Role of Vacuolar ATPase in the Trafficking of Renal Type IIa Sodium-phosphate Cotransporter

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Key Words

NpT2a • Opossum Kidney (OK) cells • Lysosomal degradation • PTH • Low Pi medium • Brefeldin A

Abstract

Background/Aims: Total body phosphate homeostasis is regulated by expression of type IIa sodium phosphate cotransporter (NpT2a) in the apical membrane (BBM) of renal proximal tubule cells. NpT2a expression is regulated by dietary phosphate and PTH but the mechanisms for trafficking of the protein are unknown. Based on 2D gel electrophoresis and mass spectroscopy data that changes in dietary phosphate stimulated changes in BBM expression of vacuolar H⁺-ATPase, we hypothesized that vacuolar H⁺-ATPase plays a significant role in regulation of NpT2a in opossum kidney (OK) cells, a model for renal proximal tubule transport. **Methods:** Role of vacuolar H⁺-ATPase was studied in opossum kidney (OK) cells by examining the effect of inhibition of vacuolar H⁺-ATPase on Pi uptake and NpT2a expression. **Results:** Pretreatment of OK cells with bafilomycin A₁ and concanamycin A, inhibitors of vacuolar H⁺-ATPases, blocked high phosphate- and PTH-induced degradation of NpT2a, but had no effect

on high phosphate or PTH induced inhibition of sodium-dependent phosphate transport. Exposure of the cells to bafilomycin A₁ significantly decreased phosphate transport and apical membrane expression of NpT2a. Treatment with brefeldin A, an inhibitor of Golgi transport, decreased phosphate transport and apical membrane expression of NpT2a while treatment of cells with both brefeldin A and bafilomycin A₁ had no additive effect. **Conclusion:** We conclude that vacuolar H⁺-ATPase plays a significant role in exocytosis of NpT2a into the apical membrane and in degradation of NpT2a but has no role in endocytosis.

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Introduction

The type IIa and IIc sodium-phosphate cotransporters (NpT2a, NpT2c) located on the apical membrane of proximal renal tubule represent a critical site for regulation of phosphate transport. They couple phosphate uptake with sodium uptake at the brush border membrane (BBM) of proximal tubular cells, thus allowing the movement of phosphate against an unfavorable electrochemical gradient [1]. Many stimuli regulate

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phosphate transport by altering the apical membrane expression of NpT2a [2–4]. The ability of proximal tubule to reabsorb Pi is determined by the number of NpT2a cotransporters residing in the BBM, which, in turn, is regulated by insertion and removal of these cotransporters into and out of the membrane [5].

NpT2a is constantly shuttled through a one-way path from synthesis and processing to insertion into the apical membrane, removal from membrane, and degradation [6]. Regulation of the number of active transporters is accomplished by altering either the rate or the bulk flow of proteins through this pathway. Parathyroid hormone (PTH)-induced phosphaturia is related to an endocytic retrieval of NpT2a from the BBM [7, 8]. Alterations in dietary phosphate intake also influence the renal absorption of Pi, and retrieval of NpT2a is responsible for the observed changes in Pi absorption after rats chronically fed a low Pi diet are acutely fed a high Pi diet [9, 10]. The chronic adaptation of Na/Pi co-transport to low Pi diet is dependent on de novo synthesis of NpT2a whereas the acute adaptation seems to be independent of mRNA and protein synthesis [10, 11].

Although the effect of many physiologic agonists on the expression of NpT2a is known, the mechanisms for trafficking of the protein in and out of the apical membrane are unknown. In preliminary experiments, we isolated and identified BBM proteins from rats on chronic or acute high or low phosphate diets. Proteomic analysis identified vacuolar H⁺-ATPase A subunit (ATP6V1A) as a differentially expressed protein in the BBM after acute changes in dietary phosphate (Table 1). Vacuolar H⁺-ATPases are present in the intracellular membranes of all eukaryotic cells where they are responsible for the acidification of organelles in the endocytotic/exocytotic pathways [12, 13]. Recent data suggest that H⁺ ATPases play a critical role in several renal tubular processes including vacuolar acidification, H⁺ secretion, bicarbonate reabsorption, endocytosis and plasma membrane protein trafficking [14–18]. Based on these data, we hypothesized that vacuolar H⁺-ATPase play a role in NpT2a trafficking in response to physiologic stimuli. The role of H⁺-ATPase in regulation of NpT2a was studied in opossum kidney (OK) cells, a model for renal proximal tubular cells. Our results show that vacuolar H⁺-ATPases are required for degradation of internalized NpT2a confirming the lysosomal degradation pathway for NpT2a in OK cells. The data further suggest that internalization of NpT2a stimulated by PTH and high phosphate diet is independent of vacuolar H⁺-ATPase, while exocytosis of NpT2a stimulated by low phosphate is H⁺-ATPase dependent.

Materials and Methods

Materials

Wild type OK cells were a generous gift of Dr. Steve Scheinman (Syracuse Health Science Center, Syracuse NY). Bovine PTH was obtained from Bachem (Philadelphia, PA) while Bafilomycin A₁ and Concanamycin A were obtained from Sigma Chemical Company (St. Louis, MO). [³²P] phosphoric acid was obtained from ICN Biomedicals (Irvine, CA). NpT2a antibodies were raised against the C-terminal 12 aminoacids in rabbits as described previously [19]. Rabbit anti-V-ATPase A subunit (catalogue number A00938) and B2 subunit (catalogue number A00960) antibodies were purchased from Genscript (Piscataway, NJ).

Low/high phosphate dietary conditions

The experiments were performed with 6–8-wk-old male Sprague-Dawley rats, as described previously [10]. After arrival at the Animal Care Facility, the animals were first stabilized on a control diet for 5 d, and they were then placed on high phosphate (1.2% Pi) or low phosphate (0.1% Pi) diets, which were otherwise identical in their mineral, electrolyte, protein, carbohydrate, fat, and calorie content (Teklad, Madison, WI). The rats were trained to consume their diet between 10 am and 12 PM each day. By the second day all rats consumed the amount of diet (16–20 g) which they usually eat during a 24-h period when fed *ad libitum*. The rats were pair-fed the high or low Pi diets for seven consecutive days.

On the eighth day, the following four groups of rats were studied: group 1, rats that were chronically fed 1.2% Pi diet and continued on 1.2% Pi diet (chronic high Pi diet); group 2, rats that were chronically fed 1.2% Pi diet and on the day of the experiment were acutely fed 0.1% Pi diet for 2 h (acute low Pi diet); group 3, rats that were chronically fed 0.1% Pi diet and continued on 0.1% Pi diet (chronic low Pi diet); group 4, rats that were chronically fed 0.1% Pi diet and on the day of the experiment were acutely fed 1.2% Pi diet for 2 h (acute high Pi diet).

Preparation of BBM vesicles from rat kidneys

On the day of the experiment at the end of the 2-h feeding period, the rats were anesthetized with intra-peritoneal administration of pentobarbital, blood was drawn from the aorta, and the kidneys were rapidly removed. Thin slices were cut at 4° C from the superficial cortex and homogenized with a Polytron in a buffer consisting of (mM): 300 DL-mannitol, 5 EGTA, 0.5 phenyl methylsulfonyl fluoride, and 16 HEPES (pH 7.5 with Tris). BBM were precipitated from this homogenate by Mg²⁺ precipitation and differential centrifugation as described [11, 16]. The final pellet was resuspended in a buffer of 300 mM mannitol, and 16 mM HEPES-Tris, pH 7.5. Purity of BBM preparations was determined by measuring the activity of BBM-specific enzymes as published [9, 20].

Two dimensional (2D) gel electrophoresis: First Dimension of 2D PAGE

Immobilized pH gradient (IPG) strips, nonlinear pH 3–10, 18 cm long (Amersham Pharmacia Biotech Inc., New Jersey)

Ref No.	Protein	GenInfo	Fold decrease (Chronic High to Acute Low Pi)	PI	M _w
1	H ⁺ -transporting ATPase (EC 3.6.3.6) chain A, vacuolar (VA68 type)	gi 1082432	0.4	5.3	68.63
2	DnaK-type molecular chaperone HSP72-ps1	gi 347019	0.4	5.4	71.14
3	Class I beta-tubulin	gi 3551054	0.58	4.8	50.11
4	Chain B, F1-ATPase	gi 6729935	0.57	4.9	51.33
5	Mid-1-related chloride channel 4	gi 17736806	0.52	5.6	42.21
6	NHERF-1 Mouse Isoform	gi 6755566	0.45	5.6	38.87
7	Apolipoprotein A-I	gi 6978515	0.46	5.5	30.10
8	Fructose-1,6-bisphosphatase	gi 119740	0.47	5.5	40.05
9	Serine/threonine kinase receptor associated protein	gi 6755682	0.53	5.0	38.78
10	Lipocortin V (annexin V)	gi 2981437	0.53	5.0	33.95
11	Chloride intracellular channel 1	gi 15617203	0.46	5.1	27.34
12	Actin, beta	gi 71620	0.33	5.3	42.08
13	Blood vessel epicardial substance	gi 13195598	0.61	6.9	41.51
14	Intracellular chloride ion channel protein p64H1	gi 13929166	0.4	5.9	28.84
15	Sodium channel protein pCSC-1 (fragment)	gi 321336	0.55	5.3	37.24
16	NHERF-3 rat isoform (PDZK1, Diphor-1)	gi 2331224	0.31	5.3	52.63
17	HSP60 protein (chaperonin 60)	gi 1334284	0.56	5.3	58.08
18	H ⁺ -transporting ATPase (EC 3.6.1.14) beta chain, mitochondrial (fragment)	gi 92350	0.38	4.9	50.75
19	ERM-binding phosphoprotein	gi 11024674	0.39	5.7	39.15
20	Tropomyosin 5; TM-5	gi 9653293	0.32	4.7	29.16
21	Solute carrier family 5 (Slc5a6) sodium-dependent multivitamin transporter	gi 18543353	0.47	8.9	69.54

Table 1. Differentially expressed proteins in BBMs of rats shifted from chronic high Pi to acute low Pi diet, as identified by proteomic analysis. Rats were maintained on different dietary conditions, as described in ‘Methods’. The reference number assigned to each protein is the same that is used in Fig. 1 to identify the corresponding spot on a representative 2-d gel. Only significantly matched proteins by peptide mass fingerprinting are included. The identifier of each protein is the GenInfo number in the NCBI protein database. M_w and pI indicate the theoretical position of each protein spot on two-dimensional gel based on the protein database.

were rehydrated overnight with 150 µg of proteins in rehydration buffer containing 8 M urea, 2% CHAPS, 0.01 M DTT, 2% ampholytes and bromophenol blue. The strips were then focused with maximal 5000 V and 80 µA at 17 °C for 24 h to reach 100,000 volt.hours. After completion of focusing, the samples were first equilibrated with buffer containing 6 M urea, 130 mM DTT, 30% glycerol, 112 mM Tris base, 4% SDS, 0.002% bromophenol blue and acetic acid, and then with buffer containing 6 M urea, 135 mM iodoacetamide, 30% glycerol, 112 mM Tris base, 4% SDS, 0.002% bromophenol blue and acetic acid.

Second Dimension of 2D PAGE

The strips were loaded onto pre-cast 10% homogeneous, 22 x 22 cm slab gels (Genomic Solutions Inc., Ann Arbor, MI). The system was run with maximal 500 V and 20,000 mW per gel using 0.2 M Tris base, 0.2 M tricine, 0.4% SDS and 0.625 M Tris/acetate lower running buffer.

Staining and Visualization

The gel slabs were fixed in 10% methanol and 7% acetic acid for 30 min. The fixing solution was removed and 500 mL of SYPRO ruby gel stain (Bio-Rad Laboratory, Hercules, CA) was added to each gel and incubated on continuous rocker at room temperature for 18 h. A high-resolution 12-bit camera with UV light box system (Genomic Solutions Inc.) was used to visualize the protein spots. The optimal light exposure time at 3 sec was set to scan all the gels.

Matching and Analysis of the Protein Spots

Investigator HT analyzer (Genomic Solutions Inc.) software was used for matching and analysis of the protein spots expressed on gels. A reference gel was created by combining all of the spots from different gels into one image. This was then used for determination of existence and difference of protein expression between each group. Average mode of background subtraction was used for normalization of intensity volume of each spot and for compatibility of the intensity between each gel. Normalized spot intensity, which represents protein amount per spot, was used as a parameter to determine differential protein expression.

In-Gel Tryptic Digestion, MALDI-TOF Mass Spectrometry, and Peptide Mass Fingerprinting

In-gel tryptic digestion and MALDI-TOF mass spectrometry were performed as described previously [21]. Peptide mass fingerprinting was performed to identify protein identities from tryptic fragment sizes by using the MASCOT search engine (<http://www.matrixscience.com>) queried to the entire NCBI protein database using the assumption that peptides are monoisotopic, oxidized at methionine residues and carbamidomethylated at cysteine residues. Up to 1 missed trypsin cleavage was allowed although most matches did not contain any missed cleavages. Mass tolerance of 150 ppm was the window of error to be allowed for matching the peptide mass values. Probability based MOWSE scores were estimated by comparison of search results against estimated random

match population and were reported as $-10 \cdot \log_{10}(P)$, where P is the absolute probability. Scores greater than 71 were considered significant ($p < 0.05$). The identity that did not meet these criteria was not reported.

Cell Culture

Wild type OK cells, passages 83-89, were grown as monolayers in 175-cm² plastic flasks (Falcon) or 3 mm by 10 mm tissue culture plates (Nunc) in Eagle's medium with Earle's salts (Gibco BRL; Life Technologies, Grand Island, NY), supplemented with 10% heat inactivated fetal calf serum, 4mM glutamine, 100µg/ml streptomycin, and 100 IU/ml penicillin, pH 7.4 in a humidified 5% CO₂-95% air environment at 37°C, as described previously [3]. They were fed three times a week and split 1:4 once per week by trypsinization and dispersal. Cells were used for experiments at 100% confluence.

Preparation of Low and High Phosphate Medium

Low phosphate medium was prepared by mixing 90 ml MEM without phosphate and 10 ml MEM containing 1 mM NaH₂PO₄. High phosphate media was prepared by adding 6 mM NaH₂PO₄ to regular MEM containing 1 mM NaH₂PO₄ such that the total phosphate concentration of NaH₂PO₄ was 7 mM. MEM without phosphate was prepared by adding 10 ml MEM-non essential aminoacid solution (Invitrogen, no. 11140), 20 ml MEM-essential aminoacid solution (Invitrogen, no. 11130) and 10 ml MEM-vitamin solution (Invitrogen, no. 11120) to a liter of phosphate free solution containing 200 mg CaCl₂, 400 mg KCl, 97.67 mg MgSO₄, 6.8 g NaCl, 1 g glucose, and 2.2 g NaHCO₃. The pH of the medium was adjusted to 7.4 with NaHCO₃.

Vacuolar H⁺-ATPase Activity

Bafilomycin A₁-sensitive ATP hydrolysis was assayed by measuring the production of inorganic phosphate as described by Lu et al. [22]. Cells were treated for 30 min at 37°C with indicated concentrations of bafilomycin A₁ or concanamycin A, lysed, crude membranes were prepared and ATPase activity was measured in the presence or absence of 1 mM bafilomycin A₁, a maximal H-ATPase inhibitory concentration. The difference between ATPase activity in the absence and presence of 1 mM bafilomycin represents H-ATPase activity. Briefly, crude membranes were incubated in ATPase buffer containing 150 mM NaCl, 2 mM MgCl₂, 1 mM sodium vanadate, and 1 mM sodium azide (pH 6.75) for 15 min at room temperature in the presence and absence of 1 mM bafilomycin A₁. The reaction was initiated by addition of ATP at a final concentration of 3 mM and incubated for 30 min at 37°C. The reaction was stopped by addition of trichloroacetic acid. The samples were extracted with equal amount of chloroform to remove lipid and detergent. The samples were centrifuged at 10,000 rpm for 20 min at room temperature. The upper aqueous phase was transferred to clean test tubes and inorganic phosphate released was measured as described previously [23]. Briefly, 0.5 ml of the supernatant was diluted to 0.9 ml with glass-distilled water, and 0.6 ml of ferrous sulfate reagent (5 gm FeSO₄ dissolved in 10% (w/v) ammonium molybdate in 10 N H₂SO₄) was added. A calibration curve was prepared simultaneously with the test samples, using known

concentrations of KH₂PO₄ (9-180 nmol P_i) and 4.8 mM ATP. The blue color obtained was read at 820 nm after 20 min incubation at room temperature in a Hewlett Packard 8453 spectrophotometer against a reagent blank. The difference in the ATPase activity assayed in the absence and presence of 1 mM Bafilomycin A₁ is taken as a measure of vacuolar H⁺-ATPase activity. Vacuolar H⁺-ATPase activity is expressed as µmoles Pi released per milligram protein per hour.

Phosphate Uptake

Phosphate transport was measured by determination of radiolabeled phosphate uptake as previously described [19]. Each assay was performed in triplicate, averaged and the mean was considered as a single data point.

Preparation of BBM vesicles from OK cells

OK cells grown on transwell filters were treated with 100 nM PTH for 6h to remove all NpT2a from the membranes. Cells were then grown in low phosphate medium for 24 h in the presence or absence of 170 nM bafilomycin A₁. Cells were lysed at 4° C and homogenized with a Polytron type homogenizer in a buffer consisting of (mM): 50 DL-mannitol, 5 EGTA, 0.5 phenyl methylsulfonyl fluoride, and 16 Hepes (pH 7.5 with Tris). BBM were precipitated from this homogenate by Mg²⁺ precipitation and differential centrifugation as described [3, 24]. The final pellet was resuspended in a buffer of 300 mM mannitol, and 16 mM Hepes-Tris, pH 7.5. Purity of BBM preparations was determined by measuring the activity of BBM-specific enzymes as published [19].

Immunoblot assay

The cells, grown to confluence in 6-well trays, were washed twice with HBSS and homogenized in 300 mM mannitol, 5 mM HEPES-Tris buffer pH 7.6 using 27½ g needle. The cell lysate was centrifuged at 2000 rpm for 10 min to remove the cell debris and crude membranes were isolated by centrifugation of the supernatant at 20,000xg for 30 min. The pellet was resuspended in 300 mM mannitol 5 mM HEPES-Tris buffer pH 7.6. This was subjected to 10% SDS-PAGE, and transferred electrophoretically to nitrocellulose membrane (Trans-Blot; Bio-Rad Laboratories, Hercules, CA). The membrane was incubated in 5% milk in 20 mM Tris, 150 mM NaCl, and 0.05% Tween 20 (TTBS) at room temperature for 1 h to inhibit non-specific binding and then incubated over night at 4°C with primary antibody (NpT2a; 1:5000) in 5% milk in TTBS. Location of specific antibodies was detected by incubation with peroxidase-labeled anti-rabbit IgG at 1:5000 dilution in 5% milk in TTBS, followed by development with enhanced Chemiluminescence (Renaissance; DuPont-NEN). The films were scanned using a Personal Densitometer SI (Molecular Dynamics).

Statistics

Data are shown as Mean ± SE. The n values shown represent the number of separate experiments. Each experiment was done in triplicate. P value is calculated using SigmaStat software utilizing student's t-test or by ANOVA followed by Bonferroni analysis using GraphPad Prism software. A p value

Fig. 1. A, A representative proteome map of rat brush border membrane. The proteins were resolved by differential pI for the first dimension and by differential M_w for the second dimension of two-dimensional PAGE. Protein spots were visualized by SYPRO ruby staining, picked, and in-gel digested with trypsin. Proteins were identified by MALDI-TOF mass spectrometry followed by *peptide mass* fingerprinting using the MASCOT search engine. The significantly matched proteins (scores > 71, $p < 0.05$) are labeled as the same spot number as shown in Table 1. B, Effect of Acute High Phosphate Media on Vacuolar H^+ -ATPase Expression. OK cells grown on transwell filters were treated for 3 days with high phosphate media followed by acute shift for 4 h to low phosphate media. Expression of vacuolar H^+ -ATPase A and B subunits was determined in BBM by western blot. A representative blot from 3 individual experiments is shown.

less than 0.05 was *a priori* considered statistically significant.

Results

Differential expression of proteins in BBMs of rats shifted acutely to low/high phosphate diet from chronic high/low phosphate diet

To identify potential protein candidates involved in renal phosphate homeostasis, we performed a proteomic analysis of BBM proteins. Rats kept on chronic high phosphate diet were shifted acutely to low phosphate diet for 4 hours or maintained on the high phosphate diet. Conversely, rats kept on a chronic low phosphate diet were shifted acutely to a high phosphate diet for 4 hours or maintained on the low phosphate diet. We compared the pattern of protein spots obtained from the BBMs of the chronic high vs acute low and from the BBMs of the chronic low vs acute high rats. Protein spots showing a greater than 2-fold change in expression from 6 separate paired gels were picked, digested with trypsin, and identified by peptide mass fingerprinting. Differences in BBM protein expression were detected when any two of the four groups of rats were compared. For this study,

we chose to focus on identification of BBM proteins that were differentially expressed exclusively when rats on a high phosphate diet were either maintained on a high phosphate diet or acutely shifted to low phosphate diet. It would be worth mentioning that while looking for proteins expressed differentially under different phosphate conditions, we excluded the proteins that showed significant change in more than one group and only those proteins were listed which were unique to a single group. In the group representing a shift from chronic low to acute high phosphate diet, only one protein (serine-threonine kinase receptor associated protein), was found to be unique to the group in terms of significant change in expression level. A shift from chronic high to acute low phosphate diet, however, resulted in differential expression of a variety of proteins including ATPases, scaffolding/binding proteins, ion channels and several unrelated proteins such as Annexin V and serine-threonine kinase. The results are given in Table 1 and a representative gel image is shown in Fig. 1A. An interesting observation was that the expression of all the listed proteins in Table 1 was found to be decreased in the BBM of rats shifted to acute low Pi diet. To confirm the proteomics data, OK cells grown on transwell filters were treated with high

Fig. 2. Effect of Bafilomycin A₁ and Concanamycin A on Vacuolar H⁺-ATPase Activity. OK cell monolayers were treated for 30 min with different doses of bafilomycin A₁ (25-250 nM) or concanamycin A (10-100 nM). Vacuolar H⁺-ATPase activity was measured as described in Methods. Each bar represents results as Mean ± SE from 4 independent experiments performed in triplicate. * indicates $p < 0.05$ by Students t-test.

phosphate media for 24 h followed by treatment with low phosphate media for 4 h. BBM were analyzed by western blot for vacuolar H⁺-ATPase A or B subunit. As shown in Fig. 1B, treatment with high phosphate media increased both A and B subunits of vacuolar H⁺-ATPase. Acute treatment of cells in high phosphate media with low phosphate media decreased the expression levels of both A and B subunits of vacuolar H⁺-ATPase compared to high phosphate treated cells.

Effect of Bafilomycin A₁ and Concanamycin A on Vacuolar H⁺-ATPase Activity

To determine whether we could identify a mechanism for regulation of NpT2a expression by one

Fig. 3. Role of Vacuolar ATPase in PTH inhibition of phosphate uptake in OK cells. OK cell monolayers were incubated A) for 30 min with 170 nM bafilomycin A₁ or B) for 15 min with 50 nM concanamycin A followed by treatment with 100 nM PTH 4 h. Phosphate uptake was determined using radiolabeled [³²P]phosphoric acid, as described in 'Methods'. Results shown are Mean±SE of three different experiments and are expressed as phosphate uptake (pmoles/mg protein/min). * $p < 0.001$ vs. control, † $p < 0.05$ vs. control, # $p < 0.05$ vs. bafilomycin A₁ or concanamycin A alone by ANOVA followed by Bonferroni analysis.

of the identified proteins, we turned to the model of the opossum kidney, OK cell. We chose vacuolar H⁺-ATPases for further analysis because of the previously published studies [16, 17] implicating vacuolar H⁺-ATPases in the process of proximal renal tubule endocytosis and degradation. We hypothesized that NpT2a is degraded in lysosomes by vacuolar H⁺-ATPases. We investigated the effect of vacuolar H⁺-ATPase on NpT2a trafficking using two chemically dissimilar inhibitors of vacuolar H⁺-ATPase, bafilomycin A₁ and concanamycin A [15, 22, 25, 26]. To determine the optimum dose of bafilomycin A₁ and concanamycin A required for inhibition of vacuolar H⁺-ATPase activity as described by Lu et al. [22], OK cells were

Fig. 4. Role of Vacuolar H^+ -ATPase in PTH dependent degradation of NpT2a in OK cells. OK cell monolayers were incubated A) for 30 min with 170nM bafilomycin A_1 or B) for 15 min with 50 nM concanamycin A followed by treatment with 100 nM PTH 4 h. Cells were lysed and crude membranes prepared. 50 μ g of membrane proteins were separated by 10% SDS PAGE, transferred to solid matrix and blotted for NpT2a. Blot shown is representative of five separate experiments. The densitometric data shown are Mean $\pm$ SE of the analysis on five different blots and is represented as ratio of NpT2a band density to β -actin band density. * p <0.01 vs. control, # p <0.05 vs. Bafilomycin alone (A) or Concanamycin alone (B) by ANOVA followed by Bonferroni analysis.

Fig. 5. Role of Vacuolar H^+ -ATPase on Phosphate Uptake in OK cells shifted from Low to High Phosphate Medium. OK cell monolayers were maintained in low phosphate medium, LP (0.1%Pi) for 3 days and/or acutely shifted to high phosphate medium, HP (1.2%Pi) for 4 h (see 'Methods' for details). Cells were treated A) with 170nM bafilomycin A_1 30 min prior to acute shift to high phosphate medium or B) with 50 nM concanamycin A 15 min prior to acute shift to high phosphate medium. Phosphate uptake was determined using radiolabeled [32 P] phosphoric acid, as described in 'Methods'. Results shown are Mean $\pm$ SE of three different experiments and are expressed as phosphate uptake (nmoles/mg protein/min). * p <0.05 vs. control, # p <0.005 vs. LP, ** p <0.001 vs. LP, ANOVA followed by Bonferroni analysis.

treated for 30 min with different concentrations of bafilomycin A_1 (25-250 nM) or concanamycin A (10-100 nM). As shown in Fig. 2, both bafilomycin A_1 (Fig. 2A) and concanamycin A (Fig. 2B) inhibited bafilomycin A_1 -sensitive H^+ -ATPase activity in dose-dependent manner. Bafilomycin A_1 inhibited the activity maximally at 170 nM and concanamycin A at 25 nM concentration. Based on these results we used 170 nM bafilomycin A_1 or 50 nM concanamycin A to study the role of vacuolar H^+ -ATPase in NpT2a regulation for the remainder of our studies.

Fig. 6. Role of Vacuolar H^+ -ATPase in Degradation of NpT2a in OK cells shifted from low to high phosphate medium. OK cell monolayers were maintained in low phosphate medium, LP (0.1%Pi) for 3 days and/or acutely shifted to high phosphate medium, HP (1.2%Pi) for 4 h (see 'Methods' for details). Cells were treated A) with 170nM bafilomycin A_1 30 min prior to acute shift to high phosphate medium or B) with 50 nM concanamycin A 15 min prior to acute shift to high phosphate medium. Cells were lysed and crude membranes prepared. 50 μ g of membrane proteins were separated by 10% SDS PAGE, transferred to solid matrix and blotted for NpT2a. Blot shown is representative of five separate experiments. The densitometric data shown are Mean $\pm$ SE of the analysis on five different blots and is represented as ratio of NpT2a band density to β -actin band density. * p <0.01 vs. vehicle, # p <0.05 vs. LP alone by ANOVA followed by Bonferroni analysis.

Role of vacuolar H^+ -ATPases in PTH-mediated regulation of NpT2a

To determine if vacuolar H^+ -ATPases were required for NpT2a regulation by PTH, OK cells were pretreated for 30 min with 170 nM bafilomycin A_1 (Fig. 3A) or for 15 min with 50 nM concanamycin A (Fig. 3B), followed by treatment for 4 h with 100 nM PTH. As shown in Fig. 3A, PTH decreased Pi uptake in both bafilomycin treated (38.7%) and untreated cells (34.9%). As shown in Fig. 3B, PTH significantly decreased Pi uptake in both concanamycin treated (45.3%) and untreated cells (35.3%). Both bafilomycin A_1 and concanamycin alone significantly inhibited Pi uptake by 24.3% and 27% respectively as compared to control.

To determine the effects of bafilomycin A_1 and concanamycin A on NpT2a expression, OK cells were treated for 4 h with PTH in the presence or absence of bafilomycin A_1 or concanamycin A as above. Crude membrane fractions were prepared, resolved on 10% SDS gel, transferred to nitro-cellulose membrane and analyzed by western blot for NpT2a. As shown in Fig. 4, PTH treatment, predictably, resulted in the disappearance of the NpT2a in crude membrane preparations (lane 2). Degradation of NpT2a was prevented by pretreatment with bafilomycin A_1 (lane 4, Fig. 4A) or concanamycin A (lane 4, Fig. 4B). Treatment with either bafilomycin A_1 or concanamycin A alone resulted in an increase in NpT2a expression in crude membrane preparations compared to vehicle-treated cells.

Role of vacuolar H^+ -ATPase in dietary phosphate-induced regulation of NpT2a expression and function

To determine the role of vacuolar H^+ -ATPase in high phosphate-stimulated decrease in Pi uptake, OK cells were maintained in low phosphate medium for 72 h followed by an acute shift to high phosphate medium for 4 h in the presence or absence of 170 nM bafilomycin A_1 or 50 nM concanamycin A. As seen in Fig. 5, growth of OK cells in chronic low phosphate medium predictably stimulated Pi uptake by about 77% compared to cells grown in normal phosphate medium. An acute shift to high phosphate medium significantly decreased this transport (22.5% vs acute low phosphate; p <0.05). Pretreatment with bafilomycin A_1 (Fig. 5A) or concanamycin A (Fig. 5B) had no effect on high phosphate-stimulated decrease in Pi uptake.

The effect of acute changes in ambient phosphate concentration on expression of NpT2a was also studied by western blot in OK cells subjected to changes in phosphate concentration and pretreated with bafilomycin A_1 or concanamycin A as above. As shown in Fig. 6, low phosphate medium resulted in an expected increase in NpT2a expression. Acute shift from low phosphate to high phosphate medium decreased NpT2a expression in crude membranes. Pretreatment with bafilomycin A_1 or concanamycin A completely prevented high Pi-stimulated NpT2a degradation. Of note, neither bafilomycin A_1 nor concanamycin had any

Fig. 7. Effect of Bafilomycin A₁ on low phosphate-induced increase in apical membrane expression of NpT2a. OK cell grown on transwell filters were treated with PTH for 6h followed by incubation for 24 h in low phosphate medium in the presence or absence of Bafilomycin A₁. Cells were lysed and BBM was prepared as described in Methods. 20μg of BBM proteins were separated by 10% SDS PAGE, transferred to solid matrix and blotted for NpT2a. Blot shown is representative of five separate experiments. The densitometric data shown are Mean±SE of the analysis on five different blots and is represented as ratio of NpT2a band density to β-actin band density. *p<0.01 vs. vehicle, #p<0.01 vs. low phosphate medium alone + p<0.01 vs Bafilomycin A1 alone by ANOVA followed by Bonferroni analysis.

significant effect on NpT2a expression in crude membranes.

The above experiments suggest that bafilomycin A₁ alone decreases phosphate transport without changing the expression of NpT2a in crude membrane preparations. To determine if the decrease in phosphate transport is due to a decrease in apical membrane expression of NpT2a, brush border membranes were prepared from cells grown on transwell filters. As shown in Fig. 7, treatment with PTH or bafilomycin A₁ decreased BBM expression of NpT2a. As expected, treatment with low phosphate medium increased BBM expression of NpT2a compared to PTH treated cells. However, treatment with bafilomycin A₁ prevented low phosphate medium-induced increase in apical membrane expression of NpT2a both under basal conditions and after treatment with PTH.

Effect of Bafilomycin A₁ and Brefeldin A on phosphate uptake and NpT2a expression

The previous experiments showed that inhibition of vacuolar H⁺-ATPase partially inhibited phosphate uptake and apical membrane expression of NpT2a despite an increase in crude membrane expression of NpT2a, suggesting that inhibition of vacuolar H⁺-ATPase blunted apical membrane trafficking of NpT2a. To confirm that inhibitors of vacuolar H⁺-ATPase decrease phosphate uptake by decreasing apical protein expression, cells were treated for 30 min with 170 nM bafilomycin A₁ and/or

a known inhibitor of Golgi trafficking brefeldin A (10 μM). As shown in Fig. 8A, both bafilomycin A₁ and brefeldin A inhibited Pi uptake by about 30%. Treatment of cells with both bafilomycin A₁ and brefeldin A resulted in a similar 35% inhibition of Pi uptake suggesting minimal if any additive effect. This finding suggested that brefeldin A and bafilomycin A₁ inhibit Pi uptake (and therefore apical membrane NpT2a expression) by a similar mechanism. However, while treatment of cells with bafilomycin A resulted in an increase in crude membrane expression of NpT2a, treatment with brefeldin A had no effect on crude membrane expression of NpT2a. Furthermore, treatment with brefeldin A also blocked the previously demonstrated increase in NpT2a expression stimulated by bafilomycin A₁. To determine if brefeldin A-mediated decrease in phosphate transport was due to a decrease in apical membrane expression of NpT2a, BBM were prepared and analyzed by western blot for NpT2a expression. As shown in Fig. 8C, treatment with brefeldin A alone or in combination with bafilomycin significantly decreased NpT2a expression in BBM (Fig. 8B).

Discussion

In this study we identified vacuolar H⁺-ATPase as a differentially expressed BBM-associated protein in rat BBMs under conditions of acute change in dietary

Fig. 8. Effect of Bafilomycin A₁ and Brefeldin A on sodium-dependent phosphate uptake and NpT2a expression. A, OK cell monolayers were treated for 30 min with 170 nM bafilomycin A₁ or 10 μM brefeldin A. Cells were washed and phosphate uptake was determined using radiolabeled [³²P] phosphoric acid, as described in 'Methods'. Each bar represents Pi uptake as mean±SE from 4 independent experiments. * indicates *p* < 0.05 from vehicle treated cells and # indicates *p* < 0.05 from Brefeldin A alone treated group by ANOVA followed by Bonferroni analysis. B and C, Brush border membranes (B) or Crude membranes (C) were prepared and analyzed by Western blot for NpT2a expression. Blot shown is representative of five separate experiments. The densitometric data shown are Mean±SE of the analysis on five different blots and is represented as ratio of NpT2a band density to β-actin band density. **p*<0.01 vs. control #*p*<0.01 vs. Bafilomycin A₁ alone by ANOVA followed by Bonferroni analysis.

phosphate ingestion and hypothesized that this protein played a role in the trafficking of NpT2a in response to physiologic stimuli such as alterations in dietary or ambient phosphate. Previously published studies have described the lysosomal routing of NpT2a by demonstrating the presence of these proteins in lysosomal preparations and by demonstrating the inhibition of NpT2a degradation by nonspecific lysosomal chaotropic agents [8, 27, 28]. Our results confirm that vacuolar H⁺-ATPases play a role in NpT2a degradation but are not required for PTH⁻ and high ambient Pi-mediated endocytosis of NpT2a from the membrane. The decrease in Pi transport stimulated by both PTH and high ambient Pi was unaffected by inhibitors of vacuolar H⁺-ATPases, suggesting that the cellular mechanisms for regulation of NpT2a expression by PTH and dietary Pi are similar and independent of vacuolar H⁺-ATPase activity.

Treatment of the OK cells with vacuolar H⁺-ATPase inhibitors alone led to a significant decrease in basal Pi transport despite an increase in crude membrane expression of NpT2a, suggesting that vacuolar H⁺-ATPase inhibitors decreased apical membrane expression of NpT2a either through stimulation of NpT2a endocytosis or through inhibition of NpT2a exocytosis. Studies in isolated brush border membranes confirmed that treatment with bafilomycin or concanamycin alone blunted low phosphate-mediated trafficking of NpT2a to the apical membrane. Treatment of cells with the inhibitor of Golgi trafficking, brefeldin A produced a decrease in phosphate transport of a similar degree as did vacuolar H⁺-ATPase inhibitors. Simultaneous treatment with both brefeldin A and a vacuolar

ATPase inhibitor produced a similar and nonadditive decrease in phosphate uptake, suggesting that brefeldin A and inhibitors of H⁺ ATPase decreased phosphate uptake through the same mechanism or pathway. Because NpT2a is not recycled and therefore would not undergo trafficking through recycling endosomes, our findings suggest that vacuolar H⁺-ATPase inhibitors and Brefeldin A block exocytosis at a similar location in the exocytic pathway.

The effect of the vacuolar H⁺-ATPase inhibitors on NpT2a expression is of interest. Pretreatment with either inhibitor increased basal expression of NpT2a and did not prevent the increase in NpT2a expression stimulated by low Pi (Fig. 4 and 6). However, the increase in NpT2a expression stimulated by low phosphate and the vacuolar H⁺-ATPase inhibitors was not additive, suggesting that while some constitutively endocytosed NpT2a undergoes degradation through the lysosomal pathway, NpT2a may also undergo degradation through an alternative mechanism. This suggestion is supported by our demonstration that brefeldin A treatment blocked the increase in NpT2a expression stimulated by bafilomycin A₁. As brefeldin A presumably prevents normal trafficking, NpT2a in brefeldin A-treated cells may be diverted to an alternative degradation pathway such as the proteasomal pathway. Previously published reports have shown that inhibition of the proteasomal pathway leads to an increase in NpT2a expression [8].

These findings argue for a multifaceted role for vacuolar H⁺-ATPases in the trafficking of NpT2a in and out of the apical membrane. Vacuolar H⁺-ATPases are present in the intracellular membranes of all eukaryotic cells where they are responsible for the acidification of organelles in the endocytotic/exocytotic pathways [13-15, 29]. They are increasingly recognized

as critical components of several renal tubular processes including vacuolar acidification, H⁺ secretion, bicarbonate reabsorption, endocytosis and plasma membrane protein trafficking. Similar to Na-K ATPase, vacuolar H⁺-ATPases may also function as an energy source to drive epithelial transport [15]. Vacuolar H⁺-ATPase activity has been linked to Na independent bicarbonate reabsorption and co-localizes with NHE3 [12]. These findings coupled with the previous data raise the possibility that the changes in NpT2a expression seen with the inhibition of vacuolar H⁺-ATPases may be due to mechanisms other than simple inhibition of degradation and accumulation of proteins. Our data do not permit us to draw a definitive conclusion.

To summarize, we have identified a novel trafficking role for vacuolar H⁺-ATPases in the regulation of NpT2a expression and function. Further studies are required to determine the precise mechanisms responsible for this effect of vacuolar H⁺-ATPases. Identification of proteins involved in regulation of phosphate transport and the mechanisms of their effects will enable novel therapeutic approaches to disorders of phosphate homeostasis.

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