Biochemical and Biophysical Research Communications xxx (2015) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

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ARTICLE INFO

Article history:
Received 1 May 2015
Accepted 14 May 2015
Available online xxx

Keywords:
PTH1R
β-catenin
G protein
ERK1/2
cAMP
Intracellular calcium

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,

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http://dx.doi.org/10.1016/j.bbrc.2015.05.082
0006-291X/

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.

Please cite this article in press as: Y. Yang, B. Wang, Disruption of β-catenin binding to parathyroid hormone (PTH) receptor inhibits PTH-stimulated ERK1/2 activation, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.05.082