Parathyroid Hormone (1-14) and (1-11) Analogs

Conformationally Constrained by \( \alpha \)-aminoisobutyric Acid Mediate Full Agonist Responses via the Juxtamembrane Region of the PTH–1 Receptor

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Running Title: Aib-substituted PTH Analogs

Abbreviations: PTH, parathyroid hormone; r, rat; h, human; PTHrP, PTH-related peptide; P1R, type-1 parathyroid hormone receptor; IBMX, 3-isobutyl-1-methylxanthine; Aib, \( \alpha \)-aminoisobutyric acid; Har, homoarginine; Nle, norleucine; other amino acids are in either the conventional one- or three-letter codes, TFE, 2,2,2-Trifluorethanol; CD circular dichroism.
Abstract: The N-terminal portion of PTH is critical for PTH-1 receptor (P1R) activation and has been postulated to be α-helical when bound to the receptor. We investigated whether substitution of the sterically hindered and helix-promoting amino acid α-aminoisobutyric acid (Aib) in N-terminal PTH oligopeptides would improve the capacity of the peptide to activate the P1R. Analysis of the effects of individual Aib substitutions at each position in [Ala, Gln, Har, Trp]PTH(1-14)amide ([M]PTH(1-14)) on cAMP-stimulating potency in HKRK-B28 cells revealed that Aib at most positions diminished potency, however, Aib at positions 1 and 3 enhanced potency. Thus [Aib, M]PTH(1-14) was ~100-fold more potent than [M]PTH(1-14) (EC50s = 1.1±0.1 nM and 100±20 nM, respectively), ~100,000-fold more potent than native PTH(1-14) and two-fold more potent than PTH(1-34). The shorter peptide, [Aib, M]PTH(1-11) was also fully efficacious and 1,000-fold more potent than [M]PTH(1-11) (EC50 4±1 nM vs. 3±1 μM). In cAMP stimulation assays performed in COS-7 cells expressing P1R-delNt, a receptor which lacks most of the N-terminal extracellular domain, [Aib, M]PTH(1-14) was 50-fold more potent than [M]PTH(1-14) (EC50s = 0.7±0.2 nM vs. 40±2 nM) and 1,000-fold more potent than PTH(1-34) (EC50 = 700 nM). [Aib, M]PTH(1-14), but not PTH(1-34), inhibited the binding of 125I-[Aib, Nle, Gln, Har, Ala, Trp, Arg, Tyr]PTH(1-21)NH2 to hP1R-delNt (IC50 = 1,600±200 nM). The Aib substitutions in otherwise unmodified PTH(1-34) enhanced potency and binding affinity on hP1R-delNt but they had no effect for this peptide on hP1R-WT. Circular dichroism spectroscopy demonstrated that the Aib-1,3 substitutions increased helicity in all peptides tested, including PTH(1-34). The overall data thus suggest that the N-terminal residues of PTH are intrinsically disordered but become
conformationally constrained, possibly as an $\alpha$-helix, upon interaction with the activation domain of the PTH-1 receptor.
Introduction

Parathyroid hormone (PTH), an 84 amino acid peptide, is the principal regulator of ionized blood calcium in the human body (1). Synthetic PTH(1-34) exhibits full bioactivity in most cell-based assay systems and can have potent anabolic effects on bone mass in animals. Recently, PTH(1-34) has been shown to reduce the risk of bone fracture in postmenopausal osteoporotic women (2,3). PTH acts on the PTH/PTHrP receptor (P1R), a class II G protein-coupled receptor which stimulates the adenylyl cyclase/cAMP and phospholipase C/inositol phosphate (IP) signaling pathways. Peptide deletion studies have shown that the amino-terminal residues of PTH play a crucial role in P1R activation (4,5) and crosslinking and receptor mutagenesis studies have revealed that the amino-terminal residues of PTH interact with the portion of the P1R that contains the extracellular loops and extracellular ends of the transmembrane helices (the juxtamembrane or J domain) (6-11). The structure of PTH as it is bound to the P1R is not known, but NMR analyses of PTH(1-34) analogs in a variety of polar and non-polar solvents suggest that the N-terminal portion of PTH (within residues Ser-3 to Lys-13) contains a short segment of α-helix, and this segment is connected by a flexible bend or turn segment to a longer α-helix in the C-terminal portion of the molecule (within residues Ser-17 to Val-31 (12-18). A recent crystallographic study of PTH(1-34) reveals a continuous helical structure that extends nearly the full length of the molecule (Ser-3 to Asn-33) with only a slight (15°) bend at the midsection (19).

Short amino-terminal native PTH peptide fragments, such as PTH(1-14)amide, exhibit very weak signaling potency (EC₅₀ for stimulation of cAMP accumulation ~ 100 µM) (11). The weak activity of such amino-terminal peptides is due, at least in part, to
their weak receptor-binding affinity. In the case of PTH(1-34), most of the receptor-binding energy is derived from interactions between the (15-34) portion of the ligand and the large (~170 amino acid) amino-terminal extracellular domain of the receptor (N domain) (20,21) (22) (6). These N domain interactions are thought to position the N-terminal residues of the ligand within proximity of the J domain of the receptor, and thereby enable the interactions that are required for the induction of transmembrane signaling (6,7). In a recent series of studies on the PTH(1-14) scaffold peptide we showed that certain substitutions at several sites in the peptide resulted in enhanced potency (23-25). The most potent PTH(1-14) analog thus identified was [Ala_{3,12},Gln_{10},Har_{11},Trp_{14}]PTH(1-14)amide ([M]PTH(1-14)), which is 1,600-fold more potent than native PTH(1-14) for stimulating cAMP formation in LLC-PK1-derived HKRK-B28 cells, and only 60-fold weaker than PTH(1-34) (23). At least some component of the improvement in signaling capability observed in [M]PTH(1-14) can be attributed to improvements in binding affinity, as the peptide could inhibit the binding of $^{125}$I-PTH(3-34) to HKRK-B28 cells (IC$_{50}$ ~ 90 µM), whereas native PTH(1-14) could not (23).

Each PTH(1-14) peptide (native or modified) studied to date exhibits approximately the same signaling potency on a mutant P1R construct that lacks most of the N-terminal domain (P1R-delNt), as it does on the wild-type P1R; in contrast, PTH(1-34) is 100-fold less potent on P1R-delNt as it is on P1R-WT (11,23,24). Thus, unlike PTH(1-34), which utilizes both N and J domain interactions and thereby achieves high potency, the N-terminal PTH peptide fragments appear to utilize only J domain interactions. These findings suggest that high potency could be attained by an N-terminal PTH analog that interact only with the J domain, providing that the problem of weak binding affinity is
overcome. In experimental support of this notion is the finding that N-terminally truncated PTH-1 receptor constructs which have the N-terminal residues of PTH (e.g. residues 1-11) covalently tethered to TM 1 of the J domain exhibit robust basal cAMP-signaling activity (10).

In the current study, we extended our work on N-terminal PTH analogs and investigated the functional consequences of introducing \(\alpha\)-aminoisobutyric acid (Aib) substitutions into [M]PTH(1-14) and related analogs. This sterically hindered amino acid constrains the peptide backbone conformation often as an \(\alpha\)-helix (26). Our functional data show that Aib substitutions at positions 1 and 3 of N-terminal PTH oligopeptide analogs result in considerable improvements in the capacities of the peptides to interact with the P1R, specifically with the receptor's juxtamembrane region. These functional effects are associated with changes in the spectroscopic properties of the peptides that are indicative of increased \(\alpha\)-helicity.
Material and Methods

Peptides: All peptide analogs contained a free amino terminus and a carboxamide at the C-terminus. The peptide indicated as PTH(1-34) is [Nle\textsuperscript{8,21},Tyr\textsuperscript{34}]ratPTH(1-34)NH\textsubscript{2} (the native rat PTH(1-34) sequence is Ala\textsuperscript{1}-Val\textsuperscript{2}-Ser\textsuperscript{3}-Glu\textsuperscript{4}-Ile\textsuperscript{5}-Leu\textsuperscript{6}-Met\textsuperscript{8}-His\textsuperscript{9}-Asn\textsuperscript{10}-Leu\textsuperscript{11}-Gly\textsuperscript{12}-Lys\textsuperscript{13}-His\textsuperscript{14}-Leu\textsuperscript{15}-Ala\textsuperscript{16}-Ser\textsuperscript{17}-Val\textsuperscript{18}-Glu\textsuperscript{19}-Arg\textsuperscript{20}-Met\textsuperscript{21}-Gln\textsuperscript{22}-Trp\textsuperscript{23}-Leu\textsuperscript{24}-Arg\textsuperscript{25}-Lys\textsuperscript{26}-Lys\textsuperscript{27}-Leu\textsuperscript{28}-Gln\textsuperscript{29}-Asp\textsuperscript{30}-Val\textsuperscript{31}-His\textsuperscript{32}-Asn\textsuperscript{34}-Phe\textsuperscript{34}). The [M]PTH(1-14) and shorter-length [M]PTH(1-X) derivatives are rat PTH analogs in which, "M" refers to the amino acid modifications: Ala\textsuperscript{3,12},Gln\textsuperscript{10},Har (homoarginine)\textsuperscript{11},Trp\textsuperscript{14}, unless a residue position is deleted by C-terminal truncation, or replaced by Aib, as indicated. The peptide indicated as [M]PTH(1-21) is [Ala\textsuperscript{3,12},Nle\textsuperscript{8},Gln\textsuperscript{10},Har\textsuperscript{11},Trp\textsuperscript{14},Arg\textsuperscript{19},Tyr\textsuperscript{21}]ratPTH(1-21)NH\textsubscript{2}; [Aib\textsuperscript{1,3},M]PTH(1-21) is the same peptide with alanine-1 and alanine-3 replaced by Aib. The peptide indicated as [Aib\textsuperscript{1,3},M]PTH(1-34) is [Aib\textsuperscript{1,3},Nle\textsuperscript{8,21},Gln\textsuperscript{10},Har\textsuperscript{11},Ala\textsuperscript{12},Trp\textsuperscript{14},Arg\textsuperscript{19},Tyr\textsuperscript{21}]ratPTH(1-34)NH\textsubscript{2}. The peptide indicated as hPTH(1-34) is [Tyr\textsuperscript{34}]hPTH(1-34)NH\textsubscript{2} and [Aib\textsuperscript{1,3}]hPTH(1-34) is the same peptide with serine-1 and serine-3 replaced by Aib. Peptides were prepared on automated peptide synthesizers (model 430A PE Applied Biosystems, Foster City, CA, or Model 396 MBS Advanced ChemTect, Louisville, Kentucky) using Fmoc main-chain protecting group chemistry, HBTU/HOBt/DIEA (1:1:2 molar ratio) for coupling reactions, and TFA-mediated cleavage/sidechain-deprotection (MGH Biopolymer Synthesis Facility, Boston, MA). All peptides were desalted by adsorption on a C18-containing cartridge; PTH(1-34), [M]PTH(1-21), and those [M]PTH(1-14) and shorter analogs which were ≤70% pure at this stage were purified further by HPLC. The dry peptide powders were reconstituted in 10 mM acetic acid and stored at −80°C. The purity, identity, and stock concentration for each peptide was secured by analytical HPLC, Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry and amino acid analysis.
Radiolabeling of [M]PTH(1-21) and [Aib\textsuperscript{1,3},M]PTH(1-21) was performed using \textsuperscript{125}I-Na\textsubscript{2} (2,200 Ci/mmol, NEN) and chloramine-T; the resultant radioligands were purified by HPLC.

\textit{Cell Culture:} The cell line HKRK-B28 (27) is derived from LLC-PK\textsubscript{1} (a porcine kidney cell line) by stable transfection with plasmid DNA encoding the human P1R and expresses ~ 280,000 receptors per cell. These cells, as well as COS-7 cells and SaOS-2-B10 (SaOS-2) cells, were cultured at 37°C in T-75 flasks (75 mm\textsuperscript{2}) 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\textsubscript{2}. Stock solutions of EDTA/trypsin and antibiotics were from GIBCO; fetal bovine serum was from Hyclone Laboratories (Logan, UT). Plasmid DNA was purified by cesium chloride/ethidium bromide density gradient centrifugation. COS-7 cells sub-cultured in 24-well plates were transfected with FuGENE 6 transfection reagent (Roche, Indianapolis, IN) and plasmid DNA (200 ng/well) according to the manufacturer's recommended procedure. Plasmids utilized in COS-7 cell transfections encoded either the wild-type human P1R (hP1R-WT) (28), the truncated human P1R deleted for residues (24-181) (hP1R-delNt) (24), the rat PTH-1 receptor containing an HA epitope tag in the N-terminal extracellular domain but otherwise wild-type (rP1R-WT) or similarly tagged mutant rP1Rs containing either the mutation Phe184→Ala (rP1R-F184A) (23,29) or Trp437→Ala (rP1R-W437A) (30) and have been described previously. All cells, in 24-well plates, were treated with fresh media and shifted to 33°C for 12 to 24 hours prior to assay (31).
cAMP Stimulation: Stimulation of cells with peptide analogs 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 analog (final volume = 300 µL). The medium was removed after incubation for 30 to 60 minutes 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. The EC$_{50}$ response values were calculated using nonlinear regression (see below).

Competition Binding: Binding reactions were performed with HKRK-B28 cells or in COS-7 cells in 24-well plates. The cells were rinsed with 0.5 mL of binding buffer, and then treated successively with 100 µL 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-[M]PTH(1-21) or $^{125}$I-[Aib$^{1,3}$,M]PTH(1-21) (ca. 26 fmol; final volume = 300 µL). Incubations were 4 to 6 hours at 4°C, at which time near equilibrium conditions were attained, as judged by association time-course experiments performed with each radioligand and HKRK-B28 cells or transfected COS-7 cells. 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 5 M NaOH and counted for radioactivity. For each tracer and in each experiment, the non-specific binding was determined as the radioactivity that bound in the presence of the same unlabeled peptide at a concentration of 1 µM, and was ~ 1 to 2
% of total radioactivity added for each tracer. The maximum specific binding ($B_0$) was the total radioactivity bound in the absence of competing ligand, corrected for nonspecific binding, and, for each tracer, ranged from 8% to 20% of the total radioactivity added. Nonlinear regression was used to calculate binding IC$_{50}$ values (see below).

**Stimulation of Inositol Phosphate Production:** COS-7 cells transfected as above with hP1R-WT or hP1R-delNt were treated with serum-free, inositol-free DMEM containing 0.1% bovine serum albumin and [3H]myo-inositol (NEN, Boston MA) (2 µCi/mL) for 16 hours prior to assay. At the time of the assay, the cells were rinsed with binding buffer containing LiCl (30 mM) and treated with the same buffer with or without a PTH analog. The cells were then incubated at 37°C for 40 minutes, after which the buffer was removed and replaced by 0.5 mL of ice cold 5% trichloroacetic acid solution. After 3 hours on ice, the lysate was collected and extracted twice with ethyl ether. The lysate was then applied to an ion exchange column (0.5 mL resin bed) and the total inositol phosphates were eluted as described previously (32) and counted in liquid scintillation cocktail.

**Inhibition of Chondrocyte Differentiation in Mouse Metatarsals:** Metatarsals from embryonic (gestational day 15.5) mice were excised and cultured in a 37°C humidified incubator (5% CO$_2$) in serum-free αMEM media in 24 well plates. Sixteen hours later, a PTH analog or vehicle was added, and the samples were incubated for an additional 48 hours at 37°C with peptide or vehicle added again at the 24-hour time point. At the end of the incubation period (64 hours total), the samples were fixed with 10% formalin/phosphate-buffered saline, then directly visualized on a dissecting
microscope using white light. Sections were processed for in-situ hybridization analysis using $^{35}$S-labeled riboprobes specific for collagen type X mRNA, a developmental marker gene expressed only in hypertrophic chondrocytes of the growth plate.

Circular Dichroism: Circular Dichroism spectra were recorded on a Jasco model 710 spectropolarimeter; peptides were analyzed at a concentration of 20 µM in 50 mM sodium phosphate buffer pH 7.4, or the same buffer containing 2,2,2-trifluoroethanol at 20% (v/v). Spectroscopic scans were performed at 20°C and at wavelengths between 185 nm and 255 nm, with data recorded at each 1 nm interval. Eight (Fig. 6) or six (Fig 7) scans were accumulated and averaged for each sample. At each wavelength, the mean residue ellipticity $[\theta]$ (in dimensions of deg x cm$^2$/dmol) was calculated by the equation: $[\theta] = \theta \times 100/l \times C \times n$; where $\theta$ is the raw ellipticity value (in dimensions of millidegree), $l$ is the sample path length in cm, $C = $ is the molar peptide concentration, and $n$ is the number of residues in the peptide (33). The helical content of each peptide was estimated by dividing $[\theta]$ observed at 222 nm for that peptide by –28,100, which is the reported $[\theta]_{222}^{obs.}$ for a model helical decapeptide (33).

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: $y_p = \text{Min} + \left[\frac{(\text{Max} - \text{Min})}{(1 + (\text{IC}_{50}/x)^{\text{slope}})}\right]$. The Excel Solver function was utilized for parameter optimization, as described previously (34,35). Differences between paired data sets were statistically evaluated using a one-tailed Student’s t-test, assuming unequal variances for the two sets.
Results

*Aib-scan in [M]PTH(1-14)*

We first analyzed the effect of introducing individual Aib substitutions at each position in the scaffold peptide [Ala3,12,Gln10,Har11,Trp14]ratPTH(1-14)NH2 ([M]PTH(1-14)) (23). In cAMP stimulation assays in HKRK-B28 cells, the parent peptide [M]PTH(1-14) stimulated an approximate 70-fold maximum increase (EMax(obs.)) in intracellular cAMP, which was equal to that attained by PTH(1-34), but the potency of the shorter peptide was 40-fold less than that of PTH(1-34) (EC50s = 100±20 nM and 2.5±0.4 nM, respectively; Fig 1 and Table 1). Severe reductions in [M]PTH(1-14) potency occurred with Aib substitutions at positions 6, 8 and 9 (all > 2,300-fold), moderate reductions occurred with substitution at positions 2, 4, 5 and 11 (all 170- to 670-fold) and minor reductions occurred with substitutions at positions 7, 10, 12, 13 and 14 (all <30-fold; Table 1). Single substitution of Aib at position 1 or 3 improved potency 10- and 8-fold, respectively, relative to [M]PTH(1-14) (P < 0.01). Combining these Aib substitutions revealed an additive effect, as [Aib1,3,M]PTH(1-14) was 90-fold more potent in stimulating cAMP formation than was [M]PTH(1-14) (EC50s = 1.1±0.1 nM, 100±20 nM, respectively), and even more potent than PTH(1-34) (EC50 = 2.5±0.4 nM, P = 0.01, Fig. 2A and Table 1).

The P1R-binding properties of these analogs were assessed in competition studies performed in HKRK-B28 cells. In previous studies we evaluated the binding of PTH(1-14) analogs using 125I-PTH(3-34) as a tracer radioligand (7,23); however, in the present study, we sought to use a tracer radioligand that was structurally more homologous to [M]PTH(1-14). We evaluated 125I-[Ala3,12,Nle8,Gln10,Har11,Trp14,Tyr15]PTH(1-15)amide, but this did not bind detectably to HKRK-B28 cells (data not shown). Extending this
peptide to position 21 and including the affinity-enhancing substitution of Glu\(^{19} \rightarrow \text{Arg}\) (5,36), yielded\(^{125}\text{I-}[\text{Ala}^{3,12},\text{Nle}^{8},\text{Gln}^{10},\text{Har}^{11},\text{Trp}^{14},\text{Arg}^{19},\text{Tyr}^{21}]\text{rPTH(1-21)}\text{NH}_2\) (\(^{125}\text{I-}[\text{M}]\text{PTH(1-21)})\), which bound adequately to HKRK-B28 cells. The amount of specifically bound radioactivity (that which could be inhibited by excess unlabeled [M]PTH(1-21) peptide) observed in these cells was 9±1 % (n=7) of total radioactivity added, and the amount of non-specifically bound radioactivity (that bound in the presence of excess of unlabeled ligand) was 1.9±0.1 % of total added. The binding of \(^{125}\text{I-}[\text{M}]\text{PTH(1-21)}\) to HKRK-B28 cells was inhibited strongly by PTH(1-34) (IC\(_{50}\) ~ 18 nM) and more weakly by [M]PTH(1-14) (IC\(_{50}\) ~ 13,000 nM, Table 1). Relative to [M]PTH(1-14), most of the Aib substitutions reduced affinity, in accordance with the corresponding effects on cAMP-signaling potency (Table 1). The Aib substitutions at positions 1 and 3 improved affinity 13- and 8-fold, respectively. The effects of these two substitutions on receptor-binding affinity were additive, as [Aib\(^{1,3},\text{M}\)]PTH(1-14) bound with an apparent affinity (IC\(_{50}\) = 1.1±0.1 nM) that was 100-fold higher than that of [M]PTH(1-14) (Fig. 2B and Table 1).

In previous studies we showed that native PTH peptides shorter than PTH(1-14) are devoid of cAMP-stimulating activity (11), but that [Ala\(^3\),Gln\(^{10}\),Har\(^{11}\)]PTH(1-11)amide ([M]PTH(1-11)) can induce a full cAMP response in HKRK-B28 cells, albeit with a potency (EC\(_{50}\) = 3 \(\mu\)M) nearly 1,000-fold weaker than that of PTH(1-34) (23). We thus prepared [Aib\(^{1,3},\text{M}\)]PTH(1-11) to determine if the paired Aib substitutions could enhance activity of this shorter-length peptide. In cAMP stimulation assays in HKRK-B28 cells, [Aib\(^{1,3},\text{M}\)]PTH(1-11) exhibited an EC\(_{50}\) of ~4.0 nM and was thus 1,000-fold more potent than [M]PTH(1-11) and nearly as potent as PTH(1-34) (Fig. 2A, Table 1). The Aib-1,3 substitutions also enhanced potency of a PTH(1-10) analog, as
[Aib\textsuperscript{1,3},Gln\textsuperscript{10}]PTH(1-10)amide ([Aib\textsuperscript{1,3},M]PTH(1-10)) was \textasciitilde 50-fold more potent than [M]PTH(1-10) (EC\textsubscript{50} \textasciitilde 16 \mu M and \textasciitilde 800 \mu M, respectively; Fig. 2A, Table 1 and (23)). The 4,000-fold weaker potency of [Aib\textsuperscript{1,3},M]PTH(1-10) relative to [Aib\textsuperscript{1,3},M]PTH(1-11) also indicated the importance of the position 11 residue (homoarginine) in the activities of these Aib-containing peptides. Little or no stimulation of cAMP accumulation was observed with [Aib\textsuperscript{1,3}]PTH(1-9) (Fig. 2A and Table 1). In competition assays, [Aib\textsuperscript{1,3},M]PTH(1-11) effectively inhibited \textsuperscript{125}I-[M]PTH(1-21) binding to HKRK-B28 cells (IC\textsubscript{50} \textasciitilde 1,000 nM), whereas [Aib\textsuperscript{1,3},M]PTH(1-10) and [Aib\textsuperscript{1,3}]PTH(1-9) did not bind detectably (Fig. 2B and Table 1).

\textit{Analog activity in COS-7 cells}

To investigate whether or not the activity-enhancing effects of the Aib substitutions at positions 1 and 3 were mediated through the juxtamembrane (J) region of the receptor, we utilized COS-7 cells transiently transfected with hP1R-delNt, a truncated P1R that lacks most of the amino-terminal extracellular domain (24). With this receptor construct, PTH(1-34) is a much weaker agonist than it is with P1R-WT, while previous PTH(1-14) analogs exhibit approximately the same potency with P1R-delNt as they do with P1R-WT (24). Consistent with these previous data, the cAMP-stimulating potency of [Aib\textsuperscript{1,3},M]PTH(1-14) on hP1R-delNt (EC\textsubscript{50} = 0.73\pm0.16 nM) was comparable to its potency on COS-7 cells expressing hP1R-WT (1.2\pm0.6 nM, Table 2). With hP1R-delNt, [Aib\textsuperscript{1,3},M]PTH(1-14) was \textasciitilde 60-fold more potent than [M]PTH(1-14) (EC\textsubscript{50} \textasciitilde 0.7 nM vs. \textasciitilde 40 nM, Fig. 3A and Table 2). This \textasciitilde 60-fold enhancement in potency was comparable to that observed for these analogs with the intact P1R (\textasciitilde 40-fold, Table 2). The enhancing effects of the Aib-1,3 substitutions are therefore exerted through the J domain of the receptor. Remarkably, [Aib\textsuperscript{1,3},M]PTH(1-14) was as potent and as efficacious on
hP1R-delNt as PTH(1-34) was on hP1R-WT (EC$_{50}$ = 0.73±0.16 nM and 1.4±0.7 nM, respectively, $P = 0.4$; EMaxs = 250±20 picomole/well and 240±50 picomole/well, respectively, $P = 0.7$, Table 2). As expected, PTH(1-34) was only weakly active on hP1R-delNt (EC$_{50}$ ~ 700 nM Fig. 3A and Table 2), but [Aib$^{1,3}$,M]PTH(1-34) was fully potent on this receptor (EC$_{50}$ = 1.9±0.6 nM). Notably this modified PTH(1-34) analog did not exhibit enhanced potency on hP1R-WT, as compared to PTH(1-34) (Table 2).

In COS-7 cells expressing hP1R-WT, [Aib$^{1,3}$,M]PTH(1-14) was 66-fold more potent than [M]PTH(1-14) in stimulating the production of total inositol phosphates (IPs) (EC$_{50}$ = 71±9 nM, and 4,700±2,000 nM, respectively). The corresponding EMax values for these responses were comparable to each other, and to that observed for PTH(1-34) (~4 times basal, Table 2). The potency of [Aib$^{1,3}$,M]PTH(1-11) on hP1R-WT (EC$_{50}$ = 460±110 nM, Table 2) was ~240-fold greater than that which we recorded previously for [M]PTH(1-11) on this receptor (EC$_{50}$ = 110±20 µM, (23). Figure 3B shows that while PTH(1-34) and [M]PTH(1-14), each at a concentration of 1 µM, were inactive for IP production in COS-7 cells expressing hP1R-delNt, [Aib$^{1,3}$,M]PTH(1-14) stimulated an approximate 3-fold increase in IP accumulation. The Aib-1,3-containing PTH(1-34), PTH(1-21) and PTH(1-11) analogs also stimulated PLC responses in cells expressing the truncated receptor with EC$_{50}$s that were comparable to those observed for these peptides in cells expressing hP1R-WT (Table 2). Thus, the Aib-1,3 substitutions enhance the ligand’s capacity to stimulate PLC activity via the P1R, and this enhancement is mediated through the juxtamembrane region of the receptor.

In our previous studies, the lack of a radioligand which could bind detectably to P1R-delNt prevented us from assessing ligand affinity at the truncated receptor. The
radioligand $^{125}$I-[M]PTH(1-21) used in the above binding studies with HKRK-B28 cells also did not bind detectably to hP1R-delNt. When the paired Aib-1,3 modifications were introduced into this peptide, however, the resulting radiolabeled analog, $^{125}$I-[Aib$^{1,3}$,M]PTH(1-21), bound to COS-7 cells expressing hP1R-delNt nearly as well as it did to COS-7 cells expressing hP1R-WT. The specific binding of this radioligand (that which could be inhibited by excess unlabeled [Aib$^{1,3}$,M]PTH(1-21)) to the two receptors was 13 ±2% and 19±2 % (n= 6) of the total radioactivity added, respectively. The total specific binding observed in COS-7 cells transfected with vector DNA alone was negligible (0.4±0.2% of total radioactivity added, n=3). This radioligand therefore enables competition binding experiments to be performed with hP1R-delNt, as well as with hP1R-WT. Homologous competition binding experiments performed with this tracer radioligand and varying amounts of unlabeled [Aib$^{1,3}$,M]PTH(1-21) indicated that the peptide bound to the two receptors with comparable affinities (IC$_{50}$s ~ 30 nM), and it was a potent agonist for each receptor (EC$_{50}$ for cAMP formation $< 0.8$ nM, EC$_{50}$ for IP$_3$ formation $< 26$ nM, Table 2).

The binding of $^{125}$I-[Aib$^{1,3}$,M]PTH(1-21) to hP1R-delNt was also inhibited by [Aib$^{1,3}$,M]PTH(1-14), [Aib$^{1,3}$,M]PTH(1-11) and [M]PTH(1-14), as well as by [Aib$^{1,3}$,M]PTH(1-34) (Fig. 3C and Table 2). The apparent binding affinities observed for these N-terminally modified peptides at hP1R-delNt were comparable to their affinities observed at hP1R-WT; unmodified PTH(1-34), however, bound effectively to hP1R-WT (IC$_{50}$ = 22±6 nM), but not at all to hP1R-delNt (Table 2). At both hP1R-delNt and hP1R-WT, [Aib$^{1,3}$,M]PTH(1-14) bound with an affinity that was ~10-fold stronger than that of [M]PTH(1-14). These results indicate that the modified ligands bind mainly to the J
domain of the receptor, and that the Aib-1,3 substitutions enhance the affinity of this J domain binding interaction.

**Activity in bone cells**

The number of PTH-1 receptors expressed on the surface of PTH target cells in bone or kidney may be considerably lower than that found in the transfected HKRK-B28 or COS-7 cells. We therefore evaluated several of the Aib-modified PTH analogs using the SaOS-2 cell line, a human osteosarcoma derivative in which the P1R is endogenously expressed at relatively low levels (~20,000 receptors/cell (37)). As in the previous transfected cell systems, [Aib\(^{1,3}\),M]PTH(1-14) was 130-fold more potent than [M]PTH(1-14) in stimulating cAMP formation in SaOS-2 cells (Fig. 4 and Table 3). Thus, the Aib-1,3 modifications enhance activity in this human bone cell line. While [Aib\(^{1,3}\),M]PTH(1-14) was 13-fold less potent than PTH(1-34) in these cells, it was at least five-orders of magnitude more potent than native PTH(1-14), for which no activity could be detected, even at a dose of 10 \(\mu\)M (Fig. 4 and Table 3).

We investigated whether or not [Aib\(^{1,3}\),M]PTH(1-14) activity could be detected in a more intact bone system using a tissue explantation assay. In this assay cartilaginous metatarsal rudiments from fetal mice were isolated at gestational day 15.5 and subsequently cultured in multi-well plates containing serum-free media. At 16 hours, and again at 24 hours, after explantation a PTH peptide analog, or vehicle control, was added to the culture. The incubation was terminated 24 hours later for a total of 48 hours of treatment over a 64-hour incubation period. In the absence of PTH, chondrocyte differentiation occurred, such that by the end of the experiment, dense mineralization was apparent at the bone’s mid-section (Fig. 5A). Differentiation was
inhibited by the presence of PTH(1-34) (0.1 µM) or [Aib\textsuperscript{1,3},M\]PTH(1-14) (1 µM), as no mineralization was observed (Fig. 5, B and C). Mineralization was also inhibited in these assays by [Aib\textsuperscript{1},M\]PTH(1-14) (data not shown), whereas no effect could be detected for native PTH(1-14) (2 µM) (Fig. 5D). Comparable results were obtained in each of three replicate experiments. In addition, mRNA in-situ hybridization analysis performed on the explanted metatarsals demonstrated that both PTH(1-34) and [Aib\textsuperscript{1,3},M\]PTH(1-14) inhibited expression of the collagen X gene, a bone developmental marker gene (data not shown). These inhibitory effects are consistent with the known capacity of PTHrP to retard chondrocyte differentiation in the growth plate cartilage of developing long bones (38).

Circular Dichroism of N-terminal PTH analogs

Circular dichroism (CD) spectroscopy was used to analyze the effects that the Aib substitutions had on secondary structure of the N-terminal peptide analogs in the free solution phase. The peptides were analyzed in phosphate buffer containing 2,2,2-trifluoroethanol (20% v/v), an organic solvent that promotes helical structure in oligopeptides, including PTH peptide fragments (25,39,40), or in phosphate buffer without TFE. In the resulting CD spectra, plotted as mean molar residue ellipticity ([θ]) vs. wavelength, the double minimum at ~209 nm and ~222 nm and the maximum at 192 nm indicated that the peptides contained helical structure, and the amount of helix in each peptide was estimated from the observed [θ]_{222} value (33). The two most helical peptide fragments were [Aib\textsuperscript{1,3},M\]PTH(1-14) and [Aib\textsuperscript{1,3},M\]PTH(1-11) (56% and 57%, respectively); the corresponding peptides containing alanine at positions -1 and -3, [M\]PTH(1-14) and [M\]PTH(1-11), exhibited 42% and 35%, helicity, respectively (Fig. 6A and Table 4). A small (4% to 5%) increase in helicity was observed in comparing
[M]PTH(1-14) to PTH(1-14), as well as in comparing [M]PTH(1-11) to native PTH(1-11) (Table 4). In phosphate buffer, [Aib\textsuperscript{1,3},M]PTH(1-14) and [Aib\textsuperscript{1,3},M]PTH(1-11) were again the two most helical N-terminal peptides (16% and 13%, respectively) and these exhibited nearly twice the helical content as the corresponding peptides containing alanine at positions -1 and -3 (8.1% and 7.5%, respectively, Fig. 6B and Table 4). In this buffer, no difference in helicity was observed between [M]PTH(1-14) and native PTH(1-14), or between [M]PTH(1-11) and native PTH(1-11) (Table 4). Thus, the Aib-1,3 modifications increase the helical content of the N-terminal PTH oligopeptides even in a purely aqueous buffer.

*Aib-1,3 substitutions in PTH(1-34).*

The above results suggested a possible correlation between the increases in potency of the N-terminal PTH analogs containing the Aib-1,3 substitutions and the increases in peptide helicity. We examined this possibility further in the context of a relatively unmodified PTH(1-34) peptide, hPTH(1-34) ([Tyr\textsuperscript{34}]hPTH(1-34)NH\textsubscript{2}). In COS-7 cells expressing hP1R-WT, [Aib\textsuperscript{1,3}]hPTH(1-34) and hPTH(1-34) were approximately equipotent for stimulating cAMP formation (EC\textsubscript{50}s = 0.67±0.18 nM and 0.44±0.02 nM, \(P = 0.3\), Fig. 7A). On hP1R-delNt, however, [Aib\textsuperscript{1,3}]hPTH(1-34) was ~65-fold more potent than hPTH(1-34) (EC\textsubscript{50}s = 43±24 nM and 2,800±300 nM, respectively, \(P = 0.001\), Fig. 7A). CD analysis performed on the two peptides in phosphate buffer containing 2,2,2-trifluoroethanol (20%), demonstrated that [Aib\textsuperscript{1,3}]hPTH(1-34) contained a greater amount of helix than did hPTH(1-34) (62% and 47% helix, respectively) (Fig. 7B). The helical content of [Aib\textsuperscript{1,3},M]PTH(1-34) (61%) was similar to that of [Aib\textsuperscript{1,3}]hPTH(1-34) (data not shown). The higher helical contents seen in these PTH(1-34) peptides, relative to those seen in the N-terminal PTH(1-14) and PTH(1-11) analogs (Table 4) is likely due
to the helical contribution of the (15-34) binding domain (39,40). These data show that Aib-1,3 substitutions in a relatively unmodified PTH(1-34) peptide increase peptide helicity and the capacity of the ligand to activate the N-terminally truncated P1R, but not the intact P1R.

Receptor interaction sites

Our binding studies showing that unmodified PTH(1-34) could inhibit the binding of an Aib-modified radioligand to the P1R (Table 2), suggested that the Aib-modified ligands interact at the same receptor site as native PTH. To investigate this further, we utilized two rat P1R mutants, rP1R-F184A and rP1R-W437A, that are altered at two sites which have been shown to be important for interaction with non-constrained PTH analogs. Phenylalanine-184 is at the carboxy-terminal end of the amino-terminal extracellular domain and is within the region crosslinked by a PTH(1-34) analog containing a photolabile benzophenone moiety attached to lysine-13 (41). Tryptophan-437 is in the third extracellular loop and is required for efficient binding of PTH(1-34) but not PTH(3-34) radioligands; this residue is thus considered to be a candidate interaction site for residues 1-2 in PTH (30). Alanine mutation at either site reduces PTH(1-34) binding affinity and cAMP-stimulating potency by ~100-fold, relative to the wild-type receptor, without affecting cell surface expression levels, as judged by antibody binding to the HA epitope-tagged receptors (29,30). As shown in Figure 8, like PTH(1-34), [Aib\textsuperscript{1,3},M]PTH(1-34), [Aib\textsuperscript{1,3},M]PTH(1-14) and [Aib\textsuperscript{1,3},M]PTH(1-11) each exhibited impaired capacity to stimulate cAMP formation with rP1R-F184A and rP1R-W437A. The dose of each peptide (5 nM) used in this analysis was sufficient to elicit a near maximal (16- to 20-fold above basal) cAMP response with rP1R-WT. These results thus indicate that, as with PTH(1-34), the capacities of the Aib-containing PTH analogs
to interact with the receptor are dependent on the residues at positions 184 and 437 in the receptor.
Discussion

In this study we showed that single Aib substitutions at either position one or three in a PTH(1-14) analog resulted eight- to ten–fold improvements in cAMP-signaling potency, and when combined resulted in an approximate 100-fold improvement in potency. Thus, [Aib\(^{1,3}\),M]PTH(1-14), with an EC\(_{50}\) of ~ 1 nM in HKRK-B28 cells, was 100-fold more potent than [M]PTH(1-14), five-orders of magnitude more potent than native PTH(1-14) (23) and two-fold more potent than PTH(1-34) (Table 1). Our competition binding studies performed with \(^{125}\text{I}([M]PTH(1-21)) indicated that these effects on potency were exerted, at least in part, by improvements in PTH-1 receptor-binding affinity. None of the peptides in our study elicited a detectable cAMP response in untransfected LLC-PK\(_1\) cells, which endogenously express the related calcitonin receptor (27), or in untransfected COS-7 cells (data not shown). The responses observed with the modified peptides were therefore P1R-dependent. The lack of binding of \(^{125}\text{I}([Aib^{1,3},M]PTH(1-21)) to untransfected COS-7 and LLC-PK\(_1\) cells also indicated that the Aib-modified peptides maintain specificity for the P1R.

Although our studies permit only indirect conclusions regarding the structure of PTH as it is bound to the receptor, the activity-enhancing effects that occurred with the Aib modifications at positions -1 and –3 in our N-terminal PTH analogs and the increases in helicity that we observed for these Aib-substituted peptide by CD spectroscopy are consistent with the hypothesis that the Aib modifications stabilize helical conformation in the ligands and thereby enable more efficient interaction with the receptor. Alternative possibilities, such as altered sidechain interactions between the Aib residues and the receptor, the induction of a different backbone conformation, such as a turn (26,42), or the promotion of more favorable initial interactions with the
lipid membrane (43), while not excluded, seem less likely, given the propensity of Aib to stabilize helical structure in oligopeptides (26,42), and the indication from many structural studies on isolated PTH peptides that residues near the N-terminal portion of the ligand can form an $\alpha$-helix (12-17,19,44,45) and our CD data. Recently, we showed that the introduction of a model helical sequence (Glu-Ala-Ala-Ala-Lys) into the relatively tolerant 10-14 region of PTH(1-14) resulted in a peptide with high helical content (89% in TFE (20%)-containing buffer) but potency of this peptide was very weak ($EC_{50}$ for cAMP stimulation $> 100 \mu M$, (25). This finding, together with our current data showing that Aib substitutions at sites other than position –1 or –3 in PTH(1-14) reduced potency (Table 1), demonstrate that helix-stabilizing modifications per se in the (1-14) domain do not necessarily result in enhanced activity on the P1R.

As a means to assess the mechanism by which the Aib-1,3 modifications enhance ligand interaction with the P1R, we utilized a truncated P1R that lacks most of the N-terminal domain. These studies demonstrated that the Aib-1,3 substitutions exert their effect principally, if not exclusively, through the J domain of the receptor, as the modified peptides exhibited the same increases in potency on hP1R-delNt, as they did on hP1R-WT. These observations are consistent with the hypothesis that the N-terminal domain of PTH interacts with the receptor's J domain while residues in the (15-34) portion of the ligand interact predominantly with the N domain (7). In two recently reported computer models of the PTH(1-34)/P1R complex that were developed from structural data on isolated PTH ligand and P1R peptide fragments, together with PTH/P1R crosslinking data, the N-terminal portion of the ligand is shown to be in $\alpha$-helical conformation and to make multiple contacts with the extracellular surface of the receptor's J domain (19,46). We tested for the possibility that our Aib-modified PTH
analogs might utilize a docking site in the receptor that is different from that used by native PTH by employing two P1R mutants altered by alanine substitutions at two key J domain residues known to be important for interaction with native PTH(1-34). Each of these point mutations: Phe184→Ala near the boundary of the N-domain and TM1 and Trp437→Ala in extracellular loop 3, impaired interaction with [Aib\textsuperscript{1,3},M]PTH(1-34), [Aib\textsuperscript{1,3},M]PTH(1-14) and [Aib\textsuperscript{1,3},M]PTH(1-11), as they did for PTH(1-34) (Fig. 8). Thus, the contact surface utilized by the Aib-modified peptides does not appear to be radically different from that used by the native hormone. This conclusion is further supported by the capacity of unmodified PTH(1-34) to inhibit the binding of \textsuperscript{125}I-[Aib\textsuperscript{1,3},M]PTH(1-21) to the receptor (Table 2).

Our studies with hP1R-delNt also demonstrated that the juxtamembrane region of the receptor can function autonomously, at least at the level of interaction with small agonist peptides and the induction of transmembrane signaling. Thus, hP1R-delNt mounted sensitive and robust cAMP responses to oligopeptides as small as [Aib\textsuperscript{1,3},M]PTH(1-14) and [Aib\textsuperscript{1,3},M]PTH(1-11) (EC\textsubscript{50} ≤ 2 nM; EMax ≥ 23-fold, relative to basal) and elicited adequate PLC responses to these ligands (Table 2). In addition, we showed, for the first time, that by using the Aib-containing radioligand, \textsuperscript{125}I-[Aib\textsuperscript{1,3},M]PTH(1-21), the binding affinity of a ligand to the J domain of the P1R can be directly assessed by competition methods. Thus, we could detect binding of a modified N-terminal PTH peptide as short as [Aib\textsuperscript{1,3},M]PTH(1-11) to hP1R-delNt (IC\textsubscript{50} = 13 µM). We also confirmed by this method that, in the absence of the receptor’s N domain, the affinity with which unmodified PTH(1-34) interacts with the receptor’s J domain is very weak (IC\textsubscript{50} > 30 µM), thereby accounting for, in large part, the weak cAMP-signaling potency that PTH(1-34) exhibits on hP1R-delNt (EC\textsubscript{50} ~700 nM, as compared to ~0.7 nM
for [Aib\textsuperscript{1,3},M]PTH(1-14). Our findings with hP1R-delNt also suggest that it should be possible for a small non-peptide molecule that interacts only with the J domain of the P1R to behave as a potent P1R agonist. Such a molecule has not been reported, but would be of considerable medical interest, since PTH(1-34) has been shown to be effective in treating osteoporosis (2). Moreover, the availability of small conformationally constrained PTH agonist peptides, such as those described herein, could provide a step towards the rational design of non-peptide mimetic compounds for the P1R, as the potential for structural variability in such ligands is considerably reduced, relative to that in non-constrained peptides (26), like PTH(1-34).

Structural studies on unmodified PTH(1-34) have not detected secondary structure for residues N-terminal to position-3 (19,47). These findings, together with our data showing that Aib substitutions at positions 1 and 3 in PTH enhance activity, suggest that the N-terminal residues of the native hormone are inherently dynamic but become ordered upon interacting with the receptor. When we analyzed the effects of the Aib-1,3 substitutions in unmodified hPTH(1-34), we observed a marked increase in helicity (Fig. 7B) together with a strong (~65-fold) enhancement in potency on hP1R-delNt, as was seen for these substitutions in the shorter N-terminal PTH fragment analogs. In contrast to the effects seen in the N-terminal peptides, however, the Aib-1,3 substitutions in hPTH(1-34) did not increase potency on hP1R-WT (Fig. 7A). The apparent binding affinity of [Aib\textsuperscript{1,3}]hPTH(1-34) to hP1R-WT (IC\textsubscript{50} \(27\pm 8\) nM, n=3, data not shown), was comparable to that observed for PTH(1-34) (IC\textsubscript{50} = \(22\pm 6\) nM, Table 2). Similarly, [Aib\textsuperscript{1,3},M]PTH(1-34) was approximately equipotent to PTH(1-34) in cAMP, PLC and competition binding assays performed with hP1R-WT, but was several hundred-fold more potent than PTH(1-34) in these assays performed with hP1R-delNt.
(Table 2). While we currently are not certain of the molecular mechanisms by which the Aib-1,3 substitutions exert their effects on peptide potency, one possible explanation for these results with PTH(1-34) analogs is that the effects of the Aib-1,3 substitutions in this peptide are redundant with effects that occur in the native hormone when it interacts with the wild-type P1R, but not when it interacts with hP1R-delNt. For example, the Aib-1,3 substitutions might pre-organize the same bioactive structure (possibly helical) in the (1-14) portion of the ligand that is induced in native PTH(1-34) as a result of the interaction between residues in the (15-34) domain of the ligand and the N-domain of the receptor. Thus, any potential entropic benefit provided by pre-organizing the (1-14) domain structure in PTH(1-34) would not be realized when the ligand interacts with hP1R-WT, whereas it would result in enhanced potency when the ligand interacts with hP1R-delNt, since the (15-34)/N-domain interaction would not occur. The same domain pre-organization effect could account for the enhanced potency seen in the Aib-1,3-substituted (PTH 1-14) fragment analogs with either hP1R-WT or hP1R-delNt, since these ligands lack the (15-34) domain altogether. In a recent study on pituitary adenylate cyclase-activating peptide, another class II G protein-coupled receptor ligand, NMR methods were used to analyze the ligand in the free and receptor-bound state and a modest conformational difference (random vs. β-coil) was detected between the N-terminal portions of the free and bound peptide (48). Such physical studies on the PTH/P1R complex are not yet possible, as the PTH receptor has not been purified in functional form, and so further work is clearly needed to assess any potential differences between the conformations of free and receptor-bound PTH.

In summary, we have shown that potent PTH(1-14) and even PTH(1-11) analogs can be obtained by introducing the conformationally constraining amino acid, Aib, at
the N-terminus of the peptides. Importantly, these peptides were active in bone cells and in an integrated bone tissue *in vitro*. The Aib modifications stabilized helical structure in the peptides and they produced marked increases in potency on hP1R-delNt, results which suggest the possibility that the N-terminal portion of PTH assumes an α-helical conformation when interacting with the activation domain of the receptor, although other conformations are not excluded. The overall information provides new insights into the mechanisms by which PTH interacts with its class II G protein-coupled receptor and could potentially open new paths for developing smaller and more potent P1R agonists.
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References:


5. Takasu, H, Gardella, TJ, Luck, MD, Potts Jr., JT and Bringhurst, FR. (1999) Amino-terminal modifications of human parathyroid hormone(PTH) selectively alter phospholipase C signaling via the type 1 PTH receptor: implications for design of signal-specific PTH ligands. Biochemistry 38, 13453-13460


interaction on the ligand binding mechanism and receptor conformation. *J. Biol. Chem.* 276, 7741-7753


12. Marx, UC, Adermann, K, Bayer, P, Forssmann, WG and Rosch, P. (2000) Solution structures of human parathyroid hormone fragments hPTH(1 34) and hPTH(1 39) and bovine parathyroid hormone fragment bPTH(1 37). *Biochem Biophys Res Commun* 267, 213-220


(PTH) receptor functionally interact with the amino-terminal portion of PTH (1-34). J. Biol. Chem. 274, 31955-31960.


Figure Legends:

Figure 1  Aib-scan of a modified PTH(1-14) analog in HKRK-B28 cells. The peptide [Ala\textsuperscript{3,12},Gln\textsuperscript{10},Har\textsuperscript{11},Trp\textsuperscript{14}]-PTH(1-14)amide ([M]PTH(1-14)), and derivatives of that peptide containing a single Aib substitution at one of each residue position, were evaluated for the capacity to stimulate intracellular cAMP accumulation in HKRK-B28 cells, as described in Materials and Methods. The peptides with substitutions at positions 1 through 7 are shown in panel A, and those with substitutions at positions 8 through 14 are shown in B. Shown are combined data (mean±S.E.M.) from 3 to 10 experiments, each performed in duplicate. Symbols are defined in the key.

Figure 2  cAMP-signaling and binding properties of PTH analogs in HKRK-B28 cells. Peptides were evaluated in HKRK-B28 cells for the capacity to stimulate intracellular cAMP accumulation (A) and the capacity to inhibit binding of \textsuperscript{125}I-[M]PTH(1-21) (B), as described in Materials and Methods. Shown are combined data (mean±S.E.M.) from 3 or 4 experiments, each performed in duplicate. Peptides and corresponding symbols are identified in the key.

Figure 3  Signaling and binding properties of PTH analogs in COS-7 cells expressing an N-terminally truncated P1R. COS-7 cells were transiently transfected with hP1R-delNt, a truncated hP1R that is deleted for most of the amino-terminal extracellular domain, and subsequently used to evaluate the capacities of the indicated PTH analogs to stimulate intracellular cAMP accumulation (A); stimulate formation of \textsuperscript{3}H-inositol phosphates (IP\textsuperscript{1}+IP\textsuperscript{2}+IP\textsuperscript{3}) (B); and inhibit the binding of \textsuperscript{125}I-[Aib\textsuperscript{1,3},M]PTH(1-21) (C). Each curve shows data combined (mean±S.E.M.) from 3 to 6 experiments, each performed in duplicate. The mean basal level of \textsuperscript{3}H-inositol phosphates (2,929±877
cpm/well, n=3) is indicated by the dashed line. Peptides and corresponding symbols are identified in the key.

Figure 4  cAMP-signaling properties of PTH analogs in SaOS-2 cells. The peptides, PTH(1-34), native PTH(1-14), [M]PTH(1-14) and [Aib\textsuperscript{1,3},M]PTH(1-14) were evaluated in the human osteosarcoma-derived cell line SaOS-2 for the capacity to stimulate intracellular cAMP accumulation, as described in Materials and Methods. Shown are combined data (mean±S.E.M.) from 3 or 4 experiments, each performed in duplicate. Symbols are defined in the Key.

Figure 5  Effect of PTH analogs on bone mineralization in embryonic mouse metatarsals. Cartilaginous metatarsal bone rudiments were excised from fetal mice at 15.5 days post-conception, transferred to tissue culture plates containing serum–free media and cultured at 37°C for a total of 64 hours. At 16h and again at 24h after explantation, samples were treated with vehicle alone (A) or vehicle containing PTH(1-34) (0.1 µM) (B); [Aib\textsuperscript{1,3},M]PTH(1-14) (1 µM) (C) or native PTH(1-14) (2 µM) (D). At the end of the 64h incubation (48 of treatment), the samples were fixed and directly visualized under white light using a dissecting scope. In the vehicle- and native PTH(1-14)-treated samples mineralization can be detected as dark material at the center of the bone rudiment. Both PTH(1-34) and [Aib\textsuperscript{1,3},M]PTH(1-14) inhibited mineralization. Shown are data from a single experiment, comparable results were obtained in three other replicate experiments.

Figure 6  Cicular dichroism spectroscopy. Spectra were recorded for the indicated N-terminal PTH oligopeptides, each at 20 µM, in 50 mM sodium phosphate buffer, pH
7.4 containing the helix-promoting organic solvent 2,2,2-trifluoroethanol (20% v/v) (A), or in purely aqueous 50 mM sodium phosphate buffer, pH 7.4 (B) as described in Materials and Methods. The negative deflections in the spectra at ~209 nm and ~222 nm, and of the positive deflection at ~192 nm, are more apparent with the Aib-containing PTH analogs, as compared to the non-Aib-containing peptides and are indicative of helical content.

**Figure 7  Effect of Aib-1,3 substitutions in hPTH(1-34).** The peptides hPTH(1-34) ([Tyr^{34}]hPTH(1-34)NH$_2$) and [Aib$_{1,3}$]hPTH(1-34) ([Aib$_{1,3}$,Tyr$^{34}$]hPTH(1-34)NH$_2$) were analyzed for the capacity to stimulate cAMP formation in COS-7 cells transfected with either hP1R-WT or hP1R-delNt (A), and for secondary structure by CD spectroscopy (B). In panel A, the cAMP values are expressed as a percent of the maximum response elicited in each experiment by hPTH(1-34) on hP1R-WT, the average of which was 150±26 picomoles/well (n=3). The basal cAMP levels observed for hP1R-WT and hP1R-delNt were 6.2±0.1 and 3.7±0.4 picomoles/well, respectively. The data shown (mean±S.E.M.) are combined from 3 experiments, each performed in duplicate. In panel B, the peptides were analyzed at a concentration of 20 µM in 50 mM sodium phosphate buffer, pH 7.4 containing 2,2,2-trifluoroethanol (20% v/v), as described in Materials and Methods. The curve shown for each peptide is a composite of six scans.

**Figure 8  Mutational analysis of receptor interaction sites for PTH analogs.** The analogs, [Aib$_{1,3}$,M]PTH(1-34), [Aib$_{1,3}$,M]PTH(1-14) and [Aib$_{1,3}$,M]PTH(1-11) and the control, PTH(1-34), were analyzed for the capacity to stimulate formation in COS-7 cells expressing either the wild-type rat PTH-1 receptor (rP1R-WT), or mutant rP1Rs containing single alanine substitutions either at Phe184 (rP1R-F184A), located at the
boundary of the amino-terminal extracellular domain and TM1, or at Trp437 (rP1R-W437A), located in the third extracellular loop. These P1R mutations have previously been shown to impair the binding and signaling responses elicited by PTH(1-34) without affecting cell surface expression (29,30). Each of the receptors contained the HA epitope tag in the amino-terminal extracellular domain (30). Shown are combined data (mean±S.E.M.) from 3 experiments, each performed in duplicate.
<table>
<thead>
<tr>
<th>Peptide[^a]</th>
<th>EC\textsubscript{50}[^b]</th>
<th>EMax\textsubscript{obs.}</th>
<th>IC\textsubscript{50}[^c]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nM</td>
<td>pmole/well</td>
<td>nM</td>
</tr>
<tr>
<td>PTH(1-34)</td>
<td>2.5 ± 0.4</td>
<td>280 ± 11</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>[M]PTH(1-14)</td>
<td>100 ± 20</td>
<td>270 ± 8</td>
<td>13,000 ± 3,000</td>
</tr>
<tr>
<td>[M]PTH(1-21)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Aib scan in [M]PTH(1-14)

<table>
<thead>
<tr>
<th>Peptide[^a]</th>
<th>EC\textsubscript{50}[^b]</th>
<th>EMax\textsubscript{obs.}</th>
<th>IC\textsubscript{50}[^c]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nM</td>
<td>pmole/well</td>
<td>nM</td>
</tr>
<tr>
<td>Aib-1</td>
<td>10 ± 3</td>
<td>273 ± 6</td>
<td>980 ± 160</td>
</tr>
<tr>
<td>Aib-2</td>
<td>47,000 ± 13,000</td>
<td>168 ± 6</td>
<td>50,000 ± 11,000</td>
</tr>
<tr>
<td>Aib-3</td>
<td>13 ± 3</td>
<td>269 ± 7</td>
<td>1,700 ± 200</td>
</tr>
<tr>
<td>Aib-4</td>
<td>17,000 ± 3,400</td>
<td>221 ± 10</td>
<td>148,000 ± 40,000</td>
</tr>
<tr>
<td>Aib-5</td>
<td>66,000 ± 38,000</td>
<td>169 ± 18</td>
<td>N.B.</td>
</tr>
<tr>
<td>Aib-6</td>
<td>230,000 ± 78,000</td>
<td>116 ± 12</td>
<td>31,000 ± 7,000</td>
</tr>
<tr>
<td>Aib-7</td>
<td>2,600 ± 980</td>
<td>275 ± 8</td>
<td>490,000 ± 170,000</td>
</tr>
<tr>
<td>Aib-8</td>
<td>&gt; 100,000</td>
<td>34 ± 7</td>
<td>N.B.</td>
</tr>
<tr>
<td>Aib-9</td>
<td>&gt; 100,000</td>
<td>51 ± 8</td>
<td>N.B.</td>
</tr>
<tr>
<td>Aib-10</td>
<td>3,000 ± 2,100</td>
<td>214 ± 22</td>
<td>9,100 ± 1,500</td>
</tr>
<tr>
<td>Aib-11</td>
<td>67,000 ± 51,000</td>
<td>96 ± 18</td>
<td>N.B.</td>
</tr>
<tr>
<td>Aib-12</td>
<td>440 ± 300</td>
<td>263 ± 9</td>
<td>15,000 ± 3,000</td>
</tr>
<tr>
<td>Aib-13</td>
<td>480 ± 250</td>
<td>259 ± 10</td>
<td>44,000 ± 8,000</td>
</tr>
<tr>
<td>Aib-14</td>
<td>350 ± 100</td>
<td>273 ± 6</td>
<td>79,000 ± 27,000</td>
</tr>
</tbody>
</table>

Aib\textsuperscript{1,3} in [M]PTH(1-X)

<table>
<thead>
<tr>
<th>Peptide[^a]</th>
<th>EC\textsubscript{50}[^b]</th>
<th>EMax\textsubscript{obs.}</th>
<th>IC\textsubscript{50}[^c]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nM</td>
<td>pmole/well</td>
<td>nM</td>
</tr>
<tr>
<td>[Aib\textsuperscript{1,3},M]PTH(1-21)</td>
<td>4.3 ± 1.6</td>
<td>284 ± 76</td>
<td>31 ± 7</td>
</tr>
<tr>
<td>[Aib\textsuperscript{1,3},M]PTH(1-14)</td>
<td>1.1 ± 0.1</td>
<td>278 ± 20</td>
<td>130 ± 20</td>
</tr>
<tr>
<td>[Aib\textsuperscript{1,3},M]PTH(1-11)</td>
<td>4.0 ± 0.8</td>
<td>243 ± 15</td>
<td>970 ± 300</td>
</tr>
<tr>
<td>[Aib\textsuperscript{1,3},M]PTH(1-10)</td>
<td>16,000 ± 2,000</td>
<td>111 ± 8</td>
<td>N.B.</td>
</tr>
<tr>
<td>[Aib\textsuperscript{1,3},M]PTH(1-9)</td>
<td>&gt; 10,000</td>
<td>10 ± 1</td>
<td>N.B.</td>
</tr>
</tbody>
</table>

[^a] Peptides were based on the rat PTH sequence and were carboxy-amidated. [M]PTH(1-14) and shorter analogs contain the modifications: Ala\textsuperscript{3,12}, Gln\textsuperscript{10}, Har\textsuperscript{11}, Trp\textsuperscript{14}, unless C-terminally truncated or substituted by α-aminoisobutyric acid (Aib), as indicated; [M]PTH(1-21) contains the same modifications, as well as Nle\textsuperscript{8}, Arg\textsuperscript{19} and Tyr\textsuperscript{21}. ^b The basal cAMP values (not subtracted) were 4.0 ± 0.1 pmole/well (n=10). ^c Competition binding analyses were performed in whole cells at 4°C for 4h with \textsuperscript{125}I-[M]PTH(1-21) as tracer radioligand. Data are means (±S.E.M.) of the number of experiments indicated (n). N.B., no binding was detected at a peptide concentration of 10 μM; N.D; the experiment was not done.
Table 2 Functional properties of PTH analogs in COS-7 cells

<table>
<thead>
<tr>
<th>Peptide b</th>
<th>cAMP c</th>
<th>PLC d</th>
<th>Binding e</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50</td>
<td>EMax(obs.)</td>
<td>EC50</td>
</tr>
<tr>
<td></td>
<td>nM</td>
<td>pmole/well (n)</td>
<td>nM</td>
</tr>
<tr>
<td>hP1R-WT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTH(1-34)</td>
<td>1.4 ± 0.7</td>
<td>240 ± 50 3</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>PTH(1-14)</td>
<td>90,000 ± 34,000</td>
<td>140 ± 30 * 3</td>
<td>---</td>
</tr>
<tr>
<td>[M]PTH(1-14)</td>
<td>49 ± 21</td>
<td>240 ± 50 3</td>
<td>4,700 ± 2,000 3</td>
</tr>
<tr>
<td>[Aib1,3-M]PTH(1-34)</td>
<td>1.3 ± 0.4</td>
<td>290 ± 82 3</td>
<td>73 ± 10</td>
</tr>
<tr>
<td>[Aib1,3-M]PTH(1-21)</td>
<td>0.8 ± 0.1</td>
<td>300 ± 30 3</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>[Aib1,3-M]PTH(1-14)</td>
<td>1.2 ± 0.6</td>
<td>240 ± 40 3</td>
<td>71 ± 9</td>
</tr>
<tr>
<td>[Aib1,3-M]PTH(1-11)</td>
<td>2.1 ± 0.7</td>
<td>190 ± 40 3</td>
<td>460 ± 110 3</td>
</tr>
<tr>
<td>[Aib1,3-M]PTH(1-10)</td>
<td>100,000 ± 40,000</td>
<td>120 ± 10 * 3</td>
<td>N.D.</td>
</tr>
<tr>
<td>hP1R-delNt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTH(1-34)</td>
<td>680 ± 110</td>
<td>220 ± 30 3</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>PTH(1-14)</td>
<td>140,000 ± 30,000</td>
<td>110 ± 10 * 3</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>[M]PTH(1-14)</td>
<td>40 ± 2.0</td>
<td>220 ± 20 3</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>[Aib1,3-M]PTH(1-34)</td>
<td>1.9 ± 0.6</td>
<td>270 ± 40 3</td>
<td>63 ± 13</td>
</tr>
<tr>
<td>[Aib1,3-M]PTH(1-21)</td>
<td>0.38 ± 0.10</td>
<td>240 ± 20 3</td>
<td>26 ± 5</td>
</tr>
<tr>
<td>[Aib1,3-M]PTH(1-14)</td>
<td>0.73 ± 0.16</td>
<td>250 ± 20 3</td>
<td>130 ± 30</td>
</tr>
<tr>
<td>[Aib1,3-M]PTH(1-11)</td>
<td>2.0 ± 0.4</td>
<td>220 ± 20 3</td>
<td>815 ± 140 3</td>
</tr>
<tr>
<td>[Aib1,3-M]PTH(1-10)</td>
<td>53,000 ± 10,000</td>
<td>84 ± 4 * 3</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

a Peptides were evaluated in COS-7 cells transiently transfected with either the wild-type hP1R (hP1R-WT), or a truncated hP1R lacking most of the amino-terminal extracellular domain (hP1R-delNt), as described in Materials and Methods. b Peptide modifications are described in Table 1 and in Materials and Methods. c The basal levels of cAMP were 8.4±0.8 and 6.4±0.9 picomole per well for hP1R-WT and hP1R-delNt, respectively, (n=11). d The basal levels of 3H-inositol phosphates were 1,105±75 and 2,099±501 cpm per well for hP1R-WT and hP1R-delNt, respectively (n=6). The Emax(obs.) (maximum response observed) values in the cAMP and PLC assays were determined at ligand doses of 0.1 to 100 µM; an asterisk indicates that a plateau in the response curve was not attained, and the EC50 was calculated by extrapolating to the maximum response attained in the same assay with PTH(1-34) (hP1R-WT) or [Aib1,3-M]PTH(1-21) (hP1R-delNt). e Competition binding assays were performed with 125I-[Aib1,3-M]PTH(1-21) radioligand as tracer. Values are means (±S.E.M.) of data from the number of independent experiments indicated (n ), each of which was performed in duplicate. A dashed line indicates that no cAMP or PLC response was observed. N.B. indicates that no inhibition of tracer binding was observed. N.D. indicates that the experiment was not done.
Table 3  cAMP Stimulation in SaOS-2 cells

<table>
<thead>
<tr>
<th>Peptide</th>
<th>EC$_{50}$</th>
<th>EMax$_{\text{obs.}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTH(1-34)</td>
<td>0.2 ± 0.02</td>
<td>350 ± 30</td>
</tr>
<tr>
<td>[M]PTH(1-14)</td>
<td>340 ± 120</td>
<td>340 ± 30</td>
</tr>
<tr>
<td>PTH(1-14)</td>
<td>---</td>
<td>2</td>
</tr>
<tr>
<td>[Aib$^1$,M]PTH(1-14)</td>
<td>22 ± 4</td>
<td>340 ± 30</td>
</tr>
<tr>
<td>[Aib$^3$,M]PTH(1-14)</td>
<td>42 ± 8</td>
<td>330 ± 30</td>
</tr>
<tr>
<td>[Aib$^{1,3}$,M]PTH(1-14)</td>
<td>2.6 ± 0.5</td>
<td>320 ± 30</td>
</tr>
</tbody>
</table>

Peptides were evaluated for the capacity to stimulate cAMP production in the human osteoblastic cell line SaOS-2. The calculated EC$_{50}$ values and observed maximum response values are means (±S.E.M.) of data from the number of experiments indicated (n). The basal cAMP level was 6.4 ±0.8 (n= 4). A dash indicates that no cAMP response was detected.
<table>
<thead>
<tr>
<th>Peptide</th>
<th>([\theta]_{222} \text{obs} \times 10^{-3})</th>
<th>Helical residues (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phos.</td>
<td>Phos.+TFE</td>
</tr>
<tr>
<td>PTH(1-14)</td>
<td>-2.6</td>
<td>-10.6</td>
</tr>
<tr>
<td>[M]PTH(1-14)</td>
<td>-2.3</td>
<td>-11.9</td>
</tr>
<tr>
<td>[Aib(^{1,3}),M]PTH(1-14)</td>
<td>-4.6</td>
<td>-15.7</td>
</tr>
<tr>
<td>PTH(1-11)</td>
<td>-1.8</td>
<td>-8.4</td>
</tr>
<tr>
<td>[M]PTH(1-11)</td>
<td>-2.1</td>
<td>-9.9</td>
</tr>
<tr>
<td>[Aib(^{1,3}),M]PTH(1-11)</td>
<td>-3.7</td>
<td>-16.1</td>
</tr>
<tr>
<td>[Aib(^{1,3}),M]PTH(1-10)</td>
<td>-3.2</td>
<td>-13.1</td>
</tr>
</tbody>
</table>

Circular dichroism spectra were recorded in either 50 mM phosphate buffer pH 7.4, or the same buffer containing trifluoroethanol (20%), as described in Material and Methods and shown in Figure 6. The helical content of each peptide was estimated using the equation 

\[
\left(\frac{[\theta]_{222}\text{obs}}{[\theta]_{222}\text{max}}\right) \times 100
\]

where \([\theta]_{222}\text{obs}\) is the mean residue ellipticity at 222 nm observed for that peptide and \([\theta]_{222}\text{max}\) is the mean residue ellipticity at 222 nm reported for a model helical peptide of 10 amino acids (-28,100; Yang et al. 1986 Methods in Enzymol. 130, 208-269). N.D., not done.
Figure 1

Aib @ positions 1-7

Aib @ positions 8-14

[Ligand] nM

Aib-1
Aib-2
Aib-3
Aib-4
Aib-5
Aib-6
Aib-7

cAMP (pmole/well)

([M]PTH(1-14))

[M]PTH(1-14)

Aib-8
Aib-9
Aib-10
Aib-11
Aib-12
Aib-13
Aib-14

[Ligand] nM

cAMP (pmole/well)
Figure 2

(A) cAMP (pmole/well) vs. [Ligand] nM

(B) 125I-[M]PTH(1-21) bound (%) vs. [Ligand] nM

- [Aib\(^{1,3}\),M]PTH(1-14)
- PTH(1-34)
- [Aib\(^{1,3}\),M]PTH(1-11)
- [M]PTH(1-14)
- [Aib\(^{1,3}\),M]PTH(1-10)
- [Aib\(^{1,3}\)]PTH(1-9)
Figure 3

A

B

C

[Aib^{1,3}, M]PTH(1-14)
[M]PTH(1-14)
PTH(1-34)
[PTH(1-14)]

[Aib^{1,3}, M]PTH(1-21)
[Aib^{1,3}, M]PTH(1-14)
PTH(1-34)

[Ligand] nM

inorganic phosphates (mol)

[125I][Aib^{1,3}, M]PTH(1-21)

[125I][Aib^{1,3}, M]PTH(1-14)

[PTH(1-34)]

[Ligand] nM
Figure 5

A Vehicle

B PTH(1-34) 0.1 μM

C [Aib^{1,3}M]PTH(1-14) 1 μM

D PTH(1-14) 2 μM
Figure 6

**A**

Phos. + TFE

**B**

Phos.

Wavelength (nm)

[0]1000: (degrees cm²/demilole)
Figure 7

A

B

[Diagram A: Graph showing the effect of different ligands on cAMP production at various concentrations. Legend includes symbols for WT+hPTH(1-34), WT+[Aib^{1,3}]hPTH(1-34), delNT+hPTH(1-34), and delNT+[Aib^{1,3}]hPTH(1-34).]

[Diagram B: Graph showing the UV spectra of hPTH(1-34) and [Aib^{1,3}]hPTH(1-34).]
Figure 8

- PTH(1-34)
- [Aib₁,₃,M]PTH(1-34)
- [Aib₁,₃,M]PTH(1-14)
- [Aib₁,₃,M]PTH(1-11)
Parathyroid hormone (1-14) and (1-11) analogs conformationally constrained by \( \alpha \)-aminoisobutyric acid mediate full agonist responses via the Juxtamembrane region of the PTH–1 receptor

Naoto Shimizu, Jun Guo and Thomas J. Gardella

*J. Biol. Chem. published online October 16, 2001*

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