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 activation of adenylyl cyclase in the absence of NHERF to principally stimulation of phospholipase C 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 \[\text{[35S]}\text{GTP}\_x\] 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 Go\_q but have no effect on stimulation of Go\_i or Go\_s. In contrast, PTHR associations with NHERF2 enhance receptor-mediated stimulation of both Go\_q and Go\_i but decrease stimulation of Go\_s. Consistent with these functional data, NHERF2 formed cellular complexes with both Go\_q and Go\_i whereas NHERF1 was found to interact only with Go\_s. These findings demonstrate that NHERF interactions regulate PTHR signaling at the level of G proteins and that NHERF1 and NHERF2 exhibit iso-type-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 Go proteins, and the physiologic responses to PTH may result from contributions of both \(\alpha\) and \(\beta\gamma\) subunits. However, the particular G protein subunit to which the receptor couples varies in a cell-specific manner. Moreover, PTHR stimulation of PLC may arise through activation of Go\_q (4) or Go\_i\(
\_\gamma\) (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 epitheilia 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\_5 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\_5 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 Go\_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 adenylyl cyclase and phospholipase C is not known. It has been speculated that the NHERF proteins may promote Gαi-mediated signaling by tethering Gαi 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 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 protein activation. We show here that NHERF1 increases PTH-stimulated PTHR coupling to Gαq but not to Gαi. In contrast, NHERF2 decreases PTH-induced Gαq and increases Gαq and Gαi activation. These data reveal that NHERF-PTHR interactions can directly influence receptor coupling to G proteins.

EXPERIMENTAL PROCEDURES

HA.11 and His4 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 Gαq antibody was obtained from Millipore (Billerica, MA). Gαq monoclonal antibody was provided by BD Transduction Laboratories. Gαq polyclonal antibody was from NewEast Biosciences (Malvern, PA). Ni-NTA-agarose was provided by Qiagen (Valencia, CA). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was from Pierce. Horseradish peroxidase-conjugated sheep anti-mouse antibody was from GE Healthcare. Lipofectamine 2000 and geneticin, protein A-Sepharose 4B conjugate, and rec-protein G-Sepharose 4B 10 mg/ml streptomycin, and 100 μg/ml ampicillin (20–40 strokes in a “loose” Dounce homogenizer on ice. The lysates were centrifuged at 100,000 × g for 10 min to remove unbroken cells, large cell debris, and some nuclei. The supernatant was further centrifuged at 30,000 × g for 20 min. The membrane pellet was resuspended in freezing buffer (100 mM Heps, 0.1 mM EDTA, pH 7.4) at a protein concentration of 5–10 μg/μl and rapidly frozen in liquid nitrogen. Membranes were then stored at −80 °C until used.

GTPγS Binding and Immunoprecipitation of GTPγS-bound Ga Subunits—The comparative changes in Ga-[35S]GTPγS binding immunoprecipitated by specific Ga subtype-specific antisera were used to delineate PTHR coupling to distinct Ga protein subunits. [35S]GTPγS binding to Ga 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 (100 mM Heps, 5 mM MgCl2, pH 7.4) containing 5 μM GDP, 5 μM [35S]GTPγS, and 100 nM PTH(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 × g for 10 min, and the resulting supernatant was removed. Membrane pellets were solubilized, and immunoprecipitation of [35S]GTPγS bound to Ga 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 β-emission spectrometry. Nonspecific binding was determined in the presence of 100 μM GTPγS.

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...
TABLE 1

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<th>Primers used to generate rescue forms of human NHERF1 and NHERF2</th>
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Gene sequences

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<td>NHERF2 sense</td>
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</tr>
<tr>
<td>NHERF2 antisense</td>
<td>5′-GTTCTGGACATTTCGCTGCTTCCAGAAATTG</td>
</tr>
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incubated with anti-Gαq polyclonal antibody, Gαq monoclonal antibodies, or Gαq polyclonal antibody for 1 h at 4°C and then protein A or protein G-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). Membranes were blocked overnight at 4°C with 5% nonfat dried milk in Tris-buffered saline plus Tween 20 and incubated with different antibodies (anti-His antibody (1:1000); anti-Gαq (1:500); anti-Gαq (1:250); anti-Gαi (1:500); anti-HA (1:1000); anti-NHERF1 (1:1000); anti-NHERF2 (1:4000); 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.

Overlay Assay—His/S-tagged PDZ1 and PDZ2 domain proteins (1 µg/lane) were spotted on nylon membrane as described previously (42). The membranes were blocked with blot buffer for 30 min at room temperature and then overlaid with 100 nM GST-tagged C-terminal 22-amino acid fragments of wild-type PTHR (PTHR-ctETVM) or its mutant form PTHR-ctETVA in blot buffer for 1 h at room temperature. The blots were washed and incubated with horseradish peroxidase-conjugated anti-GST antibody (1:3000). Interactions of the GST fusion proteins with the PDZ domains were visualized by chemiluminescence.

NHERF Knockdown—Constitutive NHERF1 or NHERF2 expression in HEK-293R cells was silenced using RNA interference. Short hairpin RNA (shRNA) constructs against human NHERF1 (GGGAACGTCAGTTCTTCAAGAAATGCA) and NHERF2 (AACAGGAAGGTGAAATCTTCAAGCAACTT) were purchased from OriGene (Rockville, MD). HEK-293R cells were transfected with NHERF1 shRNA, NHERF2 shRNA, or scrambled shRNA. Transfections were established and described previously (34). Transfected cells were cultured for 72 h and then used for G protein binding or immunoblot.

Rescue forms of human NHERF1 and NHERF2 (resNHERF1 and resNHERF2, respectively) resistant to their respective shRNA were generated by introducing three silent mutations in the NHERF1 and NHERF2 sequences using primers listed in Table 1. The mutations were constructed by PCR using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The fidelity 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 Heps 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 Gαi and Gβγ was measured by FRET between YFP-tagged Gαi 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 (FyFp/FcyFP) (45).

Receptor Binding—Receptor binding was performed as described previously (34) using HPLC-purified [125I]-[Ne8,18,Tyr34]PTH(1–34)-NH2. 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) (10−11−10−6 M) and ~100,000 cpm of [125I]-[Ne8,18,Tyr34]PTH(1–34)-NH2, 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. Nonspecific 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.

Adenyl Cyclase—Adenyl 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-methylxanthine (1 mM) for 15 min. The reaction was terminated by addition of 1 M TCA. cAMP was isolated by the two-column method.

Intracellular Calcium—Intracellular calcium concentrations ([Ca2+]i) 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: [Ca2+]i = Kd × (F − Fmin)/(Fmax − F), where F is the measured fluorescence intensity; Fmax is the fluorescence measured after addition of 10 µM ionomycin; Fmin is the fluorescence measured after addition of 10 mM EGTA, and Kd is the dissociation constant of the dye-Ca2+ 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 Go Proteins

Abolished PTH-induced $[^{35}S]$GTPγS binding. The nonhydrolyzable ATP analogue AMP-PCP (1 mM) had no effect on PTH-induced $[^{35}S]$GTPγS binding. Similar results obtained with PTH-stimulated $[^{35}S]$GTPγS binding to Goq and Gai (data not shown). These data provide strong evidence for specificity of PTH-induced $[^{35}S]$GTPγS binding to Goq, Goqi, and Gai proteins.

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

**NHERF2 Inhibits PTH-stimulated Goq and Increases Gao, Gai Activation—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 Gai, proteins when the PTHR is bound to NHERF2. We tested the effects of NHERF2 on resting and PTH-stimulated Goq, Goqi, and Gai 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 Goq, Goqi, or Gai activity but significantly blunted PTH-stimulated Goq activation (Fig. 3B). Conversely, NHERF2 augmented Goq and Gai activation (Fig. 3B). These effects occurred without a detectable change in cell membrane PTHR receptor abundance (Fig. 3C), suggesting that the action of NHERF2 on Gai/GTP exchange is not due to altered abundance of the PTHR or of ligand binding to the PTHR.
**NHERF Regulates PTH Receptor Coupling to Gα Proteins**

**NHERF and NHERF2 Differentially Regulate PTH-stimulated PTH Receptor Coupling to Gα Proteins**

The ability of PTH to increase the coupling of the receptor to G\(\alpha_s\), G\(\alpha_q\), or G\(\alpha_i\) proteins is correlated 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 G\(\alpha_s\) protein activation. To compare the effects of NHERF1 and NHERF2 on G\(\alpha_s\) 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 G\(\alpha_s\), G\(\alpha_q\), and G\(\alpha_i\) 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 G\(\alpha_s\) was greater than that of G\(\alpha_q\), but no effects on G\(\alpha_i\) activation were detected (Fig. 4B). Neither NHERF1 nor NHERF2 affected basal G\(\alpha_s\) activity (data not shown). In the presence of NHERF1, PTH significantly enhanced G\(\alpha_s\) activity without an effect on G\(\alpha_q\) or G\(\alpha_i\) (Fig. 4B). Thus, NHERF1 selectively promotes receptor coupling to G\(\alpha_q\). In contrast, NHERF2 significantly inhibited PTH-stimulated G\(\alpha_s\) but enhanced PTH-induced activation of G\(\alpha_q\) and G\(\alpha_i\) (Fig. 4B). NHERF2 therefore influences receptor coupling to all three G\(\alpha_s\) proteins, promoting opposite effects on G\(\alpha_s\) and G\(\alpha_q\), but like NHERF1 increasing PTH-dependent G\(\alpha_q\) 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 Gaq, Gai, and Goi (Fig. 5C), consistent with a previous report that PTH promoted activation of Gaq, Goai11, and Goi in HEK-293 cells (1). The PTHR-ETVA, which does not bind NHERF (49), showed decreased PTH-stimulated Gaq activation and increased PTH-stimulated activation of Goi (Fig. 5C), although Gaq 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 Go subunits (data not shown). Knockdown of NHERF1 expression selectively inhibited PTH-stimulated activation of Gaq (Fig. 5E). Silencing NHERF2 expression, in contrast, significantly increased PTH-stimulated Gaq and inhibited Goi 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-293R cells. Expression of resNHERF1 blocked shNHERF1 inhibition of PTH-stimulated activation of Gaq (Fig. 5F). resNHERF2 abolished shNHERF2 increases of PTH-stimulated Gaq and inhibition of Goi activation.

Further and independent characterization of the dynamic interactions of NHERF1 and NHERF2 with Goi was conducted using real time FRET in living cells. PTH activated Goi 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 Goi, whereas the FRET signal was abolished with shNHERF2. These data further confirm that NHERF2 specifically increases PTH-induced Goi 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 Goi or Gaoi, whereas NHERF2 enhances receptor-mediated stimulation of both Goi and Goi 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, Goai11, and Goi. CHO-R cells 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 Goq, 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 GoqR183C, 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, an index of PLC activity, in HEK-293R 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 (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 Gaq 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 Go 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 Goq measured by [α-32P]GTP-γ-azidoanilide binding followed by immunospecific Goq subunit detection, correlated with the magnitude of ligand-induced receptor-dependent
NHERF Regulates PTH Receptor Coupling to $G_\alpha$ Proteins

A. NHERF1

<table>
<thead>
<tr>
<th>PDZ1</th>
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B. PTHR-ETVM

IB: HA

B. PTHR-ETVA

IB: NHERF1

IB: NHERF2

C. GTP\(\alpha\)S bound, Fold Increase

D. Scrambled

IB: NHERF1

IB: NHERF2

E. PTH-Stimulated GTP\(\alpha\)S bound, Fold Increase

F. PTH-Stimulated GTP\(\alpha\)S bound, Fold Increase

G. ETVM + shNHERF1

ETVM + shNHERF2

ETVM + scrambled

ETVA + scrambled

Time, sec
NHERF Regulates PTH Receptor Coupling to \( \alpha \) Proteins

To demonstrate the generality of the effects of NHERF1 and NHERF2, we studied the PTH-dependent activation of heterotrimeric \( \alpha \) 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 \( \alpha \) absolutely requires the expression of NHERF2. Furthermore, in the absence of NHERF1 and NHERF2, the receptor couples primarily to \( \alpha \). Finally, \( \alpha \) 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 \( \alpha \) and decreased \( \alpha \) activation in the presence of NHERF2. Likewise, HEK-293R robustly activate cAMP and \( \alpha^{2+} \)-dependent responses. The present findings support the conclusion that this is due to NHERF1/NHERF2-dependent modulation of the pattern of \( \alpha \) 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 PTH1R 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 Gαs or Gαi 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 Gαs and Gαi 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 Gαi4-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 Gαi 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 Gαs and Gαi, whereas NHERF1 only supports Gαq-mediated responses.

Because NHERF2 was reported to inhibit adenylyl cyclase by stimulating Gαi 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 Gα protein subunits. The results show that NHERF2 significantly inhib-

FIGURE 7. Effects of NHERF1 and NHERF2 on PTH-stimulated adenylyl cyclase activity and [Ca2+]i. 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+]i. HEK-293R cells were treated as the same as A. PTH (100 nM)-stimulated [Ca2+]i 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+]i, in the presence or absence of pertussis toxin were shown in top, middle, or bottom panel, respectively.
NHERF Regulates PTH Receptor Coupling to G\(\alpha\) Proteins

> TABLE 2
Summary of results with different cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>NHERF expression</th>
<th>Treatment</th>
<th>Coupling</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS120-R</td>
<td>Neither</td>
<td>None</td>
<td>(G_\text{q} &gt; G_\text{i} ) no (G_\text{s}) activation</td>
</tr>
<tr>
<td>CHO-R</td>
<td>Neither</td>
<td>NHERF2</td>
<td>(G_\text{i} &gt; G_\text{q} &gt; G_\text{s}) activation</td>
</tr>
<tr>
<td>HEK-293-ETVM (WT-PTHR)</td>
<td>Both</td>
<td>None</td>
<td>(G_\text{i} = G_\text{q} = G_\text{s}) activation</td>
</tr>
<tr>
<td>HEK-293-ETVA (PTHR with impaired PDZ binding)</td>
<td>Both</td>
<td>None</td>
<td>(G_\text{i} = G_\text{q} = G_\text{s}) activation</td>
</tr>
</tbody>
</table>

The precise mechanistic basis of the differential regulation of the G protein coupling of the PTHR by NHERF1 and NHERF2 remains to be explained. One plausible explanation is suggested by the data shown in Fig. 6. Both NHERF1 and NHERF2 coimmunoprecipitated with \(G_\text{q}\) but only NHERF2 was able to pull down \(G_\text{i}\). The specific regions of NHERF1 and NHERF2 that mediate these putative interactions remain unknown. The next question is as follows. How do the interactions with NHERF1 and NHERF2 with G proteins influence PTHR signaling? The simplest model involves the pre-formation of a multimeric complex involving the PTHR, NHERF1/NHERF2, and a heterotrimeric G protein. We propose that the formation of this complex, by bringing the receptor and the G protein into close proximity, facilitates G protein activation. Thus, NHERF1, which binds \(G_\text{q}\), will promote \(G_\text{i}\) activation, and NHERF2, which binds \(G_\text{q}\) and \(G_\text{i}\), will enhance the activation of these two G proteins. The differential effects of NHERF1 and NHERF2 on \(G_\text{q}\) activation may be due to the different topology of the interactions of the two PDZ proteins with the PTHR. It is conceivable that NHERF1 binding to PTHR, which is mediated by the N-terminal PDZ1 domain, does not interfere with \(G_\text{s}\) binding. Conversely, NHERF2-PTHR interactions, which involve the central PDZ2 of NHERF2, may induce conformational changes that result in reduced affinity for \(G_\text{q}\).

In summary, NHERF1 interacts with \(G_\text{q}\) and increases PTH-stimulated PTHR coupling to \(G_\text{q}\) but has no effect on \(G_\text{i}\) or \(G_\text{s}\). In contrast, NHERF2 interacts with both \(G_\text{q}\) and \(G_\text{i}\). Therefore, NHERF1 and NHERF2 control PTHR signaling by differential coupling of \(G_\text{q}\) proteins to the PTHR. These novel findings explain the differences between NHERF1 and NHERF2 on downstream regulation of PTHR signaling.

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REFERENCES
NHERF Regulates PTH Receptor Coupling to Gα Proteins

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