Spatial encoding of cyclic AMP signaling specificity by GPCR endocytosis

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G protein-coupled receptors (GPCRs) are well known to signal via cyclic AMP (cAMP) production at the plasma membrane, but it is now clear that various GPCRs also signal after internalization. Apart from its temporal impact through prolonging the cellular response, we wondered whether the endosome-initiated signal encodes any discrete spatial information. Using the β2-adrenoceptor (β2-AR) as a model, we show that endocytosis is required for the full repertoire of downstream cAMP-dependent transcriptional control. Next, we describe an orthogonal optogenetic approach to definitively establish that the location of cAMP production is indeed the critical variable determining the transcriptional response. Finally, our results suggest that this spatial encoding scheme helps cells functionally discriminate chemically distinct β2-AR ligands according to differences in their ability to promote receptor endocytosis. These findings reveal a discrete principle for achieving cellular signaling specificity based on endosome-mediated spatial encoding of intracellular second messenger production and 'location-aware' downstream transcriptional control.

RESULTS

Endocytosis promotes β2-AR-elicted transcription

We began by assessing the effects of endosome signaling on the integrated β2-AR response. To do so, we profiled receptor-mediated regulation of cellular gene expression for >20,000 human genes and asked whether endocytosis is important for this response. HEK293 cells endogenously express β2-ARs at low levels, making them a useful model for studying signaling effects without potential complications of receptor overexpression. We examined the endogenous HEK293 β2-AR–cAMP response elicited by the β2-AR agonist isoproterenol at two agonist concentrations: 1 μM, a saturating concentration, and 10 nM, a subsaturating concentration that is close to the half-maximum effective concentration (EC50) for stimulating acute cAMP accumulation. Both concentrations of isoproterenol promoted significant (P < 1.0 × 10−3 by Student's t-test) β2-AR internalization (Supplementary Results, Supplementary Fig. 1a). To examine cAMP production in response to agonist stimulation, we measured real-time accumulation of the second messenger with a previously described luminescence-based cAMP biosensor that localizes diffusely throughout the cytoplasm. Although the net cAMP produced in response to 1 μM isoproterenol was greater than that to 10 nM agonist (Fig. 1a,b), microarray analysis revealed a similar gene expression response elicited by both concentrations of isoproterenol. This indicates that even subsaturating concentrations of agonist produce net amounts of cAMP capable of triggering efficient transcriptional signaling. We identified a core set of 55 isoproterenol-responsive genes (Supplementary Table 1) that were consistently induced over 1.5-fold in response to both concentrations of isoproterenol. This set is strongly enriched for cAMP target genes (30/55, P < 1.0 × 10−14 by hypergeometric test) and spans a diverse range of biological processes based on gene ontology analysis (Supplementary Table 2). To investigate whether endocytosis affects the β2-AR-mediated transcriptional response, we first took a pharmacological approach using Dyngo, a chemical inhibitor of dynamin that blocks regulated endocytosis of β2-ARs acutely. Pretreatment of cells with Dyngo for 15 min was sufficient to strongly (>90%) and
We saw significant inhibition (genes, abolished the transcriptional response altogether (Fig. 1c and *). Remarkably, acute endocytic blockade produced a profound response to blocking receptor endocytosis. (Fig. 1d).

Isoproterenol-elicited induction of both genes (2-AR transcriptional response, we observed that 2-AR internalization was verified by fluorescence flow cytometry (Fig. 1d)). However, it markedly reduced the magnitude of isoproterenol-responsive target genes (indicated on the y-axis; Supplementary Table 1) across all microarray experiments. ND, no drug (de). Scatter plots comparing expression levels for the target genes upon treatment of cells with 1 μM (d) or 10 nM isoproterenol (e). Data represent averaged log2 ratios (ISO/no drug) from n = 2 experiments. Isoproterenol targets are in gray, and endocytosis-dependent genes are in red (Supplementary Table 3). The dashed line has a slope of 1 (y = x). Arrows indicate RNA levels for PCK1 and DACT2. (f) Confirmation by qRT-PCR of PCK1 and DACT2 expression in response to blocking receptor endocytosis. (g) Clathrin knockdown (KD) effects on 2-AR signaling in cells transfected with CHC17 or control siRNAs. RNA levels of PCK1 were analyzed by qRT-PCR. Data represent averaged log2 ratios (ISO/no drug) from n = 2 experiments. Iso, isoproterenol. **P < 0.005 and *P < 0.05, as calculated by unpaired Student’s t-test. Error bars represent s.e.m.

PK1, which encodes a CAMP-regulated enzyme that catalyzes the rate-limiting step of gluconeogenesis, was one of the most strongly isoproterenol-induced genes, as shown by the microarray experiment. DACT2, which encodes a protein implicated in the control of Wnt and Nodal signaling, was detected at a lower level on the microarray chip.

Remarkably, acute endocytic blockade produced a profound and widespread inhibition of the 2-AR-mediated transcriptional response (P < 1.6 × 10−9 by Wilcoxon signed rank test) and, for some genes, abolished the transcriptional response altogether (Fig. 1c). We saw significant inhibition (P < 5.0 × 10−2 by Student’s t-test; Online Methods) of 30 and 23 genes of the 55 2-AR–dependent targets in cells treated with saturating (Fig. 1d) and subsaturating (Fig. 1e) concentrations of agonist, respectively (Supplementary Table 3). This did not reflect an off-target or general effect of Dyngo on gene transcription because, in parallel experiments, Dyngo did not interfere with basal gene expression levels or transcriptional signaling in the unfolded protein response (Supplementary Fig. 1c.f). We validated the microarray results by quantitative PCR with reverse transcription (qRT-PCR) of two endocytosis-dependent 2-AR targets, phosphoenolpyruvate carboxykinase 1 (encoded by PCK1) and dapper antagonist of β-catenin, homolog 2 (encoded by DACT2).
the endocytosis-sensitive genes detected in our analysis are indeed considered summaries above, we anticipate that the majority of this empirically determined validation rate, together with the other considerations summarized above, might represent a secondary consequence of preventing response. This was indeed the case: a single 5-min pulse of 1 M isoproterenol and 30-min chase with 10 μM alpenolol in cells pretreated with either 30 μM Dyang or vehicle (DMSO). Data represent the average from n = 5 experiments. ND, no drug. (b) Percent recycled Flag epitope-tagged β2-AR measured by flow cytometry in cells pretreated with 0.5 mM bafilomycin A1 or vehicle (DMSO). Data represent the mean surface fluorescence (n = 2), with 10,000-20,000 cells per condition, with each time point measured in triplicate. Baf, bafilomycin A1. (c) PCK1 expression measured by qRT-PCR in cells pretreated with bafilomycin A1 and incubated with indicated doses of isoproterenol for 2 h. Data represent the average from n = 2 experiments. Iso, isoproterenol. **P < 0.005, as measured by unpaired Student’s t-test. Error bars represent s.e.m.

Endocytosis affects the G protein-dependent β2-AR response

Endosome signaling by β2-ARs may also involve a G protein–independent mechanism (or mechanisms) such as β-arrestin-mediated activation of MAP kinases40,41, but two lines of evidence indicate that many of the observed transcriptional responses are most likely mediated by cAMP. First, we observed a significant enrichment of cAMP-response element (CRE)-containing genes among the endocytosis-sensitive β2-AR targets, with 19 out of 30 (P < 1.0 × 10−16 by hypergeometric test) and 15 out of 23 (P < 1.0 × 10−13 by hypergeometric test) for 1 μM and 10 nM isoproterenol, respectively (Supplementary Table 3). Second, to investigate directly whether our results reflect a cAMP-independent response, we chose six of the endocytosis-sensitive genes that were not previously annotated as CREB targets and asked whether their expression is induced by receptor-independent activation of adenylyl cyclase by forskolin36. Four of the six genes tested (AVPR1, RhoB, SLC2A3 and DACT2) were significantly (P < 5.0 × 10−4 by Student’s t-test) upregulated by forskolin treatment (Supplementary Fig. 2). On the basis of this empirically determined validation rate, together with the other considerations summarized above, we anticipate that the majority of the endocytosis-sensitive genes detected in our analysis are indeed induced through the traditional cAMP-dependent pathway.

In principle, the pronounced signaling effects of endocytic inhibition might represent a secondary consequence of preventing β2-AR trafficking through the recycling pathway, thereby inhibiting the process of functional resensitization of plasma membrane–delimited signaling after receptor phosphorylation and desensitization37. To test this possibility, we used a pulse-chase protocol to temporally uncouple receptor endocytosis from sorting back to the membrane. β2-ARs enter the endocytic pathway within 1–2 min of agonist-induced activation38, but subsequent β2-AR trafficking through the recycling pathway requires an additional ~8 min (ref. 19); by comparison, the endocytosis-dependent component of net cytoplasmic cAMP accumulation is evident within 5 min (Fig. 1a,b). Therefore, we asked whether exposing cells to isoproterenol only for 5 min might be sufficient to elicit an endocytosis-dependent gene expression response. This was indeed the case: a single 5-min pulse of isoproterenol was sufficient to elicit PCK1 induction. Remarkably, the transcriptional response initiated by this brief β2-AR activation, which effectively circumvents receptor recycling, was completely abolished by endocytic blockade with Dyngo (Fig. 2a). As a second, independent approach to address the role of receptor recycling in transcriptional signaling, we acutely inhibited the recycling pathway with the vacuolar proton ATPase inhibitor bafilomycin A1 (Baf)39,40,41. Bafilomycin A1 did not fully block recycling but nevertheless significantly (P < 5.0 × 10−4 by Student’s t-test) diminished receptor surface recovery (Fig. 2b). Yet, this manipulation did not have any effect whatsoever on PCK1 transcriptional induction in response to agonist (Fig. 2c). Taken together, these results suggest that receptor recycling and reactivation at the plasma membrane are not required for β2-AR-mediated gene induction and that the transcriptional response most likely emanates from the endosome-localized receptor pool.

Direct demonstration of location-biased signaling

In further support of the hypothesis that endosome-based β2-AR signaling is essential for transcriptional control, we noted that, even though exposing cells to 1 μM isoproterenol in the presence of endocytic blockade produced higher overall cytoplasmic cAMP response than 10 nM isoproterenol in the absence of endocytic blockade (Fig. 3a), induction of both PCK1 and DACT2 varied in the opposite direction (Fig. 3b). Moreover, this trend held for the full repertoire of β2-AR–induced genes detected by our analysis (P = 1.1 × 10−4 by Wilcoxon signed rank test; Fig. 3c). Thus, the ability of endocytosis to promote transcriptional signaling correlates with the process of endocytosis itself rather than with quantitatively increased overall levels of cytoplasmic cAMP accumulation. Accordingly, the results strongly suggest that cells discriminate the location of cAMP generation when initiating a signaling response.

To test this hypothesis directly, we developed an optogenetic strategy based on bPAC, a bacteria-derived adenylyl cyclase that allows cAMP production to be acutely induced by light22. We transfected bPAC into HEK293 cells and localized the expressed cyclase specifically to either the plasma membrane (‘bPAC-PM’) or endosome-limiting membrane (‘bPAC-Endo’) using respective Lyn-derived23 or Hrs-derived44 targeting sequences. Lyn was chosen for plasma membrane targeting because it can partition into both raft and nonraft fractions25. For comparison, we expressed the bPAC protein not fused to a targeting domain, which localizes diffusely throughout the cytoplasm (‘bPAC-Cyto’). Protein expression

Figure 3 | Transcriptional signaling is not monotonically related to net cAMP levels. (a–c) β2-AR was activated by bath application of 10 nM or 1 μM isoproterenol (Iso) in the presence of 30 μM Dyngo to block endocytosis. (a) Maximum and integrated cAMP signal was measured with the enzyme-based biosensor pGLO-20F (Promega). Data represent the mean from n = 2–3 experiments. Luminescence is measured as the percentage of the maximum forskolin signal. (b,c) Transcriptional induction of PCK1 and DACT2 (b) or isoproterenol target genes (c) was determined by qRT-PCR or microarray analysis, respectively. Isoproterenol targets are in gray, and endocytosis-dependent genes are in red (Supplementary Table 3). The dashed line has a slope of 1 (y = x). Data represent averaged log, ratios (Iso/no drug (ND)) from n = 2 experiments for each condition. **P < 0.005 by unpaired Student’s t-test. Error bars represent s.e.m.
Figure 4 | Localized cAMP production causes distinct transcriptional responses. (a) bPAC constructs were targeted to the plasma membrane (bPAC-PM), endosomes (bPAC-Endo) or cytoplasm (bPAC-Cyto) and visualized by immunofluorescence microscopy with Alexa-conjugated anti-myc antibody in fixed HEK293 cells. Scale bars, 10 μm. (b) Maximum (left) and integrated (right) cAMP signal after bPAC activation was measured with the luminescence-based biosensor pGlo-22F (Promega). Data represent mean from n = 4–5 experiments. (c) PCK1 expression was measured by qRT-PCR before (no light) or after activation of bPACs (light) with indicated doses of light. The PDE4-specific inhibitor rolipram (Rolit) was added to final concentration of 10 μM to cells immediately before exposure to light. Data represent average from n = 4 or 5 experiments. (d) Maximum and integrated cAMP signal after treatment of cells with 10 nM epinephrine (Epi) or 10 μM dopamine (DA) was measured with the luminescence-based biosensor pGLO-2OF (Promega). Data represent average from n = 3 experiments. (e) PCK1 expression measured by qRT-PCR after treatment with 10 nM epinephrine or 10 μM dopamine. Data represent mean from n = 5-7 experiments. **P < 0.005 and *P < 0.05 by unpaired Student’s t-test. Error bars represent s.e.m.

Supplementary Fig. 3a and appropriate subcellular localization (Fig. 4a and Supplementary Fig. 3b) from each construct were verified by microscopy. We adjusted bPAC transfection conditions to achieve comparable levels of acute cAMP accumulation in response to varying light doses driving bPAC activation (Fig. 4b and Supplementary Fig. 4a,b). We then monitored the transcriptional response by measuring induction of PCK1. As expected, activation of endogenous adenyl cyclases in bPAC-transfected cells with 10 nM isoproterenol led to a robust increase in PCK1 levels across all samples (Supplementary Fig. 4c). Light-induced activation of bPAC-PM did not lead to appreciable PCK1 induction, even at the highest dose of activation tested (5 min light pulse). In contrast, activation of the bPAC-Endo with a range of light doses (including those driving less overall cytoplasmic cAMP accumulation than bPAC-PM) produced clear induction of PCK1 expression (Fig. 4c and Supplementary Fig. 4d). As expected, the bPAC-Endo–dependent transcriptional induction of PCK1 was unaffected by the endocytic inhibitor Dynoglo (Supplementary Fig. 4e). Notably, bPAC-Cyto also produced a clear transcriptional response at all activating doses tested (Fig. 4c), further supporting the spatial encoding model and indicating that cAMP generated from the plasma membrane is effectively uncoupled from downstream transcriptional control.

To corroborate the transcriptional activation data, we also examined phosphorylation of CREB in cells expressing each bPAC construct. Although stimulation of endogenous transmembrane cyclases with forskolin triggered robust CREB phosphorylation in all samples (Supplementary Fig. 5a), optogenetic stimulation of only bPAC-Endo and bPAC-Cyto, but not of bPAC-PM, produced detectable activation of the transcription factor (Supplementary Fig. 5b). On the basis of these results, it appears that cAMP generated at the plasma membrane from bPAC is relatively weakly coupled to CREB-dependent induction of gene expression, whereas comparable (and even lower) levels of cAMP produced elsewhere in the cytoplasm can efficiently trigger a transcriptional response. The bPAC results provide direct and independent support for the hypothesis that differences in transcriptional signaling depend on the subcellular location of cAMP generation.

Location bias contributes to agonist discrimination

Because chemically distinct agonists acting on the same GPCR can differ considerably in ability to promote regulated endocytosis of receptors, we wondered whether cells might use the spatial encoding scheme for functional discrimination of agonists. To investigate this, we focused on dopamine and epinephrine, two endogenously produced catecholamines that are present at comparable levels in human plasma and differ structurally by a hydroxyl and a methyl group. Epinephrine is a potent β2-AR agonist that strongly stimulates receptor endocytosis, whereas dopamine is a weaker agonist that produces relatively little internalization even when applied at saturating concentration.

Remarkably, even when respective agonist concentrations were carefully adjusted to produce closely similar levels of overall cAMP accumulation (Fig. 4d), the transcriptional response elicited by epinephrine was significantly stronger (P = 2.2 × 10⁻² by Student’s t-test) (Fig. 4e). Accordingly, the present results provide independent pharmacological support for the spatial encoding hypothesis and suggest that spatial encoding by endosomal signaling can indeed function in agonist discrimination.

DISCUSSION

The present findings reveal an essential consequence of endosome-based cAMP signaling in transducing the full repertoire of transcriptional responses elicited by activation of a prototypical GPCR. The classical dogma, based on GPCR-elicited cAMP production occurring only from the plasma membrane, accordingly assumes that downstream transcriptional control originates from the cell surface. Our findings redefine this dogma by showing that endocytosis is required for the full transcriptional response. In essence, endosomes function as flexible signal delivery vehicles that physically move, in response to receptor activation, the site of receptor-elicited cAMP production away from the plasma membrane and in proximity to the nucleus for efficient downstream control of CREB-dependent transcription.

The present results are limited to a relatively undifferentiated cell model, and it will be interesting in future studies to investigate the signaling consequences of endocytosis in native systems and in more highly differentiated cells such as neurons, which extend cell processes remarkably far from the nucleus and package GPCRs into endosomes that are capable of both restricted and long-range motility. There is already evidence that endosomes support GPCR-cAMP generation in neurons, and endosome motility is important for a subset of growth factor–elicited signaling.
responses in this cell type. Another area for future study is the role of endosome-dependent signaling in mediating functional discrimination among chemically distinct GPCR ligands and whether this might open a new door to understanding the clinically relevant phenomenon of functional selectivity or agonist bias. Finally, we note that β-adrenoceptors are also capable of a low level of constitutive (ligand-independent) endocytosis, which appears to also be mediated by clathrin, and it will be interesting to investigate whether or not the constitutively internalized receptors contribute to transcriptional control.

More generally, our results are in line with emerging appreciation of the importance of spatial organization of cAMP signaling in discrete regions of the plasma membrane as well as cytoplasm through membrane compartmentation and localized CAMP turnover. Indeed, we found that the PDE4 inhibitor rolipram rendered plasma membrane–localized adenylyl cyclase (bPAC–PM) capable of triggering a transcriptional response following brief optogenetic activation (Fig. 4c). Thus the present results identify an essential cellular consequence of GPCR signaling from endosomes and reveal a discrete spatial encoding scheme that determines functional selectivity of the downstream response. Because GPCR signaling effects are mediated by second messenger control of downstream gene expression, we suggest that the present results have widespread physiological implications.

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METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Gene Expression Omnibus. All microarray data sets were deposited in under accession number GSE57274.

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Author contributions

N.G.T. performed the experiments and analyzed the data. N.G.T. and M.v.Z. designed the study, interpreted the results and wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Correspondence and requests for materials should be addressed to M.v.Z.
ONLINE METHODS

Cell culture and transfection. Human embryonic kidney (HEK293) cells were obtained from ATCC and grown in Dulbecco's modified Eagle Medium supplemented with 10% FBS (University of California–San Francisco Cell Culture Facility). Stably transfected HEK293 cells expressing Flag-tagged β2-AR were described previously. Synthetic RNA duplexes (GHC17, AllStars Negative Control) were obtained from the validated HP GenomeWide siRNA collection (Qiagen). For transient transfections, Lipofectamine 2000 or Lipofectamine-RNAiMax (Invitrogen) was used according to manufacturer's instructions. Plasmids were transfected for 24–48 h. siRNAs were transfected for 72 h.

Dyno-4a (AbCam) was prepared as a 30-mM stock solution in DMSO and added to cells grown in serum-free medium to a final concentration of 30 μM for 15 min preceding drug treatment. Bafilomycin A1 (Tocris Biosciences) was added from a 1-mM stock in DMSO to a final concentration of 0.5 mM for 15 min. Rolipram (Tocris Biosciences) was added from a 10-mM stock in DMSO to a final concentration of 10 μM immediately before exposure to light.

bPAC cloning and activation. cDNA encoding a humanized photocactivatable adenyl cyclase (bPAC) described in ref. 22 was obtained from P. Hegemann's laboratory through Addgene. The 2s×FVyF sequence was amplified from eGFP-2xFVyF plasmid, a generous gift from H. Stenmark (Oslo University Hospital, Norway), and the Lyn sequence was amplified from LF2C plasmid obtained from T. Meyer's laboratory through Addgene. Both targeting sequences were fused to the N terminus of cytosolic bPAC. All bPAC constructs were cloned into pCDNA3.1 Hygro (+) for mammalian expression. The cyclase was activated by illuminating cells inside a temperature- and CO2-controlled incubator for various times (0.5 min, 1 min, 2 min, 3 min, 4 min or 5 min).

Protein expression for each bPAC construct and CREB phosphorylation were quantified from immunofluorescence staining in ImageJ using the formula: corrected fluorescence = integrated density of cell area − (cell area × mean fluorescence of background)39.

Luminescence-based cAMP biosensors. Plasmids pGLO-20F and pGLO-22F (Promega) encoding circularly permuted firefly luciferase cAMP reporters were transfected into HEK293 cells and assayed as described in ref. 3. The pGLO-20F biosensor was used for all cAMP measurements except for bPAC-mediated cAMP accumulation assays. Because cAMP produced from bPACs at doses of light >1 min saturated the pGLO-20F biosensor, we used the pGLO-22F biosensor, which has a higher EC50 value for activation (Promega). For every experiment, reference wells were treated with 5 μM forskolin, and all experimental cAMP measurements were normalized and displayed as percent of the maximum luminescence value measured in the presence of forskolin. 'Maximum cAMP signal' refers to the peak value from each forskolin-normalized cAMP experiment. 'Integrated cAMP signal' was calculated by numerical integration of the area under the respective cAMP curve.

DNA microarray sample preparation and processing. Human HEK293 cells were grown in six-well plates at ~90–100% confluency, washed once with serum-free medium and resuspended in serum-free medium. 30 μM Dyno or DMSO was added for 15 min, followed by addition of isoprenol for 2 h. Total RNA was isolated with RNeasy Mini Kit (Qiagen) and quantified by Nanodrop. 2–5 μg of total RNA was used as input for amplification with AminoAlyl MessageAmp aRNA kit (Ambion). Up to 5 μg of in vitro transcribed aRNA was then coupled to NHS-monoesters of either Cy5 ('Iso') or Cy3 ('no drug') dyes (GE Healthcare Life Sciences). Cy5- and Cy3-labeled aRNA were combined in 3× SSC, 50 mM Heps (pH 7.0), 20 μg of poly(A) RNA, 20 μg Cot-1 DNA, 20 μg tRNA and 0.3% SDS to a total volume of 50 μl. Samples were then heated to 70°C for 5 min, spun at 14,000 r.p.m. at room temperature in a microfuge for 5 min, then hybridized at 65°C using the MAUI hybridization system (BioMicro) for 12–16 h. After hybridization, microarrays were washed first in a solution of 2× SSC with 0.05% SDS at 70°C for 5 min, then in 2× SSC at room temperature for 2 min, then in 1× SSC at room temperature for 2 min, then dried by centrifugation. Microarrays were scanned using AxonScanner 4000B (Molecular Devices), and PMTs were manually adjusted for every slide scanned to maximize signal without saturation.

DNA microarray data analysis. Only genes with signal greater than 1.5 times the background in either Cy5 or Cy3 channel and median Cy5 or Cy3 signal greater than 100 were included for further analysis. Log2 ratios (‘Iso’/‘no drug’) from each microarray were median centered. Genes were classified as ‘Iso targets’ if their expression was induced by at least 1.5-fold by isoproterenol treatment in each of four replicates and (ii) if their averaged expression from all four isoproterenol-treated replicates showed at least a twofold increase relative to untreated samples (i.e., log2('Iso'/'no drug') ≥ 1). This analysis yielded 55 high-confidence β2-AR target genes (Supplementary Table 1). Enriched gene ontology terms were identified with GeneTrail40, using a cut-off hypogeometric P < 0.05 (corrected for multiple hypothesis testing). Wilcoxon signed rank test analysis revealed that the expression induction of all 55 β2-AR genes was affected by blockade of endocytosis with Dyno at both saturating and sub-saturating doses of isoproterenol. We used a student t-test based on statistical differences by unpaired Student’s t-test of log2(Dyno + Iso/Dyno + No Drug) versus log2(DMSO + Iso/DMSO + No Drug) with a P < 0.05 to define ‘endocytosis-dependent’ genes. Average linkage hierarchical clustering was performed using Euclidian distance as a similarity metric with the R software and visualized with Java TreeView41.

Flow cytometry. For β2-AR internalization and recycling assays, we used stably transfected Flag-β2-AR cells. For receptor internalization measurements, cells were treated with isoprenol for 20 min. For receptor recycling measurements, cells were treated with isoprenol for 20 min, washed once and resuspended in fresh medium containing 10 μM alprenolol for 40 min. For receptor recycling measurements, cells were lifted and labeled with Alexa 647-M1 antibody (1:1,000). Flow cytometry of 10,000 cells per sample was carried out using a FACS-Calibur instrument (BD Biosciences). % internalized receptors = % surface receptors after 20 min isoproterenol)/(initial no. surface receptors) × 100.

Quantitative real-time PCR. Total RNA was extracted from samples with RNeasy Mini Kit (Qiagen). Reverse transcription was carried out with SuperScript III RT (Invitrogen) and a mix of oligo(dT) and random nonamer primers following standard protocols. The resulting cDNA was used as input for quantitative PCR with StepOnePlus (ABI). Taqman Expression Mastermix (Invitrogen) and validated Taqman probe mix (cat. no. 4331182, probe ID no. Hs00915740_m1) were used for DACT2 expression measurements. SYBR Select MasterMix (Invitrogen) and primers described in Supplementary Table 4 were used in determining the expression of all other genes. Statistical significance was established with unpaired Student’s t-test. All levels were normalized to the levels of a housekeeping gene (ACTA or GAPDH).

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