

Dynamics of PTH-induced disassembly of Npt2a/NHERF-1 complexes in living OK cells

Edward J. Weinman,^{1,3} Deborah Steplock,¹ Shirish Shenolikar,² and Thomas A. Blanpied¹

¹University of Maryland School of Medicine, and ³Department of Veterans Affairs Medical Center, Baltimore, Maryland; and ²Duke-National University of Singapore, Graduate Medical School, Singapore

Submitted 10 September 2010; accepted in final form 28 October 2010

Weinman EJ, Steplock D, Shenolikar S, Blanpied TA. Dynamics of PTH-induced disassembly of Npt2a/NHERF-1 complexes in living OK cells. *Am J Physiol Renal Physiol* 300: F231–F235, 2011. First published November 3, 2010; doi:10.1152/ajprenal.00532.2010.—Parathyroid hormone (PTH) inhibits the reabsorption of phosphate in the renal proximal tubule by disrupting the binding of the sodium-dependent phosphate transporter 2A (Npt2a) to the adapter protein sodium-hydrogen exchanger regulatory factor-1 (NHERF-1), a process initiated by activation of protein kinase C (PKC). To gain additional insights into the dynamic sequence of events, the time course of these responses was studied in living opossum kidney (OK) cells. Using a FRET-based biosensor, we found that PTH activated intracellular PKC within seconds to minutes. In cells expressing GFP-Npt2a and mCherry-NHERF, PTH did not affect the relative abundance of NHERF-1 but there was a significant and time-dependent decrease in the Npt2a/NHERF-1 ratio. The half-time to maximal dissociation was 15 to 20 min. By contrast, PTH had no effect on the fluorescence ratio for GFP-ezrin compared with mCherry-NHERF-1 at the apical surface. These experiments establish that PTH treatment of proximal tubule OK cells leads to rapid activation of PKC with the subsequent dissociation of Npt2a/NHERF-1 complexes. The association of NHERF-1 with Ezrin and their localization at the apical membrane, however, was unperturbed by PTH, thereby enabling the rapid recruitment and membrane reinsertion of Npt2a and other NHERF-1 targets on termination of the hormone response.

renal phosphate transport; protein kinase C; ezrin

THE RENAL REABSORPTION of filtered phosphorus correlates with the abundance of a number of sodium-dependent phosphate transporters located in the apical membrane of the cells of the proximal convoluted tubule in the mammalian kidney. In rodents, the sodium-dependent phosphate transporter 2A, Npt2a, accounts for nearly 80% of phosphate reabsorption and is the phosphate transporter whose abundance changes most rapidly in response to physiologic stimuli such as alterations in the dietary intake of phosphate and parathyroid hormone (PTH) (1). Through its COOH-terminal TRL amino acid sequence, Npt2a binds to the NH₂-terminal PDZ domain of an adaptor scaffolding protein called the sodium-hydrogen exchanger regulator factor-1 (NHERF-1) (9). NHERF-1, via its COOH-terminal domain, also binds ezrin, thereby linking the transporter to the actin cytoskeleton (2). It is estimated that 35 to 50% of the total Npt2a present in the apical brush-border membrane of renal proximal tubule cells is bound to NHERF-1 and this association results in an extended life span of Npt2a at the apical membrane surface (16). The physiologic importance of this complex of proteins is high-

lighted by the findings that in the absence of NHERF-1, proximal tubule cells are unresponsive to the inhibitory effect of PTH and to the downstream second messenger pathways activated by PTH such as protein kinase C (PKC) and protein kinase A (3, 4). These data suggest that Npt2a bound to NHERF-1 represents a unique pool of the transporter that is responsive to PTH and other hormones. Of considerable interest are the recent observations that NHERF-1 but not Npt2a is the target of the protein kinase pathways activated by PTH (6). Recent studies from our laboratory indicated that PKC mediates the phosphorylation of serine⁷⁷ in the first PDZ domain of NHERF-1 resulting in the disruption of Npt2a/NHERF-1 complexes and that this event is a requisite step in hormone-mediated inhibition of phosphate transport (16–18). This model of PTH regulation of phosphate transport was developed using static biochemical assays, and, as such, provided little insight into the dynamics of PTH-induced Npt2a trafficking in living renal cells. Accordingly, in the present experiments, we examined the distribution of Npt2a and NHERF-1 in opossum kidney (OK) cells, a proximal tubule cell line expressing these proteins as well as the PTH1 receptor. Using live cell imaging, we analyzed the kinetics of membrane localization of Npt2a, NHERF-1, and ezrin following the activation of PKC and compared these data with the previously determined changes in the lateral membrane mobility of Npt2a induced by PTH (17). These data show that PTH dissociates Npt2a from a NHERF-1/ezrin complex at the apical surface of renal proximal tubule cells and provide new insights into the dynamics of hormonal control of renal sodium-dependent phosphate transport.

MATERIALS AND METHODS

Cell culture. OK cells, an established cell line derived from the kidney of a female American opossum, were maintained in a humidified atmosphere of 5% CO₂-95% air at 37°C. The cells were grown on glass coverslips to confluence in DMEM/F12 media supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 100 g/ml streptomycin (Invitrogen). The cells were then serum starved before transfection with 2 µg of mCherry-NHERF-1 plasmid and 2 µg of either GFP-NPT2a or GFP-Ezrin plasmids using 10 µl Lipofectamine 2000 for 18 h. The cells were grown for an additional 48 h in serum-free DMEM/F12 lacking phenol red or antibiotics. Full-length mouse NHERF-1 was cloned into the pmCherry-C1 pBS34 vector (provided by R. Tsien, University of California San Diego, and the Howard Hughes Medical Institute). Full-length wild-type mouse Npt2a was cloned into the pEGFP-C1 vector (provided by N. Hernando, University of Zurich). Human ezrin was cloned into the pEGFP-N1 vector (provided by K. Ben-Aissa and S. Shaw, National Institutes of Health).

Imaging. The cells were maintained in a static bath containing DMEM/F12 lacking penicillin, streptomycin, serum, or phenol red and were imaged after treatment with DMSO (controls) or PTH (10^{−7} M) at 37°C using the point-scanning head of a Zeiss LSM 5 Duo confocal microscope equipped with an objective heater and a heated

Address for reprint requests and other correspondence: E. J. Weinman, Dept. of Medicine, Div. of Nephrology, Univ. of Maryland School of Medicine, Rm. N3W143, UHM, 22 South Greene St., Baltimore, MD 21202 (e-mail: eweinman@medicine.umaryland.edu).

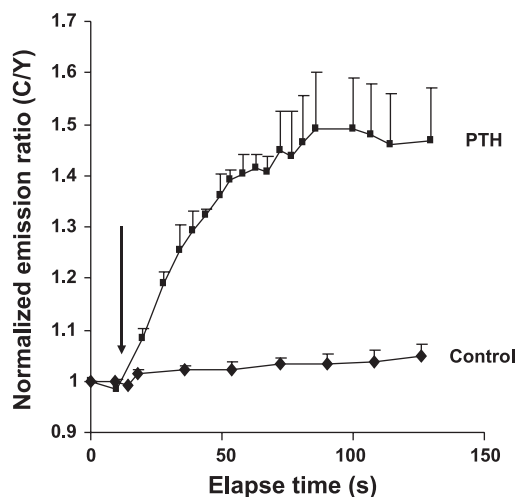


Fig. 1. Activation of protein kinase C (PKC) was determined using a FRET-based kinase activity reporter targeted to the plasma membrane in opossum kidney (OK) cells. The arrow indicates the time of the treatment with DMSO (control) or parathyroid hormone (PTH; 10^{-7} M). The results are expressed as the normalized emission ratio of CFP to yellow (means $\pm$ SE) for 3 cells in each group.

covered stage continuously superfused with humidified 5% CO_2 -95% air. Images were collected from the microvillar membrane of the cells representing the apical membrane in 5- or 10-min intervals up to 45 min using lines from a 488-nm Ar laser and a 561-nm diode laser in conjunction with a Zeiss $\times 63/1.4$ Plan-Apochromat oil-immersion objective. Z plane images were acquired at 0.5- μm intervals through

the apical extent of the cell and analyzed offline using MetaMorph software (Molecular Dynamics). Maximum projections of Z stacks were calculated, and three or more regions of interest (ROIs) were identified and the results were averaged for each cell.

Activation of PKC was determined using the FRET-based activity biosensor described by Depry and Zhang (7, 11). OK cells were transfected with a plasma membrane (lipid raft)-targeted kinase activity reporter termed MyrPalm-CKAR (provided by A. Newton, University of California San Diego). FRET, reported as the normalized ratio of cyan to yellow emission intensities, was determined using the Zeiss LSM 5 Duo point-scanning confocal microscope under control conditions or after treatment with PTH (10^{-7} M). In preliminary experiments, we established that MyrPalm-CKAR expressed in OK cells was activated by phorbol-12,13-dibutyrate (200 nM) and this activation was completely inhibited by pretreatment of the cells with the broad PKC inhibitor Gö6983 (1 μM).

Other assays. Coimmunoprecipitation experiments were performed using cell lysates from OK cells expressing mCherry-NHERF-1 and GFP-Ezrin in the absence or presence of PTH (10^{-7} M) for 45 min using polyclonal antibodies against either NHERF-1 or ezrin as previously described (14). The immunoprecipitates were resolved on 10% SDS-polyacrylamide gels, transferred to nitrocellulose, and Western immunoblots were performed using ECL. The bands of interest were quantitated and the results were expressed as the mean of means $\pm$ SE. Statistical comparison was performed using ANOVA.

RESULTS

Although PTH occupancy of the PTH1 receptor activates several protein kinase cascades, activation of PKC is the major pathway involved in PTH-mediated inhibition of renal phosphate transport (5). To study the time course of the activation

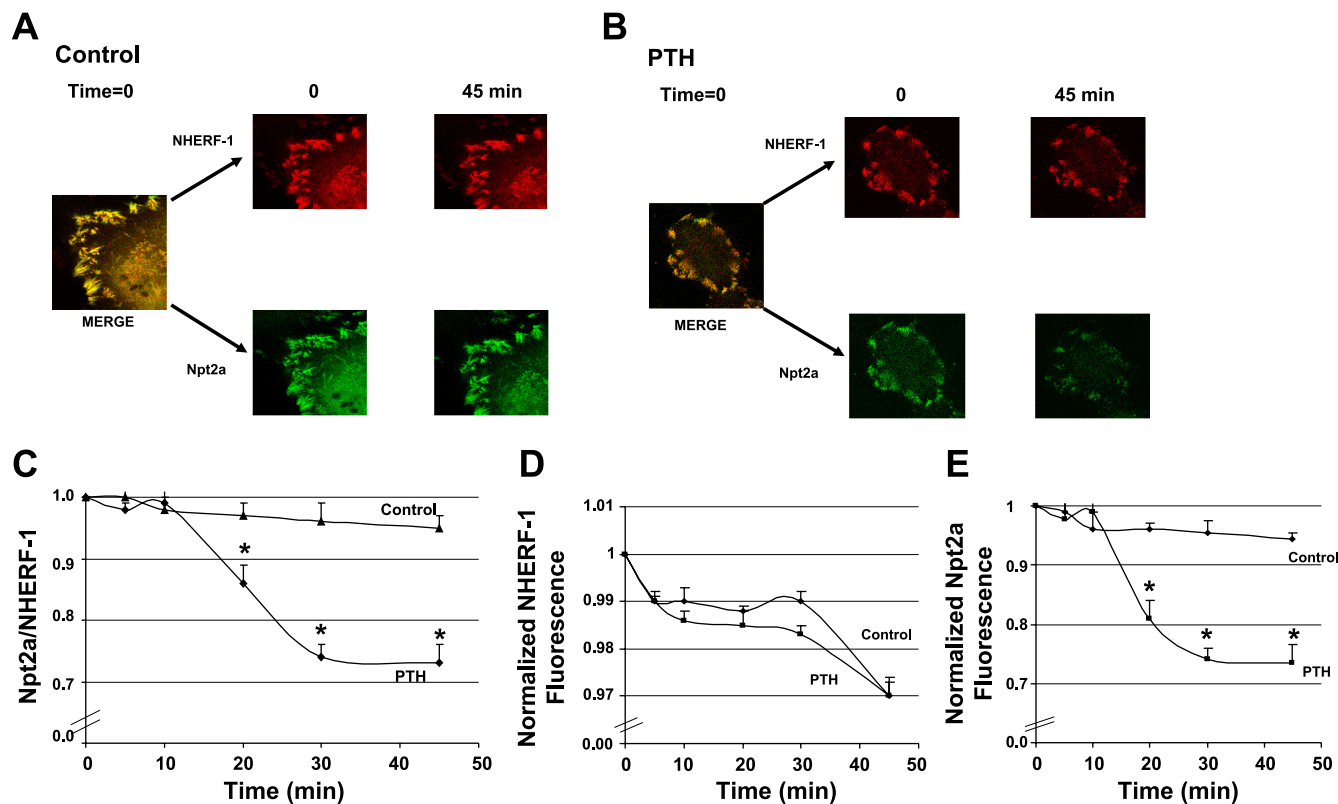


Fig. 2. OK cells expressing NH₂-terminal-labeled EGFP-Npt2a and mCherry-NHERF-1 were studied over time in the absence (A) or the presence of PTH (B). The ratio of fluorescence intensities was studied over a 45-min time period (C). D and E: same data replotted to allow direct comparison of the effect of PTH on sodium-hydrogen exchanger regulatory factor-1 (NHERF-1) and sodium-dependent phosphate transporter 2A (NPT2a) individually. The results are expressed as the mean of means $\pm$ SE of 10 cells for controls and 11 cells for PTH. * $P < 0.05$.

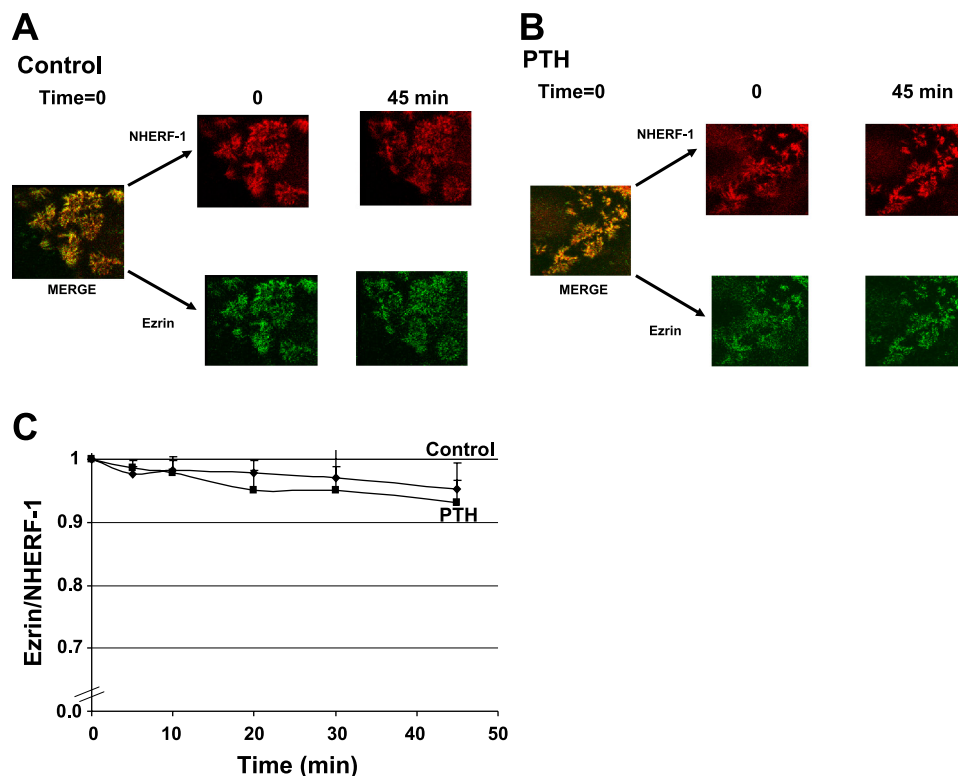


Fig. 3. OK cells expressing GFP-ezrin and mCherry-NHERF-1 were studied over time in the absence (A) or the presence of PTH (B). The ratio of fluorescence intensities was studied over a 45-min time period (C). The results are expressed as the mean of means $\pm$ SE of 8 cells for controls and 9 cells for PTH. The results are expressed as the mean of means $\pm$ SE.

of this protein kinase, the plasma membrane-targeted, FRET-based kinase activity reporter MyrPal-CKAR was expressed in OK cells (7, 11). The ROIs analyzed were chosen to include the finger-like microvillar projections of the apical plasma membrane. As shown in Fig. 1, PKC was maximally activated by 60 to 90 s following the application of PTH. These data confirmed that PTH rapidly activates PKC at or near the apical membrane.

In OK cells expressing NH₂-terminal-labeled GFP-Npt2a and mCherry-NHERF-1, both proteins colocalized in membrane patches (Fig. 2, A and B). Without addition of PTH, there was little change in either Npt2a or NHERF-1 levels in control cells (Fig. 2A). In response to PTH (10^{-7} M), the fluorescence intensity of Npt2a was decreased at 45 min compared with time 0 but there was no change in NHERF-1 (Fig. 2B). In Fig. 2C, the change in the ratio of fluorescence intensity of Npt2a to NHERF-1 is plotted as a function of time. The same data are also shown in Fig. 2, D and E, allowing direct comparison of the effect of PTH on NHERF-1 and NPT2a individually. In control cells, the small decrease in the fluorescence ratio likely reflects differential photobleaching of the two fluor. By contrast, PTH stimulated a significant decrease in the ratio that is due predominantly to the decrease in Npt2a with only a small change in NHERF-1. The decrease in Npt2a started after 10 min after treatment with PTH, reached statistical significance at 20 min, and was maximal at 30 min. The half-time to maximal change was 15 to 20 min.

PKC has also been reported to phosphorylate a COOH-terminal threonine residue in ezrin to dissociate inactive dimers (15). Thus, we analyzed the effect of PTH on the fluorescence ratio of GFP-ezrin/mCherry-NHERF-1 in apical membranes of living OK cells. These two proteins also colocalized in patches (Fig. 3A), but PTH had no effect on the ratio of their fluores-

cence intensities (Fig. 3, B and C). Since the effect of PTH on the relationship between ezrin and NHERF-1 has not previously been studied in kidney cells, we sought to confirm the lack of effect of PTH on this linkage using coimmunoprecipitation. Ezrin was immunoprecipitated from control or PTH-treated cells and the precipitates were immunoblotted for ezrin and NHERF-1 (Fig. 4). In three experiments, the ratio of ezrin to NHERF-1 was 0.17 ± 0.01 (arbitrary units) in control cells and 0.18 ± 0.01 in cells treated with PTH [P = not significant (NS)]. In the reverse experiment where NHERF-1 was immunoprecipitated, the ratio of NHERF-1 to ezrin averaged 0.54 ± 0.05 and 0.52 ± 0.07 in control and PTH-treated cells (P = NS).

DISCUSSION

PTH inhibits the reabsorption of phosphate in the renal proximal convoluted tubules by decreasing the apical membrane abundance of sodium-dependent phosphate transporters including Npt2a (3, 4). In cellular studies, Déliot et al. (6) first

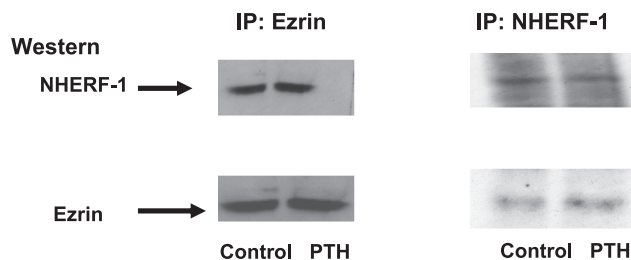


Fig. 4. Representative experiment where ezrin or NHERF-1 was immunoprecipitated from control or PTH-treated cell lysates and the precipitates were immunoblotted for NHERF-1 and ezrin.

reported that PTH-activated protein kinase cascades resulted not in the phosphorylation of Npt2a but rather NHERF-1. Our recent studies in mice confirm these observations and indicate further that activation of PKC is absolutely required for PTH to inhibit renal phosphate transport (5). This PKC-mediated modification of NHERF-1 occurs primarily at serine⁷⁷ located in the PDZ-I domain and is associated with decreased Npt2a binding (16). To determine the temporal sequence of events, we used a FRET-based kinase reporter and found that PTH activation of PKC was very rapid and near maximal within minutes after exposure to the hormone. We then analyzed the fluorescent intensity ratios for GFP-Npt2a and mCherry-NHERF-1 over time in living cells to define further the proposed model for hormonal control of phosphate transport. Confocal microscopy of OK cells expressing mCherry-NHERF-1 and GFP-Npt2a showed that both proteins localized in apical membrane patches as first described by Murer and colleagues (9). While there was a small time-dependent change in the fluorescence ratio in DMSO-treated control cells, there was a significant decrease in the GFP-Npt2a-to-mCherry-NHERF-1 ratio following PTH stimulation. This was due predominantly to the decrease in the intensity of GFP-Npt2a with no significant change in mCherry-NHERF-1. As we and others previously reported, the abundance of membrane-bound NHERF-1 in proximal tubule cells is unchanged following PTH treatment (3, 6). The decrease in the relative ratio of Npt2a to NHERF-1 was maximal after 30 min following PTH treatment with no further change seen up to 45 min.

These results, when considered in conjunction with our recent measurements of the lateral mobility of Npt2a in OK cells, allow some insights in the dynamic nature of the effects of PTH on renal phosphate transport. PTH engages PTH 1 receptors present on the apical and basolateral membranes of proximal tubule cells to rapidly activate PKC. This, in turn, results in NHERF-1 phosphorylation and the subsequent dissociation of Npt2a/NHERF-1 complexes (16). The initial dissociation of these complexes is manifested within 10 min after exposure to PTH and is reflected by an increase in the percent mobile fraction of Npt2a as determined by FRAP (17). The increased lateral mobility of Npt2a, however, is short lived and returns to normal by 10 min after PTH exposure. We ascribe this decrease in mobility to the association of Npt2a with proteins that mediate its endocytosis. Although this hypothesis remains to be tested, it is consistent with the immobilization of other membrane proteins at domains of endocytosis before their internalization (10). As Npt2a engages the endocytic machinery and is internalized, there is a decrease in the Npt2a/NHERF-1 ratio which is maximal by 30 min, consistent with the reported time course for inhibition of sodium-dependent phosphate transport in response to PTH.

Ezrin is also a PKC substrate in cells (15). Accordingly, we determined whether PTH could modulate the interaction between ezrin and NHERF-1. Prior coimmunoprecipitation studies established that ezrin assembled a complex of proteins that included NHERF-1 and, by virtue of its binding to NHERF-1, Npt2a (14). Ezrin is present in cells as inactive head to tail dimers (8). The phosphorylation of ezrin by a number of protein kinases including PKC dissociates these dimers and exposes the NH₂ terminus of ezrin thereby facilitating engagement of the COOH terminus of NHERF-1 (2, 8). The COOH terminus of NHERF-1 also contains several phosphorylation

sites that may be phosphorylated by PKC and, at least in vitro, may modulate NHERF-1 dimerization (13). However, it is currently unknown whether any of these sites are modified in response to PTH, nor is there any information about how this might impact NHERF-1 binding to ezrin. To address this question, we expressed GFP-ezrin and mCherry-NHERF-1 in OK cells and monitored their relative fluorescence intensities at the apical surface following PTH treatment. GFP-ezrin and mCherry-NHERF-1 colocalized in apical membrane patches as seen by confocal microscopy. In contrast to the changes in the GFP-Npt2a/mCherry-NHERF-1 ratio induced by PTH, we found no significant change in the fluorescence ratio for GFP-ezrin compared with mCherry-NHERF-1, suggesting that PTH did not disrupt the ezrin/NHERF-1 interaction. This was confirmed by coimmunoprecipitation experiments where we found the recovery of ezrin in anti-NHERF-1 immunoprecipitates was not affected by PTH. In the context of understanding PTH-mediated inhibition of phosphate transport in renal proximal tubule cells, these results indicate that PTH dissociates Npt2a from NHERF-1 but does not simultaneously disassemble the ezrin/NHERF-1 complexes. It might be argued that it would not be energetically efficient for a cell to totally disassemble the ezrin/NHERF-1/Npt2a complex since NHERF-1 binds a significant number of other target proteins that also require an association with ezrin (12).

In summary, the present studies in living OK cells establish the rapid time course of activation of PKC in response to PTH as well as the time course of the subsequent dissociation of Npt2a from NHERF-1 in the apical membrane of these cells. On the other hand, PTH did not disrupt the association between ezrin and NHERF-1, leaving these elements intact to interact with Npt2a and other NHERF-1-binding proteins in response to physiologic stimuli that enhance the insertion of new Npt2a transporters into the apical membrane to increase renal phosphate transport.

GRANTS

These studies were supported by National Institutes of Health Grants DK-55881 (to E. J. Weinman and S. Shenolikar) and MH-080046 (to T. Blanpied) and Research Service, Department of Veterans Affairs (to E. J. Weinman).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

1. Beck L, Karaplis AC, Amizuka N, Hewson AS, Ozawa H, Tenenhouse HS. Targeted inactivation of Npt2 in mice leads to severe renal phosphate wasting, hypercalciuria, and skeletal abnormalities. *Proc Natl Acad Sci USA* 28: 5372–5377, 1988.
2. Bretscher A, Edwards K, Fehon RG. ERM proteins and merlin: integrators at the cell cortex. *Nat Rev Mol Cell Biol* 3: 586–599, 2002.
3. Cunningham R, EX, Steplock D, Shenolikar S, Weinman EJ. Defective PTH regulation of sodium-dependent phosphate transport in NHERF-1^{-/-} renal proximal tubule cells and wild-type cells adapted to low-phosphate media. *Am J Physiol Renal Physiol* 289: F933–F938, 2005.
4. Cunningham R, Steplock D, EX, Biswas RS, Wang F, Shenolikar S, Weinman EJ. Adenoviral expression of NHERF-1 in NHERF-1 null mouse renal proximal tubule cells restores Npt2a regulation by low phosphate media and parathyroid hormone. *Am J Physiol Renal Physiol* 291: F896–F901, 2006.
5. Cunningham R, Biswas R, Brazie M, Steplock D, Shenolikar S, Weinman EJ. Distinctive signaling pathways utilized by parathyroid hormone and dopamine to inhibit phosphate transport in mouse renal proximal tubule cells. *Am J Physiol Renal Physiol* 296: F355–F361, 2009.

6. Déliot N, Hernando N, Horst-Liu Z, Gisler SM, Capuano P, Wagner CA, Bacic D, O'Brien S, Biber J, Murer H. Parathyroid hormone treatment induces dissociation of type IIa Na⁺-Pi cotransporter Na⁺/H⁺ exchanger regulatory factor-1 complexes. *Am J Physiol Cell Physiol* 289: C159–C167, 2005.
7. Depry C, Zhang J. Visualization of kinase activity with FRET-based activity biosensors. *Curr Protoc Mol Biol* In press.
8. Gary R, Bretscher A. Ezrin self-association involves binding of an N-terminal domain to a normally masked C-terminal domain that includes the F-actin binding site. *Mol Biol Cell* 6: 1061–1075, 1995.
9. Hernando N, Déliot N, Gisler SM, Lederer E, Weinman EJ, Biber J, Murer H. PDZ-domain interactions and apical expression of type IIa Na/P(i) cotransporters. *Proc Natl Acad Sci USA* 99: 11957–11962, 2002.
10. Petrini EM, Lu J, Cognet L, Lounis B, Ehlers MD, Choquet D. Endocytic trafficking and recycling maintain a pool of mobile surface AMPA receptors required for synaptic potentiation. *Neuron* 63: 92–105, 2009.
11. Violin JD, Zhang J, Tsien R, Newton AC. A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C. *J Cell Biol* 161: 899–909, 2003.
12. Voltz JW, Weinman EJ, Shenolikar S. Expanding the role of NHERF, a PDZ domain containing adaptor to growth regulation. *Oncogene* 20: 6309–6314, 2001.
13. Voltz JW, Brush M, Sikes S, Steplock D, Weinman EJ, Shenolikar S. Phosphorylation of PDZ1 domain attenuates NHERF-1 binding to cellular targets. *J Biol Chem* 282: 33879–33887, 2007.
14. Wade JB, Liu J, Coleman RA, Cunningham R, Steplock D, Lee-Kwon W, Pallone TL, Shenolikar S, Weinman EJ. Localization and interaction of NHERF isoforms in the renal proximal tubule of the mouse. *Am J Physiol Cell Physiol* 285: C1494–C1504, 2003.
15. Wald FA, Oriolo AS, Mashukova A, Fregien NL, Langshaw AH, Salas PJI. Atypical protein kinase C (iota) activates ezrin in the apical domain of intestinal epithelial cells. *J Cell Sci* 121: 644–654, 2008.
16. Weinman EJ, Biswas RS, Peng Q, Shen L, Turner CL, EX, Steplock D, Shenolikar S, Cunningham R. Parathyroid hormone inhibits renal phosphate transport by phosphorylation of serine 77 of sodium-hydrogen exchanger regulatory factor-1. *J Clin Invest* 117: 3412–3420, 2007.
17. Weinman EJ, Steplock D, Cha B, Kovbasnjul O, Frost NA, Cunningham R, Shenolikar S, Blanpied TA, Donowitz M. PTH transiently increases the percent mobile fraction of Npt2a in OK cells as determined by FRAP. *Am J Physiol Renal Physiol* 297: F1560–F1565, 2009.
18. Weinman EJ, Steplock D, Zhang Y, Biswas R, Bloch RJ, Shenolikar S. Cooperativity between the phosphorylation of Thr⁹⁵ and Ser⁷⁷ of NHERF-1 in the hormonal regulation of Renal Phosphate transport. *J Biol Chem* 285: 25134–25138, 2010.



Copyright of American Journal of Physiology: Renal Physiology is the property of American Physiological Society and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.