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Loss of acidification of anterior prostate fluids in *Atp12a*-null mutant mice indicates that nongastric H-K-ATPase functions as proton pump in vivo

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Pestov, Nikolay B., Tatyana V. Korneenko, Mikhail I. Shakhparonov, Gary E. Shull, and Nikolai N. Modyanov. Loss of acidification of anterior prostate fluids in *Atp12a*-null mutant mice indicates that nongastric H-K-ATPase functions as proton pump in vivo. *Am J Physiol Cell Physiol* 291: C366–C374, 2006. First published March 8, 2006; doi:10.1152/ajpcell.00042.2006.—The physiological functions of nongastric (colonic) H-K-ATPase (gene symbol *Atp12a*), unlike those of Na-K-ATPase and gastric H-K-ATPase, are poorly understood. It has been suggested that it pumps Na^+ more efficiently than H^+; however, so far, there is no direct evidence that it pumps H^+ in vivo. Previously, we found that the nongastric H-K-ATPase α-subunit is expressed in apical membranes of rodent anterior prostate epithelium, in a complex with the Na-K-ATPase β_1-subunit. Here we report the effects of *Atp12a* gene ablation on polarization of the β_1-subunit and secretory function of the anterior prostate. In nongastric H-K-ATPase-deficient prostate, the Na-K-ATPase α-subunit resided exclusively in basolateral membranes; however, the β_1-subunit disappeared from apical membranes, demonstrating that β_1 is an authentic subunit of nongastric H-K-ATPase in vivo and that apical localization of β_1 in the prostate is completely dependent on its association with the nongastric H-K-ATPase α-subunit. A remarkable reduction in acidification of anterior prostate fluids was observed: pH 6.38 ± 0.14 for wild-type mice and 6.96 ± 0.10 for homozygous mutants. These results show that nongastric H-K-ATPase is required for acidification of luminal prostate fluids, thereby providing a strong in vivo correlate of previous functional expression studies demonstrating that it operates as a proton pump.

hydrogen-potassium-adenosinetriphosphatase; male accessory glands; proton transport; sorting

MAMMALIAN *Atp12a* genes (previously termed *ATP1AL1*) encode the catalytic α-subunits of the nongastric H-K-ATPases (α_{ng}, also referred to as colonic HKA or $HK\alpha_2$) (13, 36). Nongastric H-K-ATPases, together with the closely related Na-K-ATPases and gastric H-K-ATPases, comprise a large family of potassium-dependent ion-transporting P_2-type ATPases (X-K-ATPases). X-K-ATPases are heteromeric proteins composed of at least two subunits. The catalytic α-subunits, large polytopic proteins of ~110 kDa, perform ATP hydrolysis and selective ion translocation. Heavily glycosylated β-subunits, type II membrane proteins of ~35 kDa, play a crucial role in formation of the active enzyme molecule and modulation of the enzyme's substrate affinities (5, 14, 22).

From a structural point of view, α_{ng} occupies an intermediate position between α-subunits of the Na-K-ATPases and gastric H-K-ATPase. Also, nongastric H-K-ATPase does not have a specific β-subunit but employs the β_1 isoform of Na-K-ATPase (8, 31, 42). This fact is relevant to the mechanisms of cellular sorting of X-K-ATPases because H-K-ATPase and Na-K-ATPase are localized to different membranes in polarized epithelial cells. The rodent prostate epithelium is a convenient tissue for studies of the polarization of α- and β-subunits in vivo because of its relatively large cells in which α_{ng} is strictly apical and associated with the β_1-subunit, whereas the Na-K-ATPase α_1-subunit is basolateral and is associated with either the β_1- or the β_3-subunit (41, 42). Consequently, the β_1-subunit is distributed between these two membrane compartments, with expression predominantly in the apical membrane (42). One of the goals of this study was to trace the fate of the α_1- and β_1-subunits in the absence of α_{ng}, especially, to determine whether β_1 can be localized apically without binding to an α-subunit.

In contrast to Na-K-ATPase and gastric H-K-ATPase, which have a long history of extensive investigations, functional properties of nongastric H-K-ATPase, the last identified member of the X-K-ATPase family, are not yet well defined. Currently available information on catalytic and transport functions is somewhat controversial, especially with respect to the cation dependence of the enzyme. Nongastric H-K-ATPase has been characterized as a ouabain-sensitive or ouabain-insensitive H-K-ATPase (1, 9, 13, 23, 32, 36, 49), and, importantly, its selectivity for both H^+ and K^+ has been questioned in several studies (10–12, 24, 44, 56). For example, NH_4^+ was shown to cause greater activation of ATPase activity than K^+ (10, 11, 56) and, importantly, Na^+ was proposed to be the predominant counterion rather than H^+ (12, 24, 44).

Analysis of nongastric H-K-ATPase expression in mammalian tissues revealed high levels of α_{ng} mRNA in distal colon, skin, and kidney (15, 25, 37, 40). At the protein level, α_{ng} has been identified in apical membranes of epithelial cells in the distal colon (45), kidney (18, 30, 58), and, more recently, rodent prostate (41). Because it was first identified in colon and kidney, all previous functional studies have concentrated on these two tissues.

Expression of nongastric H-K-ATPase was shown to be upregulated under conditions of Na^+ depletion in colon (48) or K^+ depletion in kidney (2, 7, 19) and under conditions of

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chronic diarrhea in mice lacking the NHE3 Na/H exchanger (51). Importantly, the hypothesis that the ATPase is involved in potassium reabsorption from distal colon was tested directly by disruption of the *Atp12a* gene in mice by gene targeting. Homozygous mutant mice developed fecal potassium wasting during potassium or sodium restriction (33, 52, 54). These data demonstrated that nongastric H-K-ATPase plays an important role in the maintenance of potassium homeostasis, especially under pathophysiological conditions (for recent review see Refs. 38, 52, 53).

Although the studies mentioned above clearly show that nongastric H-K-ATPase takes part in K^+ recovery, it remains unclear whether it is also involved in H^+ pumping in vivo. Is α_{ng} able to mediate net acid secretion as occurs with the gastric H-K-ATPase, or does its activity more closely resemble that of Na-K-ATPase?

Here we took advantage of the anterior prostate (AP) as a simple model organ. This exocrine gland may be regarded as a small, closed container of secretions being formed at a relatively slow rate in nonmating males. We believed that comparison of the composition of the secretions from wild-type mice and animals with a targeted disruption of the *Atp12a* gene would be a good test of hypotheses about the in vivo functional properties of nongastric H-K-ATPase and its general physiological role. Because the volume of the stored secretions is relatively small, any changes brought about by ablation of apical H-K-ATPase were expected to be of high magnitude. Specifically, we anticipated that loss of the ATPase would result, first of all, in a significant increase in pH and, second, in an increase of potassium and ammonia and/or a decrease of sodium. We anticipated that such studies would be helpful in elucidating the operational mode of the enzyme in vivo and, therefore, its physiological functions.

MATERIALS AND METHODS

Animals. C57BL/6 mice with a targeted deletion of the nongastric H-K-ATPase α-subunit gene were described previously (33). Male mice, 4–9 mo of age, and male Sprague-Dawley rats, 6–9 mo of age, were used. Animals were euthanized according to a protocol approved by the Animal Care and Use Committee of the Medical College of Ohio by intraperitoneal injection of pentobarbital, followed by cervical dislocation.

Antibodies. Preparation of rabbit polyclonal antibodies against the NH_2-terminal domain of the α_{ng} was described previously (41). Rabbit polyclonal antibodies against the human β_1-subunit ectodomain (59) were kindly provided by Dr. Pablo Martín-Vasallo (Universidad de La Laguna, La Laguna, Tenerife, Spain).

Immunohistochemistry. Tissues were fixed in Histochoice (Amresco) overnight at 4°C, washed in PBS, 50% polyethylene glycol (PEG)-300 in PBS, incubated in 100% PEG-300 at room temperature overnight, and embedded in PEG-1450 by two overnight incubations at 55°C. Four-micrometer sections were cut and transferred onto Ultrastick slides (Fisher) by the agarose block method (21). The sections were treated with chloroform for 5 min, dried, blocked by PBS containing 0.1% Triton X-100 and 10% horse serum for 5 min, then incubated with primary antibodies in PBS, washed, incubated with secondary antibodies in PBS with 1% horse serum, washed, and embedded in Prolong reagent (Molecular Probes). Images were collected with a DMIRE2 laser scanning microscope (Leica, Mannheim, Germany).

Collection of AP secretions. Anterior lobes of the prostate (coagulating glands) were gently dissected, briefly blotted on ashless filter paper, and placed in a 1.5-ml microcentrifuge tube so that the upper end was positioned on the rim, and the tube was then closed so that the cap held the end of the gland. The tube was centrifuged at 1,000 rpm for 1 min in a microcentrifuge; the secretions were then transferred to a clean tube and centrifuged for 2 min at 16,000 *g* to remove any debris. Normally, 1–4 μl of the cleared secretions was obtained from two glands of one mature mouse and 10–60 μl from one rat.

Protein electrophoresis and protein identification. Secretions from APs were solubilized in SDS sample loading buffer (XT buffer, Bio-Rad), and protein concentration was measured by a modification of the Bradford procedure that includes coprecipitation of proteins with calcium phosphate (40). Proteins (10 μg/well) were separated by electrophoresis in bis-tris 4–12% polyacrylamide gels (Bio-Rad) and stained with colloidal Coomassie gel stain (Invitrogen). Bands of interest were cut and digested with sequencing-grade modified trypsin (Promega) overnight at 37°C. Peptides were extracted with 60% acetonitrile:0.1% trifluoracetic acid and separated on a reverse-phase column (Aquasil C18 Picofrit column, New Objectives). Eluents were directly introduced into an ion-trap mass spectrometer (LCQ-Deca XP Plus, Finnigan) equipped with a nanospray source. The mass spectrometer was operated on a double-play mode in which the instrument was set to acquire a full mass spectrometry (MS) scan (400–2,000 m/z) and a collision-induced dissociation (CID) spectrum on the most abundant ion from the full MS scan. CID spectra were searched against an appropriate nonredundant database with TurboSEQUEST.

Analyses of pH, ammonia, and various cations in secretions. pH of the secretions were measured with a microelectrode (Thermo Electron). Element analysis was carried out by inductively coupled plasma (ICP)-MS on a VG PlasmaQuad 3 device, using the hot plasma program from the Chemical Analysis Laboratory (University of Georgia, Athens, GA). We measured ammonia concentrations, using oxidation of NADPH by 2-oxoglutarate in the presence of glutamate dehydrogenase as end point decrease of absorbance at 340 nm on a Hitachi U2000 spectrophotometer under conditions essentially identical to those published by Neeley and Phillipson (39). Grouped data were compared with a two-tailed Welch's corrected *t*-test. In rare cases where the data failed the Kolmogorov-Smirnov normality test (concentrations of potassium and pH of rat AP secretions), a nonparametric Mann-Whitney test was also performed.

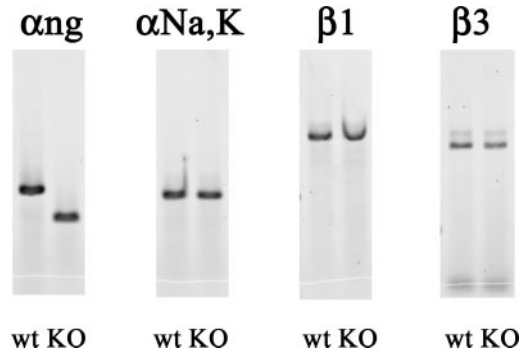

Fig. 1. Detection of transcripts of nongastric H-K-ATPase α-subunit (α_{ng}) and other X-K-ATPase isoforms in anterior prostate of *Atp12a*-knockout mice (KO). RT-PCR was performed as described previously (40–42) with primers specific for each X-K-ATPase transcript. Samples were separated by agarose gel electrophoresis and imaged with a Typhoon 8600 laser scanner (Amersham Pharmacia, Piscataway, NJ). *Lanes 1* and *2*, α_{ng} (primers complementary to exons 18 and 22); *lanes 3* and *4*, panspecific to Na-K-ATPase α_1, α_2, and α_3 isoforms; *lanes 5* and *6* and *lanes 7* and *8*, specific to Na-K-ATPase β_1 and β_3 isoforms, respectively. Anterior prostate of knockout mice has comparable levels of all detected X-K-ATPase transcripts (α_{ng}, α_1, β_1, β_3). Knockout prostate has a shortened α_{ng} product, as also occurs in colon (33, 54). WT, wild type.

RESULTS

Targeting of nongastric H-K-ATPase in mouse AP. The targeting of *Atp12a* gene by insertion of the neomycin resistance gene into exon 20 resulted in a frameshift in the transcript (33), corresponding to deletion of the COOH-terminal part of the α_{ng} protein that includes important transmembrane segments. Interestingly, the mutant transcript is present in amounts comparable to those of the wild type (Fig. 1). This indicates either that it is not efficiently degraded by RNA surveillance or that its degradation is compensated for by increased transcription. The first seems logical because the transcript is affected only in its 3′-portion and the open reading frame is still very large. Not surprisingly, levels of Na-K-ATPase α- and β-subunit mRNAs in null mutant mice appear unaffected by targeting of *Atp12a* (Fig. 1).

Immunohistochemical localization of X-K-ATPase subunit isoforms in APs of knockout mice. Nongastric H-K-ATPase α-subunit can be readily detected in mouse AP by antibodies against its NH_2-terminal domain (Fig. 2*C*), and this labeling is virtually absent from prostates of null mutant mice (Fig. 2*D*). It is not possible to elucidate the fate of the truncated α_{ng} protein because it is below the limits of detection. It may be translocated to the apical membrane and then destroyed, or its destruction may occur immediately after its synthesis.

One of the most interesting observations was the fate of the β_1-subunit, which has been shown to be the authentic partner of nongastric H-K-ATPase (8, 31, 42). Its detection in the mouse prostate turned out to be a significant problem. We tried different antibodies from several investigators and from commercial sources on both cryoembedded and paraffin-embedded sections without success (results not shown). We then fixed the

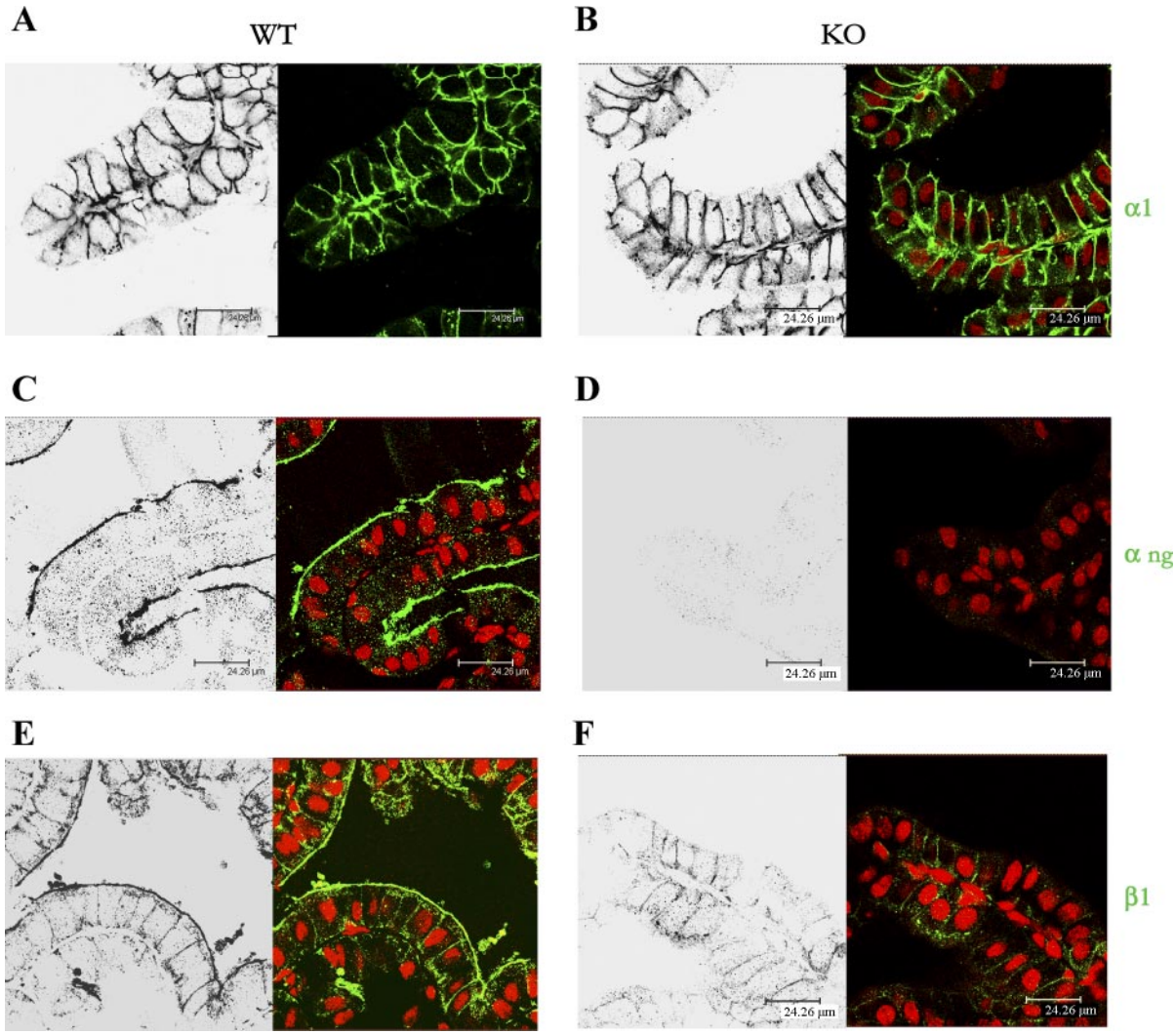

Fig. 2. Immunohistochemical detection of X-K-ATPase isoforms in knockout anterior prostate. Images were collected on a confocal microscope and are presented simultaneously as black and white negative images of green fluorescent sections (treated with antibodies labeled with Alexa Fluor 488) and color images of sections with both green fluorescence and red fluorescence (nuclei stained with ethidium bromide). *A*, *C*, *E*: wild type. *B*, *D*, *F*: knockout. *A* and *B*: labeling with anti-Na-K-ATPase α-subunit polyclonal antibodies (green). *C* and *D*: labeling with anti-α_{ng} polyclonal antibodies (green). *E* and *F*: labeling with anti-β_1 polyclonal antibodies (green).. Targeted disruption of the ATP12A1 gene results in complete loss of detectable α_{ng} protein. Distribution and expression level of Na-K-ATPase α-subunit remain unchanged. β_1 Protein disappears from apical membranes, whereas its abundance in basolateral membranes is apparently unaffected.

tissue in a proprietary solution, Histochoice, followed by embedding in PEG. With this method all but one of the available antibodies also produced no clear labeling; however, a rabbit polyclonal antibody against the recombinant ectodomain of the β_1-subunit, provided by Dr. Martín-Vasallo, gave good staining. Staining of mouse APs was similar to that observed in our previous staining of rat prostate cryosections with a monoclonal IEC antibody (41). Strong staining of the apical membrane and much weaker staining of the basolateral membrane were observed (Fig. 2*E*). It should be noted that the difference in intensities between apical and basolateral staining of mouse prostate is not as dramatic as observed previously in the rat (41). This suggests that the ratio of Na-K-ATPase on the basolateral membrane vs. nongastric H-K-ATPase on the apical membrane, each in a complex with the β_1-subunit, is higher in the rat than in the mouse.

In APs of null mutant mice, apical labeling of the β_1-subunit completely disappears and basolateral labeling appears equal to or somewhat weaker than that in wild-type prostates (Fig. 2*F*). These results provide conclusive confirmation of previous findings that apical β_1 indeed occurs in a complex with the nongastric α-subunit, thereby proving that the β_1 isoform is the authentic β-subunit of nongastric H-K-ATPase.

There are no detectable changes in labeling patterns for the Na-K-ATPase α-subunit. It resides in the basolateral compartment with apparently equal signal intensity between wild-type and knockout animals (Fig. 2, *A* and *B*). Importantly, no Na-K-ATPase α-subunit can be detected in apical membranes of the mutants; therefore, excess nascent β_1 does not drive the $\alpha_1\beta_1$ complex to apical membranes. Moreover, any unbound β_1 should be degraded, as β_1 labeling intensity is definitely not stronger in the mutant mice.

The data indicate that the β_1-subunit alone, in the absence of binding to the nongastric H-K-ATPase α-subunit, cannot be localized to the apical membrane. This observation may be also considered as an indication that the β_1-subunit does not contain strong apical localization information recognizable in the prostate epithelium.

Effect of null mutation on pH of prostate secretion. We tested the hypothesis that prostate fluid of mutant mice should have an increase in pH due to the lack of an apical proton pump encoded by *Atp12a* gene. The low amounts of secreted fluid that could be collected from mice created difficulties in their analysis, but suitable procedures were developed. The pH of the secreted fluid was directly measured with a microelectrode (Fig. 3). There was an increase in pH from 6.38 ± 0.14 in the wild type to 6.96 ± 0.10 in the knockout ($P < 0.0001$).

Also of note are the measurements of pH in secretions of ventral prostates (VPs) and APs from rat, in which the pH was 5.93 ± 0.20 in the anterior lobe and 7.24 ± 0.10 in the ventral lobe. This is in agreement with data showing that nongastric H-K-ATPase is high in the AP and completely absent in the VP. These results show for the first time that nongastric H-K-ATPase affects the acidity of a luminal fluid in vivo, consistent with previous in vitro studies indicating that it functions as a proton pump (1, 9, 13, 23, 32, 36, 49).

Other effects of null mutation on AP and its secretory function. A significant decrease in weight of APs from null mutant mice compared with those of wild-type mice is illustrated in Fig. 4. The mean AP weight and AP-to-heart ratio of mutant mice were 13.6 ± 1.6 mg and 8.7 ± 1.6%, respectively.

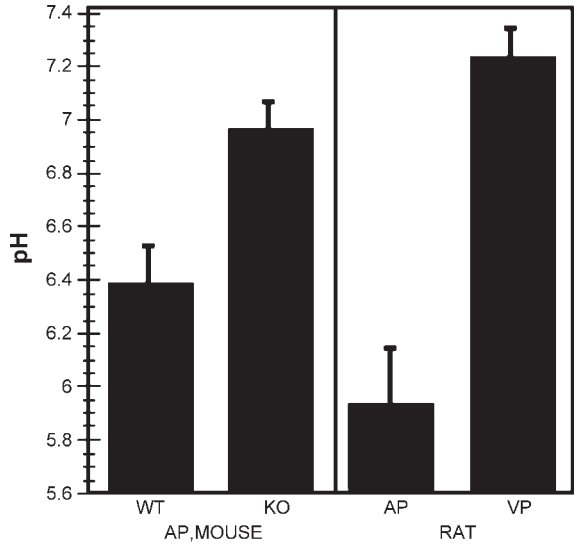

Fig. 3. pH of the prostate secretions. pH of the collected secretions cleared by centrifugation were measured directly with a microelectrode. Results are presented as means ± SD; $n = 8$ for all groups. Rat prostate lobes show a significant difference: anterior prostate (AP), which expresses nongastric H-K-ATPase, has an acidified fluid, whereas fluid of the ventral lobe (ventral prostate, VP), which lacks the ATPase, is neutral. Secretion of mouse anterior prostate (WT) is also acidified, as in the rat, but to a lesser extent. Nongastric H-K-ATPase α-subunit-null mutants (KO) have anterior prostate secretions with a significantly higher pH, showing that loss of α_{ng} causes loss of acidification.

These values were significantly lower than those of wild-type mice (20.5 ± 3.8 mg and 15.2 ± 5.6%, respectively; $P < 0.001$). Also, we were usually able to collect approximately twice as much fluid from wild-type prostates as from mutant prostates. The precise amount of secreted fluid, however, was extremely difficult to measure accurately because the collection procedure causes some destruction of the gland, so we can only speculate that the decrease in weight is caused, at least partially, by hyposecretion. On the other hand, the APs of null mutant mice appear to lack any readily observable histological changes (results not shown).

The relative simplicity of the AP secretion allowed us to analyze its protein composition by one-dimensional gel electrophoresis (Fig. 5). There were no significant differences in the intensities of two major protein bands, dorsal prostate-I [DP-I; identified as transglutaminase (17, 27, 61)] and dorsal prostate-II [DP-II; reported as either transglutaminase substrate kinesin heavy chain-like protein (17) or IgFc binding protein (IGFcBP, FcγBP) (61)]. Some high-molecular-weight bands with increased intensities were observed in the null mutants, and one of them (160 kDa) has been identified as IGFcBP.

Concentrations of major inorganic cations (Na, K, Mg, Ca, Zn) were determined with ICP-MS, which has a number of advantages, including high sensitivity and ability to evaluate several elements simultaneously (Fig. 6). Also, Ni, Al, and Mn were analyzed, and these elements were found to be below the limits of detection (<1 μM). For all determined elements except Zn, no significant changes were found. Mean values of Mg were 0.95 ± 0.37 and 1.46 ± 0.79 mM for wild-type and mutant mice, respectively ($P = 0.20$). A similar situation was observed in the case of Ca: 7.63 ± 2.31 and 6.79 ± 1.06 mM for wild-type and mutant mice, respectively ($P = 0.44$). Un-

expectedly, very high variances were observed in the case of Na and K. Values of Na had very close means, 42.3 ± 22.0 mM for wild type and 49.4 ± 31.7 mM for null mutant, and this difference is not significant ($P = 0.65$).

Measurements of K were highly variable between individual samples, and an apparent difference (30.2 ± 12.0 mM in mutant and 20.0 ± 9.7 mM in wild type) in their mean values was not statistically significant ($P = 0.49$). These data can neither confirm nor reject the hypothesis of direct influence of nongastric H-K-ATPase on K level because of the relatively high K concentrations found in AP secretions of wild-type mice (20 mM). This hypothesis would be adequately tested only if the mouse secretions had much lower K concentrations, for example, close to those measured in the case of the golden hamster (2 mM) (6). Only a severalfold increase in mutant animals could be considered to be reasonable evidence.

An increase in Zn concentration in secretions from mutant mice (23.7 ± 6.8 μM for wild type and 51.7 ± 20.3 μM for mutant animals) was statistically significant ($P < 0.02$). This unexpected observation has no readily apparent explanation.

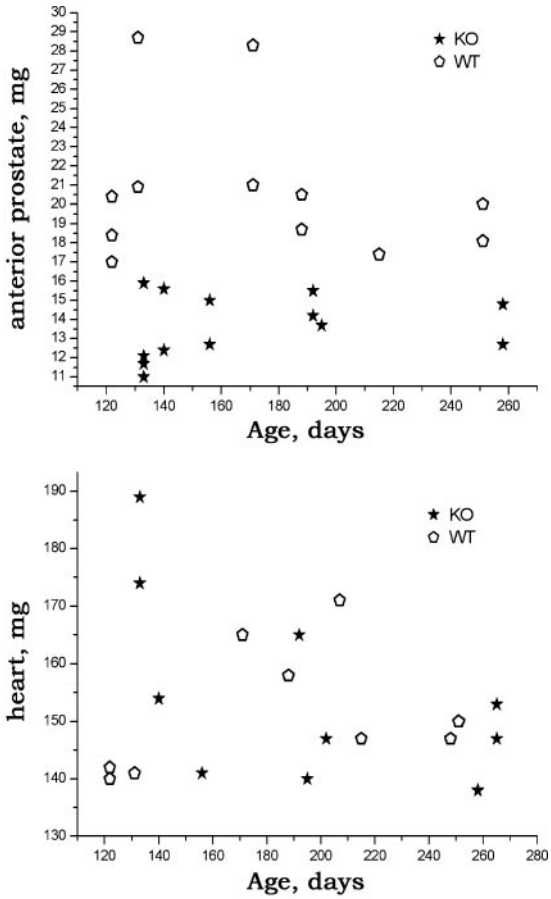

Fig. 4. Comparison of anterior prostate weights from knockout and wild-type animals. *Top*: single anterior prostates. *Bottom*: hearts. Anterior prostates were weighed before fluid collection. Results are presented as data from individual measurements of wet weights of the organs with respect to age of the animals. Weights of both anterior prostates and hearts do not change significantly in the interval of ages of the animals used. Average heart weights (control organ) of the knockout animals are unchanged. Anterior prostates of the mutant mice are significantly smaller.

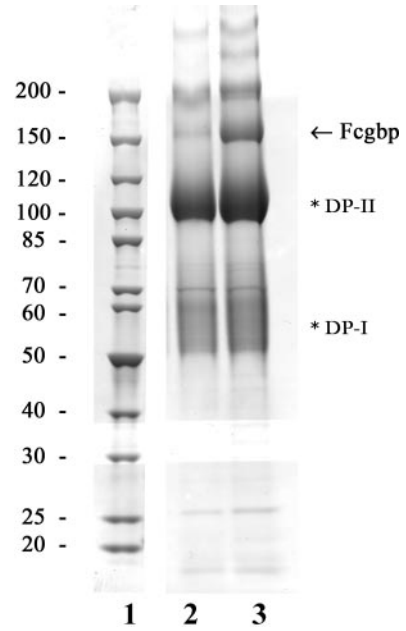

Fig. 5. Electrophoretic analysis of protein composition of anterior prostate secretion. Proteins from the secretions were separated in bis-tris 4–12% polyacrylamide gels and stained with a colloidal Coomassie stain. Protein bands corresponding to major protein constituents, dorsal prostate-I (DP-I) and dorsal prostate-II (DP-II), are indicated by asterisks. Position of the band that was subjected to protein identification (IgGFc binding protein, FcγBP) is indicated by arrow. This protein may have many different variants of different molecular masses, ranging as high as 500 kDa; here its electrophoretic mobility corresponds to a molecular mass of 150 kDa. *Lane 1*, Molecular mass standards; *lane 2*, wild type; *lane 3*, knockout.

Ammonia in the secretions was measured by an enzymatic reaction (Fig. 7). Again, data for AP secretions showed very high variability. Importantly, such variability between individual AP samples cannot be attributed entirely to experimental error because it was much higher in AP secretions than in VP secretions. This is readily seen in Fig. 7, where rat AP and VP have similar mean values but very different deviations (520 ± 380 μM for AP and 454 ± 77 μM for VP). Mean values of ammonia in mouse secretions were significantly higher than in the rat. Comparison of wild-type and knockout mice showed that differences in their mean values were not statistically significant (1,625 ± 1,008 μM for wild type and 1,527 ± 815 μM for mutant; $P = 0.86$). Hence, no differences in secreted ammonia were found in wild-type and knockout mice.

DISCUSSION

We hypothesized that the loss of active H-K-ATPase activity should have a significant effect on pH of the secreted fluid and on various ions that may be coupled to the transport of H^+. For studies of nongastric H-K-ATPase, the anterior lobe is the best region of the prostate to use because it is relatively easy to extract and it has the highest content of nongastric H-K-ATPase. Although the amount of collectable secretions is very small, it is technically possible to measure many parameters such as certain ions and pH.

Results of pH measurements demonstrated that, first, rodent AP secretions are mildly acidified (pH 6.4 in the mouse and 5.9 in the rat) and, second, loss of nongastric H-K-ATPase leads to

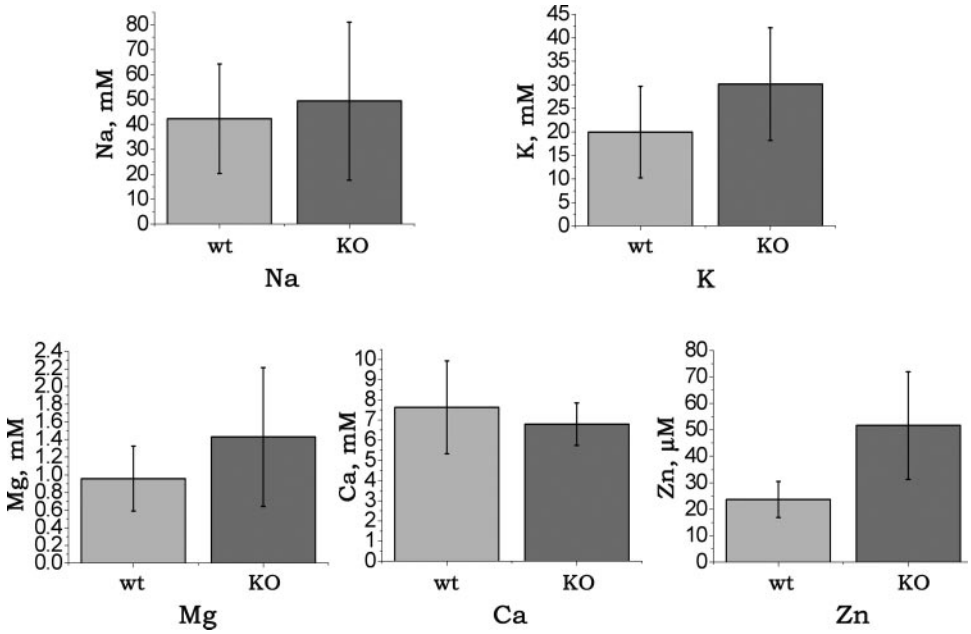

Fig. 6. Inductively coupled plasma mass spectrometry (ICP-MS) determination of some cation elements in prostate secretions. Element analysis of the collected secretions cleared by centrifugation was carried out by ICP-MS. Concentrations were calculated with the assumption that the density of mouse anterior prostate secretion is equal to that of the rat (1.09). For all elements except K, $n = 6$ for wild type and $n = 7$ for knockouts. For K, $n = 4$ for both wild-type and knockout animals. Results are presented as means ± SD. Differences of mean values for Na, K, Ca, and Mg concentrations between wild-type and mutant mice are not statistically significant. The concentration of Zn is significantly higher in anterior prostate fluid of the mutant mice.

a significant increase in pH; in fact, the pH of the secreted fluid was neutralized (7.0). This phenomenon is completely consistent with the hypothesis that nongastric H-K-ATPase operates as a proton-extruding pump in vivo. On the other hand, the physiological importance of this acidification is difficult to understand: is it a by-product of the K^+-pumping activity or is the ultimate role of H-K-ATPase to acidify the secreted fluid in the lumen of the prostate? It is worth noting that this acidification occurs only in some prostate lobes and that the relatively low volume of AP secretion constitutes only a small portion of the total ejaculate and thus cannot determine its pH. More importantly, it should be noted that prostate fluids in other species, such as humans, are alkaline, and that nongastric H-K-ATPase-deficient male (and female) mice are fertile.

A major function of the AP in rodents is believed to be that of supplying the cross-linking enzyme transglutaminase (62). This enzyme acts on protein substrates that are contained mostly in secretions of the vesicular gland, which are much larger than those of the AP. On copulation, transglutaminase contributes to formation of the vaginal copulatory plug, which is an important means of male competition (46). This interesting mechanism of evolution is present in most rodents but is not common among other mammals. It seems reasonable to hypothesize that a slightly shifted pH may be optimal for storing the fluid, and its enzymes, before mixing with other secretions.

Protein composition of AP fluids in the mutants is not significantly different from that of the wild type (Fig. 5), including the DP-I band that corresponds to secreted tranglutaminase (Fig. 5). On the other hand, intensification of the 160-kDa band was observed and this band was identified as IGFcBP. IGFcBP is rather likely to be the true molecular entity of the DP-II band (61), the major protein component of rat and mouse AP fluid. Therefore, the strong 160-kDa band in the mutants is a different variant of IGFcBP. IGFcBP is a mucin-like protein of largely unknown function (26) and uncharacterized diversity. It was hypothesized to be the major stabilizing protein of apocrine blebs (aposomes), vesicles that serve as transglutaminase stores (61). Following this logical sequence, the increase in complexity of IGFcBP species in the *Atp12a*-mutant mice may constitute an adaptive reaction to the loss of fluid acidification, which, in its turn, may be required for fine adjustment of apical secretion in normal AP epithelium.

The remarkable increase of pH observed should be safely regarded as strong evidence in favor of proton pumping by the

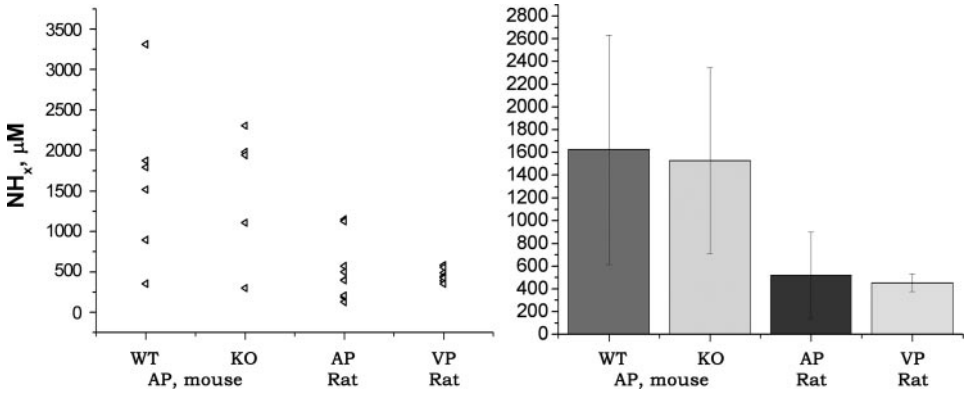

Fig. 7. Determination of ammonia concentration in prostate secretions. Ammonia concentrations in the collected secretions cleared by centrifugation were measured spectrophotometrically using oxidation of NADPH by 2-oxoglutarate in the presence of glutamate dehydrogenase. Results are presented as data from individual measurements (*left*) and as means ± SD (*right*). For mouse secretions, $n = 6$; for rat secretions, $n = 9$. Secretions of anterior prostates show a high degree of variability between individual animals. Variability for ventral prostate is much lower, although mean values are similar for both anterior and ventral prostates. Rat secretions have lower ammonia concentrations than those from mouse. Mean values for AP secretions are approximately equal in both wild-type and mutant mice.

ATPase in vivo. However, the data must be interpreted with caution. A difference in the observed concentrations does not necessarily mean that a particular element is transported by the ATPase. Indeed, adaptation to the absence of the ATPase may be a very complex phenomenon and various compensatory mechanisms may exist. This is especially true in the case of Zn, which is significantly higher in prostate secretions of the *Atp12a* knockouts. Regulation of Zn transport is very complex (20), and, moreover, most of the secretion's Zn is bound to various proteins. The total Zn measured in our case may, in fact, reflect an increase in Zn sequestration.

Also, even if no statistically significant changes were observed, this does not necessarily mean that the enzyme does not transport a particular cation. For example, AP fluid of the golden hamster was reported to have a K concentration that was half that of plasma (6). From this, we expected that loss of H-K-ATPase would result in a sharp increase in the K concentration of the secreted fluid, confirming the hypothesis that H-K-ATPase is responsible for K absorption from this fluid. However, mouse AP secretions were found to contain relatively high K concentrations, without a significant difference between wild-type and mutant mice. Therefore, the mouse is not a convenient species for studies of H-K-ATPase-mediated K transport in the prostate. With these considerations, we should conclude that no evidence either supporting or rejecting the hypothesis has been found during the comparison of K concentrations in wild-type and knockout mice.

Apparently identical mean values for ammonia concentrations in wild-type and knockout prostate secretions indicate that there is no significant change. However, a decrease in the ammonia content should be expected as a result of increasing pH in the mutants and a corresponding loss of NH_4^+ trapping. However, the decrease in trapping may be balanced by the failure to remove NH_4^+ from the fluid by H-K-ATPase, which may be responsible for the lack of even higher ammonia in the acidified wild-type secretion. Indeed, the mean values for the ammonia concentrations of rat AP and VP fluids are very similar despite the remarkable difference in their pH values. Soleimani and colleagues (38, 53) hypothesized that activity of nongastric H-K-ATPase in vivo may contribute to net NH_4^+ secretion. This, however, seems unlikely from a mechanistic point of view because NH_4^+ was shown to be a good substitute for K^+ as an activator of ATPase activity (10, 56, 60). Alternatively, the fact that high- and low-ammonia fluids were found in all AP groups may indicate that other mechanisms for regulation of secreted ammonia in prostate fluids overwhelm the effect of nongastric H-K-ATPase.

The apical or basolateral sorting of membrane proteins is differentially regulated in various cells and tissues, and these differences can be substantial. For example, certain cell lines exhibit atypical polarization of X-K-ATPases: nongastric H-K-ATPase may be targeted laterally (47), or sugar chains on the β-subunit may encode an apical signal (57). Our results show that polarization of X-K-ATPases in mouse AP epithelium is very typical, with apical H-K-ATPase and basolateral Na-K-ATPase, and this pattern is dependent on the α-subunit. May our results also be interpreted so that β_1 does not contain an apical sorting signal?

In heterologous systems, only coexpression of α- and β-subunits leads to the formation of active Na-K- and H-K-ATPases and allows their successful translocation to the plasma membrane. The lack of a β-subunit results in degradation of the free α-subunit. Similarly, it was shown previously that when the β_1-subunit was expressed without the Na-K-ATPase α-subunit in *Xenopus* oocytes, it was retained in the endoplasmic reticulum, associated with the chaperone protein BiP, and slowly degraded (3, 22, 28). In contrast, the β_g-subunit expressed alone in *Xenopus* oocytes or in the MDCK or LLC-PK renal epithelial cell lines escapes endoplasmic reticulum quality control and is routed to the plasma membrane (22, 47). Much less is known about the fates of individual subunits of X-K-ATPases in vivo when their counterparts are absent. Na-K-ATPase and nongastric H-K-ATPase have not been studied previously in this respect. The only example is represented by studies of gastric H-K-ATPase (29, 34, 50, 55). It was shown that knockout of its α-subunit gene did not abolish synthesis and accumulation of the β-subunit. This situation is different from that in prostate, where any unbound β_1 seems to be completely degraded. Indeed, isoform- and cell type-specific differences in degradation rates may be quite large.

Absence of any apical $\alpha_1\beta_1$ in both wild-type and α_{ng}-knockout mice may be explained by lack of a strong apical sorting signal in β_1 structure or, alternatively, by efficient degradation of mistargeted α_1 in the apical compartment. However, the first possibility appears more reasonable and better supported experimentally. Indeed, certain sorting signals have already been mapped in X-K-ATPase α-subunits, and their targeting can be successfully altered by chimerization (16). Moreover, apical α_1 was found in a number of tissues, including human prostate (35). However, β-dependent sorting has been well documented in only a single system in vitro (57). Therefore, mouse AP epithelium is a good example of the most common situation in vivo: only α-subunits contain structural determinants that serve as strong apical or basolateral sorting signals.

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