

Acute parathyroid hormone differentially regulates renal brush border membrane phosphate cotransporters

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Abstract Renal phosphate reabsorption across the brush border membrane (BBM) in the proximal tubule is mediated by at least three transporters, NaPi-IIa (SLC34A1), NaPi-IIc (SLC34A3), and Pit-2 (SLC20A2). Parathyroid hormone (PTH) is a potent phosphaturic factor exerting an acute and chronic reduction in proximal tubule phosphate reabsorption. PTH acutely induces NaPi-IIa internalization from the BBM and lysosomal degradation, but its effects on NaPi-IIc and Pit-2 are unknown. In rats adapted to low phosphate diet, acute (30 and 60 min) application of PTH decreased BBM phosphate transport rates both in the absence and the presence of phosphonoformic acid, an inhibitor of SLC34 but not SLC20 transporters. Immunohistochemistry showed NaPi-IIa expression in the S1 to the S3 segment of superficial and juxtamedullary nephrons; NaPi-IIc was only detectable in S1 segments and Pit-2 in S1 and weakly in S2 segments of superficial and juxtamedullary nephrons. PTH reduced NaPi-IIa staining in the BBM with increased intracellular and lysosomal appearance. NaPi-IIa internalization was most prominent in S1 segments of superficial nephrons. We did not detect changes in NaPi-IIc and Pit-2 staining over this time period. Blockade of lysosomal protein degradation with leupeptin revealed NaPi-IIa accumulation

in lysosomes, but no lysosomal staining for NaPi-IIc or Pit-2 could be detected. Immunoblotting of BBM confirmed the reduction in NaPi-IIa abundance and the absence of any effect on NaPi-IIc expression. Pit-2 protein abundance was also significantly reduced by PTH. Thus, function and expression of BBM phosphate cotransporters are differentially regulated allowing for fine-tuning of renal phosphate reabsorption.

Keywords Phosphonoformic acid · Internalization · Type II and type III phosphate transporter

Introduction

Renal phosphate excretion is mostly determined by the rate of reabsorption of filtered phosphate along the proximal tubule. To date, at least three distinct sodium-dependent phosphate transporters have been identified to be expressed in the brush border membrane (BBM) of the proximal tubule: NaPi-IIa (SLC34A1), NaPi-IIc, and more recently Pit-2 [7, 37, 43]. However, these transporters have distinct transport properties with respect to ion coupling, preferred species of phosphate, voltage sensitivity, and pH dependence [31, 32, 36, 45]. NaPi-IIa and Pit-2 mediate the electrogenic transport of inorganic phosphate coupled to three and two sodium ions, respectively, whereas NaPi-IIc transports inorganic phosphate together with two sodium ions in an electroneutral fashion [31, 32, 36, 45]. Moreover, NaPi-IIa and NaPi-IIc prefer divalent inorganic phosphate (HPO_4^{2-}). In contrast, Pit transporters preferentially transport monovalent phosphate (H_2PO_4^-) [31, 32, 36, 45].

A plethora of factors affects renal phosphate reabsorption, among them parathyroid hormone (PTH), dopamine, dietary phosphate intake, glucocorticoids, acid–base status,

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growth factors, insulin, and FGF-23 [1, 3, 6, 7, 32, 33]. The hormonal regulation of the NaPi-IIa cotransporter has been studied in much detail demonstrating that hormones such as PTH, dopamine, FGF-23, sFRP4, MEPE, glucocorticoids cause a reduction of NaPi-IIa expression [7]. Much less is known about the hormonal regulation of NaPi-IIc with only few studies showing that PTH and FGF-23 cause lower expression [24, 38]. In contrast, no hormonal regulators of Pit-2 have been reported to date. Dietary phosphate intake or potassium depletion is the only known regulatory factor of Pit-2 [10, 43].

PTH is one of the most potent acute phosphaturic factors inducing urinary phosphate excretion within 15 to 45 min after application [15, 19]. We and others have shown that PTH causes phosphate excretion at least in part by inducing the internalization of the NaPi-IIa phosphate cotransporter from the brush border membrane of proximal tubules in rats and mice [2, 3, 7, 12, 31, 51]. NaPi-IIa is then routed via clathrin-coated pits and early endosomes to lysosomes and subsequently undergoes degradation [3, 22, 35, 41]. Interestingly, the time course of renal phosphaturia and brush border membrane expression of NaPi-IIa do not strictly correlate following an acute PTH bolus [15]. Furthermore, Tenenhouse and colleagues observed that in mice lacking the NaPi-IIa transporter (*Slc34a1*^{-/-}), PTH injections in vivo (2 h) did not reduce phosphate uptake rates in isolated brush border membrane vesicles (BBMV) and had no effect on phosphaturia [51]. Indeed, Segawa et al. reported PTH-reduced brush border membrane NaPi-IIc expression only 8 h after injection [38]. Taken together, these observations strongly suggest that acute PTH may differentially affect expression and/or function of brush border membrane phosphate transporters.

Therefore, we examined the acute effects of PTH on the expression and function of all three brush border membrane sodium-dependent phosphate cotransporters in rat kidney. Our data demonstrate that PTH differentially regulates NaPi-IIa, NaPi-IIc, and Pit-2 allowing for the fine-tuning of renal phosphate reabsorption.

Materials and methods

Animals

The experiments were performed with male Wistar rats (120–150 g, Charles River, Germany). The rats were kept on a low-phosphate diet (0.1% P_i content; Kliba AG, Switzerland) for 3 days to upregulate renal phosphate reabsorption in the BBM of all proximal tubular segments as described previously [3]. The protein abundance of all three known phosphate transporters, NaPi-IIa, NaPi-IIc, and Pit-2, is increased by low phosphate diets [27, 34, 36, 37].

Moreover, all rats received 4% CaCl₂ in their drinking water 24 h prior to experiments to suppress endogenous PTH levels [3]. On the day of the experiment, all animals at 8 a.m. received either saline as control or PTH (PTH 1–34 fragment, 10 µg/100 g body weight (Sigma, St Louis, MO, USA)) injections as a bolus into the tail vein. Some rats were killed after 30 or 60 min to collect kidneys for the preparation of brush border membrane vesicles. The remaining rats were fixed by perfusion at each time point (30, 60 min). Application of leupeptine (12 mg/ml phosphate-buffered saline (PBS)) 15 min before injection of PTH was also performed as described previously [3].

All animal studies were according to Swiss Animal Welfare laws and approved by the local Cantonal Veterinary Authority of Zurich.

Fixation

After treatment, all animals were fixed by vascular perfusion via the abdominal aorta, at a pressure of 0.4 bar, as described previously [3]. Rats were anesthetized with thiopental (Pentothal, 100 mg/kg body weight) injected intraperitoneally, their abdominal cavity was opened, and the aorta and vena cava were exposed. The fixative consisted of 3% paraformaldehyde, 0.1% glutaraldehyde, and 0.05% picric acid in 0.6 M cacodylate buffer (pH 7.4; containing 3 mM MgCl₂ and adjusted to 300 mosmol/l with sucrose) and 4% hydroxyethyl starch in saline (HAES steril; Fresenius, Stans, Switzerland). After 5 min, the fixative was washed out by perfusion with hydrostatic pressure of 70 cm for 5 min with cacodylate buffer. Kidneys were removed.

Immunohistochemistry

Slices of fixed kidneys were frozen in liquid propane and cooled with liquid nitrogen. Serial sections, 4-µm thick, were cut at -22°C on a cryomicrotome, mounted on chromalum/gelatine-coated glass slides, thawed, and kept in cold PBS until use. Before immunofluorescence staining, sections were pretreated with blocking solution (Normal Goat Serum 5% in PBS with 0.1% bovine serum albumin) for 60 min. After blocking, serial sections were incubated overnight at 4°C either with a rabbit anti-rat antiserum against the NaPi-IIa protein [13] diluted 1:1,000 or with an immunopurified rabbit anti-mouse NaPi-IIc [33] diluted 1:1,500 or a rabbit anti-rat Pit-2 [43] diluted 1:250. We also tested two commercially available anti-Pit-2 antibodies (Alpha Diagnostics, San Antonio, TX, USA, and Santa Cruz Biotechnologies, Santa Cruz, CA, USA) but could not obtain specific signals by immunohistochemistry of kidneys or immunoblotting of brush border membranes from rats with phosphate depletion. All primary antibodies were

diluted in PBS with 0.1% bovine serum albumin. Sections were then rinsed three times with PBS and covered for 1 h at 4°C with the secondary antibodies: Alexa Fluor 555 goat-anti-rabbit IgG (1:500, Invitrogen), FITC-phalloidin (Molecular probes, Eugene, OR, USA, 1:50), and 4,6-diamidino-2-phenylindole (DAPI; Sigma, St Louis, MO), dilution 1:1,000). Finally, the sections were rinsed three times with PBS, coverslipped using DAKO-Glycergel (Dakopatts) containing 2.5% 1,4-diazabicyclo[2.2.2]octane (Sigma) as a fading retardant.

Immunohistochemistry images were acquired with a Leica DFC490 charged-coupled device camera attached to a Leica DM 6000 fluorescence microscope (Leica, Wetzlar, Germany) using equivalent camera parameters for kidneys sections stained with the same primary antibody. Pictures were processed using Adobe Photoshop (overlays).

Brush border membrane vesicle preparation and phosphate uptake experiments

BBMV were prepared from rat kidney cortex and outer medulla using the Mg^{2+} precipitation technique as described previously [8, 9]. The phosphate transport rate into BBMV was measured in freshly prepared BBMV at 25°C in the presence of inward gradients of 100 mM NaCl or 100 mM KCl and 0.1 mM K-phosphate. The substrate P_i was made with 0.125 mM K_2HPO_4 and ^{32}P (1 μ Ci/ml) to give a final concentration 0.1 mM close to the expected apparent $K_{m_{P_i}}$ for Na^+ -dependent transport in renal BBMV. The stop solution contained 100 mM Mannitol, 5 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM P_i . Na^+ -dependence was established by incubating BBMV in solutions in which KCl replaced NaCl equimolarly. Phosphate uptake was determined after 60 s, representing initial linear conditions, and after 120 min, to determine the equilibrium values. In order to distinguish between Na^+ -dependent P_i uptake mediated by SLC34 family members (e.g., NaPi-IIa and NaPi-IIc) and other Na^+ -dependent phosphate transporters such as SLC20 family members (e.g., Pit-1 and Pit-2), we used Trisodium phosphonoformic acid (PFA, final concentrations 0.1–6 mM) added to the same solution with 107 mM NaCl. PFA has previously been shown to have a higher selectivity for SLC34 than SLC20 phosphate transporters at these concentrations [11, 36, 43, 44]. Total protein concentration was measured using the Bio-Rad Protein Assay kit, Bio-Rad, Hercules, CA, USA. BBMV were stored at $-80^\circ C$ until further use.

Immunoblotting

Ten micrograms of renal brush border membrane proteins were solubilized in loading buffer containing DTT and separated on 8% polyacrylamide gels. For immunoblotting,

the proteins were transferred electrophoretically to polyvinylidene fluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA, USA). After blocking with 5% milk powder in Tris-buffered saline/0.1% Tween-20 for 60 min, the blots were incubated with the primary antibodies: rabbit polyclonal anti-NaPi-IIa (1:6,000) [13], rabbit polyclonal anti-NaPi-IIc (1:10,000) [33], rabbit polyclonal anti-Pit-2 (1:3,000) [43], and mouse monoclonal anti- β -actin antibody (42 kD; Sigma, St. Louis, MO; 1:5,000) either for 2 h at room temperature or overnight at 4°C. Membranes were then incubated for 1 h at room temperature with secondary goat anti-rabbit or donkey anti-mouse antibodies 1:5,000 linked to alkaline phosphatase (Promega, Madison, WI, USA) or HRP (Amersham Life Science, Little Chalfont Buckinghamshire, UK). The protein signal was detected with the appropriate substrates (Millipore Corp, Bedford, MA, USA) using the DIANA III-chemiluminescence detection system (Raytest, Straubenhardt, Germany). All images were analyzed using the software Advanced Image Data Analyser AIDA (Raytest, Straubenhardt, Germany) to calculate the protein of interest/ β -actin ratio.

Statistical analysis

All data are summarized as mean \pm SE and were analyzed using the unpaired Student's *t* test with only *p* values ≤ 0.05 considered as statistically significant.

Results

Parathyroid hormone decreases at least two types of sodium-dependent phosphate transport activities

BBMV were prepared from kidneys of rats adapted to low phosphate diet for 3 days and injected either with saline (0.9% NaCl) or PTH. Sodium-dependent phosphate uptake was measured in the absence and presence of 6 mM PFA. PFA has been known to inhibit BBMV phosphate uptake for almost three decades [39, 40] and has more recently been shown to affect only type II (i.e., NaPi-IIa and NaPi-IIc) but not type III (i.e., Pit-2) mediated phosphate transport at this concentration [43, 44]. Thirty minutes after PTH injections, sodium-dependent phosphate uptake was reduced from $3,570 \pm 294$ pmol/mg protein to $2,987 \pm 316$ pmol/mg protein ($p = 0.1$, non-significant). After 60 min, the uptake was reduced from $4,244 \pm 241$ pmol/mg protein to $3,095 \pm 403$ pmol/mg protein ($p = 0.015$, significant; Fig. 1a). In the presence of PFA, however, the total sodium-dependent phosphate uptake was reduced by about 70% indicating that type II phosphate transporters are responsible for about 70% of the total sodium-dependent phosphate transport activity in chronically low phosphate fed rats.

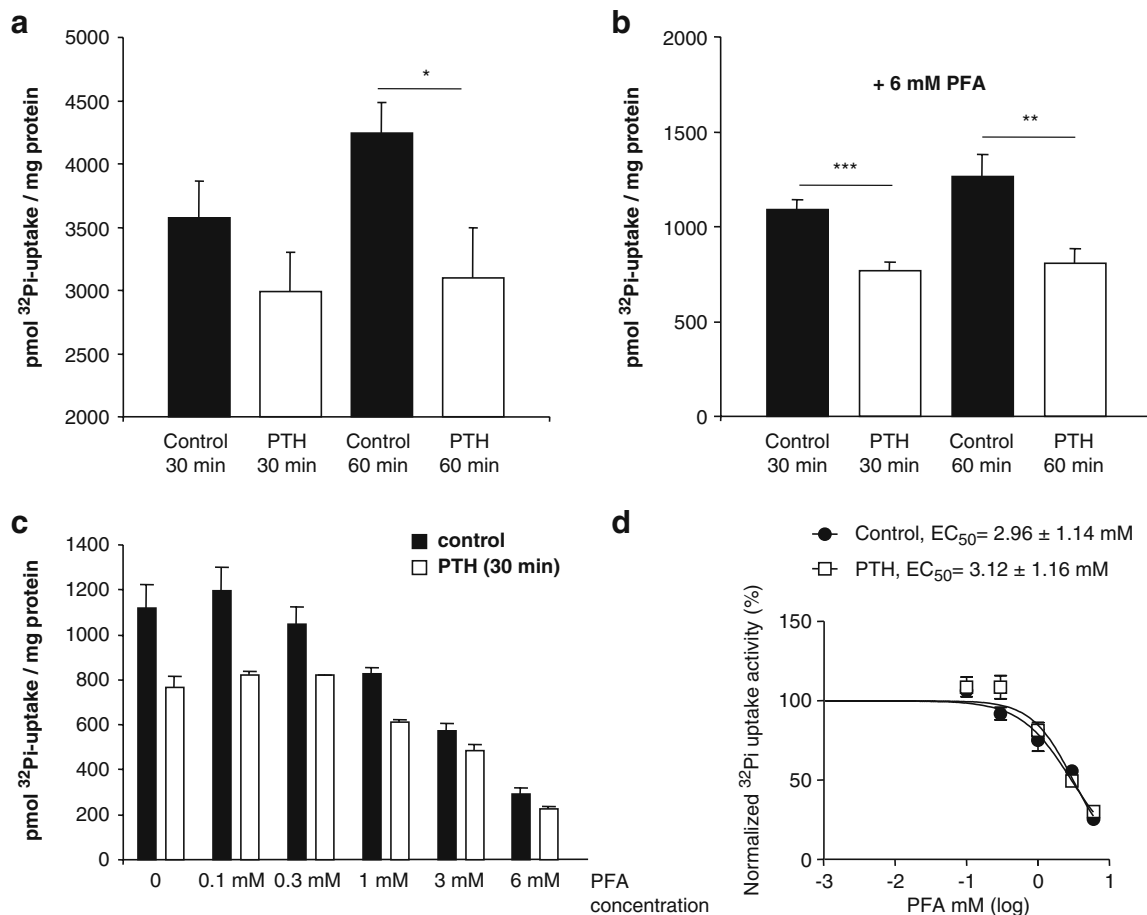


Fig. 1 Parathyroid hormone (PTH) decreases brush border membrane sodium-dependent phosphate uptake in the absence and presence of phosphonoformic acid (PFA). Rats were injected either with saline (control) or PTH, and kidneys were harvested 30 or 60 min after injections. Brush border membrane vesicles were prepared, and sodium-dependent phosphate fluxes were measured in the absence (a) or presence of 6 mM PFA (b), an inhibitor of type II but not type III phosphate cotransporters ($n = 5$ each group and time point). PTH decreased the total and PFA-resistant phosphate uptake

indicating that type II and type III transporters are regulated by PTH. c Sodium-dependent phosphate uptake rates in the presence of increasing concentrations of PFA (0.1–6 mM) into brush border membrane vesicles prepared from rats injected with saline (control) or PTH (30 min). d Phosphate transport rates were normalized against rates in the absence of PFA and the apparent half-maximal PFA concentration calculated (EC_{50}) needed to inhibit phosphate uptake ($n = 4$ each group and time point). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

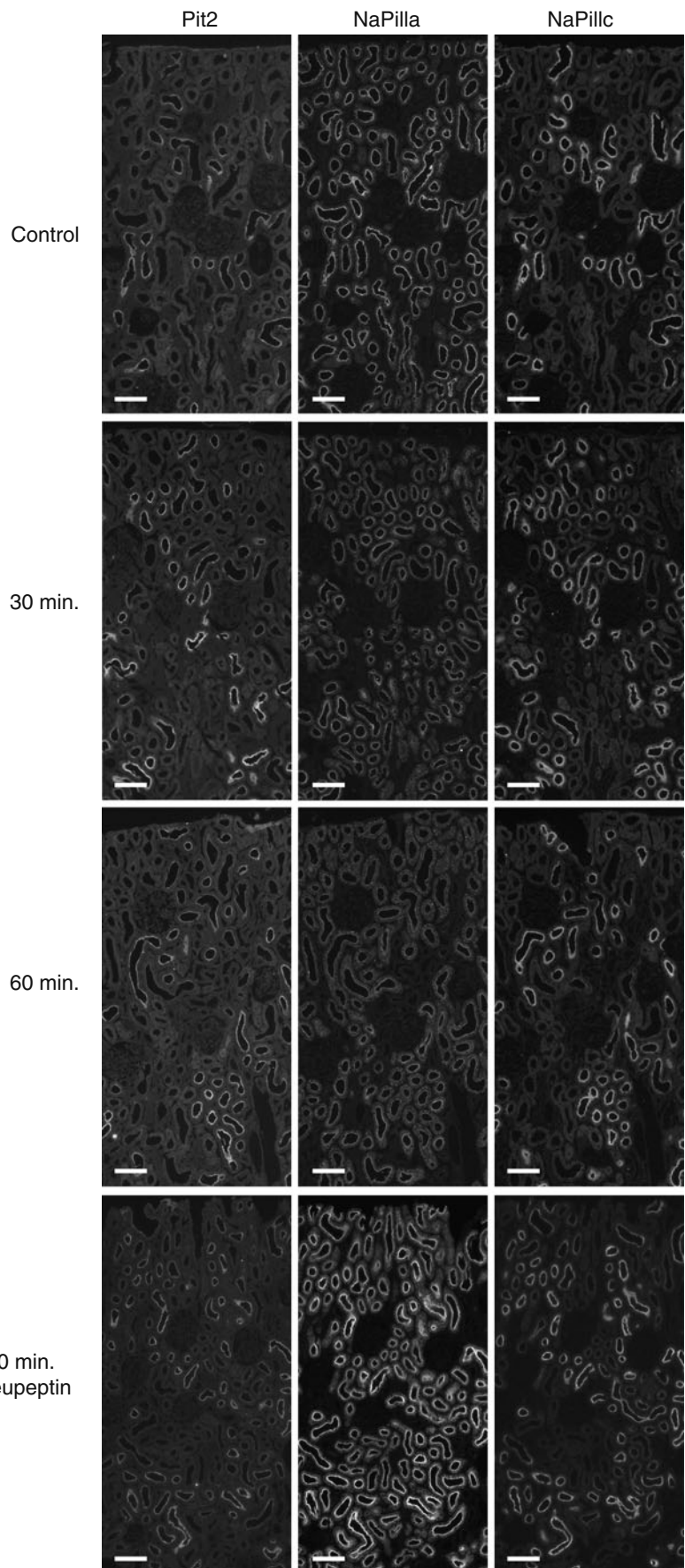
PFA-resistant phosphate uptake was further decreased from $1,091 \pm 50$ pmol/mg protein to 767 ± 46 pmol/mg protein after 30 min PTH ($p = 0.0007$, significant) and from $1,264 \pm 116$ pmol/mg protein to 808 ± 77 pmol/mg protein after 60 min PTH ($p = 0.004$, significant) (Fig. 1b). We further characterized the inhibition of sodium-dependent phosphate uptake into brush border membrane vesicles from rats injected either with saline (control) or PTH (30 min) using increasing concentrations of PFA in the range of 0.1 mM to 6 mM (Fig. 1c). Increasing concentrations decreased Na^+ -dependent phosphate uptake progressively. We did not use concentrations higher than 6 mM since preliminary experiments suggested unspecific effects on other transport systems such as for glucose (data not shown). We also estimated the apparent half-maximal concentration (EC_{50}) of PFA needed to inhibit Na^+ -

dependent phosphate uptake and obtained values of approximately 3 mM both in saline and PTH-treated brush border membranes (Fig. 1d). Thus, PTH decreases both the PFA-sensitive and the PFA-resistant sodium-dependent phosphate transport activities suggesting that type II and type III transporter(s) are downregulated.

PTH induces internalization and lysosomal degradation of the type II NaPi-IIa cotransporters

Acute application of PTH leads to the rapid internalization of the NaPi-IIa cotransporter from the brush border membrane and its subsequent routing to the lysosome and degradation [3, 22, 35, 41]. We used immunohistochemistry on consecutive sections of rat kidneys to examine the localization of NaPi-IIa, NaPi-IIc, and Pit-2 after application of

Fig. 2 Effect of acute PTH on NaPi-IIa, NaPi-IIc, and Pit-2 immunostaining. Rats were injected with NaCl (*control*), or PTH (30 and 60 min). Some rats received also leupeptin prior to PTH to prevent lysosomal degradation ($n = 5$ animals per group). Cryostat serial sections were stained for Pit-2, NaPi-IIa, and NaPi-IIc. In control kidneys, NaPi-IIa-related staining was seen along the entire length of the proximal tubule (S1–S3 segments) and in both superficial and juxtamedullary nephrons. In contrast, NaPi-IIc and Pit-2 staining was mostly observed in early convoluted proximal tubules (S1 segments) with similar intensities in superficial and juxtamedullary nephrons. After PTH injection, NaPi-IIa labeling intensity was reduced in kidneys 30 and 60 min after PTH application whereas Pit-2- and NaPi-IIc-related labeling intensity remained unchanged. Inhibition of lysosomal activity with leupeptin increased the intensity of NaPi-IIa staining in PTH injected rats; the staining for NaPi-IIc and Pit-2 appeared unaffected. *Bar size* ~40 μm



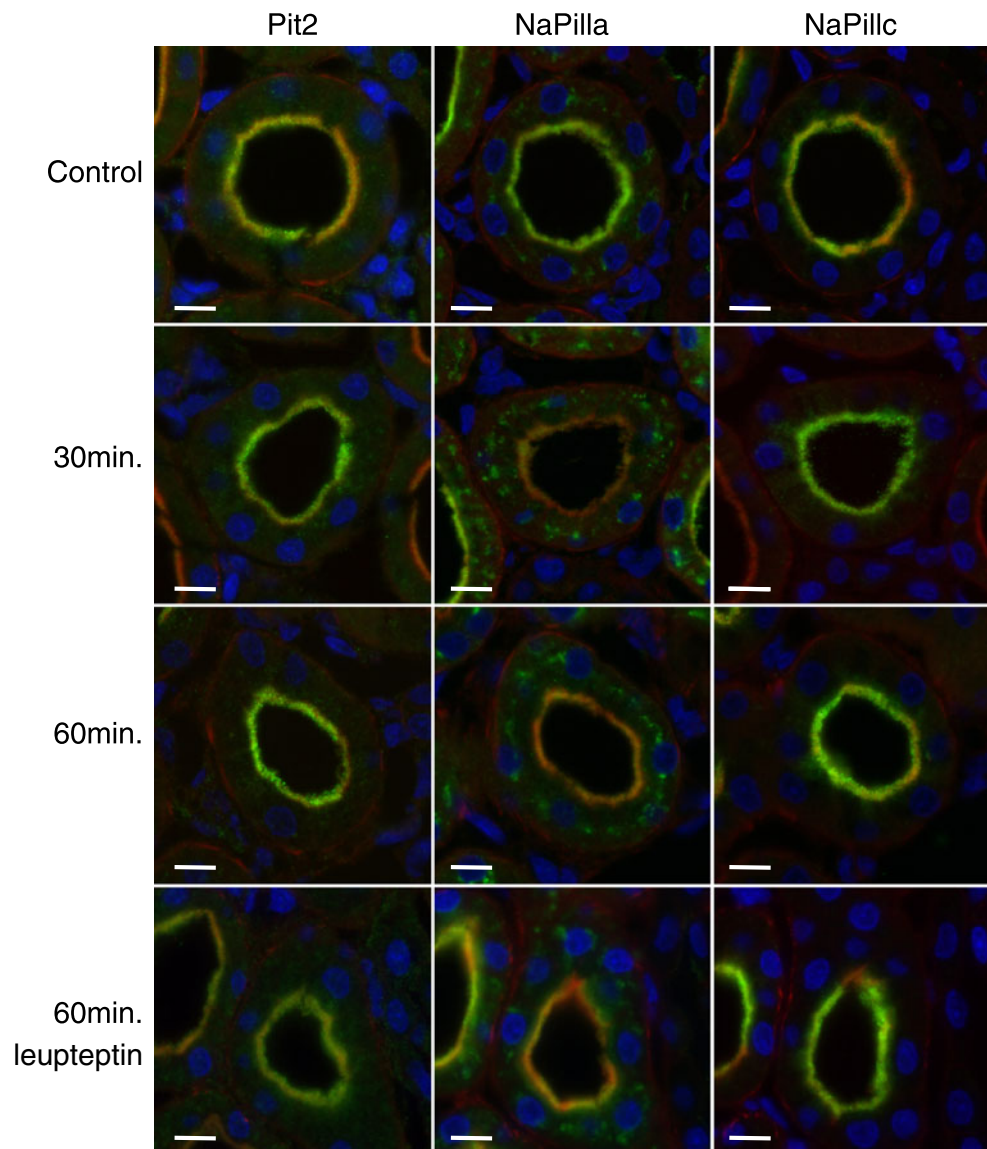
PTH or saline, respectively. In saline-treated animals, no differences in the segmental and subcellular localization of NaPi-IIa, NaPi-IIc, and Pit-2 between control and after 30 or 60 min PTH were observed (Fig. 2). In control kidneys from animals on a low phosphate diet, NaPi-IIa-related staining was seen in superficial and juxtamedullary nephrons along the entire length of the proximal tubule (S1–S3 segments). In contrast, NaPi-IIc and Pit-2 staining was mostly observed in early convoluted proximal tubules (S1 segments) with similar intensities in superficial and juxtamedullary nephrons.

After PTH injection, NaPi-IIa labeling intensity was reduced in kidneys at both time points (30 and 60 min), whereas Pit-2- and NaPi-IIc-related labeling intensity appeared unchanged (Fig. 2). Segmental differences in the response to PTH were observed. The strongest effect of PTH was seen in the early proximal tubules of superficial nephrons (S1 segments) where PTH induced a strong

decrease of NaPi-IIa-related staining in the brush border membrane and the intracellular appearance of staining (Fig. 3). We have previously shown that these intracellular structures include clathrin-coated pits, early endosomes, and lysosomes [3]. In the S2 segment of superficial nephrons, NaPi-IIa internalization was also observed; however, strong residual NaPi-IIa staining in the brush border membrane was seen (Fig. 4). In juxtamedullary nephrons, almost no difference in NaPi-IIa staining between control, saline-, and PTH-treated kidneys could be observed (data not shown). In contrast to NaPi-IIa staining, no changes in NaPi-IIc- or Pit-2-related staining (both apparent intensity and subcellular localization) could be detected on the level of light microscopy at any time point in superficial and juxtamedullary proximal tubules (Figs. 3 and 4).

In order to examine if NaPi-IIc or Pit-2 may be rapidly routed to lysosomes and degraded, we pretreated rats with

Fig. 3 Acute PTH induces internalization and lysosomal degradation of NaPi-IIa but not NaPi-IIc and Pit-2 in S1 proximal tubules. S1 segments of superficial nephrons stained with antibodies against Pit-2, NaPi-IIa, or NaPi-IIc (green label) and with rhodamine-phalloidin against β -actin filaments (red) to mark brush border membranes. Serial sections are shown. Under control conditions, Pit-2, NaPi-IIa, and NaPi-IIc stainings are predominantly localized in the BBM. Thirty minutes after PTH injection, the intensity of the NaPi-IIa staining in the BBM is reduced whereas intracellular NaPi-IIa staining (green) is strongly increased. Pit-2 and NaPi-IIc staining is only detectable in the BBM at all time points, and its intensity remained unaltered. Blockade of lysosomal degradation with leupeptin caused accumulation of NaPi-IIa staining in lysosomes whereas no NaPi-IIc- and Pit-2-related staining could be observed. $N = 5$ animals per group. Bar size $\sim 10 \mu\text{m}$



leupeptin to inhibit lysosomal degradation of proteins. Leupeptin treatment enhanced the signal for NaPi-IIa after 30 and 60 min in lysosomes whereas no staining for NaPi-IIc and Pit-2 could be found (Figs. 3 and 4).

PTH decreases brush border membrane expression of NaPi-IIa and Pit2 but not NaPi-IIc

In a next step, we prepared brush border membranes for immunoblotting to examine the relative abundance of NaPi-IIa, NaPi-IIc, and Pit-2 after 30 and 60 min PTH application. Thirty minutes after PTH application, the relative abundance of NaPi-IIa and Pit-2 was significantly reduced in the brush border membrane, whereas NaPi-IIc abundance remained unchanged (Fig. 5a, b). Similarly, 60 min after PTH application, NaPi-IIa abundance was significantly decreased. Pit-2 abundance was lower than in

the respective control group but did not reach anymore statistical significance ($p = 0.07$; Fig. 5a, b). Thus, PTH treatment acutely decreases expression of NaPi-IIa and Pit-2 in the brush border membrane.

Discussion

The acute effects of PTH on the activity and localization of the renal sodium-phosphate cotransporter NaPi-IIa has been studied in great detail using mouse and rat models, isolated perfused proximal tubules as well as the OK cell model [3, 20, 21, 26, 29, 42, 48]. Taken together, these studies demonstrated that PTH binds to receptors localized at the basolateral membrane and in the brush border membrane [12, 30, 42]. Both cAMP- and PKC-dependent pathways may at least in part converge in ERK1/2 and induce the

Fig. 4 Weak effect of PTH in S2 segments on NaPi-IIa internalization. S2 segments of superficial nephrons were stained with antibodies against Pit-2, NaPi-IIa, or NaPi-IIc (shown in green) and with rhodamine-phalloidin against β -actin filaments (red) to mark brush border membranes. Serial sections are shown. In kidneys from control animals, NaPi-IIa mostly resided in the brush border membrane. Pit-2 showed a weaker signal than in S1 segments whereas NaPi-IIc was barely detectable in superficial S2 proximal tubules. Thirty minutes after PTH injection, the intensity of the intracellular NaPi-IIa staining (green) was increased whereas Pit-2 was only detectable at the BBM. A pool of NaPi-IIa was still present in the BBM. Pretreatment with leupeptin caused a more intensive intracellular lysosomal staining for NaPi-IIa; no intracellular or lysosomal signal could be detected for NaPi-IIc and Pit-2. $N=5$ animals per group. Bar size $\sim 10 \mu\text{m}$

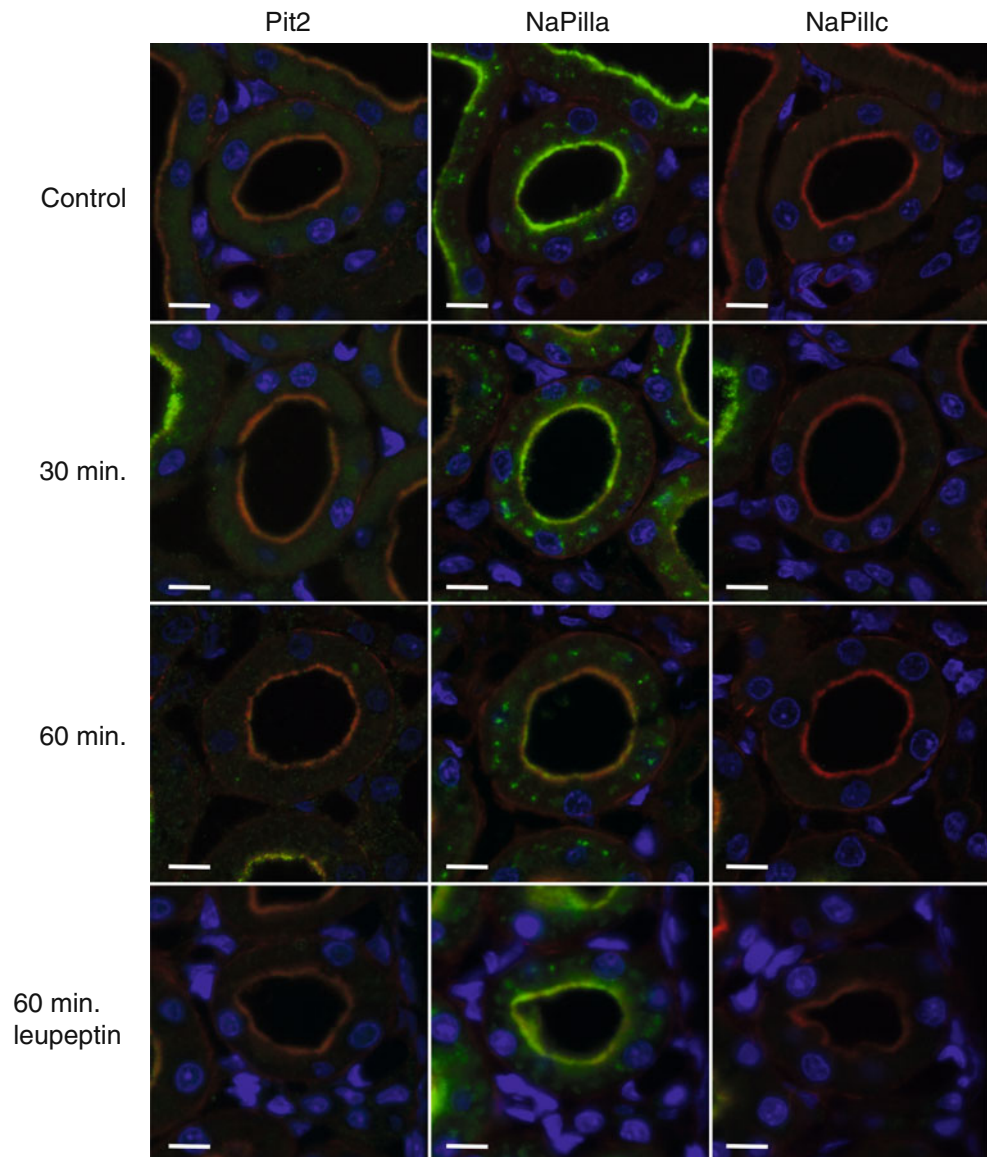
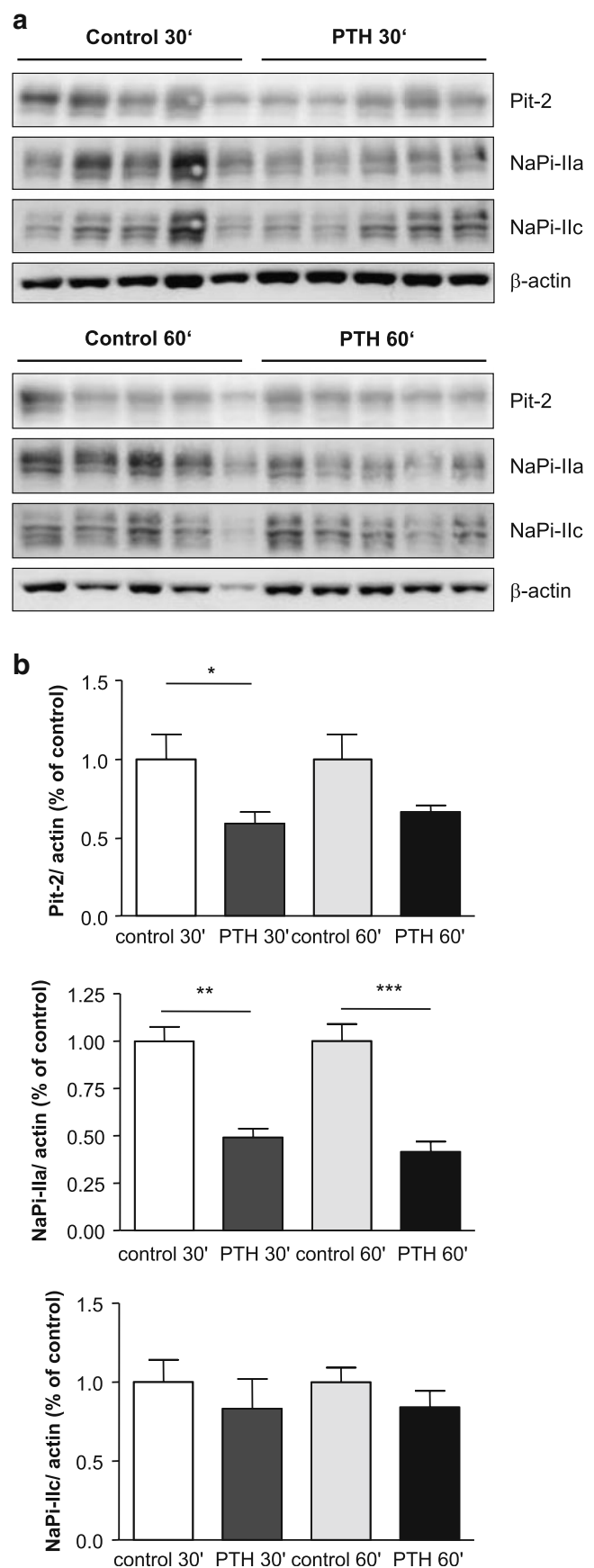


Fig. 5 Parathyroid hormone (PTH) decreases the abundance of NaPi-IIa and Pit-2 in the brush border membrane. Brush border membranes were prepared from kidneys of PTH and saline (*control*) injected rats ($n = 5$ animals per condition) and 10 μg loaded per lane for immunoblotting. **a** Membranes were tested for NaPi-IIa, NaPi-IIc, and Pit-2 and stripped to reprobe for β -actin to control for loading. **b** Densitometric analysis of membranes was performed, and the bands for the transporters of interest were normalized against β -actin and the respective control groups. PTH decreased NaPi-IIa abundance significantly 30 and 60 min after PTH injections. Pit-2 abundance was reduced significantly 30 min after PTH, whereas NaPi-IIc abundance was unaltered at both time points. $*p < 0.05$, $**p < 0.01$

internalization of NaPi-IIa [4]. NaPi-IIa localized in the brush border membrane forms part of a multiprotein complex and interacts via its PDZ-binding motif with the scaffolding protein NHERF1 [16, 18, 25, 49]. PTH enhances NHERF1 phosphorylation leading to dissociation of the NaPi-IIa–NHERF1 complex and enabling NaPi-IIa routing to clathrin-coated pits, early endosomes, and ultimately to lysosomes [14, 48]. The mechanisms and signals governing the internalization or regulation of NaPi-IIc and the recently described Pit-2 are not known to date.

Here, we examined the acute effects of PTH on all three known isoforms of renal brush border membrane sodium-phosphate cotransporters using three complimentary approaches. We demonstrate that acute application of PTH causes within 30 min a reduction in total sodium-dependent phosphate transport in isolated brush border membrane vesicles that became significant after 60 min. Importantly, the use of PFA allowed distinguishing at least two types of phosphate transport activities. Dousa and colleagues had demonstrated more than 20 years ago that brush border membrane phosphate transport could be partly blocked by PFA and related substances [40]. We and others have shown that PFA acts on type II phosphate transporters (NaPi-IIa, NaPi-IIb, and NaPi-IIc) expressed heterologously in *Xenopus* oocytes and that concentrations in the range of 3–6 mM block about 80–90% of activity [11, 44]. Importantly, type III phosphate transporters (Pit-1 and Pit-2) appear to be much less sensitive to this class of molecules and show only mild inhibition at concentrations higher than 10 mM [36, 43]. The fact that the PFA-resistant sodium-dependent uptake was also reduced by PTH strongly suggests that PTH acts also on the BBM abundance and/or activity of non-type II phosphate transporters. To further evaluate the inhibition of phosphate transport, we estimated the apparent half-maximal concentration of PFA required to inhibit phosphate transport in brush border membrane vesicles obtained from control and PTH injected rats. Our data demonstrate that PTH affected only the maximal transport rate of phosphate and the PFA inhibitable fraction but did not alter the apparent EC_{50} for PFA which remained in the range of about 3 mM. These data are in perfect agreement with previous data from rat



brush border membrane vesicles where binding of radio-labelled PFA and inhibition of phosphate transport were studied and an apparent K_m for binding of ~ 3.6 mM was found [39]. Also in agreement with the work of Dousa and colleagues, we find that about 25–30% of overall sodium-dependent phosphate uptake at the highest concentration of PFA (6 mM) is resistant to the inhibitory effect. Higher concentrations were not used since PFA starts to exert unspecific effects at concentrations of 10 mM and higher (unpublished results). Thus, these data suggest that PTH acts on at least two different populations of phosphate transporters, PFA-sensitive and PFA-insensitive ones.

Consistent with the PFA data, we observed that PTH reduced NaPi-IIa and Pit-2 abundance in isolated brush border membranes as detected by immunoblotting. NaPi-IIc expression was not altered by PTH in agreement with an earlier report showing that NaPi-IIc abundance decreased only approximately 8 h after PTH injection [38]. Immunohistochemistry confirmed that PTH reduced brush border-localized NaPi-IIa-related staining as described previously [3, 29]. Intracellular NaPi-IIa staining increased after PTH injection, and blockade of lysosomal degradation with leupeptin showed accumulation of intracellular NaPi-IIa in structures identified previously as lysosomes [3, 29]. In contrast to the findings for NaPi-IIa and the reduced abundance of Pit-2 as shown by immunoblotting, no changes in NaPi-IIc- and Pit-2-related stainings could be detected at the level of light microscopy. This discrepancy for Pit-2 might be due to our antibodies that might detect Pit-2 in immunohistochemistry only in a specific conformation. Alternatively, the binding site for the antibody might be blocked by an interacting protein that associates with Pit-2 upon PTH application. We also tested two commercially available antibodies against Pit-2 but failed to detect specific signals preventing a further investigation of this question. Clearly, the fate of Pit-2 following PTH application will require further studies.

The distinct transport properties and differential regulation by hormones and other factors with different time courses allow for a better and finer adaptation of renal phosphate reabsorption under various conditions. Differential regulation of NaPi-IIa, NaPi-IIc, and Pit-2 occurs not only in response to PTH. During dietary potassium restriction and hypokalemia, increased expression of NaPi-IIa has been observed despite phosphaturia [50]. Phosphaturia may be explained by the downregulation of NaPi-IIc and Pit-2, providing another example for differential regulation of BBM phosphate transporters [10]. However, the relative contribution of these transporters to overall renal phosphate handling and the integration of several signals on the level of the proximal under these circumstances remain an open question. PTH application elicits a rapid phosphaturic response both in humans and rodents [15, 19]. In contrast to rodents, several

lines of evidence suggest that NaPi-IIa may not be the major type II phosphate transporters in human kidney. Missense and even truncating mutations in NaPi-IIa (SLC34A1) do not cause phosphaturia in humans [23, 46], whereas mutations in NaPi-IIc (SLC34A3) are associated with massive phosphaturia, hypophosphatemia, rickets, and hypercalcuria suggesting that NaPi-IIc is an important contributor to renal phosphate reabsorption [5, 28]. The expression of Pit-2 and the relevance of a PFA-sensitive phosphate transport activity in the brush border membrane have not been studied in human kidney and remain unknown to date. If NaPi-IIa does not play a major role in human kidney and if NaPi-IIc does internalize or inactivate only with a delayed time course (as indicated by studies in rodents [38]), then other phosphate transporters must mediate the acute phosphaturic effect of PTH in humans [19]. Whether Pit-2 could be a candidate for this transport activity remains to be investigated in humans and human cell lines. Differential regulation of the three known phosphate transporters in the brush border membrane would allow to fine-tune phosphate excretion not only to PTH status but also to other regulating factors such as dietary phosphate intake, acid–base status, vitamin D₃, and others. In fact, renal phosphate excretion is almost refractory to PTH during growth or if systemic phosphate depletion exists [17, 47]. How this fine-tuning is achieved remains to be further examined.

In summary, we demonstrate that acute application of PTH affects the brush border membrane expression of NaPi-IIa and Pit-2 in rat kidney. PTH also reduces PFA-sensitive and PFA-insensitive phosphate transport activities. Therefore, our data suggest that renal brush border membrane phosphate transporters are differentially regulated by acute application of PTH.

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