Altered Selectivity of Parathyroid Hormone (PTH) and PTH-Related Protein (PTHrP) for Distinct Conformations of the PTH/PTHrP Receptor

Thomas Dean, Jean-Pierre Vilardaga, John T. Potts, Jr., and Thomas J. Gardella

PTH and PTHrP use the same G protein-coupled receptor, the PTH/PTHrP receptor (PTHR), to mediate their distinct biological actions. The extent to which the mechanisms by which the two ligands bind to the PTHR differ is unclear. We examined this question using several pharmacological and biophysical approaches. Kinetic dissociation and equilibrium binding assays revealed that the binding of [125I]PTHR(1–36) to the PTHR was more sensitive to GTPγS (added to functionally uncouple PTHR-G protein complexes) than was the binding of [125I]PTH(1–34) (~75% maximal inhibition vs. ~20%). Fluorescence resonance energy transfer-based kinetic analyses revealed that PTHrP(1–36) bound to the PTHR more slowly and dissociated from it more rapidly than did PTH(1–34). The cAMP signaling response capacity of PTHrP(1–36) in cells decayed more rapidly than did that of PTH(1–34) (t1/2 ~ 1 vs. ~2 h). Divergent residue 5 in the ligand, Ile in PTH and His in PTHrP, was identified as a key determinant of the altered receptor-interaction responses exhibited by the two peptides. We conclude that whereas PTH and PTHrP bind similarly to the G protein-coupled PTHR conformation (RG), PTH has a greater capacity to bind to the G protein-uncoupled conformation (R0) and, hence, can produce cumulatively greater signaling responses (via R0–RG isomerization) than can PTHrP. Such conformational selectivity may relate to the distinct modes by which PTH and PTHrP act biologically, endocrine vs. paracrine, and may help explain reported differences in the effects that the ligands have on calcium and bone metabolism when administered to humans. (Molecular Endocrinology 22: 156–166, 2008)

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Abbreviations: Alb, α-Aminoisobutyric acid; CFP, cyanfluorescent protein; FRET, fluorescent resonance energy transfer; Gαi2ND, negative dominant derivative of the stimulatory G protein α-subunit; IBMX, 3-isobutyl-1-methylxanthine; IP, inositol phosphate; PTH, parathyroid hormone; PTHrP, PTH/PTHrP receptor; YFP, yellow-fluorescent protein.

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different mechanisms of action of the two ligands at the PTHR.

Here we explored more closely the mechanisms by which PTH and PTHrP interact with the PTHR. We focused particularly on the capacity of the two ligands to bind to different PTHR conformations. Of interest were two high-affinity conformations of the PTHR that can be distinguished based on their differing sensitivities to the nonhydrolyzable GTP analog GTPγS that can be observed in radioligand binding assays (7, 15, 16). One conformation, termed RG, is sensitive to GTPγS and is thus presumably coupled to a heterotrimeric G protein; the other, termed R0 (17), is insensitive to GTPγS and is thus functionally uncoupled from G protein (16). The following schema describes the working hypothesis regarding the PTH-PTHrP binding mechanism and associated changes in receptor conformation that underlies our work: L + R ⇄ LR0 ⇄ LR0G, where L is ligand, R is receptor, LR0 is a low-affinity complex involving ligand interactions only to the PTHR N domain, LR0J is a high-affinity complex involving ligand interactions to both the N and J domains of the receptor, and LRRNJ is a high-affinity complex that is coupled to a heterotrimeric G protein (7, 16). One prediction of this schema is that different PTHR ligands could have different capacities to stabilize the R0 conformation, due to differences in their mode of interaction with the N and/or J domains of the receptor. We further hypothesize that R0 is an intermediary between the classical R and RG states of the two-state or ternary complex models of GPCR action (18, 19) and, as such, is a preactive receptor state that is primed to interact efficiently with a cognate heterotrimeric G protein. A prediction of this hypothesis is that the affinity with which a ligand binds to R0 will be a determinant, in part, of the overall signaling response capacity of that ligand.

We explored these hypotheses specifically for PTH(1–34) and PTHrP(1–36) ligands. The results suggest the two ligands indeed differ in their capacities to stabilize R0, in that PTHrP(1–36) binds more weakly to this conformation than does PTH(1–34). We show that this difference in conformational selectivity can result in different biological outcomes in cells, in terms of the cumulative signaling response produced, and is determined strongly by the amino acid divergence at position 5 in the ligands.

RESULTS

We first performed kinetic dissociation experiments to examine the stability of complexes formed between PTH and PTHrP radioligand analogs and the human PTHR stably expressed in membranes prepared from HKRK-B7 cells. For each radioligand, dissociation was examined in the presence and absence of GTPγS, so as to assess the effects of functionally uncoupling the receptor from heterotrimeric G proteins (Fig. 1). For 125I-PTH(1–34) (Fig. 1A), the dissociation data, both in the absence and presence of GTPγS, were better fit by a two-phase decay equation than by a single-phase equation. In the absence of GTPγS (solid symbols), 17% of the complexes were unstable and decayed rapidly (t1/2 < 1 min), whereas the remaining 83% were stable and decayed much more slowly (t1/2 ~ 4 h). Upon addition of GTPγS (open symbols), the fraction of unstable complexes increased to 21%, such that

![Fig. 1. Dissociation of PTH and PTHrP Ligands from the PTH Receptor and Effects of GTPγS](https://academic.oup.com/mend/article-abstract/22/1/156/2684081/125I-PTH(1-34)125I-PTHrP(1-36)125I-Ile5-PTHrP(1-36))

A. 125I-PTH(1–34)

B. 125I-PTHrP(1–36)

C. 125I-Ile5-PTHrP(1–36)

For each tracer radioligand, the respective values (means ± SEM) of total radioactivity (counts per minute) added, total radioactivity at t = 0, and nonspecifically bound radioactivity (averaged over the time course of each experiment) were 26,754 ± 2,652, 12,964 ± 2,476, and 522 ± 42 for 125I-PTH(1–34) (n = 4); 31,597 ± 1,679, 5,959 ± 492, and 262 ± 17 for 125I-PTHrP(1–36) (n = 5); and 51,335 ± 10,516, 22,904 ± 5,365, and 1,332 ± 337 for 125I-Ile5-PTHrP(1–36) (n = 3), respectively. Curves were fit to the data using either a two-phase (A and B) or a single-phase (C) exponential decay equation.
79% remained stable ($t_{1/2} \approx 2$ h). These findings agree closely with previous dissociation studies performed on this radioligand, and highlight the capacity of PTH(1–34) to bind to a high affinity PTHR conformation ($R^2$) that is functionally uncoupled from heterotrimeric G proteins by GTPY-S (15, 16).

Biphase kinetics were also observed for $^{[125]}$IPTHrP(1–36); however, where the complexes were again mostly stable in the absence of GTPY-S (68% decayed with a $t_{1/2}$ of $\sim 3$ h), most became unstable upon addition of GTPY-S (72% decayed with a $t_{1/2}$ of $\sim 1$ min; Fig. 1B). This strong, destabilizing effect that GTPY-S had on the binding of $^{[125]}$IPTHrP(1–36) to the PTHR mirrors that observed previously for $^{[125]}$[Nle8,21,Tyr34]PTH(1–34) and $^{[125]}$Ile5-PTHrP(1–36) to the human PTHR in HKRK-B7 cell membranes was largely unaffected by GTPY-S ($\sim 20\%$ inhibition at $1 \times 10^{-4}$ M GTPY-S), whereas the binding of $^{[125]}$IPTHrP(1–36) was strongly inhibited by GTPY-S ($\sim 70\%$ inhibition at $1 \times 10^{-7}$ M GTPY-S; $IC_{50} = 1 \times 10^{-9}$ M).

We further assessed the effects of GTPY-S on the binding of the above radioligands to the PTHR under approximate equilibrium conditions. The radioligands were thus incubated with cell membranes for 90 min in the absence or presence of varying concentrations of GTPY-S. Figure 2A shows that the binding of $^{[125]}$IPTH(1–34) and $^{[125]}$Ille5-PTHrP(1–36) to the human PTHR in HKRK-B7 cell membranes was largely unaffected by GTPY-S ($\sim 20\%$ inhibition at $1 \times 10^{-4}$ M GTPY-S), whereas the binding of $^{[125]}$IPTHrP(1–36) was strongly inhibited by GTPY-S ($\sim 70\%$ inhibition at $1 \times 10^{-7}$ M GTPY-S; $IC_{50} = 1 \times 10^{-9}$ M).

We also assessed binding to the rat PTHR using membranes prepared from the rat osteoblastic cell line ROS17/2.8 (endogenous PTHR expression). As seen with the human PTHR (Fig. 2A), the binding of $^{[125]}$Ille5-PTHrP(1–36) to the rat PTHR was again largely insensitive to GTPY-S (Fig. 2B). The binding of $^{[125]}$IPTH(1–34) to the rat PTHR appeared more sensitive to GTPY-S than was its binding to the human PTHR (Fig. 2, A vs. B), although the majority of binding was still resistant

**Fig. 2. GTPY-S Sensitivity of PTH and PTHrP Ligand Binding to the Human and Rat PTHRs**

Radioligand binding to the PTHR in membranes prepared from HKRK-B7 (A) or ROS 17/2.8 cells (B) was assessed under approximate equilibrium conditions in the presence of varying concentrations of GTPY-S. Data are expressed as a percentage of the radioactivity specifically bound (SB) in the absence of GTPY-S. The radioligands used were $^{[125]}$[Nle8,21,Tyr34]PTH(1–34)NH2, $^{[125]}$[Nle8,21,Tyr34]PTH(1–34)NH2, $^{[125]}$[Nle8,21,Tyr34]PTH(1–34)NH2, and $^{[125]}$[Nle8,21,Tyr34]PTH(1–34)NH2. Nonspecific binding was determined in wells containing the homologous unlabeled PTH or PTHrP ligand (5 × $10^{-7}$ M). In A, the values of total radioactivity (counts per minute) added, total radioactivity bound, and nonspecifically bound radioactivity were 21,105 ± 1,607, 2,806 ± 296, and 1,163 ± 29 for $^{[125]}$PTTH(1–34) (n = 3); 27,489 ± 3,507, 3,164 ± 570, and 489 ± 85 for $^{[125]}$PTTH(1–36) (n = 5); and 34,001 ± 7,111, 9,601 ± 959, and 569 ± 113 for $^{[125]}$Ille5-PTTHrP(1–36) (n = 5), respectively. In B, the corresponding values were 21,408 ± 1,245, 3,900 ± 170, and 1,214 ± 184 for $^{[125]}$PTTH(1–34) (n = 6); 20,373 ± 951, 2,079 ± 211, and 467 ± 34 for $^{[125]}$PTTHrP(1–36) (n = 6); and 23,553 ± 891, 6,570 ± 1,142, and 1,040 ± 116 for $^{[125]}$Ille5-PTTHrP(1–36) (n = 6); and 30,986 ± 4,567, 847 ± 137, and 237 ± 27 for $^{[125]}$[Nle8,21,Tyr34]PTH(1–15) (n = 6), respectively. Data are means ($\pm$ SEM) from the number of experiments indicated by n, each performed in duplicate. Note that we have shown previously that GTPY-S inhibits the binding of $^{[125]}$[Nle8,21,Tyr34]PTH(1–15) analog to HKRK-B7 cell membranes by 88% and with an $IC_{50}$ of 2.9 nm (16).
to GTPγS. As with the human PTHR, the binding of [125I][Alb1,3,M]PTH(1-36) to the rat PTHR was strongly inhibited by GTPγS and was as sensitive to GTPγS as was the binding of [125I][Alb1,3,M]PTH(1-15) (Fig. 2B). These equilibrium binding data thus further suggest that although there are clearly differences among the different species of receptor, PTH(1-34) and Ile5-PTHrP(1-36) bind more strongly to the G protein-uncoupled conformation of the PTHR, R0, than do PTHrP(1-36) or [Alb1,3,M]PTH(1-15) and that the latter two peptides bind preferentially to the G protein-coupled conformation, RG.

**Competition Analysis of R0 and RG Binding Affinity**

We then used competition methods to analyze the relative affinities with which PTH and PTHrP ligands bind to the RG and R0 conformations of the PTHR. To assess binding to RG, we used [125I][Alb1,3,M]PTH(1-15) as a tracer radioligand (binds predominantly to RG, see above) and membranes prepared from COS-7 cells cotransfected with the hPTHR and a negatively dominant Goα5 subunit (Goα5ND), which enriches for RG, vs. R or R0, receptor conformations (16, 23, 24). To assess binding to R0, we used [125I][PTH(1-34)] as a radioligand (binds predominantly to R0) and membranes prepared from COS-7 cells transfected with the hPTHR alone; we also added GTPγS (1 x 10^-5 m) to the binding reactions so as to functionally uncouple receptor-heterotrimeric G protein complexes and thus enrich for the R0 (and R) conformations vs. RG. Comparison of the apparent affinities with which an unlabeled PTH or PTHrP ligand bound to the PTHR in these two assay formats would thus provide an assessment of the selectivity with which that ligand bound to the RG vs. R0 conformation.

Figure 3A shows that PTH(1-34) bound to the R0 conformation with a 4-fold weaker affinity than it did to the RG conformation (IC50 = 4.0 vs. 0.91 nM; P = 0.001; see R0:RG ratio of Table 1). PTHrP(1-36) bound to R0 with a 66-fold weaker affinity than it did to RG (28 vs. 0.42 nM; P = 0.04; Fig. 3B). PTHrP(1-36) was thus approximately 16-fold more selective for the RG conformation vs. R0 than was PTH(1-34). Reciprocal exchange of residue 5 in these ligands reversed the pattern of conformational selectivity. Thus, His5-PTH(1-34) bound to R0 with a 670-fold weaker affinity than it did to RG (P = 0.01), whereas Ile5-PTHrP(1-36) bound to R0 with only 3-fold weaker affinity than it did to RG (P = 0.0004; Fig. 3, C and D, and Table 1).

The His5-PTH(1-34) analog was therefore approximately 220-fold more selective for the RG conformation than was the Ile5-PTHrP(1-36) analog. We also assessed the effects of the Ile5→His substitution on the binding affinity of human PTH(1-34)NH2 and rat PTH(1-34)NH2 peptides that lacked the methionine20,21→norleucine and Phe24→Tyr substitutions of our control PTH(1-34) analog, [Nle2,21,Tyr23] rat PTH(1-34)NH2, and thus contained only native rat or human PTH residues. We also assessed a human PTHrP(1-36)NH2 peptide that lacked the Ile36→Tyr substitution our control PTHrP(1-36) peptide, [Tyr36]PTHrP(1-36)NH2. The resulting competition binding data (supplemental Fig. 1, published as supplemental data on The Endocrine Society’s Journals Online web site at http://mend.endojournals.org; Table 1) revealed R0:RG selectivity profiles that closely mirrored those obtained for the corresponding PTH and PTHrP control peptides. The results thus support the view that whereas both PTH(1-34) and PTHrP(1-36) bind well to the RG receptor conformation of the PTHR, PTH(1-34) binds with higher affinity to the R0 conformation than does PTHrP(1-36) and that residue 5 in the ligand plays a key role in modulating this conformational selectivity. We note, however, that residues C-terminal of position 15 in PTH(1-34) are also likely to contribute to the capacity of the ligand to bind strongly to R0, because...
Table 1. Competition Binding to the RG and R0 Conformations of the Human PTHR

| Ligand | IC50 (nM) | n  | GTP, S | n  | R0, RG |
|--------|-----------|----|--------|----|--------|
| [Ne6,2Tyr24]PTH(1–34)NH2 | 0.91 ± 0.28 | 6  | 4.0 ± 0.6 | 6  | 4      |
| [His5,Nle5,21,Tyr24]PTH(1–34)NH2 | 0.10 ± 0.03 | 3  | 67 ± 8  | 3  | 670    |
| [Tyr26]hPTHrP(1–36)NH2 | 0.42 ± 0.09 | 3  | 28 ± 6  | 3  | 66     |
| [Ile6,Tyr26]hPTHrP(1–36)NH2 | 0.92 ± 0.07 | 3  | 2.9 ± 0.1 | 3  | 3      |
| rPTH(1–34)NH2 | 0.34 ± 0.16 | 3  | 2.3 ± 0.3 | 3  | 7      |
| [His5]PTH(1–34)NH2 | 0.19 ± 0.04 | 5  | 26 ± 5  | 5  | 138    |
| hPTH(1–34)NH2 | 0.39 ± 0.24 | 3  | 6.6 ± 2.4 | 3  | 17     |
| [His5]hPTH(1–34)NH2 | 0.76 ± 0.04 | 3  | 122 ± 35 | 5  | 160    |
| hPTHrP(1–36)NH2 | 0.59 ± 0.02 | 3  | 24 ± 3  | 3  | 42     |
| [Aib1,3,M]PTH(1–15)NH2 | 0.74 ± 0.18 | 3  | 1029 ± 148 | 3  | 1397   |

[Aib1,3,M]PTH(1–15), which contains isoleucine at position 5, bound only weakly to R0, while exhibiting strong affinity for RG (supplemental Fig. 1C; Table 1).

**Direct Recording of PTHR Activation**

The fluorescent resonance energy transfer (FRET) approach has recently been used to assess, in real time and in intact cells, the processes of ligand binding and receptor activation used by the PTHR (8, 25). We thus used this approach as an independent means to compare the time courses by which PTH and PTHrP ligands interact with the PTHR. The approach exploits an intramolecular FRET signal that occurs in a PTHR construct, PTHR-CFPIC3/YFPCT, that contains cyan-fluorescent protein (CFP) in the third intracellular loop and yellow-fluorescent protein (YFP) in the C-tail (25). The FRET signal produced by PTHR-CFPIC3/YFPCT occurs in the basal state and diminishes upon agonist binding, presumably due to conformational changes associated with receptor activation (25).

As shown in Fig. 4A, addition of hPTH(1–34) to cells expressing PTHR-CFPIC3/YFPCT induced a rapid (t1/2 = 0.7 sec) reduction (~13%) in the FRET signal. The FRET signal remained suppressed during the 15 sec of ligand application (marked by the black horizontal line above the graphs in Fig. 4) and for at least 60 sec after the ligand-containing buffer was exchanged for a ligand-free buffer. This FRET response profile obtained for hPTH(1–34) replicates that observed for this N-terminal peptide, [Aib1,3,M]PTH(1–15), which isomerize to LRG, and thus become active in terms of data obtained in the above radioligand dissociation assays and thus support the hypothesis that PTH(1–34) and PTHrP(1–36) bind predominantly to distinct PTHR conformations and that ligand residue 5 plays a role in receptor conformation selectivity.

**Duration of cAMP Signaling Capacity**

The data so far support the notion that certain PTH and PTHrP ligands can bind to a G protein-uncoupled form of the PTHR, R0, with high affinity and thereby form a stable LR0 complex. We next explored the hypothesis that this LR0 complex could, over time, isomerize to LRG, and thus become active in terms of...
Because this hypothesis predicts that a ligand that can bind stably to R0 would have the capacity to produce a more prolonged signaling response than would a ligand that binds only weakly to R0, we assessed the capacities of the ligands to stimulate cAMP production in PTHR-expressing cells at times after initially binding to the receptor. We thus treated cells with ligand at a relatively high concentration (100 or 300 nM) for 10 min, rinsed the cells thoroughly to remove unbound ligand, incubated the cells in buffer alone for various times, removed the buffer, and replaced it with a buffer containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), and after a 5-min incubation in IBMX, lysed the cells and measured intracellular cAMP. By this delayed cAMP protocol, only the intracellular cAMP generated during the 5-min IBMX phase would accumulate in the cells to measurable levels.

The experiments of Fig. 5A compare the time courses of the cAMP responses produced by PTHrP(1–36) and Ile5-PTHrP(1–36) in HKRK-B7 cells. Immediately after the wash-out step (t = 0), cells treated with either ligand produced approximately the same, near-maximal amount of cAMP (~100-fold above basal). Two hours later, the cells treated with Ile5-PTHrP(1–36) were still producing a cAMP response, which was 50% of the initial response (Fig. 5A). In contrast, the cells treated with PTHrP(1–36)
produced a signaling response at 2 h that was only 19% of the initial response, and thus about 65% less than that observed at 2 h for Ile5-PTHrP(1–36) (P < 0.003). PTH(1–34) produced responses at each time point that were not significantly different from those produced by Ile5-PTHrP(1–36) (P > 0.05, data not shown). Thus, the cAMP signaling responses induced by PTH(1–34) and Ile5-PTHrP(1–36) decayed about twice as slowly as did that of PTHrP(1–36) (t_{1/2} ≈ 1 h).

More prolonged responses for PTH(1–34) and Ile5-PTHrP(1–36), relative to PTHrP(1–36), were also observed in ROS17/2.8 cells, which express the PTHR endogenously and at a relatively low level (70,000 per cell vs. 950,000 per cell for HKRK-B7 cells), as well as in HKRK-B64 cells, which stably express the hPTHr, also at a relatively low level (90,000 per cell) (supplemental Fig. 2, A and B).

Figure 5B compares the capacity of PTH or PTHrP ligands to produce a sustained cAMP signaling responses in HKRK-B64 cells at the 60-min time point. In these experiments, the cAMP response observed at 60 min after ligand washout is expressed as a percentile of the maximal cAMP response observed for that ligand (determined by treating cells concomitantly with that ligand and IBMX for 10 min and omitting the washout phase), as shown in the figure inset. At 60 min after washout, the cAMP responses generated by PTH(1–34) and Ile5-PTHrP(1–36) were 47 and 40% of their corresponding maximal responses, respectively, whereas those of His5-PTH(1–34) and PTHrP(1–36) were 34 and 19% of their maximal responses. The response induced by [Aib^{1,3,6}M]PTH(1–15) at 60 min was 23% of its maximum and thus comparable to that of PTHrP(1–36) (P = 0.7). Thus, in HKRK-B64 cells, PTH(1–34) and Ile5-PTHrP(1–36) produce more sustained cAMP signaling responses than do PTHrP(1–36), His5-PTH(1–34), and [Aib^{1,3,6}M]PTH(1–15).

In conventional cAMP dose-response assays performed in HKRK-B64 cells, little or no difference in potency or efficacy was observed for the analogs (supplemental Fig. 3A), whereas in ROS17/2.8 cells, a modest 4.5-fold reduction in cAMP signaling potency was observed for His5-PTH(1–34), as compared with PTH(1–34) (supplemental Fig. 3B). No difference in inositol triphosphate signaling potency was observed for the analogs in COS-7 cells transiently transfected to express the hPTHr (supplemental Fig. 3C).

DISCUSSION

The studies presented here suggest intriguing differences in the mechanisms by which PTH and PTHrP interact with the PTH/PTHrP receptor, in that they point to differences in the capacities of the ligands to bind to and stabilize distinct receptor conformations. Our kinetic and equilibrium binding assays performed in cell membranes in the presence of GTPγS lead us to our main conclusion that whereas PTH(1–34) and PTHrP(1–36) bind with similar affinities to the G protein-coupled PTHR conformation, RG, PTH(1–34) binds with greater affinity to the G protein-uncoupled conformation, R0, defined here as a receptor conformation that can bind ligand with high affinity in the presence of GTPγS (15–17).

In considering the potential biological implications for such differences in the capacity of PTH or PTHrP ligands to bind to different PTHR conformations, and in particular, R0, we hypothesized that R0, although not coupled to G protein, is a preactive state that is primed to interact efficiently with a G protein as it encounters one. Thus, the LR0 complex, upon engaging a G protein, can isomerize to LRG and so become signaling competent. This hypothesis, in turn, predicts that a ligand that can bind stably to R0 will have the potential to produce a biological signal at longer times after it initially binds to the receptor than will a ligand that binds only weakly to R0. We performed the delayed cAMP time-course assays of Fig. 5 and supplemental Fig. 2 as means to test these hypotheses. The results showed that ligands that exhibited relatively high affinities for R0 in the binding assays, PTH(1–34) and Ile5-PTHrP(1–36), produced greater cAMP responses at times after initial binding than did ligands that bound more weakly to R0, PTHrP(1–36) and His5-PTH(1–34). The results thus support the hypothesis that PTH and PTHrP ligands exhibit different conformational selectivities for the PTHR and, more generally, support the notion that the capacity of a ligand to bind to R0 can determine, in part, the overall signaling capacity of that ligand in target cells.

The capacity to produce a signaling response at times after initial binding of a ligand to its receptor could be biologically relevant in situations where the cognate G proteins are in low abundance, relative to the receptor, due, for example, to differences in expression levels and/or subcellular compartmentalization. It is also possible that formation of a stable LR0 complex could enable multiple (catalytic) rounds of G protein activation (26, 27) and thereby contribute to signal amplification. In any event, our present data suggest that receptor conformational selectivity can be a factor that contributes to the functionality of a PTH receptor ligand, and this might be a property of the PTHR that can be exploited in ligand-design efforts. In this regard, we have recently developed a PTH analog, [Ala^{1,12},Ala^{3},Gln^{10},His^{1},Trp^{14},Arg^{19}]hPTH(1–28)NH2, that binds to the R0 conformation of the PTHR with considerably higher (−80-fold on the rat PTHR) affinity than does hPTH(1–34)NH2 and, when injected into mice, produces biological responses (increases in serum calcium and suppression of serum phosphate) that are significantly more prolonged than those produced by PTH(1–34) (28, 29). These findings, which are not based on differing serum concentrations of the ligands, based on pharmacokinetic data, strongly suggest that the capacity of a ligand to bind to the R0
conformation of the PTHR can contribute importantly to the biological response profile of that ligand.

Our acute dose-response signaling assays detected little if any difference in the potencies with which PTH(1–34) and PTHrP(1–36) ligands stimulate cAMP or inositol phosphate (IP) accumulation (supplemental Fig. 3; Table 2), results that, by themselves, are consistent with the view that the two ligands interact with the PTHR via largely similar mechanisms. The time-delayed cAMP assays of Fig. 5 (and supplemental Fig. 2) brought out previously unappreciated differences in the signaling properties of the two ligands, evident as differences in the signal output at times after initial binding of ligand to the receptor. Although these findings are consistent with a model involving altered selectivities for different PTHR conformations, we cannot, at present, exclude a possible role for differences in receptor desensitization mechanisms (30–33). The relationship between receptor conformational selectivity and such internalization/desensitization processes will be an interesting and important matter to explore in future studies.

The capacity of ligand to bind stably to LR0 might also facilitate coupling to secondary G proteins that presumably have lower affinity for the ligand-receptor complex than does the primary G protein. For the PTHR, this could involve coupling to G proteins of the Go12/13, Go11, or Go12/13 subclasses, which have been shown to be activated by the PTHR in response to PTH(1–34). More studies are needed to assess the relationship between PTHR ligand conformational selectivity and activation of these other G protein signaling pathways. It is also interesting to note that some capacity to form a stable LR0 complex might be an intrinsic property of the class B GPCRs, most if not all of which use a two-site ligand-binding mechanism involving interactions to both the N and J receptor domains. Thus, several others of these receptors, including the receptors for calcitonin (34), CRH (17), and glucagon (35) have been shown to form a stable complex with their cognate peptide ligand in the presence of a nonhydrolyzable guanine nucleotide analog.

We do not know whether differing capacities to bind to the R0 state of the receptor explain any of the differences in pharmacological or physiological effects attributed to the two ligands, PTH and PTHrP. It has been of interest to speculate how two physiologically different systems, one paracrine/autocrine (PTHrP) and the other endocrine (PTH) can effectively use one common receptor, widely present in cells, yet produce different effects. PTHrP is produced locally with high regional concentrations likely (36), whereas PTH is secreted into the circulation. Stabilization or formation of distinctive PTHR conformations that differ in terms of duration of signaling could be one factor that underlies, in part, the different modes of action. For example, a shorter duration of signaling might be a useful mechanism for a paracrine factor involved in the timing of cell differentiation programs. PTHrP(1–36) peptide, in the limited human studies reported so far, does seem to differ from PTH(1–34) in the extent of hypercalcemia induced after a single sc injection (14), stimulation of 1,25(OH)2 vitamin D3 production after iv infusion (13), and stimulation of bone resorption after several months of daily sc administration (12). The differences in the capacities of PTH and PTHrP ligands to bind to the R0 conformation of the PTHR described herein could potentially explain some of these differing pharmacological properties of the ligands and may be of value to explore therapeutically.

**MATERIALS AND METHODS**

**Peptides**

Peptides used were as follows (abbreviated, full structural name): PTH(1–34), [Nle6,21,Tyr36]rat(r)PTH(1–34)NH2; His5-PTH(1–34), [His5,Nle8,21,Tyr34]rPTH(1–34)NH2; PTHR(1–36), [Tyr36]human(h)PTHrP(1–36)NH2; Ile5–PTHrP(1–36), [Ile5,Tyr36]rPTHrP(1–36)NH2; [Aib1,3,M]PTH(1–15), [Aib1,3,Nle6,Gln10,Har11,Ala12,Trp14,Tyr15]rPTH(1–15)NH2 (37); hPTH(1–34), hPTH(1–34)NH2; His5-hPTH(1–34) [His5]-hPTH(1–34), [His5]-hPTH(1–34)NH2; rPTH(1–34), [rPTH(1–34)]NH2; and His5-rPTH(1–34), [His5]-rPTH(1–34)NH2. These peptides were synthesized by the Massachusetts General Hospital Biopolymer Core facility, as described (38). The human PTH(1–34) (free carboxy terminus) used in FRET analyses was purchased from Bachem California (Torrance, CA). Peptide quality was verified by analytical HPLC, matrix-assisted laser desorption/ionization mass spectrometry and amino acid analysis, and peptide concentrations of stock solutions were established by amino acid analysis.

**Table 2. cAMP and IP Signaling Properties of PTH and PTHrP Ligands**

|                | cAMP         | IP            |
|----------------|--------------|---------------|
|                | EC50 (nM) Emax (pmol/well) | EC50 (nM) Emax (cpm/well) |
| HKRK-B64 Cells |              |               |
| [Nle6,21,Tyr31]PTH(1–34)NH2 | 5.1 ± 0.5 | 55 ± 12 | 0.60 ± 0.1 | 353 ± 54 |
| [His5,Nle8,21,Tyr34]PTH(1–34)NH2 | 2.7 ± 0.6a | 59 ± 12 | 2.8 ± 0.2a | 398 ± 68 |
| [Tyr36]rPTHrP(1–36)NH2 | 5.6 ± 1.3 | 62 ± 15 | 0.78 ± 0.23 | 366 ± 43 |
| [Ile5,Tyr36]rPTHrP(1–36)NH2 | 5.4 ± 1.9 | 61 ± 14 | 1.0 ± 0.6 | 353 ± 51 |
| ROS 17/2.8 Cells |              |               |
| [Nle6,21,Tyr31]PTH(1–34)NH2 | 5.1 ± 0.5 | 55 ± 12 | 0.60 ± 0.1 | 353 ± 54 |
| [His5,Nle8,21,Tyr34]PTH(1–34)NH2 | 2.7 ± 0.6a | 59 ± 12 | 2.8 ± 0.2a | 398 ± 68 |
| [Tyr36]rPTHrP(1–36)NH2 | 5.6 ± 1.3 | 62 ± 15 | 0.78 ± 0.23 | 366 ± 43 |
| [Ile5,Tyr36]rPTHrP(1–36)NH2 | 5.4 ± 1.9 | 61 ± 14 | 1.0 ± 0.6 | 353 ± 51 |
| COS-7 Cells (hPTHrP) |              |               |
| [Nle6,21,Tyr31]PTH(1–34)NH2 | 5.1 ± 0.5 | 55 ± 12 | 0.60 ± 0.1 | 353 ± 54 |

* Data are means (±SEM) from four experiments; Emax for basal cAMP (not subtracted) was 5.2 ± 0.9 pmol/well.
* Data are means (±SEM) from three experiments; Emax for basal cAMP (not subtracted) was 21 ± 1 pmol/well.
* Data are means (±SEM) from five experiments; Emax for basal IP value (not subtracted) was 330 ± 8 cpm/well.
* P vs. [Nle6,21,Tyr34]PTH(1–34)NH2 ≤ 0.02.
Radiolabeled peptide variants were prepared by the oxidative chloramine-T procedure using Na$_{2}$O$_{2}$ (specific activity, 2200 Ci/mmol; PerkinElmer/NEN Life Science Products, Boston, MA) and were purified by reversed-phase HPLC.

Cell Culture

Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO$_2$ in DMEM supplemented with 10% fetal bovine serum (HyClone, Logan UT), 100 U/ml penicillin G, and 100 µg/ml streptomycin sulfate (Invitrogen Corp., Carlsbad, CA). The PTHR-expressing cell lines used were HKRK-B7, HKRK-B64, ROS 17/2.8, and HEK-PTHR-CFP$_{IC3}$/YFP$_{CT}$. The HKRK-B7 and HKRK-B64 lines are derivatives of the porcine kidney cell line LLC-PK$_1$ and are stably transfected to express the human PTHR at approximate surface densities of 950,000 and 90,000 PTH-binding sites per cell, respectively (39). ROS 17/2.8 cells are rat osteosarcoma cells (40) and express the endogenous rat PTHR at an approximate surface density of 70,000 PTH-binding sites per cell (41). HEK-PTHR-CFP$_{IC3}$/YFP$_{CT}$ cells are derived from HEK-293 cells and stably express PTHR-CFP$_{IC3}$/YFP$_{CT}$, a human PTHR construct previously called PTHR-Cam (25), that contains CFPS inserted at Gly$_{5}$ in the third intracellular loop and YFP inserted into the C-terminal tail. Cells were propagated in 175 flasks and divided into 24-well plates for assays with intact cells, six-well plates for membrane preparations, or onto glass coverslips for FRET studies.

COS-7 cells were transiently transfected using Fugene-6 (Roche Diagnostics, Indianapolis, IN) and CsCl-purified plasmid DNA (3 µl Fugene-6 per 1 µg DNA). The wells of six- and 24-well plates were transfected with 1 µg and 250 ng DNA per well, respectively. Cells were transfected with the PTHR alone or cotransfected with the PTHR and a negative-dominant G protein subunit, G$_{a9}$ND. This G$_{a9}$ND subunit binds more effectively, but unproductively, to receptors than does wild-type G$_{a9}$ (24) and thus enhances binding of [125I][Alb$_{1,3,5}$]MPTH(1–15)NH$_2$ radioligand to the PTHR in an R$_{G}$ conformation (see below) (23).

Binding Studies

Binding studies were performed using cell membranes as described (16). Reactions were incubated at room temperature in membrane assay buffer (20 mM HEPES (pH 7.4), 0.1 mM NaCl, 3 mM MgSO$_4$, 20% glycerol, 3 mg/ml BSA, protease inhibitor cocktail, and, at final concentrations, 1 mM AEBSF, 0.8 µM aprotonin, 20 µM leupeptin, 40 µM bestatin, 15 µM pepstatin A, and 14 µM E-64; Sigma-Aldrich Inc., St. Louis, MO). Reactions contained a total membrane protein concentration of 20–100 µg/ml and a total radioactivity concentration of approximately 150,000 cpm/ml. Unlabeled peptide ligands and/or GTP$_{S}$(S)MD (Sigma-Aldrich) were added to the reactions as indicated. At the end of the reaction, bound and free radioligand were separated by vacuum filtration using a 96-well vacuum filter plate and vacuum filter apparatus (Multi-Screen system with Durapore HV, 0.65-µm filters; Millipore Corp., Milford, MA); the air-dried filters were then eluted from the plate with 0.2-ml aliquots (~30,000 cpm) and immediately processed by vacuum filtration, as described above. Nonspecific binding was determined in parallel reaction tubes containing the unlabeled analog (5 × 10$^{-7}$ M) in both the preincubation and dissociation phases. The specifically bound radioactivity at each time point was calculated as a percent of the radioactivity specifically bound at t = 0.

Equilibrium Competition Binding and GTP$_S$ Inhibition

Binding reactions performed with [125I][Alb$_{1,3,5}$]MPTH(1–15) radioligand were assembled and incubated in the wells of the 96-well Multi-Screen vacuum filtration plates. Membranes, tracer radioligand, and various concentrations of unlabeled ligands and/or GTP$_S$ were incubated in the wells for 90 min, following which, the reaction plates were processed by rapid vacuum filtration, as described above. Binding reactions performed with [125I]PTH(1–34) radioligand were assembled and incubated in 96-well polystyrene microtiter plates (Falcon, total reaction volume = 230 µl) and at the end of the incubation were transferred to the Multi-Screen vacuum filtration plates and processed, as described above. This transfer minimized nonspecific binding of [125I]PTH(1–34) to the Multi-Screen filter membranes. For both radioligands, the nonspecific binding was determined in reaction tubes containing a saturating concentration of the unlabeled analog of the radioligand. The specifically bound radioactivity was calculated as a percentage of the radioactivity specifically bound in the absence of a competing ligand or GTP$_S$.

To assess the binding of unlabeled peptide ligands to the G protein-uncoupled PTHR conformation (R), we used membranes prepared from COS-7 cells transiently transfected with the PTHR, [125I]PTH(1–34), as a tracer radioligand and added GTP$_{S}$ to the binding reactions (1 × 10$^{-5}$ M final concentration). This binding format is based on the premise that [125I]PTH(1–34) binds predominantly to the R$_{G}$ conformation of the PTHR and that this conformation is enriched in the membranes, relative to R, by the presence of GTP$_{S}$ (15, 16). A similar approach, using radiolabeled peptide agonist and GTP$_{S}$, was used by Hoare and colleagues (17) to assess binding to the R$_{G}$ conformation of the corticotropin-releasing factor receptor-1. To assess binding to the G protein-coupled conformation (RG), we used membranes prepared from cells cotransfected with the PTHR and a negative-dominant G protein subunit (G$_{a9}$ND), and we used [125I][Alb$_{1,3,5}$]MPTH(1–15)NH$_2$ radioligand to the PTHR in an RG conformation (see below) (23).

FRET

FRET analyses using HEK-PTHR-CFP$_{IC3}$/YFP$_{CT}$ cells were performed as described (25). With PTHR-CFP$_{IC3}$/YFP$_{CT}$, excitation of the CFP donor with UV light ($\lambda_{max.ex}$ = 436 nm; $\lambda_{max.em}$ = 480 nm) produces an intramolecular FRET to the YFP acceptor, resulting in emission from that YFP ($\lambda_{max.ex}$ = 480 nm, $\lambda_{max.em}$ = 535 nm). This FRET response is observable as a decrease in intensity of CF light emission at 480 nm and an increase in intensity of YFP light emission at 535 nm. The FRET signal occurs in the ground-state receptor and decreases upon binding of a PTHR agonist ligand (25). PTH ligands were applied to and washed from the cells using a computer-assisted, solenoid valve-controlled, rapid superfusion device (ALA Scientific Instruments). When the exchange phase was initiated, the solutions containing the labeled and unlabeled analog of the radioligand (5 × 10$^{-7}$ M final concentration), with or without GTP$_S$(S) (5 × 10$^{-5}$ M). Immediately before this addition (t = 0), and at successive time points thereafter, 0.2-ml aliquots

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Stimulation of Intracellular cAMP and Inositol Phosphate

Intracellular cAMP levels were measured by RIA (38). The capacity of a ligand to produce a delayed cAMP response in cells was assessed as follows (33, 42). The cells in 24-well plates were rinsed in binding buffer [50 mM Tris-HCl (pH 7.7), 100 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 5% heat-inactivated horse serum, 0.5% heat-inactivated fetal bovine serum] and incubated further in binding buffer for varying times (1–120 min). The buffer was then replaced by binding buffer containing IBMX (2 mM), and after an additional 5-min incubation, the intracellular cAMP was quantified. By this approach, only the cAMP produced during the final IBMX stage of the incubation is measurable, because that produced before IBMX addition is degraded by cellular phosphodiesterases.

The stimulation of intracellular IPs was measured in transiently transfected COS-7 cells that were prelabeled (16 h) with [\(^{3}H\)myo-inositol] (38). Cells were treated with ligand in DMEM containing fetal bovine serum (10%) and LiCl (30 mM) for 30 min. The cells were then lysed with ice-cold, trichloroacetic acid (5%), and IPs were extracted from the acid-lysates by ion-exchange filtration.

Data Calculations

Data were processed using Microsoft Excel and GraphPad Prism 4.0 software packages. Dissociation time-course data were analyzed using a biexponential decay equation, except when an F test analysis indicated a monoequponential equation provided a better fit (\(P_{\alpha} < 0.02\)). Data from equilibrium binding, cAMP, and IP dose-response assays were analyzed using a sigmoid dose-response equation with variable slope, which yielded values of \(EC_{50}\) for concentration of ligand producing the half-maximal effect, and \(E_{\text{max}}\) (maximal cAMP or IP response). Paired data sets were statistically compared using the Student\'s t test (two tailed) assuming unequal variances for the two sets.

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Address all correspondence and requests for reprints to: Thomas J. Gardella, Endocrine Unit, Massachusetts General Hospital, Boston, Massachusetts 02114. E-mail: Gardella@helix.mgh.harvard.edu.

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