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Disruption of β-catenin binding to parathyroid hormone (PTH) receptor inhibits PTH-stimulated ERK1/2 activation

Yanmei Yang, Bin Wang*

Center for Translational Medicine, Department of Medicine, Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia, PA, USA

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ABSTRACT

The type I parathyroid hormone receptor (PTH1R) mediates PTH and PTH-related protein (PTHrP) actions on extracellular mineral ion homeostasis and bone remodeling. These effects depend in part on the activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2). Sequences located within or at the carboxyl-terminus of PTH1R control its activation and trafficking. β-catenin regulates PTH1R signaling and promotes chondrocyte hypertrophy through binding to the intracellular carboxyl-terminal region of the receptor. How the interaction of PTH1R with β-catenin affects PTH-stimulated ERK1/2 is unknown. In the present study, human embryonic kidney 293 (HEK293) cells, which do not express the PTH1R, were used to investigate whether the disruption of β-catenin binding to PTH1R affects PTH-stimulated ERK1/2 activation. We demonstrated that β-catenin interacted with wild-type PTH1R but this interaction was markedly reduced with mutant PTH1R (L584A/L585A). PTH stimulated less cAMP formation and increased more intracellular calcium in HEK293 cells transfected with wild-type PTH1R compared with mutant PTH1R, indicating β-catenin switches PTH1R signaling from Gαs activation to Gαq signaling. In addition, ERK1/2 activation in HEK293 cells transfected with PTH1R exhibited time and concentration dependence. PTH-stimulated ERK1/2 activation was mostly mediated through Gαq/PLC signaling pathway. Importantly, transfection of mutant PTH1R decreased PTH-induced ERK1/2 activation by inhibiting Gαq-mediated signaling. This study shows for the first time that the interference of β-catenin binding to PTH1R inhibits PTH-stimulated ERK1/2 phosphorylation.

1. Introduction

The parathyroid hormone (PTH)/PTH-related protein (PTHrP) receptor (PTH1R) belongs to Class B of the superfamily of G protein-coupled receptors (GPCRs) [1]. PTH1R is present primarily in the kidney and bone. Interaction with its cognate ligands, PTH, PTHrP, or biologically active peptide fragments, such as PTH(1–34), results in activation of Gαs and Gαq with consequent stimulation of adenylate cyclase and phospholipase C (PLC) [1,2]. The action of PTH is also mediated through phospholipase D [3] and mitogen-activated protein (MAP) kinases (MAPKs), which include extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun-NH_2-terminal kinase, and p38 kinase [4,5]. A cascade of cell-specific events of PTH leads to regulating extracellular mineral ion homeostasis and bone remodeling. These effects depend in part on the activation of ERK1/2 [6,7].

The PTH1R activation, desensitization, endocytosis, and recycling proceed in a cyclical manner [8,9]. Sequences located within or at the carboxyl-terminus of PTH1R control its endocytic sorting and recycling [10,11]. Many GPCRs including PTH1R possess carboxyl-terminal motifs that interact with PSD-95/Discs large/ZO-1 (PDZ) scaffolding proteins, such as, Na/H exchanger regulatory factors, NHERF1 and NHERF2 [10]. Previously, we demonstrated that NHERF1 increased PTH-stimulated PTH1R coupling to Gαq but not to Gαs or Gαi. In contrast, NHERF2 decreased PTH-induced PTH1R coupling to Gαs and increased Gαq and Gαi activation [2]. Recently, Yano et al. reported that β-catenin regulated PTH1R signaling and promoted chondrocyte hypertrophy through its binding to the intracellular carboxyl-terminal region of the receptor [12]. How the interaction of PTH1R with β-catenin affects PTH-stimulated ERK1/2 activity is unknown.

In the present study, we demonstrated that transfection of mutant PTH1R, which decreased its interaction with β-catenin,

* Corresponding author. The Center for Translational Medicine, Department of Medicine, Sidney Kimmel Medical College, Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA 19107, USA.
E-mail address: bin.wang@jefferson.edu (B. Wang).

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switched PTH1R signaling from Gαq signaling to Gαs activation, and resulted in reducing PTH-induced ERK1/2 activation. This study reports a novel role for the interaction of PTH1R with β-catenin in regulating PTH-stimulated ERK1/2 phosphorylation.

2. Materials and methods

2.1. Materials

Human $Nle^{8,18}$,Tyr^{34}-PTH(1–34) was purchased from Bachem (Torrance, CA). Anti-p44/p42 MAP kinase (ERK1/2) and phospho-p44/42 MAP kinase (pERK1/2) (Thr202/Tyr204) polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). HA.11 ascites monoclonal antibody (mAb) and HA.11 monoclonal affinity matrix were obtained from Covance (Berkeley, CA). β-catenin polyclonal antibody was from Millipore (Billerica, MA). Horseradish peroxidase-conjugated goat anti-rabbit antibody was from Pierce Chemical (Rockford, IL). Horseradish peroxidase-conjugated sheep anti-mouse antibody was from GE Healthcare (Little Chalfont, Buckinghamshire, United Kingdom). Bisindolylmaleimide I (Bis I), H89 and PD98059 were from Calbiochem (San Diego, CA). Geneticin (G418) was obtained from Invitrogen (Carlsbad, CA). FuGENE6 was purchased from Roche Applied Science (Indianapolis, IN). All other reagents were from Sigma-Aldrich (St.Louis, MO).

2.2. Cell culture

Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.3. Generation of mutant PTH1R

Mutation of intracellular carboxyl-terminal region of the HA-PTH1R by replacing two leucines with alanines (HA-PTH1R-L584A/L585A) was performed by PCR using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions [8]. The sequences of two primers utilized for PCR were 5′-CTGAGCGGCCACCTGCCGCGGCACAGGAAGAGTGGGAGAC (forward), and 5′-GTCTCCCACTCTTCCTGTGCCGCGGCAGGTGGCCGCTCAG (reverse).

2.4. Cell transfection

HEK293 cells were stably transfected with pcDNA3.1(+)-HA-PTH1R using FuGENE 6 and screened by geneticin (50 mg/L) to generate cell line of HEK293-R as previously described [8]. For transient transfection, HEK293 cells were transfected with empty vector, wild type receptor (HA-PTH1R), or mutant receptor (HA-PTH1R-L584A/L585A). After 48 h transfection, cells were used for measuring cAMP accumulation, intracellular calcium, and ERK1/2 phosphorylation.

2.5. Measurement of adenylyl cyclase activity

Adenylyl cyclase activity was determined by assay of cAMP accumulation as described previously [13]. Briefly, HEK293 cells transfected with wild-type or mutant PTH1R in 24-well plates were labeled with 0.5 μCi [^{3}H]-adenine for 2 h. The cells were then treated with vehicle or 100 nM PTH in the presence of phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (1 mM) in fresh medium containing 0.1% BSA for 15 min. The reaction was terminated by addition of 1 M *trichloroacetic acid*, followed by neutralization with 4 N KOH. cAMP was isolated by the two-column method. Radioactivity was measured by beta scintillation spectrometry.

2.6. Intracellular calcium $[Ca^{2+}]_i$ assay

Cells were seeded onto poly-D-lysine coated 96-well plates, grown to confluence, and loaded with 2 μM Fluo-4 AM (BD Biosciences, San Jose, CA) according to the manufacturer's protocol. PTH or vehicle was added by an automated pipetting system in triplicate, and the 525-nm signals were generated by excitation at 485 nm with a Flex Station II (Molecular Devices, Sunnyvale, CA) as previously reported [14]. The net peak Ca^{2+} response was calculated using the following equation: (maximum agonist induced fluorescence units) − (basal fluorescence units).

2.7. ERK1/2 phosphorylation assay

After PTH treatment, the 6-well plate was placed on ice and the cell culture medium was removed. Cells were lysed in 250 μl of RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.4, and 150 mM NaCl) supplemented with protease inhibitor cocktail set I and II (Calbiochem, San Diego), and incubated for 15 min on ice. The cell lysates were then drawn five times through a 21-gauge needle attached to a 1 ml syringe, and then microcentrifuged. The supernatants were used to detect total ERK1/2 and phospho-ERK1/2 levels.

2.8. Immunoprecipitation and immunoblotting analysis

To detect the interaction of β-catenin with HA-tagged wild-type and mutant PTH1R, the cells were lysed with RIPA buffer supplemented with protease inhibitor cocktail set I. The solubilized materials were incubated with HA.11 monoclonal affinity matrix overnight at 4 °C. The lysates and immunoprecipitated protein, eluted by the addition of Laemmli SDS-PAGE loading buffer (Bio-Rad Laboratories, CA) containing 5% β-mercaptoethanol, were analyzed by SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore) using the semi-dry method (Bio-Rad Laboratories). Membranes were blocked overnight at 4 °C with 5% nonfat dried milk in tris-buffered saline plus Tween-20 and incubated with different antibodies (polyclonal anti-ERK1/2 antibody at 1:1000, anti-pERK1/2 antibody at 1:500, anti-β-catenin antibody at 1:1000, or monoclonal anti-HA antibody at 1:1000) for 2 h at room temperature. The membranes were then washed and incubated with 1:5000 dilution of goat anti-rabbit IgG or sheep anti-mouse IgG conjugated to HRP, or with IRDye 800CW goat anti-Rabbit IgG or IRDye 680RD goat anti-mouse IgG at room temperature for 1 h. Protein bands were visualized with a luminol-based ECL substrate or quantified using the Licor Odyssey system.

2.9. Statistical analysis

The curve-fitting analysis and data statistics were performed using Prism (GraphPad Software, Inc., San Diego, CA). Data are presented as the mean ± S.E., where n indicates the number of independent experiments. Statistical analyses were performed by either a two-tailed Student's test or analysis of variance with post-test repeated measures analyzed by Bonferroni tests. Differences of $P \leq 0.05$ were assumed to be significant.

3. Results

3.1. Disruption of β-catenin binding to PTH1R switches receptor signaling from Gαq to Gαs activation

β-catenin regulates PTH1R signaling and facilitates chondrocyte hypertrophy through binding to the intracellular carboxyl-terminal region of the receptor [12]. To investigate whether the disruption of PTH1R binding to β-catenin affects PTH1R signaling, we generated mutant PTH1R (L584A/L585A) and transfected either HA-tagged wild-type or mutant PTH1R into HEK293 cells. We immunoprecipitated with HA affinity matrix followed by immunodetection with β-catenin antibody to measure their interactions. As shown in Fig. 1A, β-catenin interacted with wild-type PTH1R and this interaction was markedly reduced with mutant PTH1R. Consistently, PTH increased cAMP formation but reduced $[Ca^{2+}]_i$, an index of PLC activity, in HEK293 cells transfeted with mutant PTH1R compared with cells transfected with wild-type PTH1R (Fig. 1B and C) [12]. Together, the interference of PTH1R binding to β-catenin switches PTH1R signaling from Gαq/PLC signaling to Gαs/cAMP activation in HEK293 cells.

3.2. PTH induces ERK phosphorylation

ERK1/2 activation displays in a cell-specific manner. PTH-induced ERK1/2 phosphorylation in HEK293 cells stably transfected with PTH1R exhibited in a time- and concentration-dependent manner. Maximal ERK1/2 activation was achieved at 10 min (Fig. 2A) and declined gradually over a 60-min time period. At the 10-min time point, PTH elicited concentration-dependent increases of ERK1/2 phosphorylation over the range of 10^{-11} M to 10^{-6} M (Fig. 2B). Therefore, treatment with 100 nM PTH for 10 min was used for the determination of ERK1/2 activation in the following experiments.

3.3. PTH-stimulated ERK1/2 activation is Gαq/PLC dependent

It has been reported that ERK1/2 activation cannot be activated by a cAMP signaling-selective PTH1R ligand in HEK293 cells [5]. To assess whether the Gαq-mediated signaling is necessary for PTH-induced ERK1/2 activation, different specific inhibitors were applied to HEK293-R cells. As before, treatment with PTH induced ERK1/2 activity. This activation was significantly reduced by a specific PKC inhibitor bisindolylmaleimide I (Bis I, 10 μM), which was similar to the effect of MAPK/ERK kinase 1 and 2 (MEK1/2) inhibitor PD98059 (20 μM) (Fig. 3). In contrast, the PKA inhibitor H89 (10 μM) had no significant effect on ERK1/2 activation. These data indicate that PTH-stimulated ERK1/2 activation requires Gαq binding to PTH1R, whereas Gαs-mediated signaling is not sufficient for this activation.

3.4. Disruption of β-catenin binding to PTH1R inhibits PTH-stimulated ERK1/2 activation

PTH stimulates ERK1/2 activation in HEK 293 cells through Gαq-mediated signaling pathway. We hypothesized that the disruption of PTH1R binding with β-catenin affected PTH-stimulated ERK1/2 activation. To test this idea, either wild-type or mutant PTH1R was transfected into HEK293 cells. Indeed, transfection of mutant PTH1R significantly reduced PTH-stimulated ERK1/2 activation compared with that of wild-type PTH1R (Fig. 4). Taken together, the interference of β-catenin binding to PTH1R inhibits PTH-stimulated ERK1/2 phosphorylation by decreasing Gαq-mediated signaling pathway.

4. Discussion

The signaling and trafficking of PTH1R is influenced by its interacting proteins in a cell-specific manner. In most cells and

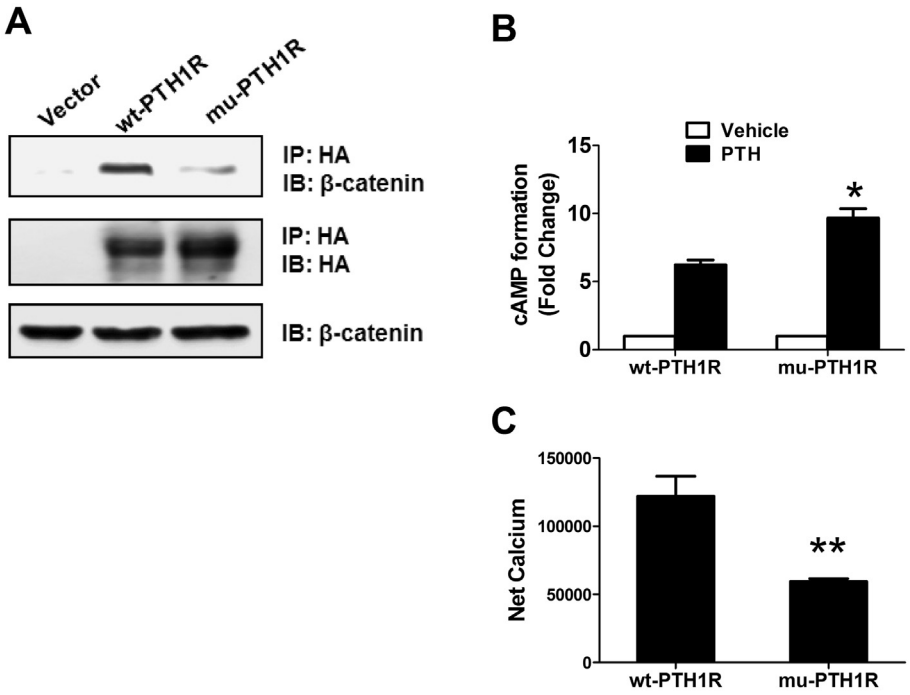

Fig. 1. Disruption of β-catenin binding to PTH1R switches receptor signaling from Gαq to Gαs activation. HEK293 cells were transfected with vector, HA-tagged wild-type PTH1R (wt-PTH1R) or mutant PTH1R (mu-PTH1R). A, interaction of β-catenin with wt-PTH1R or mu-PTH1R. HA-tagged receptors were precipitated with HA monoclonal affinity matrix. The precipitated protein was then immunoblotted (IB) with β-catenin antibody or HA antibody. A representative experiment is shown. IP, immunoprecipitation. B, the intracellular cAMP accumulation after treatment with 100 nM PTH. C, $[Ca^{2+}]_i$ concentration after treatment with 100 nM PTH. Data are summarized as the mean ± S.E. of three or four independent experiments. *, $P < 0.05$, **, $P < 0.01$, compared with wt-PTH1R treated with PTH.

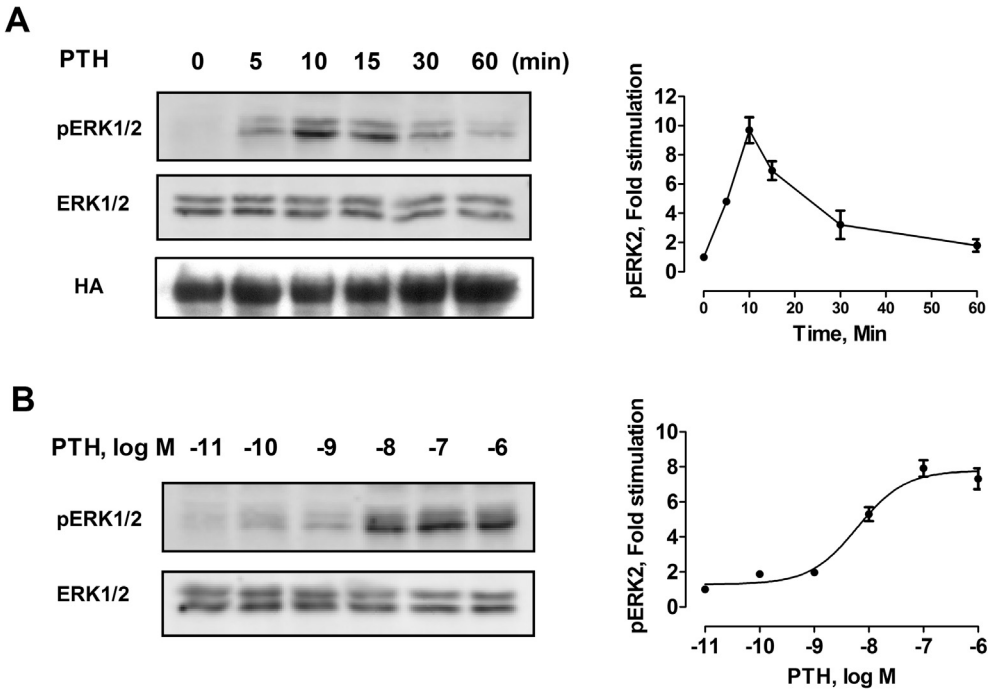

Fig. 2. PTH time- and concentration-dependently induced ERK1/2 phosphorylation. Confluent HEK293-R cells were serum-starved overnight and then treated for the indicated time and concentration of PTH. ERK1/2 phosphorylation was measured as detailed in Material and methods. Data from three independent time course and concentration dependence experiments were quantified, normalized to total ERK2, and expressed as the fold change of basal pERK2. Data are summarized as mean ± S.E. A, PTH stimulated ERK1/2 phosphorylation in a time-dependent manner. B, at the 10-min time point, PTH (10^{-11}–10^{-6} M) induced concentration-dependent increases of ERK1/2 phosphorylation. The immunoblotting and fold-stimulation of pERK2 are shown on the left and right panels, respectively.

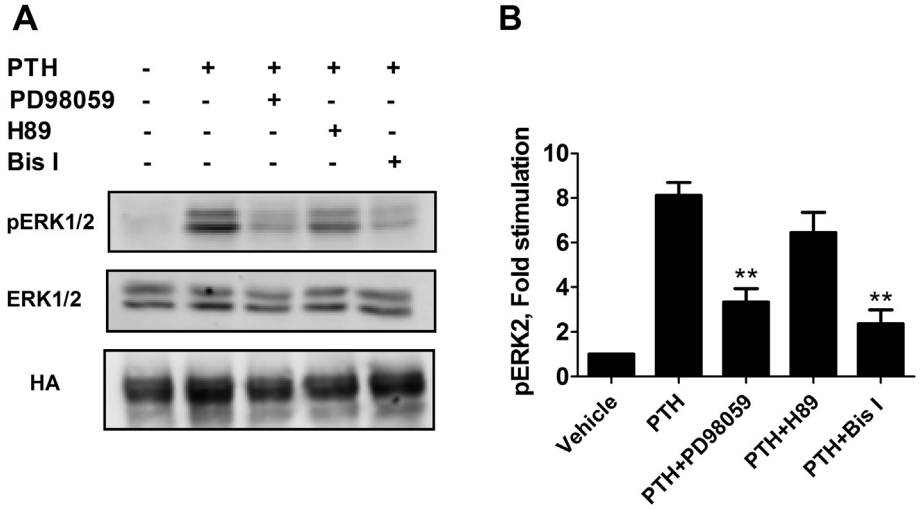

Fig. 3. PTH-stimulated ERK1/2 activation is Gαq/PLC dependent. Confluent HEK293-R were serum-starved overnight and preincubated with 10 μM of PKC inhibitor bisindolylmaleimide I (Bis I), 10 μM of PKA inhibitor H89, 20 μM of MEK1/2 inhibitor PD98059, or vehicle for 10 min, followed by a 10-min treatment with 10^{-7} M PTH. ERK1/2 phosphorylation was measured. A, a representative experiment is shown. B, data from three independent experiments were quantified, normalized to total ERK2, and expressed as the fold change of basal pERK2. Data are summarized as mean ± S.E. **, $P < 0.01$, compared with PTH control.

tissues, the PTH1R signals through both adenylyl cyclase and PLC activation. However, in some cells, the PTH1R activation occurs only to one signaling pathway. For example, in rat osteosarcoma ROS 17/2.8 cells and vascular smooth muscle cells, PTH stimulates adenylyl cyclase but not PLC [15,16], whereas in cardiac myocytes and lymphocytes [17,18], PLC, but not adenylyl cyclase, is activated. It is known that heterologous expression of PTH1R to HEK293 cells elicits both adenylyl cyclase and PLC [5]. In the present study, therefore, HEK293 cells were used to examine the effects of disrupting β-catenin binding to PTH1R on the receptor signaling switch between adenylyl cyclase and PLC, and on PTH-stimulated ERK1/2 activation.

Wnt/β-catenin signaling pathway plays an important role in kidney and skeletal development [19,20]. There is a cross-talk between PTH1R and frizzled receptor [21]. Yano et al. reported that β-catenin regulated PTH1R signaling and facilitated chondrocyte hypertrophy. They identified that the intracellular carboxyl-terminal region of the receptor interacted with β-catenin, and

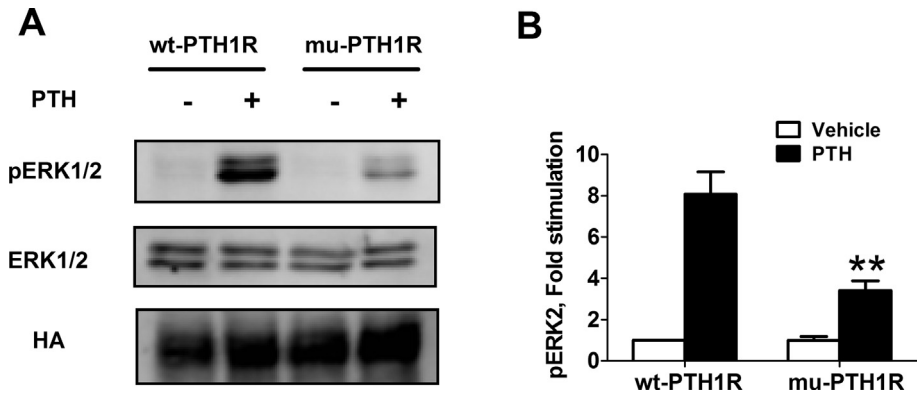

Fig. 4. Disruption of β-catenin binding to PTH1R inhibits PTH-stimulated ERK1/2 activation. HEK293 cells were transfected with either wild-type PTH1R (wt-PTH1R) or mutant PTH1R (mu-PTH1R). After transfection for 36 h, the cells were cultured in 0.1% FBS overnight, and treated with vehicle or PTH (100 nM) for 10 min. A, a representative experiment is shown. Data from three independent experiments were quantified, normalized to total ERK2, and expressed as the fold change of basal pERK2. B, data are summarized as mean ± S.E. **, $P < 0.01$, compared with wt-PTH1R treated with PTH.

deletion of the last 10 amino acids from the carboxyl-terminus of PTH1R switched Gαq/PLC signaling to Gαs/cAMP activation. Human PTH1R has a PDZ motif located in the last four amino acids [E(590) T(591)V(592)M(593)] that interact with NHERF1 and NHERF2 [10,11]. Both NHERF1 and NHERF2 control PTH1R signaling by promoting differential activation of Gα protein subunits [2]. NHERF1 also inhibits PTH-stimulated ERK1/2 activation in distal tubule cells and CHO cells heterologously expressing the PTH1R [4,22]. In order to keep the intact PDZ recognition domain in the carboxyl-terminus of PTH1R, we replaced two leucines with alanines located in the carboxyl-terminal region to generate mutant PTH1R(L584A/L585A). The immunoprecipitation data showed that β-catenin associated with wild-type PTH1R and this interaction was notably reduced with mutant PTH1R. We further found that PTH-stimulated more cAMP formation and less cytoplasmic free calcium in HEK293 cells transfected with mutant PTH1R compared with wild-type PTH1R. Previous finding indicated that cAMP signaling-selective PTH1R ligand failed to induce ERK1/2 phosphorylation in HEK293 cells [5]. We showed that PKC inhibitor blocked PTH-stimulated ERK1/2 activation but PKA inhibitor had no significant effect on PTH-induced ERK1/2 phosphorylation, indicating ERK1/2 activity induced by PTH is mediated via Gαq/PLC signaling pathway. To investigate whether the disruption of PTH1R binding to β-catenin affects PTH-stimulated ERK1/2 activation, we transfected either wild-type or mutant PTH1R to HEK293 cells. We identified that transfection of mutant PTH1R markedly reduced PTH-stimulated ERK1/2 activation compared with that of wild-type PTH1R. These data clearly indicate that the interference of β-catenin binding to PTH1R suppresses PTH-induced ERK1/2 phosphorylation by inhibiting Gαq-mediated signaling pathway.

The molecular mechanism by which β-catenin interaction with PTH1R promotes switching of receptor signaling between adenylyl cyclase and PLC is unknown. It is very likely that β-catenin-PTH1R interaction may induce conformational change after PTH treatment. The outcome favors Gαq binding to PTH1R and reduces the affinity for Gαs binding to the receptor. Further studies will be necessary to determine the influence of β-catenin on PTH1R coupling to Gα protein subunits by directly measuring GTPγS binding and immunoprecipitation of GTPγS-bound Gα subunits using specific anti-Gαs and Gαq antibody, respectively [2].

In conclusion, we demonstrate that the disruption of β-catenin binding to PTH1R switches receptor signaling from Gαq activation to Gαs signaling and inhibits PTH-stimulated ERK1/2 activation. Such an effect of β-catenin on PTH1R signaling extends previous finding that β-catenin promotes chondrocyte hypertrophy. In osteoblasts and kidney cells, PTH-induced ERK1/2 activation is correlated with cell proliferation, differentiation, and apoptosis. Understanding of the interplay between PTH1R and β-catenin may provide insights that how PTH regulates extracellular mineral ion homeostasis and bone remodeling.

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Conflicts of interest

The authors have no conflict of interest to disclose.

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Transparency document

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