Parathyroid Hormone Regulation of Na^+,K^+-ATPase Requires the PDZ 1 Domain of Sodium Hydrogen Exchanger Regulatory Factor-1 in Opossum Kidney Cells

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It was demonstrated that expression of murine sodium hydrogen exchanger regulatory factor (NHERF-1) lacking the ezrin-binding domain blocks parathyroid hormone (PTH) regulation of Na^+,K^+-ATPase in opossum kidney (OK) cells. The hypothesis that the NHERF-1 PDZ domains contribute to PTH regulation of Na^+,K^+-ATPase was tested by comparison of PTH regulation of Na^+,K^+-ATPase in wild-type OK (OK-WT) cells, NHERF-deficient OKH cells, OK-WT transfected with siRNA for NHERF (NHERF siRNA OK-WT), and OKH cells that were stably transfected with full-length NHERF-1 or constructs with mutated PDZ domains. OKH cells and NHERF siRNA OK-WT showed decreased expression of NHERF-1 but equivalent expression of ezrin and Na^+,K^+-ATPase α_1 subunit when compared with OK-WT cells. PTH decreased Na^+,K^+-ATPase activity and stimulated phosphorylation of the Na^+,K^+-ATPase α_1 in OK-WT cells but not in NHERF-deficient cells. Rubidium (^{86}Rb) uptake was equivalent in OK-WT, OKH, and OKH cells that were transfected with all but the double PDZ domain mutants. PTH decreased ^{86}Rb uptake significantly in OK-WT but not in OKH cells. PTH also significantly inhibited ^{86}Rb uptake in OKH cells that were transfected with full-length NHERF-1 or NHERF-1 with mutated PDZ 2 but not in OKH cells that were transfected with mutated PDZ 1. Transfection with NHERF expressing both mutated PDZ domains resulted in diminished basal ^{86}Rb uptake that was not inhibited further by PTH. PTH stimulated protein kinase Cα activity and α_1 subunit phosphorylation in OK-WT but not in NHERF-deficient cells. Transfection of OKH cells with NHERF constructs that contained an intact PDZ1 domain restored PTH-stimulated protein kinase Cα activity and α_1 subunit phosphorylation. These results demonstrate that NHERF-1 is necessary for PTH-mediated inhibition of Na^+,K^+-ATPase activity and that the inhibition is mediated through the PDZ1, not PDZ2, domain.

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Na^+,K^+-ATPase is a highly abundant basolateral membrane (BLM) protein in proximal renal tubule that is responsible for the maintenance of intracellular sodium and potassium balance (1). The activity of this protein creates the driving force for the transport of sodium from the tubular lumen to the renal vasculature, a determining factor in regulation of total body sodium homeostasis, extracellular fluid volume status, and BP control. Abnormalities in the regulation of Na^+,K^+-ATPase activity have been implicated in the pathogenesis of some forms of salt-sensitive hypertension. Na^+,K^+-ATPase activity is regulated by a multitude of hormones and neuropeptides through activation of several signaling pathways (1). One of the major regulators of proximal renal tubule sodium pump activity is parathyroid hormone (PTH) (2,3).

Mahon *et al.* (4,5) demonstrated a critical role for sodium hydrogen exchanger regulatory factor (NHERF) in the regulation of PTH receptor signaling. NHERF are PDZ (PSD-95, *Drosophila* discs large, ZO-1) proteins that are centrally involved in regulation of proximal renal tubule cell transport processes (6–11). Two members of the NHERF family have been described to exhibit unique expression patterns and functions in kidney (12–15). In mouse kidney, NHERF-1 is expressed predominantly in the microvilli of the proximal tubule in association with NHE-3, the type IIa sodium-phosphate co-transporter (NaPi IIa), and ezrin and is responsible for facilitation of cAMP-mediated regulation of NHE-3 and apical membrane targeting of NaPi IIa. NHERF-2 is expressed at the base of the microvilli associated with NHE-3 and ezrin but not NaPi IIa and facilitates calcium-mediated regulation of NHE-3.

The two distinct PDZ domains of NHERF exhibit divergent

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amino acid sequences and binding specificities that permit the construction and localization of extended heterogeneous protein interaction units (16–18). For example, the N terminal PDZ domain (amino acids 77 to 82; PDZ1) binds to the β2 adrenergic receptor (19), NaPi IIa (20), phospholipase C (PLC) β1,2 (21), and RACK-1 (22), whereas the C terminal PDZ domain (amino acids 217 to 222; PDZ2) binds to NHE-3 (23) and ROMK (24). Some proteins, notably the cystic fibrosis conductance regulator, are capable of binding either PDZ domain (25,26).

We recently demonstrated that expression of a murine NHERF-1 construct lacking the C-terminal ezrin-binding domain (amino acids 325 to 355) completely abolishes regulation of Na^+,K^+-ATPase activity by PTH in opossum kidney (OK) cells, a model of mammalian renal proximal tubule (27). The role of the two PDZ domains of NHERF-1 in the regulation of Na^+,K^+-ATPase activity and expression remains unknown. Binding of NHE-3 to the PDZ2 domain of NHERF-1 is necessary for its regulation by PTH (23). In addition, Breton *et al.* and others (28,29) have shown that the B1 subunit of H^+-ATPase, a member of the V-type ATPases, co-localizes through its PDZ-binding domain with NHERF. On the basis of these observations, we hypothesized that one or both PDZ domains of NHERF are also required for PTH regulation of Na^+,K^+-ATPase.

To address this hypothesis, we compared the ability of PTH to regulate α_1 subunit phosphorylation and Na^+,K^+-ATPase activity in wild-type OK cells (OK-WT) and in two models of NHERF-deficient OK cells. OK-WT cells express only NHERF-1 and not NHERF-2, making them an excellent model to investigate the role of this specific NHERF isoform in regulation of transport in the proximal renal tubule cell (30). To examine the specific role of the two PDZ domains, we stably transfected a NHERF-deficient OK cell line, OKH cells (31–33), with either full-length human NHERF-1 or NHERF-1 constructs expressing nonfunctional PDZ 1, PDZ 2, or both PDZ domains. We demonstrated that the ability of PTH to phosphorylate the α1 subunit and to regulate Na^+,K^+-ATPase activity was dependent on the expression of NHERF-1 with a normal PDZ 1 domain.

Materials and Methods

PTH (1-34) was purchased from Bachem Biosciences Inc. (King of Prussia, PA). Polyclonal antibodies against Na^+,K^+-ATPase α_1-subunit (for immunoprecipitation [IP]) and protein kinase C (PKC) activity kit were purchased from Upstate Biotechnology (Waltham, MA). MAb against Na^+,K^+-ATPase α_1-subunit (for Western blots) were purchased from Sigma-RBI (Natick, MA). Antibodies against ezrin were purchased from Sigma (St. Louis, MO). Antibodies against PKCα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphoserine antibodies were purchased from Zymed (San Francisco, CA). Geneporter transfection and Genesilencer siRNA transfection reagents were purchased from Gene Therapy Systems Inc. (San Diego, CA). All other chemicals were purchased from Sigma unless otherwise specified. Anti-NHERF antibodies were previously characterized by E.J.W. and his colleagues (14).

NHERF Mutations

The human NHERF-1 cDNA was inserted into pET-30-(a)$^+$ (Novagen, Madison, WI) to generate a hexahistidine-fused NHERF-1 (His-NHERF-1), which then was transferred to pcDNA 3.1Hygro$^+$ for expression in mammalian cells. Similarly, cDNA encoding NHERF-1 with alanine substitutions (GAGA) in the core peptide-binding sequence, GYGF (amino acids 77 to 82 and 217 to 222), that inactivates the individual PDZ domains were inserted into pcDNA 3.1Hygro$^+$. All cDNA were confirmed by double-stranded DNA sequencing (23).

Cell Culture

The OK cells are a continuous cell line derived from Virginia opossum and a widely used model for mammalian renal proximal tubule (34). OKH cells are a clonal subline of the parental OK cell line that lacks the expression of NHERF (31–33). Vector (pcDNA 3.1Hygro$^+$) transfected wild-type OK and OKH cells and OKH cells that were stably transfected with full-length human and PDZ domains–mutated NHERF-1 [hNHERF-1 (1-355)] were maintained at 37°C in a humidified atmosphere with 5% CO_2 in DMEM/F12 medium (1:1) supplemented with 10% vol/vol FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml) in presence of 600 U/ml hygromycin. The cells were fed twice a week and split once a week at a 1:4 ratio. All experiments were carried out using cells at 90 to 95% confluence. Cells that were grown on six-well culture plates were washed with serum-free medium 24 h before use.

Preparation and Purification of NHERF siRNA

NHERF siRNA sense and antisense oligonucleotides were designed by using Ambion's web-based siRNA converter software (Austin, TX). The software identified a unique sequence (5′-AAGUCUAAGCCAGGCCAGGUUC-3′) in the N-terminal region of NHERF against which siRNA was designed. The siRNA was prepared by using siRNA construction kit from Ambion according to the manufacturer's protocol. Briefly, sense and antisense oligonucleotides were hybridized separately to T7 promoter primer followed by a Klenow DNA polymerase reaction. The sense and antisense siRNA templates were transcribed independently using T7 enzyme and mixed to form double-stranded siRNA (dsRNA). The siRNA was digested with RNase and DNase to remove the 5′ overhanging leader sequences and DNA template. The resulting siRNA was purified by column purification.

Transfection of siRNA

One microgram of NHERF siRNA was transfected into OK cells using Genesilencer Transfection reagent according to the manufacturer's protocol. Briefly, 5 μl of Genesilencer reagent was diluted with 25 μl of serum-free EMEM. In another tube, 1 μg of siRNA was diluted in 25 μl of siRNA diluent and 15 μl of serum-free EMEM. The siRNA-containing mixture was mixed with diluted Genesilencer reagent and incubated at room temperature for 15 min. The siRNA Genesilencer reagent mixture was added onto growing cells in serum-free EMEM and incubated at 37°C in 95% air/5% CO_2 for 6 h, after which 1 ml of EMEM that contained 20% serum was added and further incubated for 24 h at 37°C in 95% air/5% CO_2.

NHERF cDNA Transfection

Vector (pcDNA 3.1Hygro$^+$) or human NHERF-1 and PDZ mutated human NHERF cDNA constructs in pcDNA 3.1Hygro$^+$ were transfected into OKH cells using Geneporter transfection reagent according to the manufacturer's protocol. Briefly, the cDNA and the Geneporter reagent were diluted separately in serum-free medium. The diluted cDNA was mixed with diluted Geneporter reagent and incubated at room temperature for 30 min. The culture medium from the cells was replaced with the mixture that contained Geneporter and NHERF cDNA and incubated for 24 h at 37°C in 95% air/5% CO_2. The trans-

fected cells were selected by growing them in 1000 U/ml hygromycin in a 96-well cell culture plate for 2 wk. Cells from the hygromycin-resistant wells were grown in DMEM/F12 that contained 600 U/ml hygromycin and 10% FCS. Expression of NHERF was confirmed by Western blot analysis.

Treatment with PTH

Unless otherwise stated, cells were treated for 15 min with 10^{-7} M PTH 1 to 34 at 37°C in 95% air/5% CO_2.

Membrane Preparation

The cells were treated with 10^{-7} M PTH (bovine, 1 to 34) or vehicle, washed twice with PBS, and homogenized in 50 mM mannitol/5 mM Tris (pH 7.4), and the crude membranes were prepared as described previously (27).

Western Blot

Western blot analysis for NHERF, Na^+,K^+-ATPase α_1 subunit, PKCα, and ezrin was performed exactly as described previously (27).

Determination of Na^+,K^+-ATPase Activity as K^+-Dependent pNPPase Activity

Na^+,K^+-ATPase activity was measured as described previously (27) following the method of Hird *et al.* (35) with slight modifications as described by Tran and Farley (36).

Immunoprecipitation

The crude membranes were solubilized in IP buffer that contained 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 20 mM NaF, 1 mM EDTA, 1 mM EGTA, 5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1 mM sodium pyrophosphate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1% Triton X-100, 0.5% NP40, and 0.5% SDS and centrifuged at 70,000 $\times$ *g* for 1 h in a Beckman ultracentrifuge. Na^+,K^+-ATPase α_1-subunit or PKCα was immunoprecipitated from 100 μg of supernatant protein overnight at 4°C as described previously (37). Western blotting was performed as previously reported (27).

Rubidium Uptake

Ouabain-sensitive rubidium (^{86}Rb) uptake was measured as an index of Na^+,K^+-ATPase–mediated ion transport as described previously (37) following the method of Okafor *et al.* (38).

Determination of PKCα Activity

OK cells were incubated in the presence or absence of 10^{-7} M PTH for 15 min. The cells were washed two times with PBS (pH 7.4) and lysed in IP buffer. PKCα was immunoprecipitated from 100 μg of whole-cell lysate protein as described above. The beads were washed three times with IP buffer, and PKC activity was determined as described previously (37).

Confocal Imaging

Multichambered coverglass wells (Nunc, Naperville, CT) were seeded with OK cells. Cells were washed with serum-free medium 24 h before fixation. Cells were rinsed three times with PBS that contained calcium and magnesium, incubated in 4% paraformaldehyde in PBS for 10 min, rinsed five times with PBS, solubilized with 0.025% saponin in PBS for 15 min, incubated with an appropriate dilution of primary antibody (1:250 in PBS-Saponin for both NHERF and Na^+,K^+-ATPase α_1 subunit antibodies) at 20°C, rinsed five times with PBS-Saponin, and incubated with appropriate Alexaflour secondary antibody (1:1000) conjugated to different fluorescence tags at 20°C. Alexaflour488 was used for identification of NHERF, and Alexaflour555 was used for identification of the α subunit. The cells were rinsed five times with PBS-Saponin, incubated with 300 nM DAPI for 5 min, rinsed three times with PBS, and mounted with 300 μl/well PBS. Images were acquired using a Zeiss confocal microscope and analyzed using LSM510 software. Z scan analysis on single cells was performed by scanning at 1-μm intervals and three-dimensional reconstruction of the fluorescence images. The images for NHERF and for the α subunit were merged in a single image to compare the cellular distribution of the two proteins.

Brush border membranes (BBM) and BLM were prepared from the same cell preparations as described previously (3,39). Protein concentration was measured by BCA method (Sigma) using BSA as standard.

Statistical Analyses

Data are shown as mean ± SEM. All experiments were repeated at least three times unless otherwise stated to document reproducibility. *P* value was calculated using SigmaStat software using paired *t* test. $P < 0.05$ was *a priori* considered statistically significant.

Results

Characterization of Model Systems

We previously demonstrated that OK-WT express NHERF-1 (27). OKH cells, initially identified as OK cells with aberrant PTH signaling, exhibit significantly diminished NHERF-1 expression, as shown in Figure 1, confirming findings previously shown by other investigators (31); however, expression of Na^+,K^+-ATPase α_1 subunit and ezrin in OKH cells was equivalent to OK-WT. The Western blot also indicates that PTH did not change the expression of Na^+,K^+-ATPase α_1 subunit and ezrin in the crude membranes from OK-WT and OKH cells. We also developed from WT-OK strains with diminished NHERF-1 expression through the use of siRNA technology. Immunoblot analysis of NHERF siRNA OK-WT shows a marked reduction in NHERF-1 expression but intact expression of Na^+,K^+-ATPase α_1 subunit and ezrin.

Effect of NHERF Deficiency on PTH Regulation of Na^+,K^+-ATPase Activity

To examine the effect of NHERF deficiency on regulation of the sodium pump, we compared Na^+,K^+-ATPase activity in OK-WT cells, OKH cells, and NHERF siRNA OK-WT cells. As seen in Figure 2A, treatment with 10^{-7} M PTH for 15 min produced an approximately 40% reduction in Na^+,K^+-ATPase activity in OK-WT cells but no reduction in OKH cells. PTH also had no effect on activity in NHERF siRNA OK-WT cells (Figure 2B).

Effect of NHERF Deficiency on Na^+,K^+-ATPase α_1 Subunit Phosphorylation

PTH regulation of Na^+,K^+-ATPase activity requires phosphorylation of the α_1 subunit accompanied by endocytosis of the subunit, resulting in decreased enzyme expression on the plasma membrane and therefore decreased activity (40). We previously demonstrated that expression of the NHERF construct lacking the ezrin-binding domain blocked PTH-stimulated phosphorylation of the α_1 subunit (27). To determine whether NHERF deficiency had a similar inhibitory effect on

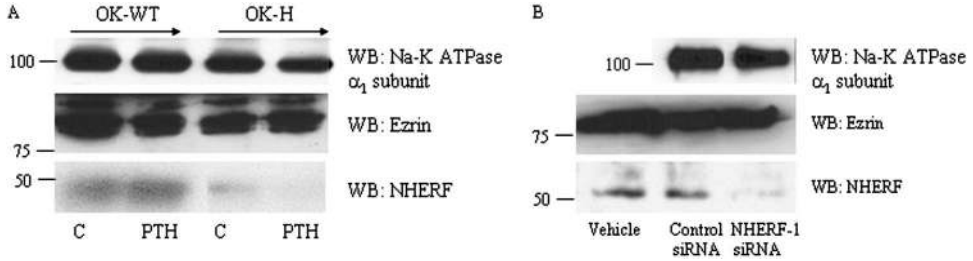

Figure 1. Comparison of expression of sodium hydrogen exchanger regulatory factor (NHERF), ezrin, and Na^+,K^+-ATPase α_1 subunit in wild-type opossum kidney OK cells (OK-WT) and NHERF-deficient OK cells. (A) Crude membranes from OK-WT or NHERF-deficient OKH cells were separated by SDS-PAGE; transferred to solid matrix; and blotted for NHERF, Na^+,K^+-ATPase α_1 subunit, and ezrin. (B) Crude membranes from OK-WT or OK-WT cells that were treated with siRNA for NHERF-1 (NHERF siRNA OK-WT) were separated by SDS-PAGE; transferred to solid matrix; and blotted for NHERF, Na^+,K^+-ATPase α_1 subunit, and ezrin. Representative Western blots from three independent experiments are shown.

PTH regulation, we measured PTH-stimulated phosphorylation of the α_1 subunit in OK-WT, OKH, and NHERF siRNA OK-WT cells. As shown in Figure 3, PTH stimulated phosphorylation of Na^+,K^+-ATPase α_1 subunit in OK-WT cells but not in NHERF-deficient models.

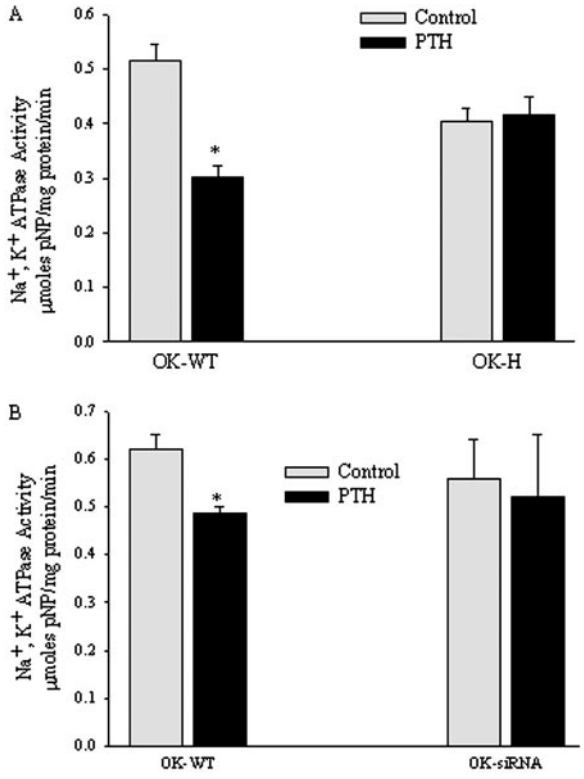

Figure 2. Comparison of Na^+,K^+-ATPase activity in OK-WT and NHERF-deficient OK cells. OK cell monolayers were treated for 15 min with either vehicle or 10^{-7} M parathyroid hormone (PTH). The cells were lysed, and membrane fractions were isolated and assayed for Na^+,K^+-ATPase activity as described in Materials and Methods. Each bar represents Na^+,K^+-ATPase activity as μmol p-nitrophenol released/mg protein per min and is represented as mean ± SEM from three independent experiments. *Significantly different from control with $P < 0.05$. (A) Effect of PTH on OK-WT and OKH cells. (B) Effect of PTH on OK-WT cells that were treated with control siRNA for NHERF or active siRNA for NHERF.

Effect of NHERF Deficiency on PTH Activation of Extracellular Signal-Regulated Kinase and PKCα

We have shown that PTH stimulation of PKCα through an extracellular signal-regulated kinase (ERK)-dependent pathway is critical for regulation of Na^+,K^+-ATPase activity (37). To determine whether PTH-stimulated activation of these signaling pathways was impaired in NHERF-deficient OKH cells, we examined the ability of PTH to stimulate ERK phosphorylation and PKCα translocation to the membrane fragment. As shown in Figure 4A, PTH stimulation of ERK phosphorylation is intact in the OKH model system. Western blot analysis of PKCα in the membrane fraction (Figure 4B) shows PTH-stimulated increased membrane expression of PKCα in OK-WT but not in NHERF-deficient OKH cells. A concomitant decrease in the PKCα expression was observed in the cytosol from OK-WT cells (data not shown).

Role of PDZ Domains in Regulation of Na^+,K^+-ATPase Activity by PTH

For determining the roles of the PDZ domains in NHERF regulation of Na^+,K^+-ATPase by PTH, OKH cells were transfected with human NHERF-1 constructs, including full-length NHERF-1 and constructs expressing mutations in PDZ1, PDZ2, or both PDZ domains. Figure 5A shows successful transfection of these constructs into OKH cells and similar expression of Na^+,K^+-ATPase α_1 subunit. Treatment with 10^{-7} M PTH for 15 min produced a significant inhibition in ouabain-sensitive ^{86}Rb uptake, a measure of Na^+,K^+-ATPase activity, in OK-WT but not in OKH cells (Figure 5B). Transfection of full-length NHERF to OKH cells restored normal inhibition of ^{86}Rb uptake by PTH, confirming that the defect in PTH regulation of Na^+,K^+-ATPase activity in OKH cells was due to NHERF deficiency. Basal Na^+,K^+-ATPase–mediated ^{86}Rb uptake was significantly decreased in OKH cells that were transfected with NHERF construct expressing both PDZ mutations compared with the OK-WT or OKH cells and did not change significantly upon treatment with PTH. Similar to OKH cells that were transfected with full-length NHERF, cells expressing the PDZ2

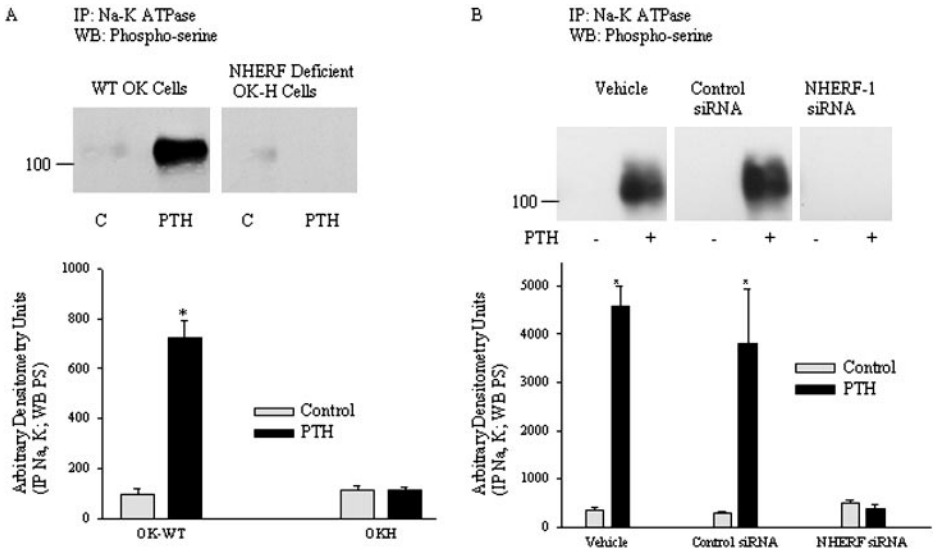

Figure 3. Comparison of PTH-stimulated phosphorylation of the Na^+,K^+-ATPase α_1 subunit in OK-WT and NHERF-deficient OK cells. OK cells were treated for 15 min with vehicle or 10^{-7} M PTH followed by lysis of cells and preparation of crude membranes. The crude membranes were immunoprecipitated with antibody directed against the Na^+,K^+-ATPase α_1 subunit and blotted for phosphoserine. A representative Western blot is shown. (A) Effect of PTH on α_1 subunit phosphorylation in OK-WT and OKH cells. (B) Comparison for OK-WT cells that were treated with siRNA for NHERF or control siRNA. Bar diagrams show densitometry data as mean ± SEM from three independent experiments.

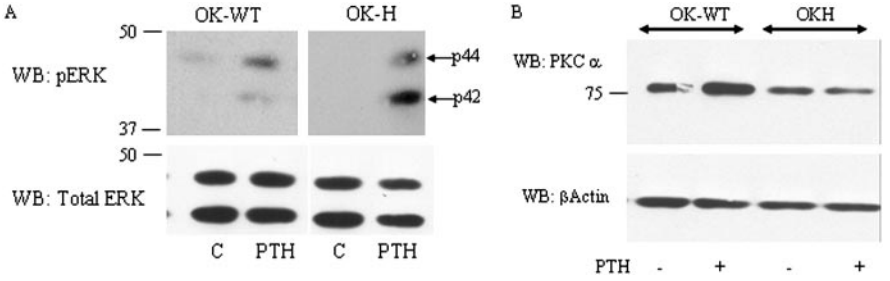

Figure 4. Comparison of PTH-mediated extracellular signal-regulated kinase (ERK) phosphorylation and protein kinase Cα (PKCα) translocation in OK-WT and OKH cells. OK cell monolayers were treated for 15 min with vehicle or 10^{-7} M PTH. (A) Cells were lysed in immunoprecipitation buffer, and lysate proteins were separated by SDS-PAGE, transferred to solid matrix, and blotted for phospho-ERK (active form) and total ERK. (B) Cells were washed and lysed in PKC lysis buffer, and membrane and cytosolic fractions were separated as described in Materials and Methods. The membrane proteins were separated by SDS-PAGE and transferred onto a solid phase, and the expression of PKCα was determined by Western blot analysis. Representative Western blots from three independent experiments are shown.

mutation showed normal PTH-mediated inhibition of ^{86}Rb uptake.

Figure 6 shows the effect of transfection of the NHERF constructs on the ability of PTH to stimulate phosphorylation of the α_1 subunit of Na^+,K^+-ATPase (Figure 6A) and activate PKCα (Figure 6B). As expected, PTH stimulated phosphorylation in OK-WT but not in OKH cells. Cells that were transfected with full-length NHERF or the NHERF construct expressing the mutated PDZ2 domain showed partial restoration of PTH-stimulated phosphorylation of the α_1 subunit. In contrast, OKH cells that were transfected with the construct expressing the mutated PDZ1 domain failed to undergo α_1 subunit phosphorylation in response to PTH. OKH cells that were transfected with the NHERF construct expressing both PDZ domain mutations have significantly increased basal phosphorylation of the α_1 subunit that did not change with PTH treatment, indicated by densitometric analysis. PTH caused an approximately 50% increase in PKCα activity in OK-WT but not in OKH cells. PTH also significantly increased the activity of PKCα in OKH cells that were transfected with full-length NHERF-1 and PDZ2 mutated NHERF-1 but not in PDZ1 or both PDZ domain–mutated NHERF-1–transfected OKH cells.

Comparison of Cellular Distribution of NHERF and Na^+,K^+-ATPase α_1 Subunit

Figure 7 shows the confocal images of OK cells stained with antibody for NHERF (green fluorescence) and the Na^+,K^+-ATPase α_1 subunit (red fluorescence). OK-WT cells (Figure 7A)

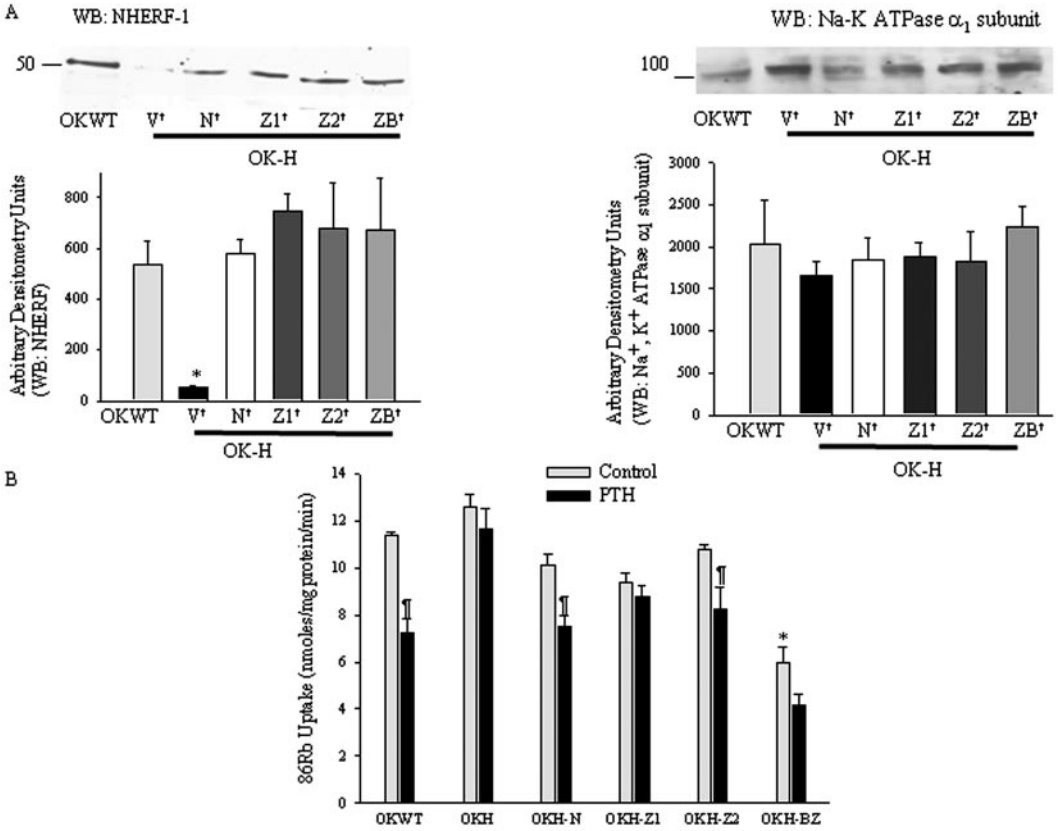

Figure 5. Comparison of Na^+,K^+-ATPase–mediated rubidium (^{86}Rb) uptake in OKH cells that were transfected with NHERF-1 constructs. (A) Expression of full-length human NHERF-1 (OKH-N) or NHERF-1 constructs expressing null mutations in the PDZ1 domain (OKH-Z1), the PDZ2 domain (OKH-Z2), or both domains (OKH-BZ) stably transfected into OKH cells. (Bottom) Expression of Na^+,K^+-ATPase α_1 subunit. Bar diagram shows densitometric data as mean ± SEM from three independent experiments. (B) OK cell monolayers of OK-WT, OKH, and OKH cells that were transfected with full-length NHERF-1 or NHERF-1 constructs and expressed mutations in the PDZ domains were treated for 15 min with vehicle or 10^{-7} M PTH. Ouabain-sensitive ^{86}Rb uptake was performed in the intact cell monolayers as described in Materials and Methods. Each bar represents nM ^{86}Rb/mg protein per min as mean ± SEM from three independent experiments. *Statistically significant difference between basal uptake in OK-WT and OKH cells expressing null mutations in NHERF-1 both PDZ domains; ¶statistically significant difference between PTH-treated and vehicle-treated, $P < 0.05$.

demonstrate a pattern of predominantly basolateral distribution of the α_1 subunit. NHERF staining was observed at both the apical and the basolateral sides. OKH cells show a similar distribution for the α_1 subunit but no detectable NHERF (Figure 7B). Figure 7C shows the distribution of NHERF and the α_1 subunit in OKH cells that were transfected with full-length NHERF, demonstrating that the transfected protein assumes a similar distribution as the OK-WT cells. Similar distribution of NHERF and Na^+,K^+-ATPase α_1 subunit was observed in NHERF mutant construct–transfected (PDZ1, PDZ2, and both PDZ domains) cells as seen in WT cells (data not shown). As other investigators have not consistently demonstrated basolateral expression of NHERF, BBM and BLM were prepared and subjected to Western blot analysis. As shown in Figure 7D, NHERF expression is observed in both the BBM and BLM preparations. BBM and BLM preparations were confirmed by Western blot analysis of NaPi-4 and Na^+,K^+-ATPase α_1 subunit (Figure 7D, bottom).

Discussion

We have demonstrated in two different models of NHERF-deficient OK proximal tubule cells that NHERF-1 is required for regulation of Na^+,K^+-ATPase activity by PTH. We also demonstrated that the PDZ1 domain is critical for this function. Transfection of NHERF-deficient cells with human NHERF-1 constructs that contained an intact PDZ 1 domain, specifically either full-length NHERF-1 or NHERF-1 with a null mutation in the PDZ2 domain, restored PTH regulation of the sodium pump. In contrast, expression of constructs that exhibited a null mutation in the PDZ1 domain failed to rescue PTH regulation of Na^+,K^+-ATPase activity. The requirement for an intact PDZ1 domain in PTH regulation of the sodium pump is consistent with previously published studies by Mahon and Segre (4) demonstrating that PTH activates an apical membrane complex in renal proximal tubule cells assembled through PTH1 receptor interaction with the PDZ1 domain of NHERF and consisting of the PTH1 receptor, PLCβ1, and actin. The authors

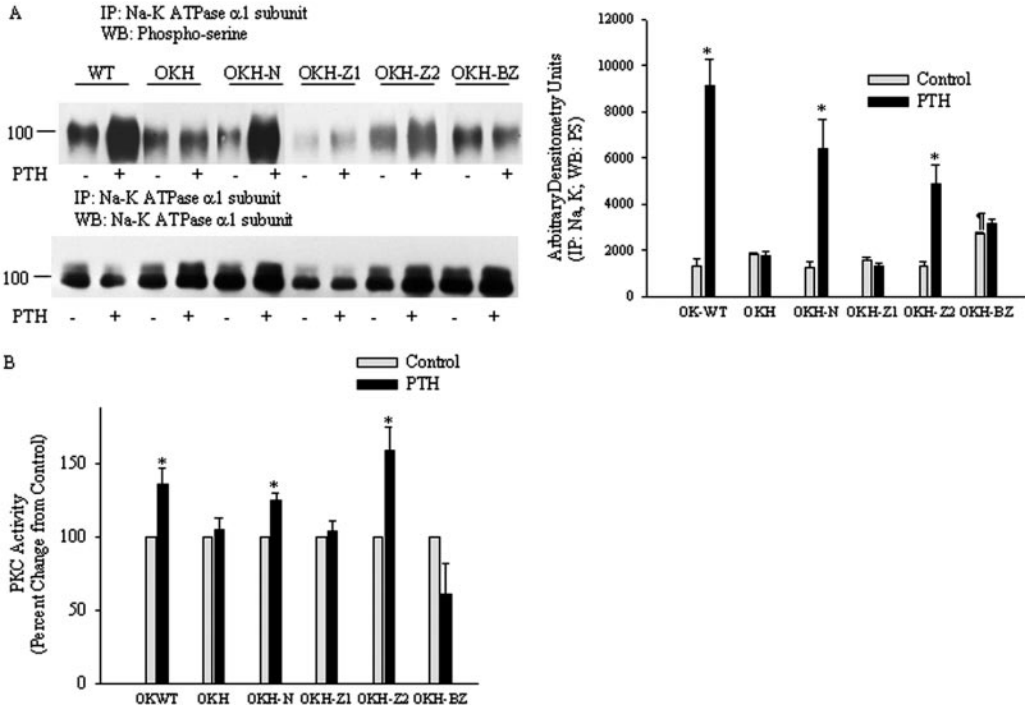

Figure 6. Comparison of PTH-stimulated Na^+,K^+-ATPase α_1 subunit phosphorylation in OK cells expressing variable NHERF constructs. OK cell monolayers of OK-WT, OKH, and OKH cells that were transfected with full-length human NHERF-1 or NHERF-1 constructs expressing PDZ domain mutations were treated with vehicle or 10^{-7} M PTH for 15 min. The cells were lysed, and Na^+,K^+-ATPase α_1 subunit was immunoprecipitated from crude membranes using anti–Na^+,K^+-ATPase α_1 subunit antibody and blotted for phosphoserine. A representative Western blot is shown. (Bottom) Total Na^+,K^+-ATPase α_1 subunit immunoprecipitated. Bar diagram shows densitometric data for phospho-serine blot as mean ± SEM from three independent experiments. *Statistically significant difference between PTH-treated and vehicle-treated cells ($P < 0.05$); ¶significantly different from OK-WT control ($P < 0.05$). (B) Cells were washed and lysed, and PKCα was immunoprecipitated from whole-cell lysates with antibody directed against PKCα. The immunoprecipitated PKCα was assayed for activity as outlined in Materials and Methods. Activity is measured as ability to radiolabel a peptide substrate and expressed as pmol phosphate/min per mg protein. Each bar represents percentage change in PKCα activity (mean ± SEM) from three independent experiments. *Statistically significant difference between PTH-treated and vehicle-treated cells ($P < 0.05$).

showed that introduction into the cells of a peptide sequence identical to the PDZ1 domain of NHERF impaired apical membrane PTH1 receptor localization presumably through competition for binding of the PTH1 receptor and blocked PTH-stimulated intracellular calcium release.

Failure of PTH to inhibit Na^+,K^+-ATPase activity in the NHERF-deficient cells was accompanied by a failure to stimulate phosphorylation of the α_1 subunit, which could be explained by a decrease in α_1 subunit expression, failure to activate appropriate kinases, or inability of the kinases to reach the appropriate substrate. Both models of NHERF-deficient OK cells exhibited α_1 subunit expression and baseline Na^+,K^+-ATPase activity that was equivalent to OK-WT. These findings, coupled with the confocal imaging studies, suggest that NHERF very likely does not play a dominant role in the plasma membrane localization of the components of the sodium pump. The diminished basal rubidium uptake in PDZ double-mutant cells despite the equivalent expression of the α_1 subunit in the crude membrane preparation is somewhat at variance with this conclusion. As rubidium uptake tends to correlate with surface expression of the α_1 subunit, this finding suggests that in OKH cells with the double PDZ mutation, α_1 subunit may not be as efficiently expressed in the plasma membrane or may not be as fully active as WT. Further studies will be required to determine the effect of double mutations in NHERF PDZ domains in regulation of Na^+,K^+-ATPase activity and expression.

Our demonstration that the inability of PTH to inhibit sodium pump activity is correlated with an inability to activate PKCα in NHERF-deficient cells is consistent with previously published data from the laboratories of Cole (32,33) and of Mahon and Segre (4,5). Cole *et al.* (32) demonstrated that PTH stimulation of PKA in NHERF-deficient OKH cells was intact but that stimulation of intracellular calcium transients was not. Mahon *et al.* (4) furthered these observations by showing that NHERF-deficient OKH cells exhibit impaired activation of PLC through disruption of the protein complex required for PTH activation of this pathway. Whether the BLM of OK cells express the membrane protein complexes similar to those described in the apical membrane is not known. We showed recently that PTH activation of PKCα is essential for regulation of sodium pump activity and that the activation is ERK dependent (37). PTH stimulation of ERK phosphorylation is intact in NHERF-deficient cells, suggesting that

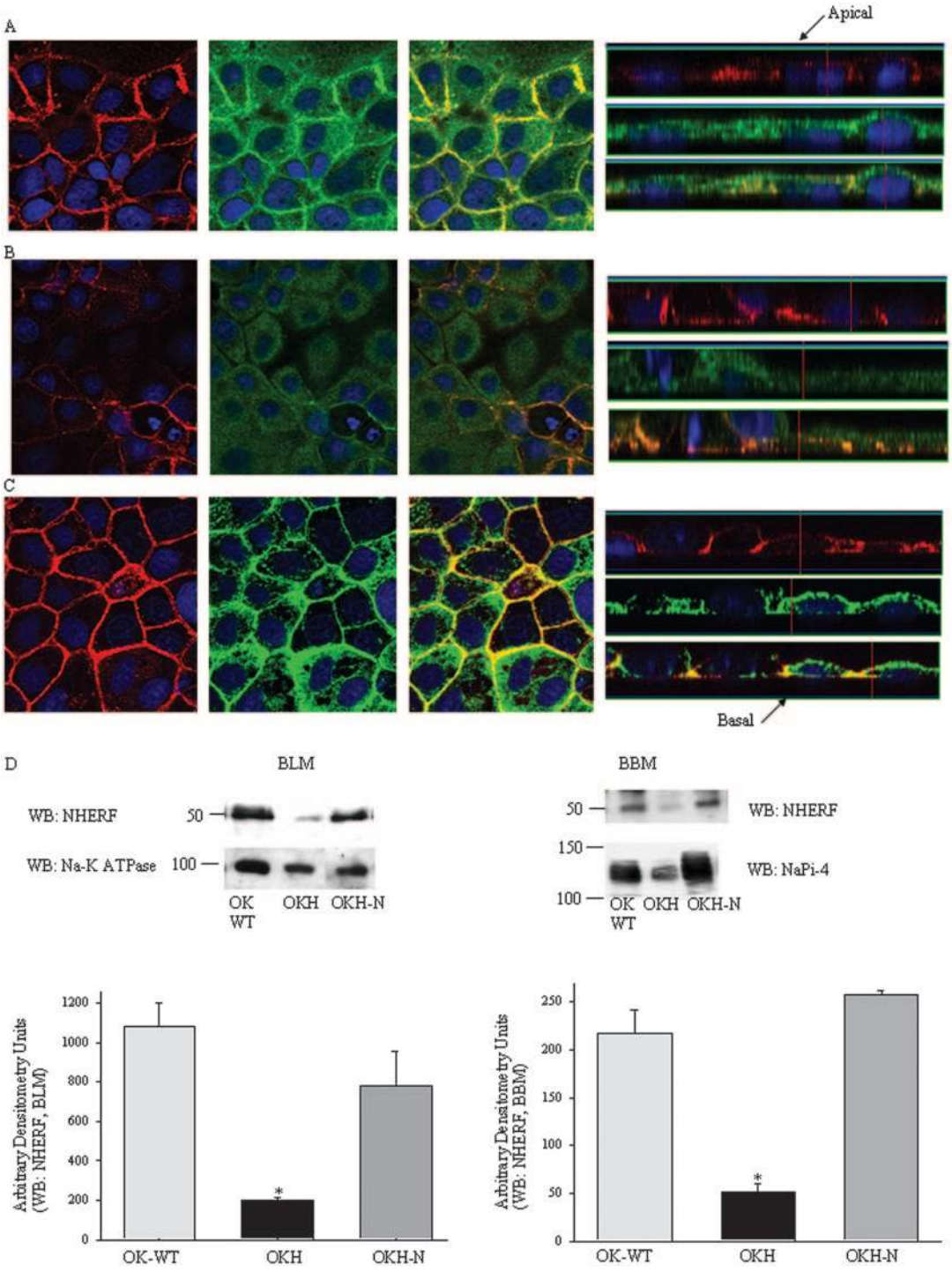

Figure 7. Comparison of the cellular distribution of NHERF and Na^+,K^+-ATPase α_1 subunit. OK cell monolayers were double labeled with antibodies directed against NHERF (green fluorescence) and the Na^+,K^+-ATPase α_1 subunit (red fluorescence). (A) Distribution of Na^+,K^+-ATPase α_1 subunit (left), NHERF (middle), and merged image of Na^+,K^+-ATPase α_1 subunit and NHERF (right) in OK-WT cells. (B) Distribution in OKH cells. (C) Distribution in OKH cells that were transfected with full-length NHERF-1. Representative confocal images from three independent experiments are shown. The far right panels show *z*-axis images of Na^+,K^+-ATPase α_1 subunit (red staining), NHERF (green staining), and merged image of Na^+,K^+-ATPase α_1 subunit and NHERF (yellow staining) with the apical side on the top and the basal side on the bottom. (D) Expression of NHERF in brush border membranes (BBM) and basolateral membranes (BLM). A representative Western blot is shown. Bar diagram shows densitometric data as mean ± SEM from three independent experiments.

activation of PKCα by PTH may depend on the simultaneous additive effects of both ERK and PLC.

From our data, we cannot determine the molecular mechanisms by which the PDZ1 domain mutation abolishes PTH regulation of the sodium pump. The mutation in the PDZ1 domain could result in a change in protein binding specific to the domain, or the mutation could result in misfolding that alters functions of the protein beyond the PDZ domain. Our confocal data show that the mutated NHERF traffics to the membrane in a normal distribution, suggesting that any misfolding that might occur is not as profound as to result in aberrant localization or in premature degradation. The question remains as to whether NHERF-1 binds the α_1 subunit of Na^+,K^+-ATPase. Our confocal images and Western blots of isolated BLM show some localization of NHERF to the basolateral surface, suggesting that a direct NHERF-α subunit interaction could exist, contrasting the findings of Hernando *et al.* (20). These results, however, confirm the previous results by Bernardo *et al.* (41), who showed NHERF expression in BLM from rabbit kidney proximal tubules. From our data, we cannot determine whether NHERF binds directly to the α_1 subunit of Na^+,K^+-ATPase. Scan of the sequence for the α_1 subunit fails to disclose any known PDZ binding domain. These results do not exclude the possible presence of a nonclassical binding motif. Alternatively, the PDZ1 domain could mediate the association of the α_1 subunit with the PTH1 receptor through interactions with other intermediary proteins, such as the β subunit or the γ subunit, or some other component of a signaling complex such as Gαq (42–46).

In conclusion, we have demonstrated that expression of the PDZ1 domain of NHERF-1 is critical for PTH regulation of Na^+,K^+-ATPase activity in renal proximal tubule cells. The role of this domain is to facilitate PTH receptor activation of the PLC/PKC pathway, a pathway that is critical for the phosphorylation and endocytosis of the α_1 subunit of the sodium pump. Our findings also highlight contrasts between the mechanisms by which NHERF-1 mediates PTH regulation of two different transporters, the sodium pump and NHE-3. For the sodium pump, the PDZ1 domain of NHERF is required for PLC/PKC-mediated phosphorylation of the α subunit by facilitating the activation of this signaling pathway by PTH. For NHE-3, the PDZ2 domain of NHERF is required for PKA-mediated phosphorylation of the protein. These findings open the door to new mechanisms by which PTH can exert individualized regulation on its target proteins.

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