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Review

PTH/PTHrP Receptor Signaling, Allostery, and Structures

Ieva Sutkeviciute,[1,5] Lisa J. Clark,[1,2,5] Alex D. White,[1,3] Thomas J. Gardella,[4] and Jean-Pierre Vilardaga[1,*]

The parathyroid hormone (PTH) type 1 receptor (PTHR) is the canonical G protein-coupled receptor (GPCR) for PTH and PTH-related protein (PTHrP) and the key regulator of calcium homeostasis and bone turnover. PTHR function is critical for human health to maintain homeostatic control of ionized serum Ca^{2+} levels and has several unusual signaling features, such as endosomal cAMP signaling, that are well-studied but not structurally understood. In this review, we discuss how recently solved high resolution near-atomic structures of hormone-bound PTHR in its inactive and active signaling states and discovery of extracellular Ca^{2+} allosterism shed light on the structural basis for PTHR signaling and function.

Highlights

Resolution of the subatomic structure of the PTH receptor in complex with Gs and long acting PTH (LA-PTH).

Identification of the signaling LA-PTH–PTHR–Gs complex in three conformations.

Physiological and disease relevance of endosomal cAMP production as it relates to Ca^{2+} homeostasis.

Extracellular Ca^{2+} and ATP as novel allosteric modulators of PTHR signaling.

Physiological and Disease Relevance of Endosomal PTHR Signaling via cAMP

The **parathyroid hormone (PTH)** (see Glossary) type 1 receptor (PTHR) is expressed in many tissues [1], primarily in bone (osteoblasts and osteocytes), the kidney (proximal and distal tubules), and mammary glands. PTHR is indispensable for maintaining normal Ca^{2+}, phosphate, and active vitamin D levels in blood as well as extracellular fluids in response to PTH. The receptor also regulates growth and development in various tissues such as bone and mammary glands in response to **PTH-related peptide (PTHrP)**. Additionally, PTH and PTHrP are implicated in bone remodeling processes, mediating both anabolic and catabolic effects, but the synthetic N-terminal PTH(1–34) fragment stimulates more prolonged increases in serum levels of 1,25-dihydroxy-vitamin-D, calcium, and bone resorption markers than does PTHrP(1–36), when the ligands are injected by continuous infusion [2–4]. Despite exerting distinct physiological functions, PTH and PTHrP mediate their biological effects via identical **heterotrimeric G protein (Gαβγ)** pathways involving primarily Gs/cAMP/**protein kinase A (PKA)** and Gq/PLC/Ca^{2+}/PKC signaling (Figure 1A), and also $G_{12/13}$/RhoA/phospholipase D and the mitogen-activated protein kinase (MAPK) (extracellular signal-regulated kinase, $ERK_{1/2}$) signaling cascades. These distinct biological effects are attributed to the ability of the PTHR to adopt two distinct active conformations that differ markedly in signaling duration and localization, termed R_G and R_0 (Box 1). Ligands selective for the G protein-dependent R_G state give rise to only transient cAMP production that is confined to the cell surface due to the rapid action of cAMP-specific phosphodiesterases and ligand–receptor internalization mediated by **β-arrestins (βarrs)**. Conversely, ligands that bind efficiently to the G protein-independent R_0 state promote both acute cAMP responses at the plasma membrane and sustained cAMP generation originating from active signaling complexes that remain stable within endosomal compartments (Figure 1B and Box 2).

PTHR is as a major clinical target for the treatment of osteoporosis. Remarkably, the only bone-building drugs directly acting via the PTHR that have been so far approved by the US FDA to effectively treat severe osteoporosis when injected subcutaneously once daily, PTH(1–34) (teriparatide or PTH) [5], or **abaloparatide (ABL)**, a modified PTHrP(1–34) [6], trigger distinct modes of cAMP signaling. While both PTH and ABL stimulate transient cAMP derived from ligand-PTHR complexes at the plasma membrane, only PTH triggers sustained cAMP/PKA signaling via the internalization of PTHR in complex with βarrs and G protein βγ (Gβγ) subunits [7,8]. Given that ABL can stimulate bone anabolic responses that are comparable to those seen for PTH, but with a significantly reduced hypercalcemic effect, we propose the hypothesis that promoting short plasma membrane cAMP responses as opposed to sustained endosomal cAMP provide the osteoanabolic benefit without enduring hypercalcemia. Thus, transient cAMP production from the cell surface might favor the formation of new bone, while sustained cAMP production from endosomes is thought to promote bone

[1]Laboratory for GPCR Biology, Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA

[2]Graduate Program in Molecular Biophysics and Structural Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA

[3]Graduate Program in Molecular Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA

[4]Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA

[5]These authors contributed equally to this work.

*Correspondence: jpv@pitt.edu

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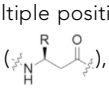
breakdown. Consistent with this view, the first reliable link between endosomal cAMP response and its physiological outcome is provided by a finding that a modified PTH/PTHrP chimera with high affinity for the R_0 state of the PTHR (hereafter referred to as long acting PTH or LA-PTH), which triggers substantially longer endosomal cAMP responses than PTH also induces enhanced and prolonged blood Ca^{2+} elevation in mice and in monkeys compared with PTH [9,10]. This link is further strengthened by a recent study demonstrating that chronic hypocalcemia occurring in patients with idiopathic hypoparathyroidism can be caused by a single point mutation in residue 25 of PTH (PTH-R25C) that cannot engage endosomal PTHR signaling in cells, or maintain blood Ca^{2+} homeostasis in animals [11]. Implicit in these observations is that endosomal cAMP production in PTHR signaling has both physiological and disease relevance.

Allostery at the PTH Receptor

A series of optical, biochemical, and cellular approaches uncovered that extracellular Ca^{2+} serves as a positive allosteric modulator of the PTHR by increasing the residence time ($1/\tau_{off}$, where τ_{off} is the time constant of ligand dissociation) of PTH or ABL on an active PTHR conformation, resulting in prolonged receptor activation and enhanced endosomal signaling via cAMP [12]. This discovery is supported by the finding that the aforementioned single point PTH-R25C mutant associated with severe idiopathic hypoparathyroidism (hypocalcemia) in humans lacks sensitivity to extracellular Ca^{2+} and is defective for endosomal cAMP signaling. High-resolution mass spectrometry experiments identified negatively charged clusters in the receptor's first extracellular loop (ECL1) that are critical for mediating Ca^{2+} allostery, possibly by acting as coordination sites within the receptor. Furthermore, previous studies using purified receptor in nanodiscs have shown that extracellular Ca^{2+} enhances ligand affinity. These findings uncover Ca^{2+} allostery as a novel temporal determinant regulating the duration of PTHR signaling. The role of extracellular Ca^{2+} as a feed-forward modulator of PTHR signaling might be particularly important in the bone microenvironment where dynamic fluctuations in Ca^{2+} concentration can reach up to 40 mM due to the release of calcium stored in bone [13]. Promoting the catabolic actions of PTH by a Ca^{2+} allostery mechanism could thus ensure calcium rebalancing even with very low levels of secreted hormone. However, this feed-forward mechanism of Ca^{2+} might favor detrimental hypercalcemia with pathologic hyperparathyroidism or with excess PTH administration by accelerating the osteocatabolic actions of PTH.

Interestingly, allosteric modulation of PTHR signaling may extend to endogenous organic small molecules, as extracellular ATP was shown to increase the potency (i.e., decreases half maximal effective concentration EC_{50} to induce a response) of PTH to stimulate the production of cAMP in cultured osteoblastic cells and the recruitment of βarrs to the recombinant PTHR expressed in HEK293 cells [14]. Given that PTH can trigger the release of ATP from osteoblasts [15], presumably by stimulating aerobic glycolysis via the production of the insulin-like growth factor 1 [16], an autocrine mechanism whereby the release of ATP by PTH acts on the PTH-bound PTHR might act as a positive feedback loop of PTHR signaling in bone cells. Direct binding of ATP to the receptor and structural determination of the Ca^{2+} and ATP binding sites remain to be demonstrated; however, allosteric modulation constitutes an emerging and significant mechanism by which PTHR biology can be tightly controlled.

Extending PTH Ligand Utility by Peptide Backbone Modification

In a new approach being developed by Gellman and colleagues, PTH ligand analogs are synthesized with non-natural β-amino acids incorporated at multiple positions in the peptide chain. The underlying rationale is that the introduced β-amino acids (), each of which carries an extra methylene spacer between the backbone amine and carbonyl groups of the residue, as compared with the single α-carbon that lies between these groups in a conventional α-amino acid (), change the configuration of the peptide backbone such that the peptide is rendered less

Glossary

Abaloparatide (ABL): N-terminal analog of PTHrP(1–34) and second generation of an osteoanabolic drug to treat osteoporosis.
β_2-adrenergic receptor (β_2AR): first cloned GPCR for a diffusible ligand in 1986 that binds epinephrine to stimulate the stimulatory G protein for adenylate cyclase.
β-arrestins (βarrs): cytosolic adaptor proteins interacting with agonist activated GPCR to regulate receptor endocytosis and signaling.
G protein-coupled receptor (GPCRs): most abundant cell surface receptors in the human genome for hormones, chemical neurotransmitters, sensory stimuli (light, odors, taste molecules), and 30–50% of current clinical drugs. Contain seven α-helical transmembrane domains. Ligand (agonist) binding to GPCRs triggers activation of heterotrimeric G proteins (Gαβγ), which in turn engage and/or regulate intracellular signaling pathways.
Heterotrimeric G proteins (Gαβγ): guanine nucleotide (GTP and GDP)-binding proteins constituted of three subunits, Gα, Gβ, and Gγ, with GDP bound to Gα in their inactive states. GPCR activation catalyzes the exchange of GDP by GTP on the Gα, an event, which engages conformational or dissociation events between the Gα and Gβγ dimer. Both GTP-bound Gα and Gβγ regulate the activity of various effectors.
N-terminal domain of PTHR (N-PTHR): the large amino-terminal extracellular domain of the PTH receptor.
Parathyroid hormone (PTH): 84 amino-acid endocrine hormone secreted by the parathyroid glands to regulate serum calcium (Ca^{2+}) and phosphate (PO_4^{3-}) ion homeostasis via binding to the PTH receptor type 1 in bone and kidney cells. The recombinant N-terminal amino acid sequence of human PTH, PTH(1–34) (teriparatide), is fully functional and the first generation of an osteoanabolic drug to treat osteoporosis.
PTH-related peptide (PTHrP): paracrine or autocrine hormone with similar N-terminal amino acid

susceptible to protease attack and thus can achieve greater bioavailability and efficacy *in vivo*. The general strategy has been to introduce multiple β-amino acids into the peptide chain in an alternating pattern with the native α-amino acids. In a systematic application of this strategy to PTH, β-amino acids were first targeted to the C-terminal binding region of PTH(1–34) [17]. One prominent analog resulting from these studies, called D6, has six β residues incorporated at the positions of His14, Glu18, Glu22, Lys26, Asp30, and Phe34. The side chain at each of these positions was preserved in the β residue replacement, with the exception of postion-14 at which a β-tryptophan was used due to the difficulty in generating a β-histidine precursor amino acid. Results of cell-based functional assays demonstrated that D6 binds to the PTHR with an affinity that is within twofold of that measured for the parent PTH(1–34) peptide and it stimulates cAMP signaling response with comparable potency. This preservation of affinity and potency on the receptor is somewhat remarkable, given that the native PTH peptide binds to the PTHR in an α-helical configuration (see below), and the six extra methylene spacers inserted into the peptide backbone chain of D6 could be expected to at least moderately distort the normal helical configuration. Nevertheless, the D6 structure is apparently able to mimic the native PTH(1–34) peptide sufficiently to allow high-affinity binding to the PTHR as well as potent activation of signal transduction responses. Importantly, peptide D6, relative to PTH(1–34), was indeed found to be highly stable in the presence of the digestive protease, trypsin, *in vitro*. Moreover, when injected into mice, D6 exhibited a half-life in the circulation that was much more prolonged than that of PTH(1–34) and it induced a calcemic response that was similarly more prolonged.

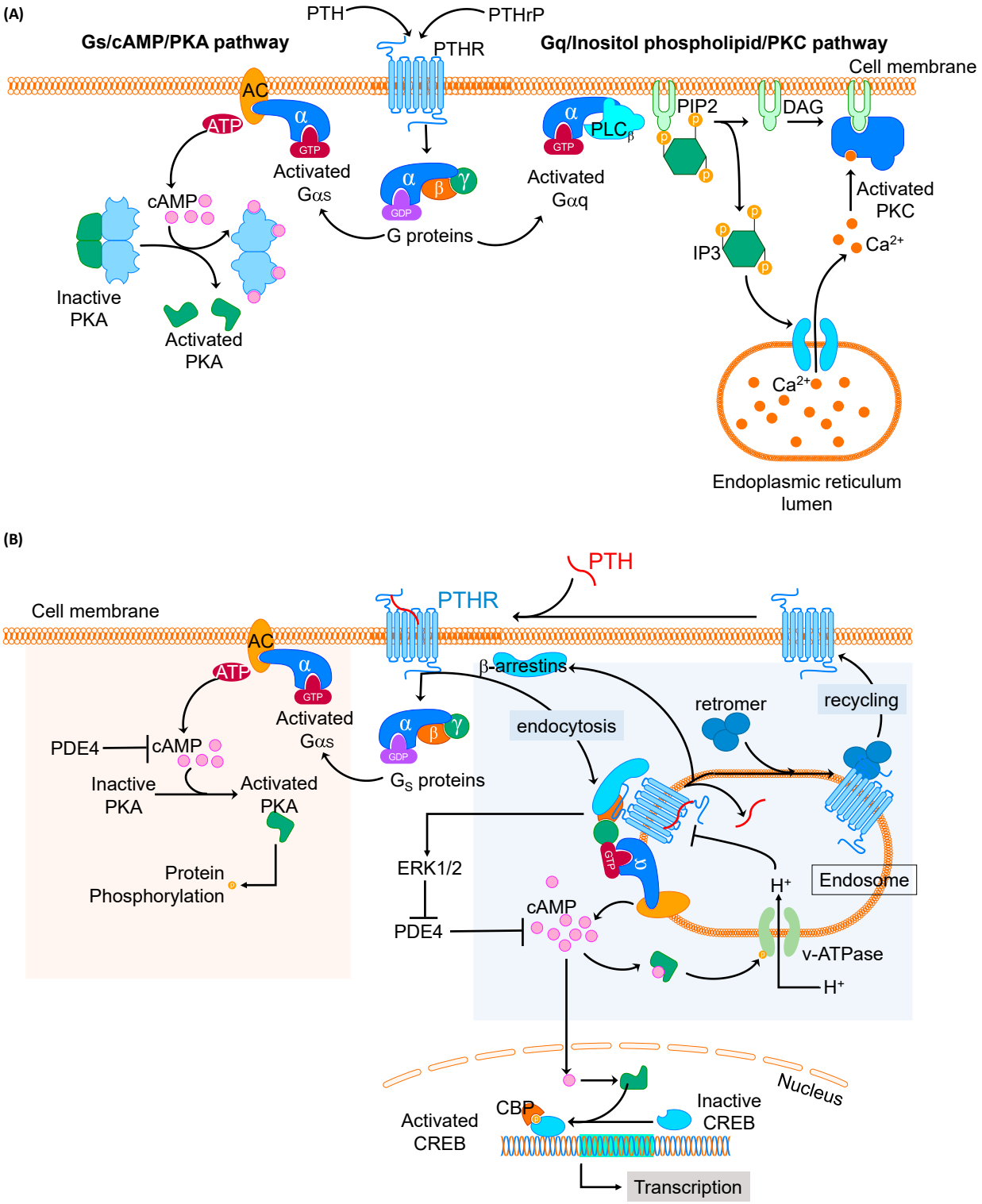
Based on these proof-of-concept findings with D6, additional analogs were generated that contained modifications extending into the N-terminal region of PTH. One such analog, called peptide 35, contained the substitutions present in D6 plus three additional replacements of the constrained β-amino acid, amino-cyclopentyl-carboxylic acid (AC5C) for Ser1, Ile5, and Leu7 [18]. This PTH analog, with a total of nine backbone modifications, again exhibited high binding affinity and signaling potency at the PTHR, extremely high stability in the presence of proteases, and it induced prolonged calcemic response when injected into mice. Although in this case, measurements of peptide 35 in the circulation suggested a disappearance rate more rapid than that of PTH(1–34). Whether or not the failure to detect an extended presence of peptide 35 in the circulation (i.e., to parallel the extended calcemic response) reflects limitation of the cell-based cAMP bioassay used for detection or some alternative mode of action (e.g., sequestration by binding to PTHR in target cells in bone and kidney or to some other unknown protein or compartment) was not established. Nevertheless, the results overall support the use of this approach for the development of new PTH ligands with useful applications, including analogs with signaling bias [19] or to be potentially used as therapeutics. In this regard, it is worth noting that the β-amino acid approach has been used to generate highly stable PTH(7–34)-based antagonists and inverse agonist analogs for the PTHR [20]. Such analogs could potentially be used to treat conditions of hormone excess, as occurs in certain cases of cancer in which a tumor secretes excessive PTHrP, or even in Jansen's metaphyseal chondrodysplasia, an ultra-rare disease of skeletal development that is caused by activating mutations in the PTHR [21].

PTH Receptor Structures

Near Atomic Structures of the PTH Receptor Signaling Complex

The structural studies of the PTHR signaling system have started long ago, first with the resolution of the peptide hormone ligand structures (summarized in Box 3), however, the structure of the receptor itself has been elusive only until recently, and the structural determinants of PTHR responsible for ligand selectivity and function are still not known. This is an obstacle for the development of clinically relevant PTH analogs for bone and mineral diseases. As a first step toward understanding the structural basis for molecular recognition and receptor activation, cryo-electron microscopy (cryo-EM) has been recently used to elucidate the near-atomic (3–4 Å) resolution structures of three active conformations of the human PTHR bound to LA-PTH and in complex with a stimulatory G protein (Figures 2 and 3) [22]. The structure of the PTHR complex in one of the states

sequence of PTH(1–34) and binding the same PTH receptor type 1.
Protein kinase A (PKA): protein kinase consisting of two regulatory (R) and two catalytic subunits (C) that is activated by cAMP elevation.
Receptor transmembrane domain (TMD): the PTHR's transmembrane domain comprising the seven α-helices and connecting extracellular and intracellular loops.



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Box 1. PTH Receptor Signaling Conformations, R_0 and R_G

The PTHR can adopt at least two distinct active conformations. One of these conformations, named R_0, is a high-affinity PTHR conformation stabilized by PTH and certain PTH analogs such as the long-acting PTH (LA-PTH) [10]. The affinity to the R_0 conformation is not dependent on G protein coupling, but can nevertheless maintain extended periods of Gs protein coupling and activation, resulting in sustained cAMP production when the receptor internalizes and redistributes in early endosomes. The R_0 conformation is thus distinct from the classical G protein-dependent high-affinity receptor conformation, hereafter referred to as R_G, which is preferentially stabilized by PTHrP and analogs such as abaloparatide [47]. The R_0 and R_G conformations of PTHR are differentiated by using membrane-based equilibrium competition binding assays [48] or Forster resonance energy transfer (FRET) experiments done in live cells [49]. For binding assays, R_0 is detected using ^{125}I-PTH(1–34) as a tracer radioligand in the presence of GTPγS (a nonhydrolyzable GTP) that prevents PTHR and G protein coupling, R_G is assessed using a modified PTH analog ^{125}I-M-PTH(1–15) (where M is Ala/Aib1, Aib3, Gln10, Har11, Ala12, Trp14, Arg19) that binds weakly when GTPγS is present but displays high affinity for PTHR when a dominant negative $G\alpha_s$ mutant (G_S-ND) is present. In FRET assays, R_0 is differentiated from R_G by recording dissociation time courses of tetramethylrhodamine (TMR)-labeled ligands from PTHR N-terminally tagged with GFP. R_0-selective ligands form stable ligand–PTHR complexes, whereas those induced by R_G-selective ligands are reversible after ligand washout. Expression of a G_S-ND has no effect on dissociation of R_0 selective ligands, but blocks the dissociation of R_G selective ligands. R_G-selective ligands usually induce short cAMP responses, whereas ligands stabilizing preferentially the R_0 state of PTHR trigger prolonged Gs activation and sustained cAMP production originated from early endosomes.

(state 1) revealed an extensive binding interface of the LA-PTH with the PTHR (Table S1 in the supplemental information online) as well as the contacts of the receptor's cytosolic core with the Gs protein. The LA-PTH is bound to the PTHR as a continuous α-helix, with the C-terminal fragment of the peptide (residues 16–34, ^{C}LA-PTH) interacting with the **N-terminal domain of PTHR (N-PTHR)** in a 'hot-dog in a bun' mode, closely resembling the crystal structure of PTHrP-bound N-PTHR [23]. The N-terminal fragment of LA-PTH (residues 1–15, ^{N}LA-PTH) is inserted deep into the core of the **receptor transmembrane domain (TMD)** and serves as a trigger for the receptor activation. Such a binding mode for LA-PTH superposes the N-PTHR perpendicularly to the cell membrane plane in a structurally dynamic manner, as indicated by extensive cryo-EM data analysis (Figure 3A). Gs primarily interacts with PTHR through C-terminal α5 helix of the Gα subunit, and the Gβ subunit makes additional contacts with intracellular loop 1 (ICL1) and helix 8 of the receptor (Figure 2B).

While the 15–34 segment of PTH is responsible for an initial high affinity receptor binding via a rapid interaction with N-PTHR that only depends on hormone concentration (Box 4), the N-terminal peptide portion contains the signaling moieties and binds the receptor's TMD domain with slower kinetics [24]. Thus, PTHR activation has been described by a two-step model: first, the C-terminal peptide fragment associates with the N-PTHR, and second, the low-affinity N-terminal peptide fragment binds to PTHR TMD, which triggers receptor activation and intracellular signaling events. The three structures of the LA-PTH–PTHR–Gs complex differing mostly by the positioning of LA-PTH on N-PTHR indicate that this domain is more dynamic than initially thought and permits us to revise the two-state model of PTHR activation (Figure 3A). The description of the structure of the PTHR complex in state 1, the most prevalent of the three, is consistent with those seen for structures of other family

Figure 1. Modes of Parathyroid Hormone Type 1 Receptor (PTHR) Signaling.
(A) Major G protein signaling pathways triggered by PTHR activation. General principle of signaling by PTHR. Parathyroid hormone (PTH) or PTH-related protein (PTHrP) binding induces or stabilizes active PTHR conformations, which promote coupling and activation of heterotrimeric G proteins. G_S activates adenylate cyclases (AC), leading to cAMP synthesis and activation of protein kinase A (PKA). Gq activates phospholipase C, which cleaves phosphatidylinositol (4,5)-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (IP3). IP3 then diffuses through the cytosol and activates IP3-gated Ca^{2+} channels located in the endoplasmic reticulum membrane, releasing stored Ca^{2+} into the cytosol. An increase in cytosolic Ca^{2+} promotes PKC translocation to the plasma membrane and then activation by DAG. (B) An additional mode of cAMP signaling has been revealed, where internalized PTHR in endosomes also prolongs cAMP production, which diffuses to the nucleus to directly activate nuclear PKA (blue box). Details are discussed in Box 2.

Box 2. Modes of PTH Receptor Signaling via cAMP

Altered modes of cAMP signaling by PTHR have been discovered: PTHrP and other R_G ligands induce transient cAMP responses derived from ligand–PTHR interactions at the plasma membrane, whereas R_0 ligands, including PTH, induce prolonged cAMP responses derived from complexes associated within endosomes [7,49] (see Figure 1B in main text). The general mechanism that desensitizes GPCR signaling at the plasma membrane through βarrs is needed to promote endosomal cAMP production in PTHR signaling. Biochemical and cellular analyses demonstrated that PTH-bound PTHR–arrestin complexes accelerate the rate of G_S activation and increase the steady-state levels of activated G_S, thus extending the generation of cAMP by PTH [50]. Internalized PTHR signaling complexes containing βarrs promote rather than terminate cAMP signaling by activating ERK1/2, leading to the inhibition of cAMP-specific phosphodiesterases such as PDE4. Despite these results, the detailed structural mechanism by which βarrs promote endosomal cAMP is largely unknown. Termination of endosomal cAMP signaling is dependent on the exchange at the PTHR complex of βarrs for the retromer complex composed of vesicle protein sorting (Vps) proteins 26, 29, and 35. This exchange is promoted by vATPase-mediated endosomal acidification [51]. The vATPase is activated by PKA phosphorylation, thereby establishing a negative feedback loop. PTHR activation of cAMP signaling that differs in duration and location of origin within the cell thus provides a potential mechanism for ligand-directed diversification of cellular responses. The endosomal cAMP signaling model is now considered a paradigm for peptide hormone GPCRs [52–55] with the PTHR as a prototypical example [56].

B **G protein-coupled receptor (GPCR)** structures where the ligand peptide adopts an α-helical conformation [25–27]. The third LA-PTH–PTHR–Gs state shows a release of the C-terminal portion of the ligand interacting with N-PTHR, while the position of the N-terminal portion remains unchanged (Figure 3A). Structurally, the closer proximity of D137 and E177 to LA-PTH residue D17 in state 3 may induce dissociation via negative charge repulsion (Figure 3B). This observation coupled to early kinetics studies of the PTHR activation suggest that the C-terminal portion of the peptide can repeatedly and rapidly bind and unbind from the N-PTHR before its N-terminal portion can be fully released from the receptor's TMD, thus enabling persistent activation. This extended view of the two-state model for PTHR could be relevant to other class B GPCRs to explain how prolonged signaling via Gs occurs.

Structural Mechanism of PTH Receptor Activation

The structural signature of GPCR activation is the relative separation of the cytoplasmic parts of transmembrane helices 3 and 6 (i.e., TM3 and TM6), which involves induction of a sharp kink in the middle of TM6 (Figure 4A). The outward movement of TM3 and TM6 is a prerequisite for G protein coupling and activation, as it opens the cytosolic cavity of the receptor to permit the insertion of the C-terminal α5 helix of Gα. This interhelical movement has been verified for both class A (rhodopsin-like) and class B (secretin receptor-like) GPCRs via multiple approaches, including electron paramagnetic resonance and mutagenesis [28–30]. In the case of PTHR, constraining the movements between TM3 and TM6 using engineered intertransmembrane zinc ion (Zn^{2+}) bridges blocked G protein activation in response to PTH [29,31].

Box 3. Early Structural Studies of PTH Receptor

Earlier solution NMR studies [57,58] revealed that PTH(1–34) adopts flexible peptide conformations with a short α-helix at the N-terminal side and a more extended α-helix at the C-terminal part. X-ray crystallography data shows that the peptide exists as a slightly bent helix with two twisted amphipathic regions spanning residues 6–20 and 21–33 [59]. Further studies of the C-terminal PTH comprising 15–34 amino acids in complex with the N-terminal extracellular domain of PTHR indicate that a disorder-to-order transition occurs in these peptide regions upon binding to the receptor [23,60] with the hydrophobic face of the amphipathic helix of the peptide contacting the receptor. Given that the crystal structure possibly reflects the conformational state of the peptide in a protein-like environment (the crystallization partners mimic protein surface) and that replacing Gly12 with an α-helix breaker proline resulted in ≈1000-fold receptor binding affinity decrease [61], it was hypothesized that a full α-helix might be a plausible conformational state of the receptor-bound hormone. This assumption is now fully supported by the recent structural studies showing α-helicity of the LA-PTH when bound to the PTHR in complex with Gs (see Figure 2 in main text).

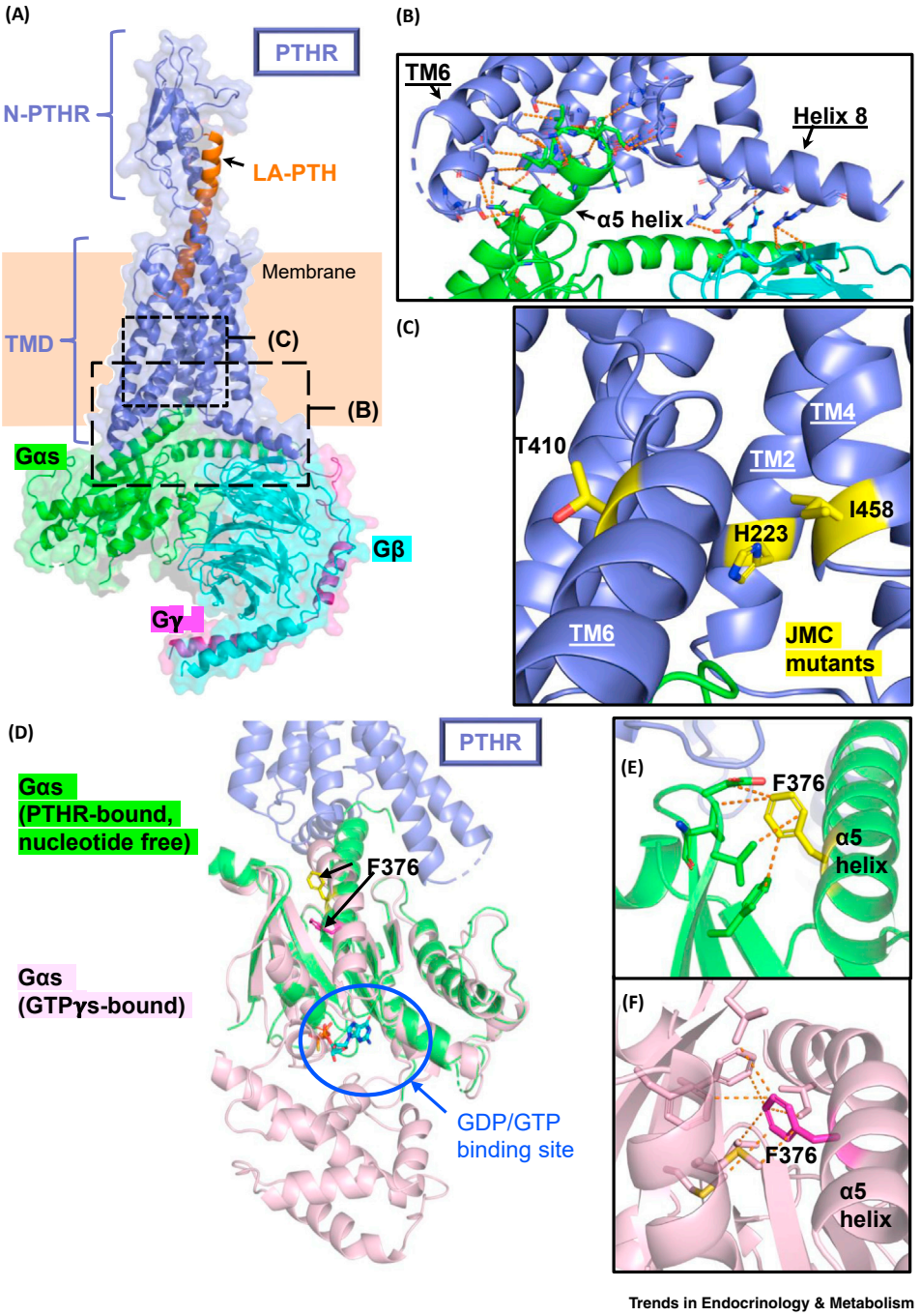

Figure 2. Cryo-Electron Microscopy Structure of Active State Parathyroid Hormone Type 1 Receptor (PTHR) Bound to Long Acting Parathyroid Hormone (LA-PTH) and in Complex with Gs Heterotrimer.
(A) Overall structure of the complex in cartoon and transparent surface representation; PTHR, LA-PTH, Gαs, Gβ, and Gγ are shown in violet, orange, green, cyan, and magenta, respectively (same color scheme in next panels). Light orange rectangle represents the putative membrane bilayer boundaries. (B) Close-up view of Gs heterotrimer–PTHR interaction interface. The interacting residues are shown as sticks and contacts are represented by orange broken lines (polar interactions ≤4 Å, hydrophobic interactions ≤5 Å). (C) Sites of PTHR mutations causing Jansen's metaphyseal chondroplasia (JMC). Wild type residues H223, T410, and I458 are

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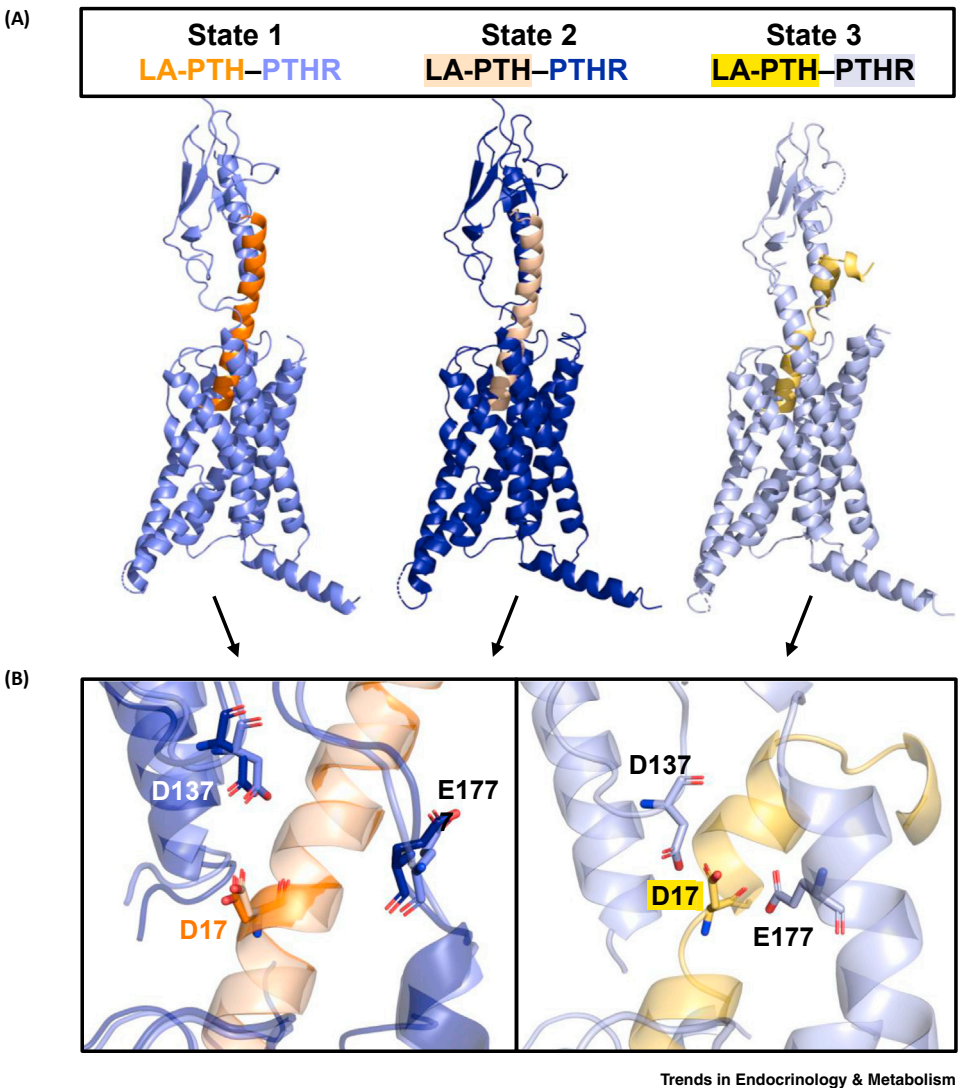

Figure 3. Structural Dynamics of Long Acting Parathyroid Hormone (LA-PTH)–Parathyroid Hormone Type 1 Receptor (PTHR) Complex.

(A) Three distinct conformational states of LA-PTH–PTHR complex obtained from cryo-electron microscopy data analysis. PTHR and LA-PTH are shown as different shades of violet and orange, respectively. The oscillation of the N-terminal domain of PTHR (N-PTHR) is evident, while the transmembrane domain (TMD) core shows higher degree of stability, likely due to tight binding of ^{N}LA-PTH. Notably, state 3 captures the event of partial dissociation of ^{C}LA-PTH from N-PTHR (yellow helix). (B) Potential mechanism of ^{C}LA-PTH dissociation, with LA-PTH D17 and PTHR D137 and E177 shown as sticks. Left, states 1 and 2. Right, state 3.

shown as yellow sticks. Single point mutation to R223, P/R410, or R/K458 causes the disease. (D) Alignment of Gαs structures. The receptor-bound state is state 1 of the LA-PTH–PTHR–Gs structure. The receptor-free state is the crystal structure of GTPγs-bound Gαs (Protein Data Bank 1AZT), colored pink. GTPγs is shown as cyan sticks. The GDP/GTP binding site of Gαs is circled blue. (E) Hydrophobic interactions between F376 (yellow) and other Gαs residues, shown as sticks, in the receptor-bound form. (F) Hydrophobic interactions between F376 (magenta) and other Gαs residues, shown as sticks, in the receptor-free form. Abbreviation: TMD, transmembrane domain.

Box 4. Kinetics of PTH Binding to the PTH Receptor

PTH (H) binding to PTHR (R) involves a two-step mechanism with an initial rapid interaction between the carboxy-terminal residues of the hormone (residues 15–34) and the amino-terminal extracellular domain of the receptor, followed by a slower interaction with the receptor's transmembrane domain comprising the seven α-helices and connecting extracellular loops [62]. Kinetics of the first step linearly depend on hormone concentrations and are defined by the bimolecular reaction $H + R \rightleftarrows HR$ and equation $k_{obs} = k_{off} + k_{on} \times [H]$, where k_{obs} is the recorded rate constant (s^{-1}). For example, at 10 μM of PTH(1–84) or PTH(1–34), $k_{obs} = 6.7 \times 10^{-3}$ s and the time constant ($\tau = \frac{1}{k}$) is 150 ms. The slower binding step involves docking the hormone's amino-terminal part (residues 1–14 for PTH) to the transmembrane domain of the receptor. Time constants follow a hyperbolic dependence on hormone concentration, indicating a complex mechanism involving not only a bimolecular interaction but also conformational changes of both hormone and receptor. At saturating concentrations of PTH or PTHrP, this second binding step is the rate limiting step for activation of the PTHR with a time constant of 1 s.

The cryo-EM structures of active PTHR–Gs complex reveal two major triggers of ligand-mediated PTHR activation: (i) interaction of peptide hormone residues 1–3 with receptor TM6, and (ii) expansion of the TMD polar network by hormone residue E4 (Figure 4B). Previous research has demonstrated that N-terminal truncations of PTH as short as two amino acids significantly reduce or eliminate biological activity of the hormone, making truncated variants potent antagonists [32,33]. Indeed, the cryo-EM structure revealed that LA-PTH residues 1–3 push against TM6 (Figure 4C), stabilizing the kink and outward movement of TM6. In a recent crystal structure of thermostabilized, inactive-like PTHR with modified PTH (ePTH), the N-terminal tip of ePTH faces TM5 (Figure 4D–F) [34]. Given that the amino acid sequence of ePTH(1–3) is structurally similar to that of LA-PTH(1–3) (Figure 4G), the different binding position of ePTH may reflect an intermediate pose prior to receptor activation. The 'inactive' receptor conformation is likely forced by protein modifications used to favor receptor crystallization: (i) thermostabilizing PTHR mutations such as, Y191C in TM1, K240M in TM2, and Q440R in TM6; and (ii) fusion of 196-residue domain of *Pyrococcus abyssi* glycogen synthase (PGS) to PTHR ICL3, which connects TM5 and TM6. The PGS likely promoted a straighter conformation of TM6 to favor crystal packing.

The LA-PTH–PTHR–Gs structure reveals that LA-PTH residue E4, a conserved residue among peptide hormone ligands of PTHR, forms two key polar interactions with Y195 and R233 (Figure 4B). These interactions lead to the formation of a more extensive hydrogen bond network within the TMD that opens the receptor's cytosolic cavity. The same polar interactions of E4 with Y195 and R233 are also observed in the structure of the thermostabilized ePTH–PTHR complex (Figure 4D,E). However, these interactions lead to the formation of a sparser polar network that fails to switch the receptor to its active state, likely due to thermostabilizing mutations and a PGS domain insert in ICL3. In the inactive structure, the positions of the peptide and the N-PTHR are both altered relative to those of the active structure (Figure 5A). However, the overall conformations of the N-PTHR are identical in the two structures (Figure 5B), indicating that the linker (residues 171–179) connecting the N-PTHR and the TMD play a key role in the flexibility of the N-PTHR.

Structural Basis for Bone/Mineral Metabolism Diseases Caused by Mutations in PTHR and Gs

Recently published structures of PTHR give insight into the molecular basis for altered PTHR signaling in bone/mineral diseases. Numerous heterozygous loss-of-function mutations have been identified in patients with primary failure of tooth eruption, consistent with a prominent role for the PTHR/PTHrP signaling system in tooth bud development [35,36], while in a compound heterozygous state, such PTHR loss-of-function alleles result in a rare neonatal-lethal condition called Blomstrand's chondrodysplasia [37]. Such loss-of-function mutations, for example, P132L, which impacts a highly conserved proline in the receptor's extracellular domain, presumably disrupt proper receptor folding and/or function.

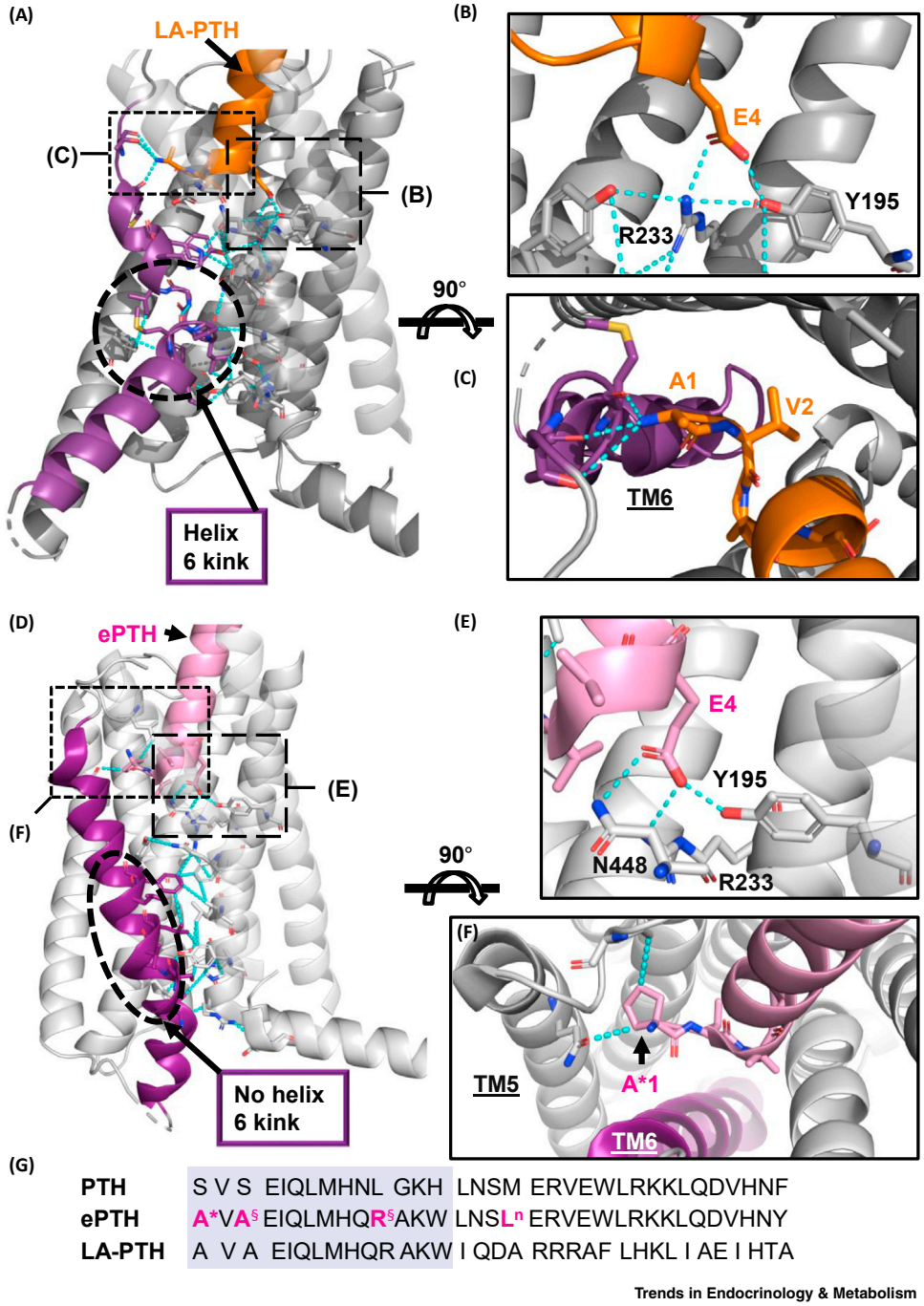

Figure 4. Structures of Parathyroid Hormone Type 1 Receptor (PTHR) in Active (A–C) and Inactive (D–F) States.

(A) Residue interaction network within the receptor transmembrane domain (TMD) core of the active structure. Interacting residues (with interaction distances ≤4 Å) are shown as sticks and contacts are represented by cyan broken lines. Long acting parathyroid hormone (LA-PTH) and TM6 of the receptor are shown in orange and violet, respectively. TM1 and TM7 are transparent cartoons for clarity. (B) Close-up view demonstrating the key activating polar interactions of E4 of LA-PTH with Y195 and R233 of the receptor. (C) Close-up top view showing

(Figure legend continued at the bottom of the next page.)

As previously discussed, the PTH-R25C mutant causes severe hypocalcemia in patients and was recently found to eliminate Ca^{2+} allosteric effects on PTHR signaling. Ligand residue 25 in the LA-PTH–PTHR active structures and in the ePTH–PTHR inactive structure, faces the flexible linker connecting the N-PTHR and the TMD (Figure 5C,D). The ePTH R25 side chain forms a polar interaction with the main chain carbonyl of PTHR L174 (Figure 5D). Based on the active PTHR structures, substitution of LA-PTH H25 to R25 would not permit interaction with L174, and no other interactions are visible. Mass-spectrometry analysis determined that acidic residues in PTHR ECL1 coordinate Ca^{2+}. Although the ECL1 did not display clear electron density in near-atomic structures of PTHR, ECL1 is long and flexible and could reach R25. In our current model, PTH R25 interacts with glutamate residues in the ECL1 to promote coordination of Ca^{2+} (Figure 5E).

Jansen's metaphyseal chondrodysplasia, characterized by short-limbed dwarfism and hypercalcemia, is caused by constitutively active PTHR mutants: H223R, T410P or R, or I458R or K located on TM2, TM6, and TM7, respectively [38–40] (Figure 2C). Based on the active state PTHR structure, the H223R and I458R mutations would generate clashes with residues in TM6. We propose these clashes would promote the opening of the receptor cytosolic core in the absence of agonist. Similarly, we anticipate that the T410P mutation triggers a kink in TM6 that is independent from the agonist-induced polar interactions (Figures 2C and 4A).

Lastly, a single point mutation in Gαs, F376V, was recently identified in two young patients presenting with skeletal abnormalities and gonadotrophin-independent precocious puberty [41]. This mutation increases PTHR-promoted signaling independent of PTHR ligands. In the crystal structure of GTPγs-bound Gαs, F376 engages in extensive contacts with nonpolar residues in α1, α5 helices, and β1, β2, β3 strands (Figure 2D,F). In the structure of the PTHR–Gs complex, the α5 helix has straightened out and rotated to engage with the cytosolic core of the receptor. Now, F376 engages in a sparser network of hydrophobic contacts with residues in β2 and β3 strands (Figure 2E). Mutation from bulky nonpolar phenylalanine to smaller nonpolar valine would disrupt these interactions in both Gαs states and is anticipated to also indirectly disrupt the GDP/GTP binding site, as supported by previous modeling studies [41]. Disruption of the Gαs catalytic site could enhance GDP/GTP exchange independent of ligand-mediated PTHR activation, although this remains to be tested experimentally.

Toward a Holistic Rather Than Reductionist View of PTH Receptor Signaling

A substantial amount of structural, molecular, and cellular information is now available regarding the role of the PTHR as an individual GPCR and its unique contributions to signal transduction and cell processes. These findings, while important on their own, have led to a reductionist view of how PTHR controls cell signaling, predominantly via a linear signaling cascade that propagates the information from the cell surface receptor to the nucleus. New findings, however, point to a far more complex and holistic view of receptor signaling networks that are tightly integrated in cells in time and space and are required for coordinated transmission of multiple extracellular signals. For example,

the position and interaction of the N-terminal tip of LA-PTH with receptor TM6. (D) Amino acid interaction network within the receptor TMD core of the inactive structure (pdb 6fj3). Interacting residues (with interaction distances ≤4 Å) are shown as sticks and contacts are represented by cyan broken lines. LA-PTH and TM6 of the receptor are shown in pink and violet, respectively. TM1 and TM7 are transparent cartoons for clarity. (E) Close-up view demonstrating the key activating polar interactions of E4 of ePTH with Y195 and R233 of the receptor. The failure to switch the receptor to its active state is likely due to multiple thermostabilizing mutations in the receptor. E4 makes an additional contact with N448 in the ePTH–PTHR structure. (F) Close-up top view showing the position and interaction of the N-terminal tip of ePTH with the TM5 of the receptor. (G) Sequence alignment of PTH, ePTH, and LA-PTH. Residues interacting with the receptor TMD are highlighted in lilac. Unnatural amino acids of ePTH: A*, aminocyclopentane-1-carboxylic acid; $A^{§}$, α-aminoisobutyric acid; $R^{§}$, homoarginine; L^{n}, norleucine.

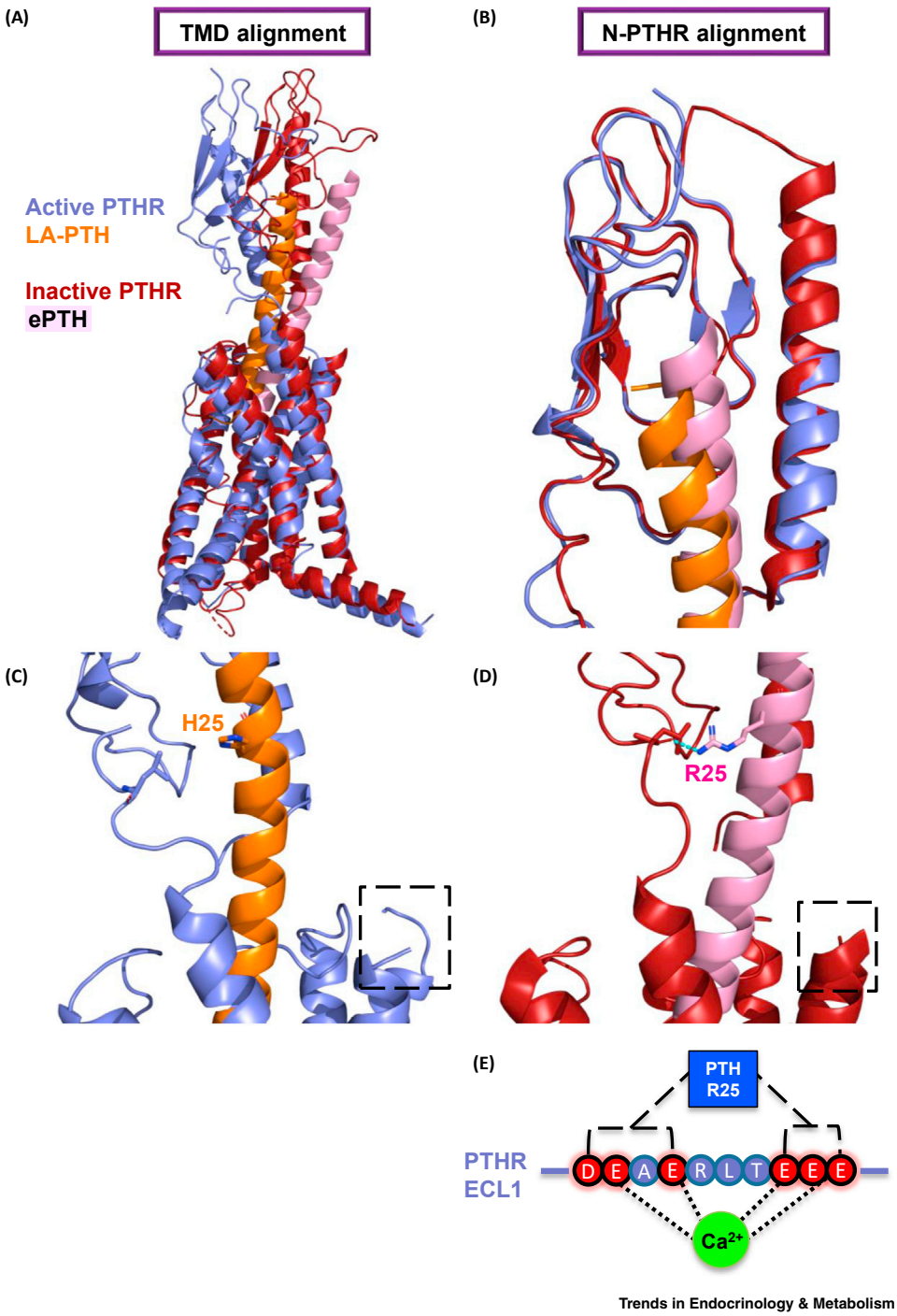

Figure 5. Overlay of Inactive and Active Parathyroid Hormone Type 1 Receptor (PTHR) Structures (A,B) and Structural Basis of Parathyroid Hormone (PTH)-R25 Contribution to Ca^{2+} Allostery (C–E).

The receptor and modified PTH (ePTH) of the inactive structure are colored dark red and pink, respectively. The receptor and long acting parathyroid hormone (LA-PTH) of the active structure are colored violet and orange, respectively. (A) Alignment of the two structures by transmembrane domain (TMD) residues 180–460. (B) Structural alignment by the N-terminal domain of PTHR (N-PTHR) residues (27–168), showcasing similar N-PTHR

(Figure legend continued at the bottom of the next page.)

β_2-adrenergic receptor (β_2AR)-deficient mice show no osteoanabolic activity after intermittent injection of PTH [42]. Thus, these studies point to an indispensable role of β_2AR signaling for the osteoanabolic activity of PTHR, at least in mice, and imply that PTH signaling is regulated by β_2AR. Additional proteomics and chemical biology studies confirmed this implication by revealing that the β_2AR, which induces a short cAMP response following agonist binding, prolongs PTH-mediated nuclear PKA and cAMP-responsive element binding protein (CREB) activation by promoting endosomal PTHR receptor signaling through the stimulatory action of G$\beta\gamma$ subunits on the adenylate cyclase type 2. These studies also unveil a new paradigm for PTHR signaling that might be extended to other GPCRs, wherein cAMP diffusion into the nucleus, and not PKA diffusion as currently thought [43,44], represents the rate-limiting step for nuclear PKA activity regulating the transcriptional activity mediated by CREB (Figure 1B) [8]. Additional functional interactions between PTHR and GPCRs, such as the frizzled and the Ca^{2+}-sensing receptors, have been reported as determinants for skeletal development and osteoanabolism [45,46]. These findings suggest that the development of a rational fine-tuned therapeutic approach for treating osteoporosis might require a comprehensive understanding of osteoblast activity resulting from the integration of signaling pathways initiated by the PTHR and multiple other GPCRs.

Outstanding Questions

What are the interacting residues between PTHR and its native ligands, PTH and PTHrP?

Do structural interfaces between PTHR and G protein differ for Gs and Gq?

What are the structures of the Ca^{2+} and ATP binding sites in PTHR?

Are there other positive or negative allosteric modulators of PTHR signaling?

Can a probabilistic network model of GPCR interactive pathways be developed to identify the signaling network that best promotes PTH-dependent bone growth?

Concluding Remarks and Future Perspectives

The new near-atomic structures of PTHR captured snapshots of the receptor bound to the peptide hormone and the nucleotide-free receptor–Gs. However, many questions still remain to be answered by the future studies (see Outstanding Questions). The new structures will serve as a basis for additional structural experiments to investigate the interactions of other G proteins (e.g., Gq) and βarrs with PTHR. Also, the structures can be used to prepare PTHR models for molecular dynamics simulations, which can reveal interactions between receptor and membrane as well as dynamics of N-PTHR and TMD in ligand-free and ligand-bound states. Computational studies will facilitate development of new minimized PTH analogs toward the treatment for a number of medical conditions such as osteoporosis and hypocalcemia. In addition, druggability screens can define the key sites of pharmacophore action and identify potential small molecule drugs that bind to PTHR in the absence or presence of native peptide. Such small molecules may exhibit improved delivery to the receptor over peptide analogs.

However, developing potent small molecules and peptidomimetics that target only the PTHR might not offer the most effective strategy for achieving optimal therapeutic efficacy.

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Supplemental Information

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conformations between the two structures. (C) Active PTHR state 1, with LA-PTH H25 and PTHR L174 shown as sticks. The receptor and LA-PTH of the active structure are colored violet and orange, respectively. Extracellular loop 1 (ECL1) start/end points indicated by a broken box. (D) Inactive PTHR structure, with ePTH R25 and PTHR L174 shown as sticks. The receptor and ePTH of the inactive structure are colored dark red and pink, respectively. ECL1 location indicated by a broken box. (E) Schematic diagram of Ca^{2+} coordination by PTH–PTHR. PTH R25 interactions with two acidic clusters in ECL1, permitting these clusters to coordinate Ca^{2+}.

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