Parathyroid hormone treatment induces dissociation of type IIa Na\(^{+}/\text{P}_i\) cotransporter-Na\(^{+}/\text{H}^+\) exchanger regulatory factor-1 complexes

Nadine Déliot,¹* Nati Hernando,¹* Zeya Horst-Liu,¹ Serge M. Gisler,¹ Paola Capuano,¹ Carsten A. Wagner,¹ Desa Bacic,² Stephen O’Brien,³ Jürg Biber,¹ and Heini Murer¹

Institutes of ¹Physiology and ²Anatomy, Zurich University, Zurich, Switzerland; and ³Genzyme Corporation, Framingham, Massachusetts

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Déliot, Nadine, Nati Hernando, Zeya Horst-Liu, Serge M. Gisler, Paola Capuano, Carsten A. Wagner, Desa Bacic, Stephen O’Brien, Jürg Biber, and Heini Murer. Parathyroid hormone treatment induces dissociation of type IIa Na\(^{+}/\text{P}_i\) cotransporter-Na\(^{+}/\text{H}^+\) exchanger regulatory factor-1 complexes. Am J Physiol Cell Physiol 289: C159–C167, 2005. First published March 23, 2005. doi:10.1152/ajpcell.00456.2004.—The type IIa Na\(^{+}/\text{P}_i\) cotransporter (NaP\(_i\)-IIa) and the Na\(^{+}/\text{H}^+\) exchanger regulatory factor-1 (NHERF1) colocalize in the apical membrane of proximal tubular cells. Both proteins interact in vitro. Herein the interaction between NaP\(_i\)-IIa and NHERF1 is further documented on the basis of coimmunoprecipitation and co-pull-down assays. NaP\(_i\)-IIa is endocytosed and degraded in lysosomes upon parathyroid hormone (PTH) treatment. To investigate the effect of PTH on the NaP\(_i\)-IIa-NHERF1 association, we first compared the localization of both proteins after PTH treatment. In mouse proximal tubules and OK cells, NaP\(_i\)-IIa was removed from the apical membrane after hormonal treatment; however, NHERF1 remained at the membrane. Moreover, PTH treatment led to degradation of NaP\(_i\)-IIa without changes in the amount of NHERF1. The effect of PTH on the NaP\(_i\)-IIa-NHERF1 interaction was further studied using coimmunoprecipitation. PTH treatment reduced the amount of NaP\(_i\)-IIa coimmunoprecipitated with NHERF antibodies. PTH-induced internalization of NaP\(_i\)-IIa requires PKA and PKC; therefore, we next analyzed whether PTH induces changes in the phosphorylation state of either partner. NHERF1 was constitutively phosphorylated. Moreover, in mouse kidney slices, PTH induced an increase in NHERF1 phosphorylation; independent activation of PKA or PKC also resulted in increased phosphorylation of NHERF1 in kidney tails in the cytosolic compartment (20).

THE TYPE IIA Na\(^{+}/\text{P}_i\) cotransporter (NaP\(_i\)-IIa) is an important regulator of inorganic phosphate (Pi) balance and mediates up to 80% of the renal reabsorption of Pi (Ref. 5; for review, see Ref. 27). NaP\(_i\)-IIa is expressed in the brush-border membrane (BBM) of the renal proximal tubules (8), where it couples the transport of one Pi with three Na\(^{+}\) (10). NaP\(_i\)-IIa contains about 650 amino acids and is predicted to contain at least eight transmembrane domains with the NH\(_2\)- and COOH-terminal tails in the cytosolic compartment (20).

NaP\(_i\)-IIa interacts with the Na\(^{+}/\text{H}^+\) exchanger-regulatory factor-1 (NHERF1), a protein of 358 residues, the mRNA of which is detected among other tissues in kidney, proximal small intestine, and liver (11, 35). Although originally identified as a factor required for cAMP-induced inhibition of the Na\(^{+}/\text{H}^+\) exchanger type 3 (NHE3) (37), NHERF1 has been shown to interact with different subsets of proteins, including membrane transporters and receptors, cytoplasmic proteins involved in intracellular signaling, and cytoskeletal proteins (for review, see Ref. 36).

NHERF1 is expressed in the apical membrane of different nephron segments of the kidney and colocalizes with NaP\(_i\)-IIa in the BBM of proximal tubules (11). It contains two PSD-95/Drosophila disk large-1/zonula occludens-1 (PDZ) domains, sequences of about 90 amino acids that are involved in protein-protein interaction (9), and a merlin-ezrin-radixin-moesin (MERM) binding domain. The apical location of NHERF1 is dependent on the MERM-binding domain, which indicates that its subcellular distribution is mediated by binding to members of the MERM family of cytoskeletal proteins. Indeed, studies in opossum kidney (OK) cells showed that the MERM-binding domain alone shows apical expression, whereas the single PDZ domains accumulate intracellularly (15).

On the basis of in vitro assays, interaction with NaP\(_i\)-IIa takes place via the first PDZ domain (PDZ1) of NHERF1 and the last three amino acids of the cotransporter (TRL), which represent a class I PDZ-biding motif S/T-X-(V/I/L), where X is any amino acid (11, 12). Moreover, in vivo experiments have shown that transfection of PDZ1 alone in OK cells has a dominant negative effect on the apical expression of the endogenous cotransporter (15). This effect is consistent with our previous finding that TRL of NaP\(_i\)-IIa impairs the apical expression of the cotransporter (17). In addition, recent reports have suggested that the NaP\(_i\)-IIa-NHERF1 association can also take place independently of the TRL motif (19, 22).

Interaction with NHERF1 is involved in different cellular events such as apical targeting and/or retention (26), regulation of the fate of retrieved proteins (6), and assembly of regulatory complexes (21, 28). As mentioned above, we have shown that the TRL of NaP\(_i\)-IIa is involved in its apical expression (17) and that this at least partially involves binding to NHERF1 (15). This finding is supported by the phenotype of the NHERF1-knockout mouse, which are characterized by reduced expression of NaP\(_i\)-IIa in BBM of proximal tubules (7, 33).

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Parathyroid hormone (PTH) induces internalization of the cotransporter (for review, see Ref. 27). This effect has been studied in animal models as well as in OK cells. In both systems, PTH induces endocytosis and lysosomal degradation of NaPi-IIa (18, 29, 34). The PTH effect is mediated by receptors localized in the apical and basolateral membranes, and activation of these receptors transduces intracellular signals involving both PKA and PKC pathways (1, 34). The apical response is mediated essentially by PKC, whereas both pathways seem to be involved in basolateral signaling (34). Both signals converge, at least partially, at the level of ERK1/2 (4).

Interestingly, NHERF1 and NHERF2 have been shown to interact with the PTH receptor 1 and PLC, and this interaction may condition the intracellular cascade activated after PTH treatment (25).

Herein we show that unlike NaP-IIa, NHERF1 remained apically expressed after PTH treatment in both proximal tubules and OK cells. Moreover, the total amount of NHERF1 was not affected by PTH. Thus the hormonal treatment seems to induce the dissociation of NaPi-IIa-NHERF1 complexes. In agreement with this hypothesis, communoprecipitation assays showed that the amount of NaP-IIa associated with NHERF1 was reduced in OK cell lysates from PTH-treated samples compared with controls. PTH induced an increase in the phosphorylation state of NHERF1 in kidney slices, whereas NaP-IIa was not phosphorylated either under basal conditions or after PTH treatment. Therefore, PTH-induced dissociation of NaPi-IIa-NHERF1 complexes takes place in the presence of increased phosphorylation of NHERF1, although further studies are required to show a cause-and-effect relationship between these phenomena.

MATERIALS AND METHODS

Cell culture and transfections. General handling of OK cells was described in detail previously (29). OK cells were transfected in the presence of Lipofectamine (GIBCO-BRL, Grand Island, NY) with empty plasmid (pcDNA) or plasmids containing myc-fused wild-type (WT) or S289A NHERF1. The generation of myc-NHERF1 was described previously (15). The S289A mutant was obtained by performing PCR-based point mutation of the cDNA encoding the WT protein. To generate the cell line expressing V5-NaPi-IIa, the V5 epitope was inserted into the second extracellular loop of the mouse cotransporter, between the two predicted glycosylation sites.

Immunostaining. Confluent OK cultures plated on glass coverslips were preincubated with or without leupeptin (100 μg/ml) for 30 min, followed by incubation with 1-34 PTH (10−8 M) for 4 h. Next, the cells were processed for immunostaining with antibodies against endogenous NaP-IIa (23) as well as with a monoclonal anti-myc antibody (Invitrogen) as previously reported (15). For actin detection, Alexa Fluor phalloidin (Molecular Probes, Eugene, OR) was added together with secondary antibodies. The coverslips were mounted into the second extracellular loop of the mouse cotransporter, between the two predicted glycosylation sites.

In vivo phosphorylation. OK cells expressing V5-fused NaPi-IIa or myc-fused NHERF1 (WT and S289A mutant) were plated on six multiwell dishes. As a negative control, mock-transfected cells were processed in parallel. Confluent cultures were first incubated for 30 min in phosphate-free medium containing (in mM) 1.8 CaCl2, 5.36 KCl, 109.5 NaCl, 0.8 MgSO4, 44 NaHCO3, 2.5 d-glucose, and 4 glutamate, plus 4x amino acids for basal MEM, 1x nonessential amino acids, and 4x vitamins for basal MEM. Cells were then incubated for an additional 30 min with 100 μCi 32PO4/ml with or without leupeptin (100 μg/ml), followed by 2-h treatment in the presence or absence of 10−3 M 1-34 PTH, 10−6 M 3-34 PTH, or 10−4 M 8-bromoadenosine 3′,5′-cyclic monophosphate (8-BrcAMP). Cells were lysed in TBS buffer (150 mM NaCl and 50 mM Tris·HCl, pH 7.4) containing 0.5% Igepal CA-630, 1% protease inhibitors (Sigma), and 1% phosphatase inhibitors. Lysates were centrifuged for 5 min at 10,000 g, and the supernatants were subjected to immunoprecipitation using monoclonal V5 or myc antibodies. Briefly, after preclearing with protein G/A-agarose beads (Oncogen Research Products, La Jolla, CA) for 1 h at 4°C, lysates were incubated overnight with either monoclonal anti-NHERF1 (Abcam, Cambridge, UK) or anti-V5 antibodies (Invitrogen, Carlsbad, CA), respectively. Immunoreactive signals were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, UK).

GST pull-down of NHERF1 and NaPi-IIa from mouse kidney samples. Glutathione-S-transferase (GST) pull-down experiments were performed as described previously (12). Briefly, glutathione agarose beads were coupled, for 30 min at 4°C with rotation, to either GST alone or GST fused to the NH2-terminal ezrin domain, the COOH-terminal cytoplasmic tail of NaPi-I, or the PDZ3 domain of PDZK1. The beads were then collected by performing centrifugation at 12,000 rpm for 30 s and incubated for 1 h at 4°C with either EBG (high) or phosphorylated lysates from kidney cortex (see Fig. 5). After several washes in Tris-buffered saline (TBS) buffer, bound proteins were eluted by incubation for 2 min at 94°C with 2X loading buffer containing DTT. Eluted proteins were separated in 10% SDS-PAGE gel, transferred to nitrocellulose membranes, and incubated with antibodies against NHERF1 or NaP-IIa.

In vivo phosphorylation. OK cells expressing V5-fused NaPi-IIa or myc-fused NHERF1 (WT and S289A mutant) were plated on six multiwell dishes. As a negative control, mock-transfected cells were processed in parallel. Confluent cultures were first incubated for 30 min in phosphate-free medium containing (in mM) 1.8 CaCl2, 5.36 KCl, 109.5 NaCl, 0.8 MgSO4, 44 NaHCO3, 2.5 d-glucose, and 4 glutamate, plus 4X amino acids for basal MEM, 1X nonessential amino acids, and 4X vitamins for basal MEM. Cells were then incubated for an additional 30 min with 100 μCi 32PO4/ml with or without leupeptin (100 μg/ml), followed by 2-h treatment in the presence or absence of 10−3 M 1-34 PTH, 10−6 M 3-34 PTH, or 10−4 M 8-bromoadenosine 3′,5′-cyclic monophosphate (8-BrcAMP). Cells were lysed in TBS buffer (150 mM NaCl and 50 mM Tris·HCl, pH 7.4) containing 0.5% Igepal CA-630, 1% protease inhibitors (Sigma), and 1% phosphatase inhibitors. Lysates were centrifuged for 5 min at 10,000 g, and the supernatants were subjected to immunoprecipitation using monoclonal V5 or myc antibodies. Briefly, after preclearing with protein G/A-agarose beads (Oncogen Research Products, La Jolla, CA) for 1 h at 4°C, lysates were incubated overnight with protein G/A-agarose beads and V5 or myc antibody. After five washes with lysis buffer, bound proteins were eluted at 94°C for 2 min. with 1X loading buffer containing 100 mM DTT. Samples were run on 9% SDS-PAGE gel and transferred to nitrocellulose membranes. Incor-
through their COOH-terminal PDZ-binding motif (i.e., TRL) and PDZ-1, respectively (11, 12), although a TRL-independent interaction also has been proposed (19, 22). In the present study, we have provided further evidence that supports an interaction between NaPi-IIa and NHERF1. Thus incubation of lysates from OK cells with an antibody against NaPi-IIa led to the coimmunoprecipitation of NHERF1 with the cotransporter (Fig. 1A, lane 3), in agreement with recent reports (24); as expected, no signal was detected when either the antibody (Fig. 1A, lane 1) or the cell lysate (Fig. 1A, lane 2) was omitted. Coimmunoprecipitation of both proteins also has been described in mouse proximal tubules (35). NHERF1 interacts with the NH2-terminal domain of ezrin (31). With the use of the NH2-terminal domain of ezrin fused to GST, NHERF1 and NaPi-IIa were pulled down together from mouse BBM (Fig. 1, B and C, lane 2), further suggesting the presence of complexes containing both proteins; GST alone produced no signal (Fig. 1, A, B, and C, lane 1). Although the in vitro nature of the immunoprecipitation and GST pull-down assays may eventually lead to misinterpretation (i.e., proteins expressed in different subcellular compartments may associate and interact in a cellular lysate), the in vitro interaction in different assays, together with the in vivo expression within the same subcellular domain (apical membranes), strongly suggests that NaPi-IIa and NHERF1 interact in their native environment. An in vivo interaction between both proteins is further supported by data obtained with NHERF1-knockout mice, which show a reduced expression of NaPi-IIa in BBM (33), and by studies in OK cells in which apical expression of NaPi-IIa was disturbed by a dominant-negative form of NHERF1 (15).

NaPi-IIa and NHERF1 localize in different compartments after PTH treatment. PTH induces downregulation of NaPi-IIa in both renal proximal tubules and OK cells. This downregulation involves megalin-dependent endocytosis (2, 3) via clathrin-coated vesicles, followed by lysosomal degradation of internalized cotransporters (18, 29, 34). In the present study, we first investigated whether the expression of NHERF1 is also regulated in response to PTH treatment. For this purpose, we stained mouse kidney slices from control and PTH-treated animals with antibodies against NaPi-IIa and NHERF1. As shown in Fig. 2A, both proteins were expressed at the BBM of untreated proximal tubules. However, 45 min after incubation with PTH, the signal for NaPi-IIa was strongly reduced, whereas no significant changes for NHERF1 could be detected. The PTH effect on the distribution of both proteins was also studied in OK cells. We previously reported the production of a myc-NHERF1-expressing cell line and showed that the pattern of expression of the myc-fused NHERF1 is similar to that of the endogenous protein (15). In our present study, we used this cell line to study the localization of NaPi-IIa (green), myc-NHERF1 (red), and actin (blue) in control cultures as well as in cells treated with PTH (Fig. 2B). In all cases, lysosomal degradation was inhibited by pretreatment with leupeptin. In control cultures, NaPi-IIa and myc-NHERF1 colocalized within apical actin patches. Colocalization of both partners is evidenced by the yellow signal of the merged image; the confocal section (Fig. 2B, rectangle) shows that both proteins were indeed expressed at the apical membrane in control cultures. Four hours after addition of PTH, NaPi-IIa was detected almost exclusively in intracellular organelles (Fig. 2B). Fractionation experiments performed in rats indicated that
PTH leads to a lysosomal routing of NaPi-IIa (18). Although similar experiments in OK cells have not been performed, the fact that intracellular accumulation is not detected upon PTH treatment in the absence of leupeptin suggests that the organelles are late endosomes and/or lysosomes. In contrast to NaPi-IIa, the pattern of expression of NHERF1 remained similar to that of actin, both in control cultures and in samples treated with PTH. The merged confocal image of NaPi-IIa and NHERF1 clearly shows that 4 h after PTH treatment, both proteins were located in different cellular compartments: NaPi-IIa localized intracellularly, whereas NHERF1 remained in apical patches. Although the overall expression of NHERF1 and actin seemed disturbed in the cultures treated with PTH, NHERF1 retained the same pattern of expression as actin. Taken together, these immunostaining studies in kidney samples and OK cells indicate that the PTH-induced endocytosis of NaPi-IIa takes place without detectable internalization of NHERF1.

The effect of PTH on the total amount of both proteins was determined using Western blot analysis. Mouse kidney slices (Fig. 3, A and B) and OK cells (Fig. 3, C and D) were incubated with PTH (in the absence of lysosomal inhibitors) for the indicated times; Western blot analysis was then performed with membrane fractions. As expected, PTH led to the degradation of NaPi-IIa in both kidney and OK samples (Fig. 3, A and C, respectively). However, the amount of either endogenous (Fig. 3, A and C) or myc-NHERF1 (Fig. 3, D) was not affected by PTH.
3, B and D, top) or transfected NHERF1 (Fig. 3D, bottom) remained constant.

The above data indicate that NHERF1 does not follow NaPi-IIa in its PTH-induced membrane retrieval; instead, NHERF1 remained attached to the actin cytoskeleton.

**PTH disturbs the interaction between NaPi-IIa and NHERF1 in OK cells.** The above finding that NaPi-IIa and NHERF1 are located in different compartments after PTH treatment suggests that prior alterations occur in the interaction between both partners. To test this hypothesis, we performed coimmunoprecipitation experiments in lysates from OK cells stably expressing V5-NaPi-IIa. Cells were pretreated with leupeptin, followed by 3-h incubation in the absence or presence of PTH. As shown in Fig. 4, lysates from control samples (−) or samples treated with PTH (+) contained similar amounts of NHERF1 (Fig. 4A) and NaPi-IIa (Fig. 4B), because lysosomal degradation of endocytosed cotransporter was prevented by leupeptin. The NHERF antibody immunoprecipitated comparable amounts of NHERF1 from both samples (Fig. 4C). However, the amount of NaPi-IIa coimmunoprecipitated with NHERF1 was reduced in the PTH-treated sample compared with control (Fig. 4D). Quantification of three independent experiments indicated a reduction of ~35% in the PTH-treated samples after coimmunoprecipitation (Fig. 4E). This finding suggests that PTH regulates (i.e., weakens) the association of NaPi-IIa and NHERF1. The apparent discrepancy between a ~35% reduction of coimmunoprecipitation compared with a virtually 100% PTH-induced downregulation of NaPi-IIa may be explained by the fact that coimmunoprecipitation experiments were performed in the presence of leupeptin to prevent degradation of the cotransporter. Therefore, the total amount of cotransporter available for interaction with NHERF1 was kept higher than physiological levels and thus may mask a stronger effect of PTH on the dissociation of both proteins.

**Phosphorylation of NaPi-IIa and NHERF1.** PTH-induced downregulation of NaPi-IIa requires activation of PKA and PKC, although the final target for phosphorylation remains unknown (4). Interaction of NHERF1 with its partners can be regulated by phosphorylation of the S/T residue at position −2 of the PDZ-binding domain (6) or by phosphorylation of the corresponding PDZ domain of NHERF1 (30). Therefore, we studied whether PTH induces changes in the phosphorylation state of the partners. For this purpose, we performed in vivo (OK cells) and ex vivo (mouse kidney slices) phosphorylation studies.

To study the phosphorylation state of NHERF1 in OK cells, we used a cell line stably expressing myc-tagged NHERF1; as negative controls, parallel experiments were performed with cells transfected with empty plasmid (pcDNA). Upon incubation in the presence of 32P, NHERF1 was immunoprecipitated with a monoclonal myc antibody. After SDS-PAGE, the total amount of immunoprecipitated protein (Fig. 5A, top) and the incorporation of 32P (Fig. 5A, bottom) were quantified. NHERF1 was detected as a phosphoprotein under basal conditions (Fig. 5A, bottom). The overall phosphorylation state was not affected by treatment with 1-34 PTH or by independent activation of PKA or PKC (data not shown). However, these data do not rule out partial changes that could be masked because of the high basal phosphorylation signal. Three serine residues located between the second PDZ domain and the MERM-binding domain have been reported to undergo phosphorylation: Ser279, Ser289, and Ser301 (Fig. 5B). The residues at positions 279 and 301 are phosphorylated by the cyclin-dependent kinase Cdc2 during the cellular cycle (14), whereas Ser289 is phosphorylated by the G protein-coupled receptor kinase GRK6A and is constitutively phosphorylated in some cell lines (13). Therefore Ser289 was mutated to alanine (S289A), and its pattern of phosphorylation was analyzed. As shown in Fig. 5A, there was a dramatic decrease in 32P incorporation on S289A compared with WT. This suggests that in OK cells, Ser289 is responsible for the bulk of constitutive phosphorylation of NHERF1, similar to human embryonic kidney HEK-293 cells (13). This reduction in phosphorylation was associated with a shift in the apparent molecular weight (Fig. 5A, top) as reported previously (13, 31). Quantification of the ratio 32P to myc provided by three independent experiments indicated that the level of phosphorylation of the S289A mutant remained unchanged after PTH treatment or after independent activation of PKA or PKC (Fig. 5C). As shown in Fig. 5D, the mutated NHERF1 showed the same pattern of expression as WT-NHERF1 when transfected in OK cells, because it was detected in actin-containing apical patches. Furthermore, degradation of NaPi-IIa proceeded according to a time course similar to that observed in cell lines expressing either WT or S289A myc-NHERF1 (Fig. 5E), suggesting that endocytosis of NaPi-IIa is not affected by the presence of the mutated NHERF1.
To study the phosphorylation state of NHERF1 in kidney, cortical slices were incubated for 30 min with $^{32}$P, followed by an additional 30-min incubation in the presence or absence of 1-34 PTH. After homogenization, NHERF1 was pulled down with the COOH-terminal tail of NaPi-I (SLC17A1; see Ref. 32) fused to GST. We had to use this construct, owing to the failure of the COOH-terminal tail of NaPi-IIa to pull down NHERF1 (unpublished observations). NaPi-I is also expressed in the apical membrane of renal proximal tubules (27). The residues required for interaction with NHERF1 are identical in NaPi-I and NaPi-IIa (TRL), and yeast trap assays have confirmed that the COOH-terminal tail of NaPi-I interacts with NHERF1 (11); as negative controls, pull-down assays were performed with GST alone. After SDS-PAGE, the amount of protein pulled down (Fig. 6A, top) and the incorporation of $^{32}$P (Fig. 6A, bottom) were analyzed. NHERF1 was readily detected using Western blot analysis in the pull-down assays performed with GST fused to the COOH-terminal tail of NaPi-I, whereas no signal was detected in the negative control (Fig. 6A, top). Furthermore, NHERF1 was detected as a phosphoprotein under basal conditions, and its overall phosphorylation state increased upon incubation of kidney slices with PTH (Fig. 6A, bottom). Quantification of the ratio of $^{32}$P to pulled-down NHERF1 obtained in seven independent experiments indicated that the level of phosphorylation almost doubled after PTH treatment (Fig. 6B). To distinguish which kinase was responsible for the increase in phosphorylation induced by PTH, kidney slices were incubated with 8-BrcAMP (to specifically activate PKA) or with DOG (to activate PKC), and samples were processed as described above. As shown in Fig. 6 (C, bottom) activation of either kinase led to stimulation of NHERF1 phosphorylation; Fig. 6D shows the quantification of the ratio $^{32}$P to pulled-down NHERF1 obtained in three independent experiments. To our knowledge, this study is the first to report constitutive as well as regulated phosphorylation of NHERF1 in the kidney.

To study the phosphorylation state of NaPi-IIa, we used OK cells stably expressing V5-tagged NaPi-IIa; as negative controls, phosphorylation was performed in cells transfected with empty plasmid (pLXIN). As shown in Fig. 7A, the V5-tagged NaPi-IIa remained apically expressed when transfected in OK cells; moreover, the tagged transporter was endocytosed and degraded in lysosomes in response to PTH (data not shown). Cells were incubated with $^{32}$P and leupeptin in the absence or presence of 1-34 PTH. Next, V5-NaPi-IIa was immunoprecipitated with a monoclonal anti-V5 antibody. After SDS-PAGE, the amount of immunoprecipitated cotransporter (Fig. 7B, top) and the incorporation of $^{32}$P (Fig. 7B, bottom) were quantified. Although NaPi-IIa was readily detected using Western blot analysis, no incorporation of $^{32}$P was detected in either the absence or presence of PTH. The phosphorylation state of NaPi-IIa was also studied in mouse kidney slices. Slices were incubated with $^{32}$P and leupeptin in the absence or presence of 1-34 PTH. After homogenization, NaPi-IIa was pulled down with GST fused to the PDZ3 domain of PDZK1 (11). As negative controls, pull-down assays were performed in parallel with GST alone. After SDS-PAGE, the amount of pulled-down cotransporter (Fig. 7C, top) and the incorporation of $^{32}$P (Fig. 7C, bottom) were analyzed. As expected, NaPi-IIa was detected using Western blot analysis in the pull-down assays.

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Fig. 5. In vivo phosphorylation of NHERF1. OK cells stably transfected with myc-tagged NHERF1 [either wild-type (WT) or S289A] or with empty plasmid (pcDNA) were phosphorylated for 2 h in the presence of $^{32}$P. NHERF1 was then immunoprecipitated with myc antibody as described in MATERIALS AND METHODS. A: representative Western blot (WB) with myc antibody (top) and $^{32}$P autoradiograph (bottom). B: schematic of NHERF1 indicating the position of the serine residues known to be phosphorylated as well as the kinases suggested to be responsible for these phosphorylations. C: cells transfected with the S289A mutant were phosphorylated in the absence (lane 1) or presence of 1-34 PTH (lane 2), 8-bromoadenosine 3',5'-cyclic monophosphate (8-BrcAMP; lane 3), or 3-34 PTH (lane 4). After being immunoprecipitated with myc antibody, samples were processed for Western blot analysis (top) and $^{32}$P incorporation (bottom). D: immunostaining of OK cells transfected with myc-fused S289A mutant. Myc signal is shown in green, and actin signal is shown in red. Square represents a focal plane, and rectangle is a confocal section. E: PTH-induced downregulation of NaPi-IIa in cell lines expressing WT (top) or S289A-mutated (bottom) myc-NHERF1. Cells were incubated for the indicated times with $10^{-8}$ M 1-34 PTH, and lysates were processed for Western blot analysis with NaPi-IIa antibodies.
performed with the PDZ3 domain of PDZK1, but not with GST alone (Fig. 7C, top). However, as was true in the experiments with OK cells, no incorporation of 32P was detected in either the absence or presence of PTH (Fig. 7C, bottom). We have no explanation for the discrepancy between these findings and those of a previous report from our laboratory in which we described a reduction in NaPi-IIa phosphorylation upon PTH treatment of OK cells (16). Because the experiments described in the present report were performed in parallel with the NHERF1 studies, the lack of phosphorylation of NaPi-IIa cannot be due to failure of the assays. Instead, they indicate that even if small phosphorylation of NaPi-IIa may occur, this modification does not play a role in the regulation of the transporter.

In summary, our data show that NaPi-IIa-NHERF1 complexes are dissociated after PTH treatment. This conclusion is based both on the different location of both partners after PTH incubation (NaPi-IIa in lysosomes, NHERF1 bound to the apical actin cytoskeleton) and on the reduced amount of NaPi-IIa coimmunoprecipitated with NHERF1 antibodies in PTH-treated samples. This dynamic behavior does not seem to be mediated by changes in the phosphorylation state of NaPi-IIa. On the other hand, NHERF1 was detected as a phosphoprotein (in OK cells and kidney samples), and PTH induced an increase in NHERF1 phosphorylation (Fig. 6).

Fig. 7. In vivo and ex vivo phosphorylation of NaPi-IIa. OK cells were stably transfected with V5-tagged NaPi-IIa or with empty vector (PLXIN). A: immunostaining with a monoclonal V5 antibody of cultures expressing V5-NaPi-IIa. Staining was analyzed using confocal microscopy. Square corresponds to focal plane; rectangle shows a confocal cross section. B: cells transfected with either empty plasmid PLXIN or V5-NaPi-IIa were phosphorylated in the presence of 32P and leupeptin with or without 1-34 PTH as described in MATERIALS AND METHODS. Subsequently, NaPi-IIa was immunoprecipitated with a monoclonal V5 antibody. Top: representative Western blot using the V5 antibody; bottom: corresponding 32P autoradiogram from the same nitrocellulose membrane. C: slices of mouse kidney cortex were phosphorylated in the presence of 32P and leupeptin with or without 1-34 PTH. Subsequently, NaPi-IIa was pulled down using GST fused to the PSD-95/Drosophila disk large-1/zonula occludens-1 (PDZ) domain 3 (PDZ3) of PDZK1 (GST-PDZ3) as indicated. Top: representative Western blot using an anti-NaPi-IIa antibody; bottom: corresponding 32P autoradiogram from the same nitrocellulose membrane.

Fig. 6. Ex vivo phosphorylation of NHERF1. Slices of mouse kidney cortex were phosphorylated in the presence of 32P for 30 min, followed by an additional 30-min incubation in absence (−) or presence (+) of 1-34 PTH (A and B) and 8-BrcAMP or 1,2-dioctanoyl-sn-glycerol (C and D). Then NHERF1 was pulled down using GST fused to the COOH-terminal tail of NaPi-II (GST-CT) as described in MATERIALS AND METHODS. Eluted material was processed for Western blot analysis with antibodies against NHERF1 (A and C, top) as well as for 32P incorporation (A and C, bottom). The Western blot and 32P signals were quantified using ImageQuant software (Molecular Dynamics). In each experiment, the incorporation of 32P was normalized to the amount of immunoprecipitated protein. B and D: quantification of seven (B) and three (D) independent experiments. *P < 0.05.
crease in phosphorylation in kidney slices. This increase was reproducible after individual activation of PKA or PKC, the two signaling pathways involved in PTH-mediated downregulation of NaPi-IIa. These findings suggest that dissociation of NaPi-IIa-NHERF1 complexes may be regulated at least partially by phosphorylation of NHERF1, although further studies must be performed to prove this hypothesis. The role of the NHERF1 constitutive phosphorylation as well as its regulation has been studied extensively in proximal cell lines in the context of PKA-mediated inhibition of NHE3. Thus Lamprecht et al. (21) showed that in OK cells, the cAMP-mediated inhibition of NHE3 involves phosphorylation of NHERF3 without changes in the phosphorylation state of NHERF1. In our present study, we found a similar lack of regulation in OK cells; however, NHERF1 phosphorylation was stimulated in kidney slices after PKA activation. This discrepancy between the OK cells and kidney samples may reflect the lack of intermediate factors in the cell culture model. In this regard, these cells do not constitutionally express PDZK1, a proximal BBM protein known to interact with NaPi-IIa (Déliot N et al., unpublished data). On the other hand, using purified proteins in an in vitro phosphorylation assay, Raghuam et al. (30) reported phosphorylation of the PDZ2 domain of NHERF1 (Ser162) in response to PKC, and this modification resulted in inhibition of the interaction between NHERF1 and CFTR. CFTR binds to both PDZ domains of NHERF1, and phosphorylation of the PDZ2 domain prevented bivalent binding and therefore activation of CFTR. NaPi-IIa binds specifically to the PDZ1 domain of NHERF1 (11); therefore, further experiments should be performed to investigate whether PKC also induces phosphorylation of the Ser162 residue in kidney samples and whether this phosphorylation has any effect on NaPi-IIa-NHERF1 binding.

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