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Endosomal generation of cAMP in GPCR signaling

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Abstract

It has been widely assumed that the production of the ubiquitous second messenger cyclic AMP, which is mediated by cell surface G protein–coupled receptors (GPCRs), and its termination take place exclusively at the plasma membrane. Recent studies reveal that diverse GPCRs do not always follow this conventional paradigm. In the new model, GPCRs mediate G-protein signaling not only from the plasma membrane but also from endosomal membranes. This model proposes that following ligand binding and activation, cell surface GPCRs internalize and redistribute into early endosomes, where trimeric G protein signaling can be maintained for an extended period of time. This Perspective discusses the molecular and cellular mechanistic subtleties as well as the physiological consequences of this unexpected process, which is considerably changing how we think about GPCR signaling and regulation and how we study drugs that target this receptor family.

Cell surface membranes are equipped with specialized seven α -helical proteins known as GPCRs¹, which are dedicated to transmitting the biological action of numerous extracellular ligands and sensorial stimuli into cells. These ligands include the majority of chemical neurotransmitters, peptide hormones, lipids and sensory stimuli (light, taste and odorant molecules) and a large variety of clinical drugs (for example, β -blockers and anti-psychotics). Signal transduction begins when a ligand (L) binds its receptor (R), shifting the inactive receptor into an active signaling state ($L + R \leftrightarrow LR \leftrightarrow LR^*$) through conformational rearrangements in the receptor occurring with kinetics varying from 1 ms to 1 s, depending on the ligand-receptor system^{2–5}: very fast (1 ms) for rhodopsin⁶, fast (50–100 ms) for small neurotransmitter receptors^{2,7} and slow (1 s) for peptide hormone receptors^{2,8}. The activated receptor then couples to inactive, GDP-bound heterotrimeric G proteins ($G\alpha\beta\gamma$) to form a transient ternary complex ($LR^* + G \leftrightarrow LR^*G$) with kinetics that depend on the expression level of G proteins and are thus determined by a diffusion-limited

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Author contributions

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collision process⁹. This interaction releases the bound GDP from the LR*G complex, which then exhibits higher affinity for the agonist ligand than the initial ligand-bound receptor state and captures GTP on G α subunits (G α). The GDP-GTP exchange on G α engages a series of conformational events in the heterotrimer G $\alpha\beta\gamma$ ¹⁰ and/or dissociation events between G α and G $\beta\gamma$ that are associated with G-protein activation. In some cases, agonist binding induces conformational reorganization of a preformed receptor–G protein complex that can also lead to G-protein activation without dissociation of G α and G $\beta\gamma$ subunits^{11,12}. Whether the interaction of G proteins to GPCRs proceeds via precoupling or diffusion-controlled mechanisms and whether their activation depends on conformational or dissociational events are thus not undisputed scenarios^{13,14}. Once activated, both G α -GTP and G $\beta\gamma$ subunits can interact with different cell membrane-bound effector enzymes (for example, adenylyl cyclases (ACs), phosphodiesterases, phospholipases and Rho GTPase) or ion channels (GIRK). These interactions initiate or suppress effector activities, thus regulating the flow of second messengers (cAMP, phosphoinositides and cGMP) or ions (Ca²⁺ and K⁺) involved in a wide range of physiological processes such as heartbeat, bone turnover and water homeostasis, among others.

To prevent overstimulation, GPCR signaling responses are attenuated within minutes by a series of reactions (Fig. 1) involving receptor phosphorylation by G protein-coupled receptor kinases¹⁵ (GRKs) that are selective for the active ligand-bound receptor conformation. Phosphorylated receptors then bind one of the arrestin isoforms, which sterically prevents coupling between receptor and G protein, thus resulting in the termination of agonist-mediated G-protein activation. The interaction with β -arrestins further promotes the transfer of ligand-bound receptor from the cell surface to early endosomes via dynamin- and clathrin-dependent endocytosis¹⁶ (Fig. 1). Receptor internalization thus serves as a means to decrease receptor number from the cell surface and directs the receptor to a compartment where the ligand and phosphates are removed (Fig. 1). Once redistributed in endosomal compartments, GPCRs can either recycle rapidly to the cell membrane, allowing resensitization, as in the case of transient receptor– β -arrestin interactions (Fig. 1), or they can move to lysosomes for degradation (Fig. 1).

A paradigm shift in classical GPCR signaling

This conventional desensitization paradigm is not consistent with recent findings showing that parathyroid hormone receptor type 1 (PTHrP) and thyroid-stimulating hormone receptor (TSHR) can sustain G-protein signaling and cAMP production after internalization of ligand–receptor complexes and their redistribution in various intracellular compartments, such as endosomes and Golgi apparatus. In the case of the PTHrP, the new concept that cAMP production occurs both at the plasma and endosomal membranes stems from a study⁸ that used FRET-based biosensors^{13,17} to compare molecular and cellular mechanisms that differentiate the functional selectivity (i.e., different biological actions) of PTH and PTH-related peptide (PTHrP), the two native ligands of the receptor. Treating kidney- or bone-derived cells with either PTH or PTHrP produced cAMP, but only PTH caused sustained cAMP production. Biochemical and FRET analyses in live cells showed that the short burst of cAMP mediated by PTHrP was well represented by the classical model for G-protein signaling (Fig. 1), with cAMP production limited to the plasma membrane and receptor and

ligand trafficking through distinct compartments. They also revealed that sustained cAMP signaling was caused by internalized PTH–PTHr complexes residing together with the stimulatory G protein (G_s) and adenylate cyclases in early endosomes. The same conclusion was concomitantly reached by studying a different receptor, the TSHR, and its cell system¹⁸. These findings were unexpected, given that it was classically thought that GPCR–G protein systems were only active on the cell surface and would only enter a cell to be degraded and/or replaced by newly synthesized GPCRs, but they laid the foundation for the new model that G_s and cAMP signaling can continue after internalization of ligand–GPCR complexes in endosomes (Fig. 1). Parallel studies on the sphingolipid S1P receptor (S1P₁R) extended this model to the inhibitory G protein (G_i) for adenylate cyclases by reporting that internalization of the S1P₁R and its trafficking in the *trans*-Golgi network contributed to the sustained G_i -dependent signaling mediated by FTY720, a S1P₁R agonist¹⁹. Initially recognized in 2009 for the PTHr and the TSHR, sustained G_s and cAMP signaling mediated by internalized GPCRs has been further reported for other peptide hormone receptors such as the glucagon-like peptide 1 receptor (GLP-1R)²⁰, the pituitary adenylate cyclase activating polypeptide (PACAP) type 1 receptor²¹ and the vasopressin type 2 receptor²² (V2R), and it has also been extended to monoamine neurotransmitter receptors including the β_2 -adrenergic and dopamine D1 receptors^{23,24}, many of which have been recently reviewed^{25–27}.

Mechanisms of prolonged signaling at GPCRs

Endosomal GPCR signaling via G proteins incites new questions about what mechanisms maintain and regulate G-protein signaling from endosomal membranes. A new approach using conformational biosensors based on single-chain antibodies, or ‘nanobodies’²⁸, which are selective for the active state of either the β_2 -adrenergic receptor (β_2 AR) or the G_{α_s} form freed of GTP or GDP nucleotides, has forwarded further experimental support for the endosomal GPCR–G protein signaling model²³. GFP-tagged nanobodies can detect activated states of β_2 AR and G_s in the plasma membrane and in early endosomes following agonist challenge in HEK-293 cells. These observations, coupled with the modest but significant ($P < 0.05$) decrease in the maximum level of cAMP mediated by isoprenaline after blocking β_2 AR internalization, are consistent with the view that internalized β_2 AR can engage new cycles of G_s activation and cAMP production from endosomes. The absence of detection of activated β_2 AR or activated G_s by nanobodies in clathrin-coated pits during the initiation step of endocytosis further supports the view that the β_2 AR induces cAMP through two episodic phases: the first takes place at the plasma membrane and is responsible for an acute but short cAMP response, and the second happens in early endosomes a few minutes after receptor internalization. This process is compatible with the actions of β -arrestins that not only uncouple the receptor from G_s but also recruit the cAMP-specific phosphodiesterase (PDE4) to the plasma membrane²⁹ and engage in the formation of clathrin-coated pits. The PTHr or the V2R differ from β_2 AR, however, because β -arrestins promote rather than attenuate cAMP production in response to PTH or vasopressin. The mechanisms by which this occurs are starting to be understood and are discussed in the following paragraph.

Clear evidence that a particular receptor conformation is needed to maintain G_S signaling from subcellular compartment has come from studies on the PTHR, a prototypical GPCR family 2 member that regulates Ca^{2+} homeostasis by its actions on bone and kidney. As for all GPCRs, the PTHR is likely to exist in a variety of different conformations that are stabilized not only by the type of interacting agonist (full, partial or inverse) but also by interacting signaling proteins^{3,30–34}. Recent studies using pharmacological and biophysical approaches provide new clues as to the nature of such altered conformational states possible for the PTHR and their relevance to endosomal receptor or G_s signaling and related biological actions^{8,35,36}. In the classical GPCR signaling paradigm, receptors were thought to exist in a low-affinity ligand-binding state when uncoupled from G proteins and to shift to a high-affinity state only upon G-protein coupling³⁷. Studies on the PTHR showed that it deviates from the classical model. It can form high-affinity complexes with PTH or its N-terminally synthetic analog PTH(1–34)^{38,39}, even in the absence of G-protein coupling, as the complexes remain stable in the presence of $GTP\gamma S$, a guanine nucleotide analog that induces receptor–G protein dissociation. In contrast, PTHrP, the other native agonist ligand for PTHR^{40,41}, forms complexes that are more like those formed with, for example, the β_2 -AR and dissociates rapidly in the presence of $GTP\gamma S$. These observations have led to the hypothesis that the PTHR can adopt at least two distinct active conformations (Box 1). One of these conformations, named R_0 , 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_s protein coupling and activation, resulting in sustained cAMP production when the receptor internalizes³⁵. This R_0 conformation is thus distinct from the classical G protein–dependent high-affinity receptor conformation, denoted R_G , which is preferentially stabilized by PTHrP.

Box 1

Differentiating distinct signaling conformations of a GPCR: the case of PTHR

Membrane-based equilibrium competition binding assays allow researchers to study and differentiate the two distinct R_0 and R_G signaling conformations of PTHR (Fig. 5a,b). For R_0 , [^{125}I]PTH(1–34) is used as a tracer radioligand, and $GTP\gamma S$ is included in the reaction; for R_G , [^{125}I]M-PTH(1–15) is used as a radioligand in the presence of a high-affinity, negative-dominant $G\alpha_S$ subunit. These assays have shown that PTH(1–34) binds with greater selectivity to R_0 versus R_G than PTHrP(1–36). For dissociation kinetics (Fig. 5b), radioligands were prebound to PTHR in membranes, and then dissociation was initiated by the addition of the homologous unlabeled analog with $GTP\gamma S$.

FRET experiments performed in live cells in real time (Fig. 5c) further confirmed that PTH and PTHrP stabilize distinct PTHR conformations by showing that the bimolecular ligand–receptor (LR) complex induced by PTH(1–34) is highly stable and resistant to washout, whereas that induced by PTHrP(1–36) is reversible. The marked difference in the stability of the ligand–receptor complex observed for PTHrP(1–36) and PTH(1–34) in these FRET assays parallels that observed in radioligand dissociation kinetic assays (Fig. 5b,c). A short pulse of the R_0 as opposed to the R_G -selective PTH analog markedly

prolongs cAMP production (Fig. 5d) even when the ligand–receptor complexes visualized by small yellow punctae are localized in endosomes (Fig. 5e).

Ligands stabilizing the R_G conformation only trigger short and transient G_s and cAMP signaling limited at the plasma membrane, as opposed to R_0 -selective ligands that can also generate sustained cAMP responses from endosomal membranes^{8,35,36,42}. The observation that stable ligand binding to R_0 does not require direct G-protein coupling but triggers a prolonged cAMP signaling response suggests that the ligand– R_0 complex can isomerize to the R_G conformation and can perhaps do so repeatedly without dissociation of the bound agonist ligand. Indeed, use of the kinetic FRET approach has confirmed that such stable binding of PTH(1–34) to the PTHR^{8,43} mediates a persistent activation of the G_s and cAMP signaling response, even following internalization of the complex to endosomal vesicles⁸.

How is endosomal cAMP production maintained and turned off? The mechanism regulating endosomal GPCR and G_s signaling has been, in part, determined for two distinct GPCRs: the vasopressin V2 receptor (a prominent GPCR regulating water homeostasis) and the PTHR for which β -arrestins promote endosomal G_s and cAMP signaling. One mechanism by which cAMP continues after receptor internalization is via the well-known capacity of β -arrestins to assemble signaling complexes that permit internalized GPCR to activate extracellular signal-regulated kinases (ERK1/2)⁴⁴. It was shown that the PTH-dependent increase in endosomal cAMP was prolonged when cells expressing PTHR were treated with cAMP-specific phosphodiesterase (PDE4) inhibitors but was damped when cells were treated with inhibitors of ERK1/2 activation. This observation, coupled with the capacity of activated ERK1/2 to phosphorylate and inhibit the enzymatic activity of PDE4 (ref. 45), support a positive feedback model where endosomal ERK1/2 signaling mediated by PTH-bound PTHR–arrestin complexes contributes to sustain a cAMP response that originates from endosomes (Fig. 2).

The capacity of β -arrestins to prolong rather than attenuate G_s -cAMP signaling by PTH or vasopressin raises two key questions: what factors turn off receptor signaling, and how does arrestin in complex with a receptor maintain G_s activation? The first question was answered by studies showing that PTHR- or V2R-containing endosomes mature through the endosomal pathway and reach a stage at which the cargo-sorting retromer complex is engaged, and this engagement coincides with the release of β -arrestins from receptors and signal termination^{22,46}. The retromer complex consists of two endosomal membrane-bound sorting nexins (Snx1 and Snx2) and a heterotrimer consisting of vesicle protein sorting (Vps) Vps26–Vps29–Vps35, which regulates the sorting of numerous cargo proteins from early endosomes to the *trans*-Golgi network^{47–49}. The retromer complex colocalizes and assembles with PTHR or V2R in endosomes after agonist-induced receptor internalization. Overexpression of the three soluble Vps subunits of the retromer complex reduces the time course of cAMP generation, whereas silencing the expression of one of them prevents the formation of the retromer complex and prolongs the duration of cAMP signaling. Binding of PTHR and the retromer causes the receptor to sort to retrograde trafficking domains. Generation of cAMP is stopped after either retromer binding in the retrograde domain or upon retromer-mediated traffic to the Golgi (Fig. 2). The mechanism responsible for shifting

signaling receptor–arrestin complexes to receptor–retromer complexes that do not signal has been recently revealed for the PTHR. This new study shows that cAMP levels that originate from internalized PTH–PTHr complexes are regulated by a negative feedback mechanism where PTH-mediated PKA activation leads to v-ATPase phosphorylation and subsequent endosomal acidification, which in turn results in the disassembly of signaling PTH–PTHr–arrestin complexes and the assembly of inactive PTHR–retromer complexes⁵⁰. Despite a better understanding of mechanisms regulating endosomal receptor deactivation and signal desensitization, ligand or receptor determinants that regulate the stability of the ligand–receptor–retromer complex in the endosomal compartment are currently unknown. Nonetheless, the structural similarity between the retromer subunit Vps26 and β -arrestins^{51,52} and the scaffolding property of the Vps35 subunit⁵³ could be involved in the direct interaction with receptors.

How can receptor–arrestin complexes continue to produce cAMP and thus couple to G_s when it has been well established that binding of arrestin and G proteins to prototypical GPCRs, such as rhodopsin and the β_2 AR, is mutually exclusive^{54–57}? Several findings provide evidence that in fact PTH assembles the formation of PTHR–arrestin– G_s complexes that prolong cAMP signaling. Initial mutational studies have shown that the binding site of $G\beta\gamma$ subunits is localized on the proximal domain of the long C-terminal tail of PTHR (132 amino acids) and does not overlap with the binding domain of β -arrestins, which includes a cluster of GRK-phosphorylated serine residues on the receptor's C-terminal tail^{58–60}. More comprehensive studies showed that $G\beta\gamma$ interacts with β -arrestins and also forms a ternary complex with the PTH-bound PTHR⁶¹. Kinetic and biochemical analyses further indicate that this ternary PTHR– $G\beta\gamma$ –arrestin complex accelerates the rate of G_s activation and increases the steady-state levels of activated G_s , leading to persistent generation of cAMP by PTH. These data provide the mechanistic basis for a new paradigm in the regulation of GPCR signaling, where β -arrestins contribute to sustaining rather than inhibiting the G-protein signaling effect of an agonist on the receptor by permitting multiple rounds of $G\alpha_s$ subunit coupling and activation or by stabilizing sustained coupling with the active state of $G\alpha_s$ (Fig. 3). This observation lets us predict the possibility that many cycles of G_s activation persist as long as PTHR and β -arrestin complexes are maintained in endosomes.

GPCR signal transduction's next steps

Sustained cAMP signaling produced by internalized receptors provides new insights into physiological processes as it may be involved in cardiac neuron excitability regulated by the PACAP type 1 receptor²¹ and in insulin secretion by internalized glucagon-like peptide 1 receptor in pancreatic beta cells. This new model also explains the physiological bias between two medically important ligands acting at the V2R, vasopressin and oxytocin, when used as a therapy for disorders of water and electrolyte transport²². The divergent antinatriuretic and antidiuretic effects produced by these ligands, which are either strong (vasopressin) or weak (oxytocin), are likely to account for the different cAMP dynamics between vasopressin (sustained endosomal cAMP production) and oxytocin (short cAMP production limited to the plasma membrane).

The emerging endosomal GPCR–G_s signaling model invites us to change our thinking about GPCR signaling and its regulation and move toward new directions to develop ligands that have improved efficacies for treating diseases by targeting GPCR in specific cellular locations such as endosomes. In the case of the PTHR, certain synthetic PTH analogs have been identified that bind with even higher affinity to R₀ than PTH(1–34)^{36,42}. This enhanced selectivity for the R₀ state of PTHR conformation is accompanied by markedly prolonged cAMP responses from endosomes in cells and, notably, prolonged hypercalcemic and hypophosphatemic responses when injected into mice. One particularly long-acting PTH analog, which is called LA-PTH and consists of a unique M-PTH(1–14)/PTHrP(15–36) hybrid structure (where M = Ala^{1,12}, Aib³, Gln¹⁰, Har¹¹, Trp¹⁴ or Arg¹⁹), can induce elevations of serum calcium in mice that persist for nearly 24 h following a single subcutaneous injection, which contrasts markedly with injections of PTH(1–34), which raise serum calcium for only 2–4 h⁴² (Fig. 4). The prolonged responses of these analogs *in vivo* can be explained by their stable binding to the PTHR in bone and kidney target cells, although a minor contribution of a low-level of systemic exposure cannot be ruled out. This class of R₀-selective PTH analogs is thus of interest as a potential new mode of therapy for patients with hypoparathyroidism (a condition that leads to abnormal low levels of ionized calcium in blood and thus affects all aspects of calcium metabolism), which is now conventionally treated with vitamin D and calcium supplements and for which *in vivo* action of injected PTH(1–34) is too short lived, due in part to rapid clearance and short action on the receptor⁶². The disease might thus be more approachable with a long-acting PTH ligand that favors endosomal PTHR signaling. Understanding its molecular and cellular basis is likely to lead to important insights into fundamental mechanisms by which the PTHR functions and may potentially lead to new strategies for PTH drug design. Indeed, because of its prolonged action, LA-PTH is now in preclinical development (formulation and toxicology) for eventual testing as a treatment for hypoparathyroidism⁶³.

Once believed to be exclusively limited at the plasma membrane, cAMP production by GPCRs is now being extended to intracellular membranes. Research efforts from various laboratories indicate that the amplitude and duration of the cAMP response to certain GPCR agonists change as the ligand–receptor complex moves along the endocytic trafficking pathway; it is maximal and transient at the cell membrane and submaximal but sustained in early endosomes. The recognition that different ligands of the PTHR trigger different physiological responses coupled to distinct duration of cAMP generation and location of receptor signaling struck researchers as another aspect of ligand-biased signaling (Fig. 5). The concept of ligand-biased signaling initially emerged to explain the property of ligands that can selectively promote the activation of some signaling pathways (for example, G protein-independent β -arrestin-dependent MAPK signaling) while preventing or bypassing other signals (for example, G protein-dependent cAMP signaling) mediated by the same receptor. That it went unrecognized that ligand-biased signaling can also involve variation in the duration and location of a same receptor signaling pathway may be one of the reasons for the apparent failure of screening approaches aimed at identifying potent small-molecule agonists or antagonists by targeting GPCR exclusively at the plasma membrane. Selective targeting of the endosomal GPCR–G protein system may offer more effective treatments than global targeting of cell surface signaling. It seems that the new paradigm discussed in

this Perspective should be considered an integral feature of biased agonism, as recently pointed out in ref. 25.

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Figure 1. Classical versus endosomal signaling models of GPCR

Activation and desensitization of a cAMP response mediated by GPCR– G_s systems proceed through a succession of biochemical and cellular events that initially take place at the cell membrane and result in the induction, propagation and termination of the second messenger molecule (steps 1–6). In the classical model, GPCR– G protein systems are only active on the cell surface and internalize to be degraded and/or replaced by newly synthesized GPCRs. In the new model, G_s and cAMP signaling can continue after internalization of ligand–GPCR complexes in endosomes (step 3'). The figure is based on ref. 64. Arr, arrestin; Ppase, protein phosphatase.

Figure 2. Regulation of endosomal GPCR signaling

Proposed model of sustained cAMP signaling and its regulation. Left, PTH-activated PTHR generates cAMP by activation of adenylyl cyclase internalizes to early endosomes in a process that involves binding of β-arrestins. Activated PTHR is then maintained in early endosomes by arrestin binding, where arrestin-mediated activation of ERK1/2 signaling causes inhibition of phosphodiesterases (PDEs) and permits sustained cAMP signaling. Right, 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. Figure adapted from ref. 46.

Figure 3. Signaling models of GPCR

(a) Classical model. The ligand (L) binds the inactive state of a GPCR (R) and stabilizes its active form (R^*), which then couples with heterotrimeric G proteins ($G\alpha\beta\gamma$) through a diffusion-controlled process (step 1). The $L-R^*-G$ complex, in turn, catalyzes GDP-GTP exchange on $G\alpha$, leading to dissociation the GTP-bound $G\alpha(G\alpha-GTP)$ along with the $G\beta\gamma$ dimer from the receptor (step 2). In the case of G_s , $G\alpha_s-GTP$ activates ACs that catalyze the synthesis of cAMP from ATP (step 3). The hydrolysis of GTP to GDP causes the reassociation of $G\alpha_s$ to $G\beta\gamma$ subunits and the termination of the cAMP production. In this model, the recruitment of β -arrestins mediate desensitization of G-protein signaling (step 4).

(b) Noncanonical model, using PTHR as an example. (i) A long-lived PTH-PTHr-arrestin complex could contribute to sustained cAMP signaling by stabilizing an interaction with the active state of G_s (i.e., the GTP-bound form of G_s); (ii) alternatively, the interaction between the activated PTHR and $G\beta\gamma$ is stabilized by β -arrestins. After the first round of activation, the initial interaction between PTHR and G_s is bypassed such that, after hydrolysis of the GTP-bound form of $G\alpha_s$, free $G\alpha-GDP$ directly reassociates with PTHR- $G\beta\gamma$ complexes to initiate a new cycle of G-protein activation. Arrestin stabilizes the G-protein cycle, resulting in prolonged cAMP production. Figure adapted from ref. 61.

Figure 4. Endosomal PTHR signaling: from bench to bedside

(a) Studies in cells led to the discovery that PTH, as opposed to PTHrP, sustains G-protein activity and cAMP production after PTHR internalization into early endosomes. (b) This observation is changing how we think about cellular signaling of the PTHR and is motivating the development of PTH analogs able to promote the endosomal cAMP signaling. One of them, LA-PTH, mediates a markedly prolonged cAMP signaling response in cells and prolonged hypercalcemic responses when injected into mice. (c) LA-PTH is now in preclinical development via the US National Institutes of Health BrIDGs program⁶³ for eventual testing as a future treatment for hypoparathyroidism. Figure adapted from refs. 36, 42.

Figure 5. Differentiating PTHR conformations

(a) Competition radioligand binding isotherms. (b) Kinetics of radioligand dissociation. (c) Real-time kinetics of ligand dissociation measured by fluorescence resonance energy transfer (FRET). (d) Time course of cAMP in live cells measured by FRET. (e) Three-dimensional view of tetramethylrhodamine (TMR)-labeled peptides (red) and a PTHR N-terminally tagged with GFP (PTHR^{GFP}, green) in live HEK-293 cells by confocal microscopy 30 min after ligand washout. Scale bars, 5 mm. The figure is based on ref. 65 and is adapted from refs. 8, 35.