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# Non-canonical signaling of the PTH receptor

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## Abstract

The classical model of arrestin-mediated desensitization of cell-surface G protein-coupled receptors (GPCRs) is thought to be universal. However, this paradigm is incompatible with recent reports that the parathyroid hormone (PTH) receptor (PTHR), a crucial GPCR for bone and mineral ion metabolism, sustains $G_S$ activity and continues to generate cAMP for prolonged periods after ligand-wash-out; during these periods the receptor is observed mainly in endosomes, associated with the bound ligand, $G_S$ and β-arrestins. In this review, we discuss possible molecular mechanisms underlying sustained signaling by the PTHR, including modes of signal generation and attenuation within endosomes, as well as the biological relevance of such non-canonical signaling.

## PTHR: a paradoxical and medically critical GPCR

Parathyroid hormone (PTH) and PTH-related protein (PTHrP) play critical and distinct physiological roles by activating a common cell-surface receptor, the PTH type 1 receptor (hereafter noted PTHR), a family 2 GPCR. Circulating and homeostatic PTH regulates blood concentrations of calcium and phosphate ions, as well as vitamin D by acting in bone (osteoblasts, osteocytes) and kidney (proximal and distal tubule cells). PTHrP, a paracrine hormone, controls cell differentiation and proliferation in developing tissues, including the skeleton, the heart, and mammary glands. PTHR, when bound by PTH or PTHrP, stimulates heterotrimeric $G_S$- and $G_{q/11}$ proteins, resulting in the activation of signaling pathways involving adenylyl cyclase/cAMP/protein kinase A (PKA) and phospholipase Cβ/inositol trisphosphate ($IP_3$)/$Ca^{2+}$/protein kinase C (PKC), respectively (1–3). PTHR can also activate other pathways that include $G_{12/13}$/RhoA/phospholipase D (PLD) and the mitogen-activated protein kinase (MAPK) (extracellular signal-regulated kinase, $ERK_{1/2}$) signaling cascades (4–6).

Defects in PTHR signaling are directly involved in human diseases of bone and mineral ions metabolism such as those associated with hyper- or hypoparathyroidism (due to a defect in PTH secretion from the parathyroid glands), hypercalcemia of malignancy (due to excessive



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PTHrP secretion), neonatal lethality in Blomstrand's chondrodysplasia (caused by a defective expression of PTHR) (7), dwarfism and hypercalcemia in Jansen's chondrodysplasia (8) or cartilage tumors of bone in Eiken Syndrome (caused by activating PTHR mutations) (9). PTHR is also a significant therapeutic target, as PTHR agonists can stimulate bone formation. The synthetic N-terminal fragment of PTH, PTH(1–34), and the intact PTH(1–84) polypeptide, are the only bone-anabolic agents currently available that can decrease fracture incidences in severe cases of osteoporosis by the stimulation of trabecular and cortical bone formation. This is accomplished through daily injections of PTH, a regimen known as intermittent PTH treatment (intPTH) (10). However, the therapeutic use for intPTH is limited by the principal side effect of hypercalcemia (elevated $Ca^{2+}$ in the blood) and a possible risk of osteosarcoma (malignant bone tumor) (11–13). In contrast with the osteoanabolic effect of intPTH, other treatment regimens, such as continuous PTH perfusion, stimulate bone resorption. Understanding molecular and cellular mechanisms by which activation of PTHR by its two native ligands triggers different biological effects and mediates the paradoxical anabolic and catabolic effects that PTH has on bone mass are thus keys for the development of new drugs for diseases of bone and mineral metabolism, such as osteoporosis, hyper- and hypoparathyroidism. Here we review recent findings that not only point to molecular mechanisms that may account for the biological differences between PTH and PTHrP, but also suggest that the internalized PTHR, in complex with $G\alpha_S$ and β-arrestin, can sustain cAMP signaling from the early endosomal compartment. The findings thus indicate a paradigm shift in our understanding of GPCR signaling.

## Kinetics of PTHR activation

A combination of biochemical, pharmacological and optical techniques, including photo-affinity cross-linking using bisphenol A (BPA)-containing PTH analogs (3, 14–18), coupled with functional assays using mutant receptors and structurally modified ligands (19–22), and Förster resonance energy transfer (FRET)-based approaches (23–26) have revealed important insights about ligand – receptor interaction mechanisms and rate-limiting reactions involved in activation of PTHR and its cognate $G_S$ protein (Box 1 and Table 1) (27, 28).

### Box 1

#### Recording activation/deactivation reactions along the PTHR signaling cascade

PTHR transmits PTH or PTHrP-induced signals via a classical sequence of reactions that takes place initially at the plasma membrane. The first step involves ligand (L) binding to a receptor (R) and its shift from an inactive to active conformation (L–Rrarr; $L–R^*$). The active $R^*$ can then bind $G_S$ protein (G) in its inactive GDP-bound form ($L–R^* + G^{GDP}$rarr;$L–R^*–G^{GDP}$). This interaction catalyses the GDP–GTP exchange on the Gα subunit, activating the G protein and triggering the dissociation of the GTP-bound Gα ($G\alpha^{GTP}$) from the receptor and from the Gβγ dimer. The dissociation follows the reaction: $L–R^* –G^{GDP}$rarr;$L–R^* –G^{GTP}$rarr;$L–R^* + G\alpha^{GTP}$+ Gβγ. Next, $G\alpha^{GTP}$ binds and activates adenylyl cyclases that convert ATP into the second messenger cAMP. The intrinsic GTPase activity of Gα hydrolyses GTP into GDP + inorganic phosphate (Pi) resulting in an inactive $G\alpha^{GDP}$, which then binds Gβγto initiate a new reaction cycle.

PTHR is so far the only receptor for which kinetics of each reaction involved in the signaling cascade from ligand binding to second messenger production has been measured in live cells (25). These kinetics have been measured by FRET-based approaches (28) (Figure I). These techniques are illustrated in the left panels of Figure I ( GFP, CFP, YFP). The right panels represent the time course of individual

reactions triggered by PTH or PTHrP. These FRET approaches in live cells, coupled with biochemical-based assays, reveal a series of unexpected findings: i) the PTH–PTHR complex internalizes rapidly into Rab5-positive endosomes (early endosomes) in association $G_S$, β-arrestins, and adenylyl cyclases; ii) the internalization of the PTH–PTHR complex is not associated with desensitization of the $G_S$ or cAMP response; iii) blocking PTH–PTHR internalization prevents a sustained cAMP response. In contrast, PTHrP actions are completely reversible and limited to the plasma membrane. The precise mechanisms that mediate the observed prolonged cAMP in response to PTH remain to be determined, but the strong colocalization of PTH with PTHR, Gs and adenylyl cyclase in early ensosomes raises the novel possibility that the internalized PTHR complexes are enzymatically active and can generate cAMP from endosomal membranes, as a means for PTHR-mediated sustained cAMP production (25, 26, 33).

We now know that the large (180 amino acid) amino-terminal extracellular domain (N) of PTHR contributes to the initial ligand – receptor (L–R) interaction by docking residues 15–34 of PTH(1–34) to the receptor with kinetics that strictly depend on agonist (A) concentrations as predicted by a simple bimolecular interaction, defined by $k_{obs}=k_{off}+k_{on}\times[A]$ where $k_{obs}$ is the recorded rate constant ($s^{-1}$) (24). High-affinity binding between PTH and PTHR depends on the subsequent step, which involves the interaction of the amino-terminal portion of the ligand to the juxtamembrane (J) region of the receptor comprising the seven transmembrane helices and connecting extracellular loops. This interaction stabilizes the active PTHR conformation with a maximal time constant ($\tau = 1/k$) of 1 s (24). This second L–R interaction involving the J region of PTHR, and not the conformational changes to the receptor that take place during activation, is the rate-limiting step for receptor activation ($\tau_{max}$ = 950 ms). Once activated, PTHR engages $G_S$ at the plasma membrane with time constants that can be as fast as that measured for PTHR activation ($\tau$ = 0.96 s for PTH, and $\tau$ = 1.6 s for PTHrP). PTHR–$G_S$ interaction kinetics are limited by the expression level of G proteins, which supports a diffusion-controlled collision process rather than a receptor-G protein precoupling model (25). The following step, which involves conformational rearrangements and disassembly events between the $G\alpha_S$ subunit and the $G\beta_1\gamma_2$ dimer, is rate-limiting for $G_S$ activation, and is only moderately faster for PTH ($\tau$ = 1.6 s) than for PTHrP ($\tau$ = 2.05 s) at saturating ligand concentrations. Cyclic AMP production is detectable a few seconds after $G_S$ activation, a delay that presumably reflects the time required for $G_S$ activation, its separation from the receptor and activation of adenylyl cyclases.

## Conformational selectivity of PTHR deactivation

As described above, the sequence of reactions involved in the activation of PTHR and $G_S$ proceed with similar kinetics and mechanisms in response to either PTH or PTHrP. By contrast, the mechanisms of signal termination are quite divergent (Box 1). Recent studies show that a brief pulse of PTH induces a long lasting active state that is characterized by prolonged $G_S$ activation and sustained cAMP production even after PTH-bound PTHR internalizes to early endosomes. PTHrP dissociates rapidly from the receptor ($\tau_{off}$ = 30 s), prompting rapid $G_S$ deactivation and cAMP signal termination at the plasma membrane (25, 26). These studies suggest that PTH and PTHrP stabilize two distinct active conformations of the PTHR. We hypothesized that one of these PTHR conformations, named $R^0$ in reference to prior studies done with the CRF receptor (29, 30), is a high affinity PTHR conformation stabilized by PTH that is not necessarily dependent on G protein coupling, but can nevertheless maintain extended periods of G protein coupling and activation. This $R^0$ PTHR conformation is thus distinct from the classical G protein-dependent high affinity

receptor conformation, hereafter noted $R^G$ and preferentially stabilized by PTHrP, as predicted by the conventional GPCR signaling paradigm (31).

To study the $R^0$ and $R^G$ conformations of PTHR, we utilized membrane-based equilibrium competition binding assays that isolate and quantify binding to each of these two conformations of the PTHR (32–34). Binding to $R^0$ was assessed using $^{125}$I-PTH(1–34) in the presence of GTPγS, a non-hydrolyzable GTP analog that antagonizes R–G protein coupling; binding to $R^G$ was assessed using a fully functional modified PTH analog that binds weakly when GTPγS is present, $^{125}$I-M-PTH(1–15) (where M is Ala/Aib1, Aib3, Gln10, Har11, Ala12, Trp14, Arg19), and membranes prepared from cells expressing PTHR and a dominant negative $G\alpha_S$ mutant ($G_S$-ND) that binds the receptor in a nearly irreversible fashion (25, 35). These approaches not only revealed that PTH(1–34) binds with greater selectivity to $R^0$, *versus* $R^G$, than does PTHrP(1–36) (Figure 1a,1b), but they also led to the identification of PTH analogs, M-PTH(1–28) and M-PTH(1–34), that bind with even higher affinity to $R^0$ than does PTH(1–34) (33, 36). The enhanced selectivity with which these analogs bind to the $R^0$ state is accompanied by markedly prolonged cAMP signaling response in cells, with clear movement of the PTHR to the internalized domain, and, importantly, prolonged hypercalcemic and hypophosphatemic responses when injected in animals (33),(37, 38).

The capacity of PTH and PTHrP to stabilize distinct PTHR conformations was also confirmed by FRET experiments done in live cells [(25)]. Here, the PTH–PTHR complex was highly stable, whereas that induced by PTHrP(1—36) was reversible after ligand washout (Figure 1c). In agreement with radioligand binding studies performed *in vitro*, live-cell FRET data showed that dominant negative $G_S$ has little or no effect on dissociation of PTH(1—34) from the receptor, but it markedly impedes the dissociation of PTHrP(1—36) (Figure 1b,1c). These results imply that the major component of the dissociation process observed for PTHrP arises from the rapid release of G proteins from the receptor, which does not occur with $G_S$-ND (Figure 1c). Taken together, these studies suggest that with certain ligands, such as PTHrP(1–36), PTHR can form conventional high-affinity complexes that are transient and depend on coupling to G proteins, whereas with other ligands, such as PTH(1–34) and M-PTH(1–34), it can form unusually high-affinity complexes that are not dependent on classical G protein coupling, but yet can sustain activation of $G_S$ proteins and cAMP production, even after receptor internalization. Generation of cAMP is abbreviated when the PTH–PTHR complex cannot internalize due to disruption of dynamin activity (25). This, and the apparently complete internalization of PTH–PTHR complexes while a high level of cAMP generation is still recorded, supports the idea that PTHR in fact requires internalization for sustained generation of cAMP.

## “Non-canonical” mode of PTHR signaling

Extensive studies of signaling by GPCRs, including but not limited to rhodopsin and the $\beta_2$-adrenergic receptor ($\beta_2$AR), have led to what is now considered a classical and general model of GPCR desensitization by arrestins (39, 40). In this “canonical” model, arrestins engage active receptors after ligand binding has stimulated G protein-coupled receptor kinases (GRK) to phosphorylate residues on the C terminus of the receptor. Arrestin binding terminates GPCR signaling by preventing receptor–G-protein coupling (41, 42), and by recruiting diverse enzymes such as phosphodiesterase 4 (PDE4) or diacylglycerol kinase (DGK) to the plasma membrane to degrade the second messengers cAMP and DAG, respectively (43, 44). Arrestin binding also promotes receptor internalization, a process that relies upon the interaction of β-arrestins with the AP-2 subunit of clathrin, a major component of the clathrin-based endocytic machinery (45). However, it is now clear that PTHR does not follow this conventional desensitization paradigm (26). β-arrestins interact

rapidly with PTH-bound PTHR (46, 47) *w*ithout inhibiting the continued generation of cAMP (26, 48). In fact β-arrestins prolong PTH-mediated cAMP in cultured cells that express either recombinant or native PTHR, and fluorescence imaging of live cells shows that PTH induces the internalization of PTHR to early endosomes along with arrestin, $G_S$ and adenylyl cyclases (25, 26). Furthermore, the time course of cAMP generation in these cells, which can be measured in real time using FRET-based fluorescent biosensors, correlates temporally with the persistence of arrestin–PTHR–$G_S$ complexes on early endosomes. Importantly, analogs of PTH, such as M-PTH(1–28) and M-PTH(1–34), which induce prolonged physiological calcemic and phosphate responses in animals (36) and prolonged cAMP generation in cultured cells, also increase the persistence of receptor-arrestin complexes on endosomes. This, and evidence that an arrestin mutant with increased affinity for active receptor also enhances cAMP generation (26), is consistent only with a model in which arrestin promotes rather than desensitizes cAMP generation by PTHR and further implicates signaling from early endosomes as a key part of the model.

A critical question arises from these findings. How can a long-lived PTH–PTHR–arrestin (L–R–arr) complex mediate prolonged $G_S$/cAMP signaling? Two observations can help to narrow the possibilities: i) there is no evidence that β-arrestins directly bind $G\alpha_S$ in any circumstance, suggesting that arrestin plays an indirect rather than a direct role in facilitating PTHR–$G\alpha_S$ coupling; and ii) recent data suggest that $G\beta\gamma$ subunits can provide a mechanism for scaffolding β-arrestin (49). If applicable in this case, a long-lived PTH–PTHR–arr ternary complex could contribute to protracted cAMP signaling mediated by PTH by two mechanisms: i) the PTH–PTHR–arr complex could stabilize an interaction with $G\beta\gamma$ that permits multiple rounds of $G\alpha_S$ subunit coupling and activation, or ii) each PTH–PTHR–arr–$G\beta\gamma$ complex could mediate sustained coupling and activation of only one, or a few molecules of $G\alpha_S$. However, this model remains entirely hypothetical and the mechanism by which arrestin promotes signaling by PTHR must be determined by future studies.

It seems possible that receptors that bind their ligand with high affinity remain competent to signal while arrestin cycles on and off the cytoplasmic tail of the receptor. If so, then arrestin turnover could leave the G-protein binding site periodically exposed for further rounds of G protein activation. Indeed, FRAP analysis of β-arrestin 1–PTHR complexes on early endosomes revealed a recovery half-life of ~30 seconds, indicating that a significant turnover of arrestin molecules does occur. However, a mutant arrestin that cycles on to and off of the PTHR much more slowly than does native arrestin, mediates prolonged, rather than abbreviated, cAMP generation, as do PTH ligands that cause wild-type β-arrestin 1 to bind the receptor with greater affinity (26). These findings argue against the possibility that sustained cAMP responses involve rapid turnover of arrestin–PTHR complexes. Thus, sustained arrestin interaction, possibly mediated by interaction with $G\beta\gamma$, is more likely to promote sustained G activation.

Another possibility is that arrestin binds the PTHR independently of G proteins. The PTHR has been shown to dimerize either constitutively or upon activation (50). In this case one protomer could bind arrestin and mediate internalization while the other continues to activate $G_S$. Alternatively, the long PTHR C-terminus contains distinct binding motifs for $G\beta\gamma$ and arrestin that theoretically could allow binding of two accessory proteins at the same time (46, 51). If this is the case, then arrestin mutants with greater affinity for activated receptor, and PTHR ligands that induce more stable arrestin binding, could prolong cAMP generation by blocking access to whichever accessory protein does decouple PTHR from G protein activation.

## PTHR signaling stopped by retromer

Depletion of β-arrestins by siRNA reduces the level and the duration of cAMP generation after PTH challenge whereas it increases cAMP induced by $\beta_2$AR in response to isoproterenol, indicating again that β-arrestins do not desensitize cAMP generation by PTHR (26, 52). If arrestin does not prevents $G_S$ coupling from PTHR by streric inhibition, then it is necessary to ask what other protein could do this job. One possibility is that, like certain receptor tyrosine kinases (53), PTHR simply continues to signal until it is sequestered in the multivesicular body prior to degradation in the lysosome. However, this is unlikely, as PTHR does not degrade but rather recycles via an unusually slow pathway (54). Other GPCRs that undergo ligand-dependent internalization such as the β2AR and μ-opioid receptor recycle directly to the plasma membrane, whereas PTHR traffics by retrograde transport to the trans-Golgi network (55) before recycling through the exocytic pathway. It is thus reasonable to suppose that the factor that sorts PTHR from the endosome to the Golgi could also stop cAMP generation by the receptor. The most likely candidate for this activity would be retromer, an endosomal heteropentameric complex that consists of two membrane-bound sorting nexins (Snx1/Snx2) and a soluble heterotrimer of vesicle protein sorting, Vps26, Vps29 and Vps35. Retromer is known to retrieve transmembrane signaling proteins, such as the mannose-6-phosphate receptor and wntless, from endosomes and return them to the Golgi (56). A particularly intriguing observation is that the structures of β-arrestins and the Vps26 subunit of retromer have a striking resemblance, although the functional significance of this similarity remains unknown (57). Regardless, there is strong evidence that retromer influences the signaling and trafficking of PTHR. Fluorescent retromer colocalized and physically interacted with internalized PTHR when co-expressed in HEK293 cells, and over-expression of the soluble Vps26/29/35 trimer both increased PTHR traffic to the Golgi and abbreviated the time course and levels of cAMP generation. It is notable that fluorescent retromer did not colocalize with PTHR immediately upon internalization of active receptors to early endosomes. Rather, three-color live imaging of cells expressing fluorescent PTHR, arrestin and retromer indicated that PTHR and arrestin occupy a distinct endosomal domain from retromer for about 20 min after challenge with PTH, after which time PTHR begins to colocalize with retromer and less strongly with arrestin. This is consistent with a model in which arrestin and retromer occupy exclusive domains of the endosome and act either to sustain (arrestin) or to block (retromer) cAMP generation by PTHR (Figure 2). Depletion of retromer by siRNA resulted in more persistent cAMP generation by PTHR but had no effect on $\beta_2$AR signaling, also supporting a role for retromer in silencing PTHR. These effects of retromer on PTHR signaling were observed both in HEK293 cells expressing transgenic PTHR as well as in rat osteosarcoma cells that natively express PTHR (26), although it remains unknown how retromer binds PTHR and decouples its signaling. The selectivity of retromer–PTHR binding is shown by the fact that neither PTH, β-arrestins, $G_S$ nor adenylyl cyclases colocalize with retromer on the Golgi, and β-arrestins did not colocalize with domains of the early endosome labeled by retromer (Figure 2) (26). A simple and coherent model to explain the unexpected roles played by retromer and arrestin in PTH-mediated cAMP generation would hold that PTHR–arrestin complexes internalize together to the early endosome while cycling between bound and unbound states; any receptor not bound to β-arrestin may instead bind retromer, preventing interactions with arrestin and Gs, or stabilizing the inactive state of the receptor and initiating traffic of PTHR to the trans-Golgi network by way of a distinct domain of the early endosome (Figure 3).

## Concluding remarks

Studies discussed in this review suggest that PTHR can adopt multiple conformations stabilized by different ligands. This conformational selectivity in turn influences the down-

stream signaling responses in target cells. Understanding how these ligand-specific events occur is critical to determine the molecular and cellular mechanisms underlying the anabolic and catabolic effects that PTHR ligands have on bone mass, depending on duration and timing of exposure. Based on available clinical data, Andrew Stewart and colleagues suggested that PTHrP(1–36) has greater efficacy in building bone mass in humans than does PTH(1–34), and thus might be a more effective treatment for osteoporosis (58). These considerations, coupled with our new findings on ligand-based conformational selectivity of PTHR, point to the prediction that $R^0$-selective ligands, due to their prolonged action via endosomal PTHR/$G_S$/cAMP signaling, would favor bone-resorption responses associated with sustained calcium release, and thus be candidate therapies for hypoparathyroidism (59, 60); conversely, RG-selective ligands, due to short and transient action at the receptor, would favor bone anabolism responses, and be candidate therapies for osteoporosis.

Initially revealed for arrestin-dependent ERK and non-receptor tyrosine kinase (src) signaling pathways (61–65), and also receptor tyrosine kinase pathways (66), endosomal signaling via G protein has been documented in yeast (67–69) and is now an emerging topic for GPCR biology in vertebrates. Indeed, sustained cAMP production mediated by endosomal G-protein signaling appears to be a new pathway not only for PTHR function but also for the class 1 GPCRs, the thyroid-stimulating hormone (TSH) receptor (70), and the dopamine D1 receptor (D1R) (67). In a few cases (PTHR, D1R) reported so far, receptor internalization appears to be necessary for sustained generation of cAMP. These recent developments put a finer point on the possibility that exceptions exist to the classic rule of arrestin preventing receptor–G protein coupling and signal termination. For the PTHR, prolonged cAMP signaling is mediated by ligands that bind to a high affinity receptor conformation, $R^0$, and thus form complexes that include $G\alpha_S$ and arrestin, and which remain stable within early endosomes. Transit of these complexes to late endosomes results in the exchange of arrestin for retromer, which correlates temporally with signal termination. Future studies will determine the molecular mechanism by which the interaction of arrestin with the PTHR permits a sustained Gs signaling, and reveal its physiological relevance for ions and mineral metabolism.

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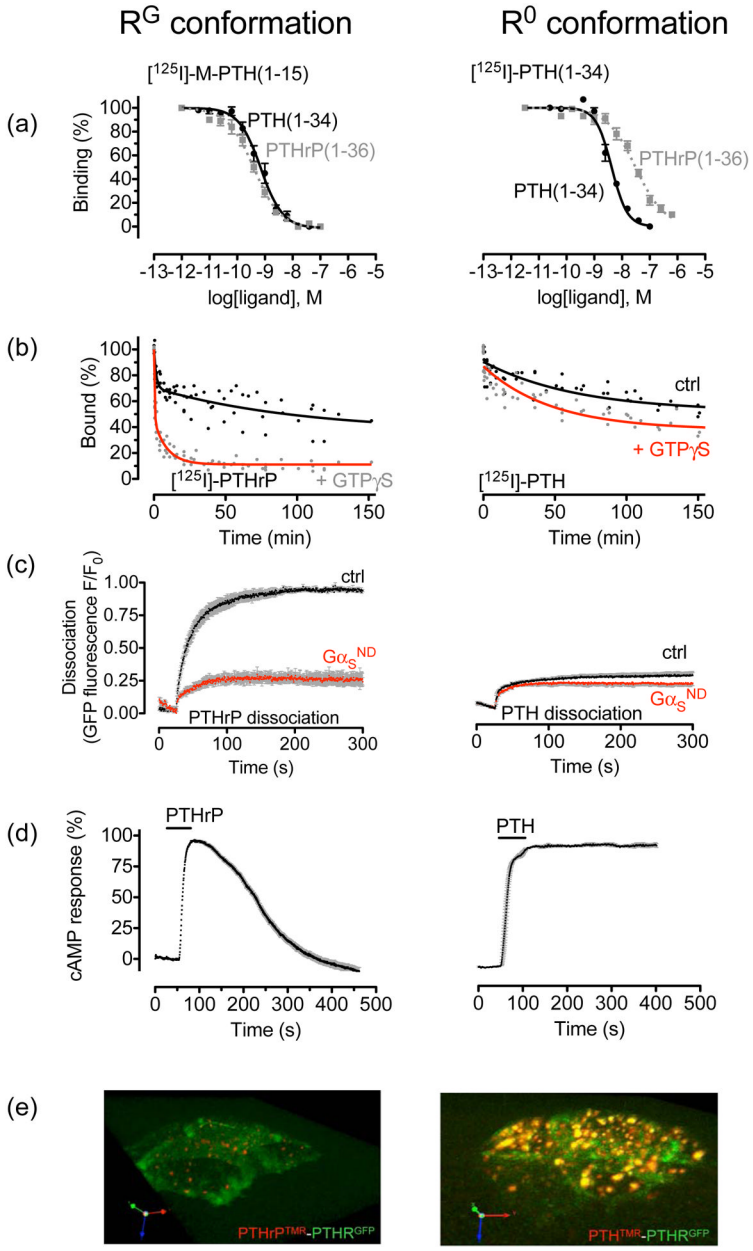


**Figure 1.**

PTHR conformations. (**a–b**) Cell membrane binding assays. Binding to the $R^0$ and $R^G$ conformations of the PTHR are determined by competition reactions. For $R^0$, [$^{125}$ I]-PTH(1—34) is used as a tracer radioligand and including GTPγS in the reaction; for RG, [$^{125}$I]-PTH(1—15) is used as a radioligand in the presence of a high-affinity, negative-dominant $G\alpha_S$ subunit (Gαs-ND). (**c–e**) Life cells FRET-based assays. Averaged dissociation time courses of TMR-labeled ligands, PTH(1–34)$^{TMR}$ (right panel) and PTHrP(1–36)$^{TMR}$ (left panel), from GFP-tagged PTHR, $^{GFP}$N-PTHR, are shown in the absence or presence of a Gαs-ND. FRET recordings from HEK-293 cells are shown as normalized ratios (c). Average time-courses of cAMP production in response to PTHrP(1–36) (left) and PTH(1–84) (right) in HEK-293 cells stably expressing PTHR and co-transfected with the cAMP biosensor, Epac$^{CFP/YFP}$. Individual cells were continuously perfused with buffer or with the

hormone for the time indicated by the horizontal bar (d). A 3D view of tetramethylrhodamine (TMR)-labeled peptides, and a PTHR N-terminally tagged with GFP ($^{GFP}$N-PTHR) in live HEK-293 cells by spinning disc confocal microscopy 30 min after ligand wash out. PTH(1–34)$^{TMR}$ (red) and $^{GFP}$N-PTHR (green) co-localized within endocytic compartments (right). In contrast, PTHrP(1–36)$^{TMR}$ alone is detected as small puntae at internalized sites (left) (e). Adapted from (25, 33).

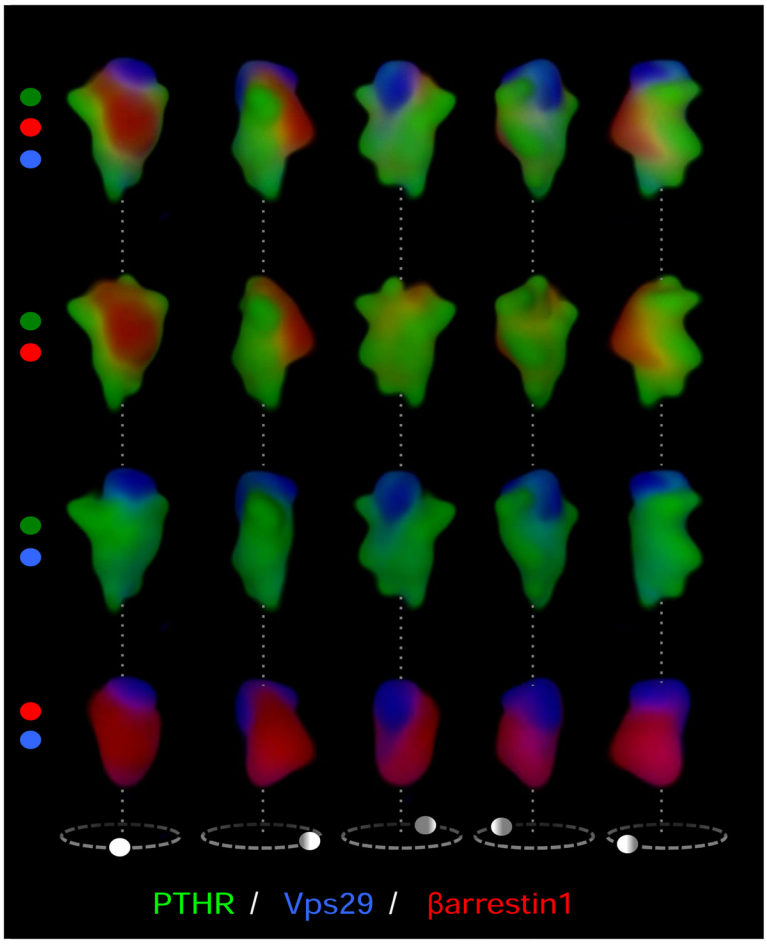


**Figure 2.**
Signaling dynamics of PTHR on the early endosome. (a) We have recently shown that complexes of β-arrestin 1 (red) and PTHR (green) internalize to a compartment of the early endosome that is labeled red-green in a 3D reconstruction of early endosomes visualized with a spectral confocal microscope (top). A second compartment labeled with the sorting complex retromer (blue) is labeled blue-green. Arrestin and retromer do not colocalize, indicating that the two proteins localize in distinct compartments of the endosome, most likely the bulk domain (arrestin) and a domain dedicated to endosome-to-Golgi retrograde traffic (retromer). (b) Persistent complexes of PTHR-arrestin generate cAMP (yellow) from endosomal membranes. However, after arrestin-receptor decoupling, PTHR is free to bind retromer and sort to a compartment that does not support cAMP generation. Retromer-bound inactive PTHR then sorts to the trans-Golgi network before recycling to the plasma membrane. Adapted from (26).

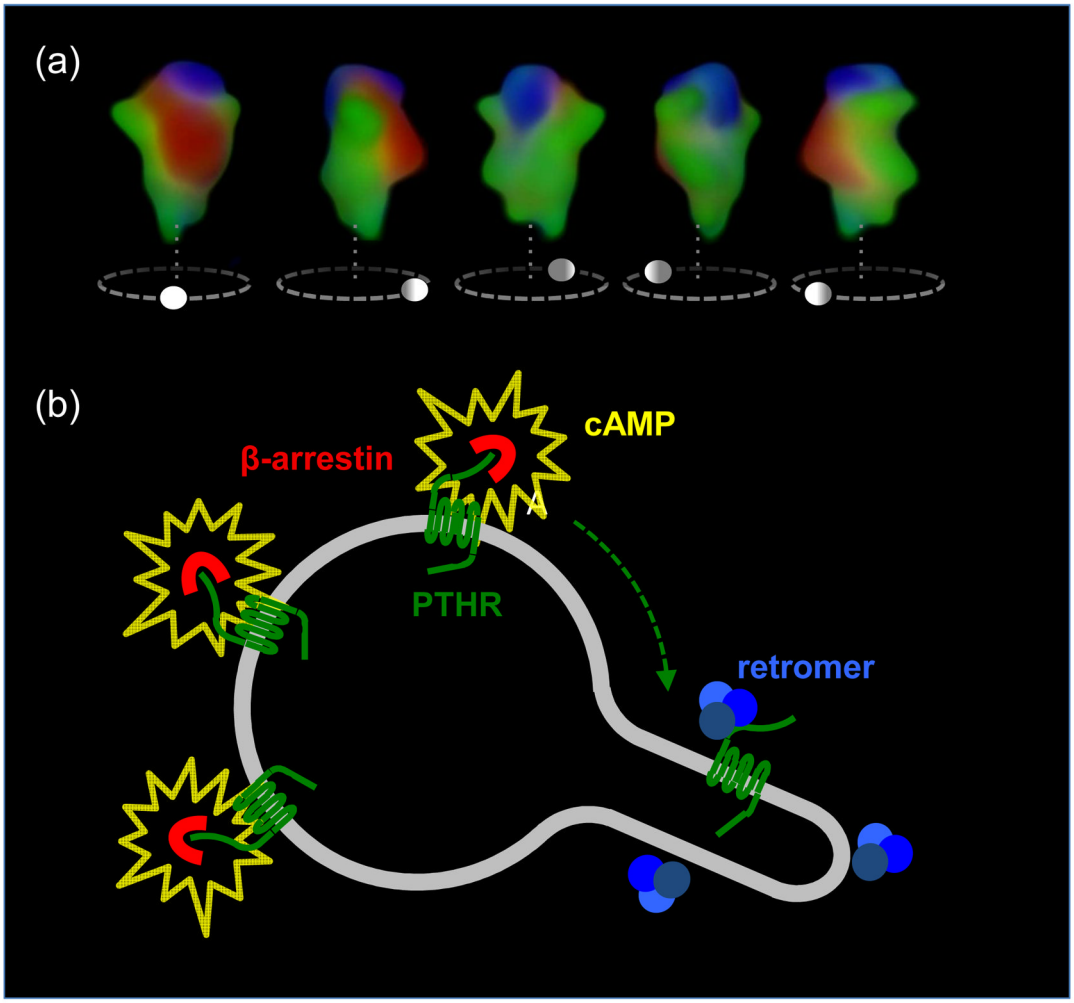


**Figure 3.**
Proposed model sustained PTHR signaling. *Left panel*, PTH-activated PTHR (green) generating cAMP (grey) by activation of adenyly cyclases internalizes to early endosomes in a process that involves binding of β-arrestin (red). Activated PTHR is then maintained in the early endosome bulk compartment by arrestin binding, where arrestin-mediated activation of ERK1/2 signaling causes inhibition of phosphodiesterases and permits sustained cAMP signaling. *Right panel,* Binding of PTHR and retromer (blue) causes sorting of the receptor to retrograde trafficking domains. Generation of cAMP is stopped after PTHR–retromer binding in the retrograde domain and retromer-mediated PTHR traffic to the Golgi. Adapted from (26).

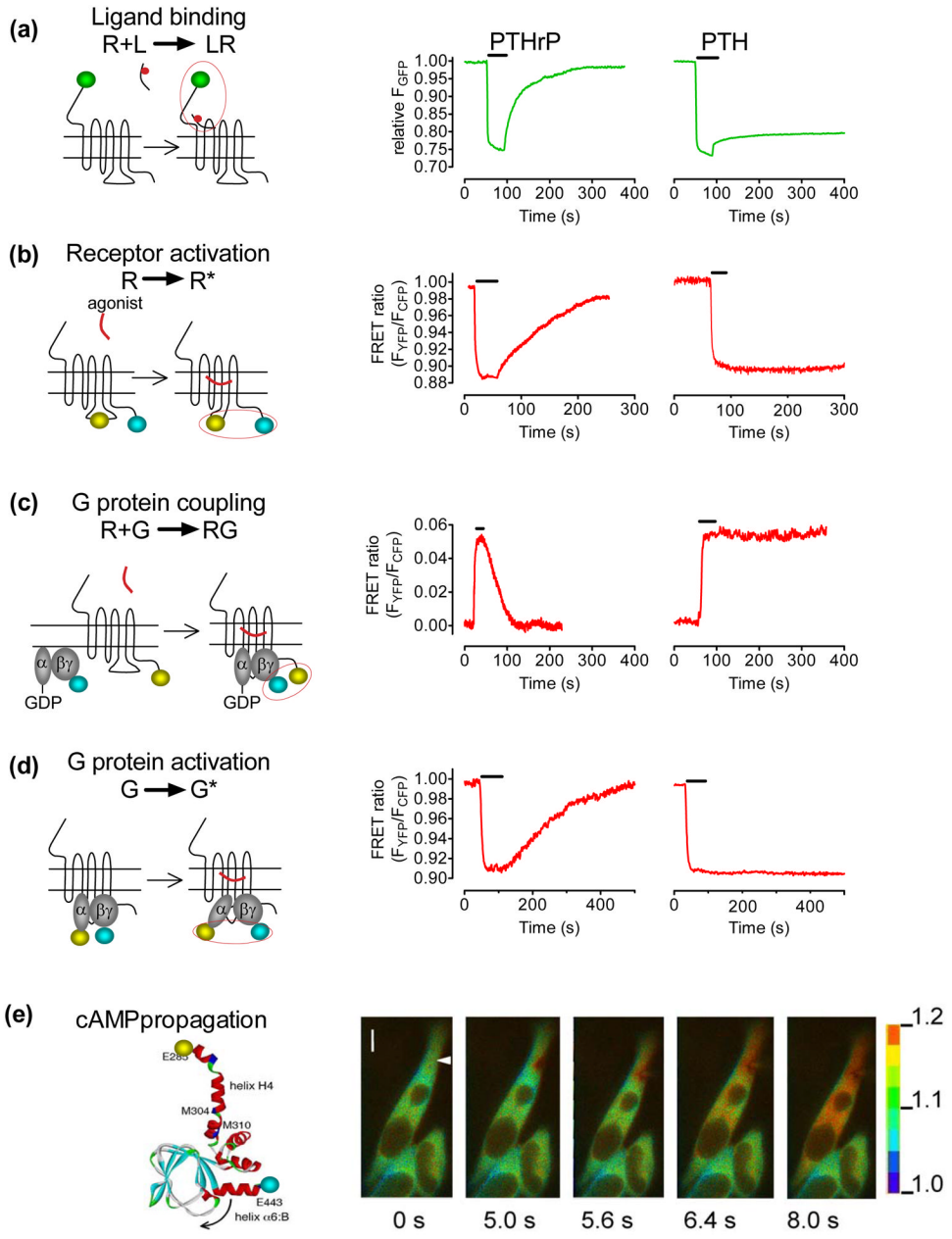


**Figure I.**

Kinetics of PTHR signaling. (**a**) Ligand/receptor interaction measured by FRET between GFP-tagged PTHR and tetramethylrhodamine-labeled PTH(1–34) or PTHrP(1–36). Shown are the changes of GFP emission by FRET in response to rapid superfusion of diverse concentrations of ligand-TMR. (**b**) Following ligand application (horizontal bar), activation of PTHR was monitored in a single HEK-293 cell by a decrease in the FRET signal of $PTHR^{CFP/YFP}$ defined as the ratio of emission intensities of YFP/CFP. (**c**) The interaction between PTHR and $G_S$ proteins in response to ligand binding is measured as an increase in FRET between YFP-labeled PTHR and CFP-labeled $G\gamma_2$ in combination with $G\alpha_S$ and $G\beta_1$ proteins. (**d**) Detection of $G_S$ activation in cells expressing the wild-type PTHR by recording FRET between YFP-labeled $G\alpha_s$ and CFP-labeled $G\gamma_2$-subunits. (**e**) PTH-mediated cAMP response upon PTHR activation in HEK-293 cells measured as a decrease of FRET in the

Epac$^{CFP/YFP}$ sensor. The panels show the propagation of the cAMP response represented as pseudocolored image of the FRET (CFP/YFP emission) ratio before and after stimulation of a single cell with PTH(1–34) via a pipette indicated by an arrow at $t = 0$ s. The scale bar on the right indicates the pseudocolored scale of the fluorescence ratios. The inner bar represents 5 μm. Adapted from (25).

## Table 1

Kinetics of PTHR–ligand association and dissociation at a ligand concentration of 10 μM (reaction 1), PTHR activation and deactivation (reaction 2), PTHR and $G_S$ interaction (reaction 3), $G_S$ activation and deactivation (reaction 4), cAMP accumulation and degradation (reaction 5). Reactions were recorded from single HEK-293 cells at a saturating concentration of ligand. Values represent the mean ± s.e.m. of the rate constant (τ) and were taken from (24, 25).

| | **Switch on (s)** | | **Turn off (s)** | |
|---|---|---|---|---|
| | **PTH(1–34)** | **PTHrP(1–36)** | **PTH(1–34)** | **PTHrP(1–36)** |
| (1) *L* + *R* Ä *LR* | $\tau^{fast}$ = 0.14 ± 0.01<br>$\tau^{slow}$ = 1.15 ± 0.10 | $\tau^{fast}$ = 0.17 ± 0.05<br>$\tau^{slow}$ = 1.54 ± 0.15 | $\tau^{fast}$ = 1.50 ± 0.27<br>$\tau^{slow}$ NA | $\tau^{fast}$ = 1.38 ± 0.23<br>$\tau^{slow}$ = 28.12 ± 0.60 |
| (2) *LR* Ä *LR** | τ = 0.95 ± 0.15 | τ = 1.59 ± 0.11 | NA | τ = 58.54 ± 6.42 |
| (3) *LR** + *G* Ä *LR*G* | τ = 0.96 ± 0.13 | τ = 1.58 ± 0.19 | NA | τ = 48.14 ± 5.29 |
| (4) *G* Ä *G** | τ = 1.58 ± 0.13 | τ = 2.04 ± 0.14 | NA | τ = 121.50 ± 6.35 |
| (5) cAMP | τ = 10.89 ± 2.26 | τ= 12.66 ± 1.06 | NA | τ = 296.70 ± 17.47 |