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NHERF1 Regulates Parathyroid Hormone Receptor Desensitization: Interference with β-Arrestin Binding[S]

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ABSTRACT

Type 1 parathyroid hormone receptor (PTH1R) activation, desensitization, internalization, and recycling proceed in a cyclical manner. The Na^+/H^+ exchange regulatory factor 1 (NHERF1) is a cytoplasmic adapter protein that regulates trafficking and signaling of several G protein-coupled receptors (GPCRs) including the PTH1R. The mineral ion wasting and bone phenotype of NHERF1-null mice suggests that PTH1R may interact with NHERF1. The objective of this study was to examine the effect of NHERF1 on PTH1R desensitization. Using rat osteosarcoma T6-N4 cells expressing the endogenous PTH1R, in which NHERF1 expression could be induced by tetracycline, PTH1R desensitization was assessed by measuring adenylyl cyclase activity after successive PTH challenges. PTH1R-mediated adenylyl cyclase responses were desensitized by repetitive PTH challenges in a concentration-dependent manner, and desensitization was inhibited by NHERF1. NHERF1 blocked PTH-induced dissociation of the PTH1R from $G\alpha_s$. Blocking PTH1R endocytosis did not mitigate PTH1R desensitization. Reducing constitutive NHERF1 levels in human osteosarcoma SAOS2 cells, which express both endogenous PTH1R and NHERF1, with short hairpin RNA directed against NHERF1 restored PTH1R desensitization. Mutagenesis of the PDZ-binding domains or deletion of the NHERF1 MERM domain demonstrated that both are required for inhibition of receptor desensitization. A phosphorylation-deficient PTH1R exhibited reduced desensitization and interaction with β-arrestin2 compared with wild-type PTH1R. NHERF1 inhibited β-arrestin2 binding to wtPTH1R but had no effect on β-arrestin2 association with pdPTH1R. Such an effect may protect against PTH resistance or PTH1R down-regulation in cells harboring NHERF1.

The parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor (PTH1R) belongs to Class B of the seven-transmembrane family of G protein-coupled receptors (GPCRs) (Gensure et al., 2005). PTH1R is present primarily in the bone and kidney. Interaction with its cognate ligands, PTH, PTHrP, or biologically active peptide fragments leads to activation of G_s and G_q with consequent stimulation of adenylate cyclase and phospholipase C (Abou-Samra et al., 1992; Friedman et al., 1996). A cascade of cell-specific events ensues that regulates virtually all aspects of extracellular calcium and phosphate homeostasis.

As with most GPCRs, the response of PTH1R to agonists is regulated by multiple mechanisms, including a well characterized and highly conserved process involving receptor phosphorylation by G protein-coupled receptor kinase 2 (GRK2) (Dicker et al., 1999; Flannery and Spurney, 2001) and arrestin recruitment (Ferrari et al., 1999; Tawfeek and Abou-Samra, 1999; Vilardaga et al., 2002). Arrestins are cytoplasmic adaptor proteins that bind to phosphorylated GPCRs and uncouple them from their cognate G proteins, thereby producing a nonsignaling, desensitized receptor (Krupnick and Benovic, 1998). β-Arrestin1 and β-arrestin2 are widely expressed and regulate the functions of many GPCRs, including the PTH1R (Malecz et al., 1998; Ferrari et al., 1999). In mice lacking β-arrestin2, PTH treatment promotes sustained cAMP signaling in primary osteoblasts in vitro and altered skeletal response to PTH in vivo (Ferrari et al., 2005). These processes contribute directly to PTH1R desensitization by facilitating the uncoupling of the receptor from its cognate G proteins.

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ABBREVIATIONS: PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide; PTH1R, type 1 PTH receptor; GPCR, G protein-coupled receptor; GRK2, G protein-coupled receptor kinase 2; NHERF1, Na^+/H^+ exchange regulatory factor 1; PDZ, postsynaptic density 95/discs large/zona occludens; MERM, merlin-ezrin-radixin-moesin; GFP, green fluorescent protein; BSA, bovine serum albumin; ROS, rat osteosarcoma; shRNA, short hairpin RNA; siRNA, small interfering RNA; IBMX, 3-isobutyl-1-methylxanthine; Tet, tetracycline; wtPTH1R, wild-type PTH1R; PKC, protein kinase C; PD, phosphorylation deficient; HA, hemagglutinin.

The interaction of β-arrestin1 or β-arrestin2 with phosphorylated PTH1R is the likely mechanism of desensitization of the PTH1R-activated responses (Tawfeek et al., 2002). After β-arrestin interactions, the PTH1R is endocytosed and either targeted for degradation, leading to receptor down-regulation (Tian et al., 1994; Ureña et al., 1994; Massry and Smogorzewski, 1998), or recycled to the membrane, leading to receptor resensitization (Chauvin et al., 2002).

Na^+/H^+ exchange regulatory factor 1 (NHERF1), also known as ezrin-radixin-moesin-binding phosphoprotein-50, is a cytoplasmic scaffolding protein. NHERF1 recruits various cellular receptors, ion transporters, and other proteins to the plasma membrane of epithelia and other cells (Voltz et al., 2001; Bretscher et al., 2002; Shenolikar et al., 2004; Tan et al., 2004). NHERF1 contains tandem postsynaptic density 95/discs large/zona occludens (PDZ) domains and a merlin-ezrin-radixin-moesin (MERM) domain. PTH1R was reported to interact with PDZ1 and PDZ2 with similar affinities (Sun and Mierke, 2005; Wang et al., 2007). The MERM domain binds to respective actin-associated MERM proteins. NHERF1 tethers the PTH1R to the actin cytoskeleton through the MERM domain.

The mineral ion-wasting and bone phenotype of NHERF1-null mice or patients with NHERF1 coding region mutations suggests that the PTH1R is the principal GPCR interacting with NHERF1 (Shenolikar et al., 2002). Humans with NHERF1 mutations present with renal stones or bone demineralization (Karim et al., 2008) underscoring the primary role of NHERF1 in associating with and modulating PTH1R activity. Expression of NHERF1 restores both PTH(1–34)-mediated inhibition of the Npt2 sodium-phosphate cotransporter (Mahon et al., 2003) and the increase of intracellular calcium (Mahon and Segre, 2004) in OK/H cells, which express low levels of NHERF1 and are resistant to the action of PTH. Our previous data established that NHERF1 inhibited PTH1R internalization without affecting receptor recycling in kidney cells and osteoblasts, and in cells heterologously expressing the PTH1R and NHERF1 (Wang et al., 2007).

The effect of NHERF1 on PTH1R desensitization has not been defined. We speculated that PTH1R desensitization may involve facilitated interaction of β-arrestin1 or β arrestin2, the PTH1R in cells lacking or with diminished expression of NHERF1 compared with cells expressing high levels of NHERF1. Using several different cell models, we show that NHERF1 potently inhibits PTH1R desensitization. This effect requires both intact PDZ and MERM domains in NHERF1. NHERF1 reduces PTH1R desensitization by preventing or displacing β-arrestin2 binding to the receptor and inhibits PTH1R dissociation from $G\alpha_s$.

Materials and Methods

Materials. NHERF1 rabbit polyclonal antibody was purchased from Affinity Bioreagents (Golden, CO). HA.11 ascites monoclonal antibody and HA.11 monoclonal affinity matrix were obtained from Covance Research Products (Princeton, NJ). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was from Pierce (Rockford, IL). Horseradish peroxidase-conjugated sheep anti-mouse antibody was from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Tetracycline hydrochloride was purchased from American Bioanalytical (Natick, MA). Lipofectamine 2000, Zeocin, blasticidin, polyclonal GFP antibody, and protein A-Sepharose 4B conjugate were obtained from Invitrogen (Carlsbad, CA). Protease inhibitor mixture Set I was from Calbiochem (San Diego, CA). Human PTH(1–34) was purchased from Bachem California (Torrance, CA). GFP agarose, polyclonal actin antibody, dynamin siRNA, and monoclonal dynamin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). cAMP-Glo assay kit was obtained from Promega (Madison, WI). FuGENE 6 was purchased from Roche Applied Science (Indianapolis, IN). Polyclonal $G\alpha_s$ antibody was obtained from Millipore (Billerica, MA). All other reagents were from Sigma-Aldrich (St. Louis, MO).

Cell Culture. Rat osteosarcoma ROS 17/2.8 cells and human osteosarcoma SAOS2 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. COS-7 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. All the cells were maintained at 37°C in a humidified atmosphere of 5% CO_2, 95% air.

Construction of pcDNA4/TO-NHERF1. His-tagged rabbit NHERF1 in pcDNA3 (provided by Dr. E. J. Weinman, University of Maryland, Baltimore, MD) was cut with KpnI and XhoI, and a 1.1-kilobase pair fragment without epitope was subcloned into the pcDNA4/TO vector (Invitrogen), which has two tetracycline operator sequences between the TATA box of the cytomegalovirus promoter and the transcriptional start site. The fidelity of the plasmids was confirmed by sequencing (ABI PRISM 377; Applied Biosystems, Foster City, CA).

Stable Expression of pcDNA6-TR and pcDNA4/TO-NHERF1. ROS 17/2.8 cells were transfected with pcDNA6-TR (Invitrogen) using Lipofectamine 2000 according to the manufacturer's instructions and screening with 10 μg/ml blasticidin and immunoblot. The cell line ROS-T6, which stably expressed tetracycline repressor protein, was obtained. ROS-T6 cells were then stably transfected with pcDNA4/TO-NHERF1 and screened by Zeocin (0.4 mg/ml) and immunoblot to generate cell line ROS-T6-N4.

Transient Transfection. Cells, as indicated, were transiently transfected with respective empty vector, plasmids of wild-type NHERF1, truncated NHERF1-(1–326) (NHERF1ΔMERM) (Li et al., 2002), mutant NHERF1, in which both PDZ1 and PDZ2 domains are scrambled (sPDZ1/2-NHERF1) (Weinman et al., 2003), wild-type receptor (GFP-wtPTH1R), phosphorylation-deficient PTH1R (GFP-pdPTH1R) (Tawfeek et al., 2002), Flag-β-arrestin2, or HA-PTH1R (Wang et al., 2007) by use of FuGENE 6 or Lipofectamine 2000 as described previously (Wang et al., 2007). Cells were used 48 h after transfection.

NHERF1 or Dynamin Knockdown. Constitutive NHERF1 expression in SAOS2 cells was silenced using RNA interference (Wang et al., 2007). Short hairpin RNA (shRNA) constructs against the human NHERF1 sequence GGAAACTGACGAGTTCTTCAAGAAATGCA mediated by a pRS shRNA vector were purchased from OriGene (Rockville, MD). Endogenous dynamin was knocked down by using siRNA against human dynamin (Santa Cruz Biotechnology). SAOS2 cells were transfected with NHERF1 shRNA, siRNA, scrambled shRNA or siRNA, which has no homology to any known human sequence. Transfections were established after the manufacturer's protocol. Transfected cells were cultured for 48 h and then used for receptor desensitization or immunoblot.

Coimmunoprecipitation and Immunoblot Analysis. Interaction of PTH1R with β-arrestin2 or NHERF1 was analyzed as described previously (Wang et al., 2008). In brief, six-well plates of COS-7 cells were transiently transfected with GFP-wtPTH1R, GFP-pdPTH1R, Flag-β-arrestin2, HA-PTH1R, or wild-type NHERF1. Forty-eight hours later, the cells were lysed with radioimmunoprecipitation assay 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 mixture I and incubated for 15 min on ice. $G\alpha_s$ interaction with the PTH1R was analyzed as described previously (Ross and Gilman, 1977; Gudi et al., 1998). COS-7 cells were harvested and solubilized with 1% Thesit detergent in 50 mM Tris-

HCl, pH 7.4, 150 mM NaCl, and 1 mM EDTA. Solubilized materials were incubated overnight at 4°C with anti-Flag M2 affinity gel (Sigma-Aldrich), GFP agarose, or $G\alpha_s$ polyclonal antibody for 1 h at 4°C, and then protein A-Sepharose 4B conjugate was added to each sample and incubated overnight at 4°C. Total lysates and immunoprecipitated protein, eluted by the addition of SDS sample buffer, were analyzed by SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore) using the semi-dry method (Bio-Rad Laboratories, Hercules, CA). 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-NHERF1 [1:1000], anti-GFP [1:1000], anti-Flag [1:2000], polyclonal anti-$G\alpha_s$ [1:500], monoclonal anti-HA [1:1000], monoclonal anti-dynamin [1:500], or anti-actin [1:2000]) for 2 h at room temperature. The membranes were then washed and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase or anti-sheep mouse IgG conjugated to horseradish peroxidase (1:5000) for 1 h at room temperature. Protein bands were revealed with a luminol-based enhanced chemiluminescence substrate.

Receptor Desensitization. PTH1R desensitization was measured by determining adenylyl cyclase activity after successive PTH challenges using cAMP-Glo assay kit following the manufacturer's instructions. The first challenge was conducted with PTH(1–1000 nM) in the absence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) for 15 min at 37°C. Residual cell surface PTH was removed by rinsing with ice-cold PBS followed by an acid-glycine wash (50 mM glycine and 100 mM NaCl, pH 3) as described previously (Chauvin et al., 2002; Wang et al., 2007). The second challenge consisted of PTH (10 nM, 15 min) at 37°C in the presence of IBMX (1 mM). The cAMP assay was performed in 96-well plate. Plates are read using a microplate-reading luminometer. Luminescence was quantified by using a cAMP standard curve. Receptor desensitization was calculated using the following equation: $[1 - (cAMP_{(PTH^1 + PTH(10\ nM^2))} - cAMP_{(PTH^1 + 0.1\%\ BSA^2)} - (0.1\%\ BSA^1 + 0.1\%\ BSA^2)/cAMP_{(0.1\%\ BSA^1 + PTH(10\ nM^2))})] \times 100\%$, where 1 indicates the first PTH or BSA challenge and 2 the second exposure to PTH or BSA.

Receptor Binding. Receptor binding was performed as described previously (Chauvin et al., 2002; Wang et al., 2007) using high-pressure liquid chromatography-purified ^{125}I-[Nle^{8,18},Tyr^{34}]PTH-(1–34)NH_2. In brief, ROS17/2.8 cells or SAOS2 cells were seeded on 24-well plates and grown to confluence. Cells were put on ice for 15 min and incubated with PTH(1–34) (10^{-11} – 10^{-6}M) and ~100,000 cpm of ^{125}I-[Nle^{8,18},Tyr^{34}]PTH(1–34)NH_2 in 250 μl of fresh media on ice for an additional 2.5 h. After incubation, cells were rinsed two twice with ice-cold PBS and then solubilized in 0.2 N NaOH. Cell surface-bound ^{125}I-PTH(1–34) was assessed by γ spectrometry. PTH1R number was analyzed by Scatchard analysis.

Statistical Analysis. All 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. Multiple comparisons were evaluated by analysis of variance with post-test repeated measures analyzed by the Bonferroni procedure. Differences of $P \leq 0.05$ were assumed to be significant.

Results

NHERF1 Inhibits PTH1R Desensitization. Previous findings indicate that rat osteosarcoma cells, ROS 17/2.8, express 5.8×10^4 PTH1R per cell but negligible NHERF1 (Sneddon et al., 2003; Wheeler et al., 2008). PTH(1–34) caused concentration-dependent cAMP accumulation over the range of 10^{-10} to 10^{-6} M to 9-fold over control levels (Fig. 1). To examine NHERF1 effects in a coherent cell model, we stably introduced NHERF1 in ROS cells under the control of a tetracycline (Tet) repressor protein. As shown in Fig. 2, Tet (1–12 ng/ml) elicited concentration-dependent NHERF1 expression in ROS-T6-N4 cells. Maximal NHERF1 expression occurred at Tet 10 ng/ml. Tet-induced NHERF1 expression in ROS-T6-N4 cells was compared with endogenous NHERF1 expression in SAOS2 cells. Immunoblot analysis using actin as an internal loading control (Supplemental Fig. 1) showed that Tet (10 ng/ml) elicited NHERF1 expression in ROS-T6-N4 cells similarly to that constitutively expressed by SAOS2 cells. Therefore, 10 ng/ml Tet was used for induction of NHERF1 expression in ROS-T6-N4 cells in the subsequent experiments. PTH-stimulated adenylyl cyclase was comparable in ROS17/2.8 and ROS-T6-N4 cells (data not shown).

PTH promoted PTH1R desensitization in ROS-T6-N4 cells in a time- and concentration-dependent manner. Desensitization began within 1 min and reached a plateau by 15 min (Fig. 3). Therefore, 15 min was used for determination of

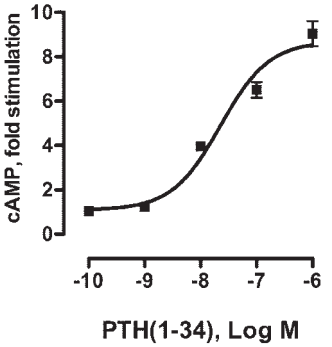

Fig. 1. PTH activates adenylyl cyclase in a concentration dependent manner. ROS17/2.8 cells were incubated with PTH(1–34) 10^{-10} to 10^{-6} M for 15 min in the presence of 1 mM IBMX. cAMP accumulation was measured as described under *Materials and Methods*. Data are summarized as the mean ± S.E. of three experiments.

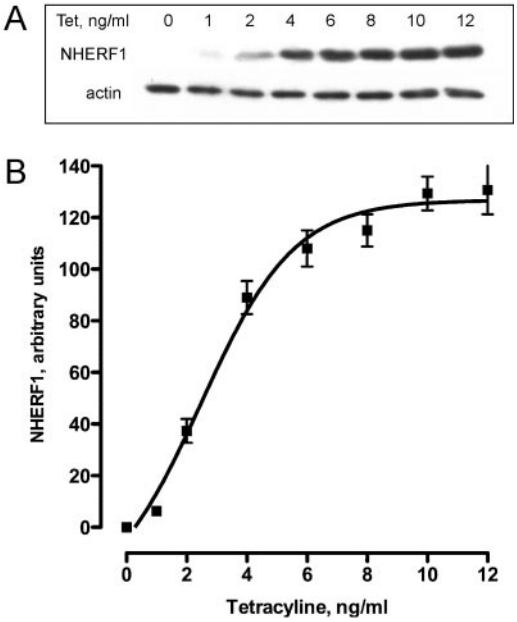

Fig. 2. Tetracycline-induced concentration-dependent increases of NHERF1 expression. Tetracycline (Tet) at the indicated concentration was added to ROS-T6-N4 cells for 48 h. Samples were treated as described under *Materials and Methods*. A, representative immunoblots of NHERF1 (top) and actin (bottom) using a specific polyclonal NHERF1 antibody and actin antibody. Actin was used as an internal control for protein loading. B, concentration-response curve for Tet-induced NHERF1 expression in ROS-T6-N4 cells.

steady-state and maximal effects. PTH (1–1000 nM) induced receptor desensitization in a concentration-dependent manner (Table 1). The EC_{50} in the absence of NHERF1 was 40 ± 3.1 × 10^{-9} M PTH. Induction of NHERF1 expression significantly reduced PTH1R desensitization (Fig. 4) for the same respective challenge with PTH and increased the EC_{50} to 282 ± 25 × 10^{-9} M ($P < 0.001$).

Complementary experiments were performed on SAOS2 cells, which constitutively express both the PTH1R and NHERF1. Here, a 15-min pretreatment with 10^{-7} M PTH desensitized the PTH1R by 40% (Fig. 5A). Incubation with NHERF shRNA, which reduced endogenous NHERF1 levels by 68% compared with a scrambled control (Fig. 5B), substantially augmented the cAMP response to a second PTH challenge to 70% of control (Fig. 5A), consistent with a role of NHERF1 to inhibit receptor desensitization.

Agonist occupancy of the PTH1R leads not only to receptor desensitization but also to receptor internalization. The latter process could contribute to diminished PTH sensitivity during the initial incubation with PTH. Therefore, we sought to dissociate PTH1R desensitization and internalization. PTH1R internalization is dynamin-dependent (Sneddon et al., 2003). Therefore, we blocked PTH1R internalization with siRNA to knock down dynamin expression in SAOS2 cells (Fig. 5C). Dynamin siRNA by itself did not affect receptor movement. However, under these conditions, dynamin siRNA inhibited PTH(1–34)-stimulated PTH1R endocytosis (Fig. 5D). It is noteworthy that PTH1R desensitization was not affected by blocking receptor internalization (Fig. 5E), suggesting that PTH receptor desensitization is not required for the receptor internalization.

NHERF1 Domains Involved in PTH1R Desensitization. NHERF1 possesses two tandem PDZ domains and a carboxyl-terminal MERM domain, linking it with actin-associated merlin, ezrin, radixin, and moesin. Previous work established that interaction of the PTH1R with either PDZ domain is sufficient to stabilize membrane localization of the PTH1R and that the effects of NHERF1 on receptor endocytosis require both intact PDZ and MERM domains (Wang et al., 2007). To determine which domains are involved in receptor desensitization, we employed NHERF1 constructs harboring mutations of the core-binding sequences in PDZ-1 and PDZ-2 (sPDZ1/2-NHERF1) and a truncated NHERF1 construct lacking the MERM domain (NHERF1ΔMERM). Transient transfection of wild-type NHERF1 in ROS17/2.8 cells inhibited receptor desensitization in response to PTH (Fig. 6A) as before. It is noteworthy that neither sPDZ1/2-NHERF1 nor NHERF1ΔMERM affected PTH1R desensitization. Thus, the effects of NHERF1 on receptor desensitization require the presence of both PDZ and MERM domains. The expression efficiency of wild-type NHERF1, sPDZ1/2-NHERF1, and NHERF1ΔMERM was confirmed by immunoblot (Fig. 6B).

NHERF1 Does Not Affect Desensitization of Phosphorylation-Deficient PTH1R. After PTH1R activation, the receptor is phosphorylated by GPCR kinases and second messenger-dependent protein kinases. PTH-dependent phosphorylation of PTH1R occurs on serines at positions 489, 491, 492, 493, 495, 501, and 504 (Tawfeek et al., 2002). Mutation to alanine abrogates phosphorylation after PTH stimulation. The phosphorylation-deficient PTH1R (pdPTH1R) exhibits enhanced PTH stimulation of adenylyl cyclase and impaired

TABLE 1

Effects of NHERF1 on PTH1R desensitization

ROS-T6-N4 cells were exposed to a first challenge for 15 min in the absence of IBMX. After ligand washout, cells were incubated a second time with 10 nM PTH (15 min) in the presence of IBMX. Cells were treated with Tet (10 ng/ml) to induce maximal NHERF1 expression, where indicated. Receptor desensitization was assayed as described under *Materials and Methods*. Data are summarized as the mean ± S.E. $n = 4$ independent experiments.

First Incubation	Second Incubation	cAMP	
		−NHERF	+NHERF
		nM	
0.1% BSA	10 nM PTH(1–34)	220 ± 30	243 ± 40
3 nM PTH(1–34)	10 nM PTH(1–34)	160 ± 2	206 ± 1.8
10 nM PTH(1–34)	10 nM PTH(1–34)	133 ± 16	192 ± 2
30 nM PTH(1–34)	10 nM PTH(1–34)	101 ± 11	156 ± 12
100 nM PTH(1–34)	10 nM PTH(1–34)	75 ± 6	134 ± 12
300 nM PTH(1–34)	10 nM PTH(1–34)	40 ± 4	90 ± 9
1000 nM PTH(1–34)	10 nM PTH(1–34)	21 ± 11	53 ± 14

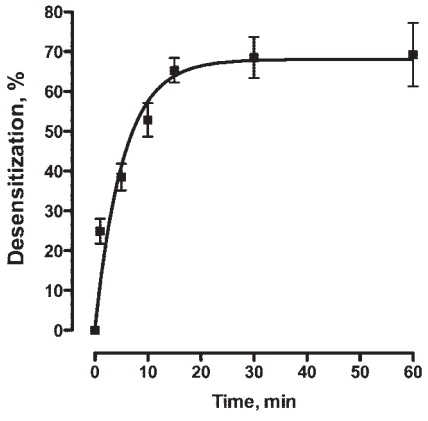

Fig. 3. Time course of PTH1R desensitization in response to PTH(1–34). ROS-T6-N4 cells were first challenged with PTH(1–34),100 nM, for 1 to 60 min in the absence of IBMX. After ligand washout, cells were exposed to PTH (10 nM, 15 min) in the presence of IBMX. cAMP accumulation was measured and receptor desensitization was calculated as detailed *under Materials and Methods*. The desensitization rate constant was 0.186 min^{-1} and the $t_{1/2}$ was 3.7 min. Data are summarized as the mean ± S.E. of three independent experiments.

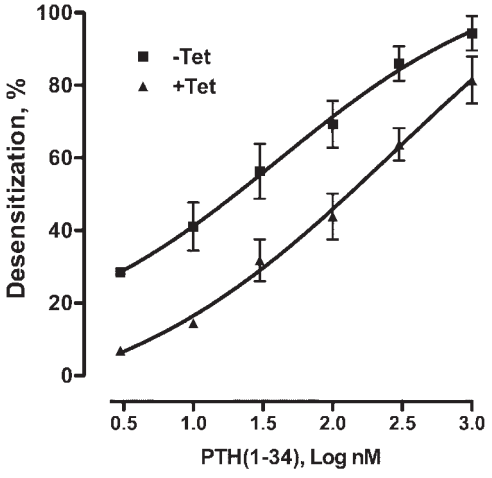

Fig. 4. NHERF1 inhibits PTH-induced PTH1R desensitization. ROS-T6-N4 cells were first challenged with PTH(1–34) (15 min, 1–1000 nM) in the absence of IBMX. After ligand washout, cells were challenged a second time with PTH (10 nM, 15 min) in the presence of IBMX. Cells were treated with Tet (10 ng/ml) to induce maximal NHERF1 expression, where indicated. Receptor desensitization was assayed as described in the legend to Fig. 3. Data are summarized as the mean ± S.E. of four independent experiments. Where not seen, error bars fall within the symbol.

PTH-dependent internalization (Tawfeek et al., 2002). COS-7 cells do not express the PTH1R or NHERF1. When cotransfected with NHERF1 and either wild-type PTH1R (wtPTH1R) or pdPTH1R, PTH-induced pdPTH1R desensitization was much lower than that of wtPTH1R (Fig. 7), suggesting that PTH1R phosphorylation is critical for receptor desensitization. Moreover, NHERF1 inhibited wtPTH1R desensitization but had no significant effect on pdPTH1R desensitization (Fig. 7).

NHERF1 Prevents β-arrestin2 Binding to the Receptor and Inhibits PTH1R Uncoupling from $G\alpha_s$. Phosphorylated PTH1R binds to β-arrestins, which uncouple the receptor, causing desensitization. Because NHERF1 has no effect on phosphorylation-deficient PTH1R desensitization, we hypothesized that NHERF1 interferes with binding of the phosphorylated receptor to β-arrestins. To investigate the potential involvement of β-arrestins in PTH1R desensitization, COS-7 cells were cotransfected with NHERF1, β-arrestin2, and with either wtPTH1R or pdPTH1R. β-Arrestin2 constitutively interacts with wtPTH1R to a greater extent than with pdPTH1R (Fig. 8A). PTH(1–34) increased β-arrestin2 association with both wtPTH1R and pdPTH1R. NHERF1 interacts with both wtPTH1R and pdPTH1R. Strikingly, NHERF1 inhibited β-arrestin2 binding to wtPTH1R but had no effect on β-arrestin2 association with pdPTH1R (Fig. 8B).

Finally, we examined the effect of NHERF1 on uncoupling PTH1R from $G\alpha_s$. COS-7 cells were cotransfected with wild-type PTH1R and NHERF1. $G\alpha_s$ constitutively coimmunoprecipitated with PTH1R (Fig. 9, top), demonstrating the coupling of the receptor with $G\alpha_s$. As expected, the interaction of PTH1R with $G\alpha_s$ was reduced in the presence of PTH as a result of binding of β-arrestin to activated receptor and subsequent uncoupling from $G\alpha_s$. NHERF1 did not affect the basal interaction of PTH1R with $G\alpha_s$ in the absence of PTH but prevented PTH-induced receptor uncoupling from $G\alpha_s$, thereby inhibiting PTH1R desensitization.

Discussion

The present results demonstrate that NHERF1 inhibits PTH1R desensitization by preventing or displacing β-arrestin2

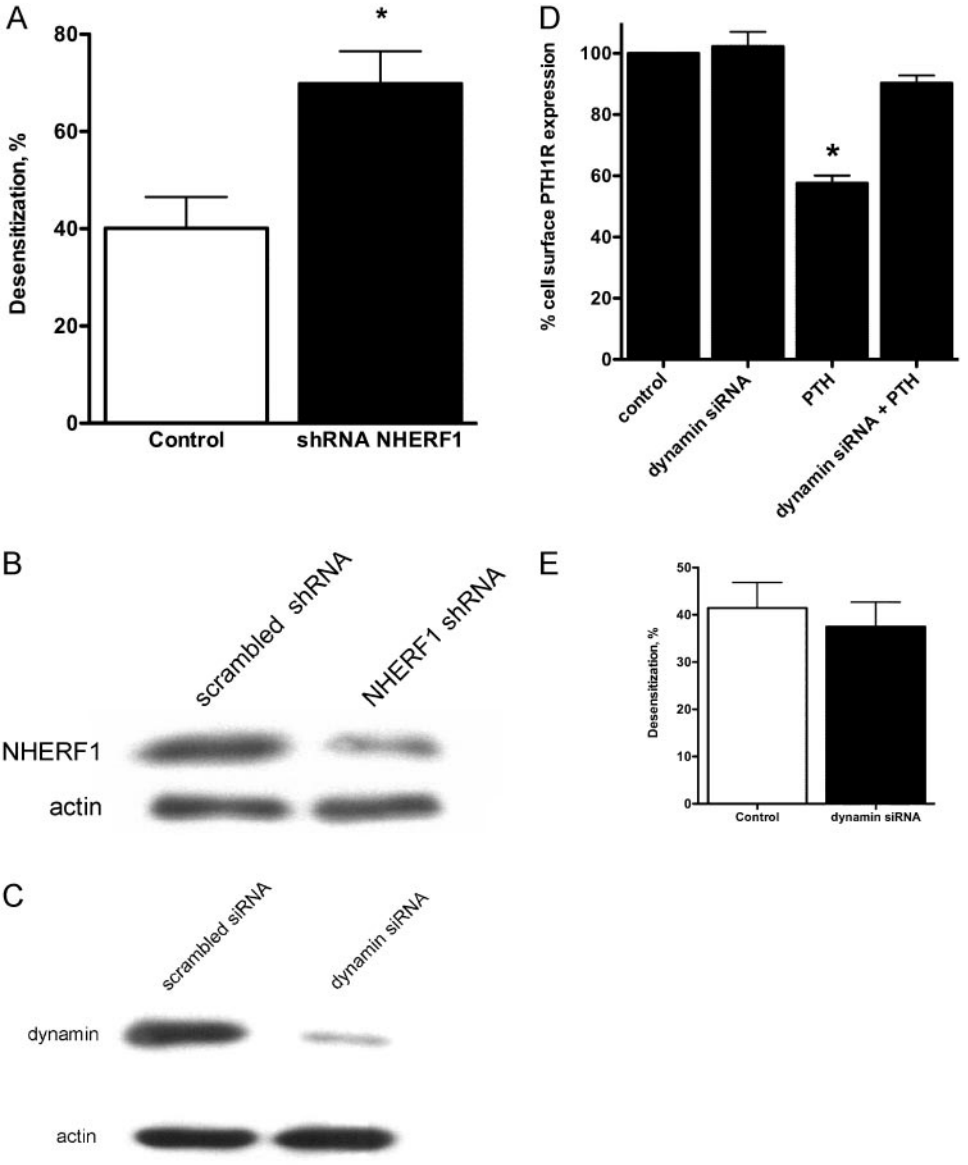

Fig. 5. Effect of NHERF1 on PTH1R desensitization. SAOS2 cells were transiently transfected with the indicated constructs or their respective scrambled controls. Data are summarized as the mean ± S.E. of three or four experiments. *, $P < 0.05$ versus control. A, NHERF1 shRNA increases receptor desensitization. Forty-eight hours after transfection with NHERF1 shRNA or scrambled shRNA (Control), receptor desensitization and cell surface receptor expression were measured as described under *Materials and Methods*. B, immunoblot showing knockdown of NHERF1 expression by shRNA; scrambled shRNA served as the control. Actin was used as a control for protein loading. C, immunoblot showing knockdown of dynamin expression by siRNA. D, dynamin siRNA blocks PTH(1–34) (100 nM, 15 min)-induced PTH1R internalization. Cells were transfected with dynamin siRNA or scrambled siRNA (control). After 48 h, cell surface receptor expression was measured as described under *Materials and Methods*. E, dynamin siRNA does not affect PTH(1–34)-induced receptor desensitization. Cells were transfected with dynamin siRNA or scrambled siRNA (control). After 48 h, receptor desensitization was measured as described under *Materials and Methods*.

binding to the receptor. The magnitude and duration of PTH action, like that of other hormones acting through GPCRs, is intimately linked to the balance between signal generation and signal termination. GPCR activation is generally followed by a rapid loss of responsiveness, termed desensitization. Receptor desensitization protects cells against excessive stimulation. GPCR desensitization occurs as a consequence of uncoupling the receptor from its cognate G protein in response to phosphorylation of Ser or Thr residues within the intracellular tail. Phosphorylation of Ser residues in the PTH1R is mediated by G protein-coupled receptor kinase-2 (GRK2) (Malecz et al., 1998; Dicker et al., 1999) and, to a lesser extent, by second messenger-dependent protein kinase C (PKC) (Castro et al., 2002). GRK2 preferentially phosphorylates the distal sites of the intracellular PTH1R tail, whereas PKC phosphorylates more upstream residues (Blind et al., 1996). Nonetheless, as we and others have shown, PTH1R phosphorylation is not required for the interaction of the PTH1R with β-arrestin and receptor internalization (Malecz et al., 1998; Dicker et al., 1999; Ferrari et al., 1999; Sneddon and Friedman, 2007).

NHERF1 plays a prominent role in regulating the signaling and trafficking of a diverse array of membrane-associated proteins including GPCRs (Voltz et al., 2001; Weinman et al., 2006). In previous work, we found that NHERF1 inhibited PTH1R internalization without affecting receptor recycling (Wang et al., 2007). The effects of NHERF1 on PTH1R desensitization and resensitization are unknown. We now show that NHERF1 regulates PTH-induced receptor desensitization. NHERF1 inhibition of PTH1R desensitization was not associated with altered receptor phosphorylation or internalization but prevented or displaced β-arrestin2 binding to the receptor.

PTH1R is abundantly expressed in osteoblasts and renal tubular cells. To investigate the mechanism of NHERF1 on receptor desensitization in a single, coherent cell model that could be experimentally manipulated, we generated a model system wherein the extent of NHERF1 expression could be controlled at defined levels of PTH1R abundance. ROS17/2.8 cells were selected as a model because they exhibit negligible NHERF1 expression, they possess PTH1R as detected by radioligand binding and function, and they are a standard osteoblast cell model (Ek et al., 2006). ROS-T6-N4 cells were engineered by using their parental cell line, ROS17/2.8 cells, to express a Tet repressor system, where application of Tet induces concentration-dependent increases of NHERF1-expression. In the present work, PTH(1–34) promoted time- and concentration-dependent PTH1R desensitization in ROS-T6-N4 cells. Addition of Tet to the cells reduced PTH1R desensitization.

Similar experiments were performed on SAOS2 cells to determine whether NHERF1 exerts comparable effects in cells constitutively expressing both PTH1R and NHERF1. After pretreatment with PTH, shRNA targeted to NHERF1 substantially augmented PTH1R desensitization. Taken together, these results provide strong evidence that NHERF1 inhibits PTH1R desensitization.

We previously reported that NHERF1 coimmunoprecipitated with full-length PTH1R and augmented PTH1R membrane retention (Sneddon et al., 2003; Wang et al., 2007). When both PDZ core-binding domains were mutated, however, NHERF1 no longer interacts with the PTH1R, and the tethering effect of NHERF1 is lost. The NHERF1 MERM domain binds to actin-associated proteins, thereby assembling a multimeric complex that includes the PTH1R, effectors such as PLCβ and protein kinase A, and indirectly link this complex with the actin cytoskeleton through direct interactions with MERM proteins. The NHERF1 MERM do-

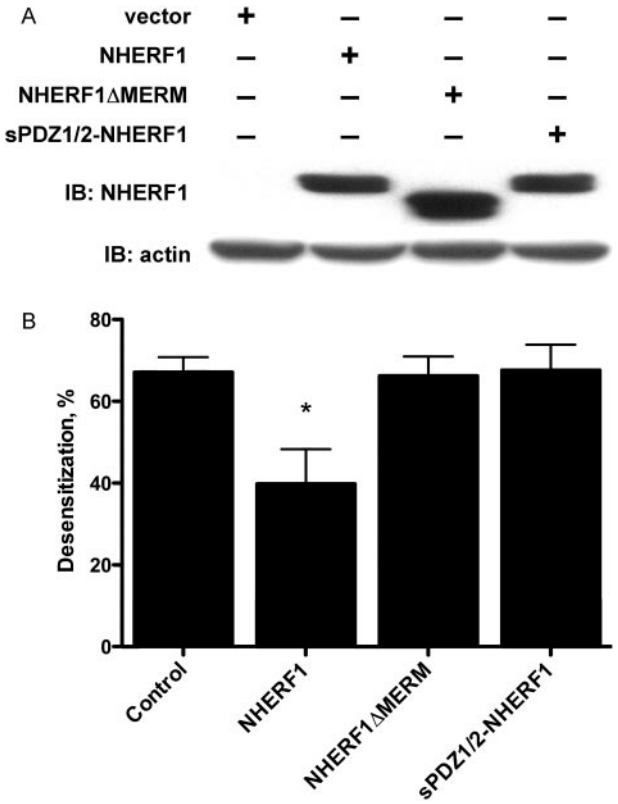

Fig. 6. Structural analysis of NHERF1 domains involved in PTH1R desensitization. A, ROS17/2.8 cells were transiently transfected with empty vector (Control), wild-type NHERF1, NHERF1 harboring mutations in PDZ1 and PDZ2 core-binding domains (sPDZ1/2-NHERF1), or truncated NHERF1 lacking the MERM domain (NHERF1ΔMERM). After 48 h, receptor desensitization was measured. Data are summarized as the mean ± S.E. of three independent experiments. *, $P < 0.05$ versus control. B, representative immunoblots of NHERF1 (top) and actin (bottom) using a specific polyclonal NHERF1 antibody and actin antibody.

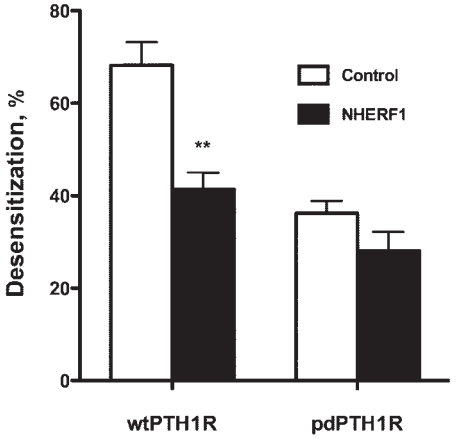

Fig. 7. Effect of NHERF1 on PTH-induced receptor desensitization. COS-7 cells were transiently transfected with empty vector (Control), NHERF1, wtPTH1R, or pdPTH1R. After 48 h, PTH1R desensitization was measured by determining adenylyl cyclase activity after successive PTH challenges as before. Data are summarized as the mean ± S.E. of three independent experiments. *, $P < 0.05$ versus control.

main is also required for PTH1R membrane tethering. When deleted, NHERF1 loses this capability. To determine which NHERF1 domains are involved in PTH1R desensitization, we compared the effects of intact NHERF1 with constructs in which both PDZ core-biding domains were disrupted, and a NHERF1 construct lacking the MERM domain. Whereas intact NHERF1-inhibited receptor desensitization in ROS17/2.8 cells, the PDZ mutants, and truncated NHERF1 had no effect on PTH1R desensitization. Together, these findings strongly support the view that the effects of NHERF1 on receptor desensitization and endocytosis require both intact PDZ and MERM domains.

Upon ligand binding to the PTH1R, signaling is activated, and the receptor is phosphorylated by G protein-coupled receptor kinases (Malecz et al., 1998). Cell surface PTH1R expression and binding were not altered in the presence of NHERF1 by measuring ^{125}I-PTH(1–34) binding in the presence and absence of increasing concentrations of unlabeled PTH (10^{-11} to 10^{-6} M) in ROS17/2.8 cells (Wheeler et al., 2008) and CHO-N10-R3 cells (Wang et al., 2007), heterologously expressing the PTH1R and NHERF1. Thus, NHERF1 does not inhibit PTH1R desensitization by blocking ligand binding to the PTH1R.

Mahon et al. (2002) reported that NHERF2, a NHERF1 homolog, markedly inhibited adenylyl cyclase by stimulating inhibitory G_i proteins in PS120 cells transfected with the PTH1R. There are no differences for PTH-stimulated cAMP formation in wild-type and NHERF1-null proximal tubule cells (Cunningham et al., 2004, 2005), and in CHO-N10-R3 cells in the presence or absence of NHERF1. In contrast, NHERF1 increases PTH-stimulated cAMP accumulation in ROS17/2.8 cells (Wheeler et al., 2008). These data indicated that NHERF1 regulates the conditional efficacy of PTH on cell-specific PTH1R activation.

We sought to determine whether the inhibitory actions of NHERF1 on PTH1R desensitization were secondary to an effect on receptor phosphorylation or β-arrestin binding to the PTH1R. Wild-type PTH1R and phosphorylation-deficient PTH1R constructs were used to determine whether NHERF1 inhibition on PTH1R desensitization involves receptor phosphorylation. COS-7 cells, which do not express endogenous NHERF1 or the PTH1R, were cotransfected with NHERF1 and either wild-type PTH1R or phosphorylation-deficient PTH1R. The results showed that PTH-induced phosphorylation-deficient PTH1R desensitization is much lower than that of wild-type PTH1R. This finding is consistent with previous reports that the phosphorylation-deficient PTH1R is impaired in PTH-dependent internalization and displays increased sensitivity to PTH stimulation (Tawfeek et al., 2002; Jones and Hinkle, 2005). NHERF1 inhibits wild-type PTH1R desensitization but has no significant effect on phosphorylation-deficient PTH1R desensitization. We previously reported that NHERF1 inhibition of PTH1R recycling was not associated with receptor phosphorylation (Wang et al., 2007). Together, these results exclude an effect of NHERF1 on PTH1R phosphorylation in mediating the inhibitory action on receptor desensitization.

Our previous findings showed that PTH-stimulated receptor internalization was greater for wild-type PTH1R compared with a truncated form of the receptor (PTH1R-480stop) lacking determinants for stable β-arrestin association (Wang et al., 2007). Overexpressing β-arrestin2 in HEK293 cells induced more PTH1R internalization than did β-arrestin1 (Vilardaga et al., 2002). Sneddon and Friedman (2007) reported that PTH(1–34)-induced PTH1R internalization in wild-type

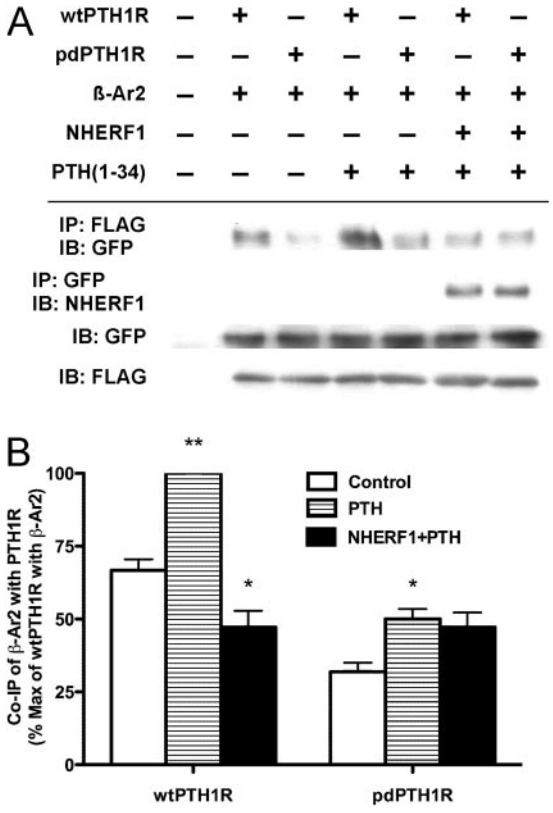

Fig. 8. Effect of NHERF1 on the interaction of wtPTH1R or pdPTH1R with β-arrestin2. COS-7 cells were transiently transfected empty vector (Control), NHERF1, β-arrestin2 (β-Ar2), GFP-wtPTH1R, or GFP-pdPTH1R. After 48 h, cells were treated with PTH(1–34), 100 nM, for 15 min where indicated. A, representative coimmunoprecipitation showing that NHERF1 inhibits β-Ar2 binding to wtPTH1R but has no effect on β-Ar2 binding to pdPTH1R. GFP-tagged receptors were expressed at equivalent levels. IP, immunoprecipitation; IB, immunoblot. B, densitometric analysis of three independent experiments showing β-Ar2 immunoprecipitated with PTH1R as a percentage of β-Ar2 immunoprecipitated with GFP-pdPTH1R, normalized for difference in protein expression. *, $P < 0.05$; **, $P < 0.01$ versus control.

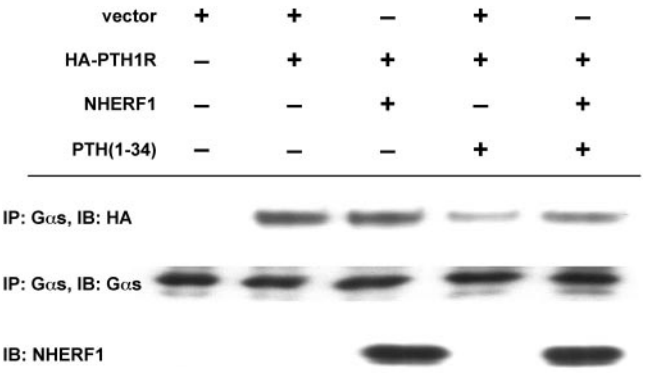

Fig. 9. Effect of NHERF1 on the interaction of PTH1R with $G\alpha_s$. COS-7 cells were transiently transfected with empty vector (Control), NHERF1, or HA-PTH1R. After 48 h, cells were treated with PTH(1–34), 100 nM, for 15 min, where indicated. A representative coimmunoprecipitation shows that NHERF1 inhibited PTH-induced receptor uncoupling from $G\alpha_s$ (top). The amount of immunoprecipitated endogenous $G\alpha_s$ in each sample was expressed at equivalent level (middle). Bottom, NHERF1 expression in each sample. IP, immunoprecipitation; IB, immunoblot.

and β-arrestin1-null mouse embryonic fibroblast cells. In contrast, the PTH1R did not internalize in β-arrestin2-null mouse embryonic fibroblast cells. β-arrestin2, therefore, plays a critical role in agonist-induced PTH1R endocytosis. Moreover, dominant negative β-arrestin1-(319–418) (Krupnick et al., 1997), which blocks arrestin-clathrin interactions, inhibited isoproterenol-induced β_2-adrenegic receptor internalization but not that of PTH on the PTH1R in hormone-responsive kidney cells (Sneddon and Friedman, 2007). Taken together, these findings indicate that β-arrestin2 regulates cell-specific PTH1R internalization.

Paing et al. (2002) reported that desensitization of protease-activated receptor-1 signaling was markedly impaired in mouse embryonic fibroblasts lacking both β-arrestin1 and β-arrestin2 compared with wild-type cells. β-Arrestins also mediate thyrotropin-releasing receptor desensitization (Jones and Hinkle, 2005). Based on these findings, we speculated that NHERF1 interferes with β-arrestin biding to the PTH1R to inhibit desensitization. The results show that β-arrestin2 preferentially interacts with wild-type PTH1R compared with phosphorylation-deficient PTH1R. PTH(1–34) increased β-arrestin2 interaction with wild-type PTH1R and phosphorylation-deficient PTH1R. These findings are compatible with the results from thyrotropin-releasing hormone receptor, where β-arrestin2 coimmunoprecipitated with wild-type receptor in the presence of thyrotropin-releasing hormone, but less β-arrestin2 was recovered with phosphorylation-deficient thyrotropin-releasing hormone receptor (Jones and Hinkle, 2005). Here, NHERF1 inhibited β-arrestin2 binding to wild-type PTH1R but not to the phosphorylation-deficient PTH1R, although NHERF1 interacts with the mutant PTH1R. These findings confirm that NHERF1 inhibits PTH1R desensitization by preventing or displacing β-arrestin2 binding to the receptor.

Phosphorylated receptors bind to β-arrestins, which uncouple the receptor from its cognate G protein and facilitates desensitization. We investigated the effect of NHERF1 on uncoupling PTH1R from $G\alpha_s$ in response to agonist stimulation. Upon PTH binding, $G\alpha_s$ is displaced as expected. However, NHERF1 prevents PTH-induced the receptor uncoupling from $G\alpha_s$, thereby stemming the inhibitory influence of β-arrestins on PTH1R desensitization.

In summary, NHERF1 regulates PTH1R desensitization in several cell models both endogenously or exogenously expressing NHERF1. Thus, the presence of NHERF1 suppresses PTH1R desensitization, and inhibiting NHERF1 restores receptor desensitization. The effect of NHERF1 on PTH1R desensitization requires PDZ and MERM domains. This action is due not to altered receptor phosphorylation but to preventing or displacing β-arrestin2 binding to the PTH1R. This action may avert PTH resistance and down-regulation of the PTH1R. Understanding these mechanisms will provide insights into developing potential drug targets for the treatment of hyperparathyroidism and osteoporosis.

References

Abou-Samra AB, Jüppner H, Force T, Freeman MW, Kong XF, Schipani E, Urena P, Richards J, Bonventre JV, and Potts JT Jr (1992) Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: a single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium. *Proc Natl Acad Sci U S A* **89:**2732–2736.

Blind E, Bambino T, Huang Z, Bliziotes M, and Nissenson RA (1996) Phosphorylation of the cytoplasmic tail of the PTH/PTHrP receptor. *J Bone Miner Res* **11:**578–586.

Bretscher A, Edwards K, and Fehon RG (2002) ERM proteins and merlin: integrators at the cell cortex. *Nat Rev Mol Cell Biol* **3:**586–599.

Castro M, Dicker F, Vilardaga JP, Krasel C, Bernhardt M, and Lohse MJ (2002) Dual regulation of the parathyroid hormone (PTH)/PTH-related peptide receptor signaling by PKC and β-arrestins. *Endocrinology* **143:**3854–3865.

Chauvin S, Bencsik M, Bambino T, and Nissenson RA (2002) PTH receptor recycling: role of receptor dephosphorylation and β-arrestin. *Mol Endocrinol* **16:**2720–2732.

Cunningham R, Steplock D, Wang F, Huang H, E X, Shenolikar S, and Weinman EJ (2004) Defective PTH regulation of NHE3 activity and phosphate adaptation in cultured NHERF-1$^{-/-}$ renal proximal tubule cells. *J Biol Chem* **279:**37815–37821.

Cunningham R, E X, Steplock D, Shenolikar S, and Weinman EJ (2005) Defective PTH Regulation of sodium-dependent phosphate transport in NHERF-1$^{-/-}$ renal proximal tubule cells and wild-type cells adapted to low phosphate media. *Am J Physiol Renal Physiol* **289:**F933–F938.

Dicker F, Quitterer U, Winstel R, Honold K, and Lohse MJ (1999) Phosphorylation-independent inhibition of parathyroid hormone receptor signaling by G protein-coupled receptor kinases. *Proc Natl Acad Sci U S A* **96:**5476–5481.

Ek ET, Dass CR, and Choong PF (2006) Commonly used mouse models of osteosarcoma. *Crit Rev Oncol Hematol* **60:**1–8.

Ferrari SL, Pierroz DD, Glatt V, Goddard DS, Bianchi EN, Lin FT, Manen D, and Bouxsein ML (2005) Bone response to intermittent parathyroid hormone is altered in mice null for β-arrestin2. *Endocrinology* **146:**1854–1862.

Ferrari SL, Behar V, Chorev M, Rosenblatt M, and Bisello A (1999) Endocytosis of ligand-human parathyroid hormone receptor 1 complexes is PKC-dependent and involves β-arrestin2. Real-time monitoring by fluorescence microscopy. *J Biol Chem* **274:**29968–29975.

Flannery PJ and Spurney RF (2001) Domains of the parathyroid hormone (PTH) receptor required for regulation by G protein-coupled receptor kinases (GRKs). *Biochem Pharmacol* **62:**1047–1058.

Friedman PA, Coutermarsh BA, Kennedy SM, and Gesek FA (1996) Parathyroid hormone stimulation of calcium transport is mediated by dual signaling mechanisms involving PKA and PKC. *Endocrinology* **137:**13–20.

Gensure RC, Gardella TJ, and Jüppner H (2005) Parathyroid hormone and parathyroid hormone-related peptide, and their receptors. *Biochem Biophys Res Commun* **328:**666–678.

Gudi S, Nolan JP, and Frangos JA (1998) Modulation of GTPase activity of G proteins by fluid shear stress and phospholipid composition. *Proc Natl Acad Sci U S A* **95:**2515–2519.

Jones BW and Hinkle PM (2005) Beta-arrestin mediates desensitization and internalization but does not affect dephosphorylation of the thyrotropin-releasing hormone receptor. *J Biol Chem* **280:**38346–38354.

Karim Z, Gérard B, Bakouh N, Alili R, Leroy C, Beck L, Silve C, Planelles G, Urena-Torres P, Grandchamp B, et al. (2008) NHERF1 mutations and responsiveness of renal parathyroid hormone. *N Engl J Med* **359:**1128–1135.

Krupnick JG and Benovic JL (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu Rev Pharmacol Toxicol* **38:**289–319.

Krupnick JG, Santini F, Gagnon AW, Keen JH, and Benovic JL (1997) Modulation of the arrestin-clathrin interaction in cells. Characterization of β-arrestin dominant-negative mutants. *J Biol Chem* **272:**32507–32512.

Li JG, Chen C, and Liu-Chen LY (2002) Ezrin-radixin-moesin-binding phosphoprotein-50/Na^+/H^+ exchanger regulatory factor (EBP50/NHERF) blocks U50,488H-induced down-regulation of the human kappa opioid receptor by enhancing its recycling rate. *J Biol Chem* **277:**27545–27552.

Mahon MJ, Cole JA, Lederer ED, and Segre GV (2003) Na^+/H^+ exchanger-regulatory factor 1 mediates Inhibition of phosphate transport by parathyroid hormone and second messengers by acting at multiple sites in opossum kidney cells. *Mol Endocrinol* **17:**2355–2364.

Mahon MJ, Donowitz M, Yun CC, and Segre GV (2002) Na^+/H^+ exchanger regulatory factor 2 directs parathyroid hormone 1 receptor signalling. *Nature* **417:**858–861.

Mahon MJ and Segre GV (2004) Stimulation by parathyroid hormone of a NHERF-1-assembled complex consisting of the parathyroid hormone I receptor, PLCβ, and actin increases intracellular calcium in opossum kidney cells. *J Biol Chem* **279:**23550–23558.

Malecz N, Bambino T, Bencsik M, and Nissenson RA (1998) Identification of phosphorylation sites in the G protein-coupled receptor for parathyroid hormone. Receptor phosphorylation is not required for agonist-induced internalization. *Mol Endocrinol* **12:**1846–1856.

Massry SG and Smogorzewski M (1998) PTH-PTHrP receptor in chronic renal failure. *Nephrol Dial Transplant* **13 (Suppl 1):**50–57.

Paing MM, Stutts AB, Kohout TA, Lefkowitz RJ, and Trejo J (2002) β-Arrestins regulate protease-activated receptor-1 desensitization but not internalization or down-regulation. *J Biol Chem* **277:**1292–1300.

Ross EM and Gilman AG (1977) Reconstitution of catecholamine-sensitive adenylate cyclase activity: interactions of solubilized components with receptor-replete membranes. *Proc Natl Acad Sci U S A* **74:**3715–3719.

Shenolikar S, Voltz JW, Cunningham R, and Weinman EJ (2004) Regulation of ion transport by the NHERF family of PDZ proteins. *Physiology (Bethesda)* **19:**362–369.

Shenolikar S, Voltz JW, Minkoff CM, Wade JB, and Weinman EJ (2002) Targeted disruption of the mouse NHERF-1 gene promotes internalization of proximal tubule sodium-phosphate cotransporter type IIa and renal phosphate wasting. *Proc Natl Acad Sci U S A* **99:**11470–11475.

Sneddon WB and Friedman PA (2007) β-arrestin-dependent parathyroid hormone-stimulated ERK activation and PTH1R internalization. *Endocrinology* **148:**4073–4079.

Sneddon WB, Syme CA, Bisello A, Magyar CE, Rochdi MD, Parent JL, Weinman EJ, Abou-Samra AB, and Friedman PA (2003) Activation-independent parathyroid

hormone receptor internalization is regulated by NHERF1 (EBP50). *J Biol Chem* **278:**43787–43796.
Sun C and Mierke DF (2005) Characterization of interactions of Na/H exchanger regulatory factor-1 with the parathyroid hormone receptor and phospholipase C. *J Pept Res* **65:**411–417.
Tan CM, Brady AE, Nickols HH, Wang Q, and Limbird LE (2004) Membrane trafficking of G protein-coupled receptors. *Annu Rev Pharmacol Toxicol* **44:**559–609.
Tawfeek HA and Abou-Samra AB (1999) Over-expression of beta arrestin-2 in LLCPK-1 cells stably expressing a green fluorescent protein-tagged PTH/PTHrP receptor increases PTH/PTHrP receptor internalization. *J Bone Miner Res* **14:** S542.
Tawfeek HA, Qian F, and Abou-Samra AB (2002) Phosphorylation of the receptor for PTH and PTHrP is required for internalization and regulates receptor signaling. *Mol Endocrinol* **16:**1–13.
Tian J, Smogorzewski M, Kedes L, and Massry SG (1994) PTH-PTHrP receptor mRNA is downregulated in chronic renal failure. *Am J Nephrol* **14:**41–46.
Ureña P, Kubrusly M, Mannstadt M, Hruby M, Trinh MM, Silve C, Lacour B, Abou-Samra AB, Segre GV, and Drüeke T (1994) The renal PTH/PTHrP receptor is down-regulated in rats with chronic renal failure. *Kidney Int* **45:**605–611.
Vilardaga JP, Krasel C, Chauvin S, Bambino T, Lohse MJ, and Nissenson RA (2002) Internalization determinants of the parathyroid hormone receptor differentially regulate β-arrestin/receptor association. *J Biol Chem* **277:**8121–8129.
Voltz JW, Weinman EJ, and Shenolikar S (2001) Expanding the role of NHERF, a PDZ-domain containing protein adapter, to growth regulation. *Oncogene* **20:**6309–6314.
Wang B, Bisello A, Yang Y, Romero GG, and Friedman PA (2007) NHERF1 regulates parathyroid hormone receptor membrane retention without affecting recycling. *J Biol Chem* **282:**36214–36222.
Wang B, Yang Y, and Friedman PA (2008) Na/H Exchange regulator factor 1, a novel Akt-associating protein, regulates extracellular signal-related signaling through a B-Raf-mediated pathway. *Mol Biol Cell* **19:**1637–1645.
Weinman EJ, Hall RA, Friedman PA, Liu-Chen LY, and Shenolikar S (2006) The association of NHERF adaptor proteins with G protein-coupled receptors and receptor tyrosine kinases. *Annu Rev Physiol* **68:**491–505.
Weinman EJ, Wang Y, Wang F, Greer C, Steplock D, and Shenolikar S (2003) A C-terminal PDZ motif in NHE3 binds NHERF-1 and enhances cAMP inhibition of sodium-hydrogen exchange. *Biochemistry* **42:**12662–12668.
Wheeler D, Garrido JL, Bisello A, Kim YK, Friedman PA, and Romero G (2008) Regulation of PTH1R dynamics, traffic and signaling by the Na^+/H^+ exchanger regulatory factor-1 (NHERF1) in rat osteosarcoma ROS 17/28 cells. *Mol Endocrinol* **22:**1163–1170.

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