The Na/H exchanger regulatory factors, NHERF1 and NHERF2, are adapter proteins involved in targeting and assembly of protein complexes. The parathyroid hormone receptor (PTHR) interacts with both NHERF1 and NHERF2. The NHERF proteins toggle PTHR signaling from predominantly adenylyl cyclase to PLC-activated cAMP formation when the NHERF proteins are expressed. We hypothesized that this signaling switch occurs at the level of the G protein. We measured G protein activation by \(^{[35S]}\text{GTP}\) binding and \(\alpha\) subtype-specific immunoprecipitation using three different cellular models of PTHR signaling. These studies revealed that PTHR interactions with NHERF1 enhance receptor-mediated stimulation of \(G_q\) but have no effect on stimulation of \(G_i\) or \(G_\alpha\). In contrast, PTHR associations with NHERF2 enhance receptor-mediated stimulation of both \(G_i\) and \(G_\alpha\), but decrease stimulation of \(G_q\). Consistent with these functional data, NHERF2 formed cellular complexes with both \(G_i\) and \(G_\alpha\), whereas NHERF1 was found to interact only with \(G_i\). These findings demonstrate that NHERF interactions regulate PTHR signaling at the level of G proteins and that NHERF1 and NHERF2 exhibit subtype-specific effects on G protein activation.

The parathyroid hormone receptor (PTHR) is a family B G protein-coupled receptor (GPCR) that regulates extracellular mineral ion homeostasis and bone growth and turnover. Interaction with its cognate ligands, PTH or the PTH-related peptide (PTHrP), stimulates adenylyl cyclase and phosphatidylinositol-specific phospholipase C (PLC) (1, 2). In some cases, occupancy of the PTHR activates only one signaling pathway. For example, in vascular smooth muscle cells, PTH stimulates adenylyl cyclase but not PLC (3, 4), whereas in keratinocytes (5, 6), cardiac myocytes (7, 8), and lymphocytes (9–11), the PTHR activates PLC but not adenylyl cyclase. In osteoblasts and kidney tubule cells, PTH activates both adenylyl cyclase and PLC (12–14). Occupancy of the PTHR activates multiple \(G_\alpha\) proteins, and the physiologic responses to PTH may result from contributions of both \(\alpha\) and \(\beta\gamma\) subunits. However, the particular \(G_\alpha\) protein subunit to which the receptor couples varies in a cell-specific manner. Moreover, PTHR stimulation of PLC may arise through activation of \(G_q\) (4) or \(G_i\) (15, 16).

The Na/H exchanger regulatory factor (NHERF) family consists of four related proteins as follows: NHERF1 and NHERF2 that contain two tandem PSD-95/Discs large/ZO-1 (PDZ) domains and an ezrin-binding domain, and NHERF3 and NHERF4 that possess four PDZ domains but no ezrin-binding domain (17). NHERF1 (also known as ezrin-binding phosphoprotein 50, EBP50) shares 52% amino acid identity with NHERF2, also called NHE3 kinase A regulatory protein (E3KARP) (18). NHERF1 and NHERF2 are implicated in protein targeting and in the assembly of protein complexes. They recruit various GPCRs, ion transporters, and other proteins to the plasma membrane of epithelia and other cells (19–22).

Despite the similarity between their PDZ domains, NHERF proteins exhibit different affinities for PDZ-binding partners. Some NHERF targets, like Taz (23), the PMCA2b \(Ca^{2+}\)-ATPase (24), and the LPA\(3\) receptor (25) preferentially bind NHERF2. Furthermore, NHERF2 may display distinct binding specificity and physiologic function that is not shared by NHERF1. NHERF2 but not NHERF1, for instance, specifically interacts with PLC-\(\beta\)3 and plays a key role in PLC-\(\beta\)3 activation by the PDZ domain-mediated interaction (26). \(Ca^{2+}\)-dependent inhibition of NHE3 requires an NHE3-NHERF2-\(\alpha\)-actinin-4 complex for oligomerization and endocytosis (27). NHERF2 specifically interacts with the LPA\(3\) receptor and defines the specificity and efficiency of receptor-mediated PLC-\(\beta\)3 activation (28).

Mahon et al. (21) reported that NHERF2 inhibited adenylyl cyclase by stimulating inhibitory \(G_i\) and increased PLC in PS120 cells transfected with the PTHR. In contrast, NHERF1 increased PTH-stimulated cAMP accumulation in ROS 17/2.8 cells (29). Adding to the variability of effects, both NHERF1 and NHERF2 increased PTH-stimulated PLC activity or intracellular calcium in PS120 cells, opossum kidney cells, and ROS 17/2.8 cells (21, 29–31), although no differences in PTH-stimulated cAMP formation were found in wild-type and NHERF1-
null proximal tubule cells (32, 33) or in CHO-N10-R3 cells in the presence or absence of NHERF1 (34).

The molecular mechanism by which NHERF association with PTHR promotes switching of receptor signaling between adenyl cyclase and phospholipase C is not known. It has been speculated that the NHERF proteins may promote $G_{q}$-mediated signaling by tethering $G_{q}$ effectors such as PLC (26, 35), PKC (36), and PKD (37) in the vicinity of receptors. However, it is also possible that NHERF-GPCR interactions might directly modulate the $G_{q}$ protein-coupling preferences of the receptors. The most direct and unambiguous way to determine the influence of NHERF1/2 on PTHR signaling is to measure effects on $G_{q}$ protein activation. We show here that NHERF1 increases PTH-stimulated PTHR coupling to $G_{q}$ but not to $G_{i}$. In contrast, NHERF2 decreases PTH-induced $G_{q}$ and increases $G_{q}$ and $G_{q}$ activation. These data reveal that NHERF-PTH$R$ interactions can directly influence receptor coupling to $G_{q}$ proteins.

**EXPERIMENTAL PROCEDURES**

HA.11 and His$_{6}$ monoclonal antibodies were obtained from Covance (Berkeley, CA). NHERF1 rabbit polyclonal antibody was purchased from Affinity Bioreagents (Golden, CO). NHERF2 rabbit polyclonal antibody was kindly provided by Dr. R. A. Frizzell (University of Pittsburgh). Polyclonal $G_{q}$ antibody was obtained from Millipore (Billerica, MA). $G_{q}$ monoclonal antibody was provided by BD Transduction Laboratories. $G_{i}$ polyclonal antibody was from NewEast Biosciences (Malvern, PA). Ni-NTA-agarose was provided by Qiagen (Valencia, CA). Horseradish peroxidase-conjugated goat antirabbit secondary antibody was from GE Healthcare. Lipofectamine 2000 and geneticin, protein A-Sepharose 4B conjugate, and rec-protein G-Sepharose 4B were obtained from PerkinElmer Life Sciences. FuGENE 6 was purchased from Roche Applied Science. All other reagents were from Sigma.

**Cell Culture**—PS120 cells were stably transfected with PTHR (PS120-R) or stably transfected with both PTHR and NHERF2 (PS120-R-N2) and were obtained from Dr. M. J. Mahon (Harvard Medical School). PS120-R cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 100 units/ml penicillin, 100 $\mu$g/ml streptomycin, 1 $\mu$g/ml puromycin. PS120-R-N2 cells were cultured in the above medium with additional 0.1 mg/ml hygromycin B. CHO cells were stably transfected with PTHR (CHO-R) (34) and cultured in Ham’s F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 $\mu$g/ml streptomycin, and 0.75 mg/ml geneticin. HEK-293 cells were stably transfected with PTHR (HEK-293R) (34) and cultured in Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 $\mu$g/ml streptomycin, and 0.75 mg/ml geneticin. All cells were maintained at 37°C in a humidified atmosphere of 5% $CO_{2}$, 95% air.

**Membrane Preparation**—Plasma membranes were isolated by differential centrifugation at 4°C as described previously (1, 39, 40). Briefly, after cells achieved confluence, they were rinsed with cold PBS and then incubated with hypotonic buffer (10 mM Hepes, 0.5 mM EDTA, pH 7.4) for 15 min. Swollen cells were harvested, collected by centrifugation (1000 $\times$ g for 10 min), and resuspended in ~9 volumes of 10 mM Tris, 1 mM EDTA, pH 7.4, with proteinase inhibitor mixture set I. Cells were disrupted with 20–40 strokes in a “loose” Dounce homogenizer on ice. The lysates were centrifuged at 10,000 $\times$ g for 10 min to remove unbroken cells, large cell debris, and some nuclei. The supernatant was further centrifuged at 30,000 $\times$ g for 20 min. The membrane pellet was resuspended in freezing buffer (10 mM Hepes, 0.1 mM EDTA, pH 7.4) at a protein concentration of 5–10 $\mu$g/µl and rapidly frozen in liquid nitrogen. Membranes were then stored at −80°C until used.

**GTP$\gamma$S Binding and Immunoprecipitation of GTP$\gamma$S-bound $G_{q}$ Subunits**—The comparative changes in $G_{q}$-[35$S$]GTP$\gamma$S binding immunoprecipitated by specific $G_{q}$ subtype-specific antisera were used to delineate PTHR coupling to distinct $G_{q}$ protein subunits. [35$S$]GTP$\gamma$S binding to $G_{q}$ proteins was performed with a modification of previously described methodologies (1, 40). Frozen membrane aliquots (150 µg) were incubated with 100 µl of assay buffer (10 mM Hepes, 100 mM NaCl, 5 mM MgCl$_2$, pH 7.4) containing 5 µM GDP, 5 µM [35S]GTP$\gamma$S, and 100 nM PTHR(1–34) at 30°C for 5 min (unless otherwise stated). Incubations were terminated by the addition of 800 µl of ice-cold assay buffer and immediate transfer to an ice bath. Cell membranes were recovered from the reaction mixture by centrifugation at 20,000 $\times$ g for 10 min, and the supernatant was removed. Membrane pellets were solubilized, and immunoprecipitation of [35$S$]GTP$\gamma$S bound to $G_{q}$ subunits was measured as described below under “Coimmunoprecipitation and Immunoblot Analysis.” After Sepharose beads were washed three times, the beads were resuspended with 100 µl of 0.5% SDS and incubated at 85–90°C for 2–3 min. The entire contents of each tube were transferred to a vial containing 5 ml of scintillation mixture, and radioactivity was measured by $\beta$-emission spectrometry. Nonspecific binding was determined in the presence of 100 µM GTP$\gamma$S.

**Coimmunoprecipitation and Immunoblot Analysis**—Interaction of G proteins with NHERF1 or NHERF2 was analyzed as described previously (41). In brief, 6-well plates of CHO-R3 cells were transiently transfected with His-NHERF1, His-NHERF2, or empty vector. Forty eight hours later, the cells were lysed with 1% Lubrol, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.4, and 150 mM NaCl. Solubilized materials were incubated overnight at 4°C with Ni-NTA-agarose or
of plasmids was confirmed by sequencing (ABI PRISM 377, Applied Biosystems, Foster City, CA). Rescue shRNA scrambled constructs containing the 29-mer scrambled cassette were purchased from OriGene.

**FRET**—

Gαq activation was measured in real time in live cells by fluorescence resonance energy transfer (FRET) as described previously (43, 44). Briefly, HEK-293 cells plated on MatTek (Ashland, MA) dishes were maintained in Hepes buffer with 0.1% (w/v) bovine serum albumin at 22 °C. Cells were observed using a 40 × 1.30 NA oil immersion objective on a Nikon A1s confocal microscope attached to a Ti-E inverted base. Subunit rearrangement of Gαq and Gβγ was measured by FRET between YFP-tagged Gαq and cerulean-tagged Gβ1γ2 bimolecular fluorescence complementation. FRET signal was measured as the normalized FRET ratio (nFRET) of the YFP and cyan fluorescent protein emission (F<sub>YFP</sub>/F<sub>CYP</sub>) (45).

**Receptor Binding**—Receptor binding was performed as described previously (34) using HPLC-purified [125I]-[Ne8,18,Tyr34]PTH(1–34)-NH<sub>2</sub>. In brief, PS120-R cells, PS120-R-N2, or CHO-R3 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<sup>−11</sup>–10<sup>−6</sup> M) and ~100,000 cpm of [125I]-[Ne8,18,Tyr34]PTH(1–34)-NH<sub>2</sub> in 250 µl of fresh media on ice for an additional 2.5 h. After incubation, cells were rinsed twice with ice-cold PBS and then solubilized in 0.2 N NaOH. Non-specific binding was measured in parallel experiments carried out in the presence of 1 µM unlabeled PTH(1–34). Cell surface-bound [125I]-PTH(1–34) was assessed by γ-spectrometry. PTHR number was analyzed by Scatchard analysis.

**Adenylyl Cyclase**—Adenylyl cyclase activity was determined by assay of cAMP accumulation as described previously (34). Briefly, HEK-293R cells transfected with scrambled shRNA, NHERF1 shRNA, or NHERF2 shRNA in 24-well plates were labeled with 0.5 µCi of [3H]adenine for 2 h. The cells were then treated with vehicle or 100 nM PTH(1–34) in the presence of phosphodiesterase inhibitor 3-isobutyl-1-methyloxanthine (1 mM) for 15 min. The reaction was terminated by addition of 1 µM TCA. cAMP was isolated by the two-column method.

**Intracellular Calcium**—

Intracellular calcium concentrations ([Ca<sup>2+</sup>]<sub>i</sub>) were measured with the calcium-sensitive dye Fluo-4/AM (Invitrogen) following the manufacturer’s protocol. Briefly, HEK-293R cells were cultured on MatTek dishes with 2 µM Fluo-4/AM in Hanks’ balanced salt solution (Invitrogen) at 22 °C for 45 min. Cells were washed three times with Hanks’ balanced salt solution and incubated with Hanks’ balanced salt solution at 22 °C for another 30 min. The calcium measurements were performed with a Nikon A1s inverted fluorescent microscope. Fluorescence was recorded at 1-s intervals for up to 20 min. At least 30–40 cells were counted under each condition. Intracellular calcium concentrations were calculated using the following equation: [Ca<sup>2+</sup>]<sub>i</sub> = K<sub>d</sub> × (F − F<sub>min</sub>)/(F<sub>max</sub> − F), where F is the measured fluorescence intensity; F<sub>max</sub> is the fluorescence measured after addition of 10 µM ionomycin; F<sub>min</sub> is the fluorescence measured after addition of 10 mM EGTA, and K<sub>d</sub> is the dissociation constant of the dye-Ca<sup>2+</sup> complex (520 nM) (46).

**Statistics**—Data are presented as the mean ± S.E., where n indicates the number of independent experiments. Multiple
NHERF Regulates PTH Receptor Coupling to G\(\alpha\) Proteins

FIGURE 1. Specificity of PTH-induced \([35S]\)GTP\(\gamma\)S binding to G protein subunit. A, cell membrane protein (20 \(\mu\)g) prepared from PS120-R or PS120-R-N2 cells was resolved on 10% SDS-polyacrylamide gels as described under “Experimental Procedures” for immunoblot analysis. Cell membrane PTHR expression was used as a loading control (55). B, membrane aliquots from PS120-R cells were incubated with \([35S]\)GTP\(\gamma\)S and 100 nM PTH(1–34) in the presence or absence of 1 mM AMP-PCP or 100 \(\mu\)M unlabeled GTP\(\gamma\)S for 5 min at 30 °C. Immunoprecipitation of \([35S]\)GTP\(\gamma\)S bound to G\(\alpha\) subunit was measured as described under “Experimental Procedures.” Data are summarized as the mean ± S.E. of three independent experiments.

A. 

|     | PS120-R | PS120-R-N2 |
|-----|---------|------------|
| G\(\alpha_s\) |            |            |
| G\(\alpha_q\) |            |            |
| G\(\alpha_i\) |            |            |

FIGURE 2. Time- and concentration-dependent PTH induction of \([35S]\)GTP\(\gamma\)S binding to G\(\alpha\) protein subunit. Cell membranes were prepared from PS120-R cells. Data are summarized as the mean ± S.E. of three independent experiments. A, time course of PTH-stimulated \([35S]\)GTP\(\gamma\)S binding to G\(\alpha\) subunit was measured in the presence or absence of 100 nM PTH(1–34). B, concentration-dependent curve of PTH-stimulated \([35S]\)GTP\(\gamma\)S binding to G\(\alpha\) subunit was measured in the presence of PTH for 5 min. Data are summarized as the mean ± S.E. of three independent experiments.

A. 

![Graph](https://via.placeholder.com/150)

B. 

![Graph](https://via.placeholder.com/150)

comparisons were evaluated by analysis of variance with post-test repeated measures analyzed by the Duncan procedure using Prism (GraphPad Software, Inc., San Diego). Differences of \(p < 0.05\) were assumed to be significant.

RESULTS

Specificity of PTH-induced G\(\alpha\) Subunit Activation—We first determined the constitutive expression of G\(\alpha\) protein subunits in membranes of PS120-R cells. Fig. 1A shows that PS120-R cells express G\(\alpha_s\), G\(\alpha_q\), and G\(\alpha_i\) proteins in cell membranes. We then measured PTH-stimulated \([35S]\)GTP\(\gamma\)S binding to G\(\alpha_s\), G\(\alpha_q\), and G\(\alpha_i\). PTH increased \([35S]\)GTP\(\gamma\)S binding to G\(\alpha\) protein by 3.6-fold (Fig. 1B). Unlabeled GTP\(\gamma\)S (100 \(\mu\)M) virtually abolished PTH-induced \([35S]\)GTP\(\gamma\)S binding. The nonhydrolyzable ATP analogue AMP-PCP (1 mM) had no effect on PTH-induced \([35S]\)GTP\(\gamma\)S binding. Similar results obtained with PTH-stimulated \([35S]\)GTP\(\gamma\)S binding to G\(\alpha_s\) and G\(\alpha_i\) (data not shown). These data provide strong evidence for specificity of PTH-induced \([35S]\)GTP\(\gamma\)S binding to G\(\alpha_s\), G\(\alpha_q\), and G\(\alpha_i\) proteins.

PTH activated G\(\alpha\) in a time- and concentration-dependent manner. Ligand-stimulated \([35S]\)GTP\(\gamma\)S binding to G\(\alpha\) occurred within 1 min (Fig. 2A). Because G\(\alpha\)-[\(35S]\]GTP\(\gamma\)S is resistant to hydrolysis by the intrinsic GTPase activity of G\(\alpha\), \([35S]\)GTP\(\gamma\)S-labeled G\(\alpha\) subunits accumulated over time under both basal and PTH-stimulated conditions. The net change of PTH-stimulated \([35S]\)GTP\(\gamma\)S binding peaked at 5 min. Therefore, we used this time point to determine the concentration dependence of PTH action. Half-maximal PTH-induced \([35S]\)GTP\(\gamma\)S binding was 6.2 nM; maximal stimulation occurred at 100 nM (Fig. 2B). Similar results were observed for PTH-stimulated \([35S]\)GTP\(\gamma\)S binding to G\(\alpha\) and G\(\alpha_i\) (data not shown). Therefore, 5 min stimulations with 100 nM PTH were used for subsequent experiments.

NHERF2 Inhibits PTH-stimulated G\(\alpha\) and Increases G\(\alpha_q\) and G\(\alpha_i\) Activity—Mahon et al. (21) reported that NHERF2 switched PTHR signaling from adenylyl cyclase to PLC in PS120-R cells stably transfected with NHERF2 (PS120-R-N2). Pertussis toxin pretreatment of PS120-R-N2 cells markedly inhibited PTH activation of PLC and enhanced activation of adenylyl cyclase, implying that PTH stimulates G\(i/\alpha\) proteins when the PTHR is bound to NHERF2. We tested the effects of NHERF2 on resting and PTH-stimulated G\(\alpha_s\), G\(\alpha_q\), and G\(\alpha_i\) exchange. PS120-R cells express small amounts of NHERF1 (47) but not NHERF2 (Fig. 3A), whereas PS120-R-N2 cells express NHERF2 mostly at cell membranes (Fig. 3A). NHERF2 did not affect basal G\(\alpha_s\), G\(\alpha_q\), or G\(\alpha_i\) activity but significantly blunted PTH-stimulated G\(\alpha\) activation (Fig. 3B). Conversely, NHERF2 augmented G\(\alpha_s\) and G\(\alpha_i\) activation (Fig. 3B). These effects occurred without a detectable change in cell membrane PTH receptor abundance (Fig. 3C), suggesting that the action of NHERF2 on G\(\alpha\) GTP exchange is not due to altered abundance of the PTHR or of ligand binding to the PTHR.
NHERF1 and NHERF2 Differentially Regulate PTH-stimulated Gq/H9251 Activation—The ability of PTH to increase the coupling of the receptor to Gq/H9251s and Gq/H9251q correlates with ligand-induced, receptor-dependent sensitivity of adenylyl cyclase and PLC signaling (1). Based on the described differences of NHERF1 and NHERF2 regulation of PTHR signaling (21, 29, 34), we hypothesized that NHERF1 and NHERF2 might differentially regulate PTH-stimulated Gq/H9251 protein activation. To compare the effects of NHERF1 and NHERF2 on Gq activation, we used CHO cells stably transfected with PTHR (CHO-R), which lack detectable expression of NHERF1 (34) or NHERF2 (data not shown) but express similar levels of Gq/H9251s, Gq/H9251q, and Gq/H9251i proteins (40). We transiently transfected CHO-R cells with His-NHERF1 or His-NHERF2, resulting in similar levels of cell membrane expression (Fig. 4A). In the absence of NHERF1 or NHERF2, PTH activation of Gq/H9251 was greater than that of Gq/H9251q, but no effects on Gq activation were detected (Fig. 4B). Neither NHERF1 nor NHERF2 affected basal Gq activity (data not shown). In the presence of NHERF1, PTH significantly enhanced Gq activity without an effect on Gq or Gq (Fig. 4B). Thus, NHERF1 selectively promotes receptor coupling to Gq.

In contrast, NHERF2 significantly inhibited PTH-stimulated Gq but enhanced PTH-induced activation of Gq and Gq (Fig. 4B). NHERF2 therefore influences receptor coupling to all three Gq proteins, promoting opposite effects on Gq and Gq, but like NHERF1 increasing PTH-dependent Gq activity. NHERF1 and NHERF2 did not affect receptor number as evidenced by comparable PTH binding to the PTHR (Fig. 4C), consistent with previous reports on CHO and ROS 17/2.8 cells (29, 34).

The PTHR, through its C-terminal ETVM PDZ recognition sequence, interacts with NHERF1 by binding to PDZ1 and
PDZ2 (34, 48). PTHR interactions with NHERF2 PDZ domains have not been described. Here, we simultaneously compared the interactions of GST-tagged C-terminal 22 amino acid peptide fragments of the wild-type PTHR (PTHR-cETVM) and a mutant form PTHR-cETVA, which cannot bind NHERF1, with PDZ1 and PDZ2 domains of NHERF1 and NHERF2 (Fig. 5A). The results show that the PTHR preferentially interacts with the PDZ1 domain of NHERF1 and PDZ2 of NHERF2. These associations were abolished with the PTHR harboring the mutated PDZ interaction motif.

Additional examination of the NHERF-mediated switch of G protein activation was undertaken in HEK-293 cells, which constitutively express NHERF1 and NHERF2. In HEK-293 cells, transfected with wild-type PTHR-ETVM (Fig. 5B), PTH significantly activated Gq, Gi, and G11, consistent with a previous report that PTH promoted activation of Goq, Gqi11, and G11 in HEK-293 cells (1). The PTHR-ETVA, which does not bind NHERF (49), showed decreased PTH-stimulated Goq activation and increased PTH-stimulated activation of G11 (Fig. 5C), although Goq activation was absent. To delineate the individual effects of NHERF1 and NHERF2 on G protein activation, endogenous NHERF1 or NHERF2 expression was silenced by RNA interference. A scrambled shRNA was used as a control. NHERF1 or NHERF2 shRNA reduced endogenous NHERF1 or NHERF2 levels by 78 and 82%, respectively, compared with a scrambled control (Fig. 5D). NHERF1 shRNA did not interfere with NHERF2 expression, and conversely, NHERF2 shRNA did not affect NHERF1 expression, demonstrating the specificity of the knockdown of endogenous NHERF1 and NHERF2 by their respective shRNAs. Neither shRNA affected basal [35S]GTPγS binding to Ga subunits (data not shown). Knockdown of NHERF1 expression selectively inhibited PTH-stimulated activation of Goq (Fig. 5E). Silencing NHERF2 expression, in contrast, significantly increased PTH-stimulated Goq and inhibited Goq activation. To rule out off-target effects of shRNA, we generated NHERF1 and NHERF2 rescue constructs (resNHERF1 and resNHERF2) harboring silent mutations to their respective shRNA and then conducted rescue experiments in HEK-293 cells. Expression of resNHERF1 blocked shNHERF1 inhibition of PTH-stimulated activation of Goq (Fig. 5F). resNHERF2 abolished shNHERF2 increases of PTH-stimulated Goq and inhibition of Goq activation.

Further and independent characterization of the dynamic interactions of NHERF1 and NHERF2 with Goq was conducted using real time FRET in living cells. PTH activated Goq in HEK-293 cells transfected with PTHR-ETVM compared with the cells transfected with PTHR-ETVA (Fig. 5G). NHERF1 shRNA did not affect PTH-induced Goq whereas the FRET signal was abolished with shNHERF2. These data further confirm that NHERF2 specifically increases PTH-induced Goq activation. Taken together, these results provide a mirror image of the effects of individual NHERF1 and NHERF2 actions on CHO-R cells and show that NHERF1 augments receptor-mediated stimulation of Goq but has no effect on stimulation of Goq or Gqi, whereas NHERF2 enhances receptor-mediated stimulation of both Goq and G11 but decreases stimulation of Goq.

NHERF but Not NHERF1 Binds Goq and Regulates Second Messenger Signaling Pathways—NHERF1 interacts directly with Goq (35). We analyzed NHERF1 and NHERF2 binding to Goq, Gi, and G11 in CHO-R cells that were transiently transfected with His-NHERF1 or His-NHERF2. Neither NHERF1 nor NHERF2 interacted with Goq (data not shown). However, both NHERF1 and NHERF2 bound G11, and the interactions were enhanced in the presence of PTH (Fig. 6A). These data are consistent with the report that NHERF1 interacts with Goq and to a greater extent with Goq-R183C, a constitutively active Goq mutant (35). We next investigated the interaction of NHERF1 and NHERF2 with Goq. NHERF2 but not NHERF1 coimmunoprecipitated with Goq by using Ni-NTA-agarose followed by immunodetection with a Goq antibody (Fig. 6B). Likewise, the interaction could be detected by using a Goq antibody for immunoprecipitation and immunodetection of NHERF2 with a His antibody. The association was also enhanced in the presence of PTH (Fig. 6B). Importantly, NHERF2 interacted with endogenous Goq in native CHO cells not expressing the PTHR (Fig. 6C), indicating that the interaction between NHERF2 and Goq is receptor-independent.

Since NHERF1 and NHERF2 differentially regulate PTH-stimulated Goq activation, their effects on PTH-induced second messenger signaling pathways in the presence or absence of pertussis toxin should differ. Therefore, we examined the effect of NHERF1 or NHERF2 on PTH-stimulated cAMP formation and [Ca2+]-i, as an index of PLC activity, in HEK-293 cells. Pertussis toxin (100 ng/ml) pretreatment for 16 h markedly increased PTH activation of adenyllyl cyclase (Fig. 7A), without affecting the magnitude of PTH-induced [Ca2+]-i (Fig. 7B, top), consistent with a previous report regarding pertussis toxin effects on PTH-stimulated cAMP formation and [Ca2+]-i (15). Silencing NHERF1 did not affect PTH-stimulated cAMP formation or maximal [Ca2+]-i. Pertussis toxin increased PTH-stimulated cAMP accumulation and decreased [Ca2+]-i, in the presence of shNHERF1 (Fig. 7, A and B, middle). As expected, pertussis toxin had no further action on PTH-stimulated cAMP production or [Ca2+]-i, after knockdown of NHERF2 (Fig. 7, A and B, bottom). Taken together, these results show that NHERF1 and NHERF2 differentially regulate PTH-stimulated G protein activation and second messenger signaling pathways.

DISCUSSION

PTH activates multiple second messenger signaling pathways that are reportedly coupled by distinct G proteins to the PTHR. Indirect approaches suggest that the PTHR is capable of coupling to Goq and to multiple Ga family members (2) and Gβγ subunits (50). However, to the best of our knowledge, only a single report analyzed PTHR activation of G proteins (1). Schwindinger et al. (1) measured PTHR coupling to Goq proteins in HEK-293 cell lines heterologously expressing the PTHR at low (C20; 40,000 receptors/cell) or high (C21; 400,000 receptors/cell) and in ROS 17/2.8 osteosarcoma cells, which constitutively express 72,000 receptors/cell (51). The ability of PTH(1–34) to activate Goq and Gaq, measured by [α-32P]GTP-γ-azidoanilide binding followed by immunospecific Ga subunit detection, correlated with the magnitude of ligand-induced receptor-dependent...
NHERF Regulates PTH Receptor Coupling to $G_\alpha$ Proteins

A. NHERF1
PDZ1  PDZ2  PDZ1  PDZ2

PTH-ctETVM

PTH-ctETVA

B. IB: HA

PTH-ETVM  PTHR-ETVA

C. IB: NHERF1

IB: NHERF2

D. G$_\alpha_s$  G$_\alpha_q$  G$_\alpha_i$

Fold Increase

E. PTHR-ETVM  PTH

PTH-ETVA  PTH

F. PTHR-Stimulated GTP$_\gamma$S bound, Fold Increase

Scrambled  shNHERF1

shNHERF2

G. ETVM + scramble

ETVM + shNHERF1

ETVM + shNHERF2

ETVA + scramble
sensitivity of adenylyl cyclase and PLC signaling pathways. PTH promoted activation of Gaq, Gq/11, and Gqi, albeit to a lesser extent, in C21 but not in C20 cells. In ROS 17/2.8 cells, PTH activated Gaq to a greater degree than in C21 cells but had no effect on Gq/11 or Gqi. NHERF proteins had not been discovered that at the time those studies were performed. Our data now permit some comparison of the results and show that the expression of NHERF1 and NHERF2 are, at least in part, responsible for the heterogeneity of the cellular responses to PTH. NHERF1 permits selective activation of Gq, whereas NHERF2 attenuates PTH-stimulated Gaq, while enhancing PTH-induced activation of Gaq and Gqi. These findings explain why PTH stimulated Gaq and Gqi in C21 cells, which express both NHERF1 and NHERF2, but not in ROS 17/2.8 cells, which express neither. Consistent with this interpretation, PTH does not stimulate [Ca2+]i, in native ROS 17/2.8 cells (29). Upon transfection of NHERF1, however, PTH significantly increased [Ca2+]i. Notably, relatively high PTHR density is required for efficient activation of PLC, in contrast to receptor activation of adenylyl cyclase that occurs at physiologic receptor concentrations (52). This may contribute to the limited PLC activation in ROS 17/2.8 cells.

To demonstrate the generality of the effects of NHERF1 and NHERF2, we studied the PTH-dependent activation of heterotrimeric G proteins in several cell lines that have been used as models to analyze the heterogeneity of the responses to PTH. We chose PS120 fibroblasts, CHO-derived cell lines, and HEK-293 cells, all of which were transfected with either the WT-PTHR or a mutated form with an impaired PDZ-binding motif. Our results are summarized in Table 2. Several conclusions can be drawn from these results. For instance, the data clearly indicate that coupling of the PTHR to Gq absolutely requires the expression of NHERF2. Furthermore, in the absence of NHERF1 and NHERF2, the receptor couples primarily to Gaq. Finally, Gaq coupling is significantly increased by either NHERF1 or NHERF2.

These results are consistent with previous reports examining the downstream effects of PTH in various cell lines and now provide a mechanism to reconcile apparent discrepancies between different cells or cell lines. Furthermore, our findings provide a solid experimental framework to explain the complex spectrum of cellular responses to PTH. For example, in PS120 cells stably transfected with the PTHR, the expression of NHERF2 decreased cAMP responses and increased PLC-dependent effects (21). Our data show that this effect is due to increased Gq and decreased Gqi activation in the presence of NHERF2. Likewise, HEK-293 cells robustly activate cAMP and Ca2+-dependent responses. The present findings support the conclusion that this is due to NHERF1/NHERF2-dependent modulation of the pattern of G protein activation downstream of the PTHR in these cells.
Despite the similarity of their PDZ domains and C-terminal ezrin-binding domain, NHERF1 and NHERF2 exhibit different affinities for PDZ-binding partners and GPCR signaling (20, 25, 53, 54). These results show that the PTHR binds preferentially to PDZ1 of NHERF1 and PDZ2 of NHERF2. Because binding to both NHERF proteins is mediated by the same C-terminal sequence, the PTHR cannot simultaneously interact with NHERF1 and NHERF2. Thus, the relative levels of expression of NHERF1 and NHERF2 may influence the differential coupling of the PTHR to different downstream signaling pathways. Importantly, although it is clear that the expression of NHERF1 and NHERF2 influences PTHR signaling, some variations among cell lines have been reported, indicating the influence of additional factors in PTHR signaling. For instance, NHERF1 increases PTH-stimulated cAMP accumulation in ROS 17/2.8 cells (29) but decreases cAMP responses in OKH cells (30). Furthermore, PTH-stimulated cAMP production in wild-type and NHERF1-null proximal tubule cells was comparable (32, 33), as it is in CHO-N10-R3 cells in the presence or absence of NHERF1 (34). Our results indicate that NHERF1 has no effects on PTH-induced Gq or Gi activation; therefore, the different effects of NHERF1 on the production of cAMP by these cell lines are probably due to other factors that remain unidentified.

The effects of NHERF2 on adenylyl cyclase activation are somewhat less diverse. NHERF2 expression markedly inhibited adenylyl cyclase in PS120 cells transfected with the PTHR, a result that is consistent with the differential activation of Gq and Gi induced by NHERF2 expression (21). Both NHERF1 and NHERF2 increase PTH-stimulated PLC activity and intercellular calcium in PS120 cells, opossum kidney cells, or ROS 17/2.8 cells (21, 29–31). These findings are consistent with the model presented here. Both NHERF1 and NHERF2 increase the activation of PLCβ and the generation of intracellular Ca2⁺ transients. However, despite the similarities between NHERF1 and NHERF2, the coupling of the PTHR to calcium signaling is mediated by distinct mechanisms. In HEK-293R cells (which express both NHERF1 and NHERF2), the PTHR stimulates Ca2⁺ release by a mechanism that is insensitive to pertussis toxin. Thus, in the presence of both NHERF1 and NHERF2, a Gq-driven mechanism predominates. Knockdown of NHERF1 has no effect on the magnitude or duration of the Ca2⁺ transients but reveals significant sensitivity to pertussis toxin. This suggests that Gq activation is important for Ca2⁺ release when only NHERF2 is present. Finally, knockdown of NHERF2 significantly reduces the magnitude of the Ca2⁺ release response, which in this case remains insensitive to pertussis toxin. A comparison of the magnitude of these responses suggests that NHERF2 is more efficient in the coupling of Ca2⁺ responses, which are mediated by the engagement of both Gq and Gi, whereas NHERF1 only supports Gq-mediated responses.

Because NHERF2 was reported to inhibit adenylyl cyclase by stimulating Go proteins in PS120 cells stably transfected with the PTHR or stably transfected with both the receptor and NHERF2 (21), we employed these cell models to investigate NHERF2 coupling of the PTHR to different Go protein subunits. The results show that NHERF2 significantly inhib-

**FIGURE 7. Effects of NHERF1 and NHERF2 on PTH-stimulated adenylyl cyclase activity and [Ca2⁺].** A, effects of NHERF1 and NHERF2 on PTH-stimulated adenylyl cyclase activity. HEK-293R cells were transfected with scrambled shRNA, shNHERF1, or shNHERF2. Pertussis toxin (PTX) (100 ng/ml) was added for 16 h as indicated. Cells were treated with 100 nM PTH for 15 min, and cAMP accumulation was measured as described under “Experimental Procedures.” Data are summarized as the mean ± S.E. of four independent experiments. ***, p < 0.01 compared with scrambled shRNA plus PTH group. B, effects of NHERF1 and NHERF2 on PTH-induced [Ca2⁺]. HEK-293R cells were treated as the same as A. PTH (100 nM)-stimulated [Ca2⁺], was measured as described under “Experimental Procedures.” Data are summarized as the mean ± S.E. of three independent experiments. The effects of scrambled shRNA, shNHERF1, and shNHERF2 on [Ca2⁺], in the presence or absence of pertussis toxin were shown in top, middle, or bottom panel, respectively.
NHERF Regulates PTH Receptor Coupling to Gα Proteins

TABLE 2
Summary of results with different cell lines

| Cell line                   | NHERF expression | Treatment                  | Coupling                        |
|-----------------------------|------------------|----------------------------|---------------------------------|
| PS120-R                     | Neither          | None                       | G_q > G_i; no G activation     |
| CHO-R                       | Neither          | NHERF2                     | G_q > G_i; no G activation      |
| HEK-293-ETVM (WT-PTHR)      | Both             | None                       | G_i = G_q = G_s no G activation |
| HEK-293-ETVA (PTHR with impaired PDZ binding) | Both             | NHERF1 shRNA               | G_i > G_q; no G activation      |
|                             |                  | NHERF2 shRNA               | G_i > G_q; no G activation      |

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