

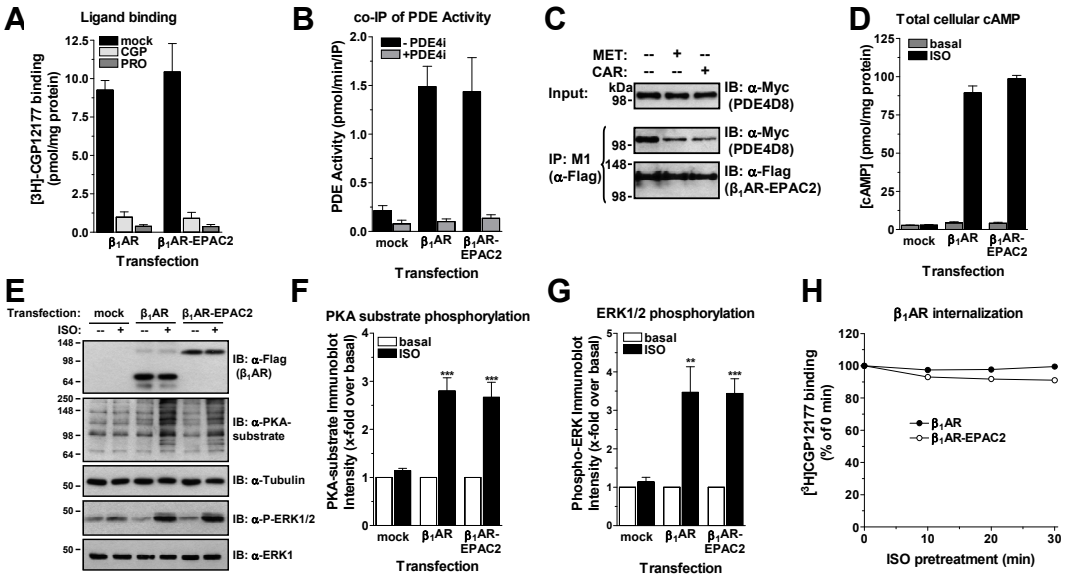
Unless otherwise noticed, all graphs show the mean ± s.e.m. of at least three experiments performed. Statistical significance was determined using Student's t-Test and is indicated as follows: NS (not significant; $P \geq 0.05$); *($P<0.05$); **($P<0.01$); ***($P<0.001$). The GraphPad Prism program (GraphPad Inc., San Diego, CA) was used for all statistical analysis. For quantification of Western blot bands, blots were scanned and the signal intensity of the immunoreactive bands was quantified as previously described [8] using the ScionImage software program (Frederick, MD).



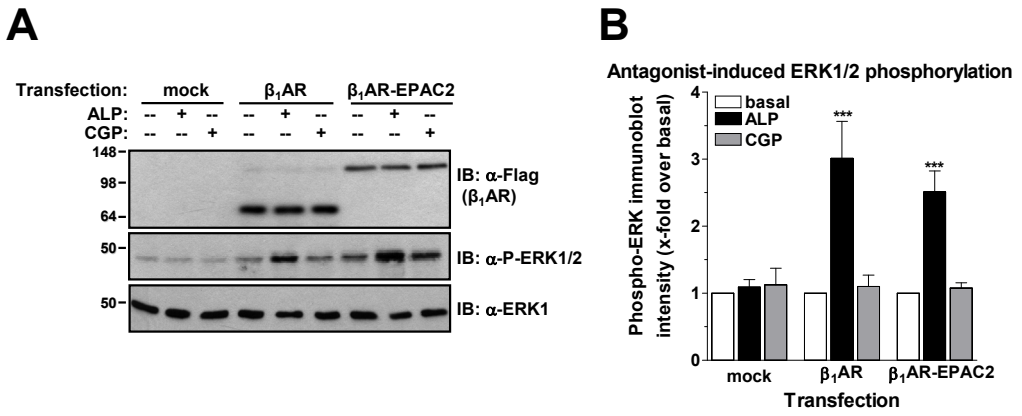
Supplementary Fig S1 | β_1AR antagonists do not affect PDE4 or total PDE activity. The graphs show cAMP-PDE activity measured in detergent extracts of β_1AR-expressing Hek293 cells. PDE4 activity is defined as the portion of total PDE activity that is sensitive to inhibition by the PDE4-selective inhibitor Rolipram (10 μM). In (**A**), the effect of Isoproterenol (ISO; 10 μM), Alprenolol (ALP; 1 μM), CGP-20712A (CGP; 1 μM) and ICI-118551 (ICI; 1 μM) on PDE activity was probed by adding these compounds directly into PDE activity assays. Conversely, in (**B**), cells were treated with the compounds for 5 min after which the cells were lysed and cAMP-PDE activity was measured in the resulting cell extracts. The data represent the mean ± s.e.m. of three experiments.



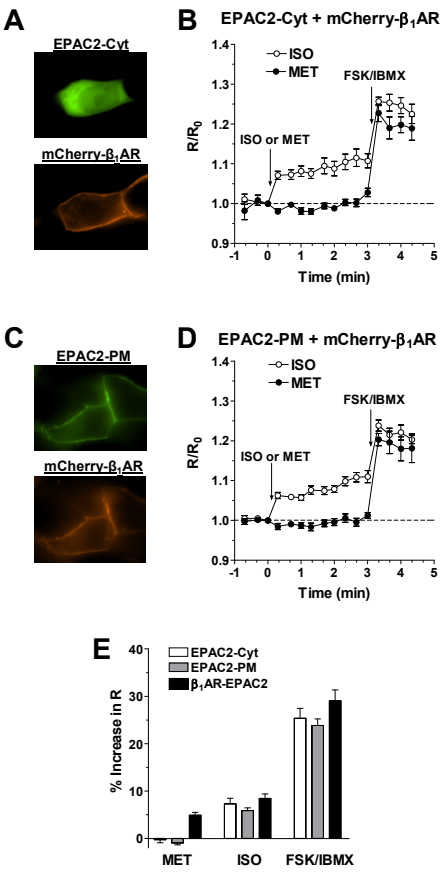
Supplementary Fig S2| β_1AR/PDE4 complex dissociation does not require β-arrestin recruitment. Exogenous β_1AR and PDE4D8-Myc were co-immunoprecipitated from extracts of mouse embryonic fibroblasts derived from mice deficient in β-arrestin 1 and 2 (βarr1/2KO-MEFs) that had been treated for 5 min with MET (1 μM), CAR (1 μM) or solvent. A representative experiment of three performed is reported.



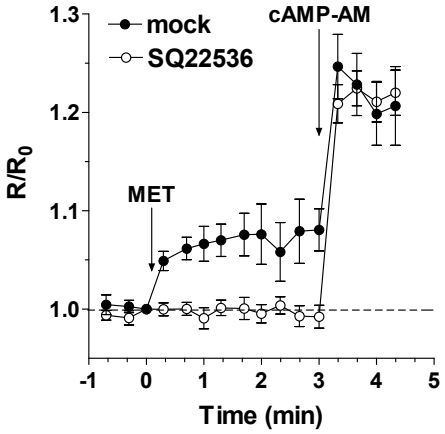
Supplementary Fig S3 | Characterization of the β_1AR-EPAC2 cAMP sensor. (**A**) When expressed at similar levels, the chimera binds [^{3}H]-CGP-12177, a β_1AR-selective antagonist, at similar levels and binding is competitively blocked by other β_1AR-ligands such as Propranolol (PRO) or CGP-20712A (CGP). Shown is binding of [^{3}H]-CGP-12177 (10 nM) to extracts of Hek293 cells expressing Flag-tagged β_1AR or β_1AR-EPAC2. Measurements were performed in the absence (mock) or presence of CGP (1 μM) or PRO (1 μM). Extracts from mock-transfected Hek293 cells showed no specific binding of tracer. (**B,C**) Fusion with the EPAC2 sensor does not impair binding of the β_1AR to PDE4 nor antagonist-dependent complex dissociation. (**B**) Shown are co-IPs of endogenous PDE activity with Flag-tagged β_1AR or β_1AR-EPAC2 from Hek293 cell extracts. cAMP-PDE activity recovered in Flag-IP pellets was measured in the absence (-PDE4i) or presence (+PDE4i) of the PDE4-selective inhibitor Rolipram (10 μM). (**C**) Shown is a representative co-IP of exogenous PDE4 with the β_1AR-EPAC2 sensor from cells pretreated for 5 min with MET (1 μM) or CAR (1 μM). (**D**) Comparable levels of cAMP accumulation in Hek293 cells expressing β_1AR or β_1AR-EPAC2. Shown is cAMP accumulation in response to a 5 min treatment with Isoproterenol (ISO; 10 nM) as measured by enzymeimmunoassay (EIA). Cell treatments were performed in the presence of ICI-118551 (ICI; 1 μM) to block signaling of endogenous β_2ARs. (**E-G**) Agonist binding to the β_1AR-EPAC2 chimera induces similar levels of PKA substrate- and ERK1/2 phosphorylation compared to wild type β_1AR. Shown is a Western blot analysis of detergent extracts prepared from control (mock) and β_1AR- or β_1AR-EPAC2-expressing Hek293 cells treated for 5 min with or without Isoproterenol (ISO; 100 nM). Molecular weight markers (in kDa) are shown on the left of the blots. The levels of PKA substrate- and ERK1/2-phosphorylation are quantified in (**F**) and (**G**), respectively. Experiments were performed in the presence of ICI-118551 (ICI; 1 μM) to block signaling of endogenous β_2ARs. (**H**) The β_1AR as well as the β_1AR-EPAC2 chimera show limited internalization upon agonist treatment. Hek293 cells expressing Flag-tagged β_1AR or the β_1AR-EPAC2 chimera were treated for the indicated times with Isoproterenol (ISO, 10 μM) after which the medium was removed, the cells were washed three times and then incubated with medium containing [^{3}H]-CGP-12177 (10 nM) for 3 min at RT. After removal of the medium, cells were lysed and [^{3}H]-CGP-12177 in the cell lysate was quantified by scintillation counting. Data shown in (A, B, D, F, G and H) represent the mean ± s.e.m. of three experiments. **(P<0.01); ***(P<0.001) Taken together, these data document that the β_1AR-EPAC2 chimera exhibits normal β_1AR receptor functions. This might be unexpected given that fusion of the EPAC2 sensor to the C-terminus of the β_1AR should likely alter or disrupt interactions of the β_1AR-EPAC2 chimera with PDZ domain scaffolds. However, given the level of receptor overexpression compared to the low expression levels of endogenous PDZ scaffolds, we expect that the majority of both wild type receptors as well as chimera are largely unbound, and thus, unaffected, by PDZ scaffolding under our experimental conditions (see [9]).



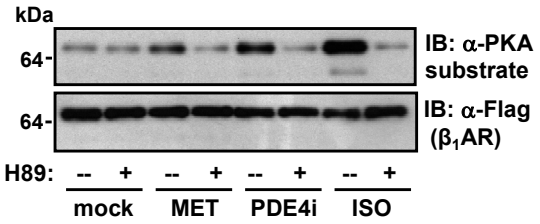
Supplementary Fig S4| β_1AR/PDE4 complex dissociation is not sufficient to induce ERK1/2 phosphorylation. Two β_1AR antagonists, Alprenolol (ALP) and Carvedilol (CAR), have been shown to induce epidermal growth factor receptor (EGFR) transactivation via a β-arrestin-mediated signaling pathway [10], whereas a range of other antagonists, including CGP-20712A (CGP), do not. Here we show that we can replicate these findings using either wild type β_1AR or the β_1AR-EPAC2 chimera and using ERK1/2 phosphorylation as a downstream readout of EGFR transactivation. The finding that all antagonists dissociate β_1AR/PDE4 signaling complexes, but only ALP (and CAR [10]) induce ERK1/2 phosphorylation, suggests that β_1AR/PDE4 complex dissociation *per se* is not sufficient to induce the β-arrestin/EGFR/ERK1/2 signaling pathway. (**A**) Shown is a Western blot analysis of detergent extracts prepared from control (mock), β_1AR- and β_1AR-EPAC2-expressing Hek293 cells treated for 5 min with ALP (1 μM) or CGP (1 μM). Molecular weight markers (in kDa) are shown on the left of the blots. The levels of ERK1/2-phosphorylation are quantified in (**B**). Experiments were performed in the presence of ICI-118551 (1 μM) to block signaling of endogenous β_2ARs. The data represent the mean ± s.e.m. of three experiments. ***(P<0.001)



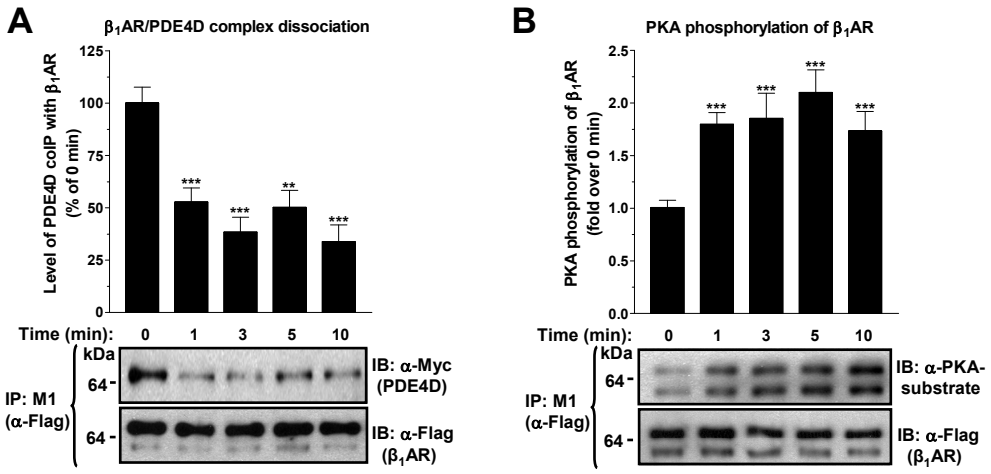
Supplementary Fig S5 | Antagonist treatment *per se* does not increase global cAMP levels. (**A-D**) To measure β_1AR-induced cAMP signals in cytosolic and submembrane compartments, Hek293 cells were co-transfected with the respective cAMP sensors (EPAC2-Cyt and EPAC2-PM) as well as an mCherry-tagged β_1AR. Visualization of the cAMP sensors in the YFP channel and mCherry-β_1AR in the RFP channel allows selection of cells that express both proteins at suitable levels. (**A**) The images show YFP (top) and RFP (bottom) emissions in Hek293 cells coexpressing the cytosolic EPAC2-Cyt sensor (YFP) and mCherry-β_1AR (RFP). (**B**) Live cell cAMP measurements in Hek293 cells co-expressing EPAC2-Cyt and mCherry-β_1AR. Shown are average traces of R/R_0 for cells treated with the β_1AR-antagonist Metoprolol (MET; 1 µM; n=16) or the βAR agonist Isoproterenol (ISO; 100 nM; n=16) followed by treatment with the adenylyl cyclase activator Forskolin (FSK; 50 µM) and the non-selective PDE inhibitor IBMX (100 µM). (**C**) The images show YFP (top) and RFP (bottom) emissions in Hek293 cells coexpressing the plasma membrane-targeted EPAC2-PM sensor (YFP) and mCherry-β_1AR (RFP). (**D**) Live cell cAMP measurements in Hek293 cells co-expressing EPAC2-PM and mCherry-β_1AR. Shown are average traces of R/R_0 for cells treated with MET (1 µM; n=16) or ISO (100 nM; n=16) followed by treatment with FSK (50 µM) and IBMX (100 µM). (**E**) The graph summarizes the effects of MET and ISO on cAMP levels measured with EPAC2-Cyt, EPAC2-PM and β_1AR-EPAC2 probes reported in (B) and (D) as well as Fig. 2D/E. Shown is the % increase in R (CFP/FRET) resulting from treatment with MET (1 µM), ISO (100 nM) or combined treatment with IBMX (100 µM) and FSK (50 µM). All measurements were performed in the presence of ICI-118551 (1 µM) to block signaling of endogenous β_2ARs.



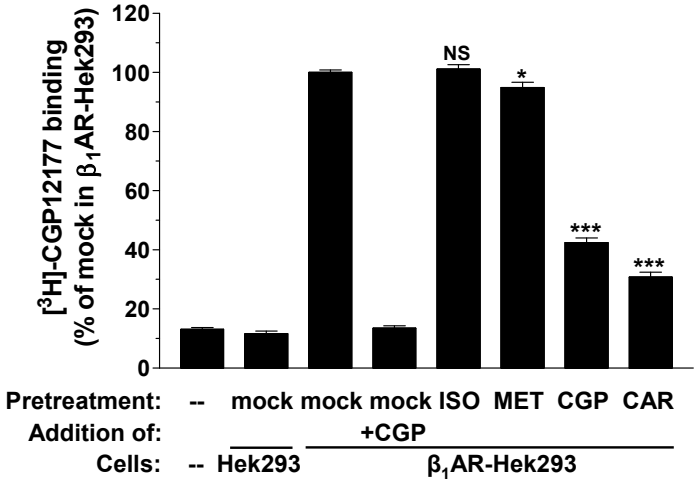
Supplementary Fig S6| Constitutive basal adenylyl cyclase (AC) activity is a prerequisite to reveal the effect of antagonist-dependent β_1AR/PDE4 complex dissociation on local cAMP levels. Hek293 cells expressing the β_1AR-EPAC2 sensor were treated for 5 min with the AC inhibitor SQ22536 (200 μM; empty circles) or solvent as a control (filled circles) followed by treatment with MET (1 μM) and the cAMP analog cAMP-AM (8-Bromo-2'-O-methyladenosine-3',5'-cyclic monophosphate . acetoxymethyl ester; 20 μM). Shown are average traces of R/R_0 for cells treated with SQ22536 (n=23) compared to mock-treated cells analyzed on the same days (n=11). Consistent with prior reports on Hek293 cells [11], and unlike what is observed in cardiac myocytes [11], treatment with SQ22536 did not lower baseline FRET emission in our hands. This is consistent with the idea that basal cAMP levels in Hek293 cells are below the detection threshold of the EPAC2 sensor.



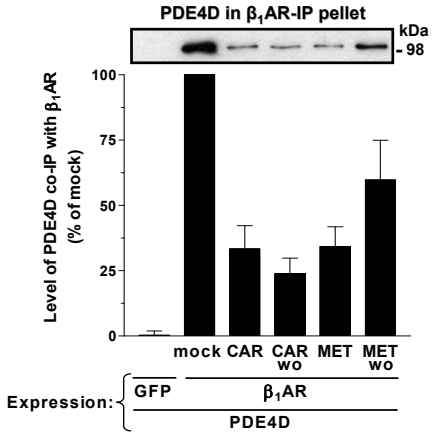
Supplementary Fig S7 | Specific detection of PKA phosphorylation using α-PKA substrate antibodies. Hek293 cells expressing β_1AR were pretreated with or without the PKA inhibitor H89 (20 μM) for 1 h followed by a 5-min treatment with either Metoprolol (MET; 1 μM), PDE4 inhibitor (PDE4i; Rolipram; 10 μM) or Isoproterenol (ISO; 10 μM). Detergent extracts prepared from these cells were subjected to α-Flag(M1)-IP and the phosphorylation level of the β_1AR recovered in IP pellets was determined by Western blotting using α-PKA-substrate antibodies. Shown is one of three experiments performed.



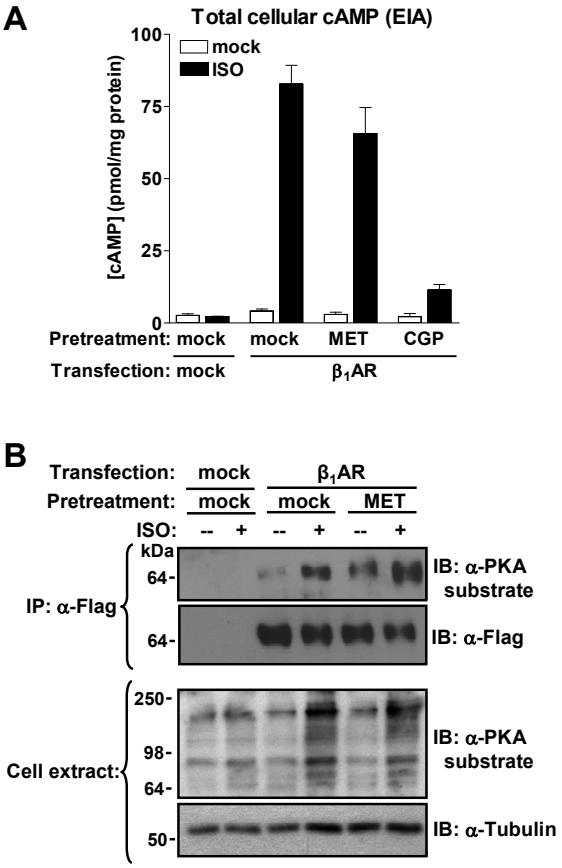
Supplementary Fig S8 | Time-dependence of antagonist-induced β_1AR/PDE4 complex dissociation. (**A**) Hek293 cells expressing Flag-tagged β_1AR and Myc-tagged PDE4D8 were treated for the indicated times with CGP-20712A (CGP; 1 μM) after which the cells were lysed and subjected to immunoprecipitation (IP) of β_1AR. The graph reports the amount of exogenous PDE4D recovered in the IP pellets. (**B**) Time-dependent increase in PKA-phosphorylation of β_1AR expressed in Hek293 cells in response to treatment with CGP (1 μM) for the indicated times. The data represent the mean ± s.e.m. of at least three experiments. **(P<0.01); ***(P<0.001)



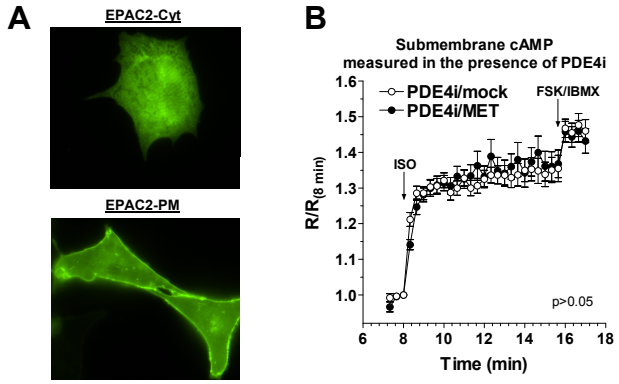
Supplementary Fig S9 | Efficacy of the washout of different ligands. Hek293 cells expressing Flag-tagged β_1AR were cultured in 12 well plates. Cells were treated for 3 min with Isoproterenol (ISO, 10 μM), Metoprolol (MET; 1 μM), CGP-20712A (CGP, 1 μM) or Carvedilol (CAR, 1 μM) after which cells were washed three times and then incubated with medium containing [^{3}H]-CGP-12177 (10 nM) for 3 min at RT. After removal of the medium, cells were lysed and [^{3}H]-CGP-12177 in the cell lysate was quantified by scintillation counting. Mock-transfected Hek293 cells (second column from the left) did not show specific binding of CGP-12177 compared to wells without cells (far left column). Also, addition of unlabelled CGP-12177 (1 μM) during the incubation with the tracer ablated [^{3}H]-CGP-12177 binding (fourth column from left). The data represent the mean ± s.e.m. of at least three experiments. NS (not significant); *(P<0.05); ***(P<0.001)



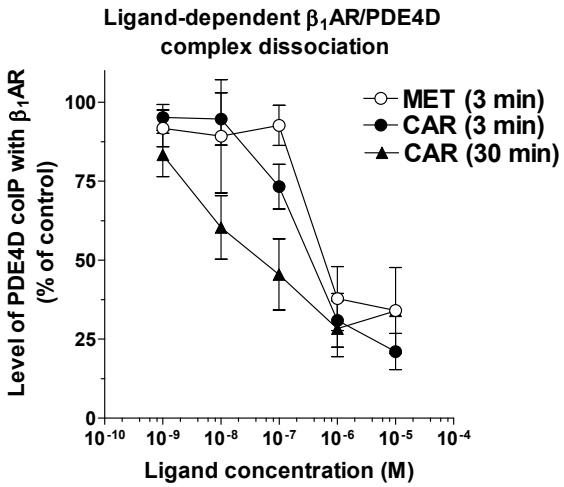
Supplementary Fig S10 | Reassociation of β_1AR/PDE4 complexes is delayed following antagonist washout. Hek293 cells expressing Flag-tagged β_1AR and Myc-tagged PDE4D8 were treated with Carvedilol (CAR; 1 μM), Metoprolol (MET; 1 μM) or solvent (mock) after which cells were lysed and subjected to immunoprecipitation (IP) of β_1AR. In the control groups (mock, CAR, MET), cells were first washed three times followed by a 7-min incubation and a 3-min treatment with antagonist or solvent. For washout groups (CAR-wo, MET-wo), cells were initially treated for 3 min with antagonists, then washed three times and incubated for another 10 min before cell harvest. The amount of exogenous PDE4D recovered in the IP pellets is shown in the immunoblot and quantified in the graph. Incomplete washout of CAR (see supplementary Fig S9) is a contributing factor that limits reassociation of β_1AR/PDE4 complexes after washout of this drug. The data represent the mean ± s.e.m. of three experiments.



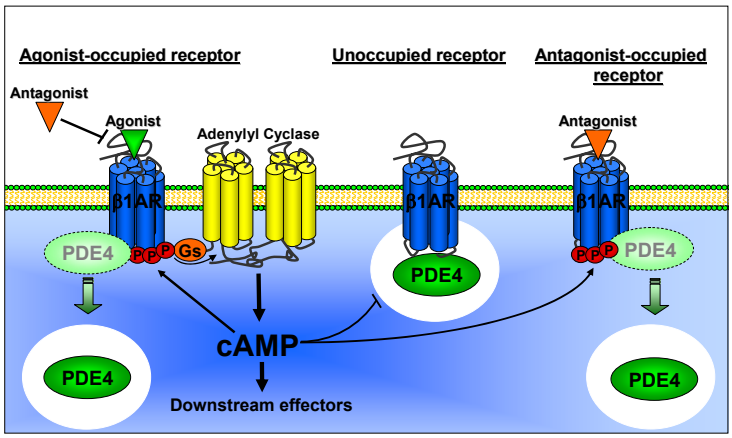
Supplementary Fig S11 | Effect of antagonist pretreatment on global cAMP accumulation and PKA phosphorylation patterns in Hek293 cells expressing wild type β_1AR. Quiescent cultures of control and β_1AR-overexpressing cells were treated for 5 min with Metoprolol (MET; 1 μM), CGP-20712A (CGP; 1 μM) or solvent (mock) after which the cells were washed three times with medium. ICI-118551 (1 μM) was then added to suppress signaling of endogenous β_2ARs and the recombinant β_1ARs were stimulated for 3 min with Isoproterenol (ISO; 10 nM). For (**A**), the cells were then lysed and total cellular cAMP accumulation was measured by enzymeimmunoassay (EIA). For (**B**), detergent extracts prepared from control or β_1AR-expressing Hek293 cells were subjected to α-Flag IP. Both total cell extracts as well as the β_1AR recovered in Flag-IP pellets were subjected to immunoblot analysis using a PKA substrate antibody. The graph in (A) shows the average ± s.e.m. of two experiments. Shown in (B) is one experiment of two performed. Incomplete washout of CGP (see supplementary Fig S9) is likely responsible for the more severe suppression of ISO-induced cAMP accumulation after washout of CGP compared to MET (A).



Supplementary Fig S12 | Antagonist-pretreatment potentiates agonist-induced cAMP accumulation in a submembrane compartment of mouse neonatal cardiac myocytes. (**A**) The images show YFP emissions in neonatal cardiac myocytes expressing the cytosolic cAMP sensor EPAC2-Cyt or the plasma membrane-targeted cAMP sensor EPAC2-PM. (**B**) Pretreatment with Metoprolol (MET; 1 μM) has no further effect on Isoproterenol (10 nM)-induced submembrane cAMP levels in the presence of a PDE4-selective inhibitor (PDE4i; Rolipram; 10 μM). Shown are average traces of FRET from EPAC2-PM-expressing neonatal cardiac myocytes stimulated with ISO (10 nM) after pretreatment and washout of MET (n=22) or solvent (mock; n=28) measured in the presence of the PDE4-selective inhibitor Rolipram.



Supplementary Fig S13 | Concentration-dependent dissociation of β_1AR/PDE4 signaling complexes by Metoprolol and Carvedilol. Hek293 cells expressing Flag-tagged β_1AR and Myc-tagged PDE4D8 were treated for 3 or 30 min with different concentrations of Carvedilol (CAR) or Metoprolol (MET) before the cells were lysed and subjected to immunoprecipitation (IP) of β_1AR. The amount of exogenous PDE4D8 recovered in the IP pellets is quantified in the graph. The data represent the mean ± s.e.m. of at least three experiments. For comparison, reported peak plasma concentrations for MET and CAR range from 100 to 1000 nM [12-17] and from 20 to 400 nM [16, 18-24], respectively, depending on dosage and preparation.



Supplementary Fig S14 | Schematic representation of the mechanism of action of β_1AR agonists and antagonists. Binding of an agonist induces the receptor-mediated activation of G_s proteins, which in turn triggers activation of adenylyl cyclase and an increase in global cAMP levels. In their classical role, antagonists reduce the potency of agonists by competing for their shared receptor binding site and thereby lower global cAMP production. However, antagonist binding to the receptor can increase local cAMP levels by inducing the dissociation of β_1AR/PDE4 signaling complexes. Translocation of PDE4 away from the receptor lowers the local capacity for cAMP hydrolysis and results in accumulation of cAMP produced by other means, such as the constitutive activity of the β_1AR or other receptors coupled to G_s or through basal activity of adenylyl cyclases. Thus, ligand-dependent dissociation of β_1AR/PDE4 signaling complexes is an alternative mechanism of regulation of cAMP levels, in addition to the G protein-mediated regulation of adenylyl cyclase activity.

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