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β_2 -adrenergic receptor control of endosomal PTH receptor signaling via G $\beta\gamma$

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Abstract

Cells express several G-protein-coupled receptors (GPCRs) at their surfaces, transmitting simultaneous extracellular hormonal and chemical signals into cells. A comprehensive understanding of mechanisms underlying the integrated signaling response induced by distinct GPCRs is thus required. Here we found that the β_2 -adrenergic receptor, which induces a short cAMP response, prolongs nuclear cAMP and protein kinase A (PKA) activation by promoting endosomal cAMP production in parathyroid hormone (PTH) receptor signaling through the stimulatory action of G protein G $\beta\gamma$ subunits on adenylate cyclase type 2.

We used osteoblast-like ROS17/2.8 cells that endogenously express the parathyroid hormone receptor (PTHr) and β_2 -adrenergic receptor (β_2 AR) as a model system to investigate the mechanism by which these two receptors functionally interact. We first probed whether β_2 AR activation induces changes in PTH-mediated cAMP production and PKA activation. To this end, FRET-based biosensors that detect cytosolic or nuclear changes in cAMP accumulation or PKA activation were expressed in live cells. Brief pulses of PTH(1–34) or isoproterenol (ISO) caused rapid increases in cAMP, to similar levels in each condition (Fig. 1a). Following this increase, the cAMP response mediated by ISO rapidly declined after

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Author contributions

F.G.J.-A. performed all the experiments with the support of V.L.W. and J.C. K.X., provided expertise and supervised proteomic experiments. M.N. provided expertise with ROS17/2.8 cells. J.M.T. provided expertise with culture of primary osteoblasts, and J.-P.V. was responsible for the overall concept of the study, as well as writing of the manuscript and data analysis with F.G.J.-A.

Competing financial interests

The authors declare no competing financial interests.

Any supplementary information, chemical compound information and source data are available in the [online version of the paper](#).

cAMP-response element binding (CREB) protein is a determinant transcription factor downstream of PTH⁵. We found that PTH stimulated CREB protein phosphorylation to a significantly greater and longer degree in cells also treated with ISO (Fig. 1b). This enhanced capacity of PTH to stimulate CREB activity in the presence of ISO correlates with the effect of ISO on PTH-mediated cAMP generation in the same cells. Taken together, these results indicate that β_2 AR activation enhances endosomal cAMP production mediated by PTH resulting in prolonged nuclear PKA activation and CREB phosphorylation.

We anticipated that regulation of PTHR activity by β_2 AR could potentially proceed via two mechanisms: (1) the presence or formation of receptor heterodimers that regulate G protein activity as previously demonstrated for certain GPCR heteromers¹¹, or (2) a functional crosstalk through a regulatory network independent of receptor heteromers. To eliminate the first possibility, we first found that artificially immobilized N-terminally GFP-tagged PTHR (PTHR^{N-GFP}) at the cell surface did not limit the mobility of a β_2 AR C-terminally tagged with CFP (β_2 AR^{CFP}), as determined by fluorescence recovery after photobleaching (FRAP) (Supplementary Fig. 5a). This result was independent of receptor activation, as neither PTH alone nor in combination with ISO modified β_2 AR^{CFP} mobility. In contrast, PTH significantly decreased the mobility of G $\beta\gamma$ tagged with cyan fluorescent protein (CFP) (G $\beta\gamma$ ^{CFP}) (Supplementary Fig. 5a). Next, intermolecular FRET between PTHR and β_2 AR C-terminally tagged with CFP or yellow fluorescent protein (YFP), respectively, was not different from non-specific FRET because of random collision between N-terminally plasma-membrane-tagged CFP and YFP molecules (Supplementary Fig. 5b).

To identify the mechanism by which the β_2 AR synergizes PTHR signaling, we used mass-spectrometry-based immunoprecipitation experiments on purified endosomes from HEK293 stably expressing PTHR, which revealed the capture of G α_s , G $\beta\gamma$ subunits, and β -arrestins by the PTH-bound PTHR located in endosomes (Supplementary Fig. 6a–c and Supplementary Table 1). Immunoprecipitation experiments (Supplementary Fig. 6d), as well as confocal imaging of cells expressing G $\beta\gamma$ ^{CFP} and tetramethylrhodamine-labeled PTH (PTH^{TMR}) or β -arrestin1 in fusion with Tomato (β -arr1^{Tom}) (Supplementary Fig. 7a,b), further support the theory of assembly of PTHR complexes with G $\beta\gamma$ and β -arrestins at time points when the receptor is localized in endosomes. Additional FRET recordings in cells expressing G $\beta\gamma$ ^{CFP} and β -arrestin2–YFP (β -arr2^{YFP}) confirmed a direct interaction between G $\beta\gamma$ and β -arrestins induced by PTH (Fig. 2b, left) when the active conformation of G α_s , detected by the nanobody Nb37^{GFP}, was located in endosomes labeled by β -arr1^{Tom} (Supplementary Fig. 7c). These data indicate that PTH maintains an endosomal PTHR signaling complex constituted by β -arrestins, G $\beta\gamma$, and activated G α_s .

To further examine the possible involvement of G $\beta\gamma$ in endosomal cAMP signaling mediated by PTH, we expressed in ROS17/2.8 cells the C-terminal domain of G-protein-coupled receptor kinase 1 (β -ARK-ct) that acts as a dominant negative G $\beta\gamma$ scavenger¹² or incubated cells with gallein, a small-molecule inhibitor of G $\beta\gamma$ ¹³. In both cases, β -ARK-ct or gallein did not block ISO-induced cAMP, but did markedly inhibit the sustained cAMP production mediated by PTH without affecting PTHR endocytosis (Supplementary Fig. 8a,b,d). Notably, control cAMP measurements in cells lacking G α_s and treated with PTH confirmed earlier studies¹⁴ that G $\beta\gamma$ subunits are not sufficient to stimulate cAMP by

themselves and need activated G_{α_s} (Supplementary Fig. 9). We then interrogated whether $G\beta\gamma$ in the PTHR complex increases the G_{α_s} -stimulated activity of certain isoforms of adenylyl cyclases¹⁵ known to be co-stimulated by $G\beta\gamma$, such as the adenylyl cyclase type 2 (AC2) (ref. 14), which was detected in ROS17/2.8 cells (Supplementary Fig. 10a). Cells treated with the selective inhibitor for AC2, SKF83566 (ref. 16), blocked the sustained cAMP response mediated by PTH (Supplementary Fig. 10b). Further support for the role of AC2 in endosomal stimulation of cAMP is provided by the selective inhibitory action of SKF83566 on cells overexpressing AC2 as opposed to those overexpressing AC5 (Supplementary Fig. 10c,d). Consistent with a model where $G\beta\gamma$ subunits are a determinant for the conditional stimulation of AC2 activity in endosomal PTHR signaling through cAMP, we found that PTH induced a substantial FRET increase in AC2 C-terminally fused with YFP (AC2^{YFP}) and $G\beta\gamma^{CFP}$ (Fig. 2a, center), which was maintained at a time point when ACs detected by forskolin-BODIPY together with $G\beta\gamma^{CFP}$ were located in endosomes (Supplementary Fig. 7c,d). These data suggest that AC2 and $G\beta\gamma$ are key factors for endosomal PTHR signaling.

We thus reasoned that the positive effect of ISO on PTH-mediated endosomal cAMP production might be accomplished by promoting interactions between $G\beta\gamma$ and AC2. Before testing this theory we examined whether $G\beta\gamma$ was involved in the synergistic action of β_2AR on PTH-mediated sustained cAMP. To this end, we expressed β -ARK-ct in ROS17/2.8 cells and in primary osteoblasts and found that β -ARK-ct blocked the action of ISO in the endosomal cAMP signaling mediated by PTH (Fig. 2b and Supplementary Fig. 2c). FRET recordings then confirmed the aforementioned hypothesis by showing that the addition of ISO significantly increased the capacity of PTH to sustain $G\beta\gamma$ interactions with both AC2 and β -arr2 (Fig. 2a, left and center). Given that these interactions were short lived or undetectable in response to ISO alone, and that ISO had no effects on PTH-mediated PTHR- β -arrestin interactions (Fig. 2a, right), we propose a new model in which $G\beta\gamma$ and AC2 not only prolong cAMP signaling in response to PTH, but also integrate the positive effect of β_2AR for promoting endosomal PTHR signaling.

To probe whether β_2AR potentiates PTHR signaling through a mechanism involving the release of $G\beta\gamma$ via activation of G_{α_i} proteins^{17,18}, we treated ROS 17/2.8 cells with pertussis toxin (PTX), an inhibitor of G_{α_i} activation. PTX significantly increased the duration and extent of ISO-induced cAMP (Supplementary Fig. 8c) and had no effect on PTH-mediated cAMP, but significantly blocked the synergistic action of ISO on PTHR signaling (Fig. 2b, right). Such PTX sensitivity is consistent with a mechanism in which G_{α_i} activation is a key determinant in the cooperative action of β_2AR in endosomal PTHR signaling because it provides an additional pool of $G\beta\gamma$, which potentiates the activity AC2. We lastly examined the generality of these results by testing the effect of a selective agonist for the G_{α_i} -coupled α_{2A} -adrenergic receptor ($\alpha_{2A}AR$) known to be expressed in bone, UK14,304 (refs. 19,20). As expected, by UK14,304 did not induce cAMP, but it promoted a significant ($P < 0.005$) increase in PTH-mediated endosomal cAMP that was dependent on AC2 stimulation (Supplementary Fig. 11a,b). This result indicates that a G_{α_i} -coupled receptor also can enhance endosomal AC2 activity stimulated by PTH, which is likely mediated by $G\beta\gamma$ and is consistent with the inability of G_{α_i} to inhibit AC2 (ref. 21).

In summary, activation of the β_2 AR markedly promotes endosomal cAMP production by PTH via the release of $G\beta\gamma$ from $G_{\alpha i}$ and conditional stimulation of cAMP via AC2, which in turn sustains nuclear PKA and CREB activation. These findings generalize a model in which the $G\beta\gamma$ exchange between GPCRs contributes to receptor crosstalk²². While our results provide a mechanistic understanding of the functional coupling between β_2 AR and PTHR, several questions remain to be explored. Can other proteins also contribute to the synergistic action of the β_2 AR on endosomal cAMP signaling mediated by PTH? For example, sorting nexin 27 (Snx27) regulating β_2 AR and PTHR recycling^{1,23,24} might be sequestered by activated β_2 AR, thus allowing extended endosomal PTHR signaling. Does the mechanism shown in this study apply to other bone cells such as chondrocytes and osteocytes, which also express these two receptors? Despite these remaining questions, our results uncover a fundamental mechanism underlying the coordinated regulation of PTHR signaling by the β_2 AR and sets the first step toward understanding the role of β_2 AR in regulating PTHR signaling in cells (Supplementary Fig. 12).

ONLINE METHODS

Cell culture and transfection

Cell culture reagents were obtained from Corning (CellGro). Human embryonic kidney cells (HEK293; ATCC, Georgetown, DC) were cultured in DMEM supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO₂. HEK293 cells stably expressing the recombinant human PTHR were grown in selection medium (DMEM, 10% FCS, penicillin–streptomycin 5%, 500 µg/ml neomycin). Rat osteosarcoma (ROS17/2.8) cells were cultured as described²⁵. Mouse embryonic fibroblast (MEF)²⁶ cells knocked out for $G_{\alpha s}$ were cultured in DMEM–F12. For transient expression, cells were cultured on glass coverslips coated with poly-D-lysine for HEK293 and ROS cells, or collagen for MEF cells in six-well plates for 24 h, and then transfected with the appropriate cDNAs using Fugene-6 (Roche) or Lipofectamine 3000 for 48–72 h before experiments. We optimized expression conditions to ensure the expression of fluorescently labeled proteins was similar in examined cells by performing experiments in cells displaying comparable fluorescence levels.

Chemicals

PTH(1–34) was purchased from Bachem and PTH(1–34)^{TMR} was synthesized and characterized as previously described²⁵. Pertussis Toxin and UK14,304 were from Sigma-Aldrich; SKF83,566, and ICI118551 were from Tocris; dynasore and forskolin were from EMD-Millipore; BODIPY–FL forskolin was from Life Technologies.

Plasmids

SEP–PTHr was provided by P. Friedman (University of Pittsburgh), AC2 plasmid was a gift from D. Cooper (University of Cambridge), NB37^{GFP} was a gift from M. von Zastrow (UCSF), and AC5^{GFP} from T. Hebert (McGill University). AC2^{YFP} was generated using PCR mutagenesis and subcloning in pYFP vector between restriction site XhoI and EcoRI. pRK5– β -ARK-ct minigene was purchased from Addgene (plasmid #14695) and initially generated in RJ Lefkowitz lab (Duke University).

Laser scanning confocal microscopy

Cells plated on coverslips were mounted in Attofluor cell chambers (Life Technologies) and incubated with HEPES buffer containing 150 mM NaCl, 10 mM HEPES, 2.5 mM KCl and 0.2 mM CaCl_2 , 0.1% BSA, pH 7.4 were transferred on the Nikon Ti-E microscope (Nikon) equipped with a Z-driven piezo motor. Imaging was acquired using a Nikon A1 confocal unit, through a $60\times$ N.A. = 1.45 objective (Nikon). Fluorescent proteins or peptides containing CFP, GFP, YFP, Tomato or tetramethylrhodamine (TMR) were excited with 440-nm (CFP), 488-nm (GFP), 514-nm (YFP) or 560-nm (Tomato or TMR) lasers (Melles Griot). Data acquisitions were done using Nikon Element Software (Nikon Corporation). After acquisition, raw data were analyzed using ImageJ software. Each different analysis was done at the single-cell level.

Immunocytochemistry

ROS17/2.8 cells cultured on 13 mm glass coverslips were fixed with paraformaldehyde 4% (in PBS) for 10 min. Following extensive wash by PBS, cells were incubated and permeabilized with blocking buffer (0.05% saponin, 1% BSA, 1% FBS) for 1 h. Primary antibodies were incubated overnight at 4 °C, and fluorescent secondary Alexa Fluor 568-antibody (Abcam; #ab175471) were incubated for 30 min at room temperature. Nucleus staining was done with DAPI incubation for 5 min following by PBS washes. Coverslips were mounted on glass slide using Fluoromount mounting solution (Sigma-Aldrich).

Immobilization of PTHR

Cell surface PTHR was immobilized by crosslinking specific antibodies using a strategy previously reported³. Cells were rinsed in buffer containing 150 mM NaCl, 10 mM HEPES, 12.8 mM D-glucose, 2.5 M KCl, 0.5 mM MgCl_2 and 0.5 mM CaCl_2 (pH 8.0) and then incubated at room temperature for 20 min in a 1:200 dilution of polyclonal rabbit anti-GFP antibody (Life Technologies; A-11122). Cells were washed twice and incubated for 20 min at room temperature in a 1:200 dilution of biotin-XX goat anti-rabbit IgG (H + L) (Life Technologies; B2770). Then, cells were washed two times before beginning the experiments.

Fluorescence recovery after photobleaching (FRAP)

Coverslips in Attofluor cell chambers were transferred to the stage of a Nikon A1 confocal microscope and imaged using a $60\times$ 1.45 NA objective. Experiments were done in HEK293 cells transiently expressing PTHR^{GFP}, $\text{G}\beta_1\gamma_2^{\text{CFP}}$, and the $\beta_2\text{AR}$ C-terminally tagged with CFP. Our pilot FRET experiments comparing association and dissociation kinetics between PTHR^{YFP} and $\text{G}\beta_1\gamma_2$ using either $\text{G}\beta_1\text{G}\gamma_2^{\text{CFP}}$ or $\text{G}\beta_1\gamma_2^{\text{CFP}}$ BiFC (where $\text{G}\beta_1$ is fused to the N-terminal part of CFP residues 1–158, and $\text{G}\gamma_2$ is fused to the C-terminal part of CFP residues 159–238 as described²⁷) showed no difference. CFP was photobleached for 4.4 s using a 457.9-nm laser beam focused on a circular region of interest (2 μm diameter) at the plasma membrane. Fluorescence recovery was monitored for 2 min before and after addition of ligand. Recovery curves were calculated for each value according to equation (1):

$$\text{Recovery (\%)} = \frac{(I_{\text{bleach}} - I_{\text{back}})}{(I_{\text{ref}} - I_{\text{back}})} \times 100$$

where I_{bleach} is the fluorescence intensity of the bleached spot, I_{back} is the fluorescence intensity of the background, and I_{ref} is the fluorescence intensity of control regions in other cells or regions far removed from the target cell. Data were fitted to an exponential decay and plotted using GraphPad Prism version 5.00 (GraphPad Software, San Diego CA, USA).

Measurements of cAMP and PKA activity

Time courses of cAMP and PKA activity were assessed using FRET-based assays^{28,29}. Cells were transiently transfected with intramolecular FRET-based biosensors for either cAMP (epac1-CFP/YFP) or PKA activity (AKARIIII-CFP/YFP), and measurements were performed and analyzed as previously described^{23,26}. Biosensors fused to a nuclear localization sequence (NLS) signal were used to measure cAMP or PKA activity³⁰. FRET signals were monitored after challenge with either PTH(1–34), ISO, or PTH + ISO. In brief, ROS17/2.8, primary osteoblasts from mice, or HEK293 cells plated on poly-D-lysine-coated glass were mounted in Attotfluor cell chambers (Life Technologies), maintained in HEPES buffer and transferred on a Nikon Ti-E equipped with an oil immersion 40 × N.A. 1.30 Plan Apo objective and a moving stage (Nikon Corporation). CFP and YFP were excited using a mercury lamp. Fluorescence emissions were filtered using a 480 ± 20 nm (CFP) and 535 ± 15 nm (YFP) filter set and collected simultaneously with a Luca EMCCD camera (Andor Technology) using a DualView 2 (Photometrics) with a beam splitter dichroic long pass of 505 nm. Fluorescence data were extracted from single cells using Nikon Element Software (Nikon Corporation). Individual cells were continuously perfused with buffer or with ligands for a brief time (<1 min), and the FRET ratio or FRET efficiency for single cells was calculated and corrected as previously described³¹.

Western blot analysis of cytosolic and nuclear fractionated samples

ROS17/2.8 cells were washed twice with ice-cold PBS after 20 min stimulation at 37 °C with ISO (10 μM), PTH(1–34) (100 nM) alone or in combination with ISO. The cells were then scraped into 1 ml ice-cold PBS, collected into eppendorf tubes and spin at 1,000 × *g* for 5 min at 4 °C. The cell pellets were resuspended in 800 μl of cytoplasmic extract reagent (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF, pH 8) in the presence of protease inhibitor mixture. After 5 min on ice, 40 μl of 10% Nonidet P-40 was added to the cell suspension and put on ice for another 5 min. The cells were centrifuged at 15,000 × *g* for 5 min at 4 °C. The supernatants were used as cytoplasmic fractions and kept on ice. The nuclear pellets were washed twice in ice-cold PBS and resuspended in 150 μl of urea buffer (4 M Urea, 62.5 mM Tris-HCl pH 6.8, 2% SDS, 1 mM EDTA). The nuclear fractions were sonicated for 20 s to reduce the viscosity.

Cytosolic and nuclear proteins were prepared for western blot by adding 2× Laemmli buffer (5% β-mercaptoethanol) and boiled for 5 min. The samples were resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane. The membranes were blocked for 1 h in 5% (wt/vol) nonfat dried milk in Tris-buffered saline plus Tween 20 and incubated overnight

at 4 °C with primary antibodies for PKA regulatory II β subunit (PKA-RII β) (1:1,000; BD Bioscience, #610626 clone 45), PKA catalytic subunit (PKA-C α) (1:1,000; Cell Signaling Technology, #4782) and phospho-PKA RII (Ser-96; 1:1000; EMD Millipore, #04-404). Antibodies against phospho-CREB and CREB (1:1,000, Cell Signaling Technology, #9191 and #9197, respectively) and β -tubulin (1:1,000; Cell Signaling, #2146) were used as nuclear and cytoplasmic markers, respectively, to confirm the purity of the two fractions. The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:1000; Dako #P0448) for 1 h at room temperature. Immunoreactive bands were visualized with enhanced chemiluminescence (Amersham) and autoradiography film.

Immunoprecipitation

HEK293 cells stably expressing hemagglutinin (HA)-tagged PTHR and cultured on 15-cm dish were stimulated with PTH(1–34) 100 nM for the indicated time. Cells were then washed with ice-cold PBS prior crosslinking for 2 h with dithiobis (succinimidyl propionate) DSP (Covachem) in PBS at 4 °C. The reaction was stopped by addition of 10 mM Tris–HCl for 10 min and cell lysates were prepared using lysis buffer (0.5% Triton X-100, 50 mM Tris–HCl pH 7.4, 140 mM NaCl, 0.5 mM EDTA) containing protease and phosphatase inhibitors (Roche). Protein concentration was determined using BCA protein assay kit (Pierce) and lysates were incubated with anti-HA agarose antibody beads (Sigma-Aldrich; #A2095 clone HA-7) overnight at 4 °C. Elution was done using LDS loading buffer (Life Technologies) and samples loaded on 10% SDS–PAGE and transferred to nitrocellulose membrane. We used antibodies against HA (Covance, clone 16B12), G β (EMD Millipore; #06-238), and β -arrestin1/2 (Cell Signaling; #4674, clone D24H9). Immunoreactive bands were visualized with Luminata Forte (EMD Millipore) and autoradiography film.

Reverse-transcription polymerase chain reaction

Adenylate cyclase isoforms expressed in ROS17/2.8 cells were identified by reverse transcription PCR. Total RNA was extracted from 70–80% confluent cells using TRIzol reagent (Ambion, Life Technologies). Purified RNAs were reverse transcribed using Accuscript High Fidelity cDNA synthesis kit (Agilent Technologies) and Oligo(dT)₁₈ primer. PCR for each AC isoform was performed using primer pairs designed according (Supplementary Table 2) to the IDT Primer QuestTool software and amplified by using Phusion High-Fidelity Polymerase (New England BioLabs). PCR products were identified by agarose gel electrophoresis and visualized under UV excitation with SYBR Safe gel staining (Life Technologies).

Endosomal PTHR proteomics

We described this method in detail in ref. 32. and its schematic flowchart is shown in Supplementary Figure 6. We briefly described the three steps involved:

1) Endosomal isolation—The HA-tagged PTHR was subcloned into the pAC-MV–tetO vector, and the resultant construct was transfected into HEK293S cells to generate an adhesion cell line stably overexpressing HA–PTHR. This stable cell line was adapted to a suspension cell culture system in Invitrogen FreeStyle medium. Endosomes were isolated 15 min after stimulation using a sucrose density gradient centrifugation using four volumes of

homogenization buffer (HB) (250 mM sucrose, 3 mM imidazole, pH 7.4, 1 mM EDTA, and protease and phosphatase inhibitors) added to the cell pellet, which then was disrupted by Dounce homogenization on ice for approximately 50 strokes. The cell lysate was centrifuged at 2,000g for 10 min at 4 °C. Post-nuclear supernatant (PNS) was carefully transferred to a 15 ml falcon tube and 62% (w/w) sucrose solution (2.351 M sucrose, 3 mM imidazole, pH 7.4, 1 mM EDTA, in ddH₂O. Refractive index ~1.4463 at 20 °C) was added to the PNS to a final sucrose concentration of 40.6%. The diluted PNS was loaded on the bottom of an ultracentrifuge tube. 1.5 volume of 35% sucrose (1.177 M sucrose, 3 mM imidazole, pH 7.4, 1 mM EDTA, in ddH₂O. Refractive index ~1.3904 at 20 °C), 1 volume of 25% sucrose (0.806 M sucrose, 3 mM imidazole, pH 7.4, 1 mM EDTA, ddH₂O. Refractive index ~1.3723 at 20 °C) and 0.5 volume of HB were sequentially overlaid on the top of the PNS and centrifuged at 210,000g at 4 °C for 1.5 h. The endosome fraction was collected for endosomal PTHR signaling complex isolation.

2) Isolation of endosomal PTHR signaling complex—*n*-Dodecyl- β -D-maltoside (DDM) was added to the endosome fraction and stirred for 1 h. The DDM concentration was diluted to 0.5% before the addition of anti-HA-agarose beads (Pierce #26181). After 4 h of incubation with agitation, the anti-HA-agarose antibody beads were collected by centrifugation and the endosomal PTHR signaling complexes were eluted with 1 mg/ml HA peptides. The HA peptides were removed by using an Amicon Ultra 0.5 ml centrifuge filter (MWCO, 10 kDa, Sigma #Z677108). The endosomal PTHR signaling complexes were digested using an in-solution digestion protocol as described previously^{32–34}. Tryptic peptides were subjected to LC–MS/MS analyses.

3) Proteomic analysis—LC–MS/MS analyses were performed on a Thermo Scientific LTQ Orbitrap XL (Thermo Scientific) with a Finnigan Nanospray II electrospray ionization source. Tryptic peptides were injected onto a 75 μ m $\times$ 150 mm BEH C18 column (particle size 1.7 μ m, Waters) and separated using a Waters nanoACQUITY Ultra Performance LC (UPLC) System (Waters, Milford, MA). The LTQ Orbitrap XL was operated in the data-dependent mode using the TOP10 strategy. In brief, each scan cycle was initiated with a full MS scan of high mass accuracy (400–2,000 *m/z*, acquired by the Orbitrap XL at 6×10^4 resolution setting and automatic gain control (AGC) target of 10^6) (ref. 5), which was followed by MS/MS scans (AGC target 5,000; threshold 3,000) in the linear ion trap on the 10 most abundant precursor ions. Selected ions were dynamically excluded for 30 s. Singly charged ions were excluded from MS/MS analysis. MS/MS spectra were searched using the Mascot (Matrix Sciences, Inc.) algorithm against a composite database containing the SwissProt *Homo sapiens* forward and reverse protein sequences. Search parameters allowed two missed tryptic cleavages, a mass tolerance of ± 10 p.p.m. for precursor ions, a mass tolerance of ± 0.02 Da for product ions, a static modification of 57.02146 Da (carboxyamidomethylation) on cysteine and a dynamic modification of 15.99491 Da (oxidation).

Osteoblast isolation

Primary osteoblasts were obtained from calvarias of 10 days old mouse C57 BL6/6J pups (Jackson Laboratory). Harvested bone calverias were washed with PBS twice and downsized

into 3 mm × 3 mm pieces then incubated with digestion solution (4:1 α -MEM (Thermo Fisher; #32571101); 0.25% Trypsin solution (Thermo Fisher; # 25200056) supplemented by 3.2 mg collagenase (Sigma-Aldrich; #C1764) at 37°C on shaker during 5 min. Supernatant is then discarded and replaced by fresh digestion solution for 20 min and reaction is stopped by addition of 15% of FBS (Atlanta Biologicals; #S11550). Supernatant was collected then fresh α -MEM was added to rinse calvaria pieces. Supernatant was collected and combined with the previous collection, centrifuged for 5 min at 600 × *g*. Cell pellet was resuspended and plated in a T75 flask using cell culture medium (α -MEM, 10% FBS, 1% penicillin–streptomycin (HyClone), 100 μ g/ml kanamycin (Life technologies), 1:500 fungizone (Omega Scientific Inc.) for expansion. The digestion process is repeated three times to obtain a cell culture with enriched with differentiated osteoblasts.

Alizarin red staining

Primary osteoblasts cells were seeded in 6-well plates and treated with PTH (100 nM) alone, isoproterenol (100 nM) alone or with the combination of PTH + Isoproterenol for 3 weeks in osteogenic media (α -MEM, 10% FBS, 1% penicillin–streptomycin, dexamethasone 10 nM (Sigma-Aldrich; #D2915), β -glycerophosphate 5 mM (Sigma Aldrich #G6251) and L-Ascorbic acid 2-phosphate 100 μ M (Sigma-Aldrich; #A8960). For staining, cells were fixed with 4% formaldehyde (Thermo Fisher; #PI-28908) for 1 h, then stained with Alizarin Red solution (Amresco; #9436, filtered 10 g/L solution in H₂O, pH titrated to 4.2 using 0.5% ammonium hydroxide for 10 min and then rinsed and maintained in distilled H₂O. Plates were scanned then staining density was measured using ImageJ (NIH).

Statistical analysis

Data are expressed as mean values $\pm$ s.e.m. Statistical analyses were performed using the unpaired Student's *t*-test using GraphPad Prism 6.0 software when applicable. Differences were considered significant at *P* < 0.05. Approaches were taken to ensure robust and unbiased results.

Experimental rigor and reproducibility were ensured by the following methods: (1) Repeating basic experiments at least three times including positive as well as negative controls. Experiments were not blinded and are reported as number (*N*) of independent experiments and number (*n*) of cells tested.

(2) Validating all reagents used. Antibodies are authenticated by expressing in HEK or ROS cells the native protein or epitope against which the antiserum was raised and confirming that it is recognized by the indicated antibody. All recombinant proteins and mRNA constructs are sequenced for fidelity through the PITT Core facility <http://www.genetics.pitt.edu>. For cell lines used in the described study, that are engineered heterologous expression model, in which PTHR, adenylate cyclase 2, or G proteins are expressed, there is no concern regarding the fidelity of the cell model. If a discernible change in PTHR signaling properties arise, or if the cell aspect changes conspicuously, we then resort to an archived aliquot of cells kept in liquid nitrogen. These cells were acquired under license from ATCC. Accordingly, they have been authenticated. Again, these cells

possess a distinct phenotype and were this to change over time, we would start from a fresh aliquot of stored cells.

Data availability

Our research resources, including methods, disposables, cells, media, and protocols are available upon request to qualified academic investigators for non-commercial research purposes. All reagents developed, such as receptor constructs, as well as techniques and detailed methods, will be made available upon written request. The corresponding author adheres to the NIH Grants Policy on Sharing of Unique Research Resources including the “Sharing of Biomedical Research Resources: Principles and Guidelines for Recipients of NIH Grants and Contracts.”

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Synergistic action of β_2 AR on PTHR signaling

(a) Averaged cytosolic cAMP production in ROS17/2.8 cells mediated by ISO (10 μ M) and PTH (100 nM) alone or in combination with ISO (10 μ M) in the absence (control) or presence of dynasore. (b) Representative western blots and quantification of time courses of CREB phosphorylation (pCREB) by either, PTH, ISO, or PTH + ISO in ROS 17/2.8 cells. Full images of blots are shown in Supplementary Figure 14. Bars represent mean values $\pm$ s.e.m. of $N = 4$ experiments; statistical comparison performed by two-way ANOVA (** $P < 0.005$).

Figure 2. $G\beta\gamma$ and AC2 control of endosomal cAMP production by PTH
(a) Time courses of interactions between $G\beta\gamma^{CFP}$ and $\beta\text{-arr}2^{YFP}$, $G\beta\gamma$ and AC2 and PTHR and $\beta\text{-arr}2$ in response to PTH (100 nM) without (black) or with ISO (gray) measured by changes in FRET in HEK293 cells ($n = 10$). **(b)** Average time courses of cAMP in ROS17/2.8 cells mediated by PTH (100 nM) + ISO (10 μM) without (control) or with $\beta\text{-ARK-ct}$ (left), AC2 inhibitor (center) or PTX (right). Data represent the mean value $\pm$ s.e.m. of $N = 4$ experiments and $n = 18\text{--}27$ cells.