Detection and localization of \( \text{H}^+\text{K}^-\text{ATPase} \) isoforms in human kidney

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Kraut, Jeffrey A., Kerstin G. Helander, Herbert F. Helander, Ngozi D. Iroezi, Elizabeth A. Marcus, and George Sachs. Detection and localization of \( \text{H}^+\text{K}^-\text{ATPase} \) isoforms in human kidney. Am J Physiol Renal Physiol 281: F763–F768, 2001.—An \( \text{H}^+\text{K}^-\text{ATPase} \) contributes to hydrogen secretion and potassium reabsorption by the rat and rabbit collecting ducts. Transport of these ions appears to be accomplished by one or both of two isoforms of the \( \text{H}^+\text{K}^-\text{ATPase} \). To evaluate whether an \( \text{H}^+\text{K}^-\text{ATPase} \) is present in the human kidney, immunohistochemical studies were performed using normal human renal tissue probed with antibodies directed against epitopes of three of the known isoforms of the \( \text{H}^+\text{K}^-\text{ATPase} \), and the V-type \( \text{H}^-\text{ATPase} \). Cortical and medullary tissue probed with antibodies against \( \text{HK}_{\alpha_1} \) showed cytoplasmic staining of intercalated cells that was less intense than that observed in the parietal cells of normal rat stomach stained with the same antibody. Weak immunoreactivity was detected in principal cells of the human collecting ducts. Cortical and medullary tissue probed with antibodies directed against \( \text{HK}_{\alpha_3} \) revealed weak, diffuse staining of intercalated cells of the collecting ducts and occasional light staining of principal cells. Cortical and medullary tissue probed with antibodies directed against \( \text{HK}_{\alpha_4} \) revealed weak, diffuse staining of the collecting ducts and occasional light staining of principal cells. By contrast, no discernible staining was noted with the use of the antibody against \( \text{HK}_{\alpha_2} \). These data indicate that \( \text{HK}_{\alpha_1} \) and \( \text{HK}_{\alpha_4} \) are present in the collecting ducts of the human kidney. In this location, these isoforms might contribute to hydrogen and potassium transport by the kidney.

renal hydrogen-potassium-adenosinetriphosphatase; hydrogen; potassium; immunohistochemistry

\( \text{H}^+\text{K}^-\text{ATPase} \) is a \( \text{P}^-\text{type ATPase} \) that consists of an \( \alpha\)-subunit that subserves the catalytic function of the enzyme and a glycosylated \( \beta\)-subunit that plays a role in stabilization, maturation, and targeting of the enzyme to the plasma membrane and perhaps in K countertransport (12, 14, 24, 25, 33). Several additional isoforms of the \( \alpha\)-subunit of the \( \text{H}^+\text{K}^-\text{ATPase} \) have been described: \( \text{HK}_{\alpha_2a} \) was cloned from rat colon (8, 9) and truncated versions, \( \text{HK}_{\alpha_2b} \) and \( \text{HK}_{\alpha_2c} \) (4, 20), were cloned from rat and rabbit kidney, respectively; \( \text{HK}_{\alpha_3} \) was cloned from toad bladder (18); and \( \text{HK}_{\alpha_4} \) (17), also termed \( \text{ATP1AL1} \), was cloned from a human skin library. All isoforms share a high homology with each other at the amino acid level (60–87%) (5). Previous studies performed in the rat and rabbit using RT-PCR, in situ hybridization, and immunostaining have localized two of the isoforms, \( \text{HK}_{\alpha_1} \) and \( \text{HK}_{\alpha_2} \), to principal and intercalated cells of the collecting ducts (11–13, 22, 32). Furthermore, microperfusion of isolated cortical and medullary collecting ducts of both species has shown that hydrogen and potassium transport by these segments is attenuated by exposure to SCH-28080, a specific inhibitor of \( \text{HK}_{\alpha_1} \) (15, 25, 31) and ouabain, an inhibitor of \( \text{HK}_{\alpha_2a} \), \( \text{HK}_{\alpha_2b} \), and \( \text{HK}_{\alpha_2c} \), at high concentrations (1 M) (23, 25). These results indicate a probable role for the \( \text{H}^+\text{K}^-\text{ATPase} \) in the transport of hydrogen and potassium by the rat and rabbit kidney.

The potential role of an \( \text{H}^+\text{K}^-\text{ATPase} \) in the regulation of acid-base and potassium balance by the human kidney remains unexplored. The present studies were designed to examine whether various isoforms of the \( \text{H-K}^-\text{ATPase} \) are present in the human kidney, and, if so, to determine their location using immunocytochemical techniques. The results of these studies demonstrate that \( \text{HK}_{\alpha_1} \) and \( \text{HK}_{\alpha_4} \) are present in the cortical and medullary collecting ducts of the human kidney. Both isoforms are primarily present within intercalated cells and to a lesser extent in principal cells. The results of these studies are consistent with a role of the \( \text{H}^+\text{K}^-\text{ATPase} \) in regulation of potassium and hydrogen balance by the human kidney. The precise functional role of the renal \( \text{H}^+\text{K}^-\text{ATPase} \) in the human remains to be determined.

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Materials and Methods

Samples. For immunohistochemical analysis of normal human kidney, tissue samples were obtained from tumor-free regions of nephrectomy specimens removed for treatment of localized renal cell carcinoma. Sections from the tissues were examined histologically before immunostaining to confirm the absence of residual tumor tissue. The protocol was approved by the Institutional Review Board of the Greater Los Angeles Veterans Affairs Health Care System. Archival specimens of normal rat stomach were utilized to confirm the absence of residual tumor tissue. The protocol was approved by the Institutional Review Board of the Greater Los Angeles Veterans Affairs Health Care System. All procedures, except the antigen retrieval, were carried out at room temperature. For contrast, the sections were out at room temperature. For contrast, the sections were

Antibodies. For detection of HKα1, antibody 1218, a monoclonal antibody directed against the separated α-subunit (26) (generously provided by Dr. A Smolka, Univ. of South Carolina, Charleston, SC), was used in dilutions ranging from 0.4 to 10 μg/ml. For detection of HKα2, a rabbit polyclonal antibody generated against a fusion protein containing the sequence from serine-14 to leucine-39 of the human ATP1AL1 sequence was utilized (kindly provided by Dr. Michael Caplan, Yale Univ., New Haven, CT). For detection of H+-ATPase, a rabbit polyclonal antibody generated against a synthetic peptide, Cys-Pro-Gln-Asp-Thr-Glu-Ala-Asp-Thr-Ala-Leu, from the NH2 terminus of the bovine kidney 56-kDa subunit of the vacuolar H+-ATPase, was used (kindly provided by Dr. Ira Kurtz, UCLA School of Medicine, Los Angeles, CA). For detection of HKα2, a polyclonal antibody generated against a synthetic peptide consisting of amino acids 686–698 of the putative rat HKα2 was used (21) (kindly provided by Dr. A Smolka).

Immunohistochemistry. Tissue specimens were fixed in buffered 4% formaldehyde dehydrated in ethanol and embedded in paraffin. Four-micrometer sections were cut in a microtome and placed on glass slides (SuperFrostPlus, Menzel). Tissue sections were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol, followed by distilled water. Preliminary studies demonstrated weak staining or no staining with conventional immunohistochemical techniques. Therefore, studies were performed using antigen-retrieval methods that serve to unmask fixed epitopes by making them accessible to the primary antibody. Briefly, sections were placed in Target Retrieval Solution (DAKO, Carpinteria, CA), pH 6.1, in a water bath at 95°C for 20 min and then allowed to reach near-room temperature over 20 min. This procedure resulted in a considerable increase in immunoreactivity of the sections. To further enhance the immunohistochemical signal, two highly sensitive techniques were used: a streptavidin-biotin method (LSAB +; DAKO) and a streptavidin-biotin-tyramide method (CSA; DAKO). Briefly, for the biotin-streptavidin method, the sections were rinsed in Tris-buffered saline (TBS) and then covered with the primary antibody diluted with 1% BSA in TBS buffer, usually for 15 min. Biotinylated secondary antibodies were then added, followed by streptavidin conjugated to alkaline phosphatase. Visualization depended on the precipitation of fuchsia-colored New Fuchsins. The sections were thoroughly rinsed with TBS between each step. For the streptavidin-biotin-tyramide method, the sections were first exposed to 3% H2O2 to block endogenous peroxidase activity, then covered with a protein block. They were subsequently exposed for 30 min to the primary monoclonal antibody diluted with TBS, followed by biotinylated rabbit anti-mouse IgG. The streptavidin, biotin, and tyramide were then added as recommended by the manufacturer (DAKO). Finally, in the presence of H2O2, peroxidase coupled to streptavidin catalyzed the precipitation of diaminobenzidine, resulting in a brown color. Rinsing between the various steps, except for the step between the protein block and the primary antibody where no rinsing took place, was carried out using TBS containing NaCl and Tween 20.

Controls. For negative controls, primary antibodies were replaced by normal mouse serum or normal rabbit IgG, respectively, diluted to the same protein concentrations as the respective antisera. Additional negative controls were performed with DAKO’s mouse negative control antibody and with 50 mM Tris buffer, pH 7.4, replacing the primary antibodies.

All procedures, except the antigen retrieval, were carried out at room temperature. For contrast, the sections were
b Briefly stained with Mayer’s hematoxylin. Coverslips were mounted with Faramount aqueous medium (DAKO), and slides were viewed with a Zeiss microscope.

Confocal microscopy. To further examine the location of the H^+-K^+-ATPase in the cell, tissues were examined using confocal microscopy. Tissues were processed in a similar fashion to the immunohistochemistry studies described above up to the step of placing them in the Target Retrieval Solution. The slides were washed in 100 mM phosphate buffer, pH 7.4, and then exposed to the primary antibody 1218 overnight at 4°C, followed by three 15-min washes with phosphate buffer. The secondary antibody, goat anti-mouse IgG coupled to tetramethyl rhodamine isothiocyanate (American Qualex, San Clemente, CA), was utilized at a dilution of 1:100. For negative controls, the primary antibody was replaced by normal mouse serum.

Immunofluorescence of the sections was assessed with a Zeiss 510 confocal microscope (Carl Zeiss, New York, NY). The fluorophore was excited using the 543-nm line of the helium-neon laser, and emission was detected using a 560-nm long-pass filter. The detector gain and amplitude offset were set so that the negative control sections were just above the threshold of detection. All subsequent confocal observations used the same detector settings. Optical slices (1 μm) were recorded.

RESULTS

As shown in Fig. 1, A and B, sections probed with antibody 1218 directed against HKα1 revealed staining of a majority of the cells of the cortical and medullary collecting ducts. In the intercalated cells, the staining was diffuse in the cytoplasm. With a similar immunohistochemical method, but utilizing a secondary anti-mouse fluorescent antibody in the final step to allow confocal visualization, staining of renal medullary tubules was observed to be present primarily on relatively large cytoplasmic vacuoles (Fig. 1C). No staining was observed when the primary antibody was replaced by mouse serum (Fig. 1D). In the principal cells, the staining was generally weaker. Staining with this antibody in the human renal tissue was considerably less prominent than that observed in the parietal cells of positive control sections from the rat gastric mucosa stained with the same antibody (Fig. 2), but at a lower

Fig. 2. Immunoreactivity in rat gastric corpus probed with antibody 1218 (0.4 μg/ml). Immunoreactivity was prominent in the parietal cells and more intense than that observed in the sections of the human kidney. The CSA method was used. Bar = 100 μm.
As shown in Fig. 3, A and B, staining with antibodies directed against HKα4 was present primarily in intercalated cells of the cortex and medulla but was significantly less intense than with antibody against HKα1. Light staining was also occasionally observed in principal cells. By contrast, sections probed with antibody 31.7 directed against rat HKα2 (not shown) revealed no detectable staining in any portion of the kidney.

Strong immunoreactivity against H^+-K^+-ATPase (Fig. 4, A and B) was observed in the cytoplasm of the intercalated cells of the cortex and medulla, and in addition, in some cells of the proximal tubules, as reported previously (19).

Whereas sections stained with the primary antibodies revealed immunoreactivity, those stained with mouse or rabbit serum, rabbit IgG (Fig. 5, A and B), the mouse negative control antibody, or the buffer (not shown) revealed no detectable staining of the renal cells.

DISCUSSION

The results of the present studies demonstrate that two of the known α-subunit isoforms of the H^+-K^+-ATPase, HKα1 and HKα4, are present in the collecting ducts of the human kidney. This conclusion is based on the demonstration that sections probed with the primary antibodies had prominent immunoreactivity of collecting duct cells, whereas those probed with mouse or rabbit serum, rabbit IgG, negative control antibodies, or buffer had no staining. In this location, the H^+-K^+-ATPase could contribute to renal handling of protons and potassium, as has been demonstrated in the rat and rabbit (12, 14, 23, 25, 31). Confocal imaging of the renal cells demonstrated that the H^+-K^+-ATPase was primarily located in relatively large vesicles in the cytoplasm of the cell. For the enzyme to be active in the renal handling of hydrogen and potassium, it should be present in the plasma membranes of the collecting duct cells. In the resting parietal cell in concentration.

Fig. 4. Immunoreactivity of renal sections probed with antibody directed against H^+-ATPase. A: renal cortex. B: renal medulla. Staining was most intense in the intercalated cells of the collecting ducts (C). The LSAB+ method was used. Bar = 100 μm.

Fig. 5. Renal control sections probed with normal rabbit serum followed by the LSAB+ method or normal rabbit IgG followed by the CSA method. No staining was observed in either cortical (A) or medullary tissue (B). Bar = 100 μm.
the stomach, the ATPase is present in cytoplasmic tubulovesicles. Stimulation of acid secretion results in translocation of the enzyme to the microvilli of the secretory canalicus (24, 30). A similar mechanism of cycling of the H\textsuperscript{+}-K\textsuperscript{+}-ATPase between the cytoplasm and plasma membranes might exist as a means of regulation of ion transport by this protein in the kidney as it is for the H\textsuperscript{+}-ATPase (14).

Detection of both proteins was not possible using conventional immunohistochemical techniques but rather required antigen-retrieval and enhancement methods (2). A likely explanation for the difficulty in detecting these proteins is the low abundance of both proteins in the human kidney. Studies of rat and rabbit kidney have also demonstrated very low abundance of H\textsuperscript{+}-K\textsuperscript{+}-ATPase proteins (3, 7, 21). An alternative, but less likely, possibility is that the epitopes of both proteins are hidden in a confirmation in the kidney that “masks” them, as has been suggested for the base transporter AE1 (2).

HK\textsubscript{42} was not detectable in the human kidney despite the use of the antigen-retrieval and enhancement techniques. This was initially surprising because HK\textsubscript{42} and HK\textsubscript{44} are 85% homologous at the amino acid level (5). However, the HK\textsubscript{42}-specific antibody is directed against epitopes of the protein in a region in which there is less similarity among the proteins (5). The absence of immunoreactivity for HK\textsubscript{42} from the human kidney noted in the present studies might indicate the absence of expression of this isoform in the human kidney in contrast to rat and rabbit or that the epitope used for generation of the antibody is modified in the human sequence. Also, as we and others have shown, expression of this isoform is upregulated by potassium depletion (21). Although we did not know the potassium balance in the patients from whom specimens were obtained, it is very possible that potassium depletion was absent.

Immunocytochemical, biochemical, and molecular biological studies in the rat and rabbit kidney (3, 6, 7, 32, 33) have demonstrated that HK\textsubscript{41} is present in both α- and β-intercalated cells of the cortical and medullary collecting ducts. On the other hand, mRNA studies showed expression of this isoform in both principal and intercalated cells of the rat kidney (1, 25).

Microperfusion studies of the rat and rabbit have shown that exposure to SCH-28080, a specific inhibitor of HK\textsubscript{41}, reduces proton and potassium transport by the cortical and medullary collecting ducts (15, 25, 31). In the medullary collecting duct, the SCH-28080-inhibitable component comprises 40% of transport under normal acid-base conditions (29). These results suggest that HK\textsubscript{41} might play an important role in acid-base and potassium homeostasis. In support of this possibility, transgenic mice with a mutated β-subunit, which resulted in the permanence of the H\textsuperscript{+}-K\textsuperscript{+}-ATPase at the apical membrane of the collecting duct, manifested a higher serum potassium concentration and lower urinary potassium excretion than did controls (30). On the other hand, transgenic mice in which the gene encoding HK\textsubscript{41} was knocked out had similar serum concentrations of bicarbonate or potassium to those in normal controls (27).

Renal tubular acidosis is classically associated not only with reduced hydrogen secretion but also with hypokalemia (10). This constellation of findings is what might be expected with reduced renal H\textsuperscript{+}-K\textsuperscript{+}-ATPase activity if the H\textsuperscript{+}-K\textsuperscript{+}-ATPase contributed importantly to the regulation of acid-base balance. At present, only kindreds with mutations in AE1 and the H\textsuperscript{+}-ATPase (10) or patients with immunologically mediated renal disease with absent H\textsuperscript{+}-ATPase immunoreactivity (10) have been reported with full-blown renal tubular acidosis. However, recently a small group of individuals in Thailand has been described with distal renal tubular acidosis and hypokalemia attributable to exposure to vanadium, an inhibitor of P-type ATPases (28). Given the presence of HK\textsubscript{41} in the human collecting duct, it is intriguing to speculate that genetic defects and clinical disorders associated with defective H\textsuperscript{+}-K\textsuperscript{+}-ATPase activity might be described in the future, similar to those reported with genetic defects in AE1 and H\textsuperscript{+}-ATPase.

HK\textsubscript{44} (ATP1AL1) was first cloned from the skin axilla (17). Comparison with the other HK isoforms revealed that HK\textsubscript{44} was 87% identical to rat or rabbit HK\textsubscript{42}. The level of dissimilarity is greatest within the NH\textsubscript{2}-terminal domain, where identity is only ~50% (5). HK\textsubscript{44} was demonstrated in human brain, kidney, and skin by RT-PCR but not by Northern blot analysis, indicating a low abundance of this protein in these tissues (17).

Studies in oocytes by Grishin and Caplan (16) demonstrated that Na\textsuperscript{+}-K\textsuperscript{+} exchange via HK\textsubscript{44} was 10-fold that of H\textsuperscript{+}-K\textsuperscript{+}-exchange, data consistent with this isoform acting primarily as a Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. Although we have not localized this transporter to the luminal membrane, its presence in the collecting ducts of the kidney might indicate that it primarily conserves potassium under conditions of potassium depletion, as proposed for HK\textsubscript{42} in the rat kidney.

In summary, HK\textsubscript{41} and HK\textsubscript{44} are present in the collecting ducts of the human kidney, a site where they probably participate in regulating renal acid excretion and potassium reabsorption.

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REFERENCES


