Residue 19 of the Parathyroid Hormone (PTH) Modulates Ligand Interaction with the Juxtamembrane Region of the PTH-1 Receptor†

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ABSTRACT: Recent data suggest that the binding of parathyroid hormone (PTH)-(1−34) to the PTH-1 receptor (P1R) involves a high-affinity interaction between the C-terminal (15−34) domain of the ligand and the amino-terminal extracellular (N) domain of the receptor and a low-affinity interaction between the N-terminal (1−14) portion of PTH and the juxtamembrane (J) region of the receptor, with the latter interaction giving rise to signal transduction. We investigated whether residues C-terminal of position 14 in PTH(1−34) contribute to the J component of the interaction mechanism by comparing the capacity of PTH analogues N-terminally modified to improve J domain affinity and C-terminally truncated at position 14, 20, or 34 to stimulate cAMP formation in COS-7 cells transiently transfected with P1R-delNt, a P1R construct that lacks most of the N domain. In these cells, the potency of [M]PTH(1−34) (M = Ala^{1,3,12},-Gln^{10},Har^{11},Trp^{14},Arg^{19}) was 120-fold greater than that of [M]PTH(1−14) (EC_{50}s = 3.0 ± 0.8 and 360 ± 90 nM, respectively) but was equal to that of [M]PTH(1−20) (EC_{50} = 2.3 ± 0.3 nM). Reverting the Arg^{19} substitution of [M]PTH(1−20) to the native Glu reduced cAMP signaling potency on P1R-delNt by 12-fold (EC_{50} of [M]PTH(1−20)−Glu^{19} = 27 ± 4 nM), and it decreased the analog's capacity to inhibit the binding of the J domain-selective radioligand, ^{125}I-[Aib^{1,3},Nle^{8},M,Tyr^{21}]ratPTH(1−21), to the full-length P1R stably expressed in LLC−PK1 cells by 40-fold. The Glu^{19} → Arg modification, however, did not affect the capacity of PTH(15−31) to inhibit the binding of the N domain-selective radioligand ^{125}I-bPTH(3−34) to the full-length receptor. The overall data suggest that residues (15−20) of PTH, and particularly residue 19, contribute to the capacity of the N-terminal portion of the ligand to interact with the juxtamembrane region of the receptor. The NMR data presented in the accompanying manuscript suggests that this role could involve intramolecular effects on secondary structure in the N-terminal portion of the ligand.

Parathyroid hormone (PTH) is an 84-amino-acid peptide that regulates ionized calcium concentrations in the extracellular fluids; PTH-related protein (PTHrP) is a ∼139-amino-acid protein that plays a key role in the development of several organs, including the skeleton. For both peptide ligands, the first 34 amino acids contain sufficient structural information for high affinity binding to and activation of the PTH receptor type-1 (P1R). The 1−14 domains of PTH and PTHrP are highly homologous (nine identities in the rat sequences), whereas the 15−34 domains are considerably more divergent (three identities in the rat). Within each ligand, the major determinants of P1R-binding affinity reside within the 15−34 domains, whereas the major determinants of cAMP-signaling reside within the 1−14 domains. Thus, C-terminal fragments of PTH(1−34), such as PTH(15−34), bind to the P1R without inducing a cAMP signaling response (*1*−*4*), while N-terminal fragments, such as PTH(1−14), are capable of stimulating a weak cAMP response (EC_{50} of PTH-(1−14) = 200 μM), despite extremely weak binding affinity (undetectable for PTH(1−14)) (*5*, *6*). The P1R is a member of the family B subclass of G protein-coupled receptors (GPCRs) and is thus structurally related to several other receptors that bind peptide hormones that are similar in size to PTH(1−34), including calcitonin, secretin, glucagon, corticotropin-releasing hormone, and several others.

As with each family B GPCR that binds a peptide hormone, the P1R has a large (∼170 amino acid) amino-terminal extracellular (N) domain that contains six highly conserved cysteine residues. These cysteines are likely to define a specific disulfide-bonded tertiary fold (*7*), although the three-dimensional structure of this domain is currently unknown. The remaining portion of the receptor, the juxtamembrane, or J domain, includes the seven transmembrane helices, the connecting loops, and the cytoplasmic tail. On the basis of modeling considerations (*8*−*10*) and some functional data (*11*, *12*) it has been suggested that the heptahelical bundle of the P1R's J domain is organized in a

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[1] Abbreviations: PTH, parathyroid hormone; r, rat; h, human; PTHrP, PTH-related protein; P1R, type-1 PTH/PTHrP receptor; P1R-delNt, a P1R construct deleted for residues 24−181 of the N domain; IBMX, 3-isobutyl-1-methylxanthine; CD circular dichroism; NMR, nuclear magnetic resonance; Har, homoarginine; Nle, norleucine; Aib, α-amino-isobutyric acid, other amino acids in either the conventional one- or three-letter codes.

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fashion at least similar to that seen in rhodopsin, a model family A GPCR (*13*) for which the crystal structure is known (*14*). Receptor mutagenesis and chimera studies indicate that the N domain of the P1R contributes importantly to PTH binding affinity (*15*−*17*), while the J domain contributes predominantly to ligand-dependent induction of receptor activation (*5*, *11*, *15*, *18*). It has been shown for most of the family B peptide hormone-binding GPCRs that both the N and J domains are required for high affinity ligand binding and ligand-induced receptor activation (*15*, *19*−*23*). For the P1R, photoaffinity cross-linking and receptor mutational studies have provided insights into the overall topology of the bimolecular PTH(1−34)·P1R complex (*6*, *15*−*18*, *24*−*27*). The results of these studies have given rise to the two-domain hypothesis for the ligand/receptor interaction mechanism, which postulates that the C-terminal portion of PTH(1−34) interacts with the receptor's N domain to provide binding energy, and this interaction enables the weaker interaction between the N-terminal portion of the ligand and the J domain of the receptor that results in receptor activation (*28*, *29*). This two-domain hypothesis accommodates most of the current data derived from PTH/P1R interaction studies, but the precise structural components in the ligand and the receptor that participate in the N- and J-domain interactions, and the mechanistic roles that the key residues play in the interaction process are still not well defined.

One potential difficulty that can arise in analyzing the mechanism by which a family B peptide ligand interacts with its cognate receptor by the functional approach is based on the possibility that allosteric or cooperative interactions between distal portions of the ligand and/or receptor play a role in the overall interaction process. Thus, it may be difficult to identify the regions of the receptor or ligand that mediate the changes in activity that occur with a given structural change in either the ligand or receptor. As a means to circumvent this potential difficulty, we have taken a domain minimization approach in our functional analyses of the PTH/P1R interaction mechanism. This approach involves the use of short, but functional, PTH peptide analogues and an N-terminally truncated, but functional, PTH-1 receptor construct (P1R-delNt) that lacks most (residues 24−181) of the N domain. As part of these studies, we have shown that PTH(1−14) exhibits the same (albeit weak) potency ($EC_{50} \sim 200$ μM) for cAMP stimulation on P1R-delNt, as it does on the intact full-length P1R; this result established that the (1−14) domain of PTH interacts predominantly, if not exclusively, with the J domain of the receptor to induce receptor activation (*5*, *6*). These studies also demonstrated the importance of the interaction between the PTH(15−34) domain and the receptor's N domain for optimal activity of native PTH, PTH(1−34) is 1000-fold less potent on P1R-delNt than it is on P1R (*5*, *6*). These results are fully consistent with, and indeed helped establish, the two-domain hypothesis. We also observed in these studies, however, that PTH(1−34) is ~100-fold more potent on P1R-delNt than is PTH(1−14), a finding that suggests that residues C-terminal of residue 14 contribute, directly or indirectly, to the J domain interaction. In the present study, we explore this possibility by utilizing a series of PTH peptide analogues that extend C-terminally to either position 14, 20, or 34 and comparing the functional properties of these analogues in cells transfected with either P1R-delNt or the full-length P1R. The results indicate that the (15−20) region of PTH, and particularly residue 19, contribute, by direct or indirect mechanisms, to the J domain interaction.

MATERIAL AND METHODS

Peptides. The primary structures of the peptides utilized in this study are described in Table 1. The peptide bPTH-(3−34) ([$Nle^{8,18}$,Tyr^{34}]bovinePTH(3−34)NH_2) was purchased from Bachem (Torrance, CA); all other peptides were prepared on an Applied Biosystems model 430A peptide synthesizer using Fmoc main-chain protecting group chemistry, HBTU/HOBt/DIEA (1:1:2 molar ratio) for coupling reactions, and TFA-mediated cleavage/side-chain deprotection (MGH Biopolymer Synthesis Facility, Boston, MA). Nascent peptides were desalted by adsorption on a C18-containing cartridge and purified further by HPLC. Peptides were reconstituted in 10 mM acetic acid and stored at −80 °C. The purity, identity, and stock concentration of each peptide was secured by analytical HPLC, matrix-assisted laser desorption/ionization (MALDI) mass spectrometry and amino acid analysis. Radiolabeling of bPTH(3−34) and [$Aib^{1,3}$,M]rPTH(1−21) was performed using ^{125}I−Na (2,200 Ci/mmol, NEN) and chloramine-T; the resultant ^{125}I-labeled analogues were purified by HPLC.

Cell Culture. Cells were cultured at 37 °C in T-75 flasks (75 mM) in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (10%), penicillin G (20 units/ml), streptomycin sulfate (20 μg/mL), and amphotericin B (0.05 μg/mL) in a humidified atmosphere containing 5% CO_2 (Hyclone Laboratories, Logan, UT); stock solutions of trypsin/EDTA and antibiotics were from GIBCO. Cells were subcultured in 24-well plates prior to transfection and assay. COS-7 cells were transiently transfected with pCDNA1-based plasmids encoding either the intact wild-type human PTH-1 receptor (P1R) or the N-terminally truncated human P1R construct, P1R-delNt, using DEAE-dextran and 200 ng of cesium chloride-purified plasmid DNA per well, as described previously (*30*). Four days after transfection, the cells were used for assay. The HKRK-B7 and HKRK-B28 cell lines were derived from the porcine kidney cell line LLC−PK_1 by stable transfection with the pCDNA1-based plasmid encoding the full-length P1R and express ~950 000 and ~280 000 PTH receptors per cell, respectively (*31*). HKRK-B7 and HKRK-B28 cells were used for assay 24−48 h after confluency was attained.

cAMP Stimulation. Stimulation of cells with peptide analogues was performed in 24-well plates. Cells were rinsed with 0.5 mL of binding buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 5% heat-inactivated horse serum, 0.5% fetal bovine serum, adjusted to pH 7.7 with HCl) and treated with 200 μL of cAMP assay buffer (Dulbecco's modified Eagle's medium containing 2 mM 3-isobutyl-1-methylxanthine, 1 mg/mL bovine serum albumin, 35 mM Hepes-NaOH, pH 7.4) and 100 μL of binding buffer containing varying amounts of peptide analogue (final volume = 300 μL). The medium was removed after incubation for 1 h at room temperature, and the cells were frozen on dry ice, lysed with 0.5 mL 50 mM HCl, and refrozen (−80 °C). The cAMP content of the diluted lysate was determined by radioimmunoassay. EC_{50} and correspond-

Table 1: Peptide Primary Structures

	Peptide[a]
PTH(1−34)	S-V-S-E-I-Q-L-M-H-N-L-G-K-H-L-N-S-M-E-R-V-E-W-L-R-K-K-L-Q-D-V-H-N-Y-amide
PTH(1−20)	A-V-S-E-I-Q-L-M-H-N-L-G-K-H-L-N-S-M-E-R-amide
[Arg^{19}]PTH(1−20)	A-V-S-E-I-Q-L-M-H-N-L-G-K-H-L-N-S-M-R-R-amide
PTH(1−14)	A-V-S-E-I-Q-L-M-H-N-L-G-K-H-amide
	M′ Analogues
[M′]PTH(1−34)	A-V-A-E-I-Q-L-M-H-A-R-A-K-H-L-N-S-M-R-R-V-E-W-L-R-K-K-L-Q-D-V-H-N-Y-amide
[M′]PTH(1−34)−Glu^{19}	A-V-A-E-I-Q-L-M-H-A-R-A-K-H-L-N-S-M-E-R-V-E-W-L-R-K-K-L-Q-D-V-H-N-Y-amide
[M′]PTH(1−20)	A-V-A-E-I-Q-L-M-H-A-R-A-K-H-L-N-S-M-R-R-amide
[M′]PTH(1−14)	A-V-A-E-I-Q-L-M-H-A-R-A-K-H-amide
	M Analogues
[M]rPTH(1−34)	A-V-A-E-I-Q-L-Nle-H-Q-Har-A-K-W-L-A-S-V-R-R-Nle-Q-W-L-R-K-K-L-Q-D-V-H-N-Y-amide
[M]PTH(1−34)	A-V-A-E-I-Q-L-M-H-Q-Har-A-K-W-L-N-S-M-R-R-V-E-W-L-R-K-K-L-Q-D-V-H-N-Y-amide
[M]PTH(1−20)	A-V-A-E-I-Q-L-M-H-Q-Har-A-K-W-L-N-S-M-R-R-amide
[M]PTH(1−20)−Glu^{19}	A-V-A-E-I-Q-L-M-H-Q-Har-A-K-W-L-N-S-M-E-R-amide
[M]PTH(1−14)	A-V-A-E-I-Q-L-M-H-Q-Har-A-K-W-amide
	PTH(15−31) Analogues
PTH(15−31)	L-N-S-M-E-R-V-E-W-L-R-K-K-L-Q-D-V-amide
[R^{19}]PTH(15−31)	L-N-S-M-R-R-V-E-W-L-R-K-K-L-Q-D-V-amide
	Tracer Analogues
bPTH(3−34)	S-E-I-Q-F-Nle-H-N-L-G-K-H-L-S-S-Nle-E-R-V-E-W-L-R-K-K-L-Q-D-V-H-N-Y*-amide
[Aib^{1,3},M]rPTH(1−21)	Aib-V-Aib-E-I-Q-L-Nle-H-Q-Har-A-K-W-L-A-S-V-R-R-Y*-amide

[a] Peptide sequences are shown N-terminal to C-terminal, with amino acids represented by the conventional one letter code, with the exception of norleucine, homoarginine, and α-aminoisobutyric acid residues, which are represented by Nle, Har, and Aib, respectively. Peptides are derivatives of human PTH, with the exception of [M]rPTH(1−34), [Aib^{1,3},M]rPTH(1−21), and bPTH(3−34), which are rat (r) or bovine (b) PTH derivatives. The peptide designated as PTH(1−34) differs from native PTH(1−34) by having only Tyr at position 34 in place of the native Phe. The M′ set of modifications consisted of Ala^{1,3,10,12} and Arg^{11,19}, and the M set of modifications consisted of Ala^{1,3,12}, Gln^{10}, Har^{11}, Trp^{14}, and Arg^{19}, as C-terminal chain length permitted. Each peptide contained a free amino terminus and a carboxamide at the C-terminus. The asterisk on the Tyr of the tracer radioligand analogues indicates the position of the ^{125}I atom.

ing Maximum response values (Emax) values were calculated using nonlinear regression (see below).

Competition Binding Assays. Binding reactions were performed with HKRK-B7 or HKRK-B28 cells in 24-well plates. Cells were rinsed with 0.5 mL of binding buffer, and 100 μL of binding buffer, 100 μL of binding buffer containing various amounts of unlabeled competitor ligand, and 100 μL of binding buffer containing ca. 100 000 cpm of ^{125}I-bPTH(3−34) or ^{125}I-[Aib^{1,3},M]rPTH(1−21) (ca. 26 fmol; final volume = 300 μL) were added successively. Incubations were 4 h at 15 °C. Cells were then placed on ice, the binding medium was removed, and the monolayer was rinsed three times with 0.5 mL of cold binding buffer. The cells were subsequently lysed with 0.5 mL 5N NaOH and counted for radioactivity. The nonspecific binding for each experiment was determined by competition with a 1 μM dose of unlabeled [Nle^{8,21},Tyr^{34}]rPTH(1−34)NH$_2$ or [Aib^{1,3},M]rPTH(1−21). The maximum specific binding (B_0) was the total radioactivity bound in the absence of unlabeled ligand, corrected for nonspecific binding. Nonlinear regression was used to calculate binding IC$_{50}$ values (see below).

Data Calculation. Calculations were performed using Microsoft Excel. Nonlinear regression analyses of binding and cAMP dose−response data were performed using the four-parameter equation: $y_p = \text{Min} + [(\text{Max} - \text{Min})/(1 + (IC_{50}/x)^{\text{slope}})]$. The Excel Solver function was utilized for parameter optimization, as described previously (*32*, *33*). The statistical significance between two data sets was determined using a one-tailed Student's t-test, assuming unequal variances for the two sets.

RESULTS

Activities on P1R-delNT. To first determine if residues C-terminal of residue 14 of PTH might play a role in determining the ligand's capacity to interact with the juxtamembrane domain of the P1R, we tested the capacities of human PTH(1−14), PTH(1−20) and PTH(1−34) analogues to stimulate cAMP formation in COS-7 cells expressing P1R-delNt. This mutant PTH receptor construct, which is well expressed on the cell surface and couples efficiently to the cAMP signaling pathway, enables the analysis of ligand interactions that occur specifically to the J region of the receptor, as the receptor's N domain is largely absent (5). To enhance affinity/potency of the peptides on P1R-delNt and to thus broaden the range of potency changes that we could detect in our assays, we incorporated into some of the analogues either the M′ set of substitutions (Ser^1→Ala/Ser^3→Ala/Asn^{10}→Ala/Leu^{11}→Arg/Gly^{12}→Ala) or the M set of substitutions (Ser^1→Ala/Ser^3→Ala/Asn^{10}→Gln/Leu^{11}→Har(homoarginine)/Gly^{12}→Ala/His^{14}→Trp and Glu^{19}→Arg) (Table 1). We previously showed that the M′ modifications enhance the potency of PTH(1−14) analogues on P1R-delNt by ∼100-fold, in comparison to the corresponding unmodified analogues, while the M set of modifications enhance potency on P1R-delNt severalfold further (6,27). The use of P1R-delNt in these previous studies enabled us to establish that the enhancing effect that the substitutions had on PTH-(1−14) activity involved improved interactions with the J domain of the receptor. In the modified PTH(1−20) and PTH(1−34) analogues of the current study, we also included the Glu^{19} → Arg substitution (unless Glu is indicated), which we had previously found to enhance the affinity of PTH-

(1−34) (*34*) and PTH(1−28) (*35*) analogues on the intact P1R by five- to 10-fold.

In COS-7 cells expressing P1R-delNt, [M′]PTH(1−34) was 47-fold more potent in stimulating cAMP formation than was [M′]PTH(1−14) (EC_{50}s = 36 ± 8 and 1700 ± 500 nM, respectively, $P = 0.02$) and ∼ 4-fold more potent than [M′]-PTH(1−20) (EC_{50} = 130 ± 40 nM; $P = 0.02$, Figure 1A and Table 2). Similar results were obtained with the M-modified analogues, as [M]rPTH(1−34) was 120-fold more potent than [M]PTH(1−14) on P1R-delNt (EC_{50}s = 3.0 ± 0.8 and 360 ± 90 nM, respectively, $P = 0.001$) and approximately equipotent to [M]PTH(1−20) (Figure 1B and Table 2). The 13−150-fold higher potencies observed for the M′- and M-modified PTH(1−20) analogues on P1R-delNt, relative to the potencies of the structurally matched PTH(1−14) analogues on this receptor, indicated that the 15−20 regions of the PTH(1−20) analogues contributed favorably to the J domain interaction. In addition, the similar potencies that the PTH(1−20) analogues exhibited on P1R-delNt, relative to the corresponding PTH(1−34) analogues, suggested that the (21−34) regions of the modified PTH-(1−34) analogues did not contribute as strongly to the J domain interaction as did the (15−20) regions.

On the basis of the above results obtained with P1R-delNt showing that residues (15−20) of PTH contribute to the J domain interaction, as well as our previous findings that the Glu^{19} → Arg modification in PTH(1−34) (*34*) and PTH(1−28) (*35*) improves affinity at the wild-type P1R, we specifically assessed whether the Glu^{19} → Arg modification affects the ligand's capacity to interact with the J domain of the receptor. To do this, we used P1R-delNt and pairs of analogues that differed only by having Glu or Arg at position 19. In the [M′]PTH(1−34) analogue, the reversion of Arg^{19} to the native glutamate resulted in a 7-fold loss of cAMP-stimulating potency on P1R-delNt (EC_{50}s = 36 ± 8 vs 240 ± 140 nM, respectively, Table 2), and in the [M]PTH(1−20) analogue, the Arg^{19} → Glu reversion resulted in a 12-fold reduction in potency on P1R-delNt (EC_{50}s = 2.3 ± 0.3 vs 27 ± 4 nM, respectively, $P = 0.0003$; Figure 1B and Table 2). In otherwise unmodified PTH(1−20), the forward mutation of Glu^{19}→Arg resulted in an approximately 8-fold gain in cAMP-stimulating potency on P1R-delNt (EC_{50}s = 60 ± 14 and 8.3 ± 0.8 μM, respectively, $P = 0.01$; Figure 1C and Table 2). These results obtained with P1R-delNt indicate that the affinity-enhancing effect of the Arg^{19} modification is mediated, at least in part, via the J domain of the P1R.

Activities on the Wild-Type P1R. We then assessed the activities of the peptides on the wild-type P1R using the HKRK-B7 cell line, which is an LLC−PK1-derived cell line in which the intact human P1R is expressed, via stable transfection, at a density of ∼ 950 000 receptors per cell (31). As expected, the potency of unmodified PTH(1−34) on the wild-type P1R was 38 000-fold higher than its potency on P1R-delNt, whereas the potency of unmodified PTH(1−14) on the intact receptor was more similar (∼2-fold higher) than its potency on P1R-delNt (Tables 1 and 2). The potency of unmodified PTH(1−20) on the intact receptor was also similar (∼7-fold higher) to its potency on P1R-delNt. These results suggest that on the wild-type P1R, residues 21−34 of PTH contribute strong favorable interactions to the N domain of the receptor whereas residues 1−20 do not.

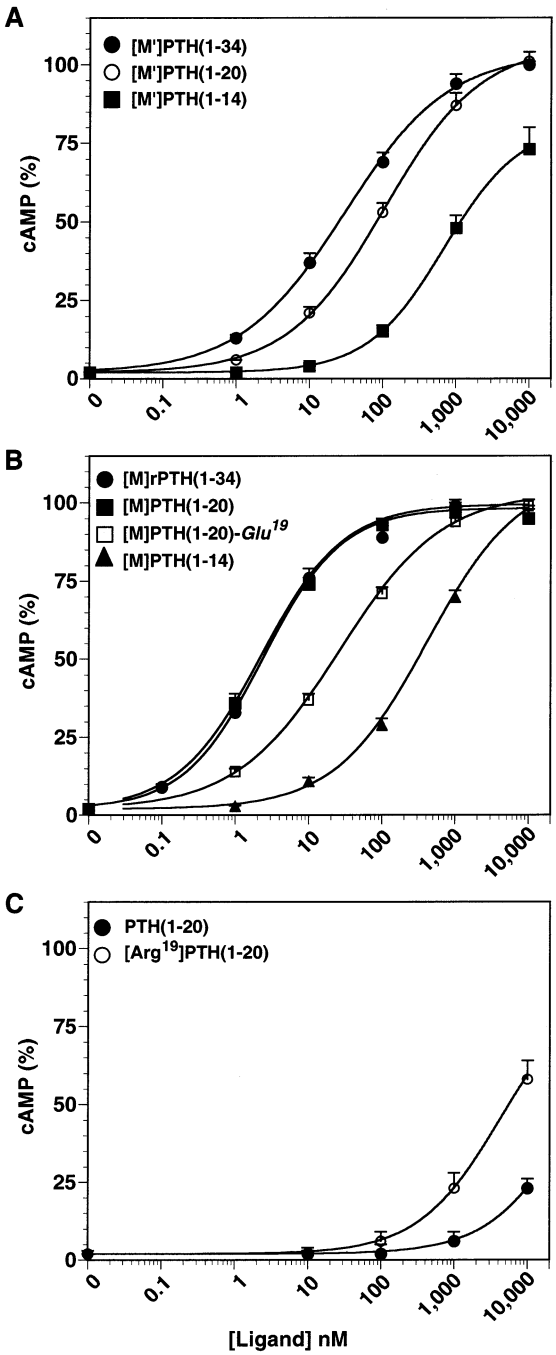

FIGURE 1: cAMP-stimulating activity of modified PTH analogues in COS-7 cells expressing an amino-terminally truncated PTH-1 receptor. COS-7 cells transiently transfected with P1R-delNt were tested for cAMP responses to varying doses of PTH analogues. The peptide structures are described in Table 1. The cAMP responses observed for each peptide were calculated as a percent of the maximum response observed in each experiment for [M′]PTH-(1−34) at 10 μM, which was 176 ± 11 pmol per well ($n = 10$); the corresponding basal cAMP level (not subtracted) was 2 ± 1 pmol/well. Shown are data (mean ± sem) combined from 5−10 separate experiments (as indicated by *n* in Table 2), each performed in duplicate.

In HKRK-B7 cells, [M]PTH(1−20) was 540-fold more potent than was [M]PTH(1−14) (EC_{50} = 0.22 ± 0.06 and 120 ± 30 nM, respectively; Figure 2A and Table 3), a difference in potency that was similar to that seen for these analogues in COS-7 cells expressing P1R-delNt (Table 2). Reversion of the Arg^{19} modification in [M]PTH(1−20) to

Table 2: cAMP Responses in COS-7 Cells Expressing P1R-delNt

peptide[a]	EC_{50}[b] (nM)	$E_{max(obs.)}$[c] (%)	*n*
PTH(1−34)	46 000 ± 800	31 ± 2	10
PTH(1−20)	60 000 ±14 000	59 ± 3	5
[Arg^{19}]-PTH(1−20)	8 300 ± 800	23 ± 2	5
PTH(1−14)[d]	313 000 ± 121 000	23 ± 5	3
M′ series			
[M′]PTH(1−34)	36 ± 8	100 ± 1	10
[M′]PTH(1−34)−Glu^{19}	240 ± 140	92 ± 5	3
[M′]PTH(1−20)	130 ± 40	101 ± 3	6
[M′]PTH(1−14)	1 700 ± 500	72 ± 7	5
M series			
[M]rPTH(1−34)	3.0 ± 0.8	100 ± 2	6
[M]PTH(1−20)	2.3 ± 0.3	97 ± 3	9
[M]PTH(1−20)−Glu^{19}	27 ± 4	99 ± 2	7
[M]PTH(1−14)	360 ± 90	95 ± 3	8

[a] Peptide structures are described in Table 1. [b] EC_{50} values were calculated by nonlinear regression. [c] The maximum response observed ($E_{max(obs.)}$) for each peptide was calculated as a percent of the $E_{max(obs.)}$ in each experiment for [M′]PTH(1−34) (10 μM), the mean of which was 176 ± 11 pmol of cAMP per well (n = 10); the corresponding basal level (not subtracted) was 4.3 ± 0.1 pmol of cAMP per well. [d] data are from ref *6*. Values are means (±sem) of the number of experiments indicated (n), each performed in duplicate.

Glu resulted in a 10-fold decrease in potency on the wild-type P1R (Figure 2A and Table 3), which was similar to the 12-fold reduction in potency seen for this reversion on P1R-delNt (Figure 1B and Table 2). Interestingly, [M]PTH(1−20) was 7-fold more potent on the P1R than was [M]PTH(1−34) (EC_{50} = 1.6 ± 0.3 nM; P = 0. 001; Figure 2A and Table 2). With the M′ series of analogues, [M′]PTH(1−20) was ∼70-fold more potent than [M′]PTH(1−14) (EC_{50}s = 5.3 ± 1.1 and 370 ± 8 nM, respectively, P = 0.0002) and 5-fold less potent than [M′]PTH(1−34) (EC_{50} = 0.96 ± 0.3 nM, P = 0.002; Table 2). Interestingly, no difference in potency was seen in comparing [M′]PTH(1−34) and [M′]PTH(1−34)−Glu^{19} (Table 3; see below and Discussion). Nevertheless, the overall results show that, as was observed on P1R-delNt, the PTH(1−20) analogues were considerably more potent on the wild-type P1R than were the corresponding PTH(1−14) analogues.

Competitive Binding with the Wild-Type P1R and PTH-(3−34) Radioligand. We then assessed the capacities of the peptides to bind to the wild-type P1R in HKRK-B7 by performing competition assays cells using ^{125}I-bPTH(3−34) as a tracer radioligand; this antagonist peptide radioligand is thought to bind predominantly, although not exclusively, to the N− domain of the P1R (29). In these assays, the capacities of the PTH analogues to inhibit binding of the tracer ligand increased as the peptide chain length extended C-terminally. Thus, inhibition was undetectable (IC_{50} > 100 μM) for the PTH(1−14) analogues; intermediate (IC_{50}s = 240 μM to 1.5 μM) for PTH(1−20) analogues, and maximal (IC_{50}s = 6 nM to 10 nM) for PTH(1−34) analogues (Figure 2B and Table 3). These binding data indicate that residues in both the 15−20 and 21−34 region of these ligands contribute to affinity at the wild-type P1R. The Arg^{19} modification clearly contributed to the P1R-binding affinity of the M-modified PTH(1−20) analogue, since the apparent affinity of [M]PTH(1−20)−Glu^{19} was ∼100-fold weaker than that of [M]PTH(1−20) (IC_{50}s = 160 ±80 and 1.5 ± 0.5 μM, respectively; Figure 2B and Table 3). In contrast, the apparent binding affinities of [M′]PTH(1−34) and [M′]-PTH(1−34)−Glu^{19} were nearly equivalent (IC_{50}s = ∼7 nM), a result consistent with the equivalent potencies observed for these two peptides in the cAMP signaling assays (Table 3).

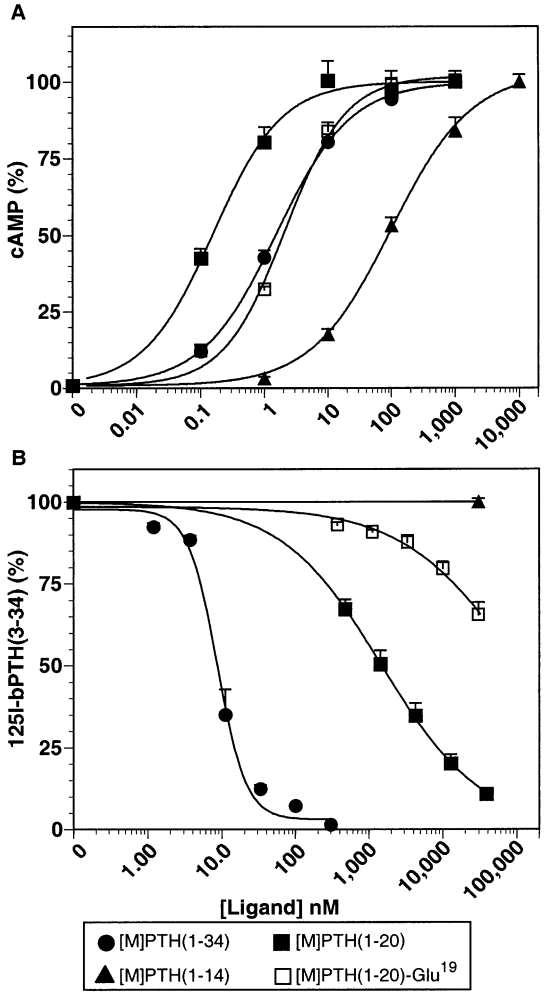

FIGURE 2: cAMP-signaling and binding properties of PTH analogues in HKRK-B7 cells. Peptides were analyzed in HKRK-B7 cells, which stably express the wild-type human P1R at high density (∼950 000 receptors/cell), for the capacity to stimulate cAMP formation (panel A) or to inhibit the binding of ^{125}I-[$Nle^{8,18}$,Tyr^{34}]-bPTH(3−34)NH_2 tracer radioligand (panel B). The peptide structures are described in Table 1. The cAMP responses were calculated as a percent of the maximum response observed for each peptide in each experiment. Data shown (mean ± sem) in panel A were combined from 3−8 separate experiments, and those shown in panel B were combined from four separate experiments; each experiment was performed in duplicate.

We then evaluated the effect of the Glu^{19} → Arg modification on the P1R-binding capacity of PTH(15−31) fragment analogue. For these assays we utilized HKRK-B28 cells, which is an LLC−PK1-derived cell line that expresses, via stable transfection, a full-length P1R at a moderate density, ∼280 000 receptors per cell (*31*). This cell line enables better detection of P1R-binding responses for low-affinity PTH fragments, such as PTH(15−31) and PTH(1−14), than does the HKRK-B7 cell line (*27*) (possibly, the lower receptor density of HKRK-B28 cells, as compared to HKRK-B7 cells, accounts for the improved capacity to detect

Table 3: cAMP and Binding Responses in HKRK-B7 Cells

	cAMP			binding	
peptide[a]	EC_{50}[b] (nM)	$E_{max(obs.)}$[c] (%)	*n*	IC_{50}[d] (nM)	*n*
PTH(1−34)	1.2 ± 0.3	100 ± 2	10	10 ± 3	6
PTH(1−20)	8 900 ± 100	59 ± 3	3	n.b.	
PTH(1−14)[e]	130 000 ± 20 000	42 ± 2	10	n.b.	
M′ series					
[M′]PTH(1−34)	0.96 ± 0.30	98 ± 3	5	6.1 ± 1.4	3
[M′]PTH(1−34)−Glu^{19}	1.5 ± 0.7	99 ± 7	3	7.3 ± 1.0	3
[M′]PTH(1−20)	5.3 ± 1.1	98 ± 3	9	240 000 ± 70 000	5
[M′]PTH(1−14)	370 ± 8	97 ± 2	3	n.b.	3
M series					
[M]PTH(1−34)	1.6 ± 0.3	100 ± 4	8	9.4 ± 2.2	3
[M]PTH(1−20)	0.22 ± 0.06	108 ± 7	7	1 500 ± 500	3
[M]PTH(1−20)−Glu^{19}	2.1 ± 0.1	103 ± 3	3	160 000 ± 80 000	3
[M]PTH(1−14)	120 ± 30	99 ± 3	3	n.b.	

[a] Peptide structures are described in Table 1. [b] EC_{50} values were calculated by nonlinear regression. [c] The $E_{max(obs.)}$ for each peptide was calculated as a percent of the $E_{max(obs.)}$ in each experiment for PTH(1−34) at 1 μM, for which the mean value was 420 ± 25 pmol of cAMP per well; the corresponding basal value (not subtracted) was 3.2 ± 0.2 pmol of cAMP per well (n =10). [d] Competition binding studies utilized ^{125}I-bPTH(3−34) as a tracer radioligand. [e] data are from ref *6*. Values are means (±sem) of the number of experiments indicated (*n*), each performed in duplicate. n.b., no binding detected.

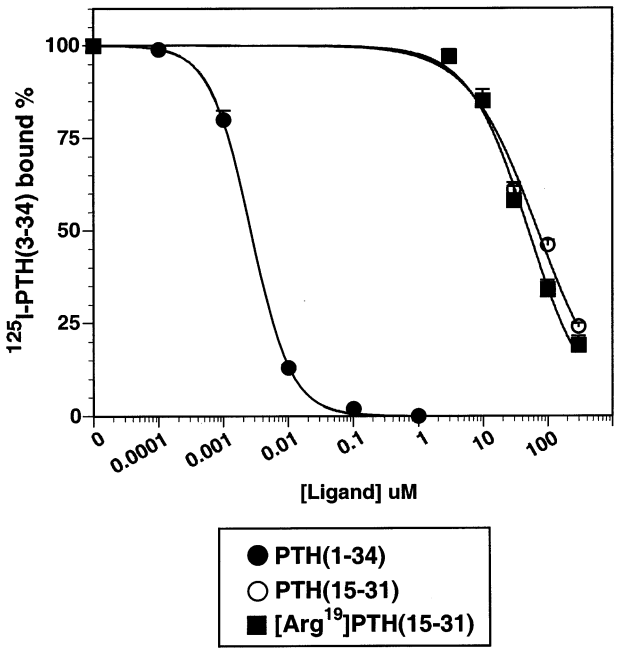

FIGURE 3: Effect of the Arg^{19} modification on the P1R-binding properties of PTH(15−31) in HKRK-B28 cells. The peptides, PTH(1−34), PTH(15−31), and [Arg^{19}]PTH(15−31), were evaluated for the capacity to inhibit the binding of ^{125}I-[$Nle^{8,18}$,Tyr^{34}]bPTH(3−34)NH_2 tracer radioligand to HKRK-B28 cells, which stably express a full-length P1R at moderate density (~280 000 receptors/cell). Shown are data (mean ± sem) combined from four separate experiments, each performed in duplicate.

weak binding interactions, but the mechanism underlying this effect is unclear[2]). As shown in Figure 3, PTH(15−31) and [Arg^{19}]PTH(15−31) inhibited the binding of ^{125}I-bPTH(3−34) to HKRK-B28 cells with comparable apparent affinities (IC_{50}s = 70 ± 3 μM and 54 ± 14 μM, respectively, P = 0.2). Thus, the Glu^{19}→Arg modification had no effect on the functional properties of this PTH(15−31) peptide fragment.

Competitive Binding with the Full-Length P1R and a PTH-(1−21) Radioligand. Finally, we examined the effect of the Arg^{19} modification on the capacity of [M]PTH(1−20) to inhibit the binding of ^{125}I-[$Aib^{1,3}$,M]rPTH(1−21) to the full-length P1R in B28 cells. According to the two-domain hypothesis, this tracer radioligand binds predominantly to the J domain of the receptor and is therefore more useful for assessing the affinity of ligands that bind mainly to the J domain of the receptor than is ^{125}I−PTH(1−34) or ^{125}I−PTH(3−34) radioligands, which bind predominantly to the N domain of the receptor (*36*). In support of this notion, the apparent binding affinities observed for [M]PTH(1−20) and [M]PTH(1−20)−Glu^{19} in assays using ^{125}I-[$Aib^{1,3}$,M]rPTH-(1−21) were 50−150-fold higher than the respective affinities observed using ^{125}I-bPTH(3−34) (compare Figure 2B and Figure 4 and Tables 3 and 4). Comparing the relative P1R-binding affinities that [M]PTH(1−20) and [M]PTH(1−20)−Glu^{19} exhibited with the ^{125}I-[$Aib^{1,3}$,M]rPTH(1−21) tracer revealed that the IC_{50} for [M]PTH(1−20) was 40-fold lower than that of [M]PTH(1−20)−Glu^{19} (28 ± 3 and 1100 ±200 nM, respectively; Figure 3 and Table 4). These results support the notion that the Arg^{19} modification increases the affinity with which the N-terminal (1−20) portion of PTH binds to the J domain of the P1R.

DISCUSSION

In the present study, we sought to determine whether residues C-terminal of position 14 in PTH(1−34) play a role in the capacity of the ligand to interact with the juxtamembrane (J) region of the receptor. This region of the receptor, which contains the seven transmembrane helices (TMs) and three extracellular loops (ECLs), has been implicated by cross-linking and mutagenesis studies to contain the major interaction determinants for residues in the 1−14 region of the ligand, whereas the amino-terminal extracellular (N)

[2] Subsequent to our current studies, we determined that our HKRK-B28 cells do not express the human P1R but, rather, a previously described (Jüppner, Gardella et al. (1994) *Endocrinology 134*, 879−884) chimeric P1R (OBR) comprised N-terminally of the opossum P1R and C-terminally of the rat P1R. The chimeric structure of this full-length P1R may contribute to its capacity to bind PTH fragment analogs with relatively high apparent affinity, but this remains to be determined.

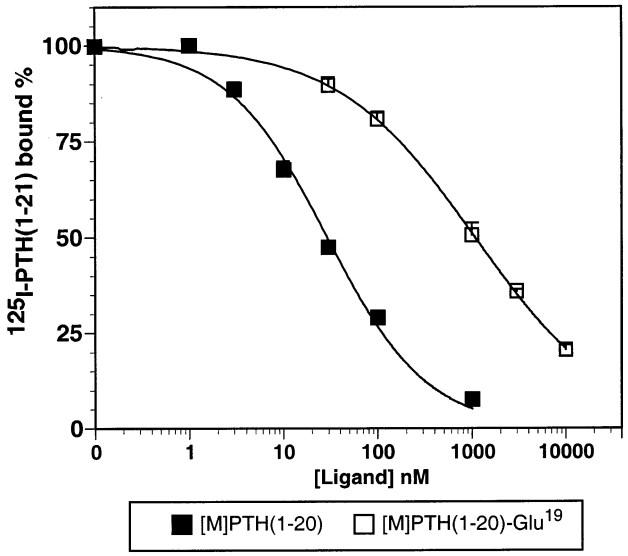

FIGURE 4: Effect of the Arg19 modification on the P1R-binding properties of [M]PTH(1−20) in HKRK-B28 cells. The peptides [M]PTH(1−20) and [M]PTH(1−20)−Glu^{19} were evaluated for the capacity to inhibit the binding of ^{125}I-[Aib^{1,3},M]rPTH(1−21) tracer radioligand to HKRK-B28 cells. Shown are data (mean ± sem) combined from four separate experiments, each performed in duplicate.

Table 4: Binding and cAMP Responses in HKRK-B28 Cells

	cAMP			binding	
peptide[a]	EC_{50}[b] (nM)	$E_{max(obs.)}$[c] (%)	*n*	IC_{50}[d] (nM)	*n*
				vs ^{125}I−PTH(3−34)	
PTH(1−34)	2.5 ± 0.4	280 ± 11	10	2.7 ± 0.3	4
PTH(15−31)−Glu^{19}	n.d.			70 000 ± 3 000	4
[Arg^{19}]PTH(15−31)	n.d.			54 000 ± 14 000	4
				vs ^{125}I−PTH(1−21)	
[M]PTH(1−20)	4.1 ± 1.7	315 ± 32	4	28 ± 3	4
[M]PTH(1−20)−Glu^{19}	9.3 ± 0.8	342 ± 35	4	1 100 ± 200	4

[a] Peptide structures are described in Table 1. [b] The EC_{50} values were calculated by nonlinear regression. The cAMP data for PTH(1−34) are from ref *37*. [c] Competition binding studies utilized ^{125}I-bPTH(3−34) or ^{125}I-[Aib^{1,3},M]PTH(1−21) as tracer radioligands. Values are means (±sem) of the number of experiments indicated (*n*), each performed in duplicate; n.d., not done.

domain of the receptor has been shown to contain the major binding determinants for the 15−34 region of PTH, as described by the two-domain hypothesis for the PTH/PTH-1 receptor interaction mechanism (*28*, *29*). A key component of our experimental design was the use of P1R-delNt, a PTH-1 receptor construct that lacks most (all but approximately eight residues) of the N domain, which in the mature wild-type receptor is approximately 170 amino acids in length. This N-terminally truncated receptor enables the analysis of ligand−receptor interactions that occur specifically to the J domain of the receptor (*5*, *6*, *37*). The results of the present study clearly indicate that residues in the 15−20 region of PTH contribute to the ligand's capacity to interact with the J region of the receptor, as we found that each of several PTH(1−20) analogues, including unmodified PTH(1−20), [M′]PTH(1−20) and [M]PTH(1−20), was 5−160-fold more potent for stimulating cAMP formation on the amino-terminally truncated PTH-1 receptor, P1R-delNt, than was each structurally matched PTH(1−14) analogue. In addition, the potency of each PTH(1−20) analogue on P1R-delNt was comparable with that of the structurally matched PTH(1−34) analogue; these latter results indicate that the 21−34 region of the ligand does not contribute strongly to the J domain interaction.

On the basis of our prior studies which showed that the Glu^{19} → Arg modification in PTH(1−34) and PTH(1−28) analogues enhances the affinity of these ligands for the wild-type P1R by severalfold (*34*, *35*), we specifically examined whether the residue at position 19 could have an effect on the J domain interaction. This was found to be the case, as [M′]PTH(1−34), [M]PTH(1−20), and [Arg^{19}]PTH(1−20), each of which contained arginine at position 19, were 7−12-fold more potent on P1R-delNt than were the Glu^{19}-containing counterpart peptides. Furthermore, in a competition binding assay that utilized the J domain-selective radioligand ^{125}I-[Aib^{1,3},M]rPTH(1−21), the apparent affinity with which [M]PTH(1−20) bound to the P1R was ∼40-fold higher than that of [M]PTH(1−20)−Glu^{19}. Thus, the mechanism by which the Arg^{19} modification increases potency involves increases in the affinity with which the N-terminal domain of PTH interacts with the J domain of the receptor.

The key question is whether ligand residues (15−20), and Arg^{19} specifically, contribute to the J domain interaction directly (e.g., via receptor contacts) or indirectly (e.g., via changes in ligand conformation). The accompanying manuscript (*36*) describes CD and NMR analyses of several peptides of the current study; the results of these spectroscopic studies reveal that, relative to Glu^{19}, the Arg^{19} modification substantially increases α-helical content in [M]-PTH(1−20), as well as in each of the other N-terminally intact PTH ligands tested. These findings, therefore, lead to the hypothesis that the beneficial effect of the Arg^{19} substitution on the receptor interaction process involves effects on peptide helicity. The possibility that the preferred bioactive conformation of the N-terminal portion of PTH is α-helical has been suggested by previous structural studies on isolated PTH(1−34) analogues (*9*, *38*), functional studies on conformationally constrained N-terminal PTH analogues (*37*), as well as hypothetical computer-generated models of the PTH−PTH receptor complex (*9*, *10*). Thus, the Arg^{19} modification could enhance the interaction of the (1−20) portion of the ligand with the J domain of the P1R by prestabilizing a bioactive (helical) conformation in the ligand.

On the other hand, the possibility that residue 19 in the ligand directly contacts the receptor is not excluded by the accompanying spectroscopic data. Furthermore, it is possible that Arg^{19} both stabilizes helical structure and contacts residues in the J domain of the receptor. Photochemical cross-linking data obtained with PTH(1−34) or PTHrP(1−36) analogues modified with benzophenone (Bp) have demonstrated proximity of other residues in the N-terminal portion of the ligand and the J domain of the P1R; thus analogues with Bp at positions one (26) or two (*25*, *39*) cross-linked to or near Met^{425} at the TM6/ECL-3 boundary; analogues with Bp at position 13 cross-linked to Arg^{186} at the TM1/N domain boundary (*40*), and analogues with Bp at position 27 cross-linked to Leu^{261} in ECL-1 (*41*). This latter result with Bp^{27} established that at least some residues in the C-terminal portion of receptor-bound PTH(1−34) can be within a few angstroms of the J domain of the receptor (*42*). We are currently pursuing cross-linking studies with Bp^{19}-

modified PTH analogues to more directly investigate the possibility that this residue comes within close proximity of the J domain of the P1R. The additional question of whether residue 19 affects the conformation of the ligand as it is bound to the receptor will ultimately require a more direct analysis of the ligand in complex with the receptor, as has recently been accomplished by NMR methods for pituitary adenylyl cyclase-activating peptide and its family B, G protein-coupled receptor (*43*). While, the chemical basis by which the arginine side chain at position 19 leads to enhanced interaction with the receptor and increased helicity is not clear at present, further experiments with additional PTH analogues modified at position 19 with side chains that are structurally homologous to arginine could help shed light on the mechanisms involved, and such experiments are currently underway.

The current functional studies provide some insights into the ligand−receptor interaction mechanism that would probably not be easy to appreciate from direct structural methods. For example, we observed that the Glu^{19} →Arg modification did not affect either the signaling potency or binding affinity of [M′]PTH(1−34) on the wild-type P1R (Table 3). Previously we showed that this substitution improves the binding affinity of unmodified PTH(1−34) on the wild-type P1R by approximately 10-fold (*34*). In our current studies, we found that the Arg^{19} enhanced the potency of [M′]PTH(1−34) on P1R-delNt by 7-fold (Table 2) and also that the Arg^{19} substitution enhanced the binding affinity of [M]PTH(1−20) on the wild-type receptor by ∼100-fold (Table 3). Taken together, these findings suggest that the enhancing effect of the Arg^{19} modification can be masked by the interaction of the 21−34 domain of the ligand with the N domain of the wild-type receptor, combined with the effects of the N-terminal modifications in [M′]PTH(1−34). These two latter effects may maximally stabilize the bioactive structure in the N-terminal portion of the ligand, such that any prestabilization by Arg^{19} would have little or no impact on affinity, at least at the wild-type P1R. We recently discussed such a possibility as a potential explanation for the observation that Aib modifications at positions 1 and 3 in PTH(1−34) increased the ligand's potency on P1R-delNt but had no effect on its potency on the wild-type P1R (*37*). We note that caution must be applied in extending our observations on the modified ligand analogues and mutant receptor to the native ligand−receptor system; however, the above considerations suggest the intriguing possibility that secondary structure is induced in the N-terminal portion of PTH as the hormone interacts with the native PTH receptor and that residue 19 plays a role in this effect.

Our studies also show that PTH(1−20) peptides, when modified, can function as highly potent agonists on both the intact P1R and P1R-delNt. In fact, we found that in HKRK-B7 cells, [M]PTH(1−20), with an EC_{50} of 0.2 nM, was 5−8-fold more potent than any of the PTH(1−34) analogues tested. In cells expressing P1R-delNt, however, [M]PTH-(1−20) exhibited an EC_{50} of 3 nM, which was more comparable with that observed for the PTH(1−34) analogues. These findings suggest the possibility that the in cells expressing high levels of the intact receptor, the 21−31 region of PTH(1−34) can attenuate the activity of PTH(1−34), perhaps by facilitating binding to uncoupled (spare) receptors. In HKRK-B28 cells, the potency of [M]PTH(1−20) (EC_{50} ∼ 4.1 nM) was comparable to that of PTH(1−34) (∼2.5 nM), and in SaOS-2 cells, the potency of [M]PTH(1−20) was ∼12-fold weaker than that of PTH(1−34) (EC_{50}s ∼ 5.9 and ∼0.2 nM, respectively, data not shown). Both HKRK-B28 and SaOS-2 cells express lower levels of P1R than do HKRK-B7 cells (∼280 000, ∼20 000, and ∼950 000 per cell respectively). Thus, the apparent super potency that [M]PTH(1−20) exhibits in HKRK-B7 cells appears to be related to the high receptor density in these cells. Nevertheless, [M]PTH(1−20) is the most potent shorter-length PTH analogue with the wild-type receptor identified to date.

From our current studies, it is now clear that residues 15−20, and particularly residue 19, of PTH contribute to the receptor interaction process via a mechanism that involves the N-terminal portion of the ligand and the juxtamembrane region of the receptor. Whether this contribution involves direct receptor contacts, effects on PTH structure, as suggested by the accompanying manuscript, or both, remains to be determined. Our overall functional results are consistent with the two-domain model of the PTH−PTH receptor interaction mechanism, and they extend this model to include a role, direct or indirect, for PTH residue 19 in the J domain component of the interaction. The current data thus provide new insights into the mechanisms by which PTH ligands interact with the PTH-1 receptor, but further investigations are needed to define these mechanisms more clearly. The overall work should lead to a better understanding of how peptide hormone/GPCR systems function and, potentially, to the design of novel PTH-based therapeutic compounds that are effective in treating diseases of bone and mineral metabolism, such as osteoporosis.

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