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Contributions of Parathyroid Hormone (PTH)/PTH-Related Peptide Receptor Signaling Pathways to the Anabolic Effect of PTH on Bone

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Abstract

PTH regulates osteoblastic function by activating PTH/PTHrP receptors (PTH1Rs), which trigger several signaling pathways in parallel, including cAMP/protein kinase A (PKA) and, via both phospholipase-C (PLC)-dependent and PLC-independent mechanisms, protein kinase C (PKC). These signaling functions have been mapped to distinct domains within PTH(1–34), but their roles in mediating the anabolic effect of intermittent PTH *in vivo* are unclear. We compared the anabolic effects in mice of hPTH(1–34) with those of two analogs having restricted patterns of PTH1R signaling. [G¹,R¹⁹]hPTH(1–28) lacks the 29–34 domain of hPTH(1–34) needed for PLC-independent PKC activation, incorporates a Gly¹ mutation that prevents PLC activation, and stimulates only cAMP/PKA signaling. [G¹,R¹⁹]hPTH(1–34) retains the 29–34 domain and activates both cAMP/PKA and PLC-independent PKC.

Human PTH(1–34) (40 µg/kg), [G¹,R¹⁹]hPTH(1–34) (120 µg/kg), and [G¹,R¹⁹]hPTH(1–28) (800 µg/kg), at doses equipotent in elevating blood cAMP at 10 min and cAMP-dependent gene expression in bone at 6 h after s.c. injection, were administered to 10 week old female C57BL/6J mice 5 days/week for 4 weeks. Acute blood cAMP responses, retested after 4 weeks, were not reduced by the preceding PTH treatment. The three PTH peptides induced equivalent increases in distal femoral bone mineral density (BMD), and, by microCT analysis, distal femoral and vertebral bone volume and trabecular thickness and mid-femoral cortical endosteal apposition. [G¹,R¹⁹]hPTH(1–34) and hPTH(1–34) increased distal femoral BMD more rapidly and augmented total-body BMD and bone volume of proximal tibial trabeculi to a greater extent than did [G¹,R¹⁹]hPTH(1–28).

We conclude that cAMP/PKA signaling is the dominant mechanism for the anabolic actions of PTH in trabecular bone and PLC-independent PKC signaling, attributable to the PTH(29–34) sequence, appears to accelerate the trabecular response and augment BMD at some skeletal sites. PTH1R PLC signaling pathway is not required for an anabolic effect of intermittent PTH(1–34) on bone.

Keywords

bone formation; PTH/PTHrP Receptor; blood cAMP; Bone densitometry; Bone QCT

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INTRODUCTION

The classical actions of parathyroid hormone (PTH) are mediated by the PTH/PTH related peptide receptor, or type I PTHR (PTH1R), a member of the B subfamily of G-protein coupled receptors (GPCRs). Experimental studies in animals and humans have demonstrated that intermittent administration of low doses of PTH by daily subcutaneous injection effectively stimulates cancellous bone formation, whereas continuous high-dose application of the hormone, as occurs during severe hyperparathyroidism, increases bone turnover and net bone resorption, thereby reducing bone mass [6,7,15,17,18,20,28].

The amino-terminal 34 amino acids of PTH have been shown to exert functions identical to the intact hormone [20,28] to fully activate PTH1R signaling. Activation of the PTH1R initiates several parallel signaling pathways, which include the G α -protein dependent cyclic AMP/PKA pathway, both PLC-dependent and PLC-independent PKC pathways [31] and an increase in intracellular Ca⁺⁺. The relationship between these signaling pathways and the anabolic and catabolic effects of PTH is of intense interest because complete understanding of the relation of specific PTH signaling to changes in bone cell function might enable the design of improved pharmacological agents for the treatment of bone diseases such as osteoporosis.

Within the PTH(1–34) amino acid sequence, several key ligand domains have been linked to activation of specific PTH1R signaling responses. Thus, the first two amino-terminal residues of hPTH(1–34) (Ser¹-Val²) are known to be required for activation of adenylyl cyclase, and use of amino-truncated analogs such as PTH(2–34), PTH(3–38) or desamino-hPTH(1–34) (N-propionyl (2–3)hPTH-amide) *in vivo* has failed to elicit a full anabolic response, leading to the conclusion that cAMP is responsible for mediating the anabolic response to PTH [11,22]. Further, carboxyl-terminal truncation, to remove the sequence His³²-Asn³³-Phe³⁴ (i.e., hPTH(1–31)), precludes activation of membrane-associated PKC(s) but does not impair stimulation of adenylyl cyclase or eliminate the anabolic response *in vivo*, which has further supported the role of cAMP in the anabolic response [13,22,26,27]. Other evidence suggests that PTH1R-dependent adenylyl cyclase activation alone may not be sufficient to elicit a full anabolic response, however [18,19], and interpretation of the studies with amino-truncated analogs was clouded by the lack of recognition that the PLC/PKC response to PTH is critically dependent upon the presence of the α -amino group of the amino-terminal Ser¹ of hPTH(1–34) – i.e., inactive amino-truncated fragments actually lack both adenylyl cyclase and PLC/PKC responses [26]. Thus, the question of the relative roles of the cAMP vs. PKC-activating PTH1R signaling pathways in mediating the anabolic action of the hormone *in vivo* remains unsettled.

Previous work from our group has demonstrated that substitution of glycine for the native serine at position 1 of hPTH(1–34) dramatically impairs PLC activation without eliminating cAMP generation and, as well, allows for retention of PLC-independent PKC activation as long as the 29–34 domain is present [27,31]. In the present experiments, we employed two signaling-selective peptides, [Gly¹, Arg¹⁹]hPTH(1–28) and [Gly¹, Arg¹⁹]hPTH(1–34), together with PTH(1–34), to investigate, *in vivo*, the anabolic effects of different signaling pathways generated by PTH and to test the hypothesis that PTH1R-generated PKC signaling, arising via either PLC-dependent or PLC-independent mechanisms, contributes to the anabolic response of bone.

MATERIALS AND METHODS

Peptides

Human PTH(1–34), [Gly¹, Arg¹⁹]hPTH(1–28) (“G¹R¹⁹ (1–28)”) and [Gly¹, Arg¹⁹]hPTH(1–34) (“G¹R¹⁹ (1–34)”) were synthesized in the Biopolymer Core Laboratory of the Endocrine Unit. All peptides were dissolved in 0.1% TFA, aliquotted, stored frozen at –80°C and

subsequently diluted to the appropriate concentrations in vehicle (2% heat-inactivated mouse serum, 0.1N HCl and 0.9% sodium chloride) immediately before injection. PTH peptide concentrations were determined after hydrolysis by amino acid analysis, and purity was confirmed by analytical mass spectroscopy to be greater than 95%.

Animals

Virgin female C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and stabilized in the MGH animal research facility for 2 weeks before starting the experiments (at age 10 weeks). Mice were housed five per cage, given free access to water, and fed with a standard diet in a room maintained at 22 °C with 60–75% humidity on a 12 h light/dark cycle. Animals were maintained in facilities operated by the Center for Comparative Medicine of the Massachusetts General Hospital in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and protocols were approved by the Institution's Subcommittee on Research Animal Care.

Blood cAMP generation

Ten week old female virgin mice were anesthetized with 1.2% avertin (100% avertin was made by dissolving 1 g of 2,2,2-tribromoethanol into 0.63 ml of 2-methyl-2-butanol) at the concentration of 25 µg/g body weight. PTH(1–34), G¹R¹⁹(1–28) or G¹R¹⁹(1–34) was injected subcutaneously (sc) into the interscapular area, and 5, 10 or 30 min later blood was isolated by transection of the carotid artery. Mice were sacrificed by cervical vertebral dislocation shortly after blood collection. Blood samples were anti-coagulated with 7.5 mM EDTA and held at 4 °C pending separation of plasma by centrifugation at 4 °C and subsequent measurement of plasma cAMP by radioimmunoassay (RIA) using a commercial cAMP assay kit (New England Nuclear Corp., Boston, MA).

Experimental protocol

Mice were randomly subdivided into four groups of 10 mice each and baseline measurements of body weight and bone mineral density (BMD - see below) were performed. PTH peptides (40µg/kg PTH(1–34), 800µg/kg G¹R¹⁹(1–28), 120µg/kg G¹R¹⁹(1–34)) or the same volume of vehicle (50 µl) alone were administered sc 5 days per week for 4 consecutive weeks. Body weight was measured every two weeks and BMD measurements by dual-energy X-ray absorptiometry (total body, total femur and central femoral metaphysis) were performed at baseline, and at the second and fourth weeks after initiating PTH treatment. Prior to sacrifice, mice in each group were subdivided into two subgroups (5 mice each) for measurement of the acute blood cAMP response to PTH challenge. For this purpose, mice in one subgroup were injected with vehicle alone while their counterparts were administered the same PTH peptide (and dose thereof) that they had received throughout the preceding 4 weeks (except that the “re-treatment” subgroup of previous control mice received PTH(1–34) at 40 µg/kg). Plasma isolation and cAMP measurement were performed as described above. Upon sacrifice, femoral and lumbar vertebral bones were separated, cleaned of soft tissue, fixed in 10% phosphate-buffered formalin (pH 7.2) for 48 h and then stored in 70% ethanol until further use.

BMD measurement

Mouse BMD was measured by dual-energy x-ray absorptiometry using a Lunar PIXImus II densitometer (GE Medical System Luna, Madison, WI) following instructions provided by the manufacturer. In brief, mice were anesthetized and placed on a measuring tray with all four limbs extended but within the field of the detector. Outcome assessments included total body (skull excluded) and total femur BMD (g/cm²) [1,5]. After sacrifice, DXA measurements of the excised right femurs were used to determine bone mineral content (BMC, g) and bone mineral density (BMD, g/cm²) of the isolated bones.

μCT analysis

Quantitative microcomputed tomography (μCT40, Scanco Medical AG, Basserdorf, Switzerland) was used *ex vivo* to assess three-dimensional (3D) trabecular bone morphology in the 5th lumbar vertebrae and distal femoral metaphysis and cortical bone geometry at the mid-femoral diaphysis using an 12 μm isotropic voxel size [2,3]. Morphometric variables were computed from the binarized images using direct, three-dimensional techniques that do not rely on any prior assumptions about the underlying structure [8–10]. For trabecular bone regions, we assessed the bone volume fraction (BV/TV, %), trabecular thickness (Tb.Th, μm), trabecular number (Tb.N, mm⁻¹), and trabecular separation (Tb.Sp, μm). For cortical bone at the femoral midshaft, we measured the average total cross-sectional area inside the periosteal envelope (TA, mm²), the cortical bone area and medullary area within this same envelope (BA, mm² and MA, mm², respectively), and the average cortical thickness (CortTh, μm).

Measurement of mRNA regulation by real-time RT-PCR

Six week old female C57BL/6J mice were injected with PTH(1–34), G¹R¹⁹(1–28) or G¹R¹⁹(1–34) as described above. Six hours later, animals were sacrificed, femurs were dissected and RNAs were rapidly isolated from bone using an Rneasy kit (Qiagen, MD). Briefly, bone tissue was extracted into 1 ml of lysis buffer using a motorized homogenizer (Brinkmann Instruments, Inc., Westbury, NY) for 20 sec. Femoral bone marrow was homogenized in lysis buffer by repeated passage through a 20-gauge syringe needle. Insoluble debris was removed by centrifugation (microcentrifuge, 1000 rpm), and total RNA in the supernatant then was isolated according to the manufacturer's instructions. Expression levels of mRNAs for RAMP3 and Nr4a2, two genes found in preliminary *in vitro* experiments to be strongly regulated in osteoblasts by PTH and forskolin but not by phorbol ester, were measured by two-step real-time RT-PCR. Briefly, the first strand of cDNA was synthesized using a SuperScript First-Strand kit (Invitrogen Life Technologies, Carlsbad, CA). For each gene, specific PCR primer pairs (RAMP3/fw, 5'-tgcaccttctccactgtg -3'; RAMP3/re, 5'-aggttgaccacttccagac -3'. Nr4a2/fw, 5'-cggtttcagaagtgcctagc -3'; Nr4a2/re, 5'-ttgcttgaacctggaatag -3'; GAPDH/fw, 5'-tgcctggagtgctactgtg-3'; GAPDH/re, 5'-gcattgctgacaatcttgag-3') were designed and synthesized by Sigma-Genosys (Woodlands, TX). The PCR reactions (94 C, 20 sec; 60 C, 20 sec; 72 C, 20 sec) were performed on an Opticon instrument (OPTICON® 2,CFD-3220; MJ Research, Inc., Waltham, MA) using a QuantiTect™ SYBR Green PCR kit (Qiagen, Valencia, CA). Gene expression was normalized to that of GAPDH measured in the same sample, and then expressed as a fold increase over control.

Tibial trabecular bone histomorphometry

Excised left tibiae were dehydrated in graded acetone and embedded in methylmethacrylate. Four-μm-thick sagittal sections of the central regions of the tibiae were cut with a Shandon microtome (Waltham, MA) and calcified bone tissue was demonstrated by von Kossa staining. Bone volume (BV/TV), trabecular bone number (TbN) and trabecular bone thickness (TbTh) were measured on the trabecular area of proximal tibia in a region between 0.5 and 2.5 fields (@200X) distal to the growth plate using a digitizing image analysis system and a morphometric program, OsteoMeasure (OsteoMetrics, Inc., Atlanta, GA), at a magnification of 200X. All parameters were calculated and expressed according to standard formulae and nomenclature.

Statistical analysis

All data analysis was carried out using GraphPad Prism software (GraphPad Software Inc, San Diego, CA). The significance of differences in BMDs, and quantitative μCT between time/treatment groups was determined based on analysis of variance (ANOVA). When significant

differences were indicated, group means were compared using Bonferroni's Multiple Comparison Test.

RESULTS

Relative *in vivo* biopotency of PTH(1–34), G¹R¹⁹(1–28) and G¹R¹⁹(1–34)

Previous *in vitro* studies from our group have demonstrated, by comparison with hPTH(1–34), that neither G¹R¹⁹(1–28) nor G¹R¹⁹(1–34) can activate PLC, that G¹R¹⁹(1–34) (but not G¹R¹⁹(1–28)) can activate PLC-independent/PKC signaling, and that both peptides retain the ability to elicit cAMP/PKA signaling via activation of rodent PTH1Rs [31]. The goal of the present studies was to assess the potential involvement of PTH1R-generated PKC signaling, arising via either a PLC-dependent or PLC-independent mechanism, in the anabolic effects of PTH *in vivo*. We first determined the doses of these two analogs required to induce a cAMP response *in vivo* equivalent to that of PTH(1–34) by measuring the initial blood cAMP response to injected peptides as a function of time and dose. As shown in Figure 1A, subcutaneous injection of PTH(1–34) (40 µg/kg) led to a rapid increase in plasma cAMP that peaked (10-fold basal) at 10 min (basal = 9.2 ± 0.99 µM; PTH = 93.8 ± 11.5 µM; $P < 0.001$). The response to PTH(1–34) at 10 min was dose-dependent up to at least 200 µg/kg (Figure 1B), indicating that 40 µg/kg is a submaximal dose of PTH(1–34). To establish their relative *in vivo* biopotency for cAMP generation, the PTH analogs G¹R¹⁹(1–34) and G¹R¹⁹(1–28) were tested in this assay at various doses below and above the submaximal (40 µg/kg) dose of hPTH(1–34). As shown in Figure 1C, comparison of responses (at 10 min after injection) showed that 120 µg/kg of G¹R¹⁹(1–34) and 800 µg/kg of G¹R¹⁹(1–28) generated plasma cAMP responses equivalent to that produced by 40 µg/kg of hPTH(1–34). In a separate experiment, plasma cAMP responses were examined using even higher doses of these analogs; 160 µg/kg of G¹R¹⁹(1–34) and 1200 µg/kg of G¹R¹⁹(1–28) produced more cAMP than did 120 µg/kg and 800 µg/kg, respectively (data not shown), which indicated that 120 µg/kg of G¹R¹⁹(1–34) and 800 µg/kg of G¹R¹⁹(1–28), like 40 µg/kg of hPTH(1–34), are submaximal doses of these peptides *in vivo*. Additional time-course studies (Figure 1D) confirmed that the kinetics of the plasma cAMP responses to the two PTH analogs, at the bioequivalent doses described above, were similar to that seen following injection of 40 µg/kg of hPTH(1–34) (Figure 1A), with a transient response in each case that was maximal at 10 min.

To determine if these acute cAMP responses were sustained throughout 4 weeks of intermittent PTH peptide administration, we retested the plasma cAMP responses to each of these PTH peptides at the conclusion of the bone-anabolic protocol, 24 h after the preceding sc dose of PTH peptide (or vehicle - see Methods). As shown in Figure 1E, the acute plasma cAMP responses to all three peptides, given at the bioequivalent doses described above (shaded bars), were equivalent to one another (fold basal: PTH(1–34) = 8.4; G¹R¹⁹(1–28) = 8.2; G¹R¹⁹(1–34) = 8.8) and to that elicited in animals previously treated with intermittent vehicle injections but now challenged for the first time with 40 µg/kg of PTH(1–34) (fold basal = 8.3). Comparison of the control groups in each case confirmed that plasma cAMP had returned to baseline by 24 h after the last dose of PTH peptide, as expected (Figure 1E, open bars).

To examine a more delayed cAMP-dependent PTH response *in vivo*, we measured the expression in femoral bones at 6 h after PTH peptide injection of mRNAs encoding Receptor activity-modifying protein (RAMP) 3 and nuclear receptor 4a2 (Nr4a2), two genes we found to be upregulated by PTH and forskolin, but not by phorbol ester, in isolated osteoblasts *in vitro*. As shown in Figure 1F, administration of hPTH(1–34) (40 µg/kg), G¹R¹⁹(1–34) (120 µg/kg) or G¹R¹⁹(1–28) (800 µg/kg) elicited equivalent increases in both of these cAMP-responsive mRNAs (approximately 3-fold the basal level measured in vehicle-injected animals). PLC activation by PTH could not be measured directly *in vivo*, and analogous experiments directed at PKC-dependent gene responses could not be performed, as genes

specifically responsive to PKC activation by PTH in osteoblasts *in vivo* have not yet been identified.

Effects of PTH(1–34), G¹R¹⁹(1–28) and G¹R¹⁹(1–34) on BMD

To assess the anabolic effect on bone of PTH(1–34) and the two analogs, G¹R¹⁹(1–34) and G¹R¹⁹(1–28), we measured total body BMD in normal female mice after 2 and 4 weeks of intermittent treatment with these peptides at doses (40, 120 and 800 µg/kg, respectively) that produced equivalent plasma cAMP responses *in vivo*. The results are depicted in Figure 2A as group means ± SEM of absolute BMD values (left panel) and of increments above same-animal pre-treatment baseline values (right panel). As expected, daily injections of PTH(1–34) (40 µg/kg) significantly increased total body BMD after 4 weeks by 12.4% (basal = 0.043 ± 0.003 g/cm²; PTH = 0.048 ± 0.0004 g/cm²; $p < 0.01$), whereas total body BMD in vehicle-treated controls increased by only 6.4% (Figure 2A, right panel). Both G¹R¹⁹-substituted analogs significantly increased total body BMD at 4 weeks, although the increase induced by G¹R¹⁹(1–34) (12.9%), like that of hPTH(1–34) (12.4%), was significantly greater than was the response to G¹R¹⁹(1–28) (8.8%) (Figure 2A, right panel).

Total femur BMD, which includes cortical bone in the mid-shaft and trabecular bone in both distal and proximal metaphyses, was measured *in vivo* (baseline, 2 and 4 weeks) as well as *ex vivo* (4 weeks) (Figure 2B). In vehicle-treated controls, average total femur BMD increased modestly (by 5.5%) over 4 weeks, as expected, (from 0.057 ± 0.0006 to 0.060 ± 0.0005 g/cm²), whereas PTH(1–34), G¹R¹⁹(1–34) and G¹R¹⁹(1–28) each increased total femur BMD to a significantly greater extent - by 13.2% (from 0.057 ± 0.0007 to 0.065 ± 0.0007 g/cm²), by 14.4% (from 0.056 ± 0.0006 to 0.065 ± 0.0005 g/cm²) and by 10.6% (from 0.057 ± 0.0008 to 0.063 ± 0.0007 g/cm²), respectively ($p < 0.05$ vs vehicle). At this site, the response to G¹R¹⁹(1–34) was seen earlier (significantly greater than controls at 2 weeks) and was significantly greater than the response to G¹R¹⁹(1–28) at 4 weeks, although this difference was not observed in *ex vivo* measurements of total femur BMD at 4 weeks, which showed significant ($p < 0.05$) increases in BMD vs. controls for all three PTH peptides but no differences between peptides (data not shown).

Compared to total femoral BMD, the responses to PTH at the distal femur were larger and occurred earlier (significant at 2 weeks) (Figure 2C). Thus, hPTH(1–34) significantly increased distal femoral BMD by 14.2% at 2 weeks and by 21% at 4 weeks, vs. increases in controls of 7.4% and 7.5% at these times. A significant response to G¹R¹⁹(1–34) also was seen at both 2 weeks (18.0%) and 4 weeks (21.6%), whereas the response to G¹R¹⁹(1–28) was significantly different from that of controls only at 4 weeks (18.9%) (Figure 2C). *Ex vivo* measurements of dissected femurs at 4 weeks were consistent with the *in vivo* results (data not shown).

Effects of PTH(1–34), G¹R¹⁹(1–28) and G¹R¹⁹(1–34) on microstructure of vertebral and femoral bone

MicroCT analysis of bone microstructure performed on trabecular bone at the 5th lumbar vertebrae from PTH(1–34)-treated mice showed expected increases, relative to vehicle-treated controls, in BV/TV (17.1%), trabecular thickness (TbTh) (6.0%) and connectivity density (ConnD) (41.7%) (Table 1). Treatment with the G¹R¹⁹(1–34) or G¹R¹⁹(1–28) analog also increased BV/TV (17.5% and 12.3%, respectively), TbTh (4.0% and 3.9%) and ConnD (44.9% and 27.6%). The effect of G¹R¹⁹(1–34) on vertebral ConnD was greater than that of G¹R¹⁹(1–28), although this difference was not seen in distal femur (see below).

Analysis of distal femurs by µCT showed even greater effects of hPTH(1–34) treatment, with increases vs. controls in BV/TV, TbTh and ConnD of 36.0%, 12.4% and 63.3%, respectively (Table 1). These effects again were mimicked by the G¹R¹⁹(1–34) and G¹R¹⁹(1–28) analogs,

and the effect of the G¹R¹⁹(1–34) analog on BV/TV and ConnD at this site was particularly prominent in comparison to either hPTH(1–34) or G¹R¹⁹(1–28) (Table 1).

Examination by μ CT of cortical architecture at the mid-femur showed that intermittent treatment with hPTH(1–34) increased bone area (8.0%) without altering total cross-sectional area (i.e. increased % bone area/total area) and also increased cortical thickness (8.1%) (Table 2). Similar changes in cortical bone occurred following treatment with the G¹R¹⁹(1–34) and G¹R¹⁹(1–28) analogs.

Effects of PTH(1–34), G¹R¹⁹(1–28) and G¹R¹⁹(1–34) on microstructure of proximal tibial trabecular bone

Bone microstructure of proximal tibia at 4 weeks was analyzed by von Kossa staining and histomorphometric quantification. As shown in Figure 3, trabecular bone volume (%BV/TV) was significantly increased in PTH(1–34)-treated mice relative to vehicle-treated controls ($25.6 \pm 2.5\%$ vs. $10.8 \pm 1.3\%$). A comparable increase in BV/TV was observed in mice treated with G¹R¹⁹(1–34) ($30.3 \pm 2.2\%$), and this effect was much greater than that seen in G¹R¹⁹(1–28)-treated mice ($18.8 \pm 1.0\%$). A similar pattern was found for trabecular number, which was increased by both PTH(1–34) and G¹R¹⁹(1–34) but not by G¹R¹⁹(1–28) in this region. All three peptides induced significant and similar increases in trabecular thickness.

DISCUSSION

In this study, we administered signal-selective analogs of PTH(1–34) to 10 week old female C57BL/6J mice to investigate the involvement of specific PTH1R signaling pathways in the anabolic effect of PTH on bone. Via PTH1Rs, PTH(1–34) activates Gas/cAMP/PKA and G α q/PLC/PKC pathways but also can activate PKC via a PLC-independent mechanism that requires the presence of the PTH(29–34) sequence. Early work with PTH analogs *in vivo* had led to the conclusion that cAMP-dependent pathways are uniquely important in the anabolic action of PTH(1–34). This concept was supported by findings that cAMP-defective analogs such as PTH(2–34) or PTH(3–38) were inactive *in vivo* whereas C-truncated analogs lacking PKC activity, such as PTH(1–31), retained the anabolic action [11,22]. At the time, however, it was not yet appreciated that an intact PTH N-terminus is required for PLC-dependent PKC activation and that PTH1Rs can increase PKC activity via multiple mechanisms, triggered by either the N- or C-terminal regions of the ligand [25,26,29,31]. In this regard, PKC signaling via PTH1Rs has been related to induction of osteoblastic bone formation [16,24], especially PKC stimulated by the PTH(29–34) portion of the ligand [14,21]. G¹R¹⁹(1–28) lacks the 29–34 domain of hPTH(1–34) required for PLC-independent PKC activation and incorporates a Ser¹ to Gly¹ mutation that blocks PLC activation (the Arg¹⁹ change improves binding affinity that otherwise is compromised by the C-terminal truncation) [27]. This peptide can no longer activate PKC via either PLC-dependent or PLC-independent mechanisms but can fully activate cAMP production via PTH1Rs, albeit with somewhat reduced potency [26,31]. The related G¹,R¹⁹(1–34) analog, which retains the 29–34 domain, was shown to activate both cAMP/PKA and (PLC-independent) PKC but is incapable of PLC activation [31]. The strategy used in the current study was to administer each of these analogs to mice, in an intermittent-treatment protocol, at doses that would activate cAMP/PKA signaling *in vivo* to the same extent as standard PTH(1–34) (40 μ g/kg/d) and then to seek differences in skeletal responses that could be attributable to loss of one or both of the PKC-activation mechanisms normally available to the PTH1R in bone cells. Bioequivalent doses of the two analogs were selected on the basis of comparable stimulation of plasma cAMP levels 10 min after sc peptide administration and of cAMP-dependent mRNA upregulation in long bones at a later time (6 h). Further, the acute cAMP responses to PTH(1–34) and each of the two G¹,R¹⁹-substituted analogs at the conclusion of the 4-week anabolic protocol were found to have remained comparable and, in

contrast to the loss of cAMP responsiveness in isolated osteoblasts induced by continuous PTH exposure *in vitro* [12,23], were not desensitized relative to the response seen in control animals not previously exposed to exogenous PTH. We have strived to carefully establish *in vivo* bioequivalent doses for the three PTH peptides used, with respect to the cAMP response that they share in common. The various approaches used are inherently limited, however, and we cannot exclude with certainty the possibility that these analogs may somehow elicit qualitatively different responses with respect to cAMP generation or action or that the effectiveness for activation of PLC-independent PKC by $G^1, R^{19}(1-34)$ *in vivo* may differ from that of a cAMP-equivalent dose of hPTH(1-34).

The effects of these PLC-deficient PTH analogs on bone were evaluated by total body, total femur and distal femur BMD measurements *in vivo*, by terminal μ CT microstructural analysis of vertebrae, distal femur and femoral midshaft and by histomorphometric quantification of proximal tibial trabeculi. The actions of all three PTH peptides tested were comparable on most standard measures of the response of trabecular bone – i.e. distal femoral BMD, %BV/TV (distal femur, vertebrae) and TbTh (distal femur, proximal tibia and vertebrae). Some differences in microarchitecture (ConnD), in favor of inducing a more plate-like structure, were observed with $G^1, R^{19}(1-34)$ versus the other two peptides (Table 1), but these were not consistent between vertebra and femur and thus are of uncertain significance. These results suggest that the cAMP/PKA response to the PTH1R activation is the major determinant of the anabolic response of trabecular bone, a conclusion concordant with observations of others [1, 11, 18, 22, 30] [4]. μ CT analysis of cortical bone at the mid-femoral shaft showed that PTH decreased medullary area and increased bone area, cortical thickness and the ratio of bone to total area without altering total cross-sectional area – changes consistent with a predominant endosteal apposition of new bone and consistent with other reports of intermittent PTH action in cortical bone of female mice [3]. These structural changes in cortical bone were induced equivalently by PTH(1-34) and the two G^1, R^{19} -substituted analogs, again consistent with a dominant role of the cAMP/PKA PTH1R signaling pathway.

Several observations did point to the possibility of unique *in vivo* effects of PKA-independent signaling on bone, however. Thus, compared with $G^1, R^{19}(1-28)$, both PTH(1-34) and $G^1, R^{19}(1-34)$ increased total body BMD to a greater extent (Figure 2A), increased distal femoral BMD more rapidly (seen at 2 weeks in Figure 2C), and led to significantly higher bone volume and trabecular numbers at the proximal tibial metaphysis. These results seem not to be attributable to a lower *in vivo* biopotency of $G^1, R^{19}(1-28)$, as the latter was fully active (at the adjusted dose employed) on other measures mentioned above in the same animals at 4 weeks. The mechanism(s) and site(s) of this PLC-independent PKC effect are unclear at this time. Whereas total body BMD reflects principally contributions from cortical bone, the three peptides were equivalent in stimulating the endosteal increase in mid-femoral cortical bone in our μ CT analysis. It may be that the PLC-independent PKC effect observed on total body BMD reflects actions at cortical sites other than the femoral mid-shaft or increased density of new bone deposited at cortical sites. It is possible also that the power of the study to detect differences among peptides in cortical microstructure was limited (there was a trend, albeit nonsignificant, toward greater mid-femoral cortical thickness with the (29-34)-containing analogs). We considered the possibility that the effects attributed to PLC-independent PKC to accelerate the trabecular response and increase total body BMD might be related to an effect on the growth plates of these growing animals. Our histomorphometric analysis of proximal tibia did show a greater effect of PTH(1-34) and $G^1, R^{19}(1-34)$ vs. $G^1, R^{19}(1-28)$ on BV/TV and trabecular number in a region subjacent to the growth plate in this weight-bearing bone. The corresponding region is not included in standard μ CT analysis of the distal femur, which could explain why these differences were not evident in that analysis. We cannot be certain that these changes occurring very close to the growth plate in the proximal tibia reflect effects on bone remodeling as opposed to alterations in primary bone formation during growth. On

the other hand, to the extent that these may be related to the differences seen in total-body BMD, the latter were only apparent at 4 weeks, by which time the rapid growth seen in control mice had slowed dramatically at least two weeks earlier. This is corroborated by the fact the 14 week old control mice in our study showed very little trabecular bone immediately adjacent to the growth plate in proximal tibia (Figure 3A). Also, we have observed similar responses in preliminary experiments in which the peptides were administered to older animals (starting at 13 weeks of age; controls = 8.99 ± 2.47 %, PTH = 15.20 ± 2.14 %, $P < 0.05$ for total-body BMD increase after 4-week treatment). More work is needed to explore these possibilities.

Collectively, these findings provide evidence that the cAMP/PKA response to PTH1R activation is the dominant mechanism that drives the anabolic response. However, the PLC-independent PKC pathway, stimulated by PTH peptides that incorporate the (29–34) sequence, appears to accelerate the pace of net bone accretion in trabecular bone, increase the extent of the response at sites captured by measurements of total body BMD and augment the size and number of trabeculae subjacent to the growth plate in weight-bearing bones like the tibia. Our data argue against an important role for PLC-dependent PTH1R signaling in the anabolic effects on bone of intermittent exogenous PTH administration. Further study of the possible mechanism of PLC-independent PKC signaling in skeletal responses to PTH is warranted.

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Figure 1. Effects of PTH(1-34), G¹R¹⁹(1-28) and G¹R¹⁹(1-34) on blood cAMP concentration
 PTH peptides were administered to groups (N=3) of anesthetized 10 week old female virgin mice by subcutaneous injection into the interscapular area. Blood was collected, plasma isolated and plasma cAMP measured as described in *Materials and Methods*. (A) PTH(1-34) (40 μg/kg) was administered 5, 10 or 30 min before sacrifice (vehicle control was given 30 min before sacrifice); (B) PTH(1-34) was given at 0, 8, 40 or 200 μg/kg and cAMP measured at 10 min; (C) Cyclic AMP was measured 10 min after PTH(1-34) (40 μg/kg), G¹R¹⁹(1-28) (200, 400, or 800 μg/kg) or G¹R¹⁹(1-34) (40, 80, or 120 μg/kg); (D) G¹R¹⁹(1-28) (800 μg/kg) or G¹R¹⁹(1-34) (120 μg/kg) were given at the indicated times prior to sacrifice. Vehicle controls were injected at - 30 min; (E) At the conclusion of the 4-week protocol of intermittent PTH peptide treatment, mice in each main treatment group (“Vehicle”, “PTH(1-34)”, “G¹R¹⁹(1-28)”) and “G¹R¹⁹(1-34)”; N=10) were subdivided into 2 subgroups (N=5), in which animals were reinjected with either vehicle alone (open bars) or PTH peptides (shaded bars) 10 min prior to sacrifice and isolation of plasma for cAMP measurement. (F) Groups (N=3) of 6-week old female mice received PTH(1-34) (40 μg/kg), G¹R¹⁹(1-28) (800 μg/kg) or G¹R¹⁹(1-34)

(120 µg/kg) 6 hr before sacrifice and isolation of RNA from femoral bone (and the included marrow) for subsequent real-time RT-PCR measurement of RAMP3 (in bone tissue) and Nr4a2 (in bone marrow, as BM in figure) expression. Data are expressed as mean $\pm$ SD, and all experiments were performed 3 times with similar results.

Figure 2. Effects of PTH(1-34), G¹R¹⁹(1-28) and G¹R¹⁹(1-34) on BMD

Four groups of ten randomly selected mice received daily (5 days/week) sc injections of 40 $\mu\text{g/kg}$ PTH(1-34) (filled squares in left panels), 800 $\mu\text{g/kg}$ G¹R¹⁹(1-28) (filled circles), 120 $\mu\text{g/kg}$ G¹R¹⁹(1-34) (open circles) or vehicle alone (open squares) for 4 weeks. BMDs of (A) total body, (B) total femur and (C) distal femur were measured at baseline, 2 and 4 weeks. Left panels show the average absolute BMDs (mean $\pm$ SE, gm/cm^2) in each group, whereas right panels show the group averages (mean $\pm$ SE, gm/cm^2) of the changes from baseline in BMD of individual animals at 2 and 4 weeks. a, $p < 0.05$ vs vehicle; b, $p < 0.05$ vs G¹R¹⁹(1-28).

Figure 3. Effects of PTH(1-34), G¹R¹⁹(1-28) and G¹R¹⁹(1-34) on proximal tibia

Mice were treated for 4 weeks as described in Figure 2. Sections (4 μm) of undecalcified tibia were stained with von Kossa, and histomorphometry of the proximal metaphysis was performed as described in Materials and Methods. Panel A shows representative histologic sections (von Kossa staining) from mice in each treatment group. Panel B shows results of histomorphometric analysis from 6 animals randomly selected from each treatment group. a, $p < 0.05$ vs vehicle; b, $p < 0.05$ vs G¹R¹⁹(1-28).

Table 1
Response of trabecular bone to intermittent vehicle, PTH(1–34), G¹R¹⁹(1–28) or G¹R¹⁹(1–34) administration (mean ± SEM).

	Vehicle	PTH(1–34)	G ¹ R ¹⁹ (1–28)	G ¹ R ¹⁹ (1–34)
<i>Distal Femur</i>				
BV/TV (%)	8.01±0.58	10.89±0.43 ^a	11.80±0.51 ^a	12.31±0.81 ^a
TbTh (μm)	49.33±0.78	55.44±1.05 ^a	55.68±1.04 ^a	55.45±1.82 ^a
TbN (mm ⁻¹)	3.61±0.15	3.71±0.09	3.78±0.09	3.93±0.11 ^a
ConnD (mm ⁻³)	49.40±6.94	80.66±5.77 ^a	88.30±5.98 ^a	97.92±4.37 ^{ab}
SMI	3.29±0.09	3.24±0.08	3.04±0.07 ^a	3.00±0.05 ^{ab}
<i>Vertebral Body</i>				
BV/TV (%)	24.68±0.78	28.91±0.99 ^a	27.74±0.82 ^a	28.99±0.58 ^a
TbTh (μm)	51.19±0.88	54.29±1.17 ^a	53.22±0.81	53.19±0.57
TbN (mm ⁻¹)	4.83±0.10	5.08±0.07	5.10±0.13	5.05±0.14
ConnD (mm ⁻³)	201.91±8.34	286.23±8.66 ^a	257.60±12.53 ^a	292.65±12.58 ^{ac}
SMI	0.73±0.08	0.40±0.11 a	0.46±0.09 ^a	0.29±0.06 ^a

Abbreviations: bone volume fraction (BV/TV), trabecular thickness (TbTh), trabecular number (TbN), connectivity density (ConnD), structure-model index (SMI).

^a p < 0.01 vs vehicle;
^b p < 0.05 vs PTH(1–34);
^c p < 0.03 vs G¹R¹⁹(1–28)

Table 2
Response of mid-femoral cortical bone to intermittent vehicle, PTH(1–34), G¹R¹⁹(1–28) or G¹R¹⁹(1–34) administration (mean ± SEM).

	Vehicle	PTH(1–34)	G ¹ R ¹⁹ (1–28)	G ¹ R ¹⁹ (1–34)
Total Area (mm ²)	1.67±0.02	1.70±0.03	1.65±0.02	1.66±0.02
Bone Area (mm ²)	0.75±0.01	0.81±0.06 ^a	0.78±0.05	0.79±0.04 ^a
Medullary Area (mm ²)	0.92±0.01	0.89±0.06	0.87±0.04 ^a	0.87±0.05 ^a
BA/TA (%)	44.75±0.42	47.70±0.67 ^a	47.30±0.46 ^a	47.68±0.61 ^a
CortThick (µm)	180.50±2.31	195.20±3.25 ^a	190.70±2.63 ^a	193.60±3.00 ^a

Abbreviations: BA/TA, bone area/tissue area.

^a p< 0.01 vs vehicle