Colonic H-K-ATPase α- and β-subunits express ouabain-insensitive H-K-ATPase

PITCHAI SANGAN, SUNDARARAJAH THEVANANTHER, SHEEELA SANGAN, VAZAHIKKURICHI M. RAJENDRAN, AND HENRY J. BINDER

Departments of Internal Medicine and Pediatrics, Yale University, New Haven, Connecticut 06520-8019

Sangan, Pitchai, Sundararajah Thevananther, Sheela Sangan, Vazhaikkurichi M. Rajendran, and Henry J. Binder. Colonic H-K-ATPase α- and β-subunits express ouabain-insensitive H-K-ATPase. Am. J. Physiol. Cell Physiol. 278: C182–C189, 2000.—Active K absorption in the rat distal colon is mediated by a P-type ATPase, the H-K-ATPase. While the H-K-ATPase α-subunit (HKα) has been cloned from rat colon, nothing is known about the other subunits of the enzyme. The present study was designed to determine whether the full-length cDNA for HKα or HKβ is expressed in rat colonic epithelial cells. Isolated rat colonic epithelial cells were transfected with the HKcα and HKcβ cDNA constructs, and the expression of the cloned cDNAs was confirmed by the detection of the HKcα protein with HKcβ cRNA when coexpressed with the HKcβ protein. In this present communication, we report that both HKcα and HKcβ proteins formed a functional enzyme complex with HKcα protein in HEK-293 cells and yielded the expression of both ouabain-insensitive H-K-ATPase activity and 86Rb uptake.

MATERIALS AND METHODS

HEK-293 cells were a gift from Dr. B. Forbush (Yale University). pcDNA 3.1+ vector was purchased from Invitrogen (Carlsbad, CA), restriction enzymes were from New England Biolabs (Beverly, MA). All other reagents were of molecular biology or analytical grade.

Plasmid construction. The full-length cDNAs encoding the rat colonic HKα (8), the colonic HKβ (23), and the rat NaKβ1 (20) were modified by PCR both at the 5′ end and the 3′ end with BamHI and EcoRI, respectively. The HKα, HKβ, and NaKβ1 cDNAs were digested separately with BamHI and EcoRI enzymes and ligated using T4 DNA ligase independently into pcDNA 3.1+. The ligations were transformed into XL1-blue Escherichia coli cells, and the plasmid DNAs were prepared according to the method described by Morele (21). The plasmid DNAs were digested with BamHI and EcoRI enzymes to release the insert, and the plasmids containing expected size inserts were sequenced by an auto-

ACTIVE POTASSIUM ABSORPTION in mammalian distal colon is mediated by one or more H-K-ATPases of the P-type gene family of ion transport ATPases (2, 10, 16, 30). P2-type ATPases are heterodimers that consist of α- and β-subunits, whereas P1-type ATPases only consist of α-subunits. Colonic H-K-ATPase α-subunit (HKα) (8) is localized in apical membranes of surface epithelial cells (15, 24), and its mRNA and protein expression are increased threefold in the distal colons of dietary Na-depleted rats (24). Functional expression of HKα cDNA without β-subunit in SF9 cells revealed H-K-ATPase activity that was insensitive to ouabain (18). In contrast, HKα cDNA when coexpressed with the β-subunit of Na-K-ATPase (NaKβ1) or gastric H-K-ATPase (HKβ) either in Xenopus oocytes or in human embryonic kidney 293 cells (HEK-293 cells) demonstrated ouabain-sensitive 86Rb uptake (5, 7). Several of these studies reported the expression of K-dependent ATP hydrolysis (i.e., H-K-ATPase activity) (5, 7).

Recently, we have isolated and identified a β-subunit from rat distal colon that we refer to as HKβ and that is expressed in both apical and basolateral membranes of rat distal colon (23). We have also demonstrated by communoprecipitation the physical association of this HKβ protein with HKα protein and its upregulation in apical membrane by dietary K depletion (23). As a result, we proposed that this is the β-subunit for the colonic H-K-ATPase. This present study was, therefore, designed to determine whether HKα, when coexpressed with HKβ cDNA in mammalian cells, exhibited ATPase activity. In this present communication, we report that both HKα and HKβ proteins formed a functional enzyme complex with HKα protein in HEK-293 cells and yielded the expression of both ouabain-insensitive H-K-ATPase activity and 86Rb uptake.

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mated fluorescence sequencer (William Keck Sequencing Facility, Yale University). Plasmids containing full-length cDNAs (hereafter referred to as pHKα, pHKβ, and pNaKβ1) with the correct sequences were used for the expression studies.

Cell culture and transfection. COS-7 cells were grown (13) in high-glucose DMEM containing 10% fetal bovine serum, 50 U/ml penicillin, 50 µg/ml streptomycin, and 2 mM l-glutamine (complete DMEM). Transfections were carried out in subconfluent COS-7 cells with vector alone, pHKα alone, pHKβ alone, pNaKβ1 alone, pHKα/pHKβ, or pHKα/pNaKβ1 in independent transfections using superfect transfection reagent (Qiagen, Chatsworth, CA) according to the manufacturer’s recommendations. In brief, plasmid DNA was mixed with serum, antibiotic-free DMEM, and the recommended amount of superfect reagent and incubated at room temperature for 30 min. After incubation, 2 ml of serum and antibiotic-free DMEM were added. The entire mixture was added to the cells that previously had been washed three times with serum and antibiotic-free medium. The plates were incubated at 37°C in a humidified CO2 incubator for 2 h. The medium was removed and washed with 5 ml medium. Finally, 6 ml of medium were added, and the cells were cultured in CO2 incubator at 37°C for 60 h. After 60 h, the medium was removed, the cells were washed with 1× PBS three times, 1× PBS was added, and the cells were scraped and harvested by centrifugation.

HEK-293 cells were grown (13) and the transfections were performed similar to that for COS-7 cells, with the exception that the cells were grown in low-glucose DMEM. To prepare stable cell lines, after 60 h of transfections, the cells were split in serial dilution (1:10, 1:50, 1:250) in the complete DMEM containing G418 (900 µg/ml; GIBCO BRL, Gaithersburg, MD). Fresh complete DMEM containing G418 was replaced every 72 h. Six weeks after transfections, colonies grown in subconfluent COS-7 cells with vector alone, pHKα alone, pHKβ alone, pNaKβ1 alone, pHKα/pHKβ, or pHKα/pNaKβ1 were processed using HKc protein or HKc protein, HKc protein, or NaKβ1 protein in cells transfected with pHKα alone, pHKβ alone, pHKα/pHKβ, or pHKα/pNaKβ1 by Western blot analysis with their respective antibodies.

Plasma membrane preparation. Cells were harvested and washed twice with 1× PBS. Cells were then resuspended in sonication buffer containing 50 mM Tris·HCl (pH 7.4), 1 mM EDTA, 250 mM sucrose, and 1 mM phenylmethylsulfon fluoride. The cells were then sonicated for 15 s each time (3×) with 30-s intervals between them. The lysate was centrifuged at 30,000 × g for 30 min at 4°C (Sorvall RC5B, SS34 rotor), and the membrane pellet was resuspended in a buffer containing 50 mM Tris·HCl (pH 7.4) and 250 mM sucrose. These crude plasma membranes were used for enzyme assay and Western blot analysis. Protein was estimated by the Bradford method using bovine γ-globulin as standard (3).

Assay of ATPases. H-K-ATPase activity was assayed in 0.5 ml of reaction volume containing 50 µg of membrane protein, 40 mM Tris·HCl (pH 7.4), 3 mM MgCl2, and 10 µM ouabain in the presence and absence of 5 mM KCl. The reaction was initiated by the addition of 3 mM ATP (Tris salt) and incubated at 37°C for 1 h; inorganic phosphate released was measured, as described previously (9). H-K-ATPase activity was calculated as the difference between activities in the presence and absence of KCl. Na-K-ATPase activity was also assayed using the above reaction condition but also included 100 mM NaCl and was calculated as the difference between activities in the presence and absence of Na. The specific activity of the enzyme is expressed as nanomoles of phosphorus liberated per milligram of protein per minute.

SDS-PAGE and Western blot analysis. SDS-PAGE was carried out, as previously described (17). Fifty micrograms of membrane protein were incubated in a sample buffer containing 10 mM Tris·HCl (pH 6.8), 2% SDS, 2% mercaptoethanol, and 10% glycerol at room temperature for 5 min and loaded on an SDS-polyacrylamide gel. Western blot analyses were performed using HKα, HKβ, and NaKβ1 antibodies, as previously described (23).

Immunofluorescence studies. Stably transfected HEK-293 cells that express HKα protein alone and express both HKα and HKβ proteins were grown on 22 × 22 mm glass coverslips for 48 h. The cells were washed with 1× PBS, fixed for 20 min in 4% paraformaldehyde prepared in 1× PBS, and processed as described previously (12). The fixed cells were incubated with appropriate antibodies for 1 h at room temperature at a dilution of 1:100. CY3-conjugated anti-rabbit IgG (Amersham) secondary antibodies were used at a dilution of 1:2,000 at room temperature for 1 h. Immunofluorescence images were visualized using a Zeiss-Axiophot microscope.

86Rb uptake studies. Tissue culture plates (24 wells) were treated with 0.1% (wt/vol) poly-L-lysine (200 µl/well) for 5 min in a tissue culture laminar flow hood. The plates were then washed with sterile deionized water and were allowed to dry for 5 min. For the untransfected cells, 1 × 106 cells/well were plated and grown in growth media (low-glucose DMEM containing 10% fetal bovine serum and 50 units penicillin/streptomycin solution). The transfected cell lines were grown in the same growth media but with the addition of 900 µg/ml G418. The cells grew at a slower rate but did not wash off during washing while performing 86Rb uptake studies in the poly-L-lysine-treated plates compared with those grown in the untreated plates.

The cells were grown for 4–5 days and fresh media were changed every 48 h. After the cells had grown to complete confluency (about 5 days), the media were aspirated and the cells were washed six times with 500 µl uptake buffer containing 145 mM NaCl, 1 mM KCl, 10 mM glucose, 1.2 mM MgCl2, 1.0 mM CaCl2, 2 mM NaH2PO4, 32 mM HEPES, and 200 µM bumetanide, pH 7.4. Bumetanide was added to block any 86Rb uptake by Na-K-2Cl cotransporter. Preliminary studies had shown that 86Rb uptake was linear for up to 30 min in both the untransfected and the transfected cell lines; therefore, all uptake studies were performed for 10 min. Additional preliminary studies in the untransfected cell line demonstrated that 10 µM ouabain reduced 86Rb uptake by ∼95%, which was attributed to uptake via Na-K-ATPase; therefore, all studies were performed in the presence of 10 µM ouabain. The cells were initially incubated for 20 min at 37°C in uptake buffer solution. The uptake buffer solution was then replaced by 200 µl uptake buffer containing 86Rb (4 µCi/ml) in the presence or absence of either 1 mM ouabain or 100 µM vanadate, and the cells were incubated for 10 min at 37°C. After incubation and removal of buffer, the cells were washed six times with 500 µl ice-cold stop buffer (10 mM HEPES-Tris, 100 mM MgCl2, pH 7.4). The cells were solubilized in 500 µl of 2% SDS/0.1 N NaOH, and 10 µl of the solubilized cell lysate were used to estimate the protein concentration by the method of Bradford (3). The remaining cell lysate was mixed with 3.5 ml scintillation solution, and radioactivity was measured in a scintillation photometer. 86Rb uptake was expressed as nanomoles per milligrams of protein per 10 min.

RESULTS

To establish that all the constructs expressed the expected size proteins, we first transiently transfected COS-7 cells using the plasmid containing full-length

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HK\(\alpha\), HK\(\beta\), and NaK\(\beta\)1 cDNAs as either individual plasmid constructs (pHK\(\alpha\)/pNaK\(\beta\)1, pHK\(\beta\)/pNaK\(\beta\)1) or as a combination of two plasmids (pHK\(\alpha\)/pHK\(\beta\) or pHK\(\alpha\)/pNaK\(\beta\)1). The results of these expression studies revealed that untransfected COS-7 cells or the cells transfected with vector alone did not express either HK\(\alpha\) or HK\(\beta\) proteins (Fig. 1A and B, lanes 1–3). Cells transfected with pHK\(\alpha\) alone, pHK\(\alpha\)/pHK\(\beta\), or pHK\(\alpha\)/pNaK\(\beta\)1 expressed HK\(\alpha\) protein, as detected by HK\(\alpha\) antibody (Fig. 1A, lanes 4, 7, and 8, respectively). Cells transfected with pHK\(\beta\) alone or pHK\(\alpha\)/pHK\(\beta\) expressed HK\(\beta\) protein, as detected by HK\(\beta\) antibody (Fig. 1B, lanes 5 and 7). Cotransfection of pHK\(\alpha\)/pHK\(\beta\) resulted in the expression of less HK\(\alpha\) protein compared with that identified in cells transfected with pHK\(\alpha\) alone. In contrast, HK\(\beta\) protein was expressed in higher amounts than HK\(\alpha\) protein whether pHK\(\beta\) was transfected alone or cotransfected with pHK\(\alpha\). Several attempts were made to alter the ratio of the \(\alpha/\beta\) constructs for transfections to result in the expression of approximately equal amounts of HK\(\alpha\) and HK\(\beta\) proteins. None of these approaches resulted in the expression of H-K-ATPase activity in cell membranes from these transfected cells. Therefore, we proceeded to establish stable cell lines that would express HK\(\alpha\) and HK\(\beta\) protein in approximately equal amounts.

Several stable HEK-293 cell lines were developed after transfection with the following cDNAs: 1) both HK\(\alpha\) and HK\(\beta\), 2) HK\(\alpha\) and NaK\(\beta\)1, 3) HK\(\alpha\) alone, and 4) HK\(\beta\) alone. Western blot analyses were performed using the crude membranes from these stable cell lines (Fig. 2). The untransfected HEK-293 cells did not express either HK\(\alpha\) protein or HK\(\beta\) protein. Cell lines stably transfected with pHK\(\alpha\) alone, pHK\(\alpha\)/pHK\(\beta\), or pHK\(\alpha\)/pNaK\(\beta\)1 expressed HK\(\alpha\) protein, as detected by HK\(\alpha\) antibody (Fig. 2A, lanes 3, 5, and 6, respectively). Cell lines stably transfected with pHK\(\beta\)
alone or with pHKα/pHKβ expressed HKβ protein, as detected by HKβ antibody (Fig. 2B, lanes 4 and 5), whereas those cell lines transfected with pHKα/pNaNKβ1 expressed NaNKβ1 as protein, as detected by NaNKβ1 antibody (Fig. 2C, lane 6).

To establish whether the expressed HKα or HKβ proteins were transported to plasma membranes, immunofluorescence studies were performed. The results of such immunofluorescence studies are presented in Fig. 3. The stable cell line expressing both HKα and HKβ proteins stained with HKα antibody (Fig. 3C) and stained with HKβ antibody (Fig. 3D) demonstrate that both HKα and HKβ proteins are transported to and localized in the plasma membranes. Staining (Fig. 3A) was not identified in the same stable cell line expressing both HKα and HKβ proteins when stained with preimmune serum, indicating that the staining produced by HKα and HKβ antibodies is specific (Fig. 3A). HKα protein in the pHKα alone stably transfected cell line was not efficiently localized to the plasma membrane (Fig. 3B). Therefore, HKα protein requires a β-subunit to be transported and localized in the plasma membrane.

ATPase activity was determined in the untransfected HEK-293 cell membranes before establishing the assay conditions for the transfected cells. Only Na-K-ATPase activity was identified in the untransfected HEK-293 cell membranes (15.8 ± 1.3 nmol Pi liberated·mg protein⁻¹·min⁻¹). This endogenous Na-K-ATPase activity was completely inhibited by 10 µM ouabain (data not presented). Similar levels of Na-K-ATPase activities (16.2 ± 0.9 and 16.1 ± 0.8 nmol Pi liberated·mg protein⁻¹·min⁻¹) were identified in the two doubly transfected cell lines, pHKα/pHKβ and pHKα/NaNKβ1, respectively. Although Na-K-ATPase activity was present endogenously in the untransfected cell line, increased levels of Na-K-ATPase were not expressed in the cell lines transfected with both α- and β-subunits. Thus all subsequent assays included 10 µM ouabain in the ATPase assay in membranes from transfected cells.

To determine whether H-K-ATPase is expressed in the stable cell lines, H-K-ATPase assay was determined in membranes prepared from the different cell lines. The maximal H-K-ATPase activity was observed in the pHKα/pHKβ stably transfected cell line (Fig. 4). In contrast, H-K-ATPase activity in the pHKα/pNaNKβ1 stably transfected cell line was 60% of that in the pHKα/pHKβ stably transfected cell line. Cells that had been stably transfected with pHKα alone expressed H-K-ATPase activity that was 21% of maximal activity. Minimal H-K-ATPase activity was noted both in cells that had been stably transfected with pHKβ alone and in the untransfected cell lines.

The effect of K⁺ concentrations on the H-K-ATPase activity in the crude plasma membranes prepared from the cell line stably transfected with pHKα/pHKβ was also studied to determine whether the in vitro expressed H-K-ATPase activity in plasma membranes of HEK-293 cells had similar or different properties from those of H-K-ATPase activity in native colonic apical membranes. Increasing K⁺ concentrations in the incubation medium stimulated and saturated H-K-ATPase activity (Fig. 5). Analysis of this data with a Lineweaver-Burk plot yielded a K_m for K of 0.63 mM. This kinetic constant is comparable to those previously reported for H-K-ATPase in native rat colonic apical membranes (K_m = 0.75 mM) (9), the rat colonic H-K-ATPase expressed as ⁸⁶Rb uptake in Xenopus oocytes (K_m = 0.73 mM) (7), and the K_m calculated for rat H-K-ATPase α-subunit expressed in SF9 cells (K_m = 1.2 mM) (18).

To establish the functional properties of the H-K-ATPase activity identified in membranes from cell lines stably transfected with pHKα/pHKβ or pHKα/pNaNKβ1, the effect of different ATPase inhibitors on H-K-ATPase activity was determined. One millimolar orthovanadate inhibited H-K-ATPase activity by ~75%. In contrast, neither 1 mM ouabain nor 0.5 mM Sch-28080 altered H-K-ATPase activity (Fig. 6).

⁸⁶Rb uptake was performed in untransfected, in pHKα/pHKβ1 transfected, and in pHKα/pNaNKβ1.
transfected HEK-293 cell lines (Fig. 7.). Preliminary studies had shown that $^{86}$Rb uptake was linear for up to 30 min in both the untransfected and the transfected cell lines; therefore, all uptake studies were performed for 10 min. Additional preliminary studies in the untransfected cell line demonstrated that 10 µM ouabain reduced $^{86}$Rb uptake by ~95%, which was attributed to uptake via endogenous Na-K-ATPase and is consistent with the studies of endogenous Na-K-ATPase in this cell line. As a result, all $^{86}$Rb studies were performed in the presence of 10 µM ouabain.

The results of these $^{86}$Rb uptake studies demonstrate that $^{86}$Rb uptake in pHKcα /pHKcβ transfected cells was 66-fold greater than that in untransfected cells (118.8 ± 4.0 vs. 1.8 ± 0.3 nmol·mg protein$^{-1}$·10 min$^{-1}$).

$^{86}$Rb uptake in pHKcα /pNaKβ1 transfected cells was also substantially greater (52-fold) than that in untransfected cells (Fig. 7). Similar to the observations of H-K-ATPase in the doubly transfected cell lines, uptake in pHKcα /pHKcβ was 26% greater than that in the pHKcα/pNaKβ1 cell line. One hundred micromolar vanadate markedly inhibited $^{86}$Rb uptake in both doubly transfected cell lines by ~90%, indicating that $^{86}$Rb uptake is mediated by an ATPase. In contrast, 1 mM ouabain did not significantly alter $^{86}$Rb uptake, establishing that $^{86}$Rb uptake in both doubly transfected cell lines is ouabain insensitive. These data establish that HKcα, when coexpressed with a β-subunit, expresses a ouabain-insensitive H-K-ATPase function.

DISCUSSION

In addition to the kidney, the mammalian large intestine contributes to the regulation of overall K balance via both absorptive and secretory processes (11, 27, 28). Active K absorption that is energized by one (or more) apical membrane H-K-ATPases has been identified in the distal colon of rat, guinea pig, and rabbit and has been the focus of several investigations during the past decade (1, 9, 26, 29). HKcα has been cloned and is a member of the gene family of P-type ATPases (8). HKcα mRNA and protein are present exclusively in surface (and ~20% of the upper crypt) epithelial cells, and its enzymatic activity is localized to the apical membrane (15, 18, 24). Considerable controversy exists regarding whether this HKcα cDNA encodes a ouabain-sensitive or ouabain-insensitive H-K-ATPase and whether a colon-specific β-subunit is required for maximal function of the colonic H-K-ATPase (5, 7, 18).

Studies of the coexpression of HKcα with either HKβ3 or NaKβ1 cRNAs in Xenopus oocytes yielded evidence of ouabain-sensitive $^{86}$Rb uptake that was consistent with H-K-ATPase function (5, 7). In contrast, expression of HKcα cDNA in Sf9 cells without any
exogenous β-subunit resulted in ouabain-insensitive H-K-ATPase activity (18). It should be noted that in these present studies with HEK-293 cells, H-K-ATPase activity in cells stably transfected with pHKα alone (Fig. 4) was 21% of its activity in cells stably transfected with pHKα/pHKβ. It is not known whether the H-K-ATPase activity in the absence of a transfected β-subunit reflects the presence of an endogenous β-subunit in both Sf9 and HEK-293 cells or the ability of HKα protein to manifest partial H-K-ATPase activity in the absence of any β-subunit.

Recent studies have established the distribution of H-K-ATPase activity in apical membranes of both surface and crypt cells (22). Although H-K-ATPase activity in surface cell apical membranes was both ouabain sensitive and ouabain insensitive, H-K-ATPase activity in apical membranes of crypt cells was exclusively ouabain sensitive. As previously noted, HKα mRNA and protein are primarily present in surface and not in crypt cells (15, 18, 24). Therefore, if HKα cDNA encodes a ouabain-sensitive H-K-ATPase, it would be necessary to postulate the presence of three distinct H-K-ATPases in the rat distal colon, two that are ouabain sensitive and one that is ouabain insensitive. Alternatively, however, the presence of only two H-K-ATPases would be required if HKα cDNA encoded the ouabain-insensitive H-K-ATPase. At the present time, there is evidence for at least two H-K-ATPase isoforms in the rat distal colon.

A β-subunit, HKβ, was recently cloned and identified from rat colon (23). A closely related isoform has been designated by others as NaKβ3 (19). Although HKβ mRNA is present in high abundance in testis and lung, several lines of evidence have been presented that provide the basis for the suggestion that HKβ is the β-subunit for the colonic H-K-ATPase (23): 1) HKβ protein is present in both apical and basolateral membranes of rat distal colon; 2) HKβ protein was coprecipitated with HKα protein from apical membranes; 3) HKβ mRNA and its apical membrane protein were increased in distal colon of K-depleted rats (23). Thus it is likely that the HKβ is the β-subunit for H-K-ATPase in native tissue. The present study sought to establish whether HKβ cDNA, when cotransfected with HKα cDNA in a mammalian cell line, expressed both H-K-ATPase activity and 86Rb uptake that were inhibited by vanadate but not by ouabain.

These present studies were designed to express HKα cDNA in a mammalian expression system in view of the conflicting observations previously reported in the expression of HKα cDNA in nonmammalian cells. The initial studies with COS-7 cells did not result in the expression of H-K-ATPase activity but did provide important information that was critical in the design of the subsequent experiments with HEK-293 cells. Such cells had been successfully used for the expression of other P-type ATPases, e.g., ATP1AL1 (13).

Immunofluorescence studies confirmed the presence of both HKα and HKβ proteins in plasma membranes (Fig. 3). H-K-ATPase activity was minimal in the nontransfected HEK-293 cells and those transfected...
with only the HKcβ cDNA. In contrast, transfection of HKcα cDNA resulted in H-K-ATPase activity both in the absence or presence of one of the β-subunits. Maximal H-K-ATPase activity was observed in those cells stably transfected with pHKcα/pHKcβ and was 60% greater than that determined in the cells stably transfected with pHKcα/pNaKβ1 (Fig. 4). Because equal amounts of HKcα protein were present in the crude plasma membranes of pHKcα/pHKcβ and pHKcα/pNaKβ1 transfected cell lines (Fig. 2A, lanes 5 and 6), the observed differences in H-K-ATPase activities in these doubly transfected cell lines cannot be attributed to differences in HKcα protein expression. Thus the higher rate of H-K-ATPase activity observed when HKcα cDNA was cotransfected with HKcβ than with NaKβ1 is consistent with the recent suggestion (23) that HKcβ is the β-subunit for colonic H-K-ATPase.

HKcβ protein is not the only β-subunit to combine with HKcα protein and to manifest H-K-ATPase activity. Codina et al. (4) recently demonstrated that HKcα assembles with NaKβ1 protein in the rat distal colon and kidney medulla. In addition, in the present studies the coexpression of HKcα with NaKβ1 resulted in H-K-ATPase function, indicating that NaKβ1 protein could act as a surrogate β-subunit for HKcα.

Our previous observations in Sf9 cells (18) are similar to the results shown in Fig. 4, in which H-K-ATPase activity was identified in the cell line stably transfected with HKcα cDNA alone. Although the experiment shown in Fig. 4 revealed evidence of H-K-ATPase activity in the absence of an obvious β-subunit, the coexpression of HKcα cDNA with HKcβ cDNA resulted in an almost fivefold higher level of H-K-ATPase expression. The expression of H-K-ATPase in the absence of a β-subunit would be consistent either with an endogenous NaKβ1 in the HEK-293 cells functioning as a surrogate or promiscuous β-subunit or with the presence of a previously unidentified β-subunit in the HEK-293 cells.

These present results demonstrate that both the expressed H-K-ATPase activity and the expressed 86Rb uptake in the cell lines stably transfected with pHKcα/pHKcβ or with pHKcα/pNaKβ1 are ouabain insensitive (Figs. 6 and 7) and conflict with prior observations in Xenopus oocytes that HKcα cDNA encodes an ouabain-sensitive function (5, 7). It should be noted that all prior demonstrations of the ouabain-sensitive HKcα cDNA expression used 86Rb uptake as the parameter of HKcα protein expression (5, 7). In contrast, these present studies directly determined both H-K-ATPase activity and 86Rb uptake as a surrogate marker of K uptake. An adequate explanation for this difference is not evident; however, it is possible that in the nonmammalian Xenopus oocyte expression system, HKcα cDNA activated an endogenous ouabain-sensitive function.

K-dependent proton secretion has been described in the guinea pig colon and has been attributed to an H-K-ATPase (26). Recently, a cDNA from guinea pig distal colon (25), which has significant (88%) sequence homology at the protein level to rat HKcα, was expressed in HEK-293 cells with Torpedo Na-K-ATPase β-subunit cDNA and was associated with ouabain-sensitive H-K-ATPase activity. The rat and guinea pig colonic H-K-ATPase α-subunit cDNAs share significant sequence homology; however, it would be important to know whether the guinea pig H-K-ATPase α-subunit is present in surface or crypt cells. Physiological studies in the rat indicate the presence of two distinct H-K-ATPases, one that is ouabain sensitive and present in crypt cells and the other that is ouabain insensitive and present in surface cells (22). The encoded proteins from rat and guinea pig respond differently to ouabain and, therefore, these two proteins likely represent different H-K-ATPase isoforms.

Two nongastric H-K-ATPase (colonic H-K-ATPase and ATP1AL1) α-subunits with a β-subunit have recently been shown to manifest Na/K exchange function (6, 14) in addition to their H/K exchange activity. Neither study assessed whether the expressed proteins had ATPase activity. Although this present study identified H-K-ATPase activity in the stably transfected HEK-293 cell lines, transfection with these α- and β-subunits did not result in an increase of the expression of Na-K-ATPase activity above that in untransfected cells. It is possible that these different results reflect the different β-subunits used in these studies.

In conclusion, this study demonstrated that HKcα and HKcβ cDNAs, when expressed in a mammalian cell system, manifest ouabain-insensitive H-K-ATPase functions, both enzymatic ATPase activity and 86Rb uptake.

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