Defective coupling of apical PTH receptors to phospholipase C prevents internalization of the Na\(^+\)-phosphate cotransporter Na\(_{\text{Pi}}\)-IIa in Nherf1-deficient mice

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In the kidney, PTH interacts with a G protein-coupled receptor (PTHR1) expressed in the apical and basolateral membrane of the proximal tubule cells (21, 22, 32). Activation of apical or basolateral PTH receptors (PTHRs) induces a strong and rapid downregulation of Na\(_{\text{Pi}}\)-IIa due to retrieval of the protein from the BBM and its subsequent routing to the lysosomes for degradation (17, 26, 31). Furthermore, several PTH fragments that selectively activate apical or basolateral PTHRs have been identified (21, 32). PTH-(1–34) is active on the apical and basolateral sides, whereas PTH-(3–34) is effective only on apically located receptors (22, 32). Apical PTHRs predominantly couple to the phospholipase C (PLC)-protein kinase C (PKC) pathway, whereas basolateral PTHRs activate the cAMP- and protein kinase A (PKA)-dependent pathways (22, 32). Both pathways ultimately lead to Na\(_{\text{Pi}}\)-IIa internalization and degradation (24).

The Na\(_{\text{Pi}}\)-IIa protein interacts via its last three COOH-terminal amino acid residues TRL with several PDZ motif-containing proteins, some of which colocalize in the BBM together with Na\(_{\text{Pi}}\)-IIa (13, 14). These proteins include, among others, the Na\(^+/\)H\(^+\) exchanger (NHE) regulatory factor (NHERF)-1 (NHERF1) and PDZK1 (6, 16). NHERF1 was first identified as a regulatory factor of NHE3 and was later shown to be identical to ezrin-binding protein 50 (EBP50) (28, 36). A second isoform, NHERF2, which is also localized in many epithelia but resides in a different compartment, has also been identified (33, 34). NHERF1 affects phosphate transporter activity and expression in the BBM in different experimental models. Na\(_{\text{Pi}}\)-IIa apical positioning is reduced in vivo in Nherf1-deficient mice (27). In addition, deletion of the COOH-terminal TRL motif in Na\(_{\text{Pi}}\)-IIa or overexpression of soluble NHERF1 PDZ domain 1 (PDZ1) in the renal opossum kidney (OK) cell line disrupted apical Na\(_{\text{Pi}}\)-IIa localization (15).

NHERF1 contains two PDZ domains, PDZ1 and PDZ2; only PDZ1 is necessary for interaction with Na\(_{\text{Pi}}\)-IIa (19, 20). Moreover, NHERF1 forms part of a signaling complex in OK cells that contains PTH1R, PLC\(\beta\), and components of the actin cytoskeleton (19). It has been recently proposed that NHERF1 and NHERF2 are important for coupling of PTH1R to PLC (19, 20).

IN KIDNEY, filtered inorganic phosphate (P\(_i\)) is reabsorbed in proximal tubular cells, and the major player in this process is the type IIa Na\(^+\)-P\(_i\) cotransporter (Na\(_{\text{Pi}}\)-IIa), located in the brush border membrane (BBM) (23–25). The activity and abundance of this transporter are tightly regulated by different factors, such as dietary phosphate intake, acid-base status, and various hormones, including steroid hormones, dopamine, and parathyroid hormone (PTH) (23–25).

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To test for the function of NHERF1 in the hormonal regulation of NaPi-IIa by PTH, one of its major physiological regulators, we examined the PTH-induced internalization and signaling pathway in Nherf1-deficient mice.

Our data show that stimulation of PLC activity via the apical PTH1R was impaired in Nherf1-deficient mice, suggesting that NHERF1 in the proximal tubule is important for proper PTH-induced internalization of NaPi-IIa. Therefore, NHERF1 spe-

Fig. 1. Internalization of type IIa Na⁺-P, cotransporter (NaP-IIa) from brush border membrane (BBM) in response to parathyroid hormone (PTH). Kidney slices from Na⁺/H⁺ exchanger isoform 3 regulatory factor 1 (NHERF1)-deficient (Nherf1⁻/⁻) and wild-type (Nherf1⁺/⁺) mice were prepared and incubated for 45 min with control solution, 100 nM PTH-(1–34), or 100 nM PTH-(3–34). Sections were stained with an antibody directed against NaPi-IIa. After exposure to PTH-(1–34), NaPi-IIa-related fluorescence was similarly decreased in slices from wild-type and Nherf1-deficient mice, with a concomitant increase of the signal in the subapical compartment (bottom). After exposure to PTH-(3–34), NaP-IIa-related fluorescence was decreased in slices from wild-type, but not Nherf1-deficient, mice compared with control mice. Original magnification ×40 (top) and ×800 (bottom).
specifically couples the apical PTHR to PLC, allowing activation of PLC-dependent pathways and the subsequent regulation of major proximal tubular transport proteins.

MATERIALS AND METHODS

Animal studies. Approximately 24 wk-old sex-matched, wild-type (Nherf1+/+) and Nherf1-deficient (Nherf1−/−) mice (30–35 g body wt) of the same genetic background (C57BL6J) were used in the experiments. The generation and breeding of these mice have been described previously (27). For genotyping, tail DNA was prepared with the phenol-chloroform extraction method. For PCR, DNA samples were prepared at a concentration of 100 ng/μl. PCR was performed using Pyrocebus fuscusius DNA polymerase (Stratagene): 5′-CTCTGGTTTATTCCCAAGGATA-3′ (primer 1), 5′-CAAGAAAGCCGTAGAGGAGCGATG-3′ (primer 2), and 5′-GAGCCAGGTTCTACCAGACGGATAA-3′ (primer 3). Amplicons generated by PCR were 1,400 bp for the wild-type gene and 2,400 bp for the knockout gene; heterozygous mice showed both amplicons. Not significantly (P > 0.05). *P < 0.05. **P < 0.001.

Anatomical localization of NHERF1 and NaPi-IIa. Kidney slices were stained for immunohistochemistry or for Western blotting (see below).

Immunohistochemistry. Kidney slices were transferred for 4 h on ice to a fixation solution (3% phosphoformaldehyde) at the end of the incubation (2–5). After fixation, slices were rinsed three times with PBS, mounted onto thin cork plates, and immediately frozen in liquid propane cooled with liquid nitrogen. For NaPi-IIa immunostaining, sections were pretreated for 10 min with 3% defatted milk powder-0.02% Triton X-100 in PBS (“blocking solution”) to reduce nonspecific binding of antibodies. The sections were then incubated with anti-rat NaPi-IIa rabbit antiserum (1:500 dilution). For PTH receptor staining, the sections were pretreated with 0.5% SDS in PBS for 5 min. After they were repeatedly rinsed with PBS, the sections were incubated for 10 min with blocking solution and then with an affinity-purified polyclonal antibody against PTHR (Covance Research Products, Richmond, CA; 1:50 dilution). All primary antibodies were diluted in blocking solution and incubated overnight at 4°C. After overnight incubation, the sections were rinsed three times with PBS and covered for 45 min at room temperature. Antibody binding was detected with the peroxidase/luminal diant, and studied with an epifluorescence microscope.

Western blotting. Renal tissue for Western blotting was obtained from incubated kidney slices or from kidneys prepared directly from mice. Mice were anesthetized and perfused as described above. Kidneys were rapidly removed and frozen until further analysis. Frozen kidneys or kidney slices were used for BBM preparation as described previously using the Mg2+-precipitation technique (7). BBM protein concentration was measured (Bio-Rad protein kit), and 10 μg of protein were solubilized in Laemmli sample buffer containing 2% (vol/vol) 2-mercaptoethanol. Proteins were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). After they were blocked with 5% milk powder in Tris-buffered saline + 0.1% Tween 20 for 60 min, the blots were incubated with the primary antibodies [rabbit anti-PDK1 (14), 1:500 dilution; rabbit anti-NaPi-IIa, 1:6,000 dilution (10); mouse monoclonal anti-actin (42 kDa; Sigma); or rabbit anti-PTH receptor (Covance, 1:1,000 dilution) overnight at 4°C or for 2 h at room temperature. After they were washed and blocked again, the blots were incubated with the secondary IgG antibodies [donkey anti-rabbit (1:10,000 dilution) or sheep anti-mouse (1:10,000 dilution) conjugated with horseradish peroxidase (Amersham Life Sciences) or alkaline phosphatase (Promega), respectively] for 1 h at room temperature. Antibody binding was detected with the peroxidase/luminal
hydrophenoxazine, a sensitive fluorogenic probe for H$_2$O$_2$. Initially, phosphatidylcholine-specific PLC assay kit (Molecular Probes) and a dual-scanning fluorescence microplate reader (GENios multifunctional instrumentation). In this assay, phosphatidylcholine (lecithin) substrate was converted by PC-PLC to form choline oxidase to betaine and H$_2$O$_2$. Finally, H$_2$O$_2$ in the presence of horseradish peroxidase reacts with Amplex red reagent in a 1:1 equilibrium value.

RESULTS

In proximal tubular cells, PTH can interact with an apical or basolateral G protein-coupled receptor that triggers PLC and PKC or adenyl cyclase and PKA, respectively (21, 22). Experimentally, it is possible to discriminate between these two pathways, since PTH-(1–34) activates apical and basolateral receptors while PTH-(3–34) activates only apical receptors (21, 22, 32). We employed this differential sensitivity to distinguish the effect of PTH on apical or basolateral receptors. We examined the downstream signaling pathways after the activation of the PTH1R in Nherf1-deficient mice to test for the function of NHERF1 in the regulation of NaPi-IIa. 

Freshly isolated kidney slices were prepared from Nherf1-deficient and control mice and incubated in vitro with 100 nM PTH-(1–34) and 100 nM PTH-(3–34) for 1 h to test for PTH-induced internalization of NaPi-IIa by immunohistochemistry. This treatment has been previously shown to induce internalization of NaPi-IIa from the BBM, leading to transient accumulation in the subapical compartment and subsequent routing to lysosomes for degradation (1, 3, 4, 11). As shown in Fig. 1A, incubation of kidney slices with PTH-(1–34) allowed a normal internalization of NaPi-IIa in kidneys from Nherf1-deficient and control mice. Higher-magnification images showed clearly the subapical appearance of NaPi-IIa after treatment with the hormone, indicating that the application of PTH, in wild-type and Nherf1-deficient mice, resulted in the retrieval of the Na$^+$-Pi cotransporter. In contrast, activation of only apical PTH receptors with PTH-(3–34) failed to induce a visible internalization of NaPi-IIa in slices prepared from Nherf1-deficient mice but caused retrieval in kidney slices from wild-type mice (Fig. 1B).

BBM fractions were prepared from kidney slices treated with PTH-(1–34) or PTH-(3–34) or left untreated and submitted to immunoblotting. NaPi-IIa protein abundance was lower in control slices prepared from Nherf1-deficient than slices from wild-type mice (Fig. 2), as previously shown (27). Both PTH fragments significantly reduced NaPi-IIa protein in the BBM vesicles from wild-type mice. In Nherf1-deficient mice, PTH-(1–34) displayed a nonsignificant decrease in the BBM, whereas PTH-(3–34) was without effect (Fig. 2), consistent with the observations from immunohistochemistry (Fig. 1).

Apical PTHRs and pharmacological activation of PKC with DOG induce NaPi-IIa internalization from the BBM vesicles through a PLC- and PKC-dependent pathway (4, 32). Therefore, we tested whether activation of PKC with DOG induced NaPi-IIa retrieval in freshly isolated kidney slices. Slices were also incubated with 8-Br-CAMP to activate the PKA-dependent internalization of NaPi-IIa as a positive control. Activation of PKA and PKC by 8-Br-CAMP and DOG, respectively, induced internalization of NaPi-IIa in slices from wild-type, as well as Nherf1-deficient, mice (Fig. 3). Thus the failure of PTH-(3–34) to retrieve NaPi-IIa is not based on a loss of the endocytic machinery to respond to a PKC-dependent stimulus; rather, the

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FIG. 3. Intact PKA- and PKC-mediated NaPi-IIa internalization. Kidney slices from wild-type and Nherf1-deficient mice were prepared and incubated for 45 min with control solution, the PKA activator 8-bromo-cAMP (100 μM, 8-Br-CAMP), or the PKC activator 1,2-dioctanoyl-sn-glycerol (10 μM, DOG). Sections were stained with an antibody against NaPi-IIa (green) and with rhodamine-phalloidin against β-actin filaments (red) as a marker for BBM. A high degree of overlap (yellow) between NaPi-IIa (green) and actin (red) under control conditions indicates apical localization of NaPi-IIa in both genotypes. After exposure to 8-Br-CAMP or DOG, NaPi-IIa-related fluorescence in wild-type BBM shifted toward the subapical compartment. There was no detectable difference between wild-type and Nherf1-deficient mice. Original magnification ×800.
defect must rely on the activation of the apical PTHR itself or the transduction of the signal from the receptor to PKC.

Next, we investigated the expression levels and localization of the PTHR on the apical side of the proximal tubular cells immunoblot analysis of BBM fractions and immunohistochemical analysis of kidney slices from nontreated animals. Compared with wild-type mice, the expression level and the localization of the PTH receptor were preserved in Nherf1-deficient mice (Fig. 4). Inasmuch as NHERF1 and NHERF2 anchor PTHRs to PLC in membrane domains (19, 20), PLC activity was measured in kidney slices from Nherf1-deficient mice to test whether signal transduction of activated PTHRs depends on interaction with NHERF1. Therefore, kidney slices were incubated for 10 min with PTH-(1–34) or PTH-(3–34). Thereafter, BBM vesicles were prepared, and PLC activity was measured. The activity of PLC clearly increased in PTH-(1–34)- and PTH-(3–34)-treated samples from wild-type mice, as expected. On the contrary, no rise in PLC activity was found in kidney slices from Nherf1-deficient mice; rather, PLC activity decreased significantly (Fig. 5). This finding strongly suggests a defective coupling of the apical PTHR to PLC in Nherf1-deficient mice and a requirement of NHERF1 for PLC activation in response to stimulation of apical PTHRs.

NaPi-IIa is also rapidly downregulated and internalized after the intake of a phosphate-rich diet (8, 18), a process that is independent of PTH (30). To investigate whether NHERF1 is involved in this rapid downregulatory adaptation, NaPi-IIa localization, activity, and abundance were assessed 4 h after the mice were given a phosphate-rich diet. Mice were trained to engulf food during the morning. They were kept for 5 days on a low-phosphate diet before the diet was switched to a high-phosphate diet on the 6th day. Some mice continued on the low-phosphate diet and served as controls. As shown in Fig. 6, dietary regulation of the cotransporter was not altered in Nherf1-deficient mice compared with control mice: 1) immunohistochemistry showed a comparable localization of the NaPi-IIa protein in sections of kidney from mice chronically adapted to a low-phosphate diet and acutely adapted to a high-phosphate diet; 2) similarly, immunoblotting of BBM fractions obtained from the same groups of animals revealed no difference in the adaptation to the dietary change; and 3) Na\(^+\)-dependent Pi uptake into BBM vesicles from the same groups of mice was identical in both groups of mice. A similar reduction in the transport activity was detected in wild-type and Nherf1-deficient mice that were acutely adapted to the

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**Fig. 4.** Preserved apical PTH receptor (PTH1R) expression in proximal tubules from Nherf1-deficient proximal tubules. A: Western blot against G protein-coupled PTHR (PTH1R) and actin demonstrated that abundance in BBM is unchanged in Nherf1-deficient compared with wild-type mice. Membranes were stripped and reprobed for actin as a loading control. B: summary of PTH1R-to-actin ratios. No significant difference was found. C: immunohistochemical staining of PTH1R in the proximal tubule. No obvious difference in localization of PTH1R in BBM in Nherf1-deficient and wild-type mice was observed. Original magnification ×800.

**Fig. 5.** Loss of phospholipase C (PLC) activation in response to PTH in Nherf1-deficient mice. PLC activity was measured in BBM fractions from kidney slices incubated with control solution, 100 nM PTH-(1–34), or 100 nM PTH-(3–34) for 10 min. PLC activity increased in PTH-treated slices from wild-type mice but decreased in slices from Nherf1-deficient mice. A summary of 5 different experiments is shown. PLC activity was normalized to that in nonstimulated control slices. *P < 0.05. **P < 0.001.
high-phosphate diet compared with mice chronically adapted to the low-phosphate diet (Fig. 6).

DISCUSSION

NaPi-IIa, located in the BBM of the proximal tubular cells, represents the major renal phosphate absorptive mechanism (24, 25). Its activity is tightly regulated by dietary intake of phosphate, acid-base status, and several hormones, including PTH (24, 25). To identify interacting proteins that may be involved in regulation, membrane targeting, and retraction of NaPi-IIa, we applied previously a yeast-two-hybrid screen with the cytosolic COOH terminus of mouse NaPi-IIa that contains the canonical TRL PDZ domain-binding consensus sequence (14). Among others, we identified the PDZ proteins NHERF1 and PDZK, which highly interact with NaPi-IIa in several in vitro assays and, importantly, colocalized with NaPi-IIa in the proximal tubular BBM (12–14). Using a PDZK1-deficient mouse model, we have shown that PDZK1 is important for the expression of NaPi-IIa under conditions of high-phosphate intake, probably by stabilizing the protein in the membrane (8).

NHERF1, in contrast, appears to be important for membrane insertion or trafficking of NaPi-IIa into the membrane. Deletion of the last TRL from the COOH terminus of NaPi-IIa or overexpression of the interacting PDZ1 domain of NHERF1 impairs apical expression of NaPi-IIa in the OK cell model (15). In the Nherf1-deficient mouse, NaPi-IIa expression in the BBM is reduced, and mice present with a mild hyperphosphaturia (27). Adaptation to a low-phosphate diet involves insertion of NaPi-IIa protein into the BBM. This process is defective in Nherf1-deficient mice (35) and in a primary proximal tubular cell line derived from Nherf1-deficient mice (9).

The basis of transfected cell line models, this scaffolding protein may be important for the formation of a multiprotein complex allowing coupling of the PTHR to its downstream effector PLCβ (19, 20). Inasmuch as NaPi-IIa binds NHERF1 and is internalized by a PLC- and PKC-dependent pathway, we were interested in investigating the role of NHERF1 in the regulation of the Na"'-Pi cotransporter by PTH. The availability of a mouse model deficient in NHERF1 has enabled in vitro experiments that indicate the importance of NHERF1 in the proximal tubule for a proper PTH-induced internalization of the NaPi-IIa cotransporter from the BBM. However, ablation of NHERF1 does not generally impair internalization, as evident from three sets of experiments: 1) internalization of NaPi-IIa occurs with PTH-(1–34), which is also acting on basolateral PTHRs; 2) internalization can be induced with pharmacological activators of the PKA and PKC pathways; and 3) internalization and downregulation of NaPi-IIa were normal after an acute switch to a high-phosphate diet. Thus the impairment is specific for the activation of apical PTHRs, in contrast to recent results obtained with mouse models deficient in the endocytic receptor protein megalin or its chaperone receptor-associated protein, where internalization in general was attenuated (1, 3).

Our experiments demonstrate that the failure to internalize NaPi-IIa in response to PTH-(3–34) is caused by defective coupling of the apical PTHR to PLC. Expression and localization of apical PTHRs were not affected by the loss of NHERF1 but, rather, by their ability to increase PLC activity on stimulation. PLC activity was even slightly, but significantly, reduced, which could be due to a cAMP-mediated inhibition by a negative-feedback mechanism on PLC activity (29). Stimulation of PKC, one of the downstream effectors of PLC, could still induce NaPi-IIa internalization. Thus NHERF1 is most likely required for the coupling of PLC to apical PTHRs in the proximal tubule. In contrast, basolateral PTHRs stimulate adenylate cyclase activity and induce NaPi-IIa internalization via a cAMP/PKA-dependent pathway (22, 32). This alternative coupling allows basolateral PTHRs to regulate NaPi-IIa, even in the absence of a functional apical PTHR-NHERF1-PLC-NaPi-IIa complex.

In proximal tubule cell models, NHERF1 assembles a PDZ-based multiprotein-signaling complex, including ezrin, NHE3, and PKA, which facilitates the phosphorylation of NHE3 by PKA and, thereby, inhibits the activity of this transporter (6, 16, 28, 36). In contrast, our data obtained from experiments on freshly isolated kidney slices in vitro and from whole animals in vivo suggest that the PKA-ezrin-NHERF1 complex is not essential for the regulation of NaPi-IIa by PKA, inasmuch as a normal PKA-mediated internalization of NaPi-IIa and residual phosphaturia were observed. The compensatory upregulation of other proteins involved in the PKA-dependent regulation cannot be completely ruled out, but defective PKA-dependent regulation of NHE3 activity has been demonstrated in Nherf1-
deficient mice, thereby pointing to the requirement of NHERF1 in this pathway (37). The normal internalization of NaPi-IIa following the PKA-dependent pathway most likely explains the partially preserved phosphaturic effect of PTH-(1–34) in Nherf1-deficient mice. However, because of the loss of the PKC-dependent pathway, PTH cannot exert its full phosphaturic effect.

In summary, loss of NHERF1 affects the PTH-induced internalization of the major renal Na\(^+\)/Pi cotransporter NaPi-IIa in vitro. The disturbance is most likely caused by the defective coupling between the apical PTHR and PLC. Ablation of NHERF1 fails to bring PTHR in close proximity to PLC and, hence, fails to activate the subsequent PKC-dependent cascade, which leads to NaPi-IIa internalization and its degradation. This is the first ex vivo in vitro evidence that NHERF1 affects the function of a G protein-coupled receptor, underlining the importance of scaffolding proteins for the organization of polarized signaling in epithelia.

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