Molecular Regulation and Physiology of the H⁺,K⁺-ATPases in Kidney

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Two H⁺, K⁺-adenosine triphosphatase (ATPase) proteins participate in K⁺ absorption and H⁺ secretion in the renal medulla. Both the gastric (HKα₂) and colonic (HKα₂) H⁺,K⁺-ATPases have been localized and characterized by a number of techniques, and are known to be highly regulated in response to acid-base and electrolyte disturbances. Both ATPases are dimers of composition α/β that localize to the apical membrane and both interact with the tetraspanin protein CD63. Although CD63 interacts with the carboxy-terminus of the α-subunit of the colonic H⁺,K⁺-ATPase, it interacts with the β-subunit of the gastric H⁺,K⁺-ATPase. Pharmacologically, both ATPases are distinct; for example, the gastric H⁺,K⁺-ATPase is inhibited by Sch-28080, but the colonic H⁺,K⁺-ATPase is inhibited by ouabain (a classic inhibitor of the Na⁺-pump) and is completely insensitive to Sch-28080. The α-subunit of the colonic H⁺,K⁺-ATPase is the only subunit of the X⁺,K⁺-ATPase superfamily that has 3 different splice variants that emerge by deletion or elongation of the amino-terminus. The messenger RNA and protein of one of these splice variants (HKα₂) is specifically up-regulated in newborn rats and becomes undetectable in adult rats. Therefore, HKα₂, in addition to its role in potassium and acid-base homeostasis, appears to play a significant role in early growth and development. Finally, because chronic hypokalemia appears to be the most potent stimulus for upregulation of HKα₂, we propose that the HKα₂ participates importantly in the maintenance of chronic metabolic alkalosis.

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The intercalated cells (IC) of the collecting tubule are responsible for regulation of urinary acidification, and therefore play a central role in maintaining acid-base homeostasis. Two types of intercalated cells have been recognized: type A and type B intercalated cells. Type A intercalated cells secrete protons into the lumen of the tubule, thereby acidifying the urine. Stochiometrically, for each proton secreted, one bicarbonate ion is returned to systemic circulation, thereby acidifying the urine. Stochiometrically, for each proton secreted, one bicarbonate ion is returned to systemic circulation, thereby acidifying the urine. Stochiometrically, for each proton secreted, one bicarbonate ion is returned to systemic circulation, thereby acidifying the urine. Therefore, HKα₂, in addition to its role in potassium and acid-base homeostasis, appears to play a significant role in early growth and development. Finally, because chronic hypokalemia appears to be the most potent stimulus for upregulation of HKα₂, we propose that the HKα₂ participates importantly in the maintenance of chronic metabolic alkalosis.

**Colonic H⁺,K⁺-ATPase**

The colonic H⁺,K⁺-ATPase assembles and functions as an α/β heterodimer. The α-subunit, hereafter designated as HKα₂, was cloned by Crowson and Shull from a rat distal colon complementary DNA library. Therefore, it is often referred to as the colonic H⁺,K⁺-ATPase α-subunit. In addition to expression in distal colon, HKα₂ also is expressed at low levels in other tissues, including kidney, where it is highly regulated.1,2
HKα2 has a molecular weight of approximately 100 kDa, and in common with the other members of the X,K+-ATPase family, is predicted to span the plasma membrane 10 times and has intracellular carboxy and amino termini (http://www.ch.embnet.org/software/TMPRED_form.html). HKα2 also contains binding domains for ouabain, is responsible for potassium/hydrogen exchange, and requires association with a β-subunit for functionality (ATPase enzymatic activity and transport function).

Pharmacologic and Transport Properties of Colonic H⁺,K⁺-ATPase

Our laboratory has shown that rat HKα2 supports ⁸⁶Rb⁺-uptake when expressed in *Xenopus laevis* oocytes in a β-subunit–dependent manner. ⁸⁶Rb⁺-uptake was insensitive to Sch-28080 (10 μmol/L) but partially sensitive to ouabain (IC₅₀ ~ 250 μmol/L). Cougnon et al. showed, using a similar approach, that HKα2 functions as an H⁺/K⁺ and Na⁺/K⁺ exchanger, and is sensitive to ouabain. A similar observation was made by Grishin et al. by cotransfecting HEK-293 cells with human ATP1AL1 (assumed to be the homolog of rat HKα2) and the β-subunit of the rabbit gastric H⁺,K⁺-ATPase. The transport activity of the expressed pump was more efficient in transporting sodium than protons at a coupling ratio of approximately 10 to 1. To show that HKα2 can function as a Na⁺-pump it would be necessary to stably transfet HEK-293 cells with rat HKα2 and to select transfected cells in the presence of low concentrations of ouabain because the endogenous Na⁺-pump of HEK-293 is very sensitive to low concentrations of ouabain. However, an experiment of this type has not been reported to date.

Splice Variants of HKα2

Alignment of the amino acid sequences of the α₁-₁, α₂-₂, and α₃-subunits of Na⁺,K⁺-ATPase, the gastric H⁺,K⁺-ATPase α-subunit (HKα₁), and HKα₂ (PileUp program from SeqWeb (Accelrys, San Diego, CA) reveals a high degree of sequence similarity. Although splice variants have not been identified for any of the -subunits of the Na⁺-pump or for HKα₁, splice variants for HKα₂ have been reported. Kone and Higham were the first to identify a splice variant of HKα₂ that was truncated by 108 amino acids at the amino-terminus. This variant, HKα₂β₁, was expressed in HEK-293 cells and displayed pharmacologic properties identical to HKα₂. A second splice variant of HKα₂ was identified by Campbell et al. in rabbit renal medulla. HKα₂C encodes a 61-residue amino-terminal extension to rabbit HKα₂ and has not been expressed in heterologous systems thus far.

Immunolocalization of HKα₂

HKα₂ messenger RNA (mRNA) and protein are expressed at low levels in the renal medulla but abundantly in the distal colon. A rabbit polyclonal antibody increased against the sequence of rat HKα₂ that extends from amino acid 686 to 698 was developed by our laboratory. Gallardo et al. used this antibody to show by immunolocalization that HKα₂ protein is expressed in the apical membrane of the distal colon (Fig 2).

Verlander et al. used a chicken HKα₂C-specific polyclonal antibody in immunolocalization experiments to show that...
HKα2, is expressed in the apical membranes of type A IC, type B IC, and principal cells. These findings are compatible with the observation11 that HKα2 also was immunolocalized to rat principal cells. The physiologic significance of localization in principal cells has not been elucidated completely, however, because these cells have not been viewed as participants in urinary acidification.

In addition to expression in kidney and distal colon, HKα2 also is expressed abundantly in prostate. Pestov et al.18 showed HKα2 protein in the apical membrane of rat anterior prostate, where it colocalizes with β1-Na⁺,K⁺-ATPase (NKβ1).19

β1-Na⁺,K⁺-ATPase is the Physiologic β-Subunit for HKα2

The different α-subunits of Na⁺,K⁺-ATPase and HKα1 require assembly with a specific β-subunit for protection from degradation, trafficking out of the endoplasmic reticulum (ER) to the plasma membrane, and for function. Although a unique β-subunit has not been identified for HKα2, immunoprecipitation experiments performed by Kraut et al20 and by our laboratory21 have revealed that HKα2 co-immunoprecipitates with NKβ1. These data are consistent with the observation that NKβ1 is expressed in the apical membrane of the distal colon15 and rat anterior prostate.16 These results also are consistent with the observation that NKβ1 is more efficient than NKβ2 (also expressed in kidney and distal colon) in supporting 86Rb⁺-uptake when cotransfected with HKα2 in HEK-293 cells (HKα plus NKβ1 versus HKα2 plus NKβ2).15 By addition of enhanced green fluorescent protein to the amino terminus of HKα2, we also showed that NKβ1 is more efficient than NKβ2 in the translocation of HKα2 to the plasma membrane.15 Therefore, abundant evidence supports the view that β1-Na⁺,K⁺-ATPase is the physiologic β-subunit for HKα2 in kidney, colon, and prostate.

Molecular Regulation of HKα2 mRNA and Protein Expression

Studies performed in several laboratories consistently have shown that HKα2 mRNA and protein expression increases in the renal medulla during chronic potassium depletion.13,22 This finding supports the view that the colonic H⁺,K⁺-ATPase plays a central role in potassium conservation. However, this interpretation has been challenged by the absence of the expected phenotype in the HKα2−/− deficient mouse model. The reason is to say that HKα2−/− mice developed normally and did not display easily discernable abnormalities in potassium homeostasis. Nevertheless, with dietary potassium deprivation HKα2−/− mice developed a lower plasma K⁺ and displayed inappropriate and persistent fecal and urinary K⁺ wasting. Based on knowledge of the means by which the HKα2−/− deficient mouse model was generated,23 it would be predicted that if a mutated HKα2 protein were synthesized in HKα2−/−, the last 84 amino acids at the carboxy-terminus should be truncated. Because these mice did not display the anticipated phenotype, we considered the possibility that the carboxy-terminus of HKα2, although absent in the HKα2−/− mouse, might not be required for functionality. To determine whether the terminal 84 amino acids were critical for functionality, we created a deletion mutation of HKα2 that truncated these terminal amino acids (ΔHKα2). This mutation, which was expressed in HEK-293 cells, assembled poorly with NKβ1, and degraded more rapidly. Moreover, in keeping with these findings, the addition of enhanced green fluorescent protein to the amino-terminus of HKα2 showed that the protein failed to migrate to the plasma membrane efficiently. Therefore, we concluded that the Menetion mutation was indeed nonfunctional. These arguments opened the possibility that alternative potassium transporters might compensate for the absence of HKα2 in the HKα2−/− deficient mouse model, or that other potassium-retaining transporters might be upregulated.

Association of CD63 With the Carboxy-Terminus of HKα2

In an attempt to identify potential modifier proteins that might interact with the HKα2 carboxy-terminus, we subsequently used this 84 amino acid sequence to screen a mouse kidney complementary DNA library, using the yeast 2-hybrid method.24 A tetraspanin protein, CD63, was identified and shown by co-immunoprecipitation to assemble with the HKα2 carboxy-terminus. A current model of HKα2/NKβ1/CD63 interaction is displayed in Figure 3. By specifically suppressing the expression of endogenous CD63 by small interfering RNA (siRNA) in transiently transfected HEK-293 cells, we showed in CD63 knockdown cells that HKα2/NKβ1, migrated more efficiently to the plasma membrane, and 86Rb⁺ uptake was significantly greater than in cells expressing CD63 protein.24 From this finding it appears reasonable to conclude that CD63 interacts with HKα2/NKβ1 to regulate endocytosis from the cell surface. This view is consistent with the findings of Duffield et al.25 In these studies it was shown that CD63 assembles with the β-subunit of the rabbit gastric H⁺,K⁺-ATPase. These investigators concluded that CD63 participates importantly in the regulation of the trafficking of the gastric H⁺,K⁺-ATPase. Therefore, these 2 studies, when taken together, suggest a previously unappreciated protein-trafficking role for the tetraspanin protein, CD63. In our studies CD63 appears to function additionally as a suppressor protein.

Potential Role for HKα2 in Growth and Development

Postnatal growth is associated with an increase in total body potassium.20 Over the first 15 days of life a newborn rat will increase in body weight from approximately 1 to about 10 to 15 g. This means that during a short period the newborn rat must increase its total body potassium 10- to 15-fold. Potassium is derived from maternal milk; the kidneys and, to a lesser extent, the distal colon play a critical role in minimizing K⁺ excretion. Therefore, growth requires a state of avid potassium conservation. Net positive potassium balance is achieved by 2 mechanisms: secretion is reduced,27-29 and
and carboxy termini. The putative 10 transmembrane domain of HKα2 (thin continuous line) represents a membrane-spanning protein (T1-T10) with cytosolic amino and carboxy termini. The β-subunit (discontinuous thick line) extends once across the cell surface. The amino-terminus is cytosolic and the carboxy-terminus is extracellular. The tetraspanin CD63 is represented by branches. The model proposes that the β-subunit interacts with the extracellular domain of HKα2 between T7 and T8 and the carboxy-terminus of CD63 interacts with the carboxy-terminus of HKα2.

Metabolic Alkalosis

Because chronic hypokalemia is a frequent accompanying feature of chronic metabolic alkalosis, it is likely that upregulation of HKα2 may be an important participant in the maintenance phase of metabolic alkalosis. In addition to enhanced urinary acidification, chronic hypokalemia also participates importantly in the maintenance of metabolic alkalosis through increased production and excretion of ammonium. This combined effect of chronic hypokalemia, upregulation of HKα2, and ammonium production and excretion, would greatly augment net acid excretion during the maintenance phase of chronic metabolic alkalosis, and persist until the hypokalemia was corrected.

Gastric H⁺,K⁺-ATPase

The gastric H⁺,K⁺-ATPase is abundant in gastric acinar cells and is responsible for gastric acid secretion. The functional protein is a heterodimer composed of α/β subunits. The α-subunit (HKα1) internalizes potassium in exchange for hydrogen and is inhibited by low concentrations of Sch-28080 and omeprazole, but is insensitive to ouabain. The unique β-subunit (HK βG) is heavily glycosylated and has a molecular weight of approximately 35 kd. Both HKα1 and HKβG protein and mRNA are expressed in the kidney, but much less abundantly than in the stomach.

Renal Regulation of HKα1

Although expressed in renal cortex and medulla, HKα1 protein, unlike HKα2, is not upregulated during chronic hypokalemia. Nevertheless, others have shown by in situ hy-

**Pathophysiologic Role of HKα2**

Distal Renal Tubular Acidosis (dRTA)

The colonic H⁺,K⁺-ATPase has been implicated in several pathophysiologic conditions. First, there was 1 infant reported with severe chronic hypokalemia and a hyperchloremic metabolic alkalosis as well as other typical clinical features of classic distal renal tubular acidosis. The investigators suggested that abnormalities of the colonic H⁺,K⁺-ATPase might be the explanation for the severe hypokalemia and renal acidification defect. To sustain growth and development, large amounts of potassium and bicarbonate supplementation were required. Nevertheless, a specific defect in HKα2 has not been proven to exist in this or other forms of classic distal RTA associated with hypokalemia. Whether an endemically form of classic distal RTA associated with striking hypokalemia, as reported in northeastern Thailand, represents failure of adaptation of HKα2 is possible, but has not been proven unequivocally.

![Proposed model for membrane localization of the HKα2/HKβ1/CD63 complex. HKα2 (thin continuous line) is represented as a membrane-spanning protein (T1-T10) with cytosolic amino and carboxy termini. The β-subunit (discontinuous thick line) extends once across the cell surface. The amino-terminus is cytosolic and the carboxy-terminus is extracellular. The tetraspanin CD63 is represented by branches. The model proposes that the β-subunit interacts with the extracellular domain of HKα2 between T7 and T8 and the carboxy-terminus of CD63 interacts with the carboxy-terminus of HKα2.](image)

![Gastric H⁺,K⁺-ATPase](image)

![Renal Regulation of HKα1](image)
bridization that HKα1 mRNA abundance in the renal cortex increases during chronic hypokalemia. Ahn et al\textsuperscript{31,42} showed that chronic potassium restriction results in modestly enhanced renal cortical expression of HKα1 and suggested that this isoform may participate in potassium conservation by the connecting tubule (CNT) and cortical collecting duct during potassium deprivation. This contrasts with HKα2 mRNA, which is preferentially upregulated in the renal medulla, as is HKα2 protein.\textsuperscript{2} In addition, in isolated inner medullary collecting tubules, Wall et al\textsuperscript{43} showed that J\textsubscript{CO2} increased in tubules harvested from chronically hypokalemic rats. A component of bicarbonate absorption was inhibited by low concentrations of Sch-28080, suggesting that HKα1 or a HKα1-type ATPase was upregulated during these experimental conditions. The residual component of bicarbonate absorption (~20%) was inhibited by high concentrations of ouabain, suggesting that the ouabain-sensitive fraction of J\textsubscript{CO2} could be attributable to HKα2.\textsuperscript{44,45}

**Enzymatic Activities of HKα1- and HKα2-Deficient Mouse Models**

For a number of years Doucet and Barlet,\textsuperscript{46} Buffin-Meyer et al.\textsuperscript{47} and Cheval et al\textsuperscript{48} have studied the properties of different K\textsuperscript{+}-ATPase activities in isolated nephron segments. This group defined 3 distinct types of K\textsuperscript{+}-ATPase activities according to pharmacologic response and augmentation in response to chronic hypokalemia: types I, II, and III. According to the nomenclature adopted by these investigators, K\textsuperscript{+}-ATPase type I is sensitive to Sch-28080 and insensitive to ouabain, and is present in collecting ducts predominately; K\textsuperscript{+}-ATPase type II is ouabain sensitive and is localized to proximal tubules and thick ascending limbs; K\textsuperscript{+}-ATPase type III is observed only during chronic hypokalemia in the collecting duct and is sensitive to ouabain and Sch-28080. To determine whether the molecular entity responsible for rat K\textsuperscript{+}-ATPase type III is HKα1, HKα2, or an unknown new α-subunit, Dherbecourt et al\textsuperscript{49} evaluated K\textsuperscript{+}-ATPase activity in the absence or presence of ouabain or Sch-28080 from tubules harvested from wild-type mice or HKα1,\textsuperscript{50} or HKα2-deficient mice.

Collecting ducts isolated from wild-type mice (expressing HKα1 and HKα2 protein) with a normal plasma potassium level displayed K\textsuperscript{+}-ATPase activity that was inhibited by Sch-28080 but not by ouabain. However, the K\textsuperscript{+}-ATPase activity in collecting ducts harvested from mice maintained on a low-potassium diet was inhibited by both ouabain and Sch-28080.

In the second set of experiments, HKα1-deficient mice (assumed to express HKα2 protein) were maintained on a diet containing potassium and displayed no K\textsuperscript{+}-ATPase activity in collecting ducts. However, in chronically hypokalemic HKα1-deficient mice, K\textsuperscript{+}-ATPase activity was inhibited by Sch-28080 and by ouabain. Therefore, although HKα1 protein is not expressed, a Sch-28080-sensitive component was observed. This observation is in agreement with the findings of these investigators that HKα2 is ouabain and Sch-28080 sensitive.

Finally, these investigators performed similar experiments using HKα2-deficient mice (assumed to express HKα1 protein). When consuming a diet containing potassium, K\textsuperscript{+}-ATPase activity was inhibited in HKα2-deficient animals by Sch-28080 but not by ouabain. This observation is consistent with expression of HKα1 protein in HKα2-deficient mice. However, during chronic hypokalemia no H\textsuperscript{+}, K\textsuperscript{+}-ATPase activity was observed, compatible with findings in our laboratory that HKα1 does not show a regulatory response to chronic hypokalemia.

Taken together, the experiments performed with wild-type, HKα1-, and HKα2-deficient mice support the view that chronic hypokalemia upregulates expression of HKα2 protein that in some conditions is sensitive to both ouabain and Sch-28080 (type III K\textsuperscript{+}-ATPase).

The findings of Doucet et al\textsuperscript{23,46,50} also are compatible with previous studies from our laboratory\textsuperscript{43} and from others\textsuperscript{31} in the field who observed an increase in J\textsubscript{CO2} in the collecting duct during chronic potassium depletion. Furthermore, the increment in J\textsubscript{CO2} was sensitive to both Sch-28080 and ouabain.\textsuperscript{43}

The observation that Sch-28080-sensitive K\textsuperscript{+}-ATPase was not present in HKα2-deficient mice during chronic hypokalemia suggests that chronic potassium depletion may downregulate expression of HKα1 in HKα2-deficient mice. Quantification of HKα1 mRNA and/or protein in potassium-deficient HKα2-deficient mice has not been reported.

**Summary**

HKα2 plays a critical role in potassium and acid-base homoeostasis through regulated H\textsuperscript{+}/K\textsuperscript{+} exchange in intercalated cells of the mammalian collecting duct. HKα2 assembles specifically with NKβ1, and is inhibited by high concentrations of ouabain. Although insensitive to Sch-28080 in vitro, there is evidence that HKα2 may be sensitive to Sch-28080 in vivo. These features are unique among members of the X\textsuperscript{+}, K\textsuperscript{+}-ATPase family. Furthermore, the molecular regulation of HKα2 appears to depend on assembly with the tetraspanin CD63. In addition to participation in potassium and acid-base homeostasis, HKα2 participates uniquely in growth and development in the early postnatal period. Although specific examples of genetic forms of distal renal tubular acidosis have not established an abnormality in H\textsuperscript{+}, K\textsuperscript{+}-ATPase expression or function, isolated case reports have suggested that an H\textsuperscript{+}, K\textsuperscript{+}-ATPase might be abnormal in certain forms of this disease. In addition, it appears likely that the colonic H\textsuperscript{+}, K\textsuperscript{+}-ATPase in the collecting duct may play a pivotal role in the maintenance of metabolic alkalosis when accompanied by chronic hypokalemia.

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