Current antagonists for the parathyroid hormone (PTH)/PTH-related protein (PTHR) receptor (PTHR) are N-terminally truncated or N-terminally modified analogs of PTH(1–34) or PTHrP(1–34) and are thought to bind predominantly to the N-terminal extracellular (N) domain of the receptor. We hypothesized that ligands that bind only to PTHR region comprised of the extracellular loops and seven transmembrane helices (the juxtamembrane or J domain) could also antagonize the PTHR. To test this, we started with the J domain-selective agonists [Gln10,Ala12,Har14,Trp14,Arg19,M]PTH(1–21), [M]PTH(1–15), and [M]PTH(1–14), and introduced substitutions at positions 1–3 that were predicted to dissociate PTHR binding and cAMP signaling activities. Strong dissociation was observed with the tri-residue sequence diethylglycine (Deg)1-para-benzoyl-l-phenylalanine (Bpa)2-Deg2. In HKRR-B7 cells, which express the cloned human PTHR, [Deg1,3,Bpa2,M]PTH(1–21), [Deg1,3,Bpa2,M]PTH(1–15), and [Deg1,3,Bpa2,M]PTH(1–14) fully inhibited (IC50 = 100–700 nm) the binding of 125I-[α-aminoisobutyric acid1,3]PTH(1–15) and were severely defective for stimulating cAMP accumulation. In ROS 17/2.8 cells, which express the native rat PTHR, [Deg1,3,Bpa2,M]PTH(1–21) and [Deg1,3,Bpa2,M]PTH(1–15) antagonized the cAMP agonist action of PTH(1–34), as did PTHrP(5–36) (IC50 = 0.7 μM, 2.6 μM, and 36 μM, respectively). In COS-7 cells expressing PTHR-delNt, which lacks the N domain of the receptor, [Deg1,3,Bpa2,M]PTH(1–21) and [Deg1,3,Bpa2,M]PTH(1–15) inhibited the agonist actions of [α-aminoisobutyric acid1,3]PTH(1–34) and [M]PTH(1–14) (IC50, s = 1 μM), whereas PTHrP(5–36) failed to inhibit. [Deg1,3,Bpa2,M]PTH(1–14) inhibited the constitutive cAMP-signaling activity of PTHR-tethered PTH(1–9), in which the PTH(1–9) sequence is covalently linked to the PTHR J domain, as well as that of PTHrP(5–36). Thus, the J-domain-selective N-terminal PTH fragment analogs can function as antagonists as well as inverse agonists for the PTHR. The new ligands described should be useful for further studies of the ligand binding and activation mechanisms that operate in the critical PTHR J domain.

Parathyroid hormone (PTH)1 is a major regulator of ionized calcium and phosphate concentrations in the blood and extracellular fluids, and PTH-related protein (PTHR) is a vital developmental morphogen (1, 2). These two peptide ligands mediate their actions by binding to the same receptor, the PTHrP receptor (PTHR) or PTHR (or PTHR subtype 1). The PTHR is abundantly expressed in bone and kidney, the principal target organs of PTH, and in a variety of developing tissues (e.g., skeleton, heart, and mammary glands), where it mediates the actions of PTHrP. Excessive circulating levels of PTH, as occurs in cases of hyperparathyroidism, or PTHrP, as frequently occurs in cancer because of secretion by malignant tumors, produces a hypercalcemic state, which can be severely debilitating and potentially fatal (1, 2). For both PTH and PTHrP, the first 34 amino acids encode all of the information needed for binding to and activating the PTHR.

The PTHR is a class 2 G protein-coupled receptor that activates the adenyl cyclase/cAMP signaling pathway. Like all class 2 G protein-coupled receptors that bind peptide hormones, the PTHR has a relatively large (~160 amino acid) N-terminal extracellular domain, herein termed the N domain (3), that plays a major role in hormone binding (4, 5). The portion of the PTHR comprised of the extracellular loops and seven helical transmembrane domains (membrane, termed the juxtamembrane or J domain (3), is thought to mediate most, or all, of the ligand interactions that are involved in receptor activation and G protein coupling (5–8). The molecular mechanism by which PTH (and PTHrP) binds to the PTHR has been extensively analyzed through the approaches of receptor mutagenesis (4–6, 9–11), photochemical cross-linking (12–14), and molecular modeling (15–17). These studies have led to a “two-site” model for the ligand-receptor interaction mechanism, according to which the C-terminal portion of PTH(1–34) (i.e. residues in the 23–34 region) binds to the N domain of the receptor to provide the majority of the binding energy to the complex, and the N-terminal portion of the ligand interacts with a lower affinity with the J domain of the receptor to induce activation (3, 5, 18). A similar two-site binding mechanism appears to be used by other class 2 G protein-coupled receptors that bind peptide hormones (19–27).

As part of our investigations into the ligand binding and activation mechanisms used by the PTHR, we have employed N-terminal PTH fragment analogs as functional probes of the bimolecular complex. In particular, we have used the PTH(1–14) fragment as a scaffold peptide for performing substitution analysis of the principal signaling domain of the hormone.

1 The abbreviations used are: PTH, parathyroid hormone; PTHrP, PTH-related protein; PTHR, PTH receptor; TM, transmembrane domain; J, juxtamembrane; Ac,c, aminocyclopentane-1-carboxylic acid; Aib, α-aminoisobutyric acid; Har, homoarginine; M, Gln10,Ala12,Har14,Trp14,Arg19; Bpa, para-benzoyl-l-phenylalanine; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high pressure liquid chromatography; IBMX, 3-isobutyl-1-methylxanthine; IP, inositol phosphate; Deg, diethylglycine; CRF, corticotropin-releasing factor; h, human.

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Native PTH(1−14) binds only poorly to the PTHR and is a very weak agonist (EC_{50} for stimulating cAMP formation = 1 × 10^{−3} M) (6). A number of affinity-enhancing substitutions in the PTH(1−14) scaffold have been identified that, when combined, increase the cAMP-stimulating potency of the peptide by as much as 100,000-fold (7, 28–30). These substitutions include Ser{\textsuperscript{1}} → Asp{\textsuperscript{1}}c (aminocyclopentane-1-carboxylic acid), Ser{\textsuperscript{3}} → Glu{\textsuperscript{3}} (α-aminoisobutyric acid), Asn{\textsuperscript{10}} → Gln{\textsuperscript{10}}, His{\textsuperscript{11}} → Ala{\textsuperscript{11}}, Trp{\textsuperscript{14}} → Tyr{\textsuperscript{14}}-amide (30), herein termed [Asp{\textsuperscript{1}}, Gln{\textsuperscript{1}}, Ala{\textsuperscript{11}}, Trp{\textsuperscript{14}}]-PTH(1−14) (30) and introduced into this scaffold substitutions at positions 1−3 (30, 35, 38, 40) that were predicted to dissociate binding affinity and signaling activity. Most of these substitutions had the predicted effects. We thus identified several new analogs of [MIPTH(1−14), [MIPTH(1−15), and [MIPTH(1−21)] that function as antagonists and, in some cases, inverse agonists for the PTHR. We show that these new antagonists indeed bind predominantly, if not exclusively, to the PTHR J domain.

**MATERIALS AND METHODS**

**Peptides**—The peptides used in the study are described in Table I and are synthesized using Fmoc chemistry and either a small-scale, multiple peptide synthesizer (Advanced Chemtech Model 396 MBS) with HBTU/HOBt/DIEA (O-benzotriazol-1-yl-N,N,N′,N′-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole/N,N-diisopropylethylamine; 1:1:2 molar ratio) chemistry, a 10-fold molar excess relative to substrate of Fmoc amino acid, and double couplings, or a large-scale synthesizer (Applied Biosystems model 431A) with DCC/HOBt chemistry. All double-fold molar excess of Fmoc amino acids to achieve coupling cycles. Peptides were desalted (C18 Sep-Pak), purified by reverse-phase HPLC, and verified by analytical HPLC, matrix-assisted laser desorption/ionization mass spectrometry, and amino acid analysis. The lyophilized peptides were reconstituted as stock solutions in 10 mM acetic acid; the purity, identity and exact peptide concentration of each stock solution (3 × 10^{−5} M) was secured by amino acid analysis. The stock solutions were stored at −80 °C. The radioligand [\textsuperscript{125}I]-[Leu{\textsuperscript{1}}, Asn{\textsuperscript{3}}, Glu{\textsuperscript{3}}, His{\textsuperscript{11}}, Ala{\textsuperscript{12}}, Trp{\textsuperscript{14}}]-hPTH(1−15))(3M) was prepared using [\textsuperscript{125}I]-Na and the chloramine-T-based oxidation reaction followed by HPLC purification.

**Cell Culture and DNA Transfection**—The cell lines HRK-K28 and HRK-K27 are clonal derivatives of the porcine kidney cell line, LLC-PK{\textsubscript{1}}, and were transfected with pCDNA1-based plasmid DNA to stably express recombinant PTH receptors at surface densities of ~280,000 and ~950,000 receptors/cell, respectively (44). HRK-K28 cells express a fully functional PTHR chimera comprised of the opsum PTHR from the N terminus to the middle of TM3 and the rat PTHR from the middle of TM3 to the C terminus (11); HRK-K27 cells express the wild-type human PTHR (44). COS-7 cells were transfected to transiently express the PTHR using the FuGENE 6 (Roche Applied Science) reagent and human PTHR (44). COS-7 cells were transfected to transiently express the PTHR using the FuGENE 6 (Roche Applied Science) reagent and human PTHR (44). COS-7 cells were transfected to transiently express the PTHR using the FuGENE 6 (Roche Applied Science) reagent and human PTHR (44). COS-7 cells were transfected to transiently express the PTHR using the FuGENE 6 (Roche Applied Science) reagent and human PTHR (44).

**Competition Binding Assays**—Binding studies were performed using [\textsuperscript{125}I]-[Leu{\textsuperscript{1}}, Tyr{\textsuperscript{14}}]-hPTH(1−15) as the tracer radioligand and varying concentrations of unlabelled test peptides as competitors, as described previously (29, 30). In brief, cells in 24-well plates were rinsed in binding buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM KCl, 2 mM CaCl{\textsubscript{2}},5 % heat-inactivated horse serum, 0.5 % fetal bovine serum, adjusted to pH 7.7 with HCl), and the following three components were added to the wells successively, 100 μl of binding buffer, 100 μl of binding buffer, 100 μl of binding buffer containing an unlabelled test peptide, and 100 μl binding buffer containing 125I-[Leu{\textsuperscript{1}}, Tyr{\textsuperscript{14}}]-hPTH(1−15) (~100,000 cpm/well). All solutions were ice-cold, and the plates were incubated at 4 °C in the binding buffer. The plates were then incubated in a 15 °C water bath for 4 h, after which the well contents were removed by aspiration, and the monolayers were rinsed three times by adding and rapidly removing 0.5 ml of binding buffer. Then, 0.5 ml of 5 % NaOH was added to lyse the monolayers followed by the addition of 2.0 ml of H{\textsubscript{2}}O, and the entire lysate was counted for radioactivity. Non specific binding was

\[\text{Assay Control:} \quad \text{Specific Binding} = \text{Total Binding} - \text{Nonspecific Binding}\]
IC₅₀ (or EC₅₀ in cAMP assays) is the ligand concentration (nm) at which 50% of the maximum response occurs, and n is the slope of the plot of y versus x and the Excel Solver function to optimize the parameters by nonlinear regression analysis (48). Paired data sets were statistically compared using the Student’s t test (two-tailed) assuming unequal variances for the two sets.

**RESULTS**

**PTHR Binding and cAMP Signaling Properties of PTH Anologs in LLC-PK1 Cells**—Starting with the potent agonist [Ac⁵c¹,Abb¹,B]PTH(1–14) (30) as a parent scaffold peptide, we first introduced at positions 1 or 2 single-residue modifications that were predicted to dissociate PTHR binding affinity and cAMP-signaling capacity. These modifications included the removal of the N-terminal amino function (35), as provided by des-NH₂-Ac⁵c¹, and des-NH₂-Abb¹ (Abb is structurally related to Ac⁵c when substituted at position 1 results in activity profiles that are nearly indistinguishable from those seen with Ac⁵c¹ (30)) and the substitution of valine 2 by arginine (39), tryptophan, or Bpa (38, 40) (Table I). The resulting analogs were tested for PTHR binding affinity and cAMP-stimulating potency using HKRK-B28 cells, an LLC-PK1-derived cell line in which a rat/opossum chimeric PTHR is expressed at a density of 280,000 receptors/cell (44). As summarized in Table II, each of the single-residue modifications resulted in some loss of binding affinity, as assessed in competition experiments performed with [³²P]-[Abb¹,Abb¹,M]PTH(1–15) tracer radioligand, with the strongest (830-fold) reduction occurring with the Abb⁵ substitution and the mildest (25-fold) reduction occurring with the Abb⁵ substitution. In the cAMP formation assays we used a peptide concentration of 10 μM, at which most, if not all, of the analogs could be predicted to attain at least half-maximum receptor occupancy. Each of the substituted analogs stimulated no more than 10% of the parental response. Because we recently found, and confirmed in the studies reported in Table II, that introducing the paired dialkyl amino acid substitution of Deg¹,³ in the [M]PTH(1–14) scaffold confers partial agonist behavior to the peptide (30), we combined the Deg¹,³ substitutions with Bpa² to obtain [Deg¹,³,Bpa²,M]-...
Table II

Binding properties of PTH analogs in HKRK-B7 cells

| Peptide\(^a\)  | Binding\(^b\) | cAMP\(^c\) |
|-------------|-------------|------------|
|              | IC\(_{50}\) | n     | %   | E\(_{\text{max}}\) | n |
| [Ac\(_c\)^1, Aib\(^3\), M]PTH(1–14) (parent) | 30 ± 7 | 3 | 100 ± 5 | 3 |
| des-NH\(_2\)Aib\(^3\) | 4,500 ± 700 | 4 | 25 ± 2 | 3 |
| des-NH\(_2\)Aib\(^3\), C\(^1\) | 1,800 ± 100 | 4 | 23 ± 2 | 3 |
| Arg\(^2\) | 25,000 ± 2,000 | 4 | 4.9 ± 0.2 | 3 |
| Trp\(^2\) | 770 ± 110 | 4 | 11 ± 0 | 3 |
| Bpa\(^2\) | 1,400 ± 200 | 4 | 5 ± 0 | 3 |
| Deg\(^1,3\) | 230 ± 50 | 3 | 65 ± 3 | 3 |
| Deg\(^1,3\), Trp\(^2\) | 2,700 ± 300 | 3 | 8 ± 0 | 3 |
| Deg\(^1,3\), Bpa\(^2\) | 840 ± 110 | 3 | 3.3 ± 0.4 | 3 |

\(^a\) The parent peptide and derivatives thereof containing the indicated modifications/substitutions at positions 1–3, are described further in Table I.

\(^b\) Competition binding assays were performed with \(^{125}\text{I}-[\text{Aib}^{1,3}, \text{M}]\text{PTH}(1–15)\) tracer radioligand.

\(^c\) The maximum cAMP response observed (E\(_{\text{max}}\)) for the derivative peptides, determined at a peptide concentration of 1 × 10\(^{-7}\) M, are reported as a percentile of the E\(_{\text{max}}\) attained in the assays by the parent peptide at a concentration of 1 × 10\(^{-9}\) M, which was 178 ± 18 pmol/well. The basal cAMP level, not subtracted in the calculations, was 3.3 ± 0.4 pmol/well (1.8 ± 0.1 %).

Fig. 1. cAMP and binding properties of agonist and antagonist analogs in HKRK-B7 cells. Competition binding (A) and stimulatory cAMP response (B) assays were conducted in HKRK-B7 cells with the peptide ligands identified in the symbol key. Shown are combined data (means ± S.E.) from three to five experiments, each performed in duplicate. Competition binding studies were performed with the \(^{125}\text{I}-[\text{Aib}^{1,3}, \text{M}]\text{PTH}(1–15)\) tracer radioligand and varying concentrations of the unlabeled competing ligand indicated in the figure key. Binding data show the amount of radioligand specifically bound (SB) calculated as a percentile of the total radioligand specifically bound in the absence of competitor (B0). Curves were fit to the data points using non-linear regression analysis; the corresponding IC\(_{50}\) and EC\(_{50}\) values are reported in Table III. The cAMP data were calculated as a percentile of the maximum response observed in each assay with PTH(1–34), the average of which was 394 ± 14 pmol/well. The corresponding basal cAMP value was 5.9 ± 0.7 pmol/well and was not subtracted in the calculations.

**PTH(1–14).** This analog exhibited the strongest dissociation of binding affinity and cAMP signaling efficacy (IC\(_{50}\) = ~840 nM, E\(_{\text{max}}\) = ~3% of the parental response, Table II) of any analog tested in the [M]PTH(1–14) series. In the LLC-PK1-derived cell line HKRK-B7 cells, in which the wild-type human PTHR is expressed at a surface density of ~950,000 receptors/cell (44), [Deg\(^1,3\), Bpa\(^2\), M]PTH(1–14) again showed a strong dissociation of binding affinity and cAMP-signaling efficacy (IC\(_{50}\) = 700 nM, E\(_{\text{max}}\) = ~6% of the response seen with [Ac\(_c\)^1, Aib\(^3\), M]PTH(1–14), Fig. 1, A and B and Table III). The dissociation of binding and cAMP signaling caused by the Deg\(^1,3\)-Bpa\(^2\)-Deg\(^2\) substitutions were confirmed in an independently synthesized N-terminal PTH fragment analog, [Deg\(^1,3\), Bpa\(^2\), M]PTH(1–15), which exhibited functional properties identical to those of [Deg\(^1,3\), Bpa\(^2\), M]PTH(1–14) (Fig. 1, A and B and Table III). In an attempt to further improve PTHR binding affinity without increasing signaling efficacy, we extended [Deg\(^1,3\), Bpa\(^2\), M]PTH(1–15) to position 21 and included the Arg\(^2\) substitution, as we recently showed that residues in the 15–20 region of the ligand, and particularly the Arg19 modification, improve the binding affinity on the PTHR J domain by at least severalfold (11, 33). The resulting analog [Deg\(^1,3\), Bpa\(^2\), M]PTH(1–21) exhibited a 6-fold higher apparent binding affinity in HKRK-B7 cells, as compared with the binding of [Deg\(^1,3\), Bpa\(^2\), M]PTH(1–15) (IC\(_{50}\) = 110 versus 710 nM, p = 0.02), and exhibited little or no increase in cAMP-signaling capacity (E\(_{\text{max}}\) = 8% of that seen for [Ac\(_c\)^1, Aib\(^3\), M]PTH(1–14), Fig. 1, A and B, Table III). We then assessed the antagonist properties of the Deg\(^1\)-Bpa\(^2\)-Deg\(^2\)-containing N-terminal peptides in PTHR-expressing cells, as discussed below.

**Antagonism of Agonist-induced cAMP Responses in ROS 17/2.8 Cells**—We first examined the capacity of [Deg\(^1,3\), Bpa\(^2\), M]-

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\(^3\) The C-terminal Tyr and Met\(^s\) → Nle (norleucine) modifications in this analog and in the [Deg\(^1,3\), Bpa\(^2\), M]PTH(1–21) analog were introduced to enable oxidative radiodination as part of our still ongoing efforts to develop an N-terminal PTH antagonist radioligand.
Novel Peptide Antagonists for the PTH Receptor

TABLE III
Functional properties of PTH analogs in HKRK-B7 cells

| Peptide*                      | Binding* | cAMP* |
|-------------------------------|----------|-------|
|                               | IC50 nm | n    | EC50 nm | pmol/well | Emax n |
| PTH(1–34)                     | 3.4 ± 0.7 | 4 | 9.0 ± 3.2 | 394 ± 14 | 5 |
| [Alb1,3,Bpa2,M]PTH(1–34)      | 6.0 ± 1.2 | 3 | 12 ± 1   | 439 ± 23 | 5 |
| [Ac5c1,Aib3,M]PTH(1–14)       | 24 ± 6   | 5 | 1.3 ± 1.0 | 355 ± 47 | 5 |
| [Deg1,3,Bpa2,M]PTH(1–14)      | 700 ± 160 | 3 | >>10,000 | 20 ± 2.4 | 4 |
| [Ac5c1,Bpa2,Aib3,M]PTH(1–15)  | 1800 ± 500 | 3 | >>10,000 | 27 ± 2.8 | 4 |
| [Deg1,3,Bpa2,M]PTH(1–15)      | 710 ± 140 | 3 | >>10,000 | 42 ± 14  | 3 |
| [Deg1,3,Bpa2,M]PTH(1–21)      | 110 ± 20  | 4 | >>10,000 | 32 ± 6.5 | 3 |
| PTHR-P(5–36)                  | 36 ± 8   | 3 | >>10,000 | 9.8 ± 1.0 | 4 |

* Peptide are described further in Table I.

** Competition binding assays were performed with 125I-[Alb1,3,M]PTH(1–15) tracer radioligand.

*** The EC50 values were calculated by non-linear regression analysis. The maximum cAMP responses observed in the assays (Emax values) are shown.

P[1–15] and [Deg1,3,Bpa2,M]PTH(1–21) to antagonize PTH agonist analogs in ROS 17/2.8 cells. These rat osteosarcoma-derived cells endogenously express the rat PTHR at a surface density of ~50,000 receptors/cell (49) and were found to give more discernable antagonist responses to PTH analog peptides than did HKRK-B7 and HKKK-B28 cells (data not shown), presumably because of their lower level of receptor expression. As agonist antagonists in these assays, we used PTH(1–34) to represent a two-site mode of agonist binding (see Introduction) and [Ac5c1,Aib3,M]PTH(1–14) to represent a J domain-selective mode of agonist binding. These two agonists are approximately equipotent for stimulating cAMP accumulation in ROS 17/2.8 cells, thus the 1 nM concentration of each peptide used in these experiments resulted in a 40–50-fold increase in cAMP accumulation, relative to the basal cAMP level (Fig. 2). Both [Deg1,3,Bpa2,M]PTH(1–21) and [Deg1,3,Bpa2,M]PTH(1–15) inhibited to near completion (89 and 76%, respectively) the cAMP response induced by PTH(1–34). This maximal level of inhibition was similar to that attained with PTHR-P(5–36) (94%), although 70- and 20-fold higher concentrations of the two N-terminal peptides were required to achieve 50% inhibition than was required for PTHR-P(5–36) (IC50s = 2.6 μM, 0.7 μM, and 36 nm, respectively, Fig. 2A). The 3–4-fold higher inhibitory potency of [Deg1,3,Bpa2,M]PTH(1–21), as compared with that of [Deg1,3,Bpa2,M]PTH(1–15), is consistent with the 6-fold higher apparent binding affinity that the longer analog exhibited in HKRK-B7 cells (Table III).

When [Ac5c1,Aib3,M]PTH(1–14) was used as the agonist, [Deg1,3,Bpa2,M]PTH(1–15), [Deg1,3,Bpa2,M]PTH(1–21), and PTHR-P(5–36) each again achieved ~90% inhibition, and the IC50s for the two N-terminal peptides were similar to each other and ~100-fold higher than that observed for PTHR-P(5–36) (1.3 μM, 0.9 μM, and 11 μM, respectively, Fig. 2B). These data show that, although weaker than PTHR-P(5–36), the modified PTH(1–15) and PTH(1–21) analogs can clearly function as antagonists in these osteoblast-derived cells that expresses the intact PTHR.

**Inhibition and Binding Properties in COS-7 Cells Expressing PTHR-delN**—To examine the receptor domains used by the N-terminal PTH antagonist analogs, we used COS-7 cells tran-
siently transfected with PTHR-delNt, a PTHR construct that lacks most of the N domain of the receptor. In COS-7 cells, PTHR-delNt is expressed at ~50% the level of the wild-type PTHR and couples efficiently to the cAMP-signaling pathway (6). As a PTH(1–34)-based agonist peptide in these studies we used [Aib<sup>1,3</sup>]<sub>β</sub>PTH(1–34), which is ~100-fold more potent on PTHR-delNt than is unmodified PTH(1–34) (30) (the potency of the latter peptide on PTHR-delNt was too weak to permit use in these experiments). As a PTH(1–14)-based agonist peptide we again used [A<sub>c</sub>ε<sub>c</sub>,A<sub>b</sub>β,M]<sub>β</sub>PTH(1–14), which exhibits nearly the same agonist potency on PTHR-delNt as it does on the wild-type PTHR (30). In the absence of antagonist, [Aib<sup>1,3</sup>]<sub>β</sub>PTH(1–34) (100 nM) and [A<sub>c</sub>ε<sub>c</sub>,A<sub>b</sub>β,M]<sub>β</sub>PTH(1–14) (1 nM) stimulated 11- and 21-fold increases in cAMP levels, respectively, relative to the cAMP levels in the untreated cells (Fig. 3, A and B). The cAMP response induced by [Aib<sup>1,3</sup>]<sub>β</sub>PTH(1–34) was inhibited by ~70% by both [Deg<sup>1,3</sup>,B<sub>p</sub>a<sub>2</sub>,M]<sub>β</sub>PTH(1–21) and [Deg<sup>1,3</sup>,B<sub>p</sub>a<sub>2</sub>,M]<sub>β</sub>PTH(1–15). At the indicated concentrations and after a 30-min incubation, intracellular cAMP was measured. Shown are the resulting cAMP responses (means ± S.E.) from 5 to 10 experiments, each performed in duplicate. In each experiment and for each agonist, the cAMP level observed in the absence of a test antagonist was calculated as a percentile of the cAMP level observed in cells treated with that agonist alone, the mean values of which were 100 ± 7 and 187 ± 11 pmol/well, for the experiments shown in A and B, respectively. The corresponding basal cAMP values, indicated by the dashed lines in the graphs, were 8.8 ± 0.8 and 8.9 ± 1.1 pmol/well, respectively, and were not subtracted in the calculations. In C, COS-7 cells transiently transfected to express PTHR-delNt were used in competition binding studies performed with the [<sup>125</sup>I]-[Aib<sup>1,3</sup>,M]<sub>β</sub>PTH(1–15) tracer radioligand and varying concentrations of unlabeled PTHrP(5–36) or [Deg<sup>1,3</sup>,B<sub>p</sub>a<sub>2</sub>,M]<sub>β</sub>PTH(1–15). The binding data show the amount of radioligand specifically bound (SB) calculated as a percentile of the total radioligand specifically bound in the absence of competitor (B0). Shown are combined data (means ± S.E.) from three experiments, each performed in duplicate. The IC<sub>50</sub> value obtained for the [Deg<sup>1,3</sup>,B<sub>p</sub>a<sub>2</sub>,M]<sub>β</sub>PTH(1–15) binding curve was 1400 nM. A curve was not fit to the data obtained with for PTHrP(5–36), because no response was observed.

![Figure 3](http://www.jbc.org/Downloaded from http://www.jbc.org)
the intact PTHR using the same PTH(1–14) agonist peptide (Fig. 2B). In contrast, PTHR(P5–36) failed to inhibit either agonist peptide on PTHR-delNt (Fig. 3, A and B). Consistent with the divergent capacities of these N- and C-truncated analogs to function as antagonists on PTHR-delNt, [Deg1–3, Bpa2, M]PTH(1–15) fully inhibited (IC50 = 0.8 µM) the binding of the 125I-[Aib1–3, M]PTH(1–15) radioligand to COS-7 cells expressing PTHR-delNt, whereas PTHR(P5–36) failed to inhibit this binding (Fig. 3C). These results established that the inhibitory action of both [Deg1–3, Bpa2, M]PTH(1–15) and [Deg1–3, Bpa2, M]PTH(1–21), but not that of PTHR(P5–36), is based on binding interactions that occur predominantly, if not exclusively, to the PTHR J domain.

We then examined the capacity of a single high concentration (10 µM) of [Deg1–3, Bpa2, M]PTH(1–15) to shift the cAMP dose–response curve obtained with PTH(1–34) in ROS 17/2.8 cells (Fig. 4A) and for that obtained with [Aib1–3]PTH(1–34) in COS-7 cells expressing PTHR-delNt (Fig. 4B). In ROS 17/2.8 cells, the addition of [Deg1–3, Bpa2, M]PTH(1–15) caused a 4-fold rightward shift in the PTH(1–34) dose–response curve, relative to that obtained in the absence of the antagonist (EC50 = 1.2 and 0.3 nM, respectively). This shift occurred in a
parallel fashion and resulted in approximately the same maximum cAMP level as that attained in the absence of antagonist (p = 0.5). Similarly, in COS-7 cells expressing PTHR-delNt, the addition of [Deg1,3,Bpa2,M]PTH(1–15) caused an 8-fold rightward and parallel shift in the dose-response curve obtained for [Aib1,3]PTH (1–34), compared with that obtained in the absence of antagonist (EC50s 560 and 70 nM, respectively) and caused little or no change in the Emax value (Fig. 4B, p = 0.1). The analog [Deg1,3,Bpa2,M]PTH(1–14) caused a similar 10-fold rightward shift in the dose-response curve obtained for [Aib1,3]PTH(1–34) in COS-7 cells expressing PTHR-delNt (data not shown). These effects of the antagonists on the agonist potency curves (i.e., parallel, rightward shifts with same Emax values) are consistent with a competitive mechanism of inhibition (50).

**Inhibition of Constitutively Active Mutant PTH Receptors in COS-7 Cells**—We then investigated the capacity of the antagonist analogs to inhibit the basal cAMP-stimulating activity of two constitutively active mutant PTHR receptor derivatives. The first of these derivatives was a “tethered” ligand-receptor construct in which the N-terminal domain of the PTH receptor is replaced by the (1–9) segment of native PTH, representing the minimum agonist pharmacophore of the ligand (31). In this construct, the ligand sequence is covalently joined to the body of the PTHR via a tetra-glycine linker between His9 of the ligand sequence and Glu182 of the receptor, which is located approximately nine residues N-terminal of the predicted extracellular end of TM1. When transiently transfected into COS-7 cells, this construct, in the absence of exogenously added ligand, results in basal cAMP levels that are comparable to those seen in COS-7 cells transfected with the wild-type PTHR and stimulated maximally with PTH(1–34). As shown in Fig. 5A, the addition of [Deg1,3,Bpa2,M]PTH(1–14) (10 μM) to such transfected COS-7 cells caused an ~40% diminishment in the basal cAMP levels. The analog PThrP(5–36) resulted in a small (16%) yet significant (p = 0.02) reduction in basal signaling activity (Fig. 5A).

The second constitutively active mutant PTHR utilized was PTHRcamH223R. This intact mutant PTHR contains an activating point mutation (His223 → Arg) at or near the cytoplasmic terminus of TM2 (46). We included in these studies [Deg1,3,Bpa2,M]PTH(1–14), [Ac5c1,Bpa2,Aib3,M]PTH(1–14), as
well as two C-terminally extended PTHrP ligands known to function as inverse agonists on this constitutively active PTHR, [Leu$^{1,15}$,n-Trp$^{12}$]PTHrP(5–36) and [Bpa$^{2}$]PTHrP(1–36) (43). Each of the tested analogs, applied at a concentration of 10 μM, reduced to at least some degree the intracellular cAMP levels in COS-7 cells expressing PTHRα/β2 (5–36) (Fig. 5B). The strongest reduction (72%) occurred with [Ac$^{5,12}$,Bpa$^{2}$,Aib$^{3}$,M]-PTH(1–15) (30 %) followed in rank-order by [Leu$^{1,15}$,n-Trp$^{12}$]PTHrP(5–36) (49%), [Bpa$^{2}$]PTHrP(1–36) (33%), and [Deg$^{1,3}$,Bpa$^{2}$,M]-PTH(1–14) (28%).

Inhibition of Ligand-induced Inositol Phosphate Signaling in COS-7 Cells Expressing the Wild-type PTHR—Because the wild-type PTHR can also couple to the PLC/IP signaling pathway, we analyzed the capacity of the antagonist ligands to inhibit this mode of PTHR signaling. As shown in the experiments of Fig. 6A performed in COS-7 cells transfected with the hPTHrP, PTHrP(5–36) and [Deg$^{1,3}$,Bpa$^{2}$,M]-PTH(1–15), each at a concentration of 30 μM, did elicit an IP response and inhibited by more than 90% (p < 0.001) the IP response induced by [Ac$^{5,12}$,Aib$^{3}$,M]-PTH(1–14) at a concentration of 1 μM. Similarly, [Deg$^{1,3}$,Bpa$^{2}$,M]-PTH(1–14) and [Deg$^{1,3}$,Bpa$^{2}$,M]-PTH(1–21) lacked IP signaling activity and inhibited by more than 90% the response induced by [Ac$^{5,12}$,Aib$^{3}$,M]-PTH(1–14) (data not shown). As shown in the experiments of Fig. 6B, PTHrP(5–36) (30 μM) inhibited by more than 90% (p < 0.001) the IP response induced by PTH(1–34) (300 nM), whereas [Deg$^{1,3}$,Bpa$^{2}$,M]-PTH(1–15) (30 μM) resulted in partial (22%) inhibition (p = 0.04). These IP antagonist results are fully consistent with the cAMP-antagonism data described above and are also consistent with the notion that mechanism of ligand-induced IP signaling in the PTHR is similar to the mechanism of ligand-induced cAMP signaling, in that each involves interactions between the N-terminal residues of the ligand and the juxtamembrane region of the receptor (28, 30, 51).

**DISCUSSION**

In this study we explored whether a PTHR ligand that binds exclusively to the portion of the receptor comprised of the extracellular loops and seven transmembrane helices (the J region) could function as a PTHR antagonist. Our results showed that this is indeed possible, as PTH(1–14), PTH(1–15), and PTH(1–21) agonist analogs, previously shown to interact mainly, if not exclusively, with the PTHR J domain (7, 11, 29, 30), when modified with activity-lowering substitutions at positions 1–3, exhibit antagonist properties on both the intact PTHR, as well as on PTHR-delNt, which lacks most of the N-terminal extracellular domain (N domain) of the receptor. These new antagonist ligands differ structurally and mechanistically from all previous PTHR antagonists as the latter analogs are C-terminally extended to at least position 34 and depend strongly on interactions with the N domain of the receptor to achieve their binding affinity and, hence, inhibitory potency (4, 5, 52). The modifications that conferred antagonist properties to the new N-terminal PTH analogs included the removal of the N-terminal amino group, the substitution of valine 2 by arginine, tryptophan, or Bpa, and the substitution of residues 1 (normally serine or alanine) and 3 (normally serine) by Deg. The activity-diminishing effects observed for these substitutions in the context of the modified N-terminal PTH fragment analogs are fully consistent with the known importance of the first few residues of native PTH(1–34) in inducing PTHR activation (34–36,41), particularly that of valine$^{2}$ (9, 38, 40). In the current analogs, the strongest dissociation of binding and signaling activities occurred with the valine $^{2}$ → Bpa substitution; combining Bpa$^{2}$ with the Deg$^{1,3}$ substitutions led to analogs with little or no cAMP or IP agonist activity, even when present at concentrations that would nearly saturate the receptor binding sites (Table II, Fig. 6). The tri-residue sequence, Deg$^{1}$-Bpa$^{2}$-Deg$^{3}$, thus constitutes a strong antagonist pharmacophore for the PTHR and, as such, was incorporated into the affinity-enhanced [M]-PTH(1–14), [M]-PTH(1–15), and [M]-PTH(1–21) analog scaffolds for subsequent functional studies. The functional properties of these three analogs were found to be very similar; the slightly (6-fold) higher binding affinity of the longer PTH(1–21) analog, compared with the PTH(1–14) and PTH(1–15) analogs (Table III) resulted in slightly higher inhibitory potencies, as seen in some of the antagonism assays (e.g. Fig. 2A), reflecting mostly the contribution of residue 19 to binding affinity (11).

One goal of these studies was to obtain new antagonist ligands that could be used to further explore the ligand binding and activation mechanisms that operate in the PTHR. In this regard, the results of our studies are generally consistent with and extend the two-site model that has been proposed for the PTH/PTHR interaction mechanism (3–6). The principal postulates of this model are depicted in Fig. 7 and in our current studies are perhaps best demonstrated by the observations that [Deg$^{1,3}$,Bpa$^{2}$,M]-PTH(1–15) and [Deg$^{1,3}$,Bpa$^{2}$,M]-PTH(1–21) maintained approximately the same inhibitory potency on PTHR-delNt as they did on the intact PTHR, whereas in contrast, PTHrP(5–36), a highly effective antagonist on the intact PTHR, exhibited little or no inhibitory capacity on the N-terminally truncated receptor. These divergent functional activities observed for the N- and C-terminally truncated antagonists on PTHR-delNt provided the principal support for our conclusion that the new N-terminal antagonist analogs bind purely to the J domain of the receptor, and thus differ mechanistically from the previous C-terminally extended antagonists, including the classical N-terminally truncated antagonists (36–38), which derive the major portion of their binding energy from interactions with the N domain of the receptor (4, 5, 52). Our findings with PTHR-delNt also demonstrate that interactions with the N
domain of the receptor are not necessary for antagonizing the PTHR, at least in the case where the antagonist binds with high affinity to the J domain, as do the new N-terminal PTH antagonist analogs described here.

The overall results so far on the PTH/PTHR interaction mechanism achieved with multiple PTH ligand analogues, mutant PTH receptors, and photo-affinity cross-linking studies, provide an initial definition of the regions of interaction between the ligand and the receptor. Although the new antagonists of the current study bind exclusively to the J domain, we also found evidence to suggest that the classical, N-terminally truncated antagonists, represented here by one of the strongest antagonists, PTHR-P(5–36) (38, 43), do not bind purely to the N domain of the receptor, a possibility that has been raised previously (3, 6). The new evidence for this includes the capacity of PTHR-P(5–36) to fully inhibit the cAMP-stimulating activity of [Ac$_5$c$_1$,Aib$_3$]PTH(1–14) (a J domain-specific agonist) on the PTHR (Fig. 2B), as well as the capacity of PTHR-P(5–36) to fully inhibit the binding of the 125I-[Aib$_1$,3,M]PTH(1–15) radioligand to the intact receptor (Fig. 1A). These results thus suggest that some portion of PTHR-P(5–36) directly interacts with the PTHR J domain. Consistent with this possibility, recent mutational and photo-affinity cross-linking data provide evidence for interactions or proximities between residues in the 5–27 region of the ligand and the PTHR J domain. Thus, the extension of PTH(1–14) peptides to position 20/21 and inclusion of the Glu$^{19} \rightarrow$ Arg substitution enhances binding affinity and/or signaling potency on PTHR-delNt (the isolated J domain) by as much as 100-fold (11). In cross-linking studies, a PTH(1–34) analog modified with benzophenone at position 17 cross-linked to Leu$^{261}$ in the first extracellular loop (53), a benzophenone 13-modified PTH(1–34) peptide cross-linked to Arg$^{196}$ near the N domain/TM1 boundary (13), and [Bpa$_3$]-PTHrP(1–36), as well as [Bpa$_{19}$,M]PTH(1–21), each cross-linked to the extracellular terminus of TM2 (32). At least some such proximities in the PTHR J domain must contribute to the overall binding energy with which PTHR-P(5–36) interacts with the receptor. In further support of this hypothesis we found that although PTHR-P(5–36) failed to inhibit the binding of 125I-[Aib$_1$,3,M]PTH(1–15) to PTHR-delNt or the cAMP-signaling activity of [Ac$_5$c$_1$,Aib$_3$]M-PTH(1–14) and [Aib$_3$]PTH(1–34) on this truncated receptor (emphasizing the critical dependence of the ligand on the presence of the N domain of the receptor) it did inhibit marginally, yet significantly, signaling by PTHR-tethered-PTH(1–9), which like PTHR-delNt lacks nearly all of the N domain of the receptor. Any interactions that occur between PTHR-P(5–36) and the PTHR J domain would therefore be too weak by themselves to enable the inhibition of binding of N-terminal PTH (1–14) analogs modified with affinity-enhancing substitutions yet be strong enough to enable at least partial inhibition of the binding of the unmodified N-terminal PTH (1–9) sequence to the PTHR J domain.

Another possibility not entirely excluded by the present data is that PTHR-P(5–36) inhibits the modified PTH(1–14) agonist actions on the intact PTHR via an allosteric mechanism, for example, by binding to the PTHR N domain in a way that interferes with movements in the J domain that are required for the formation of a PTHR active state (54) that is specifically recognized by the PTH(1–14) agonist. In this regard, it is worth noting that several non-peptide antagonist compounds have been identified for other class 2 G protein-coupled receptors, and some of these appear to bind outside of the receptor region occupied by the agonist pharmacophore of the native ligand. Thus, a key binding determinant for a non-peptide antagonist for the calcitonin gene-related protein receptor maps to the receptor-associated-modifying protein that modulates the ligand-binding specificity of that receptor (55), and a binding determinant for a non-peptide antagonist of the glucagon-like-peptide-1 receptor was identified as a tryptophan residue located at position 23 near the N terminus of that receptor (56). On the other hand, Hoare and colleagues (27) have shown that several small molecule antagonists of the corticotropin-releasing factor (CRF) receptor bind directly to the isolated CRF receptor J domain, and residues required for the binding of such antagonists have been identified in the midregions of TM3 and TM6 of the intact CRF receptor and within ~10 amino acids of residues at the extracellular ends of the same helices that contribute to CRF binding (57). It would thus seem that these CRF receptor antagonists could inhibit CRF action via direct, competitive mechanisms. A small molecule antagonist for the glucagon receptor also binds within the J domain of that receptor and requires for this binding resides within the midregions of TM2 and TM3; in this case, however, the data point to a non-competitive mechanism of inhibition (58).

The mechanism by which PTHR-P(5–36) inhibits the J domain-selective agonists on the PTHR is not at present clear. It is clear, however, that the PTH(1–14)-based antagonists bind to the J domain of the receptor and appear to function via a direct competitive mechanism (Fig. 4B). This mode of inhibition renders these new antagonists potentially useful probes of the region of the PTHR J domain that is involved in binding and responding to the PTH agonist pharmacophore. That the Bpa$_3$ substitution in these antagonists produces approximately the same profile of antagonism and inverse agonism as that seen with Bpa$_2$-modified PTH(1–34) or PTHR-P(1–36) further suggests that the modified, N-terminal PTH antagonists interact with the PTHR in a fashion similar to that used by the N-terminal portion of the relatively unmodified, longer length ligands and thus do not mediate antagonism via long range, allosteric effects.

The overall results of our study show that it is possible to antagonize the PTH receptor by targeting ligands specifically to the PTHR J domain. The data provide support for and extend the current two-domain model of the PTH/PTHR binding and activation mechanisms. The principal determinants of the antagonist pharmacophore of the new analogs are the photolabile Bpa$_3$ group and the conformationally constraining Deg$_{1,3}$ substitutions. The ligands should therefore be useful for further mapping sites of interaction in the PTH/PTHR receptor interface, specifically those that occur within the J domain of the receptor and are involved in activation (16), and potentially for the further design of ligands (peptidic or non-peptidic) that exhibit potent, agonist or antagonist properties on the PTH/PTHR receptor.

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