Stimulation by Parathyroid Hormone of a NHERF-1-assembled Complex Consisting of the Parathyroid Hormone I Receptor, Phospholipase Cβ, and Actin Increases Intracellular Calcium in Opossum Kidney Cells*

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Parathyroid hormone (PTH) binds its cognate G-protein-coupled receptor (PTH1R) and signals through both adenyl cyclase and phospholipase C (PLC). C-terminal determinants of the PTH1R interact with the Na+/H+ exchanger regulatory factor 1 (NHERF-1) by binding the first of two PDZ (psd95, discs-large, ZO-1) domains. Compared with wild-type opossum kidney (OK) cells, OKH cells, a sub-clone, do not display PTH-mediated increases of [Ca2+]i and express NHERF-1 at markedly lower levels. Stable expression of NHERF-1 in the OKH parent (OKH-N1) restores the PTH-mediated increase of [Ca2+]i that arises from an influx of extracellular calcium and is both PLC-dependent and pertussis toxin-sensitive. From a morphological perspective, NHERF-1 and the PTH1R co-localize to apical patches of OKH-N1 cells, an expression pattern that is absent in OKH cells and depends on a direct NHERF-1-PTH1R interaction in OKH-N1 cells. Actin and PLCβ1 and -β3 co-localize with NHERF-1 and the PTH1R in OKH-N1 cell apical patches. Actin is also an integral component of the NHERF-1-assembled complex because cytochalasin D disrupts apical localization of both NHERF-1 and the PTH1R and inhibits the PTH-mediated increase of [Ca2+]i. Expression of the first PDZ domain of NHERF-1 acts as a dominant-negative interactor by blocking apical localization of the PTH1R and inhibiting PTH-elicited increases of [Ca2+]i. Thus, NHERF-1 assembles a signaling complex in the apical domains of OK cells that contains the PTH1R, PLCβ, and the actin cytoskeleton.

Multiple signals emanate from the activated parathyroid hormone (PTH) and parathyroid hormone-related protein receptor (PTH1R) (1, 2). Activation of the various pathways and subsequent downstream processes mediated by the PTH1R depends on the cellular environment. PTH1R actions in the proximal convoluted tubule (PCT) of the kidney, for example, illustrate that its signaling depends not only on the cell type but also on the presence of the receptor on a specific membrane surface.

The PTH1R is abundantly expressed in the PCT, where it mediates PTH signaling primarily via activation of adenyl cyclase (AC) and phospholipase C and by increasing intracellular calcium ([Ca2+]i). PTH-mediated inhibition of phosphate uptake, which is caused by internalization and degradation of the type IIa sodium-phosphate co-transporter (Npt2), is an often-studied response in opossum kidney (OK) cells (3–5) and the PCT (6, 7). It has recently become appreciated that in polarized epithelia, such as the PCT, discrete microdomains are formed in the apical and basolateral compartments, a process that arises from targeted expression of specialized proteins (8–10). The actin cytoskeleton and associated proteins maintain cell polarity by blocking lateral movement and mixing of apical- and basolateral-membrane constituents. Several lines of evidence suggest that the PTH1R, although expressed in both apical and basolateral compartments of the PCT (11, 12), is differentially coupled to signaling pathways. First, the PTH1R in isolated basolateral membranes activates AC in vitro, whereas receptors expressed in apical membranes do not (13). Second, PTH administered to the apical side of OK cells, grown on membrane filters, displays an EC50 (5 μM) for the inhibition of phosphate uptake that is 100-fold more sensitive than when PTH is administered to the basolateral surface (500 μM) (14, 15). Third, PTH mediates Npt2 internalization and degradation via CAMP/protein kinase A when applied to the basolateral surface and via PLC/protein kinase C when applied to the luminal surface of isolated PCT segments (7). Last, sequential perfusion of PCT cells in primary culture with PTH at 20-min intervals transiently increases [Ca2+]i, to the same amplitude but results in a progressive decrease in the activation of AC (16). Although these results might be compatible with a model involving two PTH receptors, publication of several mammalian genomes have not revealed relevant PTH1R isoforms, suggesting that cell-specific factors are responsible for diverse signaling by this receptor. Cell-specific signaling by the PTH1R is obviously dependent on the repertoire of effector molecules expressed in a given cell.

We have recently shown that signaling properties of the PTH1R are profoundly affected by its direct binding of a “scaffolding” protein; the Na+/H+ exchanger regulatory factor 2 (NHERF-2) (17). NHERF-2 switches PTH1R signaling from AC to PLC through a direct psd95, discs large, ZO1 (PDZ)-domain-specific interaction between the more C-terminal of two PDZ domains and the C terminus of the receptor (17). NHERF-2 and
the closely related protein, NHERF-1 (also called EBP50), are PDZ domain-containing proteins that also indirectly bind actin via interactions with MERLIN (merlin, ezrin, radixin, moesin) proteins (18, 19). Like NHERF-2, NHERF-1 also interacts with the PTH1R in vitro and in mouse kidney extracts, but this seems to involve the more N-terminal of the two PDZ domains (data shown herein). NHERF-1-null mice display a complex phenotype, which includes renal phosphate wasting, hypocalemia, and osteopenia (20). Npt2 localizes to intracellular sites in PCT lacking NHERF-1, demonstrating that NHERF-1 targets and/or anchors the transporter to apical domains (20). We recently reported that OKH cells, a sub-clone of wild-type OK cells that do not display PTH-mediated inhibition of Npt2, markedly lack NHERF-1 expression compared with wild-type cells (21). It is noteworthy that stable expression of NHERF-1 in OKH cells (OKH-N1) restored the PTH-mediated inhibition of phosphate uptake (21). Together with data that show binding of NHERF-1 to Npt2 (21, 22), these findings suggest that NHERF-1 assembles a regulatory complex for Npt2.

Herein, we demonstrate that expression of NHERF-1 in the OK cell model is essential for numerous cellular functions. OKH cells also do not respond to PTH with an increase of phosphoinositide hydrolysis or Ca2+, and do not manifest apical patches, which are typical of the parental OK cell. When they stably express exogenous NHERF-1, however, OKH-N1 cells now have apical patches and respond to PTH with an increase in [Ca2+], which is caused by extracellular influx, rather than a release from internal stores. This influx is both PLC-dependent and pertussis toxin-sensitive and requires the assembly by NHERF-1 of PLCβ, actin, and the PTH1R.

EXPERIMENTAL PROCEDURES

Materials—The OK cell lines were a kind gift from Dr. Judith Cole. Plasmids containing cDNAs for PLCβ1 were from Dr. Sue Goe Hhee, for PLCβ2 from Dr. Eva Neer, and for PLCβ3 from Dr. Barbara Sanborn. NHERF-1 monoclonal antibodies were from Dr. Chris Yun. GST-Sepharose was from Amersham Biosciences. Fura-2-acetoxyethyl ester, Alexa Fluor 488 and 546 phalloidin, and secondary antibodies conjugated with Alexa Fluor 488 and 546 were from Molecular Probes (Eugene, OR). U73122, SKF96365, cytochalasin D, and thrombin were from Calbiochem. S-protein-HRP and rapid S-tag assay kit were from Novagen (San Diego, CA). Monoclonal antibodies to Na+/K+ ATPase were from Upstate Biotechnology (Charlottesville, VA). Pertussis toxin, Flag antibodies, 3,5,5’-tetramethylbenzidine liquid substrate system and general chemicals were from Sigma.

Overlay and Plate-binding Assay—The full-length C-terminal tail of the PTH1R (amino acids 463–591), the N-terminal deletions of the C-terminal tail (see Analysis of the NHERF-1 Dominant Negative—), and the C-terminal point mutations (P581A, L652K, Q684A, E695A, E696S/696A, and E685S/686K) were cloned into pEG vector (Amersham Biosciences) using PCR with PfuTurbo polymerase (Stratagene, La Jolla, CA), which generates an N-terminal GST fusion protein. The various GST-PTH1R-C-tail constructs were expressed in BI-21 Escherichia coli and purified using GST-Sepharose. Full-length NHERF-1 (amino acids 1–135), N1-PDZ1short (amino acids 1–127), N1-PDZ2short (amino acids 128–158), N1-PDZ1long (amino acids 1–162), and N1-PDZ2long (amino acids 65–358) were cloned into pET-30b (Novagen), using PCR with Pfu polymerase, which generates an N-terminal S-tag and His tag. All clones were verified using automated sequencing. These NHERF-1 constructs were expressed in BI21(DE3)plplysE. coli and partially purified using immobilized metal affinity chromatography (nickel-nitrilotriacetic acid; Qiagen, Valencia, CA), following the manufacturer’s procedures. Protein concentrations were determined using the S-tag Rapid Assay Kit from Novagen.

For overlay assays, 2 μg of purified GST and the various GST PTH1R-C-tails were run on SDS-PAGE and blotted to polyvinylidene difluoride membranes. The membranes were incubated with 5% non-fat dry milk in phosphate-buffered saline containing 0.1% Tween 20 (PBSTw) for 45 min. Full-length NHERF-1 and the various fragments were overlaid on the membrane in 5% milk in PBSTw at a concentration of 200 nm for 1 h at room temperature. Membranes were washed thoroughly and blotted with S-protein-HRP (1:5000 in 5% milk) for 20 min and washed a second time; interactions were detected using the Western Lightining Chemiluminescence reagent (PerkinElmer Life and Analytical Sciences, Boston, MA). For the plate-binding assay, 0.5 μg of either GST or the various GST-PTH1R-C-tail proteins were adhered to wells of 96-well plates overnight at 4°C. The wells were washed with PBST and blocked with 5% milk for 1 h at room temperature. The S-tagged NHERF-1 proteins were incubated in the wells at concentrations ranging from 0.01 nm to 1 μg in 5% milk/PBSTw. The wells were thoroughly washed, incubated with S-protein-HRP (1:5000 in 5% milk), and washed a second time; interactions were detected using the 3,3’,5’,5’-tetramethylbenzidine liquid substrate system (Sigma). After 20 min at room temperature, reactions were stopped by the addition of 5 mg/ml Triton X-100, and the reaction mixture was quantified by spectrophotometry. Binding to GST alone was subtracted from each value at a given concentration, and the data were reported as a percentage of the maximum binding.

Determination of [Ca2+]:—OKH cells were stably transfected with NHERF-1 (OKH-N1), as described previously (21). [Ca2+]i was monitored using the fura-2 method. In brief, cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F12 media supplemented with 10% fetal bovine serum and plated into Lab-Tek II chambered cover glasses (Nalge Nunc International, Naperville, IL). Cells were serum-starved for 4–5 h before the assay. Cells were cooled to room temperature and washed with a balanced salt solution (127 mM NaCl, 3.8 mM KCl, 1.2 mM CaCl2, 1.2 mM MgCl2, 5 mM NaHCO3, 5 mM HEPES, pH 7.4). Cells were then loaded with 5 μM fura-2/acetoxymethyl ester with 0.05% Pluronic F-127 (Molecular Probes, Eugene, OR) for 45 min at room temperature, washed in balanced salt solution, and unloaded for 30 min. Fura-2 fluorescence was diffuse without a punctate distribution pattern. Fluorescence was measured in single cells excited at 340 and 380 nm using a P/Trac Fluorimeter (Photon Technologies Incorporated, Lawrenceville, NJ) directed through the stage of an inverted Nikon Diaphot 200 microscope (Melville, NY) with a cut-off filter at 400 nm. Emissions were monitored in real time with a SeneSys charge-coupled device camera (Photometrics, Ltd., Tucson, AZ) and analyzed using the Poenie-Tsien ratio with ImageMaster 2 software (Pharmacia). The ratio of fluorescence at the end of each experiment, fura-2 fluorescence was calibrated to [Ca2+], by treating the cells with 5 μM ionomycin to achieve the Ca2+-bound dye ratio at 340/380 nm and fluorescence at 480 (Rmax and Smax), followed by treatment with 5 mM EGTA to determine Ca2+-free dye ratio at 340/380 and fluorescence at 480 (Rmin and Smin). These parameters were used to estimate [Ca2+], using the equation of Grynkiewicz et al. (23), which is provided as a function in the ImageMaster 2 software. Calcium transients monitored in the absence of extracellular calcium are reported as a ratio of fura-2 fluorescence of 340 over 380 nm.

Transient Transfection and Immunohistochemistry—Cells were cultured in chambered microscope slides (Nalge Nunc International). The DNA plasmid encoding the yellow fluorescent protein (YFP) (Clontech) was cloned in-frame with 30 amino acids from the C terminus of the PTH1R using PCR. This region of the C-terminal tail has a low degree of homology among species, and placing YFP in this location does not interfere with NHERF-1 interactions in vitro (data not shown). The flag epitope (DYKDDDDK) was cloned in-frame on the N terminus of the PTH1R. PLCβ3 and -β1 are transiently expressed in pcDNA3.1 (Invitrogen). PLCβ2 was already Flag-tagged in pcDNA3.1. Forty-eight hours before analysis, cells were transfected with Fugene 6 (Roche Applied Science) at 0.25 μg of plasmid/mwl at a Fugene6/DNA ratio of 5:1. Cells were then fixed with 2% paraformaldehyde in phosphate-buffered saline for 20 min, thoroughly washed with phosphate-buffered saline, permeobilized with 0.1% Triton X-100 for 10 min, and washed again with phosphate-buffered saline plus 0.1% Triton X-100 (PBSTx) for 30–45 min. Primary antibodies were diluted in 5% milk/PBSTx (NHERF-1 monoclonal, 1:2000; Flag monoclonal, 1:1000; Na+/K+-ATPase monoclonal, 1:5000) and incubated with the cells for 1 h. Cells then were washed in PBSTx and incubated with species-specific secondary antibodies conjugated with Alexa Fluor 488 (green) or Alexa Fluor 546 (red) and detected using Alexa Fluor 546 conjugated to phalloidin. Immunostained cells were analyzed using a Radiance 2100 confocal microscope and the associated LaserSharp 2000 operating software (Bio-Rad, Hercules, CA). Basolateral membranes were identified using the Na+/K+-ATPase antibody (data not shown). At least 250 points were counted of the NHERF-1 Dominant Negative—Amino acids of NHERF-1 corresponding to the PDZ1long (see “Overlay and Plate-binding Assay”) and β-galactosidase were cloned in-frame with the Flag epitope (DYKDDDDK) into pcDNA3.1 using PCR. Flag-PDZ1long of NHERF-1 and Flag-β-galactosidase were transiently expressed in...
RESULTS

The PTH1R and NHERF-1 Interactions in Vitro—NHERF-1 interacts with the PTH1R through an atypical PDZ interaction motif consisting of the four C-terminal amino acids of the receptor (17). Using overlay assays, full-length NHERF-1 binds the PTH1R tail with high affinity (Fig. 1A). NHERF-1 contains two PDZ domains; one is N-terminal (PDZ1) and the other is centrally located (PDZ2) in the molecule (Fig. 1B). PDZ1 was separated from PDZ2 by dividing the interdomain region in half, yielding PDZ1short and PDZ2short (Fig. 1B). The individual PDZ domains (PDZ1short and PDZ2short) unexpectedly failed to interact with the PTH1R C-tail to any significant degree (Fig. 1A), suggesting that disruption of the interdomain region severely affects interactions of NHERF-1 with target proteins. Additional PDZ domain constructs then were synthesized that included the entire interdomain region (PDZ1long and PDZ2long) (Fig. 1B). Relative binding affinities, determined with a plate-binding assay, show that full-length NHERF-1 and PDZ1long display indistinguishable apparent $K_d$ affinities for the PTH1R C-tail of $\sim$110 to 120 nM (Fig. 1C). On the other hand, PDZ2long does not interact with the PTH1R C-tail to any significant degree (Fig. 1C), demonstrating that PDZ1 of NHERF-1 is the primary binding site for the PTH1R.

We next sought to determine whether residues N-terminal to the four C-terminal amino acids that comprise the canonical PDZ interaction motif contribute to the NHERF-1 interaction. As demonstrated by an overlay assay, GST fusions containing N-terminal deletions of the C-tail up to 110 amino acids in
length did not affect the NHERF-1-receptor interaction (Fig. 2A). However, deletion of 120 amino acids from the N terminus completely disrupts the NHERF-1-receptor interaction (Fig. 2A), suggesting that the PDZ interaction motif on the PTH1R extends N-terminal to the four terminal residues to include some or all of the C-terminal 18 amino acids. Because the C-tail fused to the large GST moiety (27 kDa) might sterically impair the NHERF-1-receptor interaction, point mutations N-terminal to the PDZ interaction motif were incorporated in the full-length PTH1R C-tail, and its interactions with NHERF-1 were assessed by the plate-binding assay. Incorporation of P581A, L582K, or Q584A point mutations does not affect the NHERF-1-receptor interaction, whereas single and double point mutations of the glutamic acid residues at 585 and 586 markedly disrupt this interaction, suggesting that the acidic side chains in the C-tail stabilize the NHERF-1-receptor interaction (Fig. 2B).

**NHERF-1 Restores PTH-mediated Increases of Intracellular Calcium in OKH Cells**—OKH cells, a sub-clone of the wild-type OK cell line, do not display PTH-mediated inhibition of phosphatase uptake or increases of [Ca\(^{2+}\)] (24), although they do display a robust PTH-mediated activation of AC (25). We showed previously that OKH cells were deficient in expression of NHERF-1, and stable expression of NHERF-1 in OKH cells restored PTH-mediated inhibition of phosphate uptake (21). Fig. 3A shows that stable expression of NHERF-1 in OKH cells (OKH-N1) also restores the PTH-elicited increase of [Ca\(^{2+}\)], which is rapid, robust, and transient in nature. OKH-N1 cells in nominally calcium-free buffer plus 0.5 mM EGTA do not display PTH-mediated increases of [Ca\(^{2+}\)] (Fig. 3B), suggesting that the increase in cytoplasmic free calcium is caused by an influx from the extracellular space and not by release from intracellular stores. The receptor-mediated calcium channel blocker SKF96365 (26) inhibits the PTH-mediated increase in [Ca\(^{2+}\)] in a dose-dependent manner (Fig. 3C), which supports this conclusion, and is consistent with published data that PTH predominantly mediates an influx of calcium ions in wild-type OK cells (24) and PCT cultures (16, 27, 28). The phospholipase C inhibitor U73122 and pertussis toxin also block the PTH-mediated increase in [Ca\(^{2+}\)] (Fig. 3C), indicating that this effect depends on activation of PLC by a member of the G\(_{o}\) family of G proteins. The inactive analog of U73122, U73343, did not inhibit the PTH-mediated increase in [Ca\(^{2+}\)] (data not shown). OKE cells, another sub-clone of the OK cell line, express abundant amounts of NHERF-1, display responses to PTH similar to those of the parental OK cells, and display PTH-mediated calcium responses that are indistinguishable from that displayed by OKH-N1 cells (data not shown).

OK cells respond to thrombin with an increase in [Ca\(^{2+}\)], a response that has been attributed to an increase of inositol trisphosphates (29). To control for potential depletion of intracellular calcium in OKH-N1 cells when incubated in calcium-free media or an unexpected block of calcium release from intracellular stores by SKF96365, we assessed the response of these treatments to thrombin. Depletion of extracellular calcium (Fig. 4A) or inclusion of SKF96365 (Fig. 4B) does not inhibit the thrombin-mediated increase of [Ca\(^{2+}\)] in OKH-N1 cells, demonstrating that thrombin induces a release of calcium from intracellular stores. These data demonstrate that depletion of extracellular calcium or SKF96365 treatment does not introduce confounding artifacts and thus supports the assertion that PTH increases cytoplasmic-free calcium by increasing influx from outside the OKH-N1 cell.
NHERF-1 and the PTH1R Co-localize in Apical Membranes—NHERF-1 contains an interaction site for the actin-binding MERM proteins at the C terminus, in addition to its two PDZ domains (18). NHERF-1 is therefore likely to establish PTH1R signaling via 
$[Ca^{2+}]/H_1/O_1$ by serving as a scaffold to assemble relevant molecules, perhaps indirectly including a link to actin, as suggested by the presence of NHERF-1 in actin-rich apical patches of wild-type OK cells (22). These apical patches are distinct and readily detected on OKE cells (data not shown) and OKH-N1 cells (Fig. 5A), but are absent in OKH cells (Fig. 5C). The cDNA for YFP was cloned, in-frame, 30 amino acids from the C terminus of the PTH1R to facilitate localization. The interaction of the PTH1R C-tail/YFP chimera with NHERF-1 was indistinguishable from that of the C-terminal tails of PLC$\beta_1$, PLC$\beta_2$, and PLC$\beta_3$ and immunostained with NHERF-1 antibodies (Alexa Fluor 546, red) and flag-monoconal antibodies (Alexa Fluor 488, green). Representative confocal images of the apical domains of the cells are shown.

from the C terminus of the PTH1R to facilitate localization. The interaction of the PTH1R C-tail/YFP chimera with NHERF-1 was indistinguishable from that of the C-tail alone in vivo (data not shown). When OKH-N1 cells transiently express full-length PTH1R-YFP, it strongly co-localizes with NHERF-1 in apical patches (Fig. 5A). It is noteworthy that cells expressing a PTH1R-YFP construct lacking the C-terminal NHERF-1 in-
interaction domain (PTH1R-YFP-CΔ4) display a complex localization pattern; a small portion localizes to the apical domain with a punctate staining pattern, but most of this mutant receptor localizes to intracellular sites and the basolateral membrane (Fig. 5B). Although OK cells are deficient in endogenous NHERF-1, it can be detected by increasing both the laser power and gain function of the confocal microscope. NHERF-1 displays a punctate staining pattern in the apical portion of OKH cells (Fig. 5C), but the cells lack the apical patches that are characteristic structures in OKH-N1 cells (Fig. 5A), wild-type OK cells, and OKE cells (data not shown). The wild-type PTH1R-YFP localization pattern in OKH cells (Fig. 5C) is reminiscent of the pattern seen when PTH1R-YFP-CΔ4 is expressed in OKH-N1 cells (Fig. 5B) and includes punctate staining in the apical domain and prominent staining in intracellular sites and on the basolateral membrane.

Phospholipase Cβ Is a Component of the NHERF-1-assembled Complex—All four isozymes of PLCβ contain C-terminal consensus PDZ interaction motifs. The C-terminal tails of PLCβ1, -β2, and -β3 robustly interact with PDZ1long of NHERF1 in vitro (Fig. 6A). There is no detectable interaction between PDZ2long and the C-terminal tail of PLCβ1 and only a weak interaction between PDZ2long and the C-terminal tails of PLCβ2 and -β3 (Fig. 6A). PLCβ1 and -β3, when transiently expressed in OKH-N1 cells, co-localize with NHERF-1 in apical patches (Fig. 6B). PLCβ2, however, does not localize with NHERF-1 in the apical patches despite a strong in vitro interaction (Fig. 6B), but is prominently expressed in the cytoplasm and on basolateral membranes (data not shown). It is notable that PLCβ1 and -β3 strongly co-localize with the PTH1R-YFP in transiently transfected OKH-N1 cells (Fig. 6C), suggesting that NHERF-1 assembles the receptor with its downstream effector molecule. Transient expression of PLCβ1 and β3 in OKH cells reveals a localization pattern that is strikingly similar to that of PTH1R, which includes punctate expression in the apical domain and prominent staining of intracellular sites and the basolateral membranes (Fig. 7, A and B).

Actin Is an Essential Component of the NHERF-1-Assembled Complex—NHERF-1 indirectly interacts with the actin cytoskeleton through its direct association with MERM proteins, such as ezrin in renal epithelia. A circumferential band of actin microfilaments, representing the zonula adherens, is readily detected in both OKH and OKH-N1 cells (Fig. 8A). OKH cells, however, lack the actin-containing apical patches that are readily detected in OKH-N1 cells (Fig. 8A). Thus, NHERF-1 exactly localizes to these actin-containing apical patches in OKH-N1 cells (Fig. 8A), but this co-localization is not observed in OKH cells, presumably because of the low levels of NHERF-1 expressed and its apparent role in the generation of apical patches. Furthermore, PTH1R-YFP and PLCβ3 transiently expressed by OKH-N1 cells also co-localize with actin in these apical structures (Fig. 8B). These data suggest that NHERF-1 directly assembles the receptor and its effector and indirectly assembles this complex with the actin cytoskeleton through direct interactions with ezrin or other MERM proteins.

To further test the hypothesis that NHERF-1 and actin anchor an assembled signaling complex containing the PTH1R and PLCβ to the apical domain of OKH-N1 cells, OKH-N1 cells were treated with cytochalasin D, a fungal toxin that polymerizes actin microfilaments. Cytochalasin D induces actin aggregates, which is accompanied by loss of apical patches and

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**Fig. 7. Localization of PLCβ1 and PLCβ3 in OKH cells.** OKH cells were transfected with either flag-PLCβ1 (A) or flag-PLCβ3 (B) and immunostained with NHERF-1 antibodies (Alexa Fluor 546, red) and flag-monomoclonal antibodies (Alexa Fluor 488, green). Representative confocal images of the apical (AP) and basolateral (BL) domains of the cells are shown.

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**Fig. 8. Actin is a primary component of the NHERF-1-assembled apical complexes.** A, OKH and OKH-N1 cells were stained for actin (Alexa Fluor 546-phalloidin, red) and NHERF-1 antibodies (Alexa Fluor 488, green). Representative confocal images of the apical domains of the cells are shown. B, OKH-N1 cells were transiently transfected with either PTH1R-YFP (green) or flag-PLCβ3 and stained for actin (Alexa Fluor 546-phalloidin, red) and flag-monomoclonal antibodies (Alexa Fluor 488, green) for PLCβ3 only. Confocal images of apical domains of representative cells are shown.
co-localization of actin with NHERF-1 (Fig. 9A). Furthermore, cytochalasin D strikingly disrupts the apical localization of the PTH1R, resulting in its prominent redistribution to intracellular sites (Fig. 9B), and it also inhibits the PTH-elicited increase in \([\text{Ca}^{2+}]_i\), in a dose-dependent manner (Fig. 10), suggesting that the NHERF-1-assembled complex of PTH1R, PLC\(\beta\), and actin are necessary to mediate this hormone response. It is perhaps not surprising that cytochalasin D has no effect on PTH-mediated signaling via AC and accumulation of cAMP (data not shown).

NHERF-1, lacking the C-terminal MERM-binding domain, has been shown to function as a dominant-negative interactor with respect to regulation of Na\(^{+}/\text{H}^+\) exchanger 3 (30) and apical localization of Npt2 in OK cells (22). PDZ1long of NHERF-1 transiently expressed in OKH-N1 cells disrupts PTH1R localization to apical patches (Fig. 11A), which is associated with a striking reduction of the PTH-mediated increase in \([\text{Ca}^{2+}]_i\) (Fig. 11B).

**DISCUSSION**

Herein, we report that NHERF-1 mediates PTH-stimulated entry of extracellular calcium in OK cells, by a mechanism that is PLC-dependent, is pertussis toxin-sensitive, and requires an intact actin cytoskeleton. The calcium entry seems likely to be mediated by a receptor-operated calcium channel, which we were unable to define, probably because antibodies to these channel proteins do not cross-react well, if at all, with proteins from OK cells in our control studies (data not shown). We present evidence that NHERF-1 assembles a complex in the apical patches of OK cells that contains the PTH1R, phospholipase C, and actin, which directs the PTH-mediated influx of calcium. NHERF-1 binding to actin is essential to establish apical patches. Furthermore, NHERF-1 is essential for anchoring the PTH1R and PLC\(\beta\)s to these same apical structures.

PTH has been shown repeatedly to increase \([\text{Ca}^{2+}]_i\), either by causing calcium release from intracellular stores or by increasing calcium influx from the extracellular space in many cell models (31). In primary cultures of proximal tubule cells and cultures of the OK cell line, PTH-mediated influx of calcium is the predominant mechanism for increase in \([\text{Ca}^{2+}]_i\), but there is a substantially lesser component because of calcium release from intracellular stores (16, 24, 27, 28). We found that the PTH-elicited \([\text{Ca}^{2+}]_i\) response is pertussis toxin-sensitive in OK cells, which is consistent with earlier reports in several cell systems.
renal cell models (24, 28) and with our earlier report demonstrating that the NHERF-2-assembled PTH1R activates PLC by stimulating a member of the \(G_{\text{q}}\) family of G proteins (17). Overall, PTH signaling in OKH cells with stable expression of NHERF-1 is consistent with PTH-mediated responses displayed in PCT and wild-type OK cells.

At first glance, the PLC\(\beta\) dependence of the PTH-induced increase of \(\left[\text{Ca}^2+\right]_i\), would suggest that it is released from intracellular stores in response to the production of inositol trisphosphates. However, the inhibition of the PTH-mediated rise of \(\left[\text{Ca}^2+\right]_i\), in the absence of extracellular calcium or in the presence of a calcium channel blocker (SKF96365), treatments that we show do not inhibit thrombin-mediated \(\left[\text{Ca}^2+\right]_i\), responses, strongly suggests that the increased \(\left[\text{Ca}^2+\right]_i\), stimulated by PTH1R is the result of calcium influx, perhaps as a result of the effects of diacylglyceride directly or indirectly on a calcium channel or activation of a receptor-operated channel. The PTH-mediated rise of \(\left[\text{Ca}^2+\right]_i\), nonetheless is clearly the result of hormonal stimulation of the NHERF-1-PTH1R complex and the consequent activation of PLC\(\beta\) in the OKH-N1 cell model.

PDZ domain proteins, including NHERFs, usually contain more than one PDZ domain and often contain other motifs that associate directly with proteins (8, 9, 32) and can form homo- and hetero-oligomers (33). These properties are not mutually exclusive. Thus, several mechanisms by which PDZ-domain proteins assemble multicomponent protein complexes exist. PDZ-domain proteins are commonly expressed adjacent to the plasma membrane of the cell, where they assemble specialized protein complexes in which the concentration of components is usually high because they have limited capacity to diffuse to other locations within the cell (8, 34). A particularly well-studied PDZ-domain protein in Drosophila melanogaster, INAD, contains five PDZ domains that facilitate rhodopsin signaling in photoreceptors by assembling a complex consisting of two PLC molecules, two protein kinase C molecule(s), and a transient-receptor potential calcium channel, which is anchored to the actin cytoskeleton (35, 36). To our knowledge, the protein interactive sites of NHERF-1 are limited to two PDZ domains and a MERM binding domain. However, NHERF-1 forms both homo- and hetero-oligomers and thus potentially serves as a scaffold to assemble many diverse proteins within the microenvironment adjacent to the cell’s membrane.

Additional complexities come from the observation that the different PDZ domains within a multi-PDZ-domain protein display varying affinities for binding partners. Selective binding to a specific PDZ domain strongly suggests that determinants within a given domain dictate the interaction. Previous studies have shown preferential binding of a protein to the same PDZ domains of NHERF-1 and -2 (37). This is most probably caused by the higher degree of similarity between the same PDZ domains of the NHERFs. PLC\(\beta\)1 and the PTH1R preferentially bind to PDZ1 and PDZ2 of NHERF-2, respectively, thus allowing a single NHERF-2 molecule to form a complex in vitro that contains both the receptor and its effector (17). The PTH1R preferentially and unexpectedly binds to the PDZ1 of NHERF-1 rather than PDZ2. However, sequence comparisons reveal notable similarities between PDZ1 of NHERF-1 and PDZ2 of NHERF-2 that may dictate the binding specificities with the PTH1R. Furthermore, our data show that PDZ1 of NHERF-1 is also the preferred binding site for PLC\(\beta\)1, -\(\beta\)2, and -\(\beta\)3 (Fig. 7A). We do not have a ready explanation for the difference between our results and those of Hwang et al. (38) showing preferential binding of PLC\(\beta\)3 to PDZ2 of NHERF-2 and no binding to NHERF-1. Proteins binding to the same PDZ domain might be expected to preclude the formation of a complex. However, NHERF-1 forms homo-oligomeric complexes (39, 40) as well as complexes with NHERF-2 (33). Oligomerization coupled with high expression levels suggest that NHERF proteins establish a subapical scaffold that contains multiple binding sites.

As shown, PTH1R-C\(\alpha\), which does not bind NHERF-1, is predominantly expressed in intracellular sites and on the basolateral membrane (Fig. 5B). These findings suggest that NHERF-1 targets and/or anchors the PTH1R to the apical patches in OK cells. NHERF-1 also promotes apical localization of several membrane-bound proteins, including the cystic fibrosis transmembrane conductance regulator in Madin-Darby canine kidney cells (41), the TRPC4 calcium channel in human embryonic kidney 293 cells (42), Npt2 in OK cells (22), and mouse PCT (20). Furthermore, PTH1R complexes with NHERF-1 is insensitive to internalization mediated by the antagonist PTH (7–34), suggesting that NHERF-1 anchors the receptor more firmly to the membrane compartment (43).

As mentioned previously, all four isozymes of PLC\(\beta\) have canonical C-terminal PDZ interaction motifs. Both in vitro and intracellular interactions between NHERF-1 and PLC\(\beta\)1 and -\(\beta\)2 have been reported (44). Furthermore, NHERF-2 has been shown to augment activation of PLC\(\beta\) by the muscarinic receptor, presumably through a direct interaction (38). Our findings demonstrate that PLC\(\beta\)1 and -\(\beta\)2 co-localize with NHERF-1 in apical patches of OKH-N1 cells (Fig. 6B), but despite a strong interaction in vitro, PLC\(\beta\)2 does not co-localize with NHERF-1 in the apical domain (Fig. 6B) but is expressed in the cytoplasm and associates with basolateral membranes, suggesting that PLC\(\beta\)2 is not targeted to the apical surface of the cell. It is notable that the PTH1R co-localizes with both PLC\(\beta\)1 and -\(\beta\)3 in apical patches of OKH-N1 cells (Fig. 6C). This, together with other data, shows that NHERF-1 assembles a PTH1R/PLC complex in this polarized cell. In a function analogous to that of INAD in the D. melanogaster photoreceptor, NHERF-1 couples the PTH1R to its downstream effector molecule.

The current report documents a functional interaction between NHERF-1, the PTH1R, PLC\(\beta\), and, through binding MERM proteins, the actin cytoskeleton. The interaction of NHERF-1 with MERM proteins that bind the actin cytoskeleton establishes, and perhaps maintains, apical patches. Expression of NHERF-1 restores PTH elicited increases of \(\left[\text{Ca}^{2+}\right]_i\) in OKH cells that are deficient in NHERF-1, through calcium influx that is possibly mediated by a receptor-operated calcium channel. Most critically, NHERF-1 assembles an apical signaling complex that is essential for mediating calcium entry but plays no role in the PTH-stimulated accumulation of cAMP.

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