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Sodium-Hydrogen Exchanger Regulatory Factor 1 (NHERF-1) Transduces Signals That Mediate Dopamine Inhibition of Sodium-Phosphate Co-transport in Mouse Kidney*

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Dopamine inhibited phosphate transport in isolated renal brush border membrane vesicles and in cultured renal proximal tubule cells from wild-type but not from NHERF-1 null mice. Co-immunoprecipitation experiments established that NHERF-1 associated with D1-like receptors. In wild-type mice, dopamine stimulated cAMP accumulation and protein kinase C (PKC) activity in renal proximal tubule cells, an effect that was abolished by SCH-23390, a D1-like receptor antagonist. In NHERF-1 null kidney tissue; however, dopamine failed to stimulate either cAMP accumulation or PKC activity. Infection of proximal tubule cells from NHERF-1 null mice with adenovirus-green fluorescent protein-NHERF-1 restored the ability of dopamine to stimulate cAMP and PKC. Finally, in ^{32}P-labeled wild-type proximal tubule cells and in opossum kidney cells, dopamine increased NHERF-1 phosphorylation at serine 77 of the PDZ I domain of NHERF-1, a site previously shown to attenuate binding of cellular targets including the Npt2a (sodium-dependent phosphate transporter 2a). Together, these studies establish that NHERF-1 plays a key role in dopamine signaling and is also a downstream target of D1-like receptors in the mouse kidney. These studies suggest a novel role for the PDZ adapter protein NHERF-1 in coordinating dopamine signals that inhibit renal phosphate transport.

Parathyroid hormone (PTH)[4] inhibits the renal tubular reabsorption of phosphate by decreasing the abundance of Npt2a, a major sodium-dependent phosphate transporter, in the apical membranes of proximal tubule cells (1). PTH interaction with the PTH-1 receptor activates both protein kinase A (PKA) and protein kinase C (PKC) and results in the phosphorylation of NHERF-1, a PDZ domain-containing adaptor protein, to dissociate Npt2a/NHERF-1 complexes (1, 2). PTH-mediated inhibition of renal phosphate transport has an absolute requirement for activation of PKC (2). Activation of PKA, however, is not essential despite the fact that cell-permeable cAMP analogues result in the inhibition of phosphate transport in proximal tubule cells (2, 3). By contrast to PTH, dopamine-mediated inhibition of renal phosphate transport has an absolute requirement for activation of both PKA and PKC (2). As detailed in the current experiments, dopamine failed to modulate phosphate transport in proximal tubule cells isolated from NHERF-1 null mice. Given the increasing recognition that adapter proteins play a key role in signaling by many G-protein-coupled receptors, we undertook a detailed analysis of dopamine signaling and its impact on phosphate transport in proximal tubule cells and renal brush border membranes isolated from wild-type and NHERF-1 null mice (4–10). The current studies show that NHERF-1 is required for the generation of second messengers that activate signaling downstream of the renal dopamine D1-like receptors and that NHERF-1 itself is a target of the kinase cascades activated in renal tissue by dopamine. Our studies provide physiological evidence that although propagating dopamine signaling in renal proximal tubule cells, NHERF-1 is also phosphorylated at serine 77. This covalent modification selectively disrupts the association of targets such as Npt2a that bind to the first PDZ domain of NHERF-1, and this, in turn, plays a key role in dopamine-mediated down-regulation and inhibition of Npt2a activity in renal tissue.

EXPERIMENTAL PROCEDURES

Animals, Kidney Slices, and Cell Cultures—The animals were housed in standard cages in compliance with Association for Assessment and Accreditation of Laboratory Animal Care international guidelines in the Baltimore Veterans Affairs Animal Care Facility. All of the animal experiments were approved by the University of Maryland School of Medicine Animal Protocol Review Board. Inbred C57BL/6 wild-type mice and NHERF-1$^{-/-}$ mice bred into a C57BL/6 background were used in the present experiments (11).

The kidney slices were prepared as described previously (12). In brief, the left ventricle was perfused with 50 ml of a sucrose-phosphate buffer containing 140 mM sucrose and 140 mM

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[4] The abbreviations used are: PTH, parathyroid hormone; NHERF-1, sodium-hydrogen exchanger regulatory factor 1; BBM, brush border membrane; PKC, protein kinase C; GFP, green fluorescent protein; PKA, protein kinase A; KS, kidney slices; OK, opossum kidney.

Na_2HPO_4/NaH_2PO_4 (pH 7.4, 37 °C) to remove blood from the kidneys. The kidneys were then rapidly harvested, the adhering connective tissue and extra renal blood vessels were removed, and ~1-mm-thick coronal slices were prepared. The slices were transferred to a chamber containing prewarmed (37 °C) buffer containing 110 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 4 mM sodium acetate, 1 mM sodium citrate, 6 mM glucose, 6 mM L-alanine, 1 mM NaH_2PO_4, 3 mM Na_2HPO_4, and 25 mM $NaHCO_3$. All of the solutions were gassed with 5% CO_2, 95% O_2, and the pH was kept constant at 7.4.

Brush border membrane (BBM) vesicles were prepared using a modification of the magnesium aggregation method developed previously in our laboratory (13). The tissue was homogenized with a glass Teflon homogenizer in 200 mM mannitol, 10 mM MgSO, 50 mM Tris, 80 mM HEPES, pH 7.5, centrifuged for 5 min at 1,475 × *g*, and the resultant supernatant centrifuged for 15 min at 27,000 × *g*. The resultant pellet was resuspended in homogenizing solution followed by four alternating slow speed (2,445 × *g*) and fast speed (27,000 × *g*) centrifugations. The pellet resulting from the last high speed centrifugation representing purified membrane vesicles was suspended in a small amount of homogenizing solution and used for study. The enrichment of the BBM membranes, determined by the ratio of the abundance of alkaline phosphatase (measured by Western immunoblotting) in the isolated vesicles compared with whole kidney homogenates, was 11.1 ± 2.4 and 11.8 ± 3.2 (p = NS) in wild-type membranes harvested from control and dopamine-treated kidney slices, respectively, and 11.0 ± 2.1 and 11.2 ± 2.5 (p = NS) in NHERF-$1^{-/-}$ null membranes from control and dopamine-treated kidney slices.

OK cells, a proximal tubule cell line, and primary proximal tubule cells from wild-type and NHERF-1 knock-out mice were prepared as described previously (1–3). Proximal tubule cells from mice were plated on Matrigel-coated (BD) plastic culture dishes and maintained in an incubator at 37 °C in 5% CO_2. The cultures were left undisturbed for 36 h, after which the medium was replaced every 2 days until the cells achieved confluence. Both the OK cells and cultured proximal tubule cells were grown in Dulbecco's modified Eagle's medium/F-12 medium containing 0.9 mM phosphate.

Preparation of cDNAs—NHERF-1 constructs encoding the PDZ I domain were prepared using existing restriction sites and/or PCR. The mutations were generated by site-directed mutagenesis using single-stranded DNA and appropriate primers and confirmed by dideoxynucleotide sequencing. The cDNAs were subcloned into pcDNA3.1 for transfection into cells. All of the NHERF-1 proteins and peptides were expressed as fusion proteins containing an N-terminal hexahistidine tag. The cDNAs were transfected into cells using Lipofectin (Invitrogen). Adenovirus-mediated gene transfer was used to infect primary cultures of mouse proximal tubule cells as described previously (1, 3). Infective recombinant adenoviruses were produced using AdEasy (Stratagene). The recombinant adenoviruses were produced by inserting the cDNA into a shuttle plasmid (pShuttleCMV) and performing homologous recombination in *Escherichia coli* with this shuttle vector and a large adenovirus-containing plasmid following electroporation. The recombinants were identified from single colonies, and infective adenovirus virions were produced following transfection of the linearized recombinant adenovirus plasmid in HEK293 cells. Virus stocks were amplified in HEK293 cells on 15-cm plates and purified following lysis by CsCl banding using ultracentrifugation.

Measurement of Sodium-dependent Phosphate Uptake in BBM Vesicles and Primary Cultures of Proximal Tubule Cells—The uptake of phosphate (100 μM) in BBM vesicles harvested from control and dopamine-treated kidney slices (10^{-4} M for 45 min) was measured at 30 s (initial rate) and 90 min (steady state) by the rapid filtration technique using a transport medium containing 137 mM NaCl. 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.1 mM KH_2PO_4, and [^{32}P]orthophosphate (26). The uptake reaction was stopped by washing the BBM vesicles three times with ice-cold fresh medium in which sodium chloride was substituted with tetramethylammonium chloride, and 0.5 mM sodium arsenate was added.

Sodium-phosphate transport was also measured in primary cultures of renal proximal tubule cells (1–3). The cells were grown in serum-free medium for 24 h. The cells were studied under control conditions and after treatment with dopamine (10^{-4} M for 45 min) by incubation in transport medium consisting of 137 mM NaCl or 137 mM tetramethylammonium chloride, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 0.1 mM KH_2PO_4. Phosphate uptake was initiated by the addition of [^{32}P]orthophosphate to the transport medium. After 10 min at room temperature, the cells were washed three times with ice-cold fresh medium in which sodium chloride was substituted with tetramethylammonium chloride and 0.5 mM sodium arsenate was added. After the uptake measurements were completed, the cells were solubilized in 0.5% Triton X-100 for 90 min at room temperature and analyzed by liquid scintillation spectroscopy.

Other Biologic Assays—Immunoprecipitation studies were performed using freshly prepared kidney slices from wild-type mice under control conditions and after treatment with dopamine (10^{-4} M for 45 min). The precipitated proteins were separated using SDS-PAGE, transferred to nitrocellulose, and blotted for Npt2a, the dopamine D1-like receptor, or NHERF-1. Enhanced chemical luminescence was used to detect immune complexes, and densitometry was used for quantification.

In vivo phosphorylation of full-length NHERF-1 was assayed in wild-type cultured proximal tubule cells incubated for 3 h in phosphate-free Dulbecco's modified Eagle's medium containing [^{32}P]orthophosphate. The cells were studied under control conditions and after treatment with dopamine (10^{-4} M for 45 min). NHERF-1 was immunoprecipitated, and the precipitates were subjected to SDS-PAGE and transferred to nitrocellulose. Phosphorylation was quantitated using a PhosphorImager, and after the radioactivity decreased to base line, the gels were immunoblotted using an anti-NHERF-1 antibody. To study the phosphorylation of the PDZ I domain, OK cells were transiently transfected with the PDZ I constructs. The cells were incubated in phosphate-free Dulbecco's modified Eagle's medium containing [^{32}P]orthophosphate and studied under control conditions or after treatment with dopamine (10^{-4} M for 45 min). The PDZ I domains were recovered using nickel chromatography and resolved by SDS-PAGE. After transfer, phosphoryla-

TABLE 1

The effect of dopamine on sodium-dependent phosphate uptake in BBM vesicles harvested from wild-type and NHERF-1 null kidney slices

Sodium-dependent phosphate uptake (nmol/mg of protein) was determined in BBM vesicles harvested from freshly prepared kidney slices from control (−D) and dopamine-treated (+D) tissues from wild-type and NHERF-1 null mice. Uptake levels at 30 s representing an initial rate and at 90 min representing equilibrium are shown. n = number of animals. The values are expressed as the means ± S.E.

	Wild type ($n = 5$)		NHERF-1$^{-/-}$ ($n = 5$)	
	−D	+D	−D	+D
30 s	0.94 ± 0.19	0.39 ± 0.09[a]	0.30 ± 0.03	0.33 ± 0.09
90 min	0.06 ± 0.02	0.05 ± 0.02	0.05 ± 0.01	0.05 ± 0.01

[a] $p < 0.05$ *versus* −D.

tion was quantitated by a PhosphorImager. When the radioactivity had returned to background levels, the identity of the phosphorylated polypeptides was confirmed by Western immunoblot using an anti-His antibody.

The production of intracellular cAMP in cultured cells in response to dopamine (10^{-4} M for 45 min) was measured by nonacetylation EIA (cAMP Biotrak assay kit; Amersham Biosciences) in the presence of 0.4 mM 3-isobutyl-1-methylxanthine (2). PKC activity was assayed using a Promega SignaTECT PKC assay system containing a specific PKC substrate and capture membrane. The protein concentrations were determined using the method of Lowry *et al.* (14). Statistical analyses were performed using Peritz analysis of variance. p values less than 0.05 were considered statistically significant.

RESULTS

In many prior studies, we employed primary cultures of renal proximal tubule cells to analyze the role of NHERF-1 in the regulation of renal mineral and electrolyte metabolism (1–3). To further validate experiments that utilized adenovirus-mediated gene transfer in primary cultured cells, we performed parallel studies using freshly prepared mouse kidney slices (KS). In initial experiments, BBM vesicles were prepared from control and dopamine-treated kidney slices for measurement of sodium-dependent phosphate uptake. As shown in Table 1, dopamine significantly inhibited the 30-s uptake of phosphate by 51 ± 13% ($n = 5$, $p < 0.05$) in BBM vesicles from wild-type mice but not in BBM vesicles from NHERF-1 null mice (% change = 7 ± 7%, $n = 5$, $p <$ NS). Of note, basal sodium-dependent uptake of phosphate was significantly lower in BBM vesicles from NHERF-1$^{-/-}$ mice compared with controls ($p < 0.05$). The equilibrium values at 90 min did not differ between the groups. Cultured proximal tubule cells from NHERF-1 null animals infected with adenvirus-GFP-NHERF-1 had significantly higher base-line sodium-dependent phosphate uptake compared with NHERF-1$^{-/-}$ cells infected with control adenovirus GFP (6.8 ± 1.0 pmol/μg of protein/10 min *versus* 3.6 ± 0.6, $n = 4$, $p < 0.05$). Phosphate transport was inhibited 31.8 ± 2.1% ($p < 0.05$) by dopamine in adenovirus GFP-NHERF-1-infected cells compared with 1.9 ± 4.1% ($p =$ NS) in cells infected with control adenovirus GFP ($n = 4$) (Fig. 1). These results indicate that NHERF-1 is required for dopamine to inhibit the renal proximal tubule transport of phosphate.

In rodents, the D1-like receptor is expressed in the apical membrane of renal proximal tubule cells (15). Western immunoblots were performed on BBM vesicles from wild-type and NHERF-1 null kidneys to determine the relative abundance of D1-like receptors. Fig. 2 is a representative example. The ratio of D1-like receptors to alkaline phosphatase (used as a loading control) averaged 0.35 ± 0.05 in wild-type BBM vesicles and 0.40 ± 0.08 in BBM vesicles from NHERF-1 null mice ($p =$ NS, $n = 4$). We next sought to determine whether NHERF-1 interacts with D1-like receptors. Immunoprecipitation with preimmune sera failed to yield NHERF-1 or D1-like receptors in the precipitates (not shown). As shown in Fig. 3, an antibody to NHERF-1 co-immunoprecipitated D1-like receptors from freshly prepared kidney slices. In the reverse experiment, an anti-D1-like receptor antibody immunoprecipitated NHERF-1. The ratio of D1-like receptors to NHERF-1 was 0.16 ± 0.07 (arbitrary densitometry units) in control KS and 0.20 ± 0.10 in KS treated with dopamine ($n = 5$, $p =$ NS). Relative to untreated controls, dopamine stimulated cAMP accumulation 10.6 ± 1.0-fold ($n = 3$, $p < 0.05$) and 11.1 ± 1.7-fold ($n = 3$, $p < 0.05$) in wild-type kidney slices and cultured proximal tubule

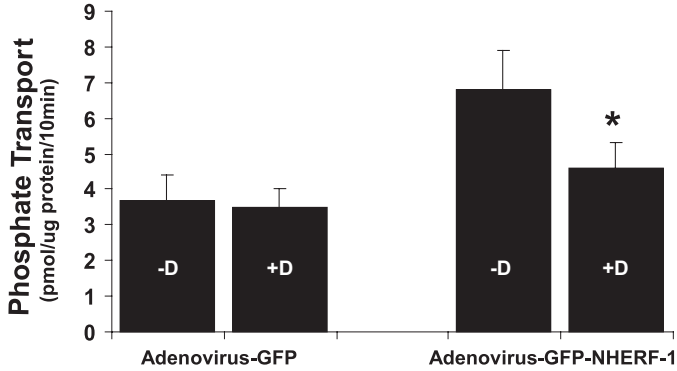

FIGURE 1. **Sodium-dependent phosphate transport was measured in the absence (−*D*) or presence (+*D*) of dopamine in cultured NHERF-1 null proximal tubule cells infected with adenovirus GFP or adenovirus GFP-NHERF-1.** *, $p < 0.05$.

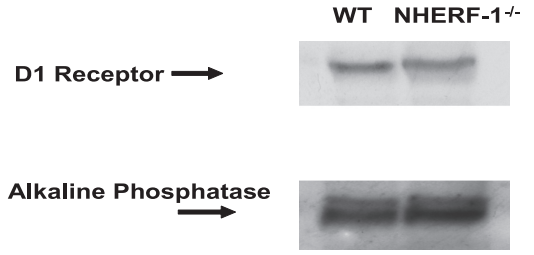

FIGURE 2. **Representative Western immunoblots of brush border membrane vesicles from wild-type (*WT*) and NHERF-1$^{-/-}$ mice using an antibody to the dopamine D1-like receptor (*upper panel*) and alkaline phosphatase (*lower panel*).**

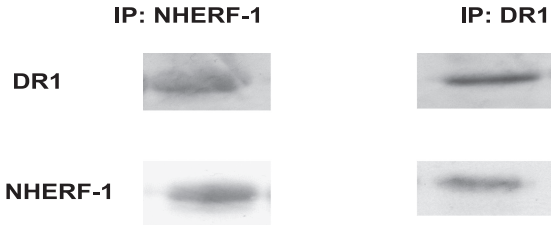

FIGURE 3. **D1-like receptors or NHERF-1 was immunoprecipitated (*IP*) from kidney slices from wild-type animals.** The precipitates were immunoblotted for D1-like receptors (*DR1*) and NHERF-1. A representative experiment is shown.

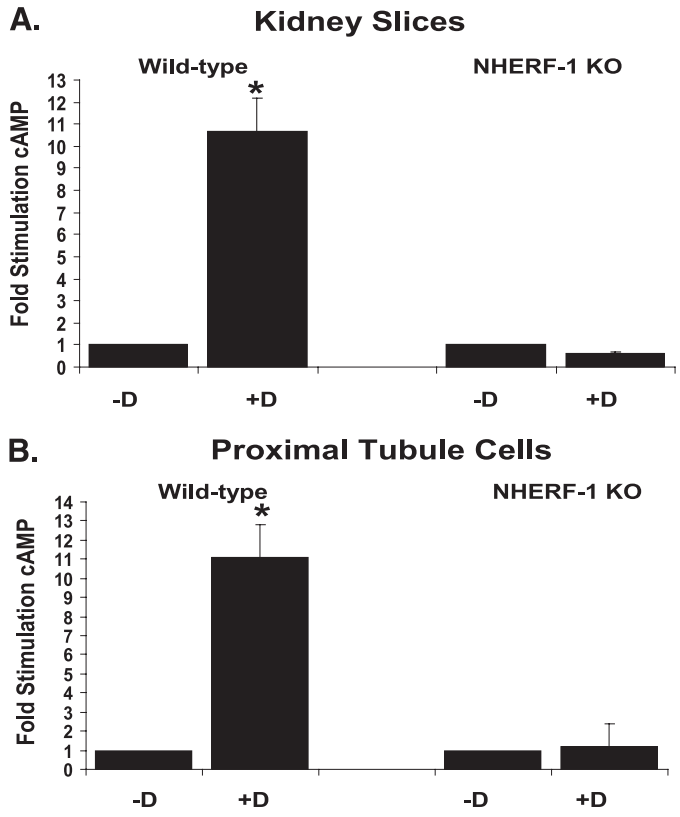

FIGURE 4. **The accumulation of cAMP was determined in control (−*D*) or dopamine-treated (+*D*) kidney slices (*A*) and cultured proximal tubule cells (*B*) from wild-type or NHERF-1 null (*NHERF-1 KO*) animals.** The results are expressed as fold increases ± S.E. relative to controls (−*D*) (shown as = 1). *, $p < 0.05$.

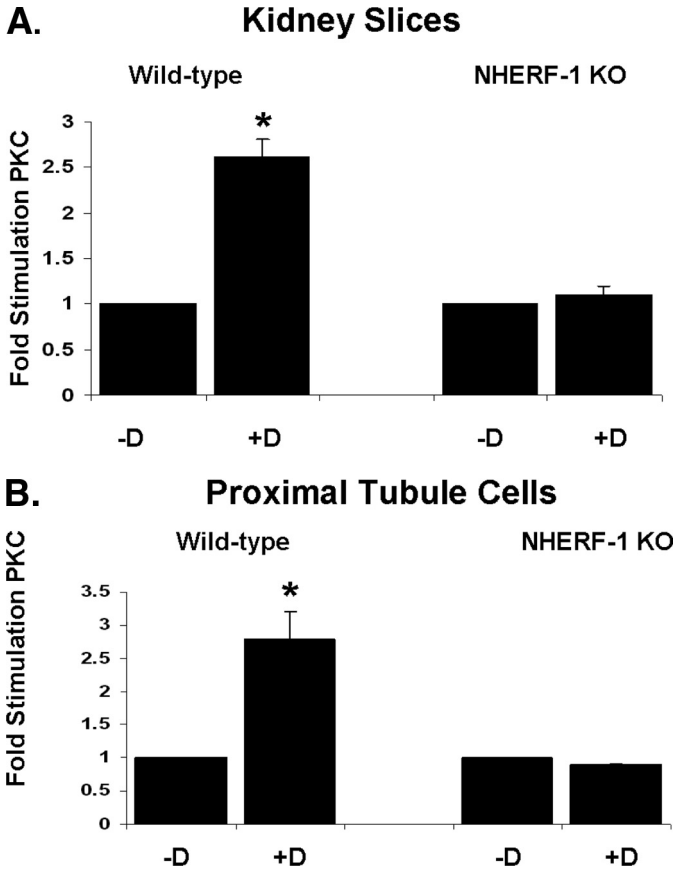

FIGURE 5. **PKC activation was determined in control (−*D*) or dopamine-treated (+*D*) kidney slices (*A*) and cultured proximal tubule cells (*B*) from wild-type or NHERF-1 null (*NHERF-1 KO*) animals.** The results are expressed as fold increases ± S.E. relative to controls (−*D*) (shown as = 1). *, $p < 0.05$.

cells from wild-type mice, respectively (Fig. 4). Dopamine also stimulated PKC activation by 2.6 ± 0.2-fold ($n = 4$, $p < 0.05$) and 2.8 ± 0.4-fold ($n = 3$, $p < 0.05$) in wild-type kidney slices and cultured proximal tubule cells from wild-type mice, respectively (Fig. 5). By contrast, neither cAMP accumulation (Fig. 4) nor PKC activity (Fig. 5) was stimulated by dopamine in KS or primary cultures of renal proximal tubule cells from NHERF-$1^{-/-}$ animals. The increase in cAMP accumulation and PKC activity in response to dopamine was restored when NHERF-1 null cultured proximal tubule cells were infected with adenovirus GFP-NHERF-1 (Fig. 6). Considered together, these data indicate the critical role of NHERF-1 in signal transduction by dopamine receptors in renal tissue.

We next measured dopamine-mediated accumulation of cAMP and PKC activity in kidney slices and cultured proximal tubule cells from wild-type mouse kidneys in the absence and presence of SCH-23390, a D1-like receptor antagonist. In the presence of the antagonist in both KS and primary cultures of renal proximal tubule cells, dopamine failed to stimulate cAMP accumulation (Fig. 7) or PKC activation (Fig. 8). To rule out an effect of SCH-23390 on PKC itself, we measured PKC activity in the presence of SCH-23390 in cultured cells treated with 8-bromo-cAMP (10^{-4} M) or 1,2-dioctanoyl-*sn*-glycerol (10^{-4} M). 8-Bromo-cAMP stimulated PKC activity 14.2 ± 1.4-fold, and 1,2-dioctanoyl-*sn*-glycerol stimulated PKC activity 15.8 ± 2.8-fold ($n = 3$). These results indicate that the D1-like receptors are the major signaling pathway in renal tubule cells of the mouse.

Treatment of mouse proximal tubule cells with PTH results in the phosphorylation of NHERF-1 (1). To determine whether NHERF-1 was also a target of dopamine receptor-activated kinase cascades, cultured mice proximal tubule cells were metabolically labeled with [^{32}P]orthophosphate and NHERF-1 immunoprecipitated from lysates of control or dopamine-treated cells. Dopamine increased the phosphorylation of full-length endogenous NHERF-1 by 34.7 ± 5.3% ($n = 5$, $p < 0.05$). A representative experiment is shown in Fig. 9*A*. Full-length NHERF-1 has many known and potential phosphorylation sites, and the above findings do not establish that serine 77 is phosphorylated by dopamine. Because anti-phosphoserine 77 antibodies are not available, we used a previously employed strategy to specifically determine whether serine 77 of the first PDZ domain of NHERF-1 was phosphorylated by dopamine (1). cDNAs representing His_6 wild-type PDZ I, PDZ I in which all four of the serine and threonine residues were mutated to alanine residues (Mutation 1), or PDZ I in which all serine and threonine residues except serine 77 were mutated (Mutation 2) were expressed in OK cells, a proximal tubule cell line that contains functional dopamine D1-like receptors. The cells were then metabolically labeled with [^{32}P]orthophosphate and treated with dopamine, and the PDZ I polypeptides were recovered using nickel chromatography. Dopamine increased the phosphorylation of wild-type PDZ I (percentage of change = +48 ± 9%, $n = 3$, $p < 0.05$), but as antici-

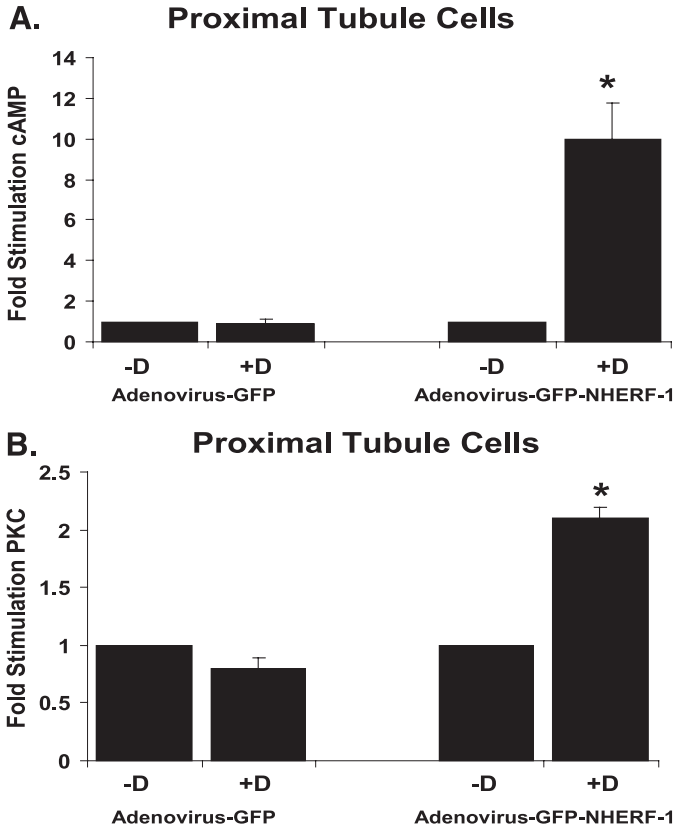

FIGURE 6. **cAMP accumulation (*A*) and PKC activation (*B*) was measured in control (−*D*) or dopamine-treated (+*D*) cultured proximal tubule cells from NHERF-1 null mice infected with adenovirus GFP or adenovirus GFP-NHERF-1.** *, $p < 0.05$.

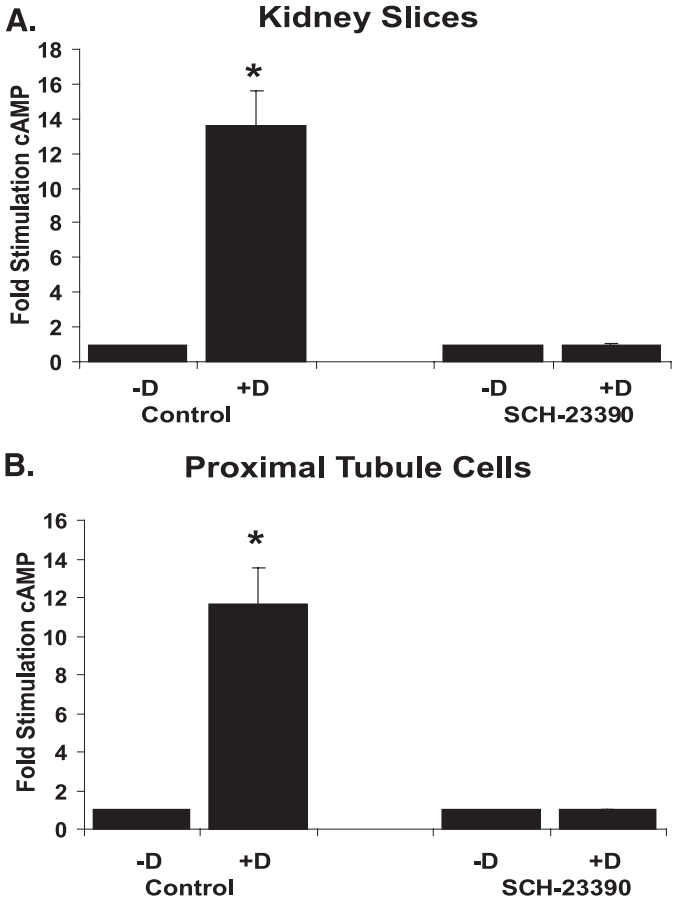

FIGURE 7. **The accumulation of cAMP was determined in control (−*D*) or dopamine-treated (+*D*) kidney slices (*A*) and cultured proximal tubule cells (*B*) from wild-type mice in the absence (*Control*) or presence of SCH-23390, a D1-like receptor antagonist.** The results are expressed as fold increases ± S.E. relative to controls (−*D*) (shown as = 1). *, $p < 0.05$.

pated, Mutation 1, the PDZ I domain containing mutations in all available phosphate acceptors, was not phosphorylated in the basal state or after treatment with dopamine. Dopamine significantly increased the phosphorylation of Mutation 2, identifying serine 77 as a key site for dopamine-stimulated NHERF-1 phosphorylation (percentage of change = +29 ± 5%, $n = 3$, $p < 0.05$) (Fig. 9*B*).

To specifically study the role of Ser^{77} on dopamine-mediated inhibition of phosphate transport, we used adenovirus GFP-NHERF-1 constructs containing either inactivating (alanine) or phosphomimetic (aspartic acid) mutations of Ser^{77} to infect NHERF-1 null proximal tubule cells in primary culture ($n = 5$) (Table 2) (1). Dopamine inhibited sodium-dependent phosphate transport by 35 ± 5% in NHERF-1 null cells infected with wild-type adenovirus GFP-NHERF-1 but not in cells infected with NHERF-1 containing a S77A mutation (percentage of change = −1 ± 6%) or a S77D mutation (percentage of change = 0 ± 10%).

We have previously demonstrated that dopamine decreases the apical membrane abundance of Npt2a in mouse kidney (2). As evidence that dopamine-mediated NHERF-1 phosphorylation at serine 77 resulted in the dissociation of apical Npt2a/NHERF-1 complexes, we measured the abundance of Npt2a and NHERF-1 in plasma membranes of control and dopamine-treated kidney slices. As shown in Fig. 10, dopamine resulted in a 34.7 ± 5.3% decrease in Npt2a abundance relative to NHERF-1 ($n = 5$, $p < 0.05$), suggesting dissociation of complexes.

DISCUSSION

Prior studies have established that dopamine, acting primarily through D1-like receptors, decreases the abundance of Npt2a in the apical membrane of renal proximal tubule cells, thereby inhibiting the renal tubular reabsorption of phosphate (2, 16, 17). The signaling pathways activated by dopamine in mouse kidney involves activation of adenylyl cyclase, generation of cAMP, and subsequent activation of PKA but not EPAC (2, 16–19). PKA, in turn, activates PKC, and both PKA and PKC are required for dopamine to maximally inhibit phosphate transport (2). The role of NHERF-1 in these cellular events has not been explored. Our current results indicate that dopamine did not inhibit phosphate transport in BBM vesicles from freshly prepared renal slices from NHERF-1 null mice or in primary cultures of proximal tubule cells derived from these animals. Viral-mediated gene transfer of NHERF-1, however, increased basal phosphate transport and restored the inhibitory effect of dopamine in these cells. These findings define a critical role for NHERF-1 in the actions of dopamine on renal phosphate transport and indicate that the defective regulation of phosphate transport in NHERF-1 null renal proximal tubule cells results from the absence of NHERF-1 rather than the consequence(s) of cellular changes secondary to the inactivation of the mouse NHERF-1 gene.

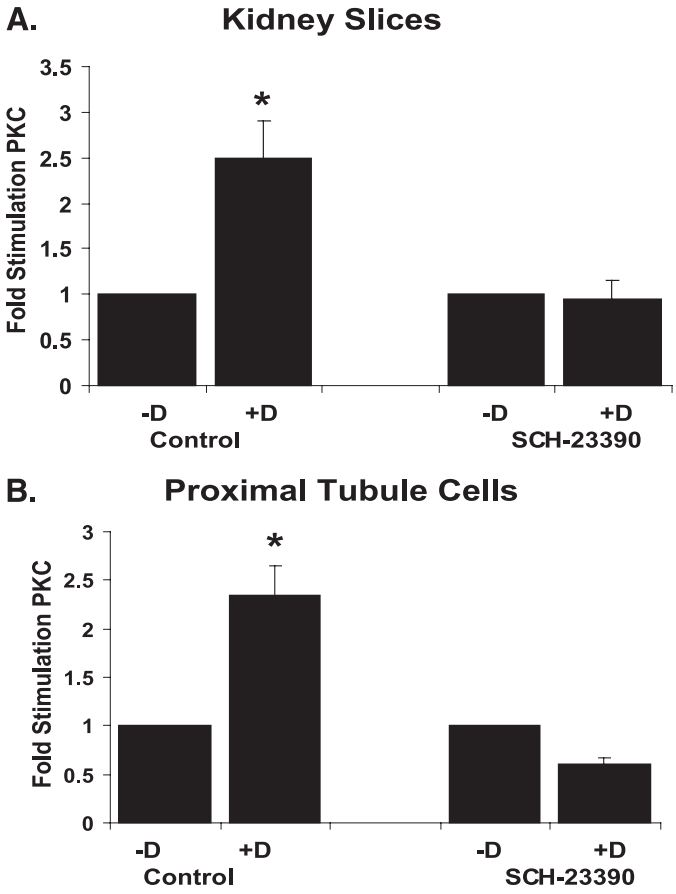

FIGURE 8. **PKC activation was determined in control (−*D*) or dopamine-treated (+*D*) kidney slices (*A*) and cultured proximal tubule cells (*B*) from wild-type mice in the absence (*Control*) or presence of SCH-23390, a D1-like receptor antagonist.** The results are expressed as fold increases ± S.E. relative to controls (−*D*) (shown as = 1). *, $p < 0.05$.

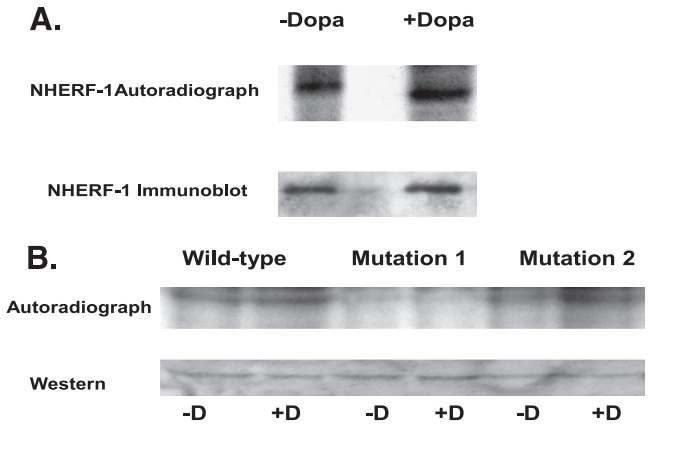

FIGURE 9. *A*, NHERF-1 was immunoprecipitated from [^{32}P]orthophosphate metabolically labeled wild-type proximal tubule cells in culture under control conditions (−*Dopa*) or treatment with dopamine (+*Dopa*). The proteins were resolved by SDS-PAGE, and a representative autoradiograph is shown. After the isotope counts dissipated, the gel was immunoblotted for NHERF-1. *B*, NHERF-1 cDNAs representing wild-type PDZ I, PDZ I in which all four serine and threonine residues were mutated to alanine residues (Mutation 1), or PDZ I in which all serine and threonine residues except serine 77 were mutated to alanine residues (Mutation 2) were expressed in [^{32}P]orthophosphate metabolically labeled wild-type proximal tubule cells in culture under control conditions (−*D*) or treatment with dopamine (+*D*). The PDZ I polypeptides were recovered using nickel chromatography. Representative autoradiographs are shown.

TABLE 2

The effect of dopamine on sodium-dependent phosphate uptake in cultured NHERF-1 null proximal cells infected with wild-type adenovirus GFP-NHERF-1 or GFP-NHERF-1 containing a S77A or S77D mutation

Sodium-dependent phosphate uptake (pmol/μg of protein/10 min) was determined in proximal tubule cells in primary culture from NHERF-1 null mice in the absence (−D) or presence of dopamine (+D). The cells were infected with adenovirus GFP-NHERF-1 constructs representing wild-type NHERF-1 or NHERF-1 with a S77A or S77D mutation. The values are expressed as the means ± S.E.

	−D	+D	Percent change
Wild type	9.2 ± 0.3	6.0 ± 0.4	35 ± 5[a]
S77A	8.0 ± 0.4	8.1 ± 0.6	−1 ± 6
S77D	5.9 ± 0.7	5.8 ± 1.0	0 ± 10

[a] $p < 0.05$.

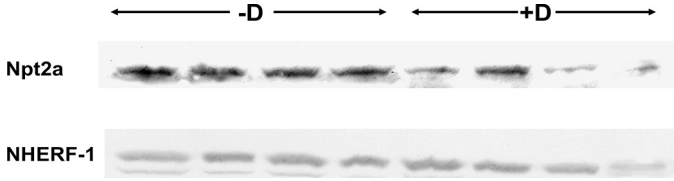

FIGURE 10. **Plasma membranes were prepared from kidney slices from wild-type animals under control conditions (−*D*) or after dopamine treatment (+*D*).** The proteins were separated by SDS-PAGE and immunoblotted for Npt2a and NHERF-1.

NHERF-1 has been shown to bind the C termini of several G-protein-coupled receptors including the β2-adrenergic receptor, the PTH 1 receptor, and the κ-opiod receptor (4–10). Emerging studies indicate, however, that there are receptor-specific as well as cell-specific interactions between NHERF-1 and selected G-protein-coupled receptors. For example, NHERF-1 binding promotes recycling of internalized ligand bound β2-adrenergic receptors to the cell surface (5). In the case of the PTH1 receptor, NHERF-1 has been proposed to connect the receptor to downstream effectors such as phospholipase C and function as a signaling switch in OK cells (8, 9). More recent studies in ROS cells, on the other hand, have reported that NHERF-1 promoted cAMP signaling by the PTH1 receptor (10). Finally, there is evidence that NHERF-1 can promote G-protein-coupled receptor signaling independent of or through direct interactions with selected heterotrimeric G-proteins (6, 20). Examination of C-terminal sequences of the D1 and D5 receptors would not readily predict that these receptors would interact with NHERF-1. In fact, *in vitro* studies have demonstrated that the D1 receptor bound to the canonical PDZ domain-containing protein, PSD-95, but not to NHERF-1 (21, 22). Nevertheless in renal proximal tubule cells, the current studies demonstrated that D1-like receptors are co-immunoprecipitated by anti-NHERF-1 antibodies and vice versa. The absence of NHERF-1, however, does not appear to influence the apical membrane abundance of the D1-like receptors in kidney tissue. This suggests a distinct role for NHERF-1 in D1-like receptor signaling as evidenced by the finding that dopamine failed to stimulate cAMP accumulation or activate PKC in NHERF-1 null cells and that infection of these cells with adenovirus GFP-NHERF-1 restored dopamine signaling. These results provide compelling evidence for a requirement for NHERF-1 in D1-like receptor signaling in renal tissue. The pre-

cise mode of binding of D1-like receptors with NHERF-1 is currently unknown, but based on prior data suggesting that D1 receptors bind PSD-95 independent of the requirement for a canonical C-terminal PDZ-binding motif, we would speculate that NHERF-1 may also utilize a non-PDZ mechanism for binding to D1-like receptors in the kidney (22).

Given that the abundance and cellular localization of NHERF-2 is unchanged in NHERF-1 null proximal tubule cells, it would appear likely that the D1-like receptor specifically requires NHERF-1. This contrasts with the PTH1 receptor in which binding and cellular signaling is supported by either NHERF-1 or NHERF-2 (23). It is noteworthy that despite the presence of multiple PDZ domain-containing proteins in the mammalian kidney, many of which show the ability to bind Npt2a, gene disruption studies clearly show that NHERF-1 is a primary regulator of Npt2a function in the mouse kidney (11, 24, 25). Prior studies from this laboratory estimated that 35–50% of Npt2a in the apical membrane of mouse proximal tubule cells is bound to NHERF-1 (1). The current experiments show that dopamine increases the phosphorylation of NHERF-1, specifically at serine 77 of the first PDZ domain that precludes the binding of canonical PDZ I targets (1, 26, 27). In this regard, these results further expand the role for NHERF-1 phosphorylation as a mechanism for regulation of Npt2a and possibly other renal targets by multiple hormones.

In conclusion, the present studies highlight a key role for NHERF-1 in second messenger generation and signaling by dopamine D1-like receptors in the mouse kidney. In addition, NHERF-1 function is itself regulated by dopamine through the phosphorylation of serine 77 in the PDZ I domain. This covalent modification may account for its release from Npt2a and the subsequent inhibition of renal phosphate transport. These studies provided new insights into the role of this adapter protein in hormone signaling in the renal tissue.

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