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The effect of β-subunit assembly on function and localization of the colonic H^+,K^+-ATPase α-subunit

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The effect of β-subunit assembly on function and localization of the colonic H^+,K^+-ATPase α-subunit.

Background. Previous experiments from our laboratory have demonstrated that $HK\alpha_2$ coimmunoprecipitated with β_1-Na^+,K^+-ATPase. Although $HK\alpha_2$ is expressed abundantly in the apical membrane of distal colon, the demonstration that β_1 localizes to this same membrane in distal colon has not been demonstrated previously.

Methods. Immunolocalization was performed in distal colon using a polyclonal antibody against $HK\alpha_2$ and a monoclonal antibody against β_1.

Results. The results demonstrate that $HK\alpha_2$ localizes to the apical membrane. Two pools of β_1-Na^+,K^+-ATPase were detected. The first localized to the apical membrane. The second pool was detected in the basolateral membrane when distal colon sections were deglycosylated with glycosidase F. Therefore, our results demonstrate that β_1 localizes to the apical membrane with $HK\alpha_2$, and supports the view that β_1 is the physiologic β-subunit for $HK\alpha_2$. We tested, therefore, the efficiency of the two β-subunits expressed in distal colon (β_1 and β_3) to support the activity of $HK\alpha_2$. Human embryonic kidney HEK-293 cells were transiently cotransfected with $HK\alpha_2$ plus β_1 or $HK\alpha_2$ plus β_3. Subsequently, $^{86}Rb^+$-uptake and plasma membrane localization were evaluated. The results demonstrate that both $HK\alpha_2/\beta_1$ and $HK\alpha_2/\beta_3$ support $^{86}Rb^+$-uptake. However, $^{86}Rb^+$-uptake measured in the cells cotransfected with $HK\alpha_2$ plus β_1 exceeded that measured in cells expressing $HK\alpha_2/\beta_3$. Fluorescence microscopy using enhanced green fluorescent protein cloned at the amino-terminus of $HK\alpha_2$ demonstrated protein migration to the plasma membrane in cells cotransfected with EGFP-$HK\alpha_2$ plus β_1. In contrast, in cells cotransfected with EGFP-$HK\alpha_2$ plus β_3, the vast majority of the protein remained confined to intracellular compartments. The significantly higher $^{86}Rb^+$-uptake corresponded to additional localization of $HK\alpha_2$ to the plasma membrane when coexpressed with β_1 compared to β_3.

Conclusion. Taken together, these and previous results from our laboratory indicate that β_1-Na^+,K^+-ATPase is likely to represent the most physiologic and efficient subunit for $HK\alpha_2$ assembly in distal colon.

Key words: molecular regulation of the colonic H+,K+-ATPase, β-subunit assembly, membrane localization.

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Six different X^+,K^+-ATPase α-subunits have been identified in the rat. One, the colonic H^+,K^+-ATPase ($HK\alpha_2$), is expressed chiefly in the distal colon and renal medulla [1]. Five different β-subunits have been identified; three of these, β_1-,β_3-Na^+,K^+-ATPase, and the β-subunit of the gastric H^+,K^+-ATPase (β_G) have been identified in kidney [2, 3]. However, β_1 and β_3 have also been identified in distal colon. While the association of the different α-subunits of the Na^+,K^+-ATPase with different β-subunits is tissue specific, the α-subunit of the gastric H^+,K^+-ATPase assembles specifically with β_G in the stomach. Nevertheless, a specific β-subunit for $HK\alpha_2$ remains controversial. Recently, a unique β-subunit (β_M) was identified in muscle by Pescov et al [4]. Studies by Crambert et al [5] investigating the function of β_m did not uncover association of this unique subunit with any of the known X^+,K^+-ATPase α-subunits, including the H^+,K^+-ATPases.

Through application of a highly specific antibody against $HK\alpha_2$ in coimmunoprecipitation experiments, we and others [6, 7] have demonstrated that $HK\alpha_2$ assembles with β_1 Na^+,K^+-ATPase in renal medulla and distal colon plasma membranes. These experiments were performed by employing a fraction enriched in plasma membranes [8]. Coimmunoprecipitation was not observed when the anti-$HK\alpha_2$ antibody was preincubated with the immunizing peptide, or when the primary antibody was omitted. Therefore, we concluded that β_1-Na^+,K^+-ATPase was the physiologic β-subunit for $HK\alpha_2$. Using an independent approach, Geering et al [9] suggested that a unique β-subunit for the human ATP1AL1 (assumed to be the human equivalent to the rat $HK\alpha_2$) has not yet been identified. In these studies, the stability of ATP1AL1 was

tested against degradation by coinjecting oocytes with cRNA for ATP1AL1 plus different β-subunits. It was found that all ATP1AL1/β complexes were less stable than the α_1-Na^+,K^+-ATPase/β_1-Na^+,K^+-ATPase complex used as a standard. Therefore, it was concluded that the functional β-subunit for ATP1AL1 remains to be identified. Nevertheless, in an independent study, Sangan et al [10] suggested that β_3-Na^+,K^+-ATPase could be the physiologic β-subunit for HKα_2. Therefore, uncertainty regarding which β-subunit functions as the physiologic β-subunit has endured to the present.

It is widely accepted that β_1-Na^+,K^+-ATPase localizes, in most cells and tissues, to the basolateral membrane, forming a complex with α_1-Na^+,K^+-ATPase [2]. However, if β_1-Na^+,K^+-ATPase functions as the physiologic β-subunit for HKα_2 in a site-specific manner in distal colon and renal medulla, it should localize to the apical membrane in these cells.

The purpose of the present study was to determine, through application of immunolocalization and functional assays, if β_1-Na^+,K^+-ATPase serves as the physiologic β-subunit for HKα_2 in the distal colon. Distal colon was chosen because HKα_2 and β_1-Na^+,K^+-ATPase are expressed abundantly in this tissue.

METHODS

Immunolocalization of β_1-Na^+,K^+-ATPase in apical membranes of distal colon and renal medulla

Rat distal colon was flash-frozen and cryosectionated at a 6-micron thickness. The sections were thaw-mounted on Fischer PLUS (Madison, WI, USA) slides, fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) (10 mmol/L Na-phosphate, pH = 7.5, 150 mmol/L NaCl) for 10 minutes, and blocked in 10% normal goat serum. The primary antibody (monoclonal anti-β_1-Na^+,K^+-ATPase) was purchased from Upstate Biotechnology (cat. # 05–382; Lake Placid, NY, USA), diluted at 1:100 and incubated for 30 minutes at room temperature. The sections were washed with PBS and incubated with affinity-purified goat antimouse immunoglobulin G (IgG) conjugated to rhodamine [25 μg/mL in 1% bovine serum albumin (BSA)-PBS; cat. # 115–025–146; Jackson ImmunoResearch, West Grove, PA, USA] for 30 minutes. The sections were washed with PBS, and postfixed with 3.7% formaldehyde PBS for 10 minutes. The sections were viewed using a Zeiss Axioplan 2 fluorescence microscope equipped with rhodamine filters and recorded using a Zeiss Axiocam CCD camera (Zeiss, Heidelberg, Germany). Control experiments were performed by omitting the primary antibody.

When needed, and before blocking nonspecific binding, the sections were incubated for 1 hour at 37°C in the presence of 500 U/mL glycosidase F containing 1% CHAPS to deglycosylate the β-subunit. The slides were rinsed with PBS, and the rest of the procedure was as described above.

A similar approach was used to ascertain the presence of HKα_2 in the apical membrane of the distal colon and that of α_1-Na^+,K^+-ATPase in the basolateral membrane. The only difference in the two procedures was that to identify HKα_2 we used a rhodamine-conjugated antirabbit antibody as a secondary antibody.

Plasma membrane preparation and immunoblot

Plasma membranes were prepared as described previously by our laboratory [6]. The organs (renal medulla, brain, and distal colon) were homogenized in the presence of 27% sucrose in homogenization buffer [10 mmol/L TrisHCl, pH 8.0, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 3 mmol/L benzamidine, and 1 μg/mL soybean trypsin inhibitor]. After removal of the nuclei at low speed centrifugation, the supernatant was applied to the top of a sucrose cushion (45%) and centrifuged at 200,000*g* for 45 minutes at 4°C. The interphase between 45/27% sucrose was collected, diluted 10-fold with homogenization buffer in the absence of sucrose, and concentrated at 20,000*g* for 30 minutes at 4°C. The membranes were resuspended in a small volume of homogenization buffer. Protein concentration was determined using the Lowry method [11], and the proteins were stored at −70° until used.

Before performing the immunoblot, membranes (50 μg) were deglycosylated with glycosidase F (cat. # P0704S; New England Biolabs, Beverly, MA, USA) following the instructions of the manufacturer. The deglycosylated proteins were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane, and probed with specific polyclonal antibodies against β_1 (cat. # 06–170; Upstate Biotechnology, Lake Placid, NY, USA) or probed with a polyclonal antibody against β_3 (cat. # 06–817; Upstate Biotechnology). The bands were visualized using an enhanced chemiluminescence (ECL) system.

Immunoblots were performed with anti-HKα_2 antibody in transiently transfected human embryonic kidney HEK-293 cells with HKα_2 plus β_1 or HKα_2 plus β_3, or in control cells transfected with pcDNA alone. HEK-293 cells were scraped and rinsed two times with 50 mL PBS at 4°C. The cells were then resuspended in 4 mL buffer (0.5 mmol/L $MgCl_2$, 10 mmol/L TrisHCl, pH 8.0, 1 mmol/L PMSF, 3 mmol/L benzamidine, and 1 μg/mL soybean trypsin inhibitor) [12] at 4°C. Homogenization was accomplished with a Dounce homogenizer (cat. # 1984–10015, pestle B, 25 strokes; Bellco Glass Co., Vineland, NJ, USA). This was followed by addition of 4 mL of the same buffer, but without $MgCl_2$ and containing 50% sucrose (w/w), and the cells were homogenized

a second time. The sample was applied to the top of a 45% (w/w) sucrose cushion in buffer A without $MgCl_2$. The sample was centrifuged for 45 minutes at 4°C at 200,000*g*. The interphase between 25/45% sucrose was collected, diluted with 30 mL of homogenization buffer without $MgCl_2$, and membranes were collected by centrifugation at 20,000*g* for 30 minutes at 4°C. The pellet was resuspended and the protein concentration was measured using the Lowry method [11].

Cloning of different subunits in pcDNA

To perform transient transfections of HEK-293 cells, HKα_2 was digested with EcoRI/XbaI from pAGA#2 [13], and cloned into the same sites of pcDNA3.1(+)-Neo (cat. # V790-20; Invitrogen, Carlsbad, CA, USA). Using similar strategy β_1 and β_3 were cloned in pcDNA3.1(+)-Zeo (cat. # V870-20; Invitrogen). These manipulations allowed transfer of the complete open reading frame of the different subunits to pcDNA3.1(+), as well as the transfer of the Kozak consensus sequence from pAGA#2 [13, 14].

Cloning of HKα_2 pEGFP-2C vector

The pAGA#2 vector containing the complete cDNA for HKα_2 was digested overnight with XhoI plus PvuI, followed by partial digestion with EcoRI. The insert (3821 bp) was cloned into EcoRI/SalI of the vector pEGFP-2C (cat. # 6083–1; Clontech, Palo Alto, CA, USA). This construct allowed generation of transient transfections of HKα_2 fused to fluorescent green protein at the amino-terminus (EGFP-HKα_2).

Cell culture and transient transfections

HEK-293 cells were grown in the presence of Dulbecco's modified Eagle's medium (DMEM) (cat. # 12100–046; Invitrogen) containing newborn calf serum (10%) (cat. # 16010–167; Invitrogen), supplemented with penicillin (10 U/mL) and streptomycin (10 μg/mL) (cat. # P-0906; Sigma, St. Louis, MO, USA) and adjusted to pH 7.4 by addition of $NaHCO_3$ (7.5%), as described previously by our laboratory [15, 16]. Cells were grown to 80% to 90% confluence at 37°C in a humidified environment in 24-well dishes (cat. # 50628; Nalge Nunc, Naperville, IL, USA). Transfections were performed using the Lipofectamine PLUS system (cat. # 10964–013; Invitrogen), and cells were grown to 80% to 90% confluency. Transfection was accomplished by mixing circular plasmid DNA (0.3 μg) with 1.5 mL PLUS and 57.5 μL serum-free medium. The mixture was incubated for 15 minutes at room temperature and added to 380 μL serum-free medium containing Lipofectamine (2.2 μL). The final mixture was added to HEK-293 cells and incubated for 3 hours at 37°C. Complete medium (1 mL) was added and incubated for 24 hours at 37°C in a humidified environment containing 5% CO_2. The medium was changed and 2 days later the experiments were performed. $^{86}Rb^+$-uptake experiments were performed as described previously by our laboratory [15].

$^{86}Rb^+$-uptake experiments in HEK-293 cells

HEK-293 cells were grown to confluency at 37°C in a humidified environment in 24-well dishes. Before the assay, the cells were washed four times (1.5 mL/each) with buffer A (145 mmol/L NaCl, 1 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 2 mmol/L Na_2HPO_4, 1 mmol/L $CaCl_2$, 100 μmol/L bumetamide, 32 mmol/L HEPES, pH 7.4) at 37°C, and then equilibrated for 15 minutes with the same buffer. The buffer was removed and replaced with fresh buffer A containing ouabain at different concentrations (see figure legends). After 15 minutes, the solution was aspirated and replaced by 250 μL of the corresponding solution containing $^{86}Rb^+$ (3–8 $\times$ 10^6 cpm). The reaction was allowed to proceed for 15 minutes at 37°C. The buffer was aspirated and washed five times with 1.5 mL of buffer B (100 mmol/L $MgCl_2$, 10 mmol/L HEPES, pH = 7.4) at 4°C. Cells were dissolved by addition of 400 to 450 μL buffer C (0.1 mol/L NaOH, 2% SDS) at 65°C for 30 minutes. Resuspended cells (450 μL) were used to determine $^{86}Rb^+$-uptake (200 μL) and protein concentration (100 μL) [11].

Fluorescence microscopy with enhanced green fluorescence protein at the amino terminus of HKα_2

HEK-293 cells, growing in 3.5-cm dishes (cat. # 08–772-20; Fisher, Madison, WI, USA), were cotransfected with EGFP-HKα_2 plus β_1, EGFP-HKα_2 plus β_3, or EGFP-HKα_2 plus pcDNA (no insert) following the protocol described above. Two days later, the cells were washed once with 5 mL of PBS (10 mmol/L Na-phosphate, pH 7.4 containing 150 mmol/L NaCl), incubated for 15 minutes with 2 mL paraformaldehyde solution (0.1 mol/L K-phosphate, 4% paraformaldehyde, 11% sucrose, pH 7.2) and washed twice with 5 mL PBS. One drop of mounting media (10% 0.5 mol/L Na-phosphate, pH 8.0, 90% glycerol, 2% n-propyl gallate) was added and the cells covered with one cover slip (cat. # 12–542-B; Fisher).

Fluorescence scanning microscopy was performed on a Zeiss LSM 510 microscope, and images were collected at 8-bit resolution using a 63 $\times$ 1.4 numerical aperture (n.a.) objective.

Antibodies

A polyclonal antibody against rat HKα_2 was raised in our laboratory [8]. Monoclonal antibody against α_1-Na^+,K^+-ATPase, monoclonal antibody against β_1-Na^+,K^+-ATPase, and the polyclonal antibody against β_1-Na^+,K^+-ATPase were purchased commercially (cats. #05–369, 05–382, and 06–170, respectively; Upstate

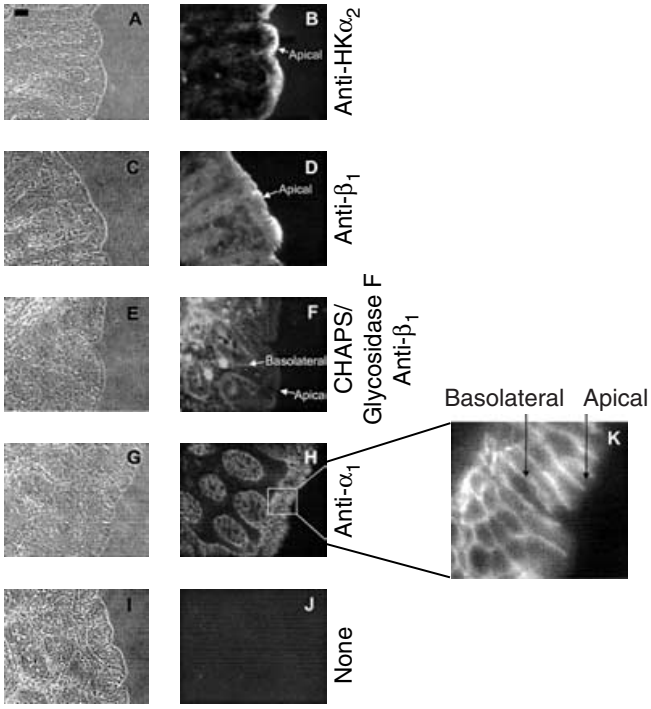

Fig. 1. β_1-Na^+,K^+-ATPase and HKα_2 localize to the apical membrane of the distal colon. Immunolocalization experiments were performed as described in **Methods**. Panels (*A*, *C*, *E*, *G*, and *I*) are bright field phase contrast images and demonstrate that antibody tissue structure was well preserved. Panel (*B*) displays immunolocalization using the anti-HKα_2 antibody. Panel (*D*) shows immunolocalization with the anti-β_1 antibody Na^+,K^+-ATPase. Panel (*F*) shows immunolocalization with the anti-β_1 antibody is slices pretreated with CHAPS and glycosidase F. Panel (*H*) shows immunolocalization with the anti-α_1 Na^+,K^+-ATPase antibody. A section of panel H is amplified in panel (*K*). In panel (*J*), the primary antibody was omitted. The bar signifies 20 μm.

Biotechnology). Rhodamine-conjugated affinity-purified goat antimouse IgG and rhodamine-conjugated affinity-purified goat antirabbit IgG were purchased from Jackson Immunoresearch (cat. # 115–025–146 and 111–025–144, respectively).

RESULTS

HKα_2 and β_1-Na^+,K^+-ATPase are expressed in the apical membrane of distal colon

We performed the experiments displayed in Figure 1 to determine if HKα_2 and β_1-Na^+,K^+-ATPase are expressed in apical membranes of distal colon, as predicted by immunoprecipitation studies [6]. The panels on the left (Fig. 1A, C, E, G, and I) correspond to the bright field phase contrast appearance of the different sections used, and demonstrate that the structure of the tissue was well preserved in all preparations. The panels on the right (Fig. 1B, D, F, H, and J) show the immunofluorescent results with the different antibodies. Figure 1B demonstrates that HKα_2 was expressed in the apical membrane of the distal colon, a finding in keeping with observations from other laboratories [17]. Figure 1D demonstrates that β_1-Na^+,K^+-ATPase was also recognized by the monoclonal antibody against β_1-Na^+,K^+-ATPase in the apical membrane of the distal colon. However, with this antibody, labeling was observed only weakly in the basolateral membrane, where β_1-Na^+,K^+-ATPase is established to be expressed abundantly [2]. Nevertheless, after preincubation with a combination of glycosidase F (5000 U/mL)/CHAPS (1%) for 1 hour at 37°C before incubation with the monoclonal antibody, β_1-Na^+,K^+-ATPase was clearly evident to be localized to the basolateral membrane. As an additional series of control experiments, we performed immunolocalization using the anti-β_1-Na^+,K^+-ATPase antibody with glycosidase F alone, with CHAPS alone, or with CHAPS plus glycosidase F. Glycosidase F alone did not enhance labeling on the basolateral membrane, CHAPS alone induced the most marked enhancement in labeling, and CHAPS plus glucosidase F resulted in the clearest delineation.

Figure 1H (and enlarged Fig. 1K) demonstrates that α_1-Na^+,K^+-ATPase (used as a control) is expressed in the basolateral membrane, as expected [2], but not in the apical membrane. In Figure 1J, no primary antibody was used.

Immunolocalization experiments using the anti-β_3-Na^+,K^+-ATPase antibody could not be performed satisfactorily. We consistently observed high background, and could not differentiate plasma membrane versus intracellular immunostaining. However, as shown below, the same anti-β_3-Na^+,K^+-ATPase antibody could be used successfully to define expression of β_3 protein in renal medulla and distal colon by immunoblot analysis when the samples were deglycosylated with glycosidase F.

An alternative approach was used to define the potential role of β_3-Na^+,K^+-ATPase in β-subunit assembly with HKα_2 in the distal colon. We performed comparative experiments in HEK-293 cells cotransfected with HKα_2 plus β_1-Na^+,K^+-ATPase or HKα_2 plus β_3-Na^+,K^+-ATPase and measured cell surface localization and $^{86}Rb^+$-uptake in transiently transfected HEK-293 cells. We reasoned that the physiologic β-subunit should support activity of HKα_2 more efficiently. A similar approach has been used by Geering et al [9].

β_1 and β_3 proteins are expressed in plasma membranes of distal colon

Plasma membranes from rat renal medulla, brain, and distal colon (50 μg each) were deglycosylated, as described in **Methods**, with glycosidase F and resolved on a 10% SDS-PAGE. The protein was transferred to a nitrocellulose membrane and blotted against a polyclonal antibody that recognized rat β_1 (Fig. 2, top) or a polyclonal antibody that recognized rat β_3 (Fig. 2, bottom). The results demonstrated that both β_1 and β_3 are present in renal medulla, distal colon, and brain (used as a positive control).

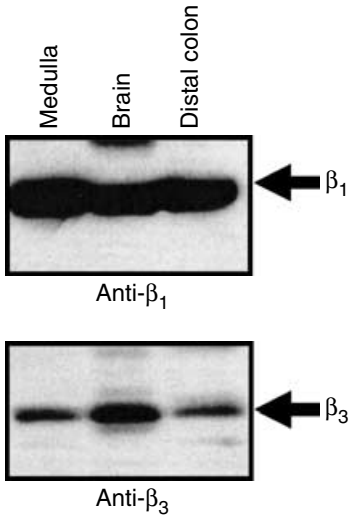

Fig. 2. β_1 and β_3 proteins are expressed in the renal medulla and in distal colon. Fifty micrograms of rat plasma membrane from renal medulla, brain, and distal colon were deglycosylated with glycosidase F, resolved on a 10% SDS-PAGE, transferred to a nitrocellulose membrane and probed with specific antibodies against rat β_1 or rat β_3. The experiment was performed two times with similar results.

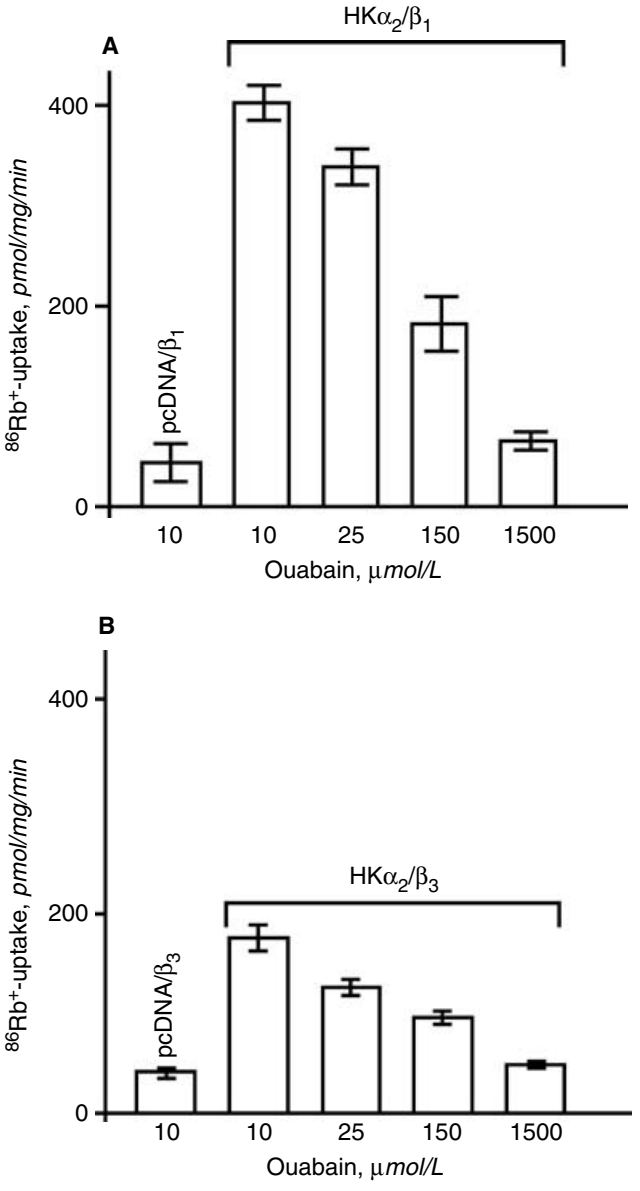

Fig. 3. $^{86}Rb^+$-uptake in human embryonic kidney HEK-293 cells transiently cotransfected with $HK\alpha_2/\beta_1$ or $HK\alpha_2/\beta_3$ is partially sensitive to ouabain. The experiment was performed as described in **Methods**, and in the presence of 10 μmol/L ouabain to block the endogenous Na^+-pump of the HEK-293 cells. The results on the ordinate (bars) are displayed as mean values for $^{86}Rb^+$ uptake (pmol/mg/min), ± SEM. The values plotted on the abscissa represent concentrations of ouabain, as indicated. The experiment was repeated 10 times with similar results. Both experiments were performed using the same pool of HEK-293 cells. Transfections and $^{86}Rb^+$-uptake were performed using the same reagents. This approach was taken to minimize group variation and to allow comparison of the results shown in the top panel with those shown in the bottom panel. Observe that the $HK\alpha_2/\beta_1$ (*A*) complex is more efficient than the $HK\alpha_2\beta_3$ (*B*) complex, as indicated by $^{86}Rb^+$-uptake. In control cells (cotransfected with pcDNA/β_1 or pcDNA/β_3, an increase in ouabain concentration from 10 μmol/L to 2 mmol/L did not result in a decrease of basal $^{86}Rb^+$-uptake, data not shown).

$^{86}Rb^+$-uptake by HEK-293 transiently cotransfected $HK\alpha_2/\beta_1$ or $HK\alpha_2/\beta_3$

Preliminary experiments (data not shown) demonstrated that $^{86}Rb^+$-uptake in HEK-293 cells was partially inhibited by ouabain concentrations as low as 10 nmol/L, and inhibition was complete at 1 μmol/L (IC_{50}~0.16 μmol/L). In order to test $HK\alpha_2$ functionality, HEK-293 cells in culture were cotransfected transiently with pcDNA3.1(+) plus β_1 or $HK\alpha_2/\beta_1$. $^{86}Rb^+$-uptake was performed three days later in the presence of low concentrations of ouabain (10 μmol/L). At this concentration, ouabain blocked endogenous Na^+,K^+-ATPase, but did not block the activity of $HK\alpha_2$ [12, 18, 19]. Figure 3A demonstrates that transient cotransfection of $HK\alpha_2$ plus β_1-Na^+,K^+-ATPase produced a dramatic increase in $^{86}Rb^+$-uptake, as expected (compare the first and second bars from the left). The activity was blocked by ouabain in a dose-dependent manner.

A similar experiment was performed by transiently transfecting HEK-293 with $HK\alpha_2$ plus β_3-Na^+,K^+-ATPase on the same day and using the same reagents. The results of a representative experiment are displayed in Figure 3B. Cotransfection with $HK\alpha_2$ plus β_3 (second bar from the left) resulted in an increase in $^{86}Rb^+$-uptake compared to controls cotransfected with pcDNA plus β_3 (first bar from the left). $^{86}Rb^+$-uptake in cells cotransfected with $HK\alpha_2/\beta_3$ was inhibited by ouabain in a dose-dependent manner. The results demonstrate that $^{86}Rb^+$-uptake was more efficient when HEK-293 cells were cotransfected with $HK\alpha_2$ plus β_1 compared to $HK\alpha_2$ plus β_3.

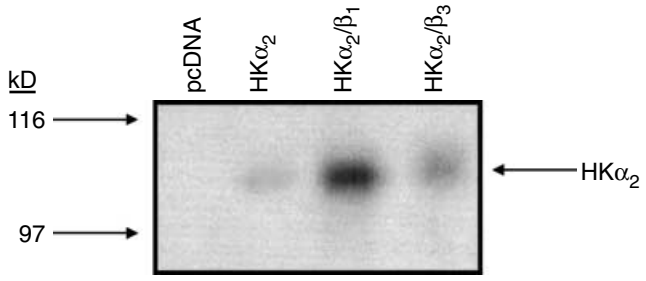

Fig. 4. HKα_2 is expressed at higher levels in HEK-293 cells when cotransfected with β_1 versus β_3. The experiments were performed as described in **Methods**. The immunoblot was performed with anti-HKα_2 (1:2000). The molecular weights are indicated at the left. The plasmids used in each transfection are indicated at the top of the autoradiograph.

We also investigated the potential effect of using different plasmid preparations. In all studies cells transfected with HKα_2 plus β_1 revealed a higher level of $^{86}Rb^+$-uptake than cells transfected with HKα_2 plus β_3.

Expression of HKα_2 protein in HEK-293 cells transiently transfected with HKα_2 plus β_1 vs. HKα_2 plus β_3

We transiently transfected HEK-293 cells with pcDNA, HKα_2 alone, HKα_2 plus β_1, or HKα_2 plus β_3 to test the efficiency of β_1 and β_3 in protecting HKα_2 against degradation. Three days after transfection, the cells were scrapped, washed with PBS, and lysed as described in **Methods**. Fifty micrograms of protein was resolved on SDS-PAGE, transferred to a nitrocellulose membrane, and probed with our anti-HKα_2 antibody (1:1000). The results displayed in Figure 4 demonstrate that the HKα_2 band was more intense when HEK-293 cells were cotransfected with HKα_2 plus β_1 than when the cells were cotransfected with HKα_2 plus β_3. These observations are compatible with the data displayed in Figure 3, and suggest that transport of $^{86}Rb^+$ was consistently higher in the HKα_2/β_1 group compared to the HKα_2/β_3 group.

Fluorescence microscopy of HEK-293 cells cotransfected with HKα_2 plus β_1 or HKα_2 plus β_3

Intracellular localization of HKα_2/β_1 was performed using fluorescence microscopy (Fig. 5). HEK-293 cells were cotransfected with EGFP-HKα_2 plus β_1 (top) or EGFP-HKα_2 plus pcDNA (bottom). In cells cotransfected with EGFP-HKα_2 plus β_1, the majority of the protein appeared in the plasma membrane (indicated by arrows). However, when the cells were cotransfected with EGFP-HKα_2 plus pcDNA, the protein accumulated in intracellular compartments and was not detected in the plasma membrane.

Similarly, we performed transient cotransfections of HEK-293 cells with EGFP-HKα_2 plus β_3. The results of a representative experiment are displayed in Figure 5 (middle). The results demonstrate EGFP-HKα_2 does not migrate efficiently to the plasma membrane when cotransfected with β_3 (compare with top and middle panels).

DISCUSSION

The colonic H^+,K^+-ATPase plays a central role in the regulation of K^+ absorption by the distal colon [20–23] and kidney [24]. However, a precise definition of the specific β-subunit that assembles with the α-subunit in vivo has been contradictory. In the present study, we used immunolocalization experiments to demonstrate for the first time that β_1-Na^+,K^+-ATPase is expressed in apical membranes of rat distal colon, the same membrane to which HKα_2 has been localized. Our results also demonstrate that β_1-Na^+,K^+-ATPase is expressed in the basolateral membrane, as expected [2]. The immunolocalization experiments also suggest that the epitope recognized by the antibody is more readily accessible in the apical than in the basolateral membrane.

Therefore, these studies were extended to utilize HEK-293 cells as an expression system to enable an investigation of the functional properties of HKα_2 when coexpressed with either β_1-Na^+,K^+-ATPase or β_3-Na^+,K^+-ATPase (the two β-subunits expressed in distal colon). The rat β_1-Na^+,K^+-ATPase was selected because in the present study we demonstrated that β_1 immunolocalized to the apical membrane of the distal colon. Additionally, in previous studies, using oocytes from *Xenopus laevis* as a heterologous expression system, we found that HKα_2 assembles stably with β_1-Na^+,K^+-ATPase [19] and supported $^{86}Rb^+$-uptake. Assembly between HKα_2 and β_1-Na^+,K^+-ATPase was also observed in coimmunoprecipitation studies using an antibody that was specifically directed against HKα_2 when membranes from renal medulla and distal colon were used [6, 7]. The rat β_3-Na^+,K^+-ATPase was chosen because it is expressed in distal colon (Fig. 2), and there has been one report suggesting that β_3-Na^+,K^+-ATPase could serve as the physiologic β-subunit for HKα_2 [10].

Using the Na^+-pump as a model, previous studies suggested that the different β-subunit isoforms could confer different K^+-activation kinetics on Na^+,K^+-ATPase. For example, coexpression of the *Bufo marinus* α_1-Na^+,K^+-ATPase with the *B. marinus* β_1-Na^+,K^+-ATPase, or β_3-Na^+,K^+-ATPase or rabbit β_G resulted in different K^+-activation kinetics for the various holoenzymes. The α_1/β_G enzyme functioned as a Na^+,K^+ pump with a much lower apparent affinity for K^+, both in the presence and absence of external Na^+, compared to α_1/β_1 or α_1/β_3 pumps [25]. From this study it was concluded that *B. marinus* α_1-Na^+,K^+-ATPase was employing β_1- or β_3-Na^+,K^+-ATPase as a physiologic β-subunit. Using similar criteria, through analysis of $^{86}Rb^+$-uptake and plasma membrane localization of HKα_2, the present

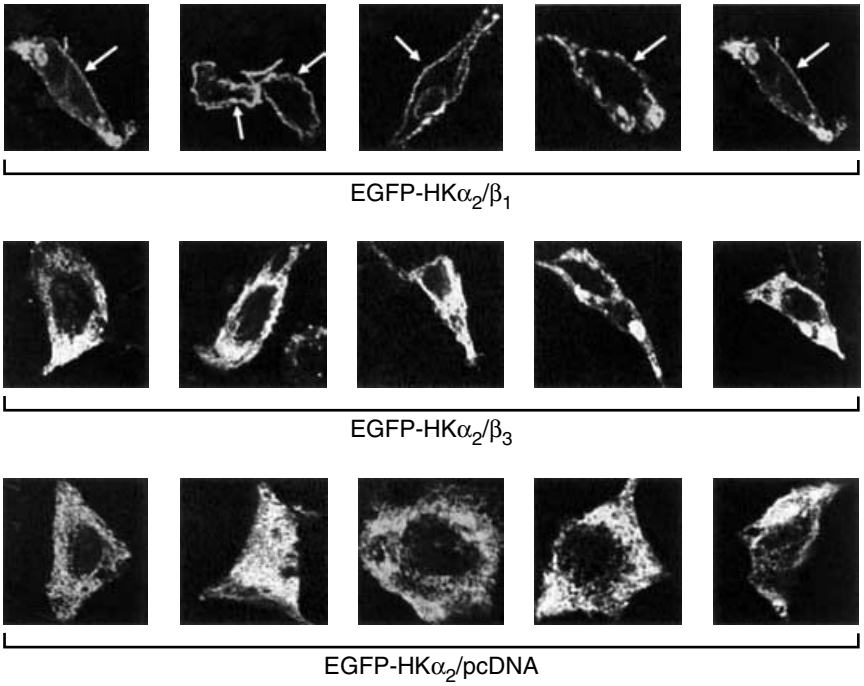

Fig. 5. Fluorescence microscopy of HEK-293 cells transiently cotransfected with EGFP-HKα_2/β_1 or EGFP-HKα_2/β_3. Top panel, HEK-293 cells were transiently cotransfected with EGFP-HKα_2 plus β_1 and fluorescence was measured three days later as described in **Methods**. The arrows demonstrate that HKα_2 localizes to the plasma membrane. The middle panel shows the same as in the top panel but the cells were transiently cotransfected with EGFP-HKα_2 plus β_3-Na^+,K^+-ATPase. Bottom panel, the cells were cotransfected with EGFP-HKα_2. Observe the requirement of β_1-Na^+,K^+-ATPase for HKα_2 translocation to the plasma membrane. The experiment was performed three times with similar results.

study provides novel evidence that β_1-Na^+,K^+-ATPase is more efficient than β_3-Na^+,K^+-ATPase in supporting HKα_2 function. Therefore, we conclude that β_1-Na^+,K^+-ATPase functions as the ideal β-subunit for HKα_2 in vitro. Such results are compatible with previous observations of stable assembly in a plasma membrane fraction enriched in HKα_2 derived from distal colon in vivo [6].

CONCLUSION

The present studies show that either β_1 or β_3-Na^+,K^+-ATPase assembles indiscriminately with HKα_2, and that each heterodimer is functional and partially sensitive to ouabain. Nevertheless, the results demonstrate that β_1-Na^+,K^+-ATPase localizes to the apical membrane of distal colon cells. Moreover, $^{86}Rb^+$-uptake is much more robust when coexpressed with β_1-Na^+,K^+-ATPase as compared to β_3-Na^+,K^+-ATPase. Furthermore, HKα_2/β_1 was translocated more efficiently to the cell surface than HKα_2/β_3. Therefore, these findings are consistent with coimmunoprecipitation experiments performed previously by our laboratory and support the view that β_1 Na^+,K^+-ATPase fulfills all necessary prerequisites to function as the physiologic β-subunit for assembly, translocation to the apical membrane, and function of HKα_2.

ABBREVIATIONS

X^+,K^+-ATPase, superfamily of proteins composed by the colonic H^+,K^+-ATPase, gastric H^+,K^+-ATPase, and the Na^+,K^+-ATPases; α_1, α_1-Na^+,K^+-ATPase; HKα_2, α-subunit of the colonic H^+,K^+-ATPase; β_1, β_1-subunit of the Na^+,K^+-ATPase; β_3, β_3-subunit of the Na^+,K^+-ATPase; EGFP-HKα_2, HKα_2 with the green fluorescent protein cloned in frame at the amino-terminus of HKα_2; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; bp = base pair.

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