Articles

Functional Evidence for an Intramolecular Side Chain Interaction between Residues 6 and 10 of Receptor-Bound Parathyroid Hormone Analogues†

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ABSTRACT: The N-terminal domain of PTH(1−34) is critical for PTH-1 receptor (P1R) activation and has been postulated to be α-helical when bound to the receptor. We investigated the possibility that the side chains of residues 6 (Gln) and 10 (Gln or Asn) of PTH analogues, which would align on the same face of the predicted α-helix, could interact and thereby contribute to the PTH/P1R interaction process. We utilized PTH(1−11), PTH(1−14), and PTH(1−34) analogues substituted with alanine at one or both of these positions and functionally evaluated the peptides in cell lines (HKRK-B7 and HKRK-B28) stably expressing the P1R, as well as in COS-7 cells transiently expressing either the P1R or a P1R construct that lacks the amino-terminal extracellular domain (P1R-DelNt). In HKRK-B7 cells, the single substitutions of $Gln^6 \rightarrow$ Ala and $Gln^{10} \rightarrow$ Ala reduced the cAMP-stimulating potency of [Ala^3,Gln^{10},Arg^{11}]rPTH(1−11)NH_2 ∼60- and ∼2-fold, respectively, whereas the combined $Ala^{6,10}$ substitution resulted in a ∼2-fold gain in potency, relative to the single Ala^6 substitution. Similar effects on P1R-mediated cAMP-signaling potency and P1R-binding affinity were observed for these substitutions in [$Aib^{1,3}$,Gln^{10},Har^{11},Ala^{12},Trp^{14}]-rPTH(1−14)NH_2. Installation of a lactam bridge between the Lys^6 and the Glu^{10} side chains of [$Ala^{3,12}$,-Lys^6,Glu^{10},Har^{11},Trp^{14}]rPTH(1−14)NH_2 increased signaling potency 6-fold, relative to the nonbridged linear analogue. Alanine substitutions at positions 6 and/or 10 of [Tyr^{34}]hPTH(1−34)NH_2 did not affect signaling potency nor binding affinity on the intact P1R; however, Ala^6 abolished PTH(1−34) signaling on P1R-DelNt, and this effect was reversed by Ala^{10}. The overall data support the hypothesis that the N-terminal portion of PTH is α-helical when bound to the activation domain of the PTH-1 receptor and they further suggest that intrahelical side chain interactions between residues 6 and 10 of the ligand can contribute to the receptor interaction process.

Parathyroid hormone (PTH)[1] is an 84 amino acid protein, the first 34 amino acids of which contain sufficient information for binding to and activating the PTH receptor (PTH receptor type 1 or P1R). The P1R is a class II G protein-coupled receptor that is expressed in bone and kidney and couples most prominently to the adenylyl cyclase/protein kinase A signaling cascade. The PTH/P1R system plays a key role in controlling the concentrations of ionized calcium in the extracellular fluids. The P1R also binds PTH-related protein (PTHrP), a protein of 139, 141, or 171 amino acids that acts in paracrine fashion during development to control the morphogenesis of several tissues, including the skeleton. As with PTH, the first 34 amino acids of PTHrP contain sufficient information for high affinity P1R binding and potent induction of P1R-mediated signaling responses. There is growing interest in the development of PTH agonist analogues as therapeutics, as a recent clinical trial has shown that PTH(1−34) is effective in treating osteoporosis (*1*).

Within the PTH(1−34) sequence, the principal determinants of receptor binding affinity and receptor activation have been broadly mapped to the C- and N-terminal portions of the peptide, respectively (*2*, *3*). Short N-terminal fragments of PTH, such as PTH(1−14) and PTH(1−11), exhibit extremely weak binding affinities ($K_d \gg 100\ \mu M$) but are nonetheless capable of eliciting cAMP-signaling responses, albeit with potencies ($EC_{50}s \geq 100\ \mu M$) that are substantially weaker than that of PTH(1−34) ($EC_{50} \sim 2$ nM) (*4*). Recently, we described modified N-terminal PTH(1−14) and PTH-(1−11) analogues that exhibit enhanced signaling potencies (*5*−*7*), and some of these analogues, such as [$Aib^{1,3}$,Gln^{10},-Har^{11},Ala^{12},Trp^{14}]rPTH(1−14)NH_2, are as potent as PTH-(1−34) in cell-based signaling assays (*6*). The mechanisms by which the substitutions in these analogues enhance potency is not entirely clear, but some, such as the replace-

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[1] Abbreviations: PTH, parathyroid hormone; r, rat; h, human; P1R, type-1 parathyroid hormone receptor; IBMX, 3-isobutyl-1-methylxanthine; Aib, α-aminoisobutyric acid; Har, homoarginine; Nle, norleucine; Ac_5c, 1-aminocyclopentane-1-carboxylic acid; other amino acids in either the conventional one- or three-letter codes; CD, circular dichroism; GPCR, G protein-coupled receptor.

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Table 1: PTH Analogues Utilized and their Amino Acid Sequences[a]

Peptide	Sequences
rPTH(1-21)NH_2 (native)	Ala-Val-Ser-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-Lys-His-Leu-Ala-Ser-Val-Glu-Arg-Met-NH_2
PTH(1-11) Analogs	
[Ala^3,Gln^{10},Arg^{11}]rPTH(1-11)NH_2	Ala-Val-Ala-Glu-Ile-Gln-Leu-Met-His-Gln-Arg-NH_2
[Ala^{3,6},Gln^{10},Arg^{11}]rPTH(1-11)NH_2	Ala-Val-Ala-Glu-Ile-Ala-Leu-Met-His-Gln-Arg-NH_2
[Ala^{3,10},Arg^{11}]rPTH(1-11)NH_2	Ala-Val-Ala-Glu-Ile-Gln-Leu-Met-His-Ala-Arg-NH_2
[Ala^{3,6,10},Arg^{11}]rPTH(1-11)NH_2	Ala-Val-Ala-Glu-Ile-Ala-Leu-Met-His-Ala-Arg-NH_2
PTH(1-14) Analogs	
[Aib^{1,3},Gln^{10},Har^{11},Ala^{12},Trp^{14}]rPTH(1-14)NH_2	Aib-Val-Aib-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH_2
[Aib^{1,3},Ala^{6,12},Gln^{10},Har^{11},Trp^{14}]rPTH(1-14)NH_2	Aib-Val-Aib-Glu-Ile-Ala-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH_2
[Aib^{1,3},Ala^{10,12},Har^{11},Trp^{14}]rPTH(1-14)NH_2	Aib-Val-Aib-Glu-Ile-Gln-Leu-Met-His-Ala-Har-Ala-Lys-Trp-NH_2
[Aib^{1,3},Ala^{6,10,12},Har^{11},Trp^{14}]rPTH(1-14)NH_2	Aib-Val-Aib-Glu-Ile-Ala-Leu-Met-His-Ala-Har-Ala-Lys-Trp-NH_2
[Ala^{3,12},Gln^{10},Har^{11},Trp^{14}]rPTH(1-14)NH_2	Ala-Val-Ala-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH_2
[Ala^{3,12},Glu^6,Lys^{10},Har^{11},Trp^{14}]rPTH(1-14)NH_2	Ala-Val-Ala-Glu-Ile-Glu-Leu-Met-His-Lys-Har-Ala-Lys-Trp-NH_2
cyclo(6,10)-[Ala^{3,12},Glu^6,Lys^{10},Har^{11},Trp^{14}]rPTH(1-14)NH_2	Ala-Val-Ala-Glu-Ile-Glu-Leu-Met-His-Lys-Har-Ala-Lys-Trp-NH_2
[Ala^{3,12},Lys^6,Glu^{10},Har^{11},Trp^{14}]rPTH(1-14)NH_2	Ala-Val-Ala-Glu-Ile-Lys-Leu-Met-His-Glu-Har-Ala-Lys-Trp-NH_2
cyclo(6,10)-[Ala^{3,12},Lys^6,Glu^{10},Har^{11},Trp^{14}]rPTH(1-14)NH_2	Ala-Val-Ala-Glu-Ile-Lys-Leu-Met-His-Glu-Har-Ala-Lys-Trp-NH_2
PTH(1-34) Analogs	
[Tyr^{34}]hPTH(1-34)NH_2	Ser-Val-Ser-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-Lys-His-Leu-Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Lys-Lys-Leu-Gln-Asp-Val-His-Asn-Tyr-NH_2
[Ala^6,Tyr^{34}]hPTH(1-34)NH_2	Ser-Val-Ser-Glu-Ile-Ala-Leu-Met-His-Asn-Leu-Gly-Lys-His-Leu-Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Lys-Lys-Leu-Gln-Asp-Val-His-Asn-Tyr-NH_2
[Ala^{10},Tyr^{34}]hPTH(1-34)NH_2	Ser-Val-Ser-Glu-Ile-Gln-Leu-Met-His-Ala-Leu-Gly-Lys-His-Leu-Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Lys-Lys-Leu-Gln-Asp-Val-His-Asn-Tyr-NH_2
[Ala^{6,10},Tyr^{34}]hPTH(1-34)NH_2	Ser-Val-Ser-Glu-Ile-Ala-Leu-Met-His-Ala-Leu-Gly-Lys-His-Leu-Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Lys-Lys-Leu-Gln-Asp-Val-His-Asn-Tyr-NH_2
^{125} *I-PTH tracer radioligands*	
[Ac_5c[1],Aib^3,Nle^8,Gln^{10},Har^{11},Ala^{12},Trp^{14},Tyr^{15}]rPTH(1-15)NH_2	Ac_5c-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-Ala-Lys-Trp-Tyr*-NH_2
[Ala^{3,12},Nle^8,Gln^{10},Har^{11},Trp^{14},Arg^{19},Tyr^{21}]rPTH(1-21)NH_2	Ala-Val-Ala-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-Ala-Lys-Trp-Leu-Ala-Ser-Val-Arg-Arg-Tyr*-NH_2

[a] Peptides are derivatives of rat (r) or human (h) PTH and contained a free amino-terminus and an amidated C-terminus. Nonconventional amino acids include homoarginine (Har), norleucine (Nle), 1-aminoisobutyric acid (Aib), and 1-aminocyclopentane-1-carboxylic acid (AC_5C). Key substituted residues at positions 6 and 10 are underlined. Residues linked via side chain lactams are indicated by the suffix "cyclo" and are bracketed. Asterisks indicate iodinated tyrosines. Native rPTH(1−21) is shown as a reference sequence.

ment of Ala[1] with the conformationally constraining amino acid α-aminoisobutyric acid (Aib), have been shown by CD analysis to increase peptide helicity; findings which suggest that an α helix may be the preferred bioactive conformation for the N-terminal portion of PTH (*6*).

Photoaffinity cross-linking and receptor mutagenesis studies (*8*−*11*) have suggested that the molecular mechanism by which PTH(1−34) interacts with the P1R involves two principal components: a high affinity binding interaction between the C-terminal PTH(15−34) domain and the amino-terminal extracellular (N) domain of the receptor, and a lower affinity interaction between the N-terminal PTH(1−14) domain and the juxtamembrane (J) region of the receptor containing the seven transmembrane helices and three extracellular loops that gives rise to receptor signaling (the two domain hypothesis) (*12*, *13*). A key point of uncertainty in the mechanism concerns the structure of the ligand as it is bound to the receptor. Solution-phase NMR studies of PTH(1−34) have generally revealed a bi-helical structure—an N-terminal helix encompassing approximately residues 3−10 and a longer C-terminal helix encompassing approximately residues 17−30 and a turn or flexible hinge region connecting the two helices (*14*−*19*). On the other hand, a recent X-ray crystallographic analysis of PTH(1−34) revealed a single continuous helix that extends from residue 3−32 (*20*). Potentially consistent with such a linear structure is the study performed by Condon et al., in which it was found that a PTH(1−31) analogue containing three lactam bridges involving three pairs of residues, each with *i*, *i* + 4 spacing (e.g., Lys^{13}−Asp^{17}; Lys^{18}−Asp^{22}; and Lys^{26}−

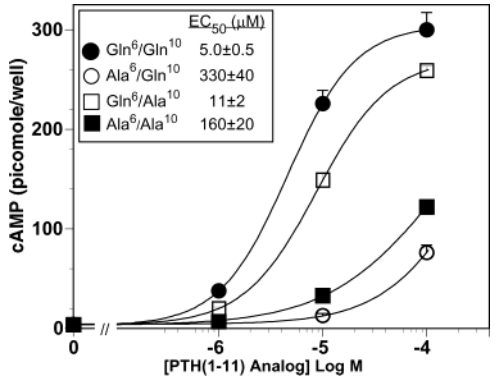

FIGURE 1: Effects of alanine substitutions at positions 6 and 10 in a PTH(1−11) analogue on cAMP-stimulating potency in HKRK-B7 cells. The parent peptide [Ala^3,Gln^{10},Arg^{11}]rPTH(1−11)NH$_2$ containing Gln at positions 6 and 10 (Gln^6/Gln^{10}, filled circles) and analogues of that peptide containing Ala substitutions at positions 6 and/or 10: [Ala^{3,6},Gln^{10},Arg^{11}]rPTH(1−11)NH$_2$ (Ala^6/Gln^{10}, open circles); [Ala^{3,10},Arg^{11}]rPTH(1−11)NH$_2$ (Gln^6/Ala^{10}, open squares); and [Ala^{3,6,10},Arg^{11}]rPTH(1−11)NH$_2$ (Ala^6/Ala^{10}, filled squares) were evaluated for cAMP-stimulating potency in HKRK-B7 cells. The corresponding EC$_{50}$ values are shown in the insets. Data shown (mean ± SEM) were combined from three separate experiments, each performed in duplicate.

Asp^{30}), was fully potent and highly helical, at least as determined by CD spectroscopy performed in buffer containing acetonitrile at a concentration of 20% (*21*).

Although the different biophysical analyses have suggested variability in the overall structure of PTH(1−34), most have provided support for the presence of at least some α-helical structure in the N-terminal region of PTH. The key question that remains, however, and one that has yet to be addressed by direct physical methods, is whether such an N-terminal α-helix is required for, or at least compatible with, a productive interaction with the PTH-1 receptor. In the current study, we utilized a functional approach to explore the possibility that the side chains of residues 6 and 10 of PTH, which are glutamine and asparagine, respectively, in the native hormone, can interact in the receptor-bound state. We reasoned that if the N-terminal domain of PTH is α-helical when bound to the receptor, then the side chains of these two residues would project in similar directions from adjacent turns of the helix because of their *i* (residue 6)−*i* + 4 (residue 10) spacing along the peptide chain and thus could interact, for example, via a hydrogen bonding mechanism and thereby contribute to bioactivity. Studies in other model helical peptide systems have shown that the side chain functional groups of Gln and Asn residues located at positions *i* and *i* + 4 can hydrogen bond and thereby stabilize helical structure (*22*). We further hypothesized that alterations to the putative interaction between residues 6 and 10 in PTH by a substitution at one site would change affinity and/or potency and that such an effect could be specifically modulated by a second substitution at the other site. We employed for our studies modified N-terminal PTH(1−11) and PTH(1−14) analogues (*5*, *6*), as well as an intact and relatively unmodified PTH(1−34) analogue, and we assessed the effects of substitutions at positions 6 and/or 10 in these analogues on their capacities to interact with the P1R in transfected cells. We used the intact P1R as well as a truncated P1R construct (P1R-DelNt) that lacks the large amino-terminal extracellular (N) domain, as this construct enables us to evaluate whether specific ligand substitutions modulate interactions to the N or J domains of the P1R (*5*). The results of the studies provide support for the notion that residues 6 and 10 of PTH can interact in the receptor-bound state, and thus, that the N-terminal portion of PTH is α-helical when bound to the activation domain of the receptor.

MATERIALS AND METHODS

Peptides. The amino acid sequences of the peptides used in the study are shown in Table 1. The scaffold peptides [Ala^3,Gln^{10},Arg^{11}]rPTH(1−11)NH$_2$ (*7*), [Aib^{1,3},Gln^{10},Har^{11},-Ala^{12},Trp^{14}]rPTH(1−14)NH$_2$ (*6*), and [Ala^{3,12},Gln^{10},Har^{11},-Trp^{14}]rPTH(1−14)NH$_2$ (*5*) have been described by us previously. Peptides were synthesized using Fmoc protecting chemistry and TFA-mediated cleavage/deprotection by the MGH Biopolymer Synthesis Facility, Boston, MA. Lactam bridge modifications between glutamic acid and lysine side chains were prepared using an orthogonal protection strategy (*23*) with allyl-protected amino acids. The nascent PTH(1−11) analogues were desalted by adsorption on a C18-containing cartridge and used directly; all other peptides were purified by reversed-phase 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. Iodination of [Ala^{3,12},Nle^8,Gln^{10},Har^{11},Trp^{14},Arg^{19},Tyr^{21}]-rPTH(1−21)NH$_2$ (*6*) and [Ac$_5$c^1,Aib^3,Nle^8,Gln^{10},Har^{11},Ala^{12},-Trp^{14},Tyr^{15}]rPTH(1−15)NH$_2$, which contains 1-aminocyclopentane-1-carboxylic acid (AC$_5$C) at position-1 (*24*), was performed using ^{125}I−Na and chloramine-T; the resultant radioligand was purified by HPLC.

Cells. The cell lines HKRK-B7 and HKRK-B28 (*25*) are derivatives of the porcine kidney cell line, LLC-PK$_1$, and express via stable transfection, recombinant, PTH-1 receptors (P1Rs) at surface densities of ∼950 000 and 280 000 receptors per cell, respectively. The HKRK-B7 cells express the wild-type human P1R. The HKRK-B28 cells, previously indicated to express the human P1R, have recently been determined to express a P1R chimera comprised of the opossum P1R from the N-terminus to the midregion of TM3 and the rat P1R from the midregion of TM3 to the C-terminus; these cells bind N-terminal PTH analogues with higher affinity than do HKRK-B7 cells (*26*). The cell line LDelNt-2 was derived from LLC-PK$_1$ cells via stable transfection with a plasmid encoding P1R-DelNt, a recombinant human PTH-1 receptor construct in which most (residues 24−181) of the amino-terminal extracellular domain is deleted (*6*). COS-7 cells in 24-well plates were transiently transfected with FuGENE 6 and CsCl$_2$ plasmid DNA (200 ng/well) encoding either the wild-type human P1R (P1R-WT) or P1R-DelNt.

cAMP Stimulation and Binding Assays. Assays were performed in whole cells in 24-well multi-well plates, as described previously (*6*). cAMP stimulation assays utilized 3-isobutyl-1-methylxanthine (2 mM) and were conducted for 60 min at room temperature. Binding reactions utilized ^{125}I-PTH(1−21) or ^{125}I-PTH(1−15) analogue (ca. 100 000 cpm per well) as a tracer radioligand and were conducted for 6 h at 4 °C (*5*).

Data Calculation. Nonlinear regression was used to calculate EC$_{50}$ and IC$_{50}$ values (*6*). Differences between

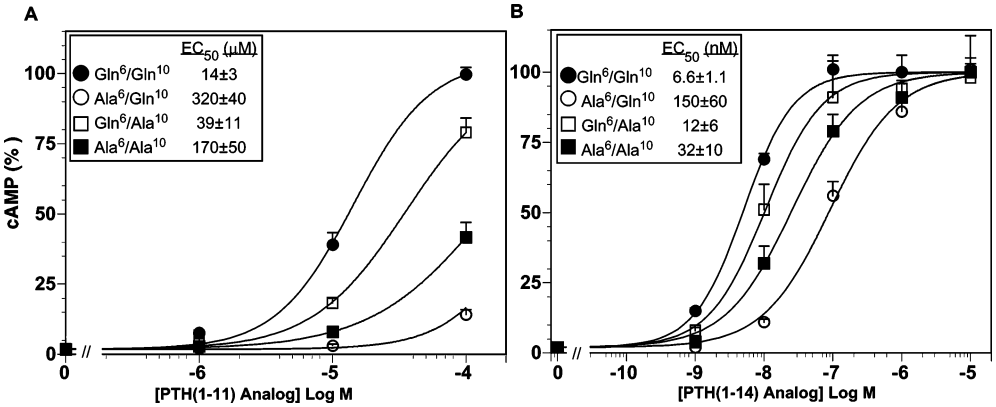

FIGURE 2: Effects of alanine substitutions at positions 6 and 10 in PTH (1−11) and PTH(1−14) analogues on cAMP-stimulating potency in LDelNt-2 cells. (A) The peptide [Ala^3,Gln^{10},Arg^{11}]rPTH(1−11)NH$_2$ containing Gln at positions 6 and 10 (Gln^6/Gln^{10}, filled circles) and analogues of that peptide containing Ala substitutions at positions 6 and/or 10: [Ala^{3,6},Gln^{10},Arg^{11}]rPTH(1−11)NH$_2$ (Ala^6/Gln^{10}, open circles); [Ala^{3,10},Arg^{11}]rPTH(1−11)NH$_2$ (Gln^6/Ala^{10}, open squares); and [Ala^{3,6,10},Arg^{11}]rPTH(1−11)NH$_2$ (Ala^6/Ala^{10}, filled squares) were evaluated for cAMP-stimulating potency in LDelNt-2 cells, an LLC-PK$_1$-derived cell line that stably expresses P1R-DelNt. (B) The conformationally constrained peptide [Aib^{1,3},Gln^{10},Har^{11},Ala^{12},Trp^{14}]rPTH(1−14)NH$_2$ containing Gln at positions 6 and 10 (Gln^6/Gln^{10}, filled circles) and the analogues: [Aib^{1,3},Ala^{6,12},Gln^{10},Har^{11},Trp^{14}]rPTH(1−14)NH$_2$ (Ala^6/Gln^{10}, open circles); [Aib^{1,3},Ala^{10,12},Har^{11},Trp^{14}]-rPTH(1−14)NH$_2$ (Gln^6/Ala^{10},open squares); and [Aib^{1,3},Ala^{6,10,12},Har^{11},Trp^{14}]rPTH(1−14)NH$_2$ (Ala^6/Ala^{10}, filled squares) were evaluated as in panel A. The data in panel A are expressed as a percent of the maximum response observed in each experiment for [Ala^3,Gln^{10},-Arg^{11}]rPTH(1−11)NH$_2$, which was 113 ± 4 pmol of cAMP per well, and the corresponding basal cAMP level was 2.0 ± 0.3 pmol per well; the data in panel B are expressed as a percent of the maximum response observed in each experiment for [Aib^{1,3},Gln^{10},Har^{11},Ala^{12},-Trp^{14}]rPTH(1−14)NH$_2$, which was 108 ± 3 pmol of cAMP per well, and the corresponding basal cAMP level was 1.8 ± 0.5 pmol per well (n = 3). The corresponding EC$_{50}$ values are shown in the insets. Data shown (mean ± SEM) were combined from three separate experiments (except for [Aib^{1,3},Ala^{10,12},Har^{11},Trp^{14}]rPTH(1−14)NH$_2$ for which two experiments were used), each performed in duplicate.

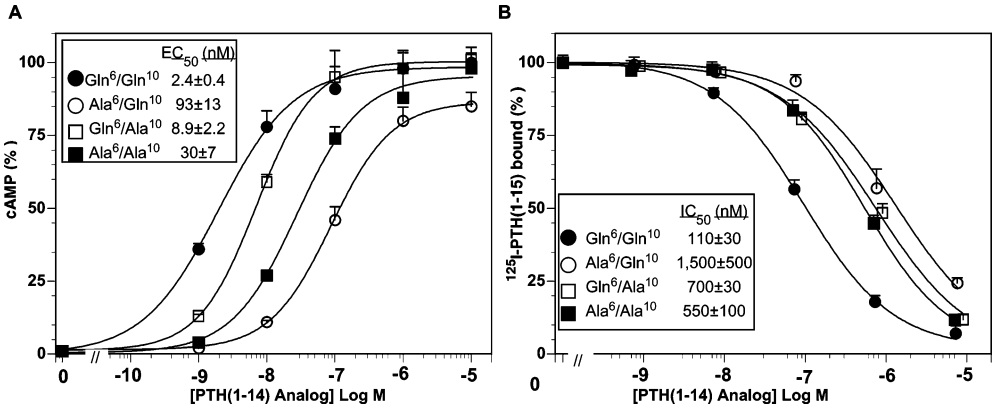

FIGURE 3: Effects of alanine substitutions at positions 6 and 10 in a PTH(1−14) analogue on cAMP-stimulating potency and binding affinity in HKRK-B28 cells. The peptide [Aib^{1,3},Gln^{10},Har^{11},Ala^{12},Trp^{14}]rPTH(1−14)NH$_2$ containing Gln at positions 6 and 10 (Gln^6/Gln^{10}, filled circles) and the analogues: [Aib^{1,3},Ala^{6,12},Gln^{10},Har^{11},Trp^{14}]rPTH(1−14)NH$_2$ (Ala^6/Gln^{10}, open circles); [Aib^{1,3},Ala^{10,12},Har^{11},Trp^{14}]-rPTH(1−14)NH$_2$ (Gln^6/Ala^{10}, open squares); and [Aib^{1,3},Ala^{6,10,12},Har^{11},Trp^{14}]rPTH(1−14)NH$_2$ (Ala^6/Ala^{10}, filled squares) were evaluated in HKRK-B28 cells for the capacity to stimulate cAMP formation (A) or to inhibit the binding of ^{125}I-[Ac$_5$c^1,Aib^3,Nle^8,Gln^{10},Har^{11},Ala^{12},-Trp^{14},Tyr^{15}]rPTH(1−15)NH$_2$ (B). The data in panel A are expressed as a percent of the maximum response observed in each experiment for [Aib^{1,3},Gln^{10},Har^{11},Ala^{12},Trp^{14}]rPTH(1−14)NH$_2$, which was 262 ± 47 pmol of cAMP per well (n = 3); the corresponding basal cAMP level was 3.5 ± 0.1 pmol per well. The data in panel B are expressed as a percent of the radioactivity specifically bound in the absence of inhibiting ligand, which was 11.6 ± 1.4% of the total radioactivity added (∼114 000 CPM/well). The corresponding EC$_{50}$ and IC$_{50}$ values are shown in the insets. Data (mean ± SEM) were combined from three separate experiments, each performed in duplicate.

paired data sets were statistically evaluated using a one-tailed Student's t test, assuming unequal variances for the two sets.

RESULTS

Substitutions at Positions 6 and 10 in PTH(1−11). We first assessed the functional effects of substitutions at positions 6 and 10 in PTH using [Ala^3,Gln^{10},Arg^{11}]rPTH-(1−11)NH$_2$ as the scaffold peptide (Table 1) (*7*). The peptides were analyzed for the capacity to stimulate cAMP formation in HKRK-B7 cells, an LLC-PK$_1$-derived cell line that expresses via stable transfection the human P1R. As shown in Figure 1, the parent peptide [Ala^3,Gln^{10},Arg^{11}]-rPTH(1−11)NH$_2$ elicited a 75-fold increase in cAMP formation with a corresponding EC$_{50}$ value of 5.0 ± 0.5 μM. Substitution of Gln^6 with Ala resulted in a marked reduction in potency, as the EC$_{50}$ observed for [Ala^{3,6},Gln^{10},Arg^{11}]-rPTH(1−11)NH$_2$ (330 ± 40 μM) was 65-fold higher than that observed for [Ala^3,Gln^{10},Arg^{11}]rPTH(1−11)NH$_2$ (P = 0.008). Replacement of Gln^{10} with Ala resulted in a modest (2-fold) reduction in potency (EC$_{50}$ of [Ala^{3,10},Arg^{11}]rPTH-(1−11)NH$_2$ = 11 ± 2 μM; P vs parent = 0.035). Substitution of both Gln^6 and Gln^{10} with Ala did not result in a simple additive reduction in potency (a 130-fold reduction would have been so predicted) but instead resulted in only a 31-fold reduction in potency. Thus, the potency of [Ala^{3,6,10},-

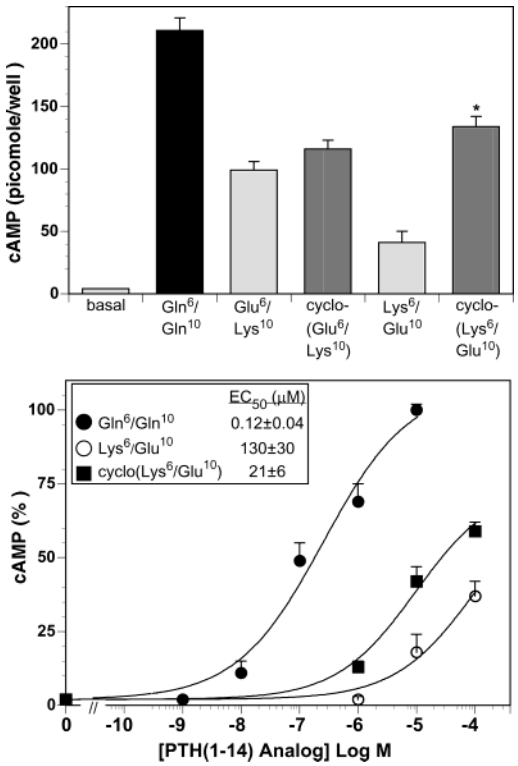

FIGURE 4: Effect of lactam bridges between positions 6 and 10 in PTH(1−14) analogues on cAMP-stimulating potency in HKRK-B28 cells. (A) The peptide [Ala^{3,12},Gln^{10},Har^{11},Trp^{14}]rPTH(1−14)-NH_2 (Gln^6/Gln^{10}) and analogues of that peptide containing Glu or Lys substitutions at positions 6 and 10, for which the side chains were either unmodified: [Ala^{3,12},Glu^6,Lys^{10},Har^{11},Trp^{14}]rPTH(1−14)NH_2 (Glu^6/Lys^{10}) and [Ala^{3,12},Lys^6,Glu^{10},Har^{11},Trp^{14}]rPTH(1−14)NH_2 (Lys^{10}/Glu^{10}), or were covalently linked via a lactam bridge: cyclo(6,10)-[Ala^{3,12},Glu^6,Lys^{10},Har^{11},Trp^{14}]rPTH(1−14)-NH_2 {cyclo(Glu^6-Lys^{10})} and cyclo(6,10)-[Ala^{3,12},Lys^6,Glu^{10},Har^{11},-Trp^{14}]rPTH(1−14)NH_2 {cyclo(Lys^6-Glu^{10})} were evaluated at doses of 10 μM for the capacity to stimulate cAMP accumulation in HKRK-B28 cells. The asterisk indicates that the response elicited by cyclo(6,10)-[Ala^{3,12},Lys^6,Glu^{10},Har^{11},Trp^{14}]rPTH(1−14)NH_2 was significantly ($P < 0.0001$) greater than that elicited by [Ala^{3,12},-Lys^6,Glu^{10},Har^{11},Trp^{14}]rPTH(1−14)NH_2. (B) The peptides [Ala^{3,12},-Gln^{10},Har^{11},Trp^{14}]rPTH(1−14)NH_2 (Gln^6/Gln^{10}, filled circles); [Ala^{3,12},Lys^6,Glu^{10},Har^{11},Trp^{14}]rPTH(1−14)NH_2 (Lys^6/Glu^{10}, open circles); and cyclo(6,10)-[Ala^{3,12},Lys^6,Glu^{10},Har^{11},Trp^{14}]rPTH(1−14)NH_2 (cyclo(Lys^6-Glu^{10}), filled squares) were evaluated at varying doses for the capacity to stimulate cAMP accumulation in HKRK-B28 cells. The data are expressed as a percent of the maximum response observed in each experiment with [Ala^{3,12},Gln^{10},Har^{11},-Trp^{14}]rPTH(1−14)NH_2, which was 359 ± 52 pmol of cAMP per well ($n = 3$); the corresponding basal cAMP level was 6 ± 1 pmol per well. The corresponding EC_{50} values are shown in the insets. The peptides [Ala^{3,12},Glu^6,Lys^{10},Har^{11},Trp^{14}]rPTH(1−14)NH_2 and cyclo(6,10)-[Ala^{3,12},Glu^6,Lys^{10},Har^{11},Trp^{14}]rPTH(1−14)NH_2 were also analyzed in these studies and yielded EC_{50} values of 40 ± 6 and 27 ± 9 μM, respectively. Data in panels A and B are means ± SEM from three separate experiments, each performed in duplicate.

Arg^{11}]rPTH(1−11)NH_2 (EC_{50} = 160 ±20 μM) was 2-fold greater than that of [Ala^{3,6},Gln^{10},Arg^{11}]rPTH(1−11)NH_2 ($P = 0.02$). The Gln^{10} → Ala substitution, therefore, partially rescued the potency defect imposed by the Gln^6 → Ala substitution, but it did not improve peptide potency when position 6 was occupied by Gln.

The two-domain hypothesis of the PTH-P1R interaction mechanism predicts that the effects of the substitutions at positions 6 and 10 observed with the above PTH(1−11) analogues involve interactions (direct or indirect) that occur within the juxtamembrane region of the receptor. To test this hypothesis, we utilized P1R-DelNt, a human P1R construct that is deleted for most of the N domain. This P1R construct exhibits normal signaling responses to PTH agonists, as long as ligand affinity is adequate, as can be achieved by modifying the N-terminal portion of PTH (*4*−*7*) or by covalently tethering the N-terminal portion of PTH to the construct (*27*). For the current studies, we established an LLC-PK_1-derived cell line, LDelNt-2, that stably expresses hP1R-DelNt. In these cells, the parent peptide, [Ala^3,Gln^{10},-Arg^{11}]rPTH(1−11)NH_2, elicited a 55-fold increase in cAMP formation, and the corresponding EC_{50} was 14 ± 3 μM (Figure 2A). The PTH(1−11) analogue having the Gln^6 → Ala substitution elicited only an 8-fold increase in cAMP accumulation, and the corresponding EC_{50} (320 ± 40 μM) was 22-fold higher than that of the parent peptide (Figure 2A; $P = 0.01$). This reduction in potency was partially rescued by the Gln^{10} → Ala substitution, as the potency of [Ala^{3,6,10},Arg^{11}]rPTH(1−11)NH_2 (EC_{50} = 170 ± 50 μM) was 2-fold lower than that of [Ala^{3,6},Gln^{10},Arg^{11}]rPTH(1−11)-NH_2 ($P = 0.05$). As was observed with the intact P1R in HKRK-B7 cells, the analogue [Ala^{3,10},Arg^{11}]rPTH(1−11)-NH_2 (Gln at position 6) was ∼3-fold less potent than [Ala^3,-Gln^{10},Arg^{11}]rPTH(1−11)NH_2 (Figure 2A); thus, the enhancing effect of the Ala-10 substitution was observed when position 6 was Ala, but not when this position was Gln. These data obtained with P1R-DelNt parallel those obtained with the intact P1R and indicate that the functional effects of the substitutions at positions 6 and 10 are mediated via the J domain of the receptor.

We examined whether the 6−10 rescue effect could be observed in the PTH(1−14) scaffold analogue [Aib^{1,3},Gln^{10},-Har^{11},Ala^{12},Trp^{14}]rPTH(1−14)NH_2, which contains the conformationally constraining substitutions of Aib at positions 1 and 3; this peptide has higher potency and higher helical content than does the corresponding PTH(1−14) analogue containing alanine at positions 1 and 3 (*6*). In LDelNt-2 cells, [Aib^{1,3},Gln^{10},Har^{11},Ala^{12},Trp^{14}]rPTH(1−14)NH_2 elicited a 50-fold increase in cAMP accumulation, and the corresponding EC_{50} value was 6.6 ± 1.1 nM (Figure 2B). Substituting Gln^6 of this peptide with alanine strongly reduced potency, as [Aib^{1,3},Ala^{6,12},Gln^{10},Har^{11},Trp^{14}]rPTH(1−14)NH_2 (EC_{50} = 150 ± 60 nM) was ∼22-fold weaker than the parent PTH-(1−14) analogue ($P = 0.08$). The potency of [Aib^{1,3},Ala^{10,12},-Har^{11},Trp^{14}]rPTH(1−14)NH_2 (EC_{50} = 12 ± 6 nM) was ∼2-fold weaker than that of the parent peptide but combining the Ala^{10} substitution with the Ala^6 substitution again resulted in partial rescue of the signaling defect imposed by the Ala^6 substitution, as the potency of [Aib^{1,3},Ala^{6,10,12},Har^{11},Trp^{14}]-rPTH(1−14)NH_2 (EC_{50} = 32 ± 10 nM) was ∼5-fold higher than that of [Aib^{1,3},Ala^{6,12},Gln^{10},Har^{11},Trp^{14}]rPTH(1−14)NH_2 ($P = 0.09$, Figure 2B).

Similar effects of these substitutions on the signaling potency of [Aib^{1,3},Gln^{10},Har^{11},Ala^{12},Trp^{14}]rPTH(1−14)NH_2 were observed on the intact P1R expressed in B28 cells (Figure 3 A). These cells express an intact rat/opossum P1R chimera that bind N-terminal PTH analogues with higher affinity than HKRK-B7 cells expressing the wild-type hP1R (*26*). We were thus able to assess the effects of the substitutions at positions 6 and 10 on the binding affinity of [Aib^{1,3},Gln^{10},Har^{11},Ala^{12},Trp^{14}]rPTH(1−14)NH_2 by performing competition analyses in these cells utilizing ^{125}I-[Ac$_5$c^1,-

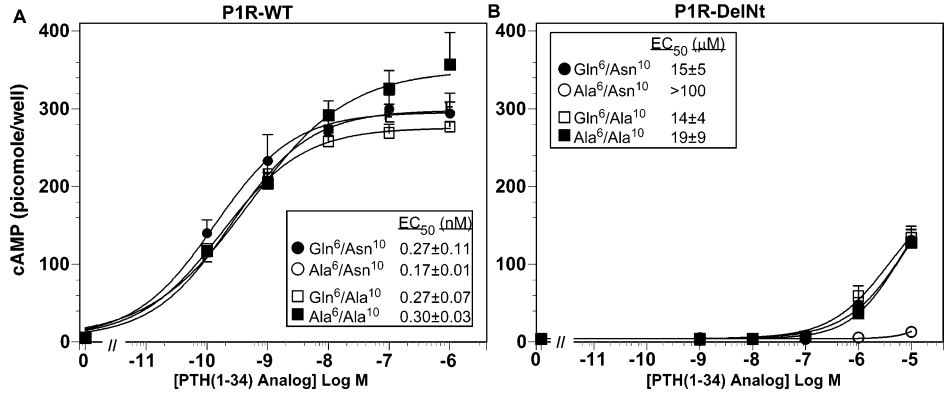

FIGURE 5: Effects of alanine substitutions at positions 6 and 10 in a PTH(1−34) analogue on cAMP-stimulating potency in COS-7 cells. The peptide [Tyr^{34}]hPTH(1−34)NH$_2$ (Gln^6/Asn^{10}; filled circles) and the analogues [Ala^6,Tyr^{34}]hPTH(1−34)NH$_2$ (Ala^6/Asn^{10}, open circles); [Ala^{10},Tyr^{34}]hPTH(1−34)NH$_2$ (Gln^6/Ala^{10}, open squares); and [Ala^{6,10},Tyr^{34}]hPTH(1−34)NH$_2$ (Ala^6/Ala^{10}) were evaluated for cAMP-stimulating potencies in COS-7 cells expressing, via transient transfection, either the wild-type hP1R (A) or hP1R-DelNt (B) (note that the weaker potencies of the analogues on P1R-DelNt, as compared to on hP1R-WT, reflect the importance of the N domain of the receptor in mediating binding interactions for PTH(1−34) peptides (*12*)). Data shown (mean ± SEM) were combined from three separate experiments, each performed in duplicate.

Table 2: Functional Properties of PTH(1−34) Analogues in HKRK-B28 Cells[a]

	cAMP		Binding
peptide	EC_{50} (nM)	E_{max} (%)	IC_{50} (nM)
[Tyr^{34}]hPTH(1−34)NH$_2$	1.9 ± 0.8	100 ± 5	6.0 ± 3.2
[Ala^6,Tyr^{34}]hPTH(1−34)NH$_2$	4.9 ± 3.6	105 ± 10	4.5 ± 1.4
[Ala^{10},Tyr^{34}]hPTH(1−34)NH$_2$	3.0 ± 1.7	106 ± 7	6.4 ± 2.8
[Ala^{6,10},Tyr^{34}]hPTH(1−34)NH$_2$	2.2 ± 0.3	107 ± 8	3.9 ± 1.1

[a] The peptide [Tyr^{34}]hPTH(1−34)NH$_2$ and analogues thereof having Gln^6 and/or Asn^{10} substituted by Ala were evaluated in HKRK B28 cells for the capacity to stimulate cAMP accumulation and to inhibit the binding of ^{125}I [Ala$^{3.12}$,Nle^8 Gln^{10},Har^{11},Trp^{14},Arg^{19},Tyr^{21}]PTH(1−21)NH$_2$ tracer radioligand. The maximum cAMP response observed (E_{max}) for each peptide at a concentration of 1×10^{-6} M is given as a percent of the maximum response observed for [Tyr^{34}]hPTH(1−34)NH$_2$, the average of which was 416 ± 51 pmol per well. Data (means ± SEM) are combined from three separate experiments, each performed in duplicate.

Aib^3,Nle^8,Gln^{10},Har^{11},Ala^{12},Trp^{14},Tyr^{15}]rPTH(1−15)NH$_2$ as a tracer radioligand (*24*). The Ala^6 and Ala^{10} single substitutions reduced the apparent affinity of the PTH(1−14) peptide ~14- and ~6-fold, respectively, relative to that of the parent analogue, whereas combining the Ala^{10} substitution with the Ala^6 substitution resulted in partial rescue of the binding defect imposed by the Ala^6 substitution, as the affinity of [Aib^{1,3},Ala^{6,10,12},Har^{11},Trp^{14}]rPTH(1−14)NH$_2$ was ~3-fold higher than that of [Aib^{1,3},Ala^{6,12},Gln^{10},Har^{11},Trp^{14}]rPTH(1−14)NH$_2$ (Figure 3B).

Effect of Lactam Bridges between Positions 6 and 10 in PTH(1−14). We then assessed the effect of installing a direct covalent linkage between the side chains of the residues at positions 6 and 10 on PTH analogue function. To do this, we introduced paired Glu^6/Lys^{10} or Lys^{10}/Glu^6 substitutions in an otherwise nonconstrained PTH(1−14) analogue scaffold [Ala^{3,12},Gln^{10},Har^{11},Trp^{14}]rPTH(1−14)NH$_2$ (*5*) and formed an amide bond between the carboxylate side chain group of the introduced glutamate residue and the amino side chain group of the introduced lysine residue. For controls, a portion of each Glu/Lys- or Lys/Glu-substituted PTH(1−14) analogue was preserved in the nonbridged form. The four peptides [Ala^{3,12},Glu^6,Lys^{10},Har^{11},Trp^{14}]rPTH(1−14)NH$_2$ (linear), cyclo(6,10)-[Ala^{3,12},Glu^6,Lys^{10},Har^{11},Trp^{14}]rPTH(1−14)-NH$_2$ (cyclized via a 6−10 lactam bridge), [Ala^{3,12},Lys^6,-Glu^{10},Har^{11},Trp^{14}]rPTH(1−14)NH$_2$, and cyclo(6,10)-[Ala^{3,12},-Lys^6,Glu^{10},Har^{11},Trp^{14}]rPTH(1−14)NH$_2$ were first compared at a single dose of 10 μM for the capacity to stimulate cAMP formation in B28 cells. As shown in Figure 4A, none of the four analogues was as effective as the parent PTH(1−14) analogue in inducing a cAMP response. For the peptides containing the Glu^6/Lys^{10} pairing, the activity of the lactam-containing analogue was comparable to that of its linear counterpart (Figure 4, panel A and legend). For the reciprocal analogue containing the Lys^6/Glu^{10} pairing, the bridged peptide was more active than its linear counterpart (Figure 4A), and in dose−response analyses, cyclo(6,10)-[Ala^{3,12},-Lys^6,Glu^{10},Har^{11},Trp^{14}]rPTH(1−14)NH$_2$ was 6-fold more potent than [Ala^{3,12},Lys^6,Glu^{10},Har^{11},Trp^{14}]rPTH(1−14)NH$_2$ (Figure 4B; $P = 0.02$).

Effects of Substitutions at Positions 6 and 10 in PTH(1−34). We then investigated the functional effects of alanine substitutions at positions 6 and/or 10 in [Tyr^{34}]hPTH(1−34)NH$_2$. The analogues of these studies contained either Gln (native) or Ala at position 6 and either Asn (native) or Ala at position 10. We first compared the potencies of these peptides on the intact P1Rs expressed in COS-7 and HKRK-B28 cells. No difference in signaling potency could be discerned for the four analogues in COS-7 cells transiently expressing the wild-type hP1R (Figure 5A), nor was any difference in signaling potency or apparent binding affinity detected in HKRK-B28 cells (Table 2). In COS-7 cells expressing P1R-DelNt, however, differences in cAMP-signaling potencies could be detected. Thus, the Gln^6 → Ala substitution abolished signaling activity, and addition of the Gln^{10} → Ala substitution, which by itself had no effect on PTH-(1−34) potency, to the Gln^6 → Ala substitution completely restored activity, such that [Ala^{6,10},Tyr^{34}]hPTH(1−34)NH$_2$ was as potent as [Tyr^{34}]hPTH(1−34)NH$_2$ (Figure 5B).

DISCUSSION

In this paper, we explored the possibility that interaction between the side chains of the residues at positions 6 and 10 in PTH could affect the capacity of the peptide to interact with the PTH-1 receptor. The results provide evidence to suggest that the side chains of these residues can interact

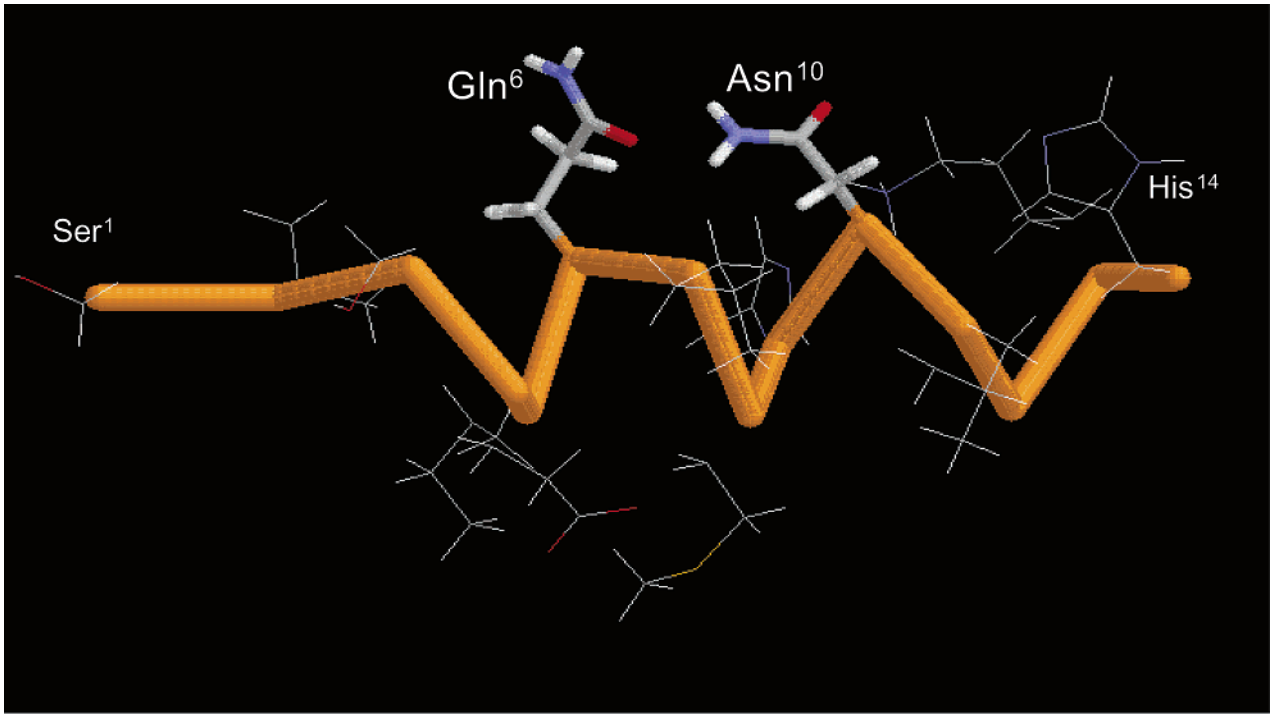

FIGURE 6: NMR structure of the PTH(1−14) domain. Shown is the (1−14) portion of the NMR structure of hPTH(1−34) obtained in 20% 2,2,2-trifluoroethonal reported by Marx et al. (*14*). The image shown was generated using the coordinate file (1HPY) deposited by the authors at the Protein Data Bank (www.rcsb.org) and the Rasmol imaging software package (*35*). The peptide backbone (orange) makes several helical turns, and the carboxamide side chain groups of Gln^6 and Asn^{10} are positioned such that they could interact (e.g., by hydrogen bonding) via the oxygen atom (red) of Gln^6 and the nitrogen atom (blue) of Asn^{10}. Our current functional data are consistent with the possibility that such a helical structure and intrahelical interaction occurs in receptor-bound PTH.

and that this interaction may contribute to the ligand's capacity to interact productively with the PTH-1 receptor. Since these residues are in an *i*, *i* + 4 configuration, the data also support the hypothesis that the N-terminal portion of PTH is α-helical when the ligand is bound to the receptor. The key observation that supports these conclusions is the rescue effect that the Gln^{10} → Ala substitution had on the signaling defect imposed by the Gln^6 → Ala substitution in several different PTH peptide scaffolds. Although the rescue effect was partial, in that the [$Ala^{6,10}$]PTH(1−11) and [$Ala^{6,10}$]-PTH(1−14) analogues were only ∼2−6-fold more potent than the [Ala^6]PTH(1−11) and [Ala^6]PTH(1−14) counterpart peptides, it was reproducible, as we observed it in several different peptide scaffolds and with several different P1R-expressing cell systems. Furthermore, the rescue effect appeared to be selective, in that the Gln^{10} → Ala substitution by itself (e.g., in the context of Gln^6) reduced potency of each analogue by 2−12-fold.

Without a direct assessment of the structures that the ligands used in our study adopt in the receptor-bound state, it is not possible to ascribe a definitive molecular mechanism by which the observed functional effects arise. One possible explanation for the observed effects is that in the parent peptides, an interaction between the side chains of Gln^6 and Gln^{10} constrains the rotational position of the Gln^{10} side chain, such that an unfavorable interaction, for example, with another residue in the ligand or the receptor, is avoided. Such an unfavorable interaction would become possible upon removal of the Gln^6 side chain but then be averted by subsequent removal of the Gln^{10} side chain. One of the functions of the Gln^6 side chain might, therefore, be to prevent unfavorable interactions of the Gln^{10} or (Asn^{10}) side chain, by an intrahelical *i*, *i* + 4 side chain interaction. The possibility that the side chains of residues 6 and 10 might interact is supported by a recent NMR analysis of PTH(1−34) reported by Marx et al. (*14*). In the atomic coordinate file (1HPY, available in the Brookhaven Protein Data Bank: rcsb.org/pdb) that accompanies this study, the side chains of Gln^6 and Asn^{10} can be seen to project from the same face of the N-terminal (Glu^4−His^{14}) α-helix such that the side chain carboxamide of Gln^6 could interact with that of Asn^{10} via a hydrogen bond (Figure 6). Interaction of these two side chains was also indicated in the recent NMR study of hPTH(1−31) (*15*). It is thus conceivable that such an interaction helps stabilize the N-terminal helix of PTH, as has been documented in other model helical peptides (*22*), and thereby contributes to peptide potency. This possibility needs further investigation, however, and other mechanisms are not to be excluded. For example, the greater helix-forming propensity of alanine, as compared to glutamine and especially asparagine (*28*), could play a role in the rescue effects observed for the combined Ala^6/Ala^{10} substitutions. In any case, that the rescue effect observed for the Gln^{10} → Ala substitution on the Gln^6 → Ala substitution was partial seems to suggest that the side chain of Gln^6 plays additional roles in PTH function, perhaps involving direct receptor interaction, as has been suggested previously from functional studies on PTH(1−34) analogues (*29*). Further substitution analyses of position 6 in various PTH scaffold peptides could help to assess this possibility. Finally, we note that while it remains possible that the Gln^{10} → Ala substitution rescues the signaling defect imposed by the Ala^6 substitution via a mechanism that is independent of residue 6, such as by altering a direct interaction with the receptor, this seems

unlikely, given that the Ala^{10} substitution selectively enhanced the activity of analogues containing alanine at position 6.

We explored further the possibility that an interaction between the side chains of residues 6 and 10 of PTH contributes to peptide activity by introducing covalent lactam bridges between the side chains of paired Glu/Lys residues substituted, in reciprocal fashion, at these positions in a PTH-(1−14) analogue. A number of prior studies on PTHrP(1−34) (*30*), PTH(1−31) (*21*, *31*), and PTH(1−28) (*32*) analogues have shown that lactam bridges at different locations can stabilize helical structure and increase affinity and/or potency. So far, the most N-terminal lactam bridge reported for a PTH analogue involved a $Lys^{13}-Asp^{17}$ pairing (*21*). The paired Glu^6/Lys^{10} or Lys^6/Glu^{10} substitutions of our PTH-(1−14) analogues were not well-tolerated, as the nonbridged control peptides, [$Ala^{3,12}$,Glu^6,Lys^{10},Har^{11},Trp^{14}]rPTH(1−14)-NH_2, and [$Ala^{3,12}$,Lys^6,Glu^{10},Har^{11},Trp^{14}]rPTH(1−14)NH_2 exhibited potencies that were 300- and 1000-fold weaker, respectively, than that of the unsubstituted PTH(1−14) parent peptide. However, relative to their corresponding linear control peptides, the bridged PTH(1−14) analogues either maintained potency (cyclo(6,10)-[$Ala^{3,12}$,Glu^6,Lys^{10},Har^{11},-Trp^{14}]rPTH(1−14)NH_2) or exhibited a 6-fold improvement in potency (cyclo(6,10)-[$Ala^{3,12}$,Lys^6,Glu^{10},Har^{11},Trp^{14}]rPTH-(1−14)NH_2). These results are further consistent with the notion that the side chains of residues 6 and 10 of PTH have the capacity to interact and are in α-helical conformation when the ligand is bound to the receptor.

An intriguing finding of our study was that in the context of PTH(1−34), the alanine substitutions at positions 6 and/or 10 did not affect potency, at least when analyzed in cells expressing the intact P1R. When analyzed in COS-7 cells transfected with the N-truncated receptor construct, P1R-DelNt, however, a marked (≥10-fold) reduction in potency was observed for [Ala^6,Tyr^{34}]hPTH(1−34)NH_2, and this defect was fully rescued by the $Gln^{10} \rightarrow$ Ala substitution. These findings suggest that the same type of putative interaction that occurs between Gln^6 and Gln^{10} in our PTH-(1−11) and PTH(1−14) analogues can also occur in intact PTH(1−34) containing the native Asn residue at position 10 but that the interaction of the C-terminal (15−34) portion of PTH(1−34) with the N domain of the wild-type P1R masks the effects that the alanine substitutions at these sites have on function. Thus, the N domain interaction mitigates the deleterious effect of the $Gln^6 \rightarrow$ Ala substitution and hence diminishes the rescue effect of the $Gln^{10} \rightarrow$ Ala substitution. Such effects could potentially indicate a cooperative interaction between the N and the J components of the ligand−receptor interaction mechanism, as we commented on recently in our studies on Aib-modified PTH analogues. In these studies, we found that Aib substitutions at positions 1 and 3 in PTH(1−34) did not enhance potency of the peptide on the wild-type P1R, but they markedly (by ∼100-fold) enhanced potency of PTH(1−34) on P1R-DelNt. We thus postulated that the binding of the C-terminal domain of PTH(1−34) to the N domain of the P1R facilitates the association of the (1−14) domain of the ligand with the J domain of the receptor, which in turn, induces folding of the (1−14) domain. By this model, pre-organization of structure by the Aib substitutions would not have a discernible effect on PTH(1−34) activity at the wild-type receptor but would enhance activity on P1R-DelNt. A similar helix-stabilizing mechanism might thus underlie the lack of effect of the position 6 and 10 substitutions on the potency of PTH-(1−34) acting on the intact P1R.

In conclusion, we have provided evidence to suggest that the side chains of residues 6 and 10 of receptor-bound PTH can interact and that the N-terminal portion of PTH is α-helical when bound to the J domain of the receptor (*6*, *20*, *33*). These hypotheses need to be directly assessed by structural analysis of PTH in complex with the P1R, as has recently been achieved for PACAP (pituitary adenylyl cyclase-activating peptide) bound to its class II GPCR (*34*), but this is not yet possible for the PTH/PTH receptor system. Nevertheless, the present data contribute to the ongoing effort to define the PTH−PTH receptor interact mechanism, and they could provide new clues for the eventual design of low-molecular weight PTH receptor agonists, peptidic or non-peptidic, that can be used to treat diseases of bone and mineral metabolism, such as osteoporosis.

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