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Molecular basis of parathyroid hormone receptor signaling and trafficking: a family B GPCR paradigm

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Abstract

The parathyroid hormone (PTH) receptor type 1 (PTHR), a G protein-coupled receptor (GPCR), transmits signals to two hormone systems—PTH, endocrine and homeostatic, and PTH-related peptide (PTHrP), paracrine—to regulate different biological processes. PTHR responds to these hormonal stimuli by activating heterotrimeric G proteins, such as G_S that stimulates cAMP production. It was thought that the PTHR, as for all other GPCRs, is only active and signals through G proteins on the cell membrane, and internalizes into a cell to be desensitized and eventually degraded or recycled. Recent studies with cultured cell and animal models reveal a new pathway that involves sustained cAMP signaling from intracellular domains. Not only do these studies challenge the paradigm that cAMP production triggered by activated GPCRs originates exclusively at the cell membrane but they also advance a comprehensive model to account for the functional differences between PTH and PTHrP acting through the same receptor.

Keywords

G protein-coupled receptor; Parathyroid hormone; Cyclic AMP; G proteins; Internalization; Signaling selectivity; Signaling endosomes

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Introduction

The parathyroid hormone receptor type 1 (PTHR) is a family B G-protein coupled receptor (GPCR) with several paradoxical properties. It binds and is activated by the endocrine ligand PTH, as well as the paracrine ligand PTHrP, to regulate vital biochemical processes in bone, kidney and other tissues (Fig. 1). A key function of PTH acting at the PTHR is the regulation of concentrations of calcium and phosphate ions, and vitamin D in blood and extracellular fluids [1]. Another important function of PTH is to stimulate bone formation when the hormone is intermittently administrated (daily injection). This anabolic action of PTH on bone contrasts with its well-known catabolic action, which is necessary to release Ca^{2+} from bone through stimulation of bone resorption. The key role of PTHrP is to regulate the development of bone, heart, mammary glands and other tissues [2–4]. PTHR is thought to regulate these cell functions by primarily activating two sub-types of heterotrimeric G-proteins: G_s and G_q, which in turn regulate the activity of adenylyl cyclases and phospholipase C (PLC) that control the flow of cAMP/PKA and IP/PKC signaling cascades, respectively (Fig. 2). PTHR can also couple to other subtypes of G proteins, including $G_{i/o,}$ which can inhibit adenylyl cyclase, and $G\alpha_{12/13}$ which activate phospholipase D and RhoA in osteosarcoma cells challenged with PTH [5, 6]. PTHR desensitization is thought to be regulated by interaction with cytosolic β-arrestins (β-arrestin-1 and -2) that coordinate in space and time receptor/G protein uncoupling as well as receptor internalization [7–9]. All these pathways, in turn, may modulate various distal signaling responses such the MAP kinase cascades [10–13].

Parathyroid hormone receptor type 1 has emerged as an important target for human therapeutics. Activating mutations in PTHR result in the human pathologies known as Jansen's disease, associated with hypercalcemia and dwarfism, and the Eiken Syndrome associated with cartilage tumors of bone [14–16]; other PTHR-inactivating mutations cause perinatal lethality, a condition known as Blomstrand's Chondrodysplasia [17–19]. The observation that intermittent injection of synthetic human PTH(1–34) acts as an anabolic signal for bone formation led the US Food and Drug Administration to approve PTH(1–34) for the treatment of osteoporosis in humans [20–26]. The therapeutic window for PTH(1–34) as a bone-anabolic drug, however, is limited by its principal side effect leading to hypercalcemia [27–29]. The importance of PTHR as a critical initiator of signals that regulate cellular responses in bone, kidney and developing tissues, and its role in pathological conditions underscores the importance of elucidating its mechanisms of signaling and trafficking. In this review, we discuss recent developments in the understanding of molecular mechanisms involved in PTHR signaling and trafficking, and the consequences these may have towards more effective treatments of bone and mineral diseases.

Structure-function relation in PTH and PTHR

The use of a number of experimental techniques, including photo-affinity cross-linking using *para*-benzoyl-L-phenyl-alanine (BPA)-containing PTH and PTHrP ligands, coupled with functional assays using mutant receptors and structurally altered ligands has yielded important insights about the general mechanism of PTH-PTHR interaction. We thus know that the receptor's large (180 amino acid) amino-terminal extracellular (N) domain plays a key role in docking the carboxy-terminal portion of PTH(1–34) to the receptor, and that the amino-terminal portion of the ligand interacts with the juxtamembrane (J) region of the receptor containing the extracellular loops and seven transmembrane domain helices. Additional experiments based on FRET approaches in live cells confirmed this two-binding step mechanism, and showed that the interaction between the ligand and the receptor's J

domain plays a key role in inducing or stabilizing the conformational changes involved in receptor activation and G protein coupling [30, 31] (Fig. 3).

The C-terminal (20–31) region of PTH is thought to contain the key determinants for receptor binding affinity, and to form an amphipathic α-helix that projects its hydrophobic face towards the receptor. The recent crystal structure determination of the N domain of the PTHR in complex with PTH(15–34) at a resolution of 1.95 Å remarkably supports this binding scenario [32]. The N-terminal (1–19) region of the ligand is thought to contain the critical residues for triggering receptor activation, most notably Val^2, which is predicted to induce folding to the (1–19) domain into a bio-active α-helix upon interaction with the Met^{425} at the extracellular end of transmembrane helix 6 of the receptor [30]. As described by the two-binding site model, these binding and activation domains of PTH interact with the N and J domains of PTHR, respectively. This general model is, however, an oversimplification. For example, mutations at positions 25 and 26 of PTH(1–34) modulate its interaction with PTHR-ΔN, a PTHR variant that lacks the N domain, suggesting that some interactions can occur between the ligand's C-terminal binding domain and the receptor's J domain [33, 34]. Such interactions between the C-terminal ligand and the receptor's J domain might also contribute to signaling. Indeed, C-terminal PTH fragments, such as PTH(13–34), can activate PKC via a PLC-independent pathway, and residues 11 and 21 of PTH(1–34) can cross-link to receptor N domain residues 165–176 [35]. These data imply, therefore, that the bound ligand adopts a complex folded, rather than a simple extended, conformation, and that the ligand-binding surface of the N and J domains of the receptor are close to each other in the ligand-occupied state.

Nature of receptor conformational changes

Early electronic paramagnetic resonance (EPR) experiments demonstrated that the molecular nature of the conformational change in rhodopsin, a family A GPCR that mediates light-induced G_t protein (transducin) activation, is associated with a relative movement and rotation of the cytoplasmic part of helix 3 away from the cytoplasmic part of helix 6 [36]. An alternative approach that engineered inter-helical zinc ion-bridges to constrain the movements between helices 3 and 6 confirmed that the helical movement model in rhodopsin is involved in G_t activation [37]. A similar strategy showed the importance of the relative motion between helices 3 and 6 in other GPCRs as different as the PTHR and the β_2-adrenergic receptor for G_S activation [38]. Altogether, these studies indicate that the conformational rearrangement between helices 3 and 6 is a common molecular switch mechanism in both GPCR family A (rhodopsin-like) and family B (PTHR-like) to activate G_S proteins. Subsequent studies showed that the agonist-induced movement between PTHR helices 3 and 6 not only triggers activation of G_S but also activation of G_q, indicating that different G protein subtypes are activated by the same helical rearrangement of the receptor [39]. This receptor's conformational change, however, is not involved in the binding and activation process of regulatory proteins such as G protein-receptor kinases (GRKs) and β-arrestins. This implies that there are different conformational requirements for G protein activation and for coupling to GRKs and arrestins and that free movements of helices 3 and 6 is specifically necessary for G protein activation.

Conformational selectivity: the R^0 concept

The transfer of information from ligand binding to its receptor to the generation and propagation of second messenger molecules involves sequential steps, where a dynamic interplay between ligands, receptors, and G proteins is critical. These steps are triggered by the binding of the ligand (L) to an inactive-state receptor (R), which in turn switches to an active-state conformation (R*). The activated receptor then interacts with heterotrimeric G

proteins (G) to form a transient L–R*–G complex. This interaction process further promotes a conformational change-induced exchange of GDP for GTP on Gα, and the subsequent activation by Gα-GTP of membrane-bound effector proteins such as adenylyl cyclase, which catalyzes the synthesis of cyclic AMP from ATP.

It is now widely accepted that GPCRs are conformationally flexible, and that structurally distinct ligands for a given receptor can selectively stabilize different receptor conformations and thereby activate different signaling cascades [40–46]. Ligand-based conformational selectivity can also modulate the routing of the activated receptor through divergent desensitization/internalization pathways [47]. Studies of the PTH–PTHR interaction mechanisms have yielded new clues that such ligand-based conformational selectivity plays a powerful role in determining the biological actions of different PTH and PTHrP ligands [48, 49]. These studies suggest that the PTHR can adopt a novel high-affinity conformation that is preferentially bound by certain ligand analogs to form complexes that are stable in the presence of GTPγS (a non-hydrolyzable GTP). This high-affinity PTHR conformation, presumably not coupled to heterotrimeric G proteins, is termed R^0 to distinguish it from the high affinity G protein-coupled conformation, RG, as described by the classical ternary complex model of GPCR activation (Fig. 3) [50]. The capacity to form this R^0 conformation seems to be a property that is common to family B GPCRs, and indeed was initially described by Hoare and colleagues in their studies of the related CRF receptor [51, 52].

Membrane-based equilibrium competition binding assays that permit the differentiation and quantification of binding to the R^0 and RG conformations [48, 49, 53] have shown that PTH(1–34) binds with greater selectivity to R^0 versus RG than does PTHrP(1–36) [49]. Additional PTH analogs have been further identified that bind with even higher affinity to R^0 than does PTH(1–34) [44]. This enhanced selectivity for R^0 is accompanied by strikingly prolonged signaling responses in cells, and, most importantly, markedly prolonged hypercalcemic and hypophosphatemic responses in mice [54, 55]. The clear correlation between these in vitro and in vivo effects suggest that hitherto unappreciated aspects of the fundamental mechanisms by which the PTHR functions are at play. The mechanisms by which these new R^0-selective ligands bind to the PTHR and mediate prolonged signaling responses are unclear, and further experimentation is needed. An emerging hypothesis is that the relative affinity with which a ligand binds to R^0 versus RG determines the duration of the signaling response induced by that ligand in target cells, and thus its biological activity in vivo [44, 48, 49, 85]. This idea has important implications for the development of new PTH analogs as anabolic therapies for osteoporosis. Although the mechanism by which PTH mediates its paradoxical, anabolic and catabolic, effects on bone are not well understood, however, one critical parameter is the periodicity and duration of ligand exposure. Indeed, a single daily injection of PTH stimulates a net increase in bone, whereas a continuous infusion of PTH stimulates a net loss of bone [22, 25, 26, 56–59]. The new findings on PTHR conformational selectivity suggest that the timing and the duration of PTH action on bone may be controlled at the level of the ligand-receptor interaction. One key prediction is that R^0-selective ligands, due to prolonged actions at the receptor, will favor bone-resorption responses with sustained calcium release, and thus be candidate therapies for hypoparathyroidism [58, 60], whereas RG-selective ligands, due to transient action at the receptor, will favor bone anabolic responses, and thus be candidate therapies for osteoporosis.

A current working hypothesis is that R^0 is a “pre-active”, intermediary state of the PTHR that is not coupled to a G protein but can isomerize to the biologically active state RG, according to the scheme illustrated in Fig. 3. The prolonged signaling responses could thus involve an extended residence time of the ligand (L) in the LR^0 complex at the cell surface, with the pre-active complex persisting in a latent state until it eventually engages a G$\alpha\beta\gamma$

heterotrimer then to proceed through an activation/desensitization cycle. Alternatively, the LR^0 complex may persist on the cell membrane in a dynamically active state that undergoes repeated (catalytic) rounds of G protein coupling and activation [61–65], thus avoiding, for a time, the desensitization responses. A third, less-conventional possibility, is that prolonged signaling involves internalized, yet still active, complexes (see following paragraph). Endosomal signaling is an emerging topic in GPCR biology, but is generally limited to the arrestin-dependent ERK and non-receptor tyrosine kinase (Src) pathways [66–68], although endosomal Gα signaling has been documented in yeast [69].

Signaling selectivity of the PTHR system

Ligand-based receptor conformational selectivity can be the mechanistic basis for signal-selective analogs [40, 41, 70]. Such ligands can serve as powerful tools for dissecting receptor mechanisms, and can lead to improved therapeutics that minimize side effects. Several signal-selective PTH ligand analogs have been reported. These include the cAMP-selective agonist Gly^1, Arg^{19}-PTH(1–28), defective for PLC-dependent and PLC-independent PKC signaling [71, 72], Bpa^1-PTHrP(1–36) [73] and its related analog Trp^1-PTHrP(1–36) [12], defective for PLC activation, receptor internalization, β-arrestin recruitment, and β-arrestin-mediated ERK signaling. PTH analogs having conserved Val^2 replaced by Bpa or Trp are defective for both cAMP and IP_3 signaling [74]. Thus, slight variations in the positioning and structure of the side chains at positions 1 and 2 lead to differential effects on receptor signaling, and presumably on PTHR conformational selection. PTH(7–34) is deficient in G_S- and G_q-mediated pathways but surprisingly can stimulate PTHR internalization independent of β-arrestin as well as ERK1/2 activation, and [$D\text{-}Trp^{12}$,Tyr^{34}]-PTH(7–34) is an inverse agonist that mediates ERK1/2 activation via β-arrestin, at least in cells lacking the adaptor protein NHERF [75–78]. These findings suggest binding to an “active” PTHR conformation that is distinct from those conformations that couple to G proteins.

In targeting new PTHR ligands for osteoporosis, ligand-based conformational selectivity potentially offers a means to minimize hypercalcemic effects. In this regard, in recent clinical studies, Stewart and co-workers suggested that PTHrP(1–36) may be more “purely anabolic” than PTH(1–34) [79], as the former can stimulate similar improvements in bone mineral density (BMD) without attendant increases in serum calcium or markers of bone turnover [80], and is relatively defective in stimulating $1,25(OH)_2$-vitamin D synthesis, effects not likely due to pharmacokinetics [81, 82]. These findings suggest differences at the level of ligand-receptor interaction, and support our hypothesis that ligand-based receptor conformational selectivity can lead to differential effects on PTHR activity in vivo, and thus to potentially new PTHR-based therapeutics.

Kinetics and rate-limiting steps of the PTHR signaling cascade

The development of FRET-based methods has permitted the kinetic analysis of key biochemical reactions—ligand binding, receptor activation, G protein coupling and activation, cAMP production—involved in the PTHR signaling system in live cells (Fig. 3). These novel quantitative FRET-based approaches, recently reviewed [83, 84], revealed the kinetics and rate-limiting reactions that proceed along the PTHR signaling cascade, and define the molecular origin of signal differences mediated by the binding of PTH(1–34) or PTHrP(1–36) to PTHR [85].

For both PTH(1–34) and PTHrP(1–36), the receptor association process involved a two-step mechanism involving fast and slow binding components [31, 85]. The fast binding step is a simple bimolecular interaction between the hormone and the N-domain of the PTHR. The kinetics of this step linearly depends on hormone concentrations with a time constant (t) of

≈150 ms at 10 μM of ligand. In contrast, the slow binding step involves ligand interactions with the receptor's J-domain, which proceeds with time constants that follow a hyperbolic dependence on ligand concentration, suggesting a more complex mechanism involving conformational changes of the ligand and the receptor. The maximal time constant of the slow binding step is ≈1 s for PTH(1–34) at a saturating concentration and is moderately slower for PTHrP(1–36). The kinetic rate for this slow binding phase of both PTH(1–34) and PTHrP(1–36) is similar to the kinetic rate for ligand-induced PTHR activation, as assessed by an intramolecular FRET-based receptor biosensor [31, 86], and is thus rate limiting for activation of the PTHR.

Although PTH(1–34) and PTHrP(1–36) associate with the PTHR by similar processes, they dissociate by distinct mechanisms. PTHrP(1–36) dissociates slowly but fully from the receptor, whereas PTH(1–34) forms a more persistent complex with the receptor. Recording PTHR activation by intramolecular FRET with a receptor bio-sensor as previously described [86] showed that, in contrast to PTHrP(1–36), PTH(1–34) induces a stable active state of PTHR that persists well after removal of ligand. The PTHR can therefore adopt a high affinity conformation that is preferentially bound by PTH to form a ligand–receptor complex that is active for a sustained period.

The kinetics of the following step, which involves PTHR and G_S interaction, are dependent on the relative expression levels of the PTHR and G_S, in accord with a diffusion-controlled process. The maximum time constant obtained at a high level of G_S are in the second scale for both PTH(1–34) (τ = 0.96 s) and PTHrP(1–36) (τ = 1.58 s) and are identical to those obtained for the corresponding PTHR activation switch. In the context of a low level of G protein, the kinetic rate of the PTHR-G_S interaction is not therefore determined by the time course of receptor activation but rather by a diffusion-controlled collision process. A few seconds after G_S activation by the activated PTHR, cAMP production is initiated and reaches its maximal level with a half time ($t_{1/2}$) of ≈10 s in cultured cells. The relative slowness of cAMP initiation and production as compared to the rapidity of the antecedent processes is presumably dependent on a series of sequential steps involving $G\alpha_S$ and adenylyl cyclase interaction, conversion of ATP into cAMP by activated adenylyl cyclases, and the action of phosphodiesterases.

Modulation of PTHR signaling and trafficking by adapter proteins

The post-binding and activation events that determine the regulation of PTHR actions and desensitization are largely consistent with the view that the agonist-activated PTHR is phosphorylated on serines on the carboxy-terminal tail, is then internalized into vesicles via a β-arrestin-/clathrin-coated pit (CCP)-mediated process, and that at least some of the internalized receptor can undergo rapid recycling to the cell surface [8, 9, 75, 87, 88]. Additional complexity regarding PTHR actions arises from modification of its behavior as a consequence of interactions with cytoplasmic proteins such as the Na/H exchanger regulatory protein factors 1 and 2 (NHERF 1 and 2), and Disheveled (Dvl) [76, 89]. These PTHR–adapter protein interactions occur along the 130 amino acids that form the intracellular tail of the PTHR, and confer a high degree of specificity on PTHR signaling through cell- and ligand-specific effects (Fig. 4) [5, 89–109].

PTHR–NHERF interaction

The best characterized of these biomolecular interactions is with the NHERF family of proteins. NHERF1, also known as the ezrin-binding protein 50 (EBP50), is a scaffolding protein that contains 2 tandem N-terminal PDZ domains and a C-terminal ezrin-radixin-moesin (ERM)-binding motif, through which it connects to actin bundles. The PTHR harbors a carboxy-terminal PDZ-like ligand domain that conforms to the D/E-S/T-X-φ motif

(where φ is a hydrophobic amino acid) [110]. Using this recognition domain, the PTHR binds preferentially to the PDZ1 domain of NHERF1 and to the PDZ2 domain of NHERF2 [111, 112]. NHERF1 assembles a signaling complex that incorporates the PTHR, phospholipase C, and actin filaments [112]. In so doing, NHERF can switch PTHR signaling from adenylyl cyclase to phospholipase C [111]. In kidneys, the PTHR is expressed both in proximal and distal tubules, whereas NHERF1 is expressed only in proximal tubules [113]. This cell-specific pattern of expression of PTHR and NHERF1 has important consequences for the actions of PTH on phosphate absorption, which occurs in proximal tubules, and on cellular calcium transport, which occurs in distal tubules. Thus, NHERF1-null mice exhibit lowered serum phosphate and elevated urinary phosphate excretion because of diminished apical membrane tethering of the Npt2a phosphate transporter. Serum calcium is unchanged, though excretion increases secondary to complexation with phosphate [114]. Similar results have been reported in patients harboring NHERF1 polymorphisms or mutations [115].

The interaction of the PTHR with NHERF1 importantly confers ligand bias on the receptor. In the presence of NHERF1, as in proximal tubules or osteoblasts, only full agonists such as PTH(1–84) or its fragment PTH(1–34) activate and internalize the PTHR [76]. In this setting, PTHR activation and internalization are coupled. In the absence of NHERF1, however, receptor activation and internalization are uncoupled, as PTH partial agonists, inverse agonists, and antagonists can induce internalization. Another important function of NHERF1 is the modulation of the PTHR coupling to different G proteins. PTHR signaling to PLC is increased in cells expressing NHERF1 [111, 112, 116].

The interactions of the PTHR with NHERF1 also affect receptor desensitization. PTHR desensitization in ROS 17/2.8 osteosarcoma cells, which lack NHERF1, proceeds in a concentration-dependent manner and was inhibited by NHERF1 [117]. Reducing NHERF1 levels with shRNA in the human SAOS2 osteoblastic cell line, which endogenously express NHERF1, inhibited PTHR desensitization. A phosphorylation-deficient PTHR exhibited reduced desensitization and interaction with β-arrestin 2 compared with wild-type PTHR. Moreover, NHERF1 inhibited β-arrestin binding to wild-type PTHR but had no effect on β-arrestin 2 association with the phospho-deficient PTHR. These findings suggest that NHERF1 may protect against PTH resistance or PTHR down-regulation.

The gene for NHERF1 also possess a non-canonical estrogen response element composed of multiple half-sites [118]. Estrogen treatment appreciably upregulates NHERF1 expression and this effect is blocked by anti-estrogens [119]. Together with the findings for PTHR, these observations suggest that PTH resistance and responsiveness may be regulated not only by the circulating forms of PTH but also by the levels of NHERF1 expression, and the status of estrogen sufficiency.

PTHR-β-arrestin interaction

Parathyroid hormone (PTH) binding to PTHR is quickly followed by its phosphorylation by G protein-coupled receptor kinases (GRKs) and the internalization of the ligand–receptor complex into endocytic vesicles with a $t_{1/2}$ <10 min through a process involving binding of β-arrestins [7–9]. This internalization process relies upon the capacity of β-arrestins to bind clathrin, a major component of the clathrin-based endocytic machinery, with high affinity and thus to serve as an adaptor that targets activated and phosphorylated receptors to clathrin-coated pits [120, 121]. The initial translocation of β-arrestins to the activated PTHR depends upon determinants in the cytoplasmic tail of the PTHR, which include a cluster of GRK-phosphorylated Serine residues (Fig. 4) [105–109]. Binding of β-arrestins to activated GPCRs is thought to uncouple receptors from heterotrimeric G proteins and thus terminate agonist-mediated G protein-signaling [122]. Arrestins also serve as adaptors that assemble

intracellular complexes between internalized GPCRs and signaling proteins to activate MAPK pathways, among others [10–13].

PTHR–Dvl interaction

A recent study has demonstrated a direct interaction between the carboxy-terminal tail of the PTHR and the PDZ adaptor protein Dishevelled 2 (Dvl2) [89]. Unlike NHERF1, however, the PDZ domain of Dvl2 does not interact with the C-terminal canonical PDZ-interaction motif of the PTHR. A mutant PTHR truncated at position 480 can still interact with Dvl2, but a truncation at position 470 eliminates Dvl2 binding [89]. Thus, these studies show that Dvl2 interacts with an internal sequence located between residues 470 and 480. It is suggested that the binding site of Dvl2 is the sequence ^{472}KSWSRW [89], located immediately downstream of the transmembrane helix 7 of the PTHR (Fig. 4). This sequence bears remarkable homology to the consensus Dvl-binding motif (KTXXXW) conserved in all members of the Frizzled (Fzd) family of receptors [97]. The complex formation between Dvl2 and the PTHR is mediated by PTH(1–34) as measured by co-immunoprecipitation experiments [89]. Furthermore, PTH(1–34) addition to cells that express the PTHR induces recruitment of Dvl2 to the plasma membrane, where it co-localizes with the PTHR [89]. However, this co-localization is only transient; as the PTHR traffics through the endocytic pathway, Dvl2 remains plasma membrane-delimited, forming larger structures consistent with the assembly of large oligomers, a phenomenon associated with the activation of the Wnt signaling pathways [90, 91]. The binding of Dvl2 to the PTHR has important consequences for the regulation of PTHR signaling. Ablation of the interactions between Dvl2 and the PTHR eliminates PTH-dependent activation of β-catenin-dependent gene expression [89]. Importantly, Dvl2 plays an important role in the regulation of frizzled (Fzd) receptor trafficking. This function stems from the direct interactions of Dvl2 with the endocytic adaptor complex AP-2 [92] and with β-arrestins [123], which are required for the internalization of Fzd4. A similar scenario occurs with the internalization of the PTHR because overexpression of a Dvl2 mutant that cannot interact with the AP-2 complex blocks ligand-induced internalization of the PTHR [89]. The physiological consequences of this regulatory role are not understood.

A paradigm shift: sustained cAMP production from internalized PTHR

The induction of cAMP production mediated by ligand-activated GPCRs traditionally is believed to originate at the plasma membrane and be rapidly extinguished within minutes by mechanisms involving receptor endocytosis, with the bound ligand being either released at the cell surface or internalized separately from receptors and G proteins, and by PKA activation of phosphodiesterase PDE4, which degrades cAMP [124]. This model, however, does not provide a satisfactory explanation for recent studies showing that PTH(1–34) and certain PTH ligands exhibit prolonged cAMP responses in cell culture, and prolonged calcemic responses in animals [43]. These persistent responses contrast with those observed for PTHrP(1–36) and related ligands, which promote short-lived signaling responses that are more consistent with the classical model for GPCR desensitization. The above model also does not provide a rational explanation as to why in clinical testing PTH(1–34) stimulates more prolonged increases in serum levels of 1,25-dihydroxyvitamin D, calcium, and bone resorption markers than does PTHrP(1–36).

By monitoring the actions of brief stimuli of PTH(1–34) or PTHrP(1–36) on PTHR activation and internalization in cultured cells, Ferrandon and colleagues found that the complex PTH(1–34) + PTHR internalizes rapidly into early endosomes in association with G proteins and adenylyl cyclases, and that this process is required for a sustained cAMP response [85]. In contrast, PTHrP(1–36) actions are completely reversible and limited to the plasma membrane. These data raise the hypothesis that PTHR in response to PTH can

generate cAMP from intracellular membranes, and that early endosomes serve as a platform for PTHR-mediated sustained cAMP production (Fig. 5). To some extent, sustained cAMP production from internal endosomes may underlie the catabolic actions of PTH on bone coupled with the unavoidable increase in calcium levels in blood. This new concept of endosomal receptor/ $G\alpha_S$ signaling, supported by another study on the TSH receptor [125], challenges the central tenet that cAMP production triggered by GPCRs originates exclusively at the cell membrane, and thus represents a major paradigm shift in our understanding of mechanisms of signaling mediated by PTHR and other peptide hormone GPCRs.

Perspectives

Despite extensive investigations by a number of groups many gaps remain in our understanding of the essential mechanisms that govern the actions of PTH and PTHrP ligands at the PTHR. We do not know the specific intermolecular interactions that define the ligand/receptor interface and the post-binding events that trigger downstream signaling and desensitization responses in bone and kidney cells, and we are just beginning to appreciate the importance of receptor conformational variation.

The strong correlation we have observed so far between the affinity with which a ligand binds to the R^0 state of PTHR and its capacity to produce prolonged responses in vitro and in vivo establishes that the R^0 concept is biologically relevant, and, indeed, poses important implications for the development of new PTHR ligands for osteoporosis and hypoparathyroidism. Key issues to address in the future include (1) identification of the ligand structural determinants of receptor binding and activation, and (2) the cell-based mechanisms of prolonged cAMP signaling and its termination, as well as the modulation of PTHR signaling by adapter proteins, and their relevance to metabolic responses in bone and kidney.

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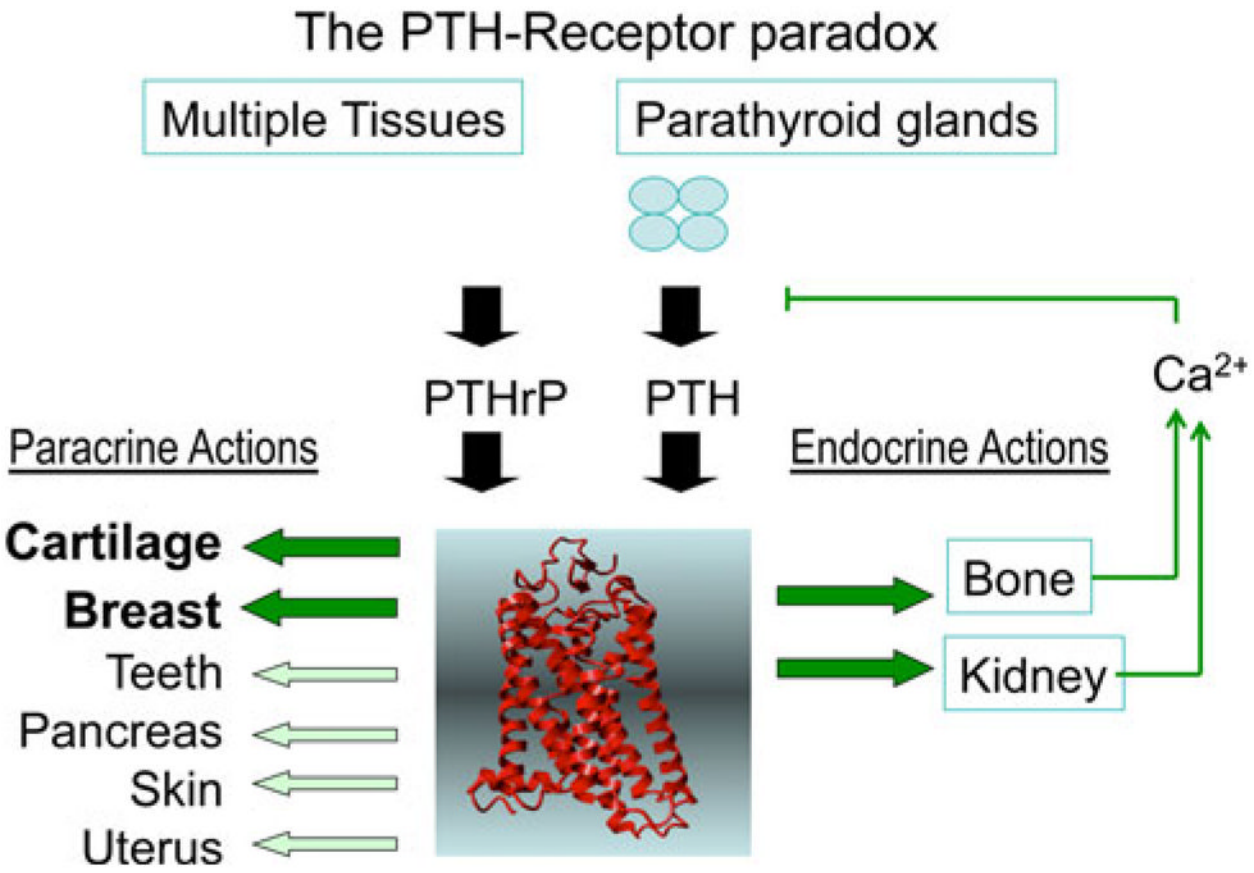

Fig. 1.

PTH and PTHrP bind and stimulate the same receptor to mediate divergent functions. PTH is rapidly secreted from the chief cells in parathyroid gland in response to small decreases in Ca^{2+} in the blood to restore a normal calcium level (≈1.2 mM) by promoting the release of calcium from bone and calcium reabsorption from the kidney. PTHrP is produced and secreted from many tissues and plays a critical role in tissue development, in particular the skeleton and mammary glands

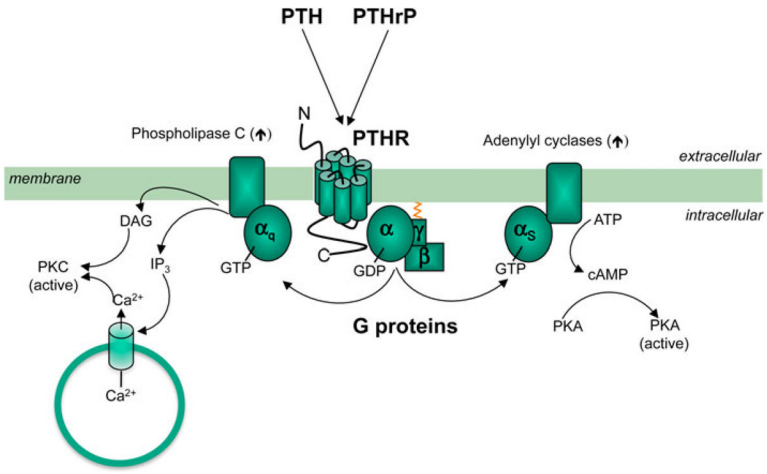

Fig. 2.

General principle of signaling by PTHR. Following ligand binding, the receptor undergoes conformational changes, which promote the coupling with heterotrimeric G proteins (G$\alpha\beta\gamma$), and catalyzes the exchange of GDP for GTP on the α-subunit. This event triggers conformational and/or dissociation events between the α- and $\beta\gamma$ subunits. Gα_S activates adenylyl cyclases leading to cAMP synthesis, which in turn activates protein kinase A (PKA). Gαq activates phospholipase C, which cleaves phosphatidylinositol (4,5)-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (IP_3). IP_3 then diffuses through the cytosol and activates IP_3-gated Ca^{2+} channels in the membranes of the endoplasmic reticulum, causing the release of stored Ca^{2+} into the cytosol. The increase of cytosolic Ca^{2+} promotes PKC translocation to the plasma membrane, and then activation by DAG

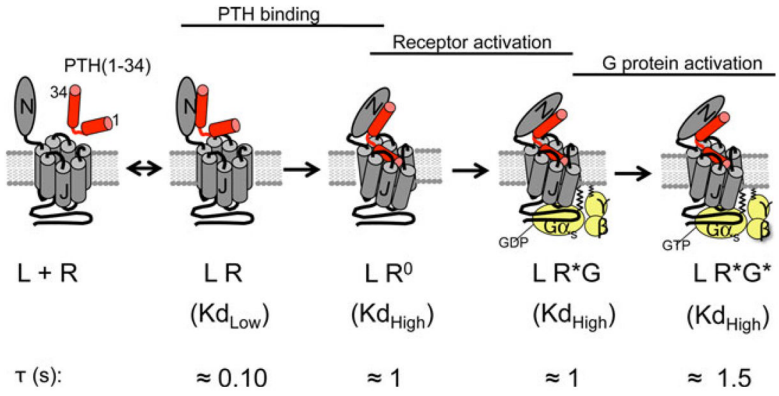

Fig. 3.

Mechanistic model for PTHR activation. Upon PTH(1–34) binding, there are two rates of association, a faster one that corresponds to agonist binding to the receptor N-domain and is strictly concentration-dependent, followed by a slower binding step to the receptor J-domain that is coupled to receptor activation. The hormone binds to and stabilizes the R^0 conformation of PTHR, which in turn locks PTH into a high-affinity complex with the receptor. *L* PTH, *R* PTHR, LR^0 a high-affinity complex between the ligand and the PTHR, *LR*G* and *LR*G** active states for receptor and G protein state, respectively. Time constants (τ) to accomplish each step are given in seconds

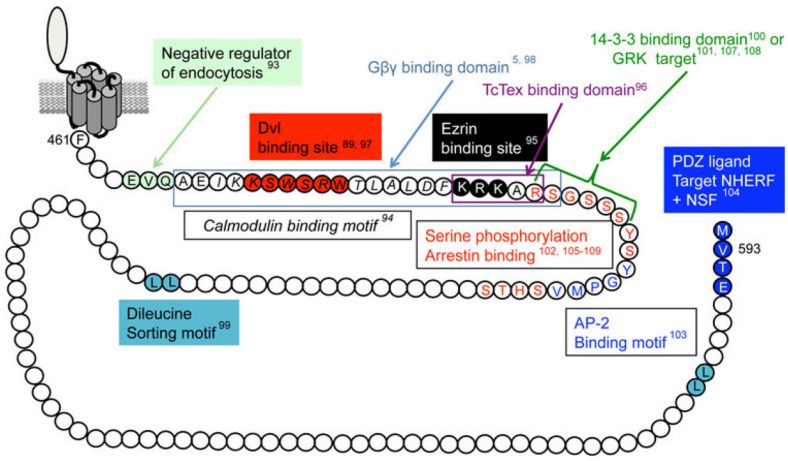

Fig. 4.

Carboxy-terminal PTHR binding motifs. The sequence of the intracellular tail of the human PTHR is shown. Described binding motifs are designated by *boxed* and *colored* notation. References are indicated in *superscript*

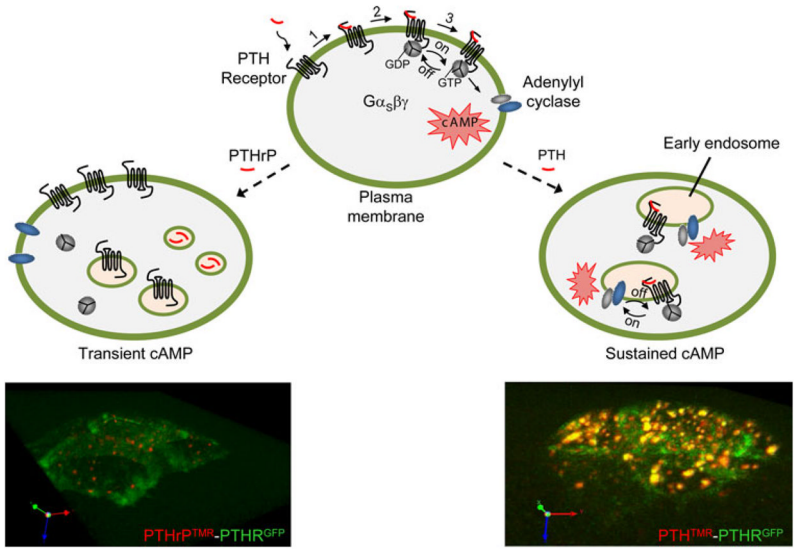

Fig. 5.

Mode of activation of the PTHR by long-acting and short-acting signaling ligands. The action of a short-acting signaling ligand is well represented by the classical model for G protein signaling. The ligand interacts first with the receptor (step 1). The receptor is then switched on to lead to G protein recruitment (step 2) and activation (step 3), which in turn initiates adenylyl cyclase activation. In the classical model—that is, the PTHrP-like hormone model—the ligand rapidly dissociates from the receptor, which deactivates and ultimately terminates the signaling. In our model for a long-acting signaling ligand such as PTH, the hormone interacts tightly with the receptor in a conformationally dependent manner. The receptor is then locked into a prolonged active state inducing sustained receptor-G protein coupling and sustained G protein activation. The ternary ligand–receptor-G protein heterocomplex is preserved during its internalization in early endosomes and persists over time to mediate the prolonged downstream signaling of PTH-like hormone. Images represent a 3D view of TMR-labeled ligands and the PTHR N terminally tagged with GFP (^{GFP}N-PTHR) captured in live HEK-293 cells by spinning disc confocal microscopy 20 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 punctae at internalized sites (*left*). Adapted from Ref. [85]