Biased GPCR signaling by the native parathyroid hormone–related protein 1 to 141 relative to its N-terminal fragment 1 to 36

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The parathyroid hormone (PTH)–related protein (PTHrP) is indispensable for the development of mammary glands, placental calcium ion transport, tooth eruption, bone formation and bone remodeling, and causes hypercalcemia in patients with malignancy. Although mature forms of PTHrP in the body consist of splice variants of 139, 141, and 173 amino acids, our current understanding on how endogenous PTHrP transduces signals through its cognate G-protein coupled receptor (GPCR), the PTH type 1 receptor (PTHR), is largely derived from studies done with its N-terminal fragment, PTHrP1–36. Here, we demonstrate using various fluorescence imaging approaches at the single cell level to measure kinetics of (i) receptor activation, (ii) receptor signaling via Gi and Gq, and (iii) receptor internalization and recycling that the native PTHrP1–141 displays biased agonist signaling properties that are not mimicked by PTHrP1–36. Although PTHrP1–36 induces transient cAMP production, acute intracellular Ca2+ (iCa2+) release and β-arrestin recruitment mediated by ligand–PTHR interactions at the plasma membrane, PTHrP1–141 triggers sustained cAMP signaling from the plasma membrane and fails to stimulate iCa2+ release and recruit β-arrestin. Furthermore, we show that the molecular basis for biased signaling differences between PTHrP1–36 and properties of native PTHrP1–141 are caused by the stabilization of a singular PTHR conformation and PTHrP1–141 sensitivity to heparin, a sulfated glycosaminoglycan. Taken together, our results contribute to a better understanding of the biased signaling process of a native protein hormone acting in conjunction with a GPCR.

Upon its activation, the parathyroid hormone (PTH) receptor (PTHR) triggers both Gs/cAMP/PKA and Gq/Ca2+/PKC signaling cascades. Developments in recording GPCR-signaling cascade in individual cells in real time using optical approaches during the decade of the ‘00s (1, 2) have revealed that PTH1–34 and PTHrP1–36 differ markedly by the duration and cellular localization of the cAMP response (3). Brief stimulation with PTHrP1–36 induces only transient cAMP production from the cell surface that is rapidly desensitized upon recruitment of β-arrestins (βarrs), cytosolic adapter proteins that canonically act to occlude further G protein coupling and promote translocation of the ligand–receptor complex from the cell surface to early endosomes. In contrast, PTH1–34 causes an additional sustained phase of cAMP generation via PTH–PTHR–βarr complexes that remain active in early endosomes. Thus, this distinction in the spatiotemporal cAMP profiles of PTH and PTHrP was proposed to be the underlying determinant responsible for their biological specificity.

Mature forms of PTH and PTHrP are originally synthesized and secreted as 84 aa and 141 aa proteins, respectively. Early reports demonstrating that their respective N-terminal part, PTH1–34 and PTHrP1–36, retain their full capacity to stimulate adenylyl cyclase in cAMP accumulation assays led to the utilization of these N-terminal fragments in most studies. Indeed, it was PTH1–34 and PTHrP1–36 that were used in the aforementioned work that revealed differences in the time courses and subcellular locations of cAMP production by these two peptides. In contrast to these earlier findings of transient signaling by PTHrP1–36, a recent publication proposed sustained endosomal cAMP generation induced by full-length PTHrP1–141 (4). The authors employed a combination of radioimmunoassays and chemical inhibitors to suggest that PTHrP1–141 induces prolonged cAMP signaling in an endocytosis-dependent manner analogous to that observed for PTH1–34; however, cAMP experiments were performed in the presence of phosphodiesterase inhibition, which provided a measure of the cumulative levels of cAMP produced during a defined time interval, as opposed to the dynamic levels of cAMP that result from the net effects of its production and breakdown. Furthermore, the chemical compounds utilized to inhibit endocytosis generated inconsistent results with experiments.
Figure 1. Signaling properties of PTHrP1-141. A, time courses of cAMP in single HEK293 cells stimulated for 30 s with 1 nM ligands. Data are the mean ± SEM of \( n = 37 \) (PTHrP1-36), \( n = 21 \) (PTHrP1-141), \( n = 6 \) (PTH1-84), and \( n = 7 \) (PTH1-34) cells. B, time courses of cAMP in HEK-293 cells after washout of ligands measured by Glo-sensor assay. Data are the mean ± SEM of \( n = 3 \) experiments. C and D, relationship between cAMP responses in HEK-293 cells after washout of a range of ligand concentrations (C) and corresponding EC50 values (D). Data represent the integrated response determined by measuring the area under the curve of experiments shown in panel (B) and are the mean ± SD of \( n = 3 \) experiments. ns, not significant with \( p = 0.097 \) by t test. E, intracellular Ca\(^{2+}\) mobilization measurements in single HEK-293 cells stably expressing PTHR. Data are the mean ± SEM for \( n = 44 \) (PTH1-34), \( n = 42 \) (PTHrP1-36), and \( n = 48 \) (PTHrP1-141) cells. F, scatter plots with the mean ± SD of data shown in panel (C). **** \( p < 0.0001 \) determined by one-way ANOVA with Tukey–Kramer post hoc test. (G and H) competition binding at equilibrium with [\(^{125}\)I]PTH1-15 and [\(^{125}\)I]PTH1-34 as radioligands to detect the R\(_{G}\) (E) and R\(_{0}\) (F) states of PTHR, respectively. Data are mean ± SD from \( N = 2 \) independent experiments with duplicate wells for each concentration. I and J, kinetics of PTHR activation. Normalized activation kinetics of PTHR determined by FRET ratio changes from HEK293 cells expressing the receptor sensor (scheme) (G), and time constant (\( \tau \)) of PTHR activation determined by fitting curves in panel (A) to a monoexponential decrease (H). Mean ± SEM of \( n = 25 \) (PTH1-34), \( n = 6 \) (PTHrP1-136), and \( n = 9 \) (PTHrP1-141) cells. * \( p = 0.0114 \), **** \( p < 0.0001 \) determined by one-way ANOVA with Tukey–Kramer post hoc test.

PTH, parathyroid hormone; PTHR, PTH receptor.
showing no reduction of sustained cAMP responses induced by PTHrP₁-141 or PTH₁-34, while others showed only reduction for PTHrP₁-141 but not for PTH₁-34. Reduction of PTH₁-34-induced sustained cAMP response by blocking receptor endocytosis is expected given this ligand’s established ability to signal via internalized PTHR from early endosomes (3, 5–9). These considerations motivated the necessity to implement alternative methods that permit analysis of real-time cAMP response kinetics in single cells. The results unveil the mechanism by which PTHrP₁-141 engages in sustained signaling and how this differs from the transient effects observed with the N-terminal fragment PTHrP₁-36.

Results and discussion

We utilized FRET to record real-time courses of cAMP production in single HEK293 cell stably expressing PTHR (HEK-PTHR). We found that brief stimulation with PTHrP₁-141 induced a sustained cAMP response that was similar in both magnitude and duration to that induced by PTH₁-84 or PTH₁-34 and clearly distinct from the short-lived cAMP response mediated by PTHrP₁-36 (Figs. 1A and S1). Next we applied Glo-sensor cAMP accumulation assays to verify that time courses of sustained cAMP production mediated by the two native hormones, PTH₁-84 and PTHrP₁-141, were similar (Fig. 1B) and without a significant difference in the hormone concentration.
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dependence (Fig. 1, C and D). We observed a striking inability of PTHrP<sub>1-141</sub> to efficiently induce the release of intracellular calcium ($iCa^{2+}$) from the endoplasmic reticulum (Fig. 1, E and F), indicating defective Gq activation by PTHrP<sub>1-141</sub>. We have previously shown that Gq activation is required for endosomal cAMP generation by PTH<sub>1-34</sub> (9), suggesting a differential location of cAMP generation by this ligand. Moreover, the molecular basis for the failure of PTHrP<sub>1-141</sub> to mimic cAMP and $iCa^{2+}$ signaling responses mediated by PTHrP<sub>1-36</sub> were unlikely to be caused by different binding affinities to either G protein coupled (R<sub>C</sub>) or uncoupled (R<sub>0</sub>) states of PTHR (Fig. 1, G and H) but were rather due to the stabilization of a distinct receptor conformation.

We tested this theory by using cells expressing a FRET-based PTHR sensor (scheme in Fig. 1I). Time-resolved determination of intramolecular FRET changes recorded from single cells allows the analysis of the kinetics of receptor activation in response to ligand binding (1). A decrease in FRET mediated by an agonist reflects receptor switching from an inactive to an active conformation, and distinct time-constants of receptor activation measured for a saturating concentration of agonists indicate the stabilization of distinct signaling receptor conformations (1, 3, 10). As expected, perfusion of a saturating concentration of PTH<sub>1-34</sub> or PTHrP<sub>1-36</sub> to individual cells triggered a decrease in FRET; however, the significantly distinct time constants ($\tau$) for receptor activation indicated the stabilization of distinct PTHR conformations (Fig. 1, I and J).

To assess the role of $\beta$arraf receptor switching via FRET in cells transiently expressing PTHR<sup>CFP</sup> and $\beta$arraf-2<sup>YFP</sup>. The $\beta$arr2 isoform was randomly selected, given that earlier studies demonstrated that PTH<sub>1-34</sub> and PTHrP<sub>1-36</sub> displayed equal potencies (EC<sub>50</sub> values) for recruitment of both $\beta$arr1 and $\beta$arr2 (8, 11, 12). Consistent with previous studies, addition of PTH<sub>1-34</sub> resulted in significant association of $\beta$arr with the receptor that was stably maintained following ligand washout (Fig. 2A). In contrast, analogous experiments using PTHrP<sub>1-141</sub> failed to promote this interaction (Fig. 2A), suggesting that the sustained signaling observed for PTHrP<sub>1-141</sub> occurs in a $\beta$arraf-independent manner. This finding led us to test the role of receptor internalization, a key step in PTHR endosomal signaling. Measurements of receptor internalization and recycling in single cells stably expressing PTHR<sup>SEP</sup>, the PTHR N-terminally tagged with a pH-sensitive GFP (super-elliptic pHluorin SEP) that exhibits fluorescence intensity reduction in the acidic environment encountered in endosomes (scheme in Fig. 2B), showed reduced internalization and faster recycling in response to PTHrP<sub>1-141</sub> or PTHrP<sub>1-36</sub> when compared to PTH<sub>1-34</sub> (Figs. 2B and S2).

We next determined whether internalized PTHrP<sub>1-141</sub>-PTHR can signal via cAMP. We have previously shown that expression of a dominant-negative dynamin mutant, DynK44A, effectively blocks translocation of PTH–PTHR complexes from the cell surface and blunts the sustained phase of cAMP generation without affecting the forskolin response (3, 8). Accordingly, we compared the cAMP response following brief stimulation with PTHrP<sub>1-141</sub> in HEK-PTHR control cells and those transiently expressing DynK44A fused to a red fluorescent protein (DynK44A<sup>REP</sup>) (Fig. 2C). Strikingly, blockade of receptor internalization significantly reduced the magnitude and duration of cAMP production by PTH<sub>1-34</sub> (Fig. 2, C and E) but had no effect on cAMP mediated by PTHrP<sub>1-141</sub> (Fig. 2, D and E), indicating that native PTHrP does not promote sustained signaling in an endocytosis-dependent manner. We recently reported on the development of G<sub>S</sub>-biased PTH analogs that

![Figure 3](image-url)
stimulate sustained cAMP production exclusively from the cell surface due to retention of active ligand–receptor complexes at the cell surface. This was experimentally confirmed via cAMP time courses using a cell-impermeable PTHR antagonist, which completely abolished the sustained phase of cAMP generation for Gs-biased peptides but not for PTH1-34, consistent with its ability to signal from intracellular compartments (8). We thus utilized this same approach to test whether PTHR1-141 likewise induces prolonged cAMP signaling via ligand–receptor complexes that are localized to the cell surface. Indeed, addition of the cell-impermeable antagonist at 15 min following agonist washout rapidly reduced cAMP levels to baseline in cells treated with PTH1-34 but had no effect in those stimulated with PTH1-141 (Fig. 2, F and G). These findings demonstrate that PTHR1-141 promotes sustained cAMP responses via active ligand–receptor complexes localized to the cell surface, which appear inconsistent with experiments showing receptor internalization.

To reconcile this apparent incompatibility, we hypothesized that the highly positively charged domain of PTHR1-141 (88KKKKGGPKQRKKKR108), not present in PTHR1-36 or PTH, permits the hormone to attach to the cell surface via interactions with polyanionic glycosaminoglycans (GAGs) present on membrane glycoproteins such as heparan sulfate proteoglycan. Consistent with this theory was the significant reduction in the magnitude and duration of cAMP production in response to PTH1-141 in the presence of soluble heparin used as a decoy to prevent potential PTHR1-141 and GAGs interactions (Fig. 3, A and B). The selective effect of heparin was verified by its lack of inhibitory action on cAMP induced by either PTH1-34 or PTHR1-36 (Fig. 3A, and Table 1).

Collectively, these data prompt a reinterpretation of our previous understanding on how hormones act on the PTHR by providing compelling evidence that native PTHR1-141 is biased toward sustained PTHR signaling via cAMP at the plasma membrane. The results support a model where PTHR1-141 stabilizes an active receptor conformation that impairs βarr coupling and Gq signaling possibly through the interaction with GAG. Future experiments are needed for an extended characterization of PTHrP and GAG interaction as a possible means to reevaluate recycled receptor by the cell surface–anchored hormone (Fig. 3C).

### Experimental procedures

Materials and methods are detailed in SI Appendix.
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