Critical role of parathyroid hormone (PTH) receptor-1 phosphorylation in regulating acute responses to PTH

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The PTHR1 is a class B G protein-coupled receptor (GPCR) expressed by target cells of several other calcium- and phosphate-regulating factors, including the renal excretion of Pi into the urine. PTH acts in concert with other hormones to regulate calcium homeostasis and phosphate homeostasis.

**Results**

**Properties of the PD-PTHR1 in Vitro.** Comparison of the PD-PTHR1 with the WT-PTHR1 in cell-membrane–based binding assays designed to assess binding to two distinct high-affinity receptor conformations: a protein–independent receptor conformation, R0, and a protein–dependent conformation, RG, as well as in cell-based assays of signaling via the cAMP/PKA, PLC/ιCa/PKC, and ERK1/2 pathways using unmodified PTH (1–34) as a probe, revealed only minor differences in the functional responses of the two receptors (Fig. 1 A–D and Fig. S1 A and B). Datasets S1, S2, S3, and S4). Of note, however, the cAMP assays revealed a higher basal, and proportionately higher PTH-induced signaling response for the PD-PTHR1 vs. the WT-PTHR1 (Fig. 1 C and D). Western blot analysis of cell membrane preparations (Fig. SIC), indicated that the PD-PTHR1 was expressed at lower levels than the WT-PTHR1; thus, the higher basal cAMP signaling levels of the PD-PTHR1 likely arose from a higher level of ligand-independent signaling activity. Assessment


The authors declare no conflict of interest.

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of ligand-induced receptor internalization using a fluorescently labeled PTH(1–34) TMR (where TMR is tetramethylrhodamine) analog and confocal microscopy revealed in cells expressing the WT-PTHR1 the formation of numerous fluorescent punctae that were dispersed within the cytoplasm at 5 min after ligand addition, and then became more perinuclear by 30 min (Fig. S1D). In cells expressing the PD-PTHR1, the fluorescence was initially weak and diffuse at 5 min, and then some punctae were apparent at dispersed locations by 30 min. The overall results of these in vitro studies are largely consistent with prior studies (5–7, 15), and thus show that the PD-PTHR1 is at least as functional as the WT-PTHR1 in terms of its capacity to bind PTH ligands and activate second-messenger signaling pathways, and exhibits at least some delay in ligand-induced internalization responses.

**Effects of PTH (1–34) on Ca²⁺ and Pi Levels in Blood and Urine of WT and PD Mice.** Before injection, the concentration of Ca²⁺ in the blood of PD mice was not different from that of WT mice (~1.25 mM, Fig. 2A). Following injection of PTH (1–34) into WT mice, blood Ca²⁺ levels increased to ~1.35 mM at 1 to 2 h post-injection, and then returned to vehicle-control levels by 4 h; in contrast, blood Ca²⁺ in PD mice did not rise following PTH (1–34) injection but rather declined moderately at 1 h postinjection and then returned to vehicle levels by 2 h (Fig. 2A). PTH (1–34) injection into WT mice lowered blood Pi levels at 1 to 2 h postinjection; in contrast, blood Pi levels did not decrease in PD mice following PTH (1–34) injection, but remained at or near vehicle-control levels for at least 4 h (Fig. 2B). Thus, both the early-phase calcemic and hypophosphatemic responses to PTH (1–34) injection were blunted in PD mice. At later times (4–8 h) after PTH injection, blood Pi levels in both WT and PD mice tended to increase above vehicle levels. Whereas the early phase hypophosphatemic response to PTH (1–34), observed here only in WT mice, can be attributed to the rapid, PTH-induced down-regulation of the Pi transporter, NPT2a, in renal proximal tubule cells (16), the later-phase hyperphosphatemic response likely involves the PTH-induced increase in bone resorption.

Little or no change was detected in the concentration of calcium in the urine collected from either WT or PD mice at times after injection of PTH (1–34) (Fig. 2C). Urine Pi levels increased strongly at 1 and 2 h following injection of PTH (1–34) in WT mice, but no increase was observed in PD mice (Fig. 2D).

**Analysis of the PD-PTHR1 with Modified PTH Analogs.** To assess further the functional properties of the PD-PTHR1, we used the N-terminally modified PTH analog M-PTH (1–34) (11), and a long-acting M-PTH/PTH₃ hybrid analog called LA-PTH. Compared with PTH (1–34), these two analogs bound with severalfold higher affinity to the Rᵣ conformation of both the WT-PTHR1 and PD-PTHR1 (Fig. S2; Dataset S1), mediated more prolonged cAMP responses following ligand wash-out in cells expressing either the WT or PD receptor (Fig. S3 A–F; Dataset S5), exhibited at least similar capacities to activate iCa signaling via either receptor (Fig. S3 G and H; Dataset S4), and, in WT mice, mediated more prolonged increases in urinary cAMP and blood Ca²⁺ (Fig. S4).

**Calcemic and Phosphaturic Actions of PTH Analogs in WT and PD Mice.** In WT mice, injection of LA-PTH induced a robust increase in blood Ca²⁺ that lasted for at least 8 h; in contrast, injection of the analog into PD mice caused blood Ca²⁺ levels to decline to below vehicle-control levels, and this reduction lasted for at least 6 h (Fig. 3A). In WT mice, LA-PTH injection caused reductions in blood Pi levels that persisted for at least 6 h; in contrast, blood Pi levels in PD mice injected with LA-PTH never decreased, but rather remained within vehicle-control levels for the initial 2 h and then rose to above vehicle-control levels at 4 and 6 h before returning to vehicle levels by 8 h (Fig. 3B). Similar paradoxical effects on blood Ca²⁺ and Pi were observed in the PD mice following injection with M-PTH (1–34) (Fig. S5 A and B). Little or no change was detected in the concentration of urine calcium in either WT or PD mice after injection of LA-PTH (Fig. 3C). Urine Pi levels in WT mice rose rapidly after injection of LA-PTH, with the peak increase occurring by 1 h after injection, and returned...
The mRNA for osteoprotegerin (OPG), an osteoblast factor ligand, a PTH-responsive osteoblast factor involved in osteoclast resorption, was elevated in PD mice 1.5-fold at 3 h after PTH (1–34) injection (P vs. vehicle = 0.002), whereas no increase in CTX was detected in WT mice (Fig. 5D).

In the kidneys of both WT and PD mice, PTH (1–34) injection resulted in five- to sevenfold increases in the levels of mRNA for CYP27B1, which encodes the 1α-hydroxylase that mediates PTH-induced synthesis of active 1,25(OH)₂-vitamin-D₃ in the proximal tubule, and two- to threefold decreases in the levels of mRNA for CYP24A1, which encodes the 1,25-dihydroxyvitamin-D₃ 24-hydroxylase that mediates the renal metabolism of 1,25-dihydroxyvitamin-D₃ (Fig. 6C and D). Overall, these findings establish that upstream cAMP signaling, as well as certain hallmark downstream gene expression responses to PTH injection observed in the PD mice—i.e., the failure to export cAMP and Pi into the urine—are thus not likely due to impaired upstream signaling responses in these target tissues.

Effects of PTH Analogs on CAMP Levels in Blood and Urine of WT and PD Mice. Injection of PTH (1–34) induced rapid increases in blood cAMP levels in both WT and PD mice, and the maximum increase in the PD mice was significantly greater than that in WT mice (10-fold vs. sixfold at 30 min postinjection, relative to initial; Fig. 4A). Urine cAMP levels in WT mice increased ~20-fold by 15 min after injection of PTH (1–34), whereas in striking contrast the urine cAMP levels in PD mice did not change following PTH (1–34) injection (Fig. 4B). Similarly, whereas injection of LA-PTH resulted in rapid and robust increases in cAMP in both blood and urine of WT mice, the analog increased cAMP levels in the blood, but not urine of PD mice (Fig. 4C and D).

Effects of PTH Analogs on Signaling in Bone and Kidney of WT and PD Mice. To assess PTHR1 signaling directly in target tissues, we measured the levels of cAMP in homogenized kidneys and calvaria isolated from mice 15 min after PTH injection. Injection of PTH (1–34) resulted in two- to sixfold increases (vs. vehicle) in cAMP levels in both kidney and bone tissues of WT mice, and similar, if not enhanced, increases in cAMP were observed in the kidney and bone tissues of PD mice (Fig. 5A and B). Injection of LA-PTH also increased cAMP levels in both target tissues of both mice, and the increases were again greater (P < 0.05) in PD mice than in WT mice (Fig. 5C and D).

We then evaluated the effects of PTH (1–34) injection on the levels of expression of several known PTH-response genes in bone and kidney tissue. Injection of PTH (1–34) into WT mice resulted in a 2.5-fold increase in the levels of mRNA for RANK-ligand, a PTH-responsive osteoblast factor involved in osteoclast activation in calvarial bone, and a significantly greater, 13-fold increase in RANK-L mRNA was observed in PD mice (Fig. 6A). The mRNA for osteoprotegerin (OPG), an osteoblast factor which inhibits RANK-L and is down-regulated by PTH, was reduced fivefold in calvaria of both WT and PD mice following PTH (1–34) injection (Fig. 6B). Consistent with a greater effect on RANK-L expression in PD mice than in WT mice, blood levels of type I collagen C-telopeptide (CTX), a marker of bone resorption, were elevated in PD mice 1.5-fold at 3 h after PTH (1–34) injection (P vs. vehicle = 0.002), whereas no increase in CTX was detected in WT mice (Fig. 6C).
vehicle = 0.008), there was no increase in blood 1,25(OH)2-vitamin-D3 levels in PD mice (Fig. S6B), despite the changes in the renal hydroxylase mRNAs (Fig. 6 C and D). As renal production of 1,25(OH)2-vitamin-D3 is dependent on the uptake of the precursor, 25-hydroxyvitamin-D3, from the urine, the failure of blood 1,25(OH)2-vitamin-D3 levels to increase upon PTH injection in PD mice could also be explained by impaired renal filtration, and hence reduced precursor availability. To assess effects on renal filtration, we injected WT and PD mice with FITC-labeled inulin, either alone or together with PTH (1–34), and then measured the rate of FITC-inulin clearance from the blood (17). Coinjection of PTH (1–34) caused little or no change in the rate of clearance of the marker in WT mice, but it markedly diminished the rate of clearance in PD mice (Fig. 7A). Without PTH coinjection, the blood clearance rates of FITC-inulin were similar in WT and PD mice. Estimations of glomerular filtration rate (GFR) from these data indicated that whereas PTH (1–34) injection did not significantly alter GFR in WT mice [215 ± 15 vs. 176 ± 24 µL/min; vehicle vs. PTH (1–34); P > 0.05], it reduced GFR in PD mice by at least 10-fold (248 ± 31 vs. 21 ± 3 µL/min; P = 0.0003). An even more prolonged reduction in FITC-inulin clearance was observed in PD mice upon coinjection of LA-PTH (Fig. 7B).

As PTH is known to have hypotensive effects on the vasculature (18), and previous studies in rats show that PTH-induced reductions in systemic blood pressure can result in reduced glomerular blood flow (19), we assessed the effects of PTH administration on systemic blood pressure in WT and PD mice (20). Following a brief i.v. infusion of PTH (1–34) (50 nmol/kg over 2 min of infusion), left ventricular (LV) end-systolic pressure (ESP) declined rapidly in both WT and PD mice, but the decline was significantly greater in PD mice than in WT mice (Fig. 7C). Injection of PTH (1–34) into PD mice did not reduce cardiac output or LV end-diastolic pressure (Fig. S7 A and B), consistent with vasodilation mediating the effects of PTH on blood pressure.

**Discussion**

It is evident from these studies that physiological responses to injected PTH ligands are acutely and severely perturbed in mice bearing the PD PTHR1 knock-in mutation, compared with responses in WT mice. The altered responses observed in the PD mice with PTH (1–34) were confirmed and indeed exaggerated with the longer-acting analogs, M-PTH (1–34) and LA-PTH. Whereas responses in bone appeared, if anything, enhanced in the PD mice, as shown by the more pronounced increases in calvarial cAMP and RANK-L mRNA, certain renal responses, namely the PTH-induced export of cAMP into the urine, and the phosphaturic response, were markedly defective. These defects in renal function were not associated with defects in either upstream PTH signaling or immediate downstream responses in the kidneys of the PD mice, as shown by the robust inductions in the levels of cAMP and CYP27B1 mRNA in kidney tissue isolated from the mice after injection. The defects in blood and urine responses observed in the PD mice were, however, associated with marked PTH-induced reductions in renal filtration, as revealed by the severely reduced rates of FITC-inulin clearance. The PD mice further exhibited an overly exuberant systemic hypotensive response to PTH injection, which could well account for the defects in renal filtration (19). This enhancement in the PTH-induced hypotensive response presumably reflects a defect in the capacity of the PD-PTHR1 to desensitize and/or down-regulate in vasculature cells following PTH activation. The PTHR1 is known to be expressed in vascular smooth muscle and endothelial cells and to mediate reductions in vascular tone (18). A defect in desensitization is also likely reflected by the higher basal and PTH-induced cAMP signaling responses observed for the PD-PTHR1 in our in vitro assays, and likely contributes to the normalization of the blood Ca2+ and PI levels observed in the

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**Fig. 5.** Effects of PTH analog injection on cAMP levels in bone and kidney of WT and PD mice. (A) Mice were injected s.c. with vehicle or PTH (1–34) (50 nmol/kg) and 15 min after injection kidneys were isolated, homogenized, and the homogenates were analyzed for cAMP. (B) Homogenized calvaria isolated from the mice used in the experiment of A were analyzed for cAMP. (C) Mice were injected s.c. with LA-PTH (10 nmol/kg) and 15 min after injection kidneys were isolated, homogenized, and the homogenates were analyzed for cAMP. (D) Homogenized calvaria from the mice used in the experiment of C were analyzed for cAMP. Values of cAMP are expressed relative to 100 mg of tissue; data are means ± SEM; n = 5 mice per group; P vs. corresponding vehicle: ***P ≤ 0.01, **P ≤ 0.001; P vs. corresponding WT: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

**Fig. 6.** Effects of PTH analog injection on PTH target gene mRNA levels in bone and kidney of WT and PD mice. (A) Mice were injected s.c. with vehicle or PTH (1–34) (50 nmol/kg) and 2 h after injection calvarial bones were isolated, homogenized, and the homogenates were analyzed by RT-PCR for mRNA encoding RANK-L. (B) Calvarial homogenates used in A were also assessed for mRNA encoding OPG. (C) Kidney isolated from the mice used in the experiment of A and B were assessed for mRNA encoding CYP27B1, the 25-hydroxyvitamin-D3–1a-hydroxylase. (D) Kidney homogenates were also analyzed for mRNA encoding CYP24A1, the 1,25-dihydroxyvitamin-D3, 24-hydroxylase. Data are means ± SEM; n = 4–5 mice per group; P vs. corresponding vehicle: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001; P vs. corresponding WT: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.
PD mice in the context of their lower endogenous levels of PTH (1–84), as found previously (21).

We also observed that whereas injection of PTH (1–34) into WT mice induced the expected reduction in NPT2a protein levels in renal brush border membranes, it failed to do so in PD mice (Fig. S8 A–D). This finding could conceivably indicate a defect in some PTHR function, as yet unknown but likely other than cAMP signaling per se, in renal proximal tubule cells that is associated with the absence of a fall in blood Pi in the PD mice (20). The mechanism by which PTH induces the export of cAMP into the renal brush border membranes that is involved in the PTH-induced down-regulation of NPT2a (16, 22). It seems at least equally likely, however, given the current findings, that the results reflect a reduced access of the ligand to renal receptors, especially on the luminal membrane surface (23), due to impaired filtration. The fact that the injected PTH ligands increased cAMP in kidney tissue of the PD mice to at least the same extent as they did in WT mice, and also increased CYP27B1 mRNA, confirms that the ligands gained access to at least the blood or basolateral surface of renal target cells. The mechanisms by which PTH induces the export of cAMP into the urine by renal cells is not known, but the lack of a urinary cAMP response in the PD mice, coupled with the increased cAMP levels in the blood and renal tissues, together with the markedly reduced GFR, further supports a model by which the injected PTH had reduced access to the apical surfaces of renal tubules.

Perhaps the most surprising finding in these studies was the hypocalcemic response to PTH (1–34) and long-acting analogs. This hypocalcemia occurred despite the findings that the increases in cAMP, as well as RANK L mRNA, in bone, and increases in CTX in blood, were greater in the PD mice than in the WT mice, confirming that the bone response to PTH was, if anything, enhanced in the PD mice. A more exuberant bone response to PTH in the PD mice is further suggested by the finding that the blood Pi levels rose to significantly above vehicle levels by 4 h following injection with LA-PTH (Fig. 3B), as this elevation in blood Pi most likely arises from a robust bone-resorption response, coupled with a defect in Pi excretion. As discussed below, it seems plausible that the failure of blood Ca levels to rise in PD mice following PTH injection is related to the failure of blood Pi levels to fall, which, in turn, is related to the impairment in GFR. In the prior study of Bounoutas et al., a frank hypercalcemic response was indeed observed in PD mice following 3 d of PTH (1–34) infusion (10). This hypercalcemic response can likely be accounted for by the lower dose and slower route of PTH administration (7 nmol/kg over a 24-h infusion) used in that study, which was likely too low to induce the marked reductions in blood pressure, GFR, and hence Pi clearance, as were observed in our studies, in which PTH was administered at higher doses (10–50 nmol/kg) and as single bolus injections.

The fact that hypocalcemia occurred in our study at 4 h after LA-PTH injection, when blood Pi levels were significantly above normal, may not be unexpected given the known capacity of blood Pi infusion to lower blood Ca (24, 25). It is the earlier fall in blood Ca in the PD mice observed at 1 to 2 h after PTH injection, when blood Pi levels were near normal, that seems particularly surprising. Earlier reports, however, highlight the importance of the rapid phosphaturic action of PTH and the associated lowering of blood Pi for the hypocalcemic response (25–27). Thus, Parsons et al. showed in dogs and rats that PTH injection first induces a hypocalcemic effect that occurs before the fall in blood Pi, after which blood Ca increases (28, 29). Raiz et al. showed in vitro a damping effect of Pi on the PTH-induced release of Ca from bone calvaria (30).

The PTH-induced hypocalcemic effect noted by Parsons was demonstrated by radio-calcium labeling methods to be due to a transient flux of Ca from blood into bone (29). Our studies do not provide direct evidence for such a mechanism, but they nevertheless serve to focus attention on the nature of the rapid response in calcium transport systems stimulated by PTH. A working hypothesis is that these transport systems are affected by ambient Pi concentrations, and that the absence of a fall in blood Pi in the PD animals, resulting from the hypotension-induced block in GFR, accounts for the early hypocalcemia. Because these systems must be an important element of the overall homeostatic regulation of calcium by PTH, efforts to further characterize them, and the cell types involved are important topics for further study.

Materials and Methods

**Peptides.** Human PTH analogs used were PTH (1–34), M-PTH (1–34) (M = Ala1,12,Aib13,Gln15,16,Arg17,18), and M-PTH (1–15), the latter containing the M modifications as well as Nle2 and Tyr13. LA-PTH contained the hPTH (1–14) sequence modified with Ala11,12,Gln15,16,Arg17,18, followed by the hPThPr (15–34) sequence modified with Ala11,12,22 and Lys24 (31). Radioligands used were hPTH(1–34) (100 nM), [125I-Nle12]–[125I-Tyr13]–[125I-Thr1(34)NH2] and [125I-M-PTH (1–15) (125I[Aib11,Nle12,Gln15,16,Arg17,18,Trp14,Y15]hPTH(1(34)NH2]) (14). (TMR)-PTH (1–34) contained TMR attached to the epsilon amino group of Lys13. Peptides contained C-terminal amides, except LA-PTH and PTH (1–34), which contained free C-terminal carboxyl groups.

**PTHr1 Binding, Signaling, and Microscopy.** Binding to the RG and R2 conformations of the rat PTHR (WT or PD) was assessed by competition methods using COS-7 cell membranes, as described in ref. 14. Binding to R2 was assessed using [125I]-PTH (1–34) tracer radioligand, and GTP<sub>S</sub> (1 × 10<sup>−7</sup>) M was added to the reactions. Binding to RG was assessed using membranes containing a high-affinity G<sub>S</sub> mutant (G<sub>S</sub>-G<sub>S</sub> and 125I-M-PTH (1–15) tracer radioligand. Signaling via the CAMPPKA pathway was assessed using HEP-293-derived cell lines that stably express the Glosensor CAMP reporter (Promega Corp.) (32), along with either the WT rat PTHR1 (GR-35 cells) or the PD rat PTHR1 (GD-4 cells). CAMP responses were also assessed using HEP-293 cells transiently transfected to express a CAMP-response-element/luciferase (Cre-Luc) reporter (33), and either the WT or PD rat PTHR1. Intracellular calcium (Ca<sup>2+</sup>) signaling was assessed in transiently PTHR1-transfected HEP-293 cells using Fura2-AM (Invitrogen, Life Tech.) (22). CAMP and Ca<sup>2+</sup> signaling assays were performed in 96-well plates and analyzed using a PerkinElmer Envision plate reader. Signaling via the ERK1/2 pathway was assessed using Western blot using antibodies (Cell Signaling Tech. Inc.) specific for phospho- or total ERK1/2. Confocal microscopy was performed using cells fixed and mounted at times after TRM-PTHR (1–34) (100 nM) addition, and a Bio-RadZeiss Radiance 2000 confocal microscope (Carl Zeiss MicroImaging Inc.).

**Physiologic Responses in Mice.** Mice (male C57BL/6, aged 8–19 wk) were treated following the ethical guidelines adopted by the Massachusetts General Hospital. WT mice were obtained from Charles River Laboratory.
Homozygous PD knock-in mice (10) were maintained at the MGH. Mice were injected s.c. with vehicle (10 mM citric acid/150 mM NaCl/0.5% Tween-80, pH 5.0) or vehicle containing PTH (1–34) or M-PTH (1–34) at doses of 50 nmol/kg or LA-PTH at 10 nmol/kg. Blood was collected from the tail vein and analyzed directly for Ca²⁺ using a Siemens RapidLab 348 Ca²⁺/pH analyzer; or as plasma for Pi using a photometric assay (Wako Laboratory); CAMP in plasma, urine, and tissue homogenates was measured by RIA; plasma levels of 1,25(OH)₂-vitamin-D₃ and CTX were measured using EIA kits (IDS Ltd.). Urine creatinine was measured using a photometric assay (Stanbio Laboratory).

**Tissue mRNA.** Mouse kidneys and calvaria were harvested, frozen in liquid nitrogen, homogenized in TRIzol (Invitrogen), and mRNA was purified using the RNeasy Plus mini kit (Qiagen), quantified using a NanoDrop 8000 UV spectrophotometer (Thermo Scientific), and processed using a QuantiTect Reverse Transcription kit (QIAGEN catalog no. 205313), a QuantFastSYBER Green PCR kit (Qiagen catalog no. 204054), and a StepOnePlus PCR instrument (Applied Biosystems). PCR primers are shown in Fig. S9.

**FITC-Inulin Clearance and Blood Pressure Measurements.** FITC-Inulin (Sigma; dialyzed (molecular weight cutoff = 1,000) vs. 150 mM NaCl, was injected via the tail vein, either alone (vehicle) or together with either PTH (1–34) (50 nmol/kg) or LA-PTH (10 nmol/kg). Tail vein blood was then collected and the plasma was assessed for FITC fluorescence (λ excitation = 480 nm; λ emission = 530 nm) using a PerkinElmer-Victor3 plate reader. GFR was calculated by fitting the data to a biexponential decay function and using the equation GFR = [1/(α + β)] where I is ligand dose, A and B are the y-intercepts of the two decay components, and α and β are the corresponding decay constants (17). Cardiac blood pressure was measured as described in ref. 20; mice were anesthetized by i.p. injection with ketamine (100 mg/kg), fentanyl (50 mg/kg), and rocuronium (2 mg/kg), intubated, and mechanically ventilated (fraction of inspired O₂ = 1, 10 mL/g, 120 breaths per minute). The chest was opened, and a pressure–volume conductance catheter (PVR-1030; Millar Instruments) was introduced through the apex into the left ventricle and used to measure ESP. A second catheter inserted into the right jugular vein was used to administer PTH (1–34) (25 nmol·kg⁻¹·min⁻¹) via a perfusor pump at a rate of 1.25 μL·g⁻¹·min⁻¹ for 2 min.

**Data Calculations.** Data were processed using Excel 2008 (Microsoft Corp.) and Prism 5.0 (GraphPad Software Inc.). Statistical analyses used the Student t test (two-tailed, unequal variances), with significance inferred from P values of 0.05 or less.

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Fig. S1. Functional properties of the PD-PTHR1 in vitro. (A) Signaling via the PLC/IP3/Ca pathway was assessed in transiently transfected HEK-293 cells using Fura2-AM; ratiometric Fura2 fluorescence was recorded in cells treated with vehicle, or PTH(1-34) (100 nM). Data are means (±SEM) of six experiments each in quadruplicate. Fura-2 signaling parameters are reported in Dataset S4. (B) PTH(1-34)-induced ERK1/2 phosphorylation was assessed in HEK-293 cells transiently transfected to express either the WT-PTHR1 or PD-PTHR1; cells were treated with PTH(1-34) (100 nM) or buffer for 15 or 30 min, then analyzed by Western blot. (C) Ligand-induced internalization was assessed by confocal fluorescent microscopy in transiently transfected HEK-293 cells treated with the fluorescent (red) ligand TMR-PTH(1-34) (100 nM) for 5 or 30 min (400× magnification). (D) HEK-293-derived cell lines stably transfected to express either the rPTHR1-WT (GR-35) or rPTHR1-PD (GPD-1, GPD-4, and GPD-10), or control cells not PTHR1-transfected (GS-22A) were treated with vehicle or PTH(1-34) (100 nM) for 10 min, and then cell membranes were prepared and analyzed by Western blotting using a primary rabbit polyclonal antiserum (PRB-620P, Covance Inc.) that recognizes an epitope in the Exon E2 region of the rat PTHR1. Detection was with a horse-radish peroxidase-conjugated secondary antibody directed towards rabbit IgG. Actin was analyzed to control for sample loading. Cell membranes were prepared as used in radioligand binding assays. Molecular weight markers (kDa) are indicated on the left.
Competition binding analysis of PTH(1–34) and modified PTH analogs. Competition binding assays were performed using membranes prepared from transiently transfected COS-7 cells; R\(^0\) assays used \(^{125}\)I-PTH(1–34) as tracer radioligand and the reactions contained GTP\(\gamma\)S (1 \(\times\) 10\(^{-5}\) M); RG assays used \(^{125}\)I-M-PTH (1–15) tracer radioligand and membranes prepared from COS-7 cells cotransfected to express a high-affinity G\(\alpha\)s mutant. (A) Binding of PTH(1–34), M-PTH (1–34), and LA-PTH to the R\(^0\) conformation of the WT-PTHR1. (B) Binding of the same analogs to the R\(^0\) conformation of PD-PTHR1. (C) Binding to the RG conformation of the WT-PTHR1. (D) Binding to the RG conformation of the PD-PTHR1. Data are means (±SEM) of six (PTH(1–34) only) or three experiments, each performed in duplicate (Dataset S1).
Fig. S3. cAMP and intracellular calcium signaling via the WT-PTHR1 and PD-PTHR1. Assays of cAMP accumulation were performed using HEK-293-derived cell lines stably transfected to express the luciferase-based cAMP glosensor protein along with either the WT-PTHR1 (GR-35 cells) or PD-PTHR1 (GPD-4 cells). (A) Dose–response analysis of PTH(1–34), M-PTH(1–34) and LA-PTH in cells expressing the WT-PTHR1. (B) Ligand dose–response analysis in cells expressing the PD-PTHR1. In the assays of A and B, luminescence was recorded 14 min after ligand addition. (C) Accumulation of cAMP in the presence of vehicle, PTH(1–34) (0.3 nM), M-PTH(1–34) (0.3 nM), or LA-PTH (0.1 nM) in cells expressing the WT-PTHR1. (D) Accumulation of cAMP in the presence of vehicle or the indicated ligands in cells expressing the PD-PTHR1. In C and D, the ligands were applied to the cells at t = 0 and luminescence was recorded for 12 min thereafter. (E) Accumulation of cAMP following ligand wash-out in cells expressing the WT-PTHR1. (F) Accumulation of cAMP following ligand wash-out in cells expressing the PD-PTHR1. In E and F, the cells of C and D, respectively, were removed from the plate reader at the end of the 12-min “ligand-on” phase, rinsed twice to remove unbound ligand, treated with fresh media containing luciferin, and luminescence was again recorded for an additional 180 min of “wash-out.” Luminescence was recorded using a PerkinElmer Envision plate reader; cells were preloaded with luciferin for 30 min before ligand addition. Data are means (± SEM) of four experiments, each performed in duplicate (A and B) or quadruplicate (C–F). cAMP assay parameters are reported in Datasets S2 and S5. (G) Intracellular calcium signaling in cells expressing the WT-PTHR1. (H) Intracellular calcium signaling in cells expressing the PD-PTHR1. iCa assays used HEK-293 cells transiently transfected to express either the WT-PTHR1 or PD-PTHR1. The cells were preloaded with the calcium-sensitive dye Fura2-AM for 30 min, and then ratiometric Fura2-AM fluorescence (lex. = 340 nm vs. lex 380 nm; lem. = 515 nm) was measured using a PerkinElmer Envision plate reader; at t = 10 s, the cells were treated with vehicle, PTH(1-34), or LA-PTH, each ligand at a concentration of 100 nM. Data are means (±SEM) of six experiments, each performed in quadruplicate.


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Fig. S4. Effect of PTH analogs on urine cAMP and blood Ca$^{++}$ in WT mice. (A) WT mice were injected with vehicle or the indicated ligands, each at a dose of 10 nmol/kg, and at times thereafter, and just before injection ($t = 0$) spot urine was collected and analyzed for cAMP. (B) Tail vein blood was collected from the same mice and analyzed for Ca$^{++}$. Data are means ± SEM; $n =$ six mice per group; $P$ vs. vehicle: *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

Fig. S5. Effects of M-PTH(1–34) on blood levels of Ca$^{++}$ and Pi in WT and PD mice. (A) WT or PD mice were injected s.c. with vehicle or M-PTH(1–34) (50 nmol/kg); tail vein blood was collected at the indicated times thereafter, or immediately before injection ($t = 0$), and assessed for the concentration of Ca$^{++}$. (B) Mice were injected as in A; tail vein blood was collected and the plasma was assessed for the concentration of Pi. Data are means ± SEM; $n =$ 3 or 4 mice per group; $P$ vs. the corresponding vehicle: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. 

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**Fig. S6.** Effects of PTH(1-34) on blood levels of CTX and 1,25(OH)$_2$-vitamin-D$_3$. (A) WT or PD mice were injected s.c. with vehicle or PTH(1-34) (50 nmol/kg) and tail vein blood was collected at 3 and 6 h after injection and assessed for concentrations of CTX. (B) Mice were treated as in A, and blood was assessed for 1,25(OH)$_2$-vitamin-D$_3$. Data are means ± SEM; n = 3 or 4 [CTX] or 4 [1,25(OH)$_2$-vitamin-D$_3$] mice per group; P vs. corresponding vehicle: *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. CTX, C-telopeptide.

**Fig. S7.** Effects of PTH(1–34) infusion on heart rate and end diastolic pressure in WT and PD mice. As described in Fig. 7C legend, mice were anesthetized and infused into the right jugular vein with PTH (25 nmol·kg$^{-1}$·min$^{-1}$) for 2 min, and effects on cardiac function were measured using a pressure probe inserted into the left ventricle: (A) heart rate; (B) end-diastolic pressure; t = 0 indicates initial conditions immediately before PTH infusion and the bracket indicates time of PTH infusion. Data are means ± SEM; n = 6 mice per group.
Fig. 58. Effects of PTH(1–34) on NPT2a protein levels in kidney brush-border membranes (BBMs). WT or PD mice were injected s.c. with vehicle or PTH(1–34) (50 nmol/kg), and BBMs were prepared from kidneys isolated 15 min (A and B) or 1 h (C and D) after injection and analyzed by Western blotting for the presence of NPT2a and actin. The graphs (B, D) show densitometric quantification of the ~70-kDa NPT2a band, normalized for each sample to the value obtained for the ~38-kDa actin band, and expressed as a percent of the mean corresponding value obtained with vehicle-injected WT mice. BBMs were prepared as described (1); 30 μg protein was loaded per lane; primary antibody for NPT2a was a gift from K. Miyamoto, Tokushima University, Japan; that for actin was from Santa Cruz (catalog no. sc-1616); secondary antibody was HRP-labeled goat anti-rabbit IgG (Cell Signaling Technology Inc. catalog no. 7074). Data are means ± SEM; n = 3 mice per group; P vs. corresponding vehicle: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.


Fig. 59. Primers used for RT-PCR analysis.
Other Supporting Information Files

Dataset S1 (XLS)
Dataset S2 (XLS)
Dataset S3 (XLS)
Dataset S4 (XLS)
Dataset S5 (XLSX)