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PTH-mediated inhibition of the renal transport of phosphate

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Introduction and brief historical overview

Phosphate homeostasis is maintained primarily by regulation of the rates of excretion of phosphate in the urine. Of the many mechanisms that regulate the urinary excretion of phosphate, the effect of parathyroid hormone (PTH) is considered to be of critical importance. This review focuses on recent studies that have begun to define a somewhat surprising but well orchestrated sequence of biochemical and biophysical events linking PTH1 receptors to the phosphate transporters through the scaffolding protein Sodium-hydrogen Exchanger Regulatory Factor-1 (NHERF-1) to affect hormone-mediated regulation of renal phosphate transport. Greenwald and Gross first documented that parathyroid gland extract increased the urinary excretion of phosphate [1]. This seminal paper, published in 1925, initiated an almost 85 year search to define the mechanism by which PTH inhibits phosphate transport in the kidney. Agus and coworkers reported in 1971 that the reabsorption of phosphate filtered at the glomerulus occurs exclusively in the proximal convoluted portion of the nephron and that PTH inhibits this process [2]. They also documented that cAMP, the only known second messenger at that time, inhibited the proximal tubular transport of phosphate. In 1991, Murer and colleagues ushered in the molecular understanding of phosphate transport when they cloned a number of apical membrane localized sodium-dependent phosphate transporters including Npt2a (NaPi IIa), the major phosphate transporter in the kidney of rodents [3]. This group would then document that phosphate transport in renal proximal tubule cells correlated with the abundance of Npt2a in the apical membrane of proximal tubule cells and that PTH decreased its abundance [4]. It was assumed that the downstream protein kinase cascades initiated by PTH, phosphorylated specific serine and/or threonine residues in Npt2a although an initial mutational analysis failed to identify known endocytic motifs that affected the trafficking of Npt2a in response to PTH [4]. In 2002, two independent lines of investigation converged on the finding that Npt2a was bound to apical membrane scaffolding PDZ-domain containing proteins including NHERF-1 and that NHERF-1 null mice exhibited hypophosphatemia and renal phosphate wasting [5,6]. Later studies would indicate that NHERF-1 bound 35 to 50% of the apical membrane Npt2a transporters and functioned as an Npt2a membrane retention signal to extend the time of Npt2a expression at the apical membrane surface of renal proximal tubule cells [7]. It would then be demonstrated that

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PTH did not inhibit the transport of phosphate in the absence of NHERF-1 indicating that Npt2a bound to NHERF-1 was uniquely the pool of the transporter that was subject to hormonal regulation [8]. Thus, it would appear that PTH, acting through the PTH1 receptor, inhibits phosphate transport in the renal proximal tubule by a NHERF-1 dependent mechanism.

Modern view of renal phosphate transport in the renal proximal tubule

Under normal circumstances, approximately 80% of phosphate filtered at the glomerulus is reabsorbed in the proximal convoluted tubule. The loop of Henle and the more distal nephron segments do not contribute to net phosphate transport and phosphate exiting the proximal tubule appears in the urine. In the proximal tubule, phosphate transport is sodium-dependent and the rate limiting step is transport across the apical membrane of these cells mediated by at least three transporters, Npt2a, Npt2c, and Pit-2. Although PTH affects the activity of several sodium-dependent transporters in the renal proximal tubule, it has been clearly demonstrated that PTH-mediated inhibition of phosphate transport is not the consequence of altered driving forces, but a rather a direct effect on sodium-coupled phosphate entry into the cell. The absorbed phosphate exits across the basolateral surface of proximal tubule cells by a mechanism yet to be defined. In rodents, Npt2a is the dominant transporter and inactivation of this gene results in an 80% decrease in phosphate transport [9]. Npt2c is developmentally regulated in rodents and was thought to be of limited importance in adult animals. Inactivation of Npt2c in mice does not affect overall phosphate transport [10]. In man, however, mutations in Npt2c results in the development of a clinical syndrome called Hereditary Hypophosphatemic Rickets with Hypercalciuria and it has been suggested that Npt2c may play a more important role in phosphate transport than previously recognized [11]. Alternatively, mutated Npt2c might affect the function of Npt2a thereby explaining the differences between knockout mice with no Npt2c and man with a mutated gene product.

The differences in the characteristics of Npt2a, Npt2c, and Pit-2 have recently been reviewed [12]. In the context of the present discussion, it is important to note that Npt2a, Npt2c, and Pit-2 are recruited to the apical membrane of renal proximal tubule cells when there are stimuli to increase the reabsorption of phosphate and retrieved from the apical membrane when there are stimuli to decrease reabsorption and increase the urinary excretion of phosphate. Although all three transporters respond to the same stimuli, the signal transduction pathways and the response times are markedly different. For example, in animals, PTH decreases the apical membrane abundance of Npt2a in minutes, Npt2c in hours, while the response time of the minor transporter, Pit-2, has not been determined. If these response times also apply to man, it would appear that Npt2a is likely the major regulated phosphate transporter in the minute to minute regulation of phosphate excretion. The pathways for retrieval of these three phosphate transporters also differ. In response to PTH, Npt2a undergoes clathrin-mediated endocytosis and is targeted to lysosomes for degradation [13]. There is no evidence for an endosomal recycling pathway for Npt2a. By contrast, Npt2c undergoes down-regulation through a microtubule-dependent pathway that does not involve lysosomal degradation. The precise pathway for the decrease in apical membrane expression for Npt2c has not been determined but recent evidence suggests that the protein is shifted to the base of the microvillar compartment where it undergoes in situ dissolution [14]. Pit-2 degradation also appears to be resistant to lysosomal inhibitors but the precise pathway for endocytosis and degradation remains to be defined.

PTH-mediated inhibition of phosphate transport

PTH, PTH1 receptor, NHERF-1, and second messenger pathways

In renal proximal tubule cells, the PTH1 receptor is expressed on both the apical as well as the basolateral surfaces of the cells [15]. This dual representation of the same receptor in a single cell is unusual but not without precedent in the kidney [16]. It would be proposed that the basolateral PTH1 receptors interact with PTH in the blood of the peritubular capillaries while the apical receptors engage small but active PTH fragments filtered at the glomerulus. Subsequent experiments would indicate that the apical membrane PTH1 receptor signaled via a protein kinase C (PKC) pathway while the basolateral receptor signaled by a protein kinase A (PKA) pathway [17]. These results, in turn, raised two important questions. First, what accounted for the differences in downstream signals from the same receptor located on opposite sides of renal proximal tubule cells? Second, which of these two signal transduction pathways, PKA or PKC, was responsible for mediating the inhibitory effect of PTH on phosphate transport in the kidney?

Evidence has been advanced to indicate that Npt2a exists in the apical membrane as part of a signaling complex that included the PTH1 receptor, an A Kinase Anchoring Protein, the catalytic and regulatory components of PKA, NHERF1, and ezrin, suggesting that structural and anchoring proteins may play a role in regulating PTH signaling and functional responses [18,19]. A specific role for NHERF-1 in PTH1 receptor signal transduction was provided by Mahon, Segre and colleagues who demonstrated that both NHERF-1 and NHERF-2 bound to the PTH1 receptor [20]. When bound to either of the NHERF proteins, the receptor signals predominantly via a PKC pathway. In the absence of NHERF, signaling is via the cAMP/PKA pathway. Friedman and colleagues have recently extended understanding of this biologic switch mechanism by demonstrating that NHERF1 not only regulates the internalization and desensitization of the PTH1 receptor but also blocks desensitization of the PTH1 receptor by impairing binding of the receptor to arrestin [21]. NHERF-1 and NHERF-2 are located in the apical membrane of renal proximal tubule cells but are not present in the basolateral membrane [22]. This unique localization of the NHERF proteins provides a plausible explanation for the differences in PTH signaling from the apical and basolateral membranes. While of considerable interest, the physiologic significance of these interactions is, as yet, unclear. In mouse renal tissue, for example, PTH activation of PKC and PKA pathways is identical in wild-type and NHERF-1 null mice suggesting that either NHERF-1 or NHERF-2 located in the apical membrane can support the apical membrane PTH1 receptor [8].

Direct activation of either PKC or PKA individually in the renal proximal tubules cells inhibits sodium-dependent phosphate transport [23]. Since PTH activates both pathways, a number of investigators have sought to determine the individual effects of each pathway. Using multiple but non-specific pharmacologic inhibitors of PKC, it has been demonstrated that these reagents inhibit PTH-mediated inhibition of phosphate transport [24]. By contrast, inhibition of PKA does not block the inhibitory effect of PTH. To a first approximation, these finding would suggest that the PKC pathway is the relevant pathway and would be consistent with the findings that PKC directly phosphorylates NHERF-1 (see below). On the other hand, PKA can also phosphorylate NHERF-1 by an as yet unknown indirect pathway. Moreover, recent studies utilizing PTH analogs which specifically activate cAMP and phospholipase C or cAMP alone demonstrated equivalent reduction in Npt2a expression suggesting that activation of the cAMP/PKA pathway is of paramount importance in the inhibition of Npt2a [25]. Suffice it to note, that the relative role of PKC and/or PKA activation in PTH-mediated inhibition of phosphate transport continues to be debated.

Targets of PTH-mediated activation of PKC and PKA. The phosphorylation of NHERF-1

By analogy to a number of ion channels and hormone receptors, it would have been predicted that Npt2a would be the target of the second messenger pathways discussed above. The C-terminus of Npt2a contains several serine and threonine residues; potential targets of the activated protein kinases. Murer and colleagues approached the question of the mechanism of PTH-mediated inhibition of phosphate transport by mutating selected residues in Npt2a and monitoring the trafficking of the transporter [4]. While this analysis was extensive, it failed to identify key target residues. Later studies by the same investigators reported that Npt2a was not a phosphoprotein in unstimulated cells and was not phosphorylated in response to PTH treatment [26]. Said in another way, Npt2a is not modified by PTH-activated second messenger pathways. In the same studies, they reported an increase in the phosphorylation of NHERF-1.

NHERF-1 was initially isolated as a phosphoprotein and earlier studies suggested that PTH increased the phosphorylation of NHERF-1 in HEK-293 cells [27,28]. Ensuing studies identified several phosphorylation sites in the C-terminus of NHERF-1 and, perhaps, in the PDZ II domain. The significance of modifications at these sites has been discussed [29]. Npt2a, however, binds to the PDZ I domain of NHERF-1 and the possibility that phosphorylation of the PDZ I domain of NHERF-1 could serve as a regulator of physiologic responses had received limited attention. Using expressed cDNAs representing only the PDZ I domain and protein phosphatase inhibitors, serine⁷⁷ was identified as the major phosphate acceptor in this domain and threonine⁹⁵ as a secondary site [30]. Serine⁷⁷ resides in the alpha helix constituting a portion of the putative Npt2a binding groove and is a target of PTH-mediated phosphorylation. Using recombinant polypeptides, *in vitro* experiments demonstrated that serine⁷⁷ was phosphorylated directly by PKC but not PKA [7]. When expressed in cells, however, both PKC and PKA activation resulted in the phosphorylation of serine⁷⁷ indicating that PKA-mediated phosphorylation of serine⁷⁷ occurs in a cell dependent context and is likely via an indirect pathway. Multiple cellular and *in-vitro* assays demonstrated that biochemical modification of serine⁷⁷ of NHERF-1 decreased its binding affinity to Npt2a resulting in the dissociation of Npt2a/NHERF-1 complexes, and that this modification was required for PTH to inhibit phosphate transport in renal proximal tubule cells [7]. Recent experiments have also demonstrated an important role for threonine⁹⁵ of the PDZ I domain of NHERF-1 [31]. Threonine⁹⁵ is also phosphorylated by PTH-activated pathways. Phosphorylation of this residue does not affect the binding of Npt2a directly but rather is a required modification for PKC to phosphorylate serine⁷⁷. Thus, there appears to be cooperativity between the phosphorylation of threonine⁹⁵, the phosphorylation of serine⁷⁷, and PTH-mediated inhibition of phosphate transport.

The temporal sequence of events in PTH-mediated inhibition of phosphate transport

Using a PKC biosensor targeted to lipid rafts in proximal tubule-like opossum kidney cells in culture, we have recently demonstrated that PKC is activated by PTH in seconds to minutes [32]. Analysis of the lateral mobility of Npt2a by Fluorescence Recovery After Photobleaching (FRAP) indicates a significant increase within the first 10 min after treatment with PTH followed by a return to baseline [33]. The increase in lateral mobility is dependent on the presence of NHERF-1 and the PDZ domain binding sequence of the C-terminus of Npt2a. We speculated that the initial increase in lateral mobility represents the dissociation of Npt2a from NHERF-1. The phosphorylation of NHERF-1 would retard its reassociation with Npt2a and facilitate the binding of Npt2a to other proteins that decreased its lateral mobility and ultimately resulted in retrieval of the transporter from the plasma membrane. The recently identified interaction between Npt2a and myosin IV may represent

an example of this secondary process [34]. By confocal microscopy, the abundance of Npt2a in the apical membrane is not altered in the first 10 min after PTH treatment, the time that the lateral mobility is increased [32]. Subsequently, there is a decrease in the abundance of Npt2a in the apical surface that correlates with inhibition of phosphate transport. Interestingly, the abundance of NHERF-1 is not altered by PTH further suggesting the dissociation of Npt2a/NHERF-1 complexes.

The larger perspective

Starting from the initial observation that NHERF-1 was required for PTH to inhibit phosphate transport in the proximal tubule of the kidney, evidence has accumulated to indicate that NHERF-1 is the target of the down-stream phosphorylation cascades initiated by the occupied PTH1 receptor. The phosphorylation of residues in the PDZ I domain of NHERF-1 results in the dissociation of Npt2a/NHERF-1 complexes; a reaction required before Npt2a endocytosis can occur. These studies indicate that not only are the Npt2a/NHERF-1 complexes active in transporting phosphate, they are also the pool of transporters affected by PTH. While PDZ domains were initially believed to form static complexes, more recent experiments including studies of the relation between Npt2a and NHERF-1 discussed above, clearly indicate that PDZ domains can function as targets for the regulation of physiologic processes. We have speculated as to whether the regulated binding of Npt2a to NHERF-1 applies only to the biology of these two proteins or whether it represents a more general model involving other NHERF-1 PDZ I target proteins. In prior studies, we have shown that protein phosphatase inhibitors decrease the binding of three other NHERF-1 PDZ I interacting proteins, namely the β 2-adrenergic receptor, the platelet-derived growth factor receptor, and the cystic fibrosis transmembrane regulator [30]. The details of these effects, however, are yet to be elucidated.

Other recent studies have demonstrated that the C-terminus of NHERF-1 binds to the PDZ II domain forming a closed loop [35]. Phosphorylation of C-terminal residues of NHERF-1 results in the breakage of this bond thereby permitting NHERF-1 to interact with all the members of the cytoskeletal protein family including ezrin, moesin, radixin, and merlin [36]. These conformational changes and binding associations are likely to have important physiologic effects on processes involving NHERF-1 and, conceivably, may have longer range effects on interactions involving the PDZ I domain.

Clinical Correlations and speculations

NHERF-1 knockout mice demonstrate mild hypophosphatemia associated with increased urinary excretion of phosphate. In addition, they have elevated levels of 1,25 (OH)₂-vitamin D, modestly decreased serum concentrations of PTH, hypercalciuria, and at one year of age, increased calcium deposition in the interstitium of the medulla of the kidney [37]. Prie and coworkers reported mutations in the NHERF-1 gene in patients with nephrolithiasis associated with a decrease in the tubular maximal reabsorption of phosphate [38]. The identified mutations were all in non-PDZ-1 domains of NHERF-1. These observations would suggest that mutations in the non-Npt2a binding domain of NHERF-1 can alter phosphate transport by affecting either the binding of NHERF-1 to ezrin or by other effects on the PDZ-1 domain of NHERF-1 as discussed above. More recently in an as yet unpublished report, the same group identified a kidney stone patient with a mutation in the PDZ-1 domain, a phenotype predicted to more accurately reproduce the phenotype of the NHERF-1 knockout mouse.

The abundance of Npt2a in the apical membrane of renal proximal tubule cells correlates with the rates of phosphate absorption. The absence of NHERF-1 and, presumably, inactivating mutations in the NHERF-1 gene results in abnormal targeting of the Npt2a

phosphate transporter to the apical membrane of renal proximal tubule cells and renal phosphate wasting, at least in mice. We think it likely that additional examples of renal phosphate wasting associated with NHERF-1 mutations will also be found in man. Most of the defined regulatory processes involving phosphate homeostasis appear to be associated with changes in the apical membrane abundance of Npt2a including the inhibitory effects of PTH, dopamine, and Fibroblast Growth Factor-23 and the adaptation to alterations in the dietary intake of phosphate [4,39,40]. It should not be concluded, however, that all processes that alter phosphate balance are related to NHERF-1. Hypokalemia and changes in the lipid composition of the apical membrane of model renal tubule cells alter phosphate transport by mechanisms not involving the trafficking of Npt2a [41,42]. The role of NHERF-1 in these physiologic and pathophysiologic conditions has not been explored. Finally, the Npt2a transporter, as well as the other sodium-dependent phosphate transporters, associates with a number of other binding proteins in addition to NHERF-1. Absence or mutations in these proteins are likely to affect phosphate transport in the kidney independent of the relation between Npt2a and NHERF-1.

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Figure 1.

A proposed model of PTH-mediated inhibition of renal phosphate transport. Approximately 35 to 50% of Npt2a in the renal microvillar apical membrane is bound to NHERF-1 and it is this pool of the transporter that is responsive to PTH. PTH binds to apical and basolateral membrane PTH1 receptor activating protein kinase cascades that result in the phosphorylation of NHERF-1 and the dissociation of Npt2a/NHERF-1 complexes. Npt2a dissociated from NHERF-1 is then free to interact with other proteins that mediate its endocytosis.