NHERF1 Regulates Parathyroid Hormone Receptor Membrane Retention without Affecting Recycling*

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Na/H exchange regulatory factor-1 (NHERF1) is a PDZ protein that regulates trafficking of several G protein-coupled receptors. The phenotype of NHERF1-null mice suggests that the parathyroid hormone (PTH) receptor (PTH1R) is the principal GPCR interacting with NHERF1. The effect of NHERF1 on receptor recycling is unknown. Here, we characterized NHERF1 effects on PTH1R membrane tethering and recycling by radioligand binding and recovery after maximal receptor endocytosis. Using Chinese hamster ovary cells expressing the PTH1R, where NHERF1 expression could be induced by tetracycline, NHERF1 inhibited PTH1R endocytosis and delayed PTH1R recycling. NHERF1 also inhibited PTH-induced receptor internalization in MC4 osteoblast cells. Reducing constitutive NHERF1 levels in HEK-293 cells with short hairpin RNA directed against NHERF1 augmented PTH1R endocytosis in response to PTH. Mutagenesis of the PDZ-binding domains or deletion of the MERM domain of NHERF1 demonstrated that both are required for inhibition of endocytosis and recycling. Likewise, an intact COOH-terminal PDZ recognition motif in PTH1R is needed. The effect of NHERF1 on receptor internalization and recycling was not associated with altered receptor expression or binding, activation, or phosphorylation but involved β-arrestin and dynamin. We conclude that NHERF1 inhibits endocytosis without affecting PTH1R recycling in MC4 and PTH1R-expressing HEK-293 cells. Such an effect may protect against PTH resistance or PTH1R down-regulation in certain cells harboring NHERF1.

The magnitude of GPCR*-mediated, ligand-induced responses is tightly linked to the balance between signal generation and signal termination. Rapid termination of GPCR signaling is mediated by receptor desensitization and internalization, whereas prolonged reduction in responsiveness is due to down-regulation and diminished receptor biosynthesis. Just as GPCR desensitization provides a mechanism to protect cells against excessive stimulation, GPCR resensitization guards cells against prolonged inactivity and hormone resistance. Sequences located within or at the carboxyl terminus of GPCRs control endocytic sorting and recycling of certain GPCRs (1, 2).

Na/H exchange regulatory factor 1 (NHERF1), also known as Ezrin-Radixin-Moesin (ERM)-binding phosphoprotein-50 (EBP50), is a cytoplasmic scaffolding protein implicated in protein targeting and in the assembly of protein complexes (3–6). NHERF1 recruits various GPCRs, ion transporters, and other proteins to the plasma membrane of epithelia and other cells (3, 4, 7, 8).

NHERF1 contains two tandem PDZ domains and a MERM domain. Class I PDZ proteins, such as NHERF1, recognize in the target protein the sequence motif (D/E)(S/T)XΦ, where Χ represents any amino acid and Φ is a hydrophobic residue, generally Leu/Ile/Val although it can also be Met (9, 10). As described below, this recognition sequence is present in the parathyroid hormone (PTH) type I receptor (PTH1R). The MERM domain binds to the actin-associated proteins, merlin, ezrin, radixin, and moesin (3), and tethers the complex to cytoskeletal elements in a phosphorylation-dependent manner and to the catalytic and regulatory subunits of protein kinase A (11).

NHERF1 binds to β2-adrenergic receptors, enhancing receptor recycling to plasma membranes (12). NHERF1 similarly binds to κ-opioid receptors, thereby increasing the rate of recycling of internalized receptors (13). Conversely, NHERF1 stabilizes epidermal growth factor receptors at the cell surface and delays their internalization upon epidermal growth factor-mediated receptor endocytosis (14). The PTH1R, which regulates extracellular mineral ion homeostasis, exhibits such recycling behavior, although it is profoundly down-regulated in disease states. The PTH1R also contains a PDZ recognition motif that interacts with NHERF1.

The PTH1R belongs to Class B of the seven-transmembrane family of GPCRs (15). PTH1R is present primarily in the bone and kidney. Interaction with its cognate ligand, PTH-(1–84), or biologically active peptide fragments leads to activation of Gs and Gq with consequent stimulation of adenylate cyclase and phospholipase C (16, 17). A cascade of cell-specific events ensue that regulate virtually all aspects of extracellular calcium and phosphate homeostasis.

The NHERF1 PDZ-I domain interacts with the carboxyl terminus of the PTH1R (18, 19). In this setting, NHERF1 tethers the PTH1R to the actin cytoskeleton through the MERM domain. Expression of NHERF1 restores both PTH-(1–34)-mediated inhibition of the Npt2 sodium-phosphate cotrans-
porter (20) and the increase of intracellular calcium (19) in OK/H cells, which express low levels of NHERF1 and are resistant to the action of PTH. Our previous data indicated that NHERF1 regulates the conditional efficacy of PTH ligands on cell-specific PTH1R sequestration (21–23).

Upon binding PTH, as with most GPCRs, the receptor is phosphorylated by G protein–coupled receptor kinases (24, 25) and by second messenger–dependent protein kinases (e.g. protein kinase A and C) (26, 27), and β-arrestin is recruited (28, 29). These processes contribute directly to PTH1R desensitization by facilitating the uncoupling of the receptor from its cognate G proteins. The PTH1R undergoes agonist-promoted endocytosis through a clathrin- and dynamin-dependent process (21, 28). Following internalization, the PTH1R is either recycled to the plasma membrane, leading to receptor resensitization (30) or degraded through lysosomal and proteosomal pathways, leading to receptor down-regulation (31, 32).

The effects of NHERF1 on PTH1R retention at the cell membrane and recycling are unknown. In the present study, we demonstrate that most of receptors are recycled to the cell membrane after inducing receptor endocytosis with PTH. Using several different cell models, we show that NHERF1 potently inhibits PTH1R endocytosis and delays PTH1R recycling. This effect requires both intact PDZ and MERM domains in NHERF1. Likewise, an intact carboxyl-terminal PDZ recognition motif in the PTH1R is also needed.

**EXPERIMENTAL PROCEDURES**

**Materials**—HA.11 monoclonal antibody was obtained from Covance (Berkeley, CA). NHERF1 rabbit polyclonal antibody was purchased from Affinity Bioreagents (Golden, CO). Horse-radish peroxidase-conjugated goat anti-rabbit secondary antibody was from Pierce. Horseradish peroxidase-conjugated sheep anti-mouse antibody was from Amersham Biosciences. Tetracycline hydrochloride was purchased from American Bioanalytical (Natick, MA). Lipofectamine 2000, zeocin, blotasticidin, and Geneticin were obtained from Invitrogen. Protease inhibitor mixture Set I and cycloheximide were from Calbiochem. Human PTH1 (1–34) was purchased from Bachem (Torrance, CA), All other reagents were from Sigma.

**Cell Culture**—CHO cells (Invitrogen) transfected with pcDNA6-TR and stably expressing the tetracycline (Tet) repressor protein were cultured in Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml blasticidin.

MC4 cells were obtained from Dr. G. Xiao (University of Pittsburgh) and cultured in minimum essential medium (catalog number R718-07; Invitrogen) with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

HEK293 cells were cultured in Dulbecco's modified Eagle's medium/F-12 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% CO₂, 95% air.

**Construction of pcDNA4/TO-NHERF1, pcDNA3.1+–HA-PTH1R, pcDNA3.1+–HA-M593A, and pcDNA3.1+–HA-480stop PTH1R**—His-tagged rabbit NHERF1 in pcDNA3.1+, pcDNA3.1+–HA-M593A, and pcDNA3.1+–HA-480stop PTH1R was cut with KpnI and Xhol, and a 1.1-kb 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. pcET30A-sPDZ2 was cut with KpnI and Xhol and was subcloned into the pcDNA3.1+ vector.

HA-tagged human PTH1R in pcDNA1 (33) (provided by Dr. T. J. Gardella (Massachusetts General Hospital, Boston) was cut by HindIII and XbaI and subcloned into the mammalian expression vector pcDNA3.1+.

Mutation of the terminal amino acid of HA-PTH1R from methionine to alanine (M593A) was performed by PCR using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

HA-PTH1R-480stop was prepared by amplifying the 1–480 sequence by PCR amplified using the forward primer with a HindIII restriction site, GCC TGT AAA ATT AAG TGG ACC CAG CTC, and the reverse primer with an XbaI restriction site, GCC GCG TCT AGA TCA TGC CAG TGT CCA GCC. The purified PCR fragment was cut by HindIII and XbaI and subcloned into the pcDNA3.1+.

The fidelity of the plasmids was confirmed by sequencing (ABI PRISM 377; Applied Biosystems, Foster City, CA) and subsequent sequence alignment (NCBI BLAST) with rabbit NHERF1 and human PTH1R (GenBank accession numbers U19815 and L04308, respectively) to assure the fidelity of the above constructs.

**Stable Expression of pcDNA6-TR, pcDNA4/TO-NHERF1, and HA-PTH1R**—T-Rex CHO cells were transfected with pcDNA4/TO-NHERF1 or pcDNA/TO vector (control) using Lipofectamine 2000 following the manufacturer's instructions and screening with zeocin (0.4%) and immunoblot. Two cell lines were obtained. The first, CHO-N10 cells, express NHERF1 when Tet is added to the cell culture medium. The other, CHO-EV6, is a control cell line, where NHERF1 cannot be induced. CHO-N10, CHO-EV6, and HEK293 cells were stably transfected with pcDNA3.1+–HA-PTH1R using Lipofectamine 2000 or FuGene 6 and screened by Geneticin (1.5%) and immunoblot to generate cell lines (CHO-N10-R3, CHO-EV6-R4, and HEK293-R25, respectively).

**Transient Transfection**—Cells, as indicated, were transiently transfected with 4.0 μg of DNA/well in 6-well plates or 1.0 μg of DNA/well in 24-well plates or with empty vector (pcDNA3.1), plasmids of wild type NHERF1, truncated NHERF1(1–326) (NHERF1ΔMERM) (13, 34), mutant NHERF1, in which PDZ1, PDZ2 or both PDZ1 and PDZ2 domains are scrambled (sPDZ1-NHERF1, sPDZ2-NHERF1, or sPDZ1/2-NHERF1) (35), wild-type receptor (HA-PTH1R), mutant receptor (HA-M593A), truncated receptor (HA-480stop), β-arrestin-3 (31–418), or HA-K44A dynamin by use of Lipofectamine 2000. Cells were used 48 h after transfection.

**NHERF1 Silencing**—Constitutive NHERF1 expression in HEK293-R25 cells was silenced using RNA interference. Short hairpin RNA (shRNA) constructs against human NHERF1 sequence GGAAGGCAAGGTTCTTCAAGAAATGC mediated by pRS shRNA vector were purchased from Origene (TR316855; Rockville, MD). HEK293-R25 cells were trans-
affected with NHERF1 shRNA or scrambled shRNA, which has no homology to any human sequence. Transfections were established following the manufacturer’s protocol. Briefly, Opti-MEM (Invitrogen) containing 0.5 µg of the respective plasmid and 1.5 µl of FuGENE 6 was added to each well of a 24-well plate (about 50% cell confluence). Transfected cells were cultured for 48 h and then used for receptor recycling or immunoblot.

Coimmunoprecipitation and Immunoblot Analysis—Interactions of NHERF1 with the indicated PTH1R constructs were analyzed as described (21). Briefly, 6-well plates of the indicated cells were transiently transfected with wild-type NHERF1, sPDZ1-NHERF1, sPDZ2-NHERF1, sPDZ1/2-NHERF1, HA-PTH1R, M593A-PTH1R, 480s-PTH1R, or the respective empty vector. Tet (50 ng/ml) was added where indicated. 48 h later, the cells were lysed with Nonidet P-40 (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40) supplemented with protease inhibitor mixture I and incubated for 15 min on ice. Solubilized materials were incubated overnight at 4 °C with HA.11 monoclonal affinity matrix. 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 semidry method (Bio-Rad). Membranes were blocked overnight at 4 °C with 5% nonfat dried milk in Tris-buffered saline plus Tween 20 (TBST) and incubated with different antibodies (polyclonal anti-NHERF1 antibody at 1:1000 and HA.11 ascites monoclonal antibody at 1:1000) for 2 h at room temperature. The membranes were then washed and incubated with goat anti-rabbit IgG or sheep anti-mouse IgG conjugated to horse-radish peroxidase at a 1:5000 dilution for 1 h at room temperature. Protein bands were visualized with a luminol-based enhanced chemiluminescence substrate.

Receptor Binding, Internalization, and Recyling—Receptor binding, internalization, and recycling assays were performed as described previously (21, 30) using high pressure liquid chromatography-purified \[^{125}\text{I}]\text{Nle}^{8,18}\text{Tyr}^{34}\text{PTH}-(1–34)\text{NH}_2. PTH1R binding was measured on cells plated on 24-well plates and grown to confluence. PTH-(1–34) or vehicle was added to the culture medium and incubated at 37 °C for 30 min. Residual cell surface binding was removed by rinsing with ice-cold PBS and acid washing twice with 50 mM glycine, 100 mM NaCl (pH 3). The medium was replenished with Ham’s F-12 containing 10% fetal bovine serum. Cells were put on ice for 15 min and incubated on ice for an additional 2.5 h with ~100,000 cpm of \[^{125}\text{I}]\text{Nle}^{8,18}\text{Tyr}^{34}\text{PTH}-(1–34)\text{NH}_2 in 250 µl of fresh media. Nonspecific binding was determined in parallel incubations of nontransfected CHO-N10 cells with \[^{125}\text{I}]\text{Nle}^{8,18}\text{Tyr}^{34}\text{PTH}-(1–34)\text{NH}_2 and was subtracted from total binding to calculate specific binding or measured in parallel experiments carried out in the presence of 1 µM unlabeled PTH-(1–34) (21, 29). After incubation, cells were rinsed twice by cold PBS and then solubilized in 0.2 N NaOH. Cell surface-bound \[^{125}\text{I}]\text{PTH}-(1–34) was assessed by γ spectrometry. Fractional plasma membrane PTH1R binding was calculated as (cpm of unstimulated control – cpm of PTH)/cpm of unstimulated control) × 100%.

Receptor recycling was determined in a similar manner. However, after the 30-min PTH treatment and washout, the cells were returned to 37 °C for the indicated time, after which PTH binding was measured. PTH1R recycling was calculated as (cpm of PTH group/cpm of unstimulated group) × 100%. Receptor number (B_m) and affinity (K_d) were measured by competitive binding as described, and parameters were determined by Scatchard analysis (Prism) (36).
In Vivo Receptor Phosphorylation—In vivo receptor phosphorylation was measured as described previously (22) using \([^{32}P]\)orthophosphate.

Adenylyl Cyclase Activity—Cyclic AMP accumulation was determined in subconfluent cells in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine as described previously (37).

Statistics—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 posttest repeated measures analyzed by the Bonferroni procedure (Prism; GraphPad). Differences greater than \(p \leq 0.05\) were assumed to be significant.

RESULTS

**NHERF1 Effects on PTH1R Internalization and Recycling**—Initial characterization of NHERF1 effects on PTH1R trafficking were conducted on an engineered CHO cell model in which NHERF1 levels could be controlled at a constant and biologically relevant number of PTH1R. These cells express 6.0 \times 10^5 PTH1R/cell, with an average \(K_d\) of 14.2 nM. Tet (50 ng/ml) induces maximal NHERF1 expression (38).

PTH promoted time- and concentration-dependent PTH1R internalization. Receptor endocytosis stimulated by PTH began within 5 min and plateaued by 30 min (Fig. 1A). Therefore, 30 min was used for subsequent determinations of steady-state and maximal effects. Receptor endocytosis proceeded in a concentration-dependent manner with half-maximal PTH1R internalization at 1.2 \times 10^{-10} M PTH.

Internalized PTH1Rs are either recycled to the plasma membrane or degraded (30–32). The influence of NHERF1 on PTH1R stabilization and recycling is unknown. In the absence of NHERF1, a 30-min challenge with PTH decreased \([^{125}\text{I}]\)PTH binding by 60%, corresponding to internalization of 40% of membrane-bound PTH1Rs (Fig. 1B). This degree of PTH1R endocytosis corresponds favorably to that in other cells and using different techniques (21, 30, 39). Following induction of NHERF1 expression, PTH1R internalization decreased with a corresponding doubling of membrane-delimited PTH1R from 40 to 78% (Fig. 1B).

PTH1R recycling was measured as the rate of recovery of PTH1R binding following maximal internalization. Recycling proceeded in a time-dependent manner with \(T_{1/2} = 52\) min in the absence of NHERF1 and 106 min in the presence of NHERF1. Virtually complete recycling was achieved by 2 h in either case. Thus, NHERF1 retards endocytosis and delays PTH1R recycling.

The addition of Tet to control CHO-EV-R4 cells lacking the Tet repressor had no effect on receptor internalization or recycling (data not shown). Thus, Tet effects result from induction of NHERF1 expression and not from toxic or nonspecific actions. Further, the rate and extent of PTH1R recycling was indistinguishable in the presence or absence of 200 \(\mu\)M cycloheximide (data not shown), suggesting that de novo PTH1R synthesis does not contribute importantly to the observed findings.

Similar experiments were performed on MC4 osteoblastic cells (subclone 4 of MCT3-E1 (40)) to determine if NHERF1 exerts comparable effects in cells constitutively expressing the PTH1R. MC4 cells express 1.4 \times 10^5 PTH1R/cell with an average \(K_d\) of 9.4 nM. After a 30-min exposure to 10^{-7} M PTH-(1–34), 48% of PTH1Rs were located at the cell surface (Fig. 2A). Receptor recycling proceeded in a time-dependent manner with complete recycling by 2 h. Endogenous NHERF1 expression in MC4 cells is negligible (Fig. 2B). Transient transfection of NHERF1 inhibited PTH1R-induced internalization without affecting the rate of receptor recycling.

Complementary experiments were performed on HEK-293 cells, which constitutively express NHERF1 and were stably transfected with the PTH1R. Here, after a 30-min pretreatment with 10^{-7} M PTH, only 25% of PTH1Rs were internalized, with 75% remaining on the cell surface (Fig. 2C). shRNA targeted to
NHERF1 substantially reduced PTH1R binding, consistent with a role of NHERF1 to stabilize receptors at the plasma membrane. shRNA reduced endogenous NHERF1 levels by 76% compared with scrambled control (Fig. 2D). The rate constants for recycling (control, 0.03367 min⁻¹; shRNA, 0.02131 min⁻¹) were statistically indistinguishable.

**NHERF1 Domains Involved in PTH1R Internalization and Recycling**—NHERF1 possesses two tandem type 1 PDZ domains. To determine which of these are involved in receptor recycling, we employed NHERF1 constructs harboring mutations of the core-binding sequences in PDZ-1 (sPDZ1-NHERF1), PDZ-2 (sPDZ2-NHERF1), or PDZ-1 plus PDZ-2, (sPDZ1/2-NHERF1). Transient transfection of wild-type NHERF1 in CHO-EV-R4 cells inhibited receptor internalization in response to PTH (Fig. 3A). Interestingly, both sPDZ1-NHERF1 and sPDZ2-NHERF1 decreased PTH1R internalization (Fig. 3A) and interact with the PTH1R (Fig. 3B). However, double PDZ1 plus PDZ2 mutations (sPDZ1/2) did not affect PTH1R membrane localization and, as might be expected, did not associate with the receptor (Fig. 3B). These data show that interaction of the PTH1R with either PDZ domain is sufficient to stabilize membrane localization of the PTH1R.

NHERF1 also possess a carboxyl-terminal MERM domain, linking it with the actin-associated proteins, merlin, ezrin, radixin, and moesin. We examined the effect of a NHERF1 construct lacking the MERM domain (NHERF1ΔMERM) on β-arrestin in PTH1R internalization (28, 29, 41).

We next determined the effects of NHERF1 on recycling of the different receptor constructs. NHERF1 did not affect recycling of the truncated or PDZ mutant receptors (Fig. 4A). These results are compatible with the finding that NHERF1 interacts only with wild-type PTH1R but not with mutant or truncated receptors (Fig. 4B) and confirms the requirement for an intact PDZ recognition motif for the interaction and effects of NHERF1 on PTH1R stabilization.

**NHERF1 Modulates β-Arrestin and Dynamin-dependent Receptor Internalization**—PTH translocates β-arrestin 2 (21, 28), and PTH1R endocytosis requires dynamin (21). However, the functional interaction between NHERF1 on β-arrestin and dynamin-dependent PTH1R internalization is unknown. To investigate the potential involvement of β-arrestins in PTH1R endocytosis, CHO-N10-R3 cells were transiently transfected with β-arrestin 1 or 2. CHO cells exhibit high constitutive levels of β-arrestin expression (42). As expected, overexpression of β-arrestin 1 or β-arrestin 2 only minimally increased receptor internalization (Fig. 5A) compared with control. However, NHERF1 inhibited receptor internalization in control cells as well as in cells overexpressing β-arrestin 1 or 2. Comparable effects have been noted in CHO cells stably transfected with the κ-opioid receptor and transiently transfected with β-arrestin 43. We reasoned that because CHO cells constitutively express high levels of β-arrestins, dominant negative β-arrestin
1-(319–418) (44, 45), which blocks arrestin-clathrin interactions, should inhibit PTH1R endocytosis. Introduction of H9252-arrestin 1-(319–418) reduced PTH-stimulated receptor endocytosis by 35% (Fig. 5B). Induction of NHERF1 further decreased PTH1R internalization by an additional 50% but no greater than that observed with NHERF1 alone. We also examined the effect of β-arrestin overexpression and inhibition on NHERF1-sensitive PTH1R recycling. Overexpression of β-arrestin 1 or 2 did not affect the magnitude or extent of PTH1R recycling, and induction of NHERF1 had no greater effect in the presence of β-arrestin 1 or 2 than alone.

Dynamin is a GTPase that regulates the formation of clathrin-coated vesicles (46). To determine NHERF1 effects on dynamin-dependent PTH1R receptor internalization, CHO-N10-R3 cells were transiently transfected with dominant negative dynamin (K44A-dynamin). K44A-dynamin inhibited PTH1R endocytosis (Fig. 5B). The inhibitory effect of K44A-dynamin was somewhat greater than that of β-arrestin-(319–418). The presence of NHERF1 augmented the inhibitory action of K44A-dynamin on PTH1R endocytosis by an additional 50%. These data suggest that NHERF1 modulates β-arrestin and dynamin-sensitive PTH1R internalization.

NHERF1 Does Not Affect PTH1R Cell Surface Expression, Ligand Binding, Activation, or Phosphorylation—Because NHERF1 regulates PTH1R signaling (18, 20), we wished to determine if the inhibitory actions of NHERF1 on PTH1R endocytosis were secondary to an effect on receptor abundance, ligand binding, receptor activation, or phosphorylation.

Cell surface PTH1R expression was measured by Scatchard analysis in CHO-N10-R3 cell in the absence or presence of Tet-induced NHERF1. $B_{max}$ was $5.68 \times 10^5$ receptors/cell in the absence of NHERF1 and $6.35 \times 10^5$ in the presence of NHERF1. $K_d$ values were $10–11$ nM in both cases. As shown in Fig. 6A, $[^{125}I]$PTH-(1–34) binding in the presence and absence of increasing concentrations of cold PTH ($10^{-11}$ to $10^{-6}$ M) were not altered in the presence of NHERF1. Thus, NHERF1 does not inhibit PTH1R endocytosis by blocking ligand binding to the PTH1R.

Mahon et al. (18) reported that NHERF2, a NHERF1 homolog, markedly inhibited adenylyl cyclase by stimulating inhibitory Gi proteins in PS120 cells transfected with the PTH1R. We examined the effect of NHERF1 on PTH-stimulated adenylyl cyclase activity. Neither NHERF1 nor pertussis toxin affected PTH-stimulated cAMP formation in CHO-N10-R3 cells (Fig. 6B) or protein kinase A activity (data not shown).

Finally, we determined the effect of NHERF1 on PTH-stimulated receptor phosphorylation. CHO EV-R4 cells were transiently transfected with HA-tagged NHERF1 and labeled with $[^{32}P]$orthophosphate. PTH-induced receptor phosphorylation was not enhanced or reduced in the presence of NHERF1 (Fig. 6C). Taken together, these findings show that the effect of NHERF1 on PTH1R recycling is not due to an alteration of PTH1R expression, an action on receptor binding, activation, or phosphorylation.

DISCUSSION

The present studies were initiated to understand better the regulatory effect of the adapter protein NHERF1 on trafficking of the PTH1R, a Class B GPCR. NHERF1 is a multifunctional protein involved in the regulation of signaling and trafficking of
**PTH Receptor Recycling**

A

![Graph A](image)

B

![Graph B](image)

**FIGURE 5. β-Arrestin- and dynamin-sensitive NHERF1 inhibition of PTH1R internalization and recycling.** CHO-N10-R3 cells in 24-well plates were transiently transfected with: A, empty vector, β-arrestin 1, or β-arrestin 2; B, β-arrestin-(319 – 418) or K44A-dynamin. NHERF1 was induced (Tet, 50 ng/ml) where indicated. After 48 h, the cells were incubated in the presence or absence of PTH-(1–34) for 30 min. Receptor internalization was assayed as described in the legend to Fig. 1. Data are summarized as ± S.E. of three experiments. **, p < 0.01 versus PTH-(1–34).

GPCRs possessing a requisite PDZ binding domain. In the case of the PTH1R, for instance, NHERF1 dictates G protein signaling (18), ligand sensitivity or recognition (22), and internalization (21). Based on the observations that NHERF1 modulates recycling of other GPCRs known to interact with NHERF1, we hypothesized that PTH1R membrane retention and recycling are regulated by NHERF1. The results establish that NHERF1, acting both through PDZ domains and the MERM domain, stabilizes receptor docking at the plasma membrane. However, recycling of internalized receptors was not appreciably altered. These findings are qualitatively comparable with those of the epidermal growth factor receptor, where NHERF1 stabilized membrane-delimited receptors without affecting the rate of recycling (14) but contrast with results for β-adrenergic and κ-opioid receptors, where NHERF1 promoted membrane retention and increased the rate of recycling (12, 13).

In the case of the β2-adrenergic and κ-opioid receptors, NHERF1 promotes rapid recycling to the plasma membrane (12, 13). δ-Opioid receptors lack a PDZ-binding domain. However, the addition of the DSLL PDZ recognition motif from the β2-adrenergic receptor to the δ-opioid receptor conferred rapid receptor recycling. These observations provide strong evidence that NHERF1 accelerates recycling of select Family A GPCRs. In the case of the PTH1R, a Family B receptor also possessing a PDZ recognition domain, recycling is inherently slow and was not substantially affected by NHERF1. Thus, in this setting, NHERF1 stabilizes the PTH1R at the cell surface without altering its rate of recycling, which was effectively complete within 2 h in the presence or absence of NHERF1.

A unifying view that may reconcile these divergent observations is that in the case of the PTH1R (21) and the epidermal growth factor receptor, a structurally unrelated tyrosine kinase receptor, NHERF1 interacts constitutively with the receptor, whereas in the case of β2-adrenergic (12) and κ-opioid (13) receptors, interaction with NHERF1 occurs only upon ligand occupancy of the receptor. Moreover, both β2-adrenergic and μ-opioid receptors exhibit rapid dissociation from arrestin and fast recycling, typical of Class A receptor recycling. These features may provide a means for discerning the actions of NHERF1 on GPCR membrane retention and recycling. Other aspects of NHERF1 effects may arise from the presence or absence of additional cis-interacting proteins that account for the cell-specific actions of NHERF1 on GPCR trafficking.

A novel finding described here is the bifunctional requirement for both PDZ and MERM domains in the regulation of receptor recycling by NHERF1. Wild-type NHERF1 or mutants harboring a single intact PDZ domain substantially augmented PTH1R membrane retention. When both PDZ domains were mutated, the tethering effect of NHERF1 was lost. Consistent with these functional observations, PTH1R coimmunoprecipitated with intact or single PDZ NHERF1 mutants but not when both PDZ core-binding domains were mutated. Thus, PDZ-1 and PDZ-2 NHERF1 domains are operationally redundant in effecting PTH1R tethering. The NHERF1 MERM domain is also required for the stabilizing action on PTH1R. When deleted, NHERF1 lost this effect. Thus, both PDZ and MERM domains are required for the biological action of NHERF1 on PTH1R membrane tethering. This conclusion is fortified by results from the complementary strategy, where the carboxyl-terminal PDZ-binding domain of the PTH1R was altered. NHERF1 interacted only with wild-type receptor (PTH1R-ETVA) to regulate PTH1R membrane retention. NHERF1 had no effect on the mutant (PTH1R-ETVA) or truncated (PTH1R-480stop) receptors. Together, these findings strongly support the view that the effects of NHERF1 on receptor endocytosis require intact PDZ and MERM domains and require an integral carboxyl-terminal PDZ recognition motif on the receptor. A variant of this scheme, where GPCR endocytosis and recycling were NHERF1-independent but MERM-dependent, has been described. Stanasila et al. (47) found that ezrin directly binds α1b adrenergic receptors, which lack a PDZ recognition domain, thereby interacting directly with cortical actin without
the involvement of NHERF1 or other intermediary scaffolding proteins.

Upon ligand binding to the PTH1R, signaling is activated, and the receptor is phosphorylated by G protein-coupled receptor kinases (48). We found that NHERF1 did not affect PTH-(1–34)-stimulated receptor phosphorylation. The PDZ-binding motif (DSLL) of the β2 adrenergic receptor contains a serine at the −2 position that is phosphorylated in response to receptor activation. Mutation of Ser111 with a phosphomimetic (S411D) or alanine (S411A) blocked interactions with NHERF1, resulting in targeting of receptors to lysosomes (12). Thus, in the case of β2-adrenergic receptors, G protein-coupled receptor kinase-mediated phosphorylation is important for receptor recycling. The PTH1R lacks an acid residue in the PDZ recognition domain. This is consistent with the absence of an effect of NHERF1 on PTH1R phosphorylation and may represent another level at which receptor-specific regulation of trafficking may be regulated by NHERF1.

The carboxyl terminus of GPCRs is a major regulatory domain controlling receptor interaction with β-arrestins (49). Truncation of this segment of the PTH1R, for instance, markedly reduced the magnitude and rate of its endocytosis. NHERF1 further enhanced the inhibitory effects of dominant negative mutants of arrestin and dynamin. This strongly suggests that the PTH1R is internalized by arrestin- and dynamin-dependent and -independent processes and that NHERF1 expression affects both mechanisms to similar extents. This interpretation is consistent with previously reported observations that NHERF1 significantly reduces the lateral diffusion of the receptor (38). Receptor internalization requires the accumulation of receptors at the site of formation of the endocytic vesicle. By reducing the rate of diffusion of the receptor, NHERF1 therefore slows the accumulation of the PTH1R at these sites. Interestingly, however, transient overexpression of β-arrestin 1 or β-arrestin 2 only minimally enhanced receptor internalization, which was still inhibited by NHERF1. These observations suggest that upon occupancy of the PTH1R by PTH-(1–34), β-arrestins are engaged and direct the receptor to endosomes that allow for receptor resensitization and recycling. These findings confirm a role for β-arrestins in PTH1R internalization independent of NHERF1.

In summary, NHERF1 promotes membrane retention of the PTH1R in several cell models both endogenously and exogenously expressing NHERF1. The effect of NHERF1 on receptor endocytosis requires both intact NHERF1 PDZ and MERM domains. Likewise, an intact carboxyl-terminal PTH1R PDZ recognition motif is needed. This action is not due to altered ligand binding, receptor activation, or phosphorylation. NHERF1 had a negligible effect on PTH1R recycling. Thus, NHERF1 stabilizes the PTH1R at the cell membrane and increases the fraction of receptor at the cell surface. This effect may prevent PTH resistance and PTH1R down-regulation of PTH1R in cells expressing NHERF1.

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**PTH Receptor Recycling**

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NHERF1 Regulates Parathyroid Hormone Receptor Membrane Retention without Affecting Recycling

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