Renal Phosphate Wasting in the Absence of Adenylyl Cyclase 6

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ABSTRACT

Parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF-23) enhance phosphate excretion by the proximal tubule of the kidney by retrieval of the sodium-dependent phosphate transporters (Npt2a and Npt2c) from the apical plasma membrane. PTH activates adenylyl cyclase (AC) through PTH1 receptors and stimulates the cAMP/PKA signaling pathway. However, the precise role and isoform(s) of AC in phosphate homeostasis are not known. We report here that mice lacking AC6 (AC6−/−) have increased plasma PTH and FGF-23 levels compared with wild-type (WT) mice but comparable plasma phosphate concentrations. Acute activation of the calcium-sensing receptor or feeding a zero phosphate diet almost completely suppressed plasma PTH levels in both AC6−/− and WT mice, indicating a secondary cause for hyperparathyroidism. Pharmacologic blockade of FGF receptors resulted in a comparable increase in plasma phosphate between genotypes, whereas urinary phosphate remained significantly higher in AC6−/− mice. Compared with WT mice, AC6−/− mice had reduced renal Npt2a and Npt2c protein abundance, with approximately 80% of Npt2a residing in lysosomes. WT mice responded to exogenous PTH with redistribution of Npt2a from proximal tubule microvilli to intracellular compartments and lysosomes alongside a PTH-induced dose–response relationship for fractional phosphate excretion and urinary cAMP excretion. These responses were absent in AC6−/− mice. In conclusion, AC6 in the proximal tubule modulates cAMP formation, Npt2a trafficking, and urinary phosphate excretion, which are highlighted by renal phosphate wasting in AC6−/− mice.

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Parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF-23) are the primary regulators of renal phosphate (P1) excretion by the proximal tubule and critically involved in the regulation of P1 balance and maintenance of plasma P1. PTH acts on the proximal tubule through the Gs protein-coupled PTH1 receptor (PTH1R) to stimulate adenylyl cyclase (AC) and, thus, the synthesis of cAMP and consecutive activation of protein kinase A (PKA).1–3 After activation of PTH1R, the sodium-phosphate cotransporters Npt2a and Npt2c (approximately 70% and approximately 30% reabsorption of filtered P1, respectively) are retrieved from the apical plasma membrane, resulting in increased P1 excretion.4–6 In addition, cAMP-independent effects of PTH have been reported,7 which may involve PTH1R action on phosphoinositide-specific phospholipase C (PLC)8,9 and mitogen-activated protein kinases.10,11 FGF-23 mediates its action in the proximal tubule through the FGF receptor/Klotho complex, which downregulates the expression of Npt2a and Npt2c.12,13 Immunohistochemistry
identified Npt2a to be localized predominantly in the apical plasma membrane of S1–S3 segments of the proximal tubule.\textsuperscript{6,14,15} Npt2c has a similar cellular distribution with greater abundance in S1 segments.\textsuperscript{5,16,17}

Genetic defects in Gs\textsubscript{a} result in impaired PTH-induced retrieval of Npt2a, resulting in a failure to respond with increased urinary P\textsubscript{i} excretion: a disease commonly referred to as pseudohyppoparathyroidism type Ia or Ib (depending on the presence or absence of skeletal defects, respectively).\textsuperscript{18–20} In comparison, less is known about the role of AC proteins in mediating the signaling between PTH\textsubscript{1}R and Npt2a and/or Npt2c. Generation of cAMP involves the activation of ACs, of which nine different membrane-bound isoforms have been identified (AC1–AC9).\textsuperscript{21} Studies on AC isoform mRNA expression in rat kidney showed that, except for AC1 and AC8, all AC isoforms are expressed.\textsuperscript{22–24} In mice where a green fluorescent protein reporter gene was expressed under the control of the AC6 promoter, green fluorescent protein-positive cells were found in cells of the proximal tubules, thick ascending limbs, distal tubules, and collecting ducts.\textsuperscript{25} Functional studies have shown a role of AC6 in the thick ascending limb, distal tubule, and collecting duct insofar as mice lacking AC6 have Bartter syndrome\textsuperscript{26} and nephrogenic diabetes insipidus (NDI).\textsuperscript{27,28} In a collecting duct-specific AC6 knockdown model, vasopressin-stimulated epithelial sodium channel open probability was abolished.\textsuperscript{29}

Based on studies indicating that vasopressin V\textsubscript{2} receptors use the AC6–cAMP signaling pathway\textsuperscript{26–30} and the relatively high abundance of AC6 in the proximal tubule,\textsuperscript{23,30,31} we proposed that AC6 may contribute to PTH-stimulated cAMP formation, P\textsubscript{i} excretion, and thus, P\textsubscript{i} homeostasis. In the present study, we examined P\textsubscript{i} homeostasis, PTH-induced renal excretion of P\textsubscript{i}, and cAMP, and trafficking and expression of Npt2a and Npt2c in mice lacking AC6 (AC6\textsuperscript{2/2}). The results show that AC6\textsuperscript{−/−} mice have renal phosphate wasting, and thus, AC6 has an essential contribution to PTH actions, cAMP formation, and renal P\textsubscript{i} excretion in vivo.

Figure 1. Increased PTH and FGF-23 levels are associated with reduced renal Npt2a and Npt2c expression in AC6\textsuperscript{−/−} mice with free access to food and water. (A) In contrast to WT mice, significantly higher urinary phosphate/creatinine, PTH, and FGF-23 levels were detected in AC6\textsuperscript{−/−} mice, whereas plasma 1,25 (OH\textsubscript{2})D levels were suppressed. No significant differences between AC6\textsuperscript{−/−} and WT mice were detected in plasma phosphate or calcium concentrations. (B) Analysis of Npt2a and Npt2c abundances in membrane fractions of renal cortex by Western blotting; densitometric analysis of Western blots was performed using β-actin expression as a reference. Increased urinary phosphate in AC6\textsuperscript{−/−} mice is associated with lower amounts of Npt2a and Npt2c compared with WT. For physiologic parameters, n=9–11/genotype; for protein abundance, n=5/genotype. *P<0.05 versus WT.
RESULTS

Basal Analysis of AC6−/− Mice with Free Access to Control Diet and Fluid

Urinary Pi/creatinine was significantly higher on control diet (0.6% Pi, 0.8% Ca2+) in AC6−/− versus wild-type (WT) mice in spontaneously voided urine, which was associated with an approximately 7-fold higher plasma PTH and an approximately 2-fold higher plasma FGF-23 in AC6−/− versus WT mice. Plasma 1,25-dihydroxy vitamin D [1,25 (OH)2D] was significantly lower (approximately 0.7-fold) in AC6−/− versus WT mice. Plasma Pi and Ca2+ were not different between genotypes (Figure 1A). Under basal conditions, higher urinary Pi/creatinine was associated with significantly reduced renal abundance of Npt2a and Npt2c (Figure 1B) and mRNA expression levels (Npt2a: 0.71 ± 0.1 and Npt2c: 0.46 ± 0.1 versus WT, P<0.05).

Immunohistochemistry (Figure 2) detected Npt2a throughout the proximal tubule of WT mice, with greater abundance in the early S1 segment. Npt2a staining was predominantly localized to the microvilli. In AC6−/− mice, the tubular distribution of Npt2a was similar to WT mice, but Npt2a staining was generally weaker and observed, in addition to microvilli, intracellularly in a punctate distribution. In AC6−/− mice, Npt2a labeling in the S3 segment was predominantly in distinct intracellular structures, with diffuse labeling of the microvilli. Semiquantitative laser-scanning confocal microscopy (Figure 3) confirmed the observed tubular and intracellular distributions of Npt2a in both genotypes.

Immunogold electron microscopy of Npt2a (Figure 4) indicated that, in contrast to WT mice (Supplemental Figure 1), gold particles in AC6−/− mice were abundantly observed in structures morphologically resembling secondary lysosomes (Supplemental Figure 2 shows double labeling with various markers), with little labeling associated with microvilli or intracellularly. Semiquantitative assessment of total gold particles showed a different distribution of Npt2a between the genotypes; AC6−/− mice had significantly lower numbers of gold particles in microvilli and higher numbers in lysosomes compared with WT. There was a trend for decreased numbers of total gold particles in AC6−/− (Figure 4).

Npt2c labeling by immunohistochemistry was weak in WT mice in the early S1 segment, with staining predominantly localized to the microvilli (Figure 5). Npt2c labeling intensity increased in late proximal tubules from both genotypes and was often detected in the microvilli, with a clear transition to thin descending limbs of Henle’s loop. Labeling intensity of Npt2c in early proximal tubules was weaker in AC6−/− versus WT mice, with no observable differences in late proximal segments. Laser-scanning confocal microscopy (Figure 5) confirmed the observed tubular and intracellular distributions of Npt2c in both genotypes and showed a trend for reduced Npt2c abundance in AC6−/− mice.

Response to Calcium-Sensing Receptor Activation, Dietary Phosphate Restriction, and FGF Receptor Blockade in AC6−/− Mice

At baseline, PTH was significantly higher in AC6−/− versus WT mice (255±60 versus 39±6 pg/ml, P<0.05), and acute oral treatment with the calcimimetic NPS R-568 suppressed plasma PTH after 30 minutes to comparable low levels (AC6−/−: 14±2 versus WT: 14±1 pg/ml, both P<0.05 versus the basal same genotype), indicating secondary hyperparathyroidism and not primary hypersecretion of PTH. To test if AC6−/− mice can respond to a reduction in dietary Pi, mice were placed for 7 days on a zero Pi diet. Both genotypes reduced urinary Pi/creatinine, plasma P1, and PTH (Figure 6A). Of note, zero Pi intake was the only condition where urinary Pi/creatinine was significantly lower in AC6−/− versus WT mice. Plasma FGF-23 was reduced in both genotypes compared with control diet; however, FGF-23 remained significantly higher in AC6−/− versus WT mice. Blocking FGF receptors by PD173074 for 6 days resulted in a comparable increase in plasma P1 (Δ increase in WT: 0.8±0.1 and AC6−/−: 0.9±0.2 mmol/L), whereas cumulative urinary Pi/creatinine was significantly lower and Npt2c expression tended to be lower in AC6−/− mice (Supplemental Figures 3 and 4).
Hyperphosphatemia

Renal clearance experiments showed a significantly higher absolute (WT: 24±8 versus AC6−/−: 133±35 nmol/min, P<0.05) and fractional urinary P_i excretion after vehicle application (Figure 7). Plasma P_i and the amount of filtered P_i (WT: 580±45 versus AC6−/−: 502±50 nmol/min, P was NS) were comparable between genotypes, whereas GFR was modestly lower in AC6−/− versus WT mice (Figure 7). Urinary cAMP excretion tended to be lower in AC6−/− mice associated with greater fractional fluid excretion, consistent with their previous reported NDI.27 Bolus application of PTH to WT mice (Figure 8) dose-dependently increased absolute and fractional urinary P_i excretion and urinary cAMP excretion. In contrast, AC6−/− mice (Figure 8) responded to PTH with a slight decrease in absolute urinary P_i excretion, and PTH did not change the higher fractional urinary P_i excretion and absolute urinary cAMP excretion (all doses not significantly different from vehicle).

**Effect of PTH on Renal Cortical cAMP Formation**

Freshly isolated renal cortical tubule suspensions from WT mice significantly increased cAMP formation after forskolin stimulation; however, this response was absent in AC6−/− mice. In similar suspensions, PTH increased cAMP formation in WT but not AC6−/− mice (Figure 8B).

**Effect of PTH on Npt2a and Npt2c Trafficking in AC6−/− Mice**

To study the in vivo response to PTH receptor activation, we administered PTH to WT and AC6−/− mice and studied kidneys by immunohistochemistry. Npt2a labeling and distribution were (Figure 9), in contrast to control conditions (Figures 2 and 3), similar between WT and AC6−/− mice in the early S1 segment, with staining observed in the microvilli and intracellularly in a punctate pattern. In the late S3 segment, no observable difference was detected in Npt2a labeling or the distribution between genotypes. Immunogold labeling showed that, in WT mice, compared with control conditions, the distribution of Npt2a gold particles after PTH treatment was different, with a reduction in the number of gold particles residing in the microvilli and an increase of gold particles in lysosomes and intracellular compartments (compare Figure 4 with Figure 9). In contrast, PTH treatment did not significantly change the distribution of Npt2a in AC6−/− mice. After PTH treatment, the distribution of Npt2c was similar in WT and AC6−/− mice with predominant labeling of the microvilli and showing a similar distribution compared with control conditions (Figure 5).

**DISCUSSION**

Much information has been learned about the molecular determinants of PTH-regulated phosphate transport, but little is known about the role and molecular identity of the AC isoform(s) involved. The present studies show, for the first time, that almost all of the PTH-stimulated cAMP formation and urinary P_i excretion are mediated by AC6. We show that AC6 plays a vital role in P_i homeostasis and that mice lacking AC6 achieve P_i homeostasis only by having compensatory elevated PTH and FGF-23 levels. This defect in urinary P_i excretion is associated with and potentially, the consequence of reduced levels of cAMP, leading to effects on the abundance and distribution of Npt2a and Npt2c.
AC6−/− mice had 7-fold higher PTH compared with WT mice. We excluded primary hyperparathyroidism, because acute activation of the calcium-sensing receptor completely suppressed PTH. In patients with pseudohypoparathyroidism, the calcium-sensing receptor agonist cinacalcet reduced PTH. Vice versa, in a mouse model of primary hyperparathyroidism, cinacalcet was ineffective in suppressing PTH. The exclusion of primary hyperparathyroidism in AC6−/− mice was supported by very low PTH when fed a zero Pι diet. 

Why do AC6−/− mice show renal phosphate wasting and not reduced Pι excretion? Hypothetically, AC6−/− mice should show renal PTH resistance, impaired Pι excretion, hyperphosphatemia, and hyperparathyroidism. Although AC6−/− mice show an absent cAMP and phosphaturic response to PTH, the elevated PTH possibly activates phosphatidylinositol-specific PLC (PI-PLC), leading to Npt2a/Npt2c redistribution. Consistent with this hypothesis, continuous infusion of PTH to mice with a PTH receptor inactive for PI-PLC signaling but an intact AC/cAMP/PKA signaling pathway can only transiently increase urinary Pι excretion, indicating that PI-PLC is important for the sustained phosphaturic effect of PTH. It is possible that AC6 is part of a negative feedback loop, which when absent, fails to suppress PTH. Alternatively, AC6 may play a secondary role to limit PI-PLC signaling events. Additionally, because FGF-23 reduces Npt2a and Npt2c expression and increases urinary Pι excretion, the approximately 2-fold increase in FGF-23 levels in AC6−/− mice might aggravate renal phosphate wasting in AC6−/− mice. Although the increased FGF-23 levels are not as severe compared with the drastically increased levels in Hyp, FGFR1−/−/FGFR4−/−, or Klotho−/− mice, even a minor elevation in the FGF-23 signaling pathway may affect urinary Pι excretion. 

Consistent with elevated PTH and FGF-23, AC6−/− mice showed a significantly reduced abundance of Npt2a and Npt2c. Using immunohistochemistry and confocal microscopy, Npt2a was localized along the entire length of the proximal tubule in both genotypes, although AC6−/− mice had less intense staining compared with WT, especially in the S1 segment. Our basal immunogold electron microscopy clearly highlights that, in the absence of AC6, the majority (approximately 80%) of Npt2a is localized in secondary lysosomes. It might reflect that the majority of Npt2a has entered the degradative pathway. The reductions of Npt2a protein abundances observed in Western blotting of membrane preparations are comparable with the reduced localization of Npt2a gold particles in microvilli of AC6−/− mice. Our previous studies in AC6−/− mice indicated a mild Bartter syndrome caused by reduced NKCC2 expression and NDI caused by impaired aquaporin-2 phosphorylation and trafficking. At this point, it is unclear if these diseases can contribute to the high PTH/FGF-23 and renal phosphate wasting in AC6−/− mice. To our knowledge, Npt2a expression has only been studied in type II Bartter syndrome (renal outer medullary potassium channel-type) and lithium-induced NDI. Both studies found reduced Npt2a expression; however, the underlying mechanism was never determined. In addition, AC6 might regulate Npt2a/Npt2c function by modulating the phosphorylation of other proteins involved in apical membrane insertion/retention of Npt2a/Npt2c. 

In contrast to studies localizing Npt2c mainly to the S1 and weaker in the S2 segment, our antibody identified Npt2c along the entire length of proximal tubules, with clear transitions from a labeled S3 segment to an unlabeled thin descending limb of Henle’s loop. Our antibody previously gave a strong signal in the outer stripe of the outer medulla in WT mice, consistent with Npt2c expression in S3.
Figure 5. Weaker Npt2c labeling in early proximal tubules of AC6−/− mice. Immunohistochemical and quantitative confocal laser-scanning microscopy analysis of Npt2c in WT and AC6−/− mice. (A and B) Weak Npt2c labeling was observed in the early (S1) segment of the proximal tubule, with staining predominantly localized to the apical microvilli (arrows). (C and D) Npt2c labeling intensity increased in the late proximal tubule in both WT and AC6−/− mice and was detected in the apical microvilli. (E and F) Npt2c labeling was observed in the late proximal tubule (pt; S3) segment with clear transitions to unlabeled descending thin limbs of Henle’s loop (tl). G–J show representative quantitative laser-scanning confocal microscopy images of Npt2c in WT and AC6−/− mice and corresponding differential image contrast (DIC) overlays. Npt2c was localized to the apical brush border in WT and AC6−/− mice. (K and L) In general, Npt2c labeling intensity in early proximal tubules (arrows) was greater in WT compared with AC6−/− mice, with no observable difference in late proximal segments. (M) Quantification of total Npt2c labeling intensity (mean fluorescent signal) in proximal tubules of WT and AC6−/− mice (n=4/genotype).

The reason for this discrepancy remains unknown; however, it might relate to species differences.

Why is FGF-23 increased and 1,25(OH)2D suppressed in AC6−/− mice? PTH and FGF-23 are part of a negative feedback loop (suppressing each other). However, our data in AC6−/− mice indicate that elevated PTH can offset this negative feedback. A recent study indicates that PTH has a stimulatory effect on FGF-23 release,7 and mice with a constitutively active PTH receptor, overexpressed in osteocytes, have significantly elevated FGF-23 compared with WT mice, a response partially mediated by a cAMP-dependent mechanism.44 Patients with constitutively active PTH receptors (e.g., Jansen metaphyseal chondrodysplasia) also show increased FGF-23, despite hypophosphatemia, possibly indicating that FGF-23 could be governed by other factor(s), including activation of PTH receptors.45 Based on our data, AC6 in bone does not seem to be the enzyme mediating this stimulatory effect, because AC6−/− mice show increased and not reduced FGF-23. The well known regulators of FGF-23 do not seem to be the culprits, and additional work is needed to identify these regulatory factors, possibly involving a kidney bone crosstalk. FGF-23 and PTH have opposite effects on 1,25(OH)2D: FGF-23 inhibits and PTH stimulates 1,25(OH)2D. In AC6−/− mice, the suppression of 1,25(OH)2D dominates (along with reduced Npt2a expression), possibly as a consequence of the unopposed activation of FGF-23. In contrast, a study in humans showed that 18-hour PTH treatment increases 1,25(OH)2D and FGF-23, indicating that 1,25(OH)2D is a potent stimulator of FGF-23 secretion.46 Additional studies are needed to better understand the interactions of these hormones or if additional factors, like circulating cleaved forms of Klotho,47 are involved in PTH/FGF-23 regulation.

To assess the role of AC6 for renal Pi transport, we used an acute oral hyperphosphatemic model. After 7 days on zero Pi intake, a situation maximizing the potential contribution for each of the Pi transport mechanisms,32 AC6−/− mice show evidence for impaired renal Pi excretion, with consecutively increased plasma Pi after 30 and 60 minutes, in addition to a tendency for lower urinary Pi/creatinine. Vice versa, when mice lacking intestinal Npt2b were exposed to a comparable acute hyperphosphatemic model, the increase in plasma Pi was impaired after 30 and 60 minutes.48

Is AC6 mediating the acute phosphaturic response to PTH and cAMP formation? Our data indicate that AC6 is the major isoform required for the acute phosphaturic effect and cAMP formation in response to PTH. The timeframe of the phosphaturic response in our experiments (15 minutes) is consistent with other studies, showing a rapid inhibition of renal Pi transport and Npt2a abundance as early as 5 minutes.49 A recent study in mice provides evidence that the cAMP/PKA signaling pathway is important for acute PTH-induced phosphaturia and reduction of Npt2a staining.6,50 The maximum effect of PTH-induced fractional Pi excretion observed in WT mice was comparable with the fractional Pi excretion observed after vehicle application and under all tested PTH concentrations in AC6−/−, possibly indicating that AC6−/− mice are at their maximum Pi excretion capacity. Confirming the in vivo results under clearance conditions, freshly
isolated renal cortical tubule suspensions from AC6^{-/-} lacked a cAMP response to forskolin or PTH. The impaired renal forskolin response in this study is consistent with the renal forskolin response of an AC6^{-/-} mouse made by another group. AC5 and AC6 are the only calcium-inhibitable AC isoforms, and PTH-simulated AC activity can be inhibited by increasing calcium concentrations, showing that AC5 and/or AC6 are mediating the PTH response. The impaired forskolin-stimulated cAMP formation in renal cortex (current study), renal inner medulla, and whole kidney of AC6^{-/-} mice indicates that AC6 plays a prominent role for renal cAMP formation.

Acute application of PTH leads to a rapid internalization of Npt2a from the brush border membrane and its subsequent routing to lysosomes. Our immunogold electron microscopy shows that, 1 hour after PTH treatment (a timeframe shown to result in almost complete retrieval of Npt2a from the brush border membrane), there is a shift of Npt2a from the microvilli to intracellular compartments and lysosomes; however, this acute PTH-induced Npt2a trafficking is absent in AC6^{-/-} mice. Moreover, 1 hour after PTH application, both genotypes had a comparable amount of Npt2a gold particles in the microvilli, which may explain why fractional urinary Pi excretion was not significantly different at the highest PTH doses used in our clearance studies. An explanation for the number of Npt2a gold particles in microvilli of WT mice after PTH application still being higher than the number of gold particles found in AC6^{-/-} mice under basal conditions might relate to the continuously elevated PTH and/or FGF-23. Our data support other studies indicating a different time course in the regulation of Npt2c versus Npt2a. Npt2c retrieval in response to PTH is not complete until 4–8 hours after treatment. Consistent with these studies, we did not see a difference in Npt2c abundance or distribution in either genotype after PTH administration.

In summary, we are just beginning to understand the roles of different AC isoforms for hormone-mediated regulation of urinary phosphate and plasma PTH in response to a zero phosphate diet; however, they show greater increases in plasma phosphate after acute oral phosphate loading. (A) One week of zero phosphate intake (Zero P) significantly reduced urinary Pi/creatinine and plasma PTH levels in both genotypes (for comparison, values of the control diet (Con P) are shown from Figure 1). Plasma FGF-23 levels remained significantly higher in AC6^{-/-}, whereas plasma phosphate was reduced to comparable levels in WT and AC6^{-/-} mice. (B) Effects of acute oral phosphate loading. AC6^{-/-} mice show a significantly greater increase in plasma phosphate after 30 and 60 minutes compared with WT mice. (C) Urinary phosphate/creatinine tended to be lower 60 minutes after phosphate loading in AC6^{-/-} versus WT mice. For diets, n=9–11/group; for acute oral phosphate loading, n=4–8/group. *P<0.05 versus WT. #P<0.05 versus basal same genotype.
renal transport. We recognize that our study has limitations because of the complex regulation of Pi homeostasis by the elevated PTH and FGF-23 in AC6$^{+/+}$ mice, thereby hindering our ability to decipher the extent of each hormone on, for example, the reduction in renal Npt2a and Npt2c expression/abundance. However, our results show that, of nine different AC isoforms, AC6 determines PTH-induced cAMP formation and Npt2a and Npt2c expression and trafficking. Furthermore, we show that these effects are functionally relevant for urinary Pi excretion and FGF-23 levels. As a result, mice lacking AC6 are a new model of renal phosphate wasting, despite having absent PTH-induced cAMP formation.

**CONCISE METHODS**

All animal experimentation was conducted in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD) and approved by the local Institutional Animal Care and Use Committee. Age-matched adult male and female AC6$^{+/+}$ and WT littermates were generated as described using heterozygous breeding; their genetic background is a mix of 129Sv/J and C57BL/6. Mice were fed either a control diet (0.6% Pi, 0.8% Ca$^{2+}$; Harlan Teklad, Madison, WI) or for studying the effect of Pi intake, a zero Pi diet (0% Pi, 0.8% Ca$^{2+}$; Harlan Teklad). Experimental male mice were between 3 and 6 months old.

**Analysis of AC6$^{+/+}$ Mice with Free Access to Fluid**

Mice were kept on control diet or zero Pi diet for 7 days with free access to food and water. Spontaneously voided urine was collected for determination of P$_i$, Ca$^{2+}$, and creatinine (all determined photometrically; Thermo Fisher Scientific, Middleton, VA). Blood was drawn from the retro-orbital plexus for determination of P$_i$, Ca$^{2+}$, PTH (1–84; ALPCO Immunoassays, Salem, NH), FGF-23 (Immutopics International, San Clemente, CA), and 1,25(OH)$_2$D (Immunodiagnostic Systems, Scottsdale, AZ). In another set of mice, kidneys were harvested on control diet to determine renal cortical abundance of Npt2a and Npt2c as described below.

**PTH Response to Ca$^{2+}$-Sensing Receptor Activation and FGF Receptor Blockade**

The calcimimetic NPS R-568 (Tocris Biosciences, Minneapolis, MN) was applied (30 mg/kg by oral gavage, 10 $\mu$L/g body wt of 0.5% carboxymethylcellulose) to activate Ca$^{2+}$-sensing receptors. At baseline (0 minutes) and after 30 minutes, blood was taken from the retro-orbital plexus under brief isoflurane anesthesia for determination of PTH levels. For FGF receptor blockade, mice were treated every 12 hours for 6 days with PD173074 (50 mg/kg in Kolliphor EL:ethanol:water [7.5:2.5:90] by oral gavage, 1% of body weight; LC Laboratories, Woburn, MA). Blood was taken before and at the end of the treatment period, and urine was collected daily.

**Acute Hyperphosphatemic Mouse Model**

Animals were placed on a zero Pi diet for 1 week. Baseline blood and urine samples were taken at 0 minutes. Animals were then gavaged with vehicle (sterile water, 1% of body weight) or a 0.5 mol/L NaH$_2$PO$_4$ solution. Blood was collected after 30 and 60 minutes, and urine was collected at 60 minutes postgavage. Plasma Pi as well as urinary Pi and creatinine were measured as described above.

**Effect of PTH on Urinary Pi and cAMP Excretion in Renal Clearance Experiments**

Mice were anesthetized with thiobutabarbital/ketamine and prepared for clearance experiments. A catheter was placed in the femoral
artery for continuous BP recording. For assessment of two-kidney GFR, the jugular vein was cannulated for continuous maintenance infusion of 2.25 g/dl BSA in 0.85% NaCl at a rate of 0.5 ml/h per 30 g body wt. [3H]inulin was added to the infusion to deliver 5 mCi/h.

After 1 hour of equilibration, intravenous bolus application of vehicle (0.5 µl/g body wt of 0.85% NaCl over 1 minute) was followed by application of increasing doses of PTH (1, 3, 10, 30, and 100 µg/kg; 1–34 Human; GenScript, Piscataway, NJ). After each bolus, allowing 5 minutes for drug distribution, urine was quantitatively collected through a bladder catheter for 15 minutes to determine urinary Pi. Plasma and urinary Pi concentrations were measured as described above. Urinary cAMP concentration was assessed by RIA.58,59

Freshly Isolated Renal Cortical Tubule Suspensions
Renal cortex was isolated using a modification of the method described by Guder,60 and cAMP accumulation was measured as described.27,59

Figure 8. PTH-induced urinary cAMP, phosphate excretion, and cAMP formation in renal cortical tubule suspensions are impaired in AC6^{-/-} mice. (A) Bolus application of PTH in WT mice dose-dependently increased fractional urinary Pi excretion and urinary cAMP excretion. In contrast to WT mice, the phosphaturic effect and cAMP formation in response to PTH are absent in AC6^{-/-} mice. (B) Stimulation of cAMP formation by forskolin (Forsk; 10 µmol/L) or PTH is absent in freshly isolated renal cortical tubule suspensions of AC6^{-/-} compared with WT mice. Protein concentration was adjusted to 60 µg/vial. Experiments were performed with phosphodiesterase inhibition (0.5 mmol/L 3-isobutyl-1-methylxanthine; n=6–8/genotype for clearance experiments and n=5/genotype for isolated cortical tubules). bw, body weight. *P<0.05 versus WT. #P<0.05 versus control same genotype.

Cortical Abundance and mRNA Expression of Npt2a and Npt2c
Kidneys were removed, and renal cortex was dissected and prepared for Western blotting.26,27 Proteins were transferred to nitrocellulose membranes and immunoblotted with Npt2a (1:1000) and Npt2c (1:2500).16,42 Chemiluminescent detection was performed with ECL Plus (Amersham, Piscataway, NJ). Densitometric analysis was
performed by ImageJ Software (National Institutes of Health). β-Actin was used as loading control (dilution 1:20,000; Sigma-Aldrich). Mice were euthanized, and renal inner medullae and brain were rapidly removed. In a different set of mice (n=3/genotype), whole-kidney RNA was isolated and reverse-transcribed into cDNA. Quantitative PCR was performed as described previously27,59 using 8 ng RNA/reaction, and it was used in conjunction with primer pairs (forward/reverse) specific for the following murine genes: Npt2a, 5'-GGCTCCAACAT-TGGCACTAC-3'/5'-ACAGTAGGATGCCCCGAGATG-3'; Npt2c, 5'-TACCCCTCTTCTGGGTTC-3'/5'-CAGTCTCAAGACAGG-CACCA-3'. Data analysis used the ΔΔ cycle threshold method, and they were normalized to 18S ribosomal RNA expression and compared with WT expression.

Immunohistochemistry and Preparation of Tissue for Microscopy
All procedures have been described in detail previously.61 Labeling was visualized by use of a peroxidase-conjugated secondary antibody for light microscopy (P448; Dako, Glostrup, Denmark).

Immunofluorescent Labeling of Kidney Sections, Confocal Laser-Scanning Microscopy, and Image Quantification
Tissue preparation, sectioning, and labeling were performed as previously described.28 Semiquantitative imaging was performed using 8–10 individual images from a labeled section per experimental mouse per genotype as described previously.28
Quantitative Immunogold Electron Microscopy
All tissue processing, staining, and counting procedures have been described in detail previously.6,9 Briefly, tissue from mice either under basal conditions or after 60 minutes of PTH treatment (200 μg/kg, 2 μl/g body wt in 0.85% NaCl intravenously)4,50 were assessed after labeling with Npt2a. A minimum of six complete proximal tubule cells from different tubules per animal was analyzed from sections oriented approximately at right angles to the apical cell membrane, and they showed negligible background over mitochondria and nuclei. Gold particles within 40 nm of the apical brush border were classified as microvilli, gold particles labeling defined structures morphologically resembling lysosomes were classified as lysosomes, and all other gold particles were classified as intracellular.

Statistical Analyses
The data are expressed as mean±SEM. Unpaired and paired t tests were performed, as appropriate, to analyze for statistical differences between groups. All quantitative data from confocal laser-scanning microscopy were analyzed using one-way ANOVA followed by Newman–Keuls multiple comparisons tests between the groups. For all statistical analyses, P<0.05 was considered significant.

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DISCLOSURES
None.

REFERENCES


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Supplemental Figure 1. Immunogold electron microscope localization of Npt2a in proximal tubule of wild-type (WT) and AC6 knockout (AC6⁻/⁻) mice. A) Low magnification cross section of early proximal tubule cell demonstrating that gold particles representing Npt2a are abundantly observed in structures morphologically resembling lysosomes (L), with little labeling associated with the microvilli (arrows) in AC6⁻/⁻ mice. B) In contrast, in WT mice gold particles representing Npt2a are abundantly observed in the microvilli (arrows), with little labeling of lysosomes. C and D) Higher magnification demonstrating little Npt2a labeling is associated with the microvilli (arrows) of AC6⁻/⁻ mice compared to WT mice. Gold particles are 10 nm in diameter.
Supplemental Figure 2. The majority of Npt2a resides in secondary lysosomes within proximal tubule cells of AC6⁻/⁻ mice. (A) Immunogold electron microscopy image demonstrating an abundance of gold particles representing Npt2a (10 nm gold particles) in electron dense structures morphologically resembling lysosomes, and distinct from other electron dense structures such as mitochondria (M). (B) Double labelling immunogold electron microscopy with Npt2a (5nm gold particles) and AtpB (beta subunit of ATP synthase, 10nm gold particles, arrows) confirms Npt2a labelling is not in mitochondria. In contrast, Npt2a (5nm gold particles) co-distributes to some extent with (C) LAMP2 or (D) Cathepsin B (10 nm gold particles, arrows) positive structures, suggesting that Npt2a is confined to a cohort of secondary lysosomes.
Supplemental Figure 3. Effect of FGF receptor blockade on plasma phosphate and urinary $P_i$ excretion in wild-type (WT) and AC6 knockout (AC6$^{-/-}$) mice. WT and AC6$^{-/-}$ mice were treated with the FGF receptor inhibitor PD173074 (50 mg/kg in Cremophor EL/ethanol/water; 7.5:2.5:90 via oral gavage, 1% of body weight) every 12 hours for 6 days.\textsuperscript{1,2} Blood was collected before and 6 days after treatment, spontaneous urine was collected daily. A) Treatment with PD173074 significantly increased plasma phosphate in both genotypes. B) Of note, cumulative urinary phosphate/creatinine remained significantly higher in AC6$^{-/-}$ versus WT mice. \textsuperscript{#}P<0.05 versus basal same genotype, \textsuperscript{*}P<0.05 versus WT.
Supplemental Figure 4. Effect of FGF receptor blockade on Npt2a and Npt2c protein expression in wild-type (WT) and AC6 knockout (AC6−/−) mice. WT and AC6−/− mice were treated with the FGF receptor inhibitor PD173074 (50 mg/kg in Cremophor EL/ethanol/water; 7.5:2.5:90 via oral gavage, 1% of body weight) every 12 hours for 6 days.1,2 After 6 days of treatment, Npt2a and Npt2c abundances in membrane fractions of renal cortex were analyzed via Western blotting. Whereas treatment with PD173074 did not significantly affect Npt2a between genotypes, Npt2c tended to be lower in AC6−/− mice, giving a possible explanation for the higher urinary phosphate/creatinine (Supplemental Figure 3).
Supplemental Figure 5. Effect of acute PTH application on urinary $P_i$ excretion in wild-type (WT) and AC6 knockout (AC6$^{-/-}$) mice. WT and AC6$^{-/-}$ mice were fed a zero $P_i$ diet for 7 days. On day 7 urine was collected 2 hours after intravenous application of vehicle (0.85% NaCl, 2 µl/g bw) or PTH (200 µg/kg bw). As comparison urinary $P_i$/creatinine on a control diet is shown on the right side. We found that all mice developed PTH resistance because urinary phosphate/creatinine did not increase after PTH administration. Renal PTH resistance in rats$^{3-5}$ and mice$^6$ on low $P_i$ intake was described before; however, it was never clarified what caused PTH-resistance under these conditions. $n=6$/genotype, *$P<0.05$ versus WT.
Supplemental References


