The effect of β-subunit assembly on function and localization of the colonic H\textsuperscript{+},K\textsuperscript{+}-ATPase α-subunit

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

**Background.** Previous experiments from our laboratory have demonstrated that HKα\textsubscript{2} coimmunoprecipitated with β\textsubscript{1}-Na\textsuperscript{+}.K\textsuperscript{+}-ATPase. Although HKα\textsubscript{2} is expressed abundantly in the apical membrane of distal colon, the demonstration that β\textsubscript{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 HKα\textsubscript{2} and a monoclonal antibody against β\textsubscript{1}.

**Results.** The results demonstrate that HKα\textsubscript{2} localizes to the apical membrane. Two pools of β\textsubscript{1},Na\textsuperscript{+}.K\textsuperscript{+}-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\textsubscript{1} localizes to the apical membrane with HKα\textsubscript{2}, and supports the view that β\textsubscript{1} is the physiologic β-subunit for HKα\textsubscript{2}. We tested, therefore, the efficiency of the two β-subunits expressed in distal colon (β\textsubscript{1} and β\textsubscript{3}) to support the activity of HKα\textsubscript{2}. Human embryonic kidney HEK-293 cells were transiently cotransfected with HKα\textsubscript{2} plus β\textsubscript{1} or HKα\textsubscript{2} plus β\textsubscript{3}. Subsequently, \textsuperscript{86}Rb\textsuperscript{+}-uptake and plasma membrane localization were evaluated. The results demonstrate that both HKα\textsubscript{2}/β\textsubscript{1} and HKα\textsubscript{2}/β\textsubscript{3} support \textsuperscript{86}Rb\textsuperscript{+}-uptake. However, \textsuperscript{86}Rb\textsuperscript{+}-uptake measured in the cells cotransfected with HKα\textsubscript{2} plus β\textsubscript{1} exceeded that measured in cells expressing HKα\textsubscript{2}/β\textsubscript{3}. Fluorescence microscopy using enhanced green fluorescent protein cloned at the amino-terminus of HKα\textsubscript{2} demonstrated protein migration to the plasma membrane in cells cotransfected with EGFP-HKα\textsubscript{2} plus β\textsubscript{1}. In contrast, in cells cotransfected with EGFP-HKα\textsubscript{2} plus β\textsubscript{3}, the vast majority of the protein remained confined to intracellular compartments. The significantly higher \textsuperscript{86}Rb\textsuperscript{+}-uptake corresponded to additional localization of HKα\textsubscript{2} to the plasma membrane when coexpressed with β\textsubscript{1} compared to β\textsubscript{3}.

**Conclusion.** Taken together, these and previous results from our laboratory indicate that β\textsubscript{1},Na\textsuperscript{+}.K\textsuperscript{+}-ATPase is likely to represent the most physiologic and efficient subunit for HKα\textsubscript{2} assembly in distal colon.

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

Through application of a highly specific antibody against HKα\textsubscript{2} in coimmunoprecipitation experiments, we and others [6, 7] have demonstrated that HKα\textsubscript{2} assembles with β\textsubscript{1} Na\textsuperscript{+}.K\textsuperscript{+}-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-HKα\textsubscript{2} antibody was preincubated with the immunizing peptide, or when the primary antibody was omitted. Therefore, we concluded that β\textsubscript{1},Na\textsuperscript{+}.K\textsuperscript{+}-ATPase was the physiologic β-subunit for HKα\textsubscript{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 HKα\textsubscript{2}) has not yet been identified. In these studies, the stability of ATP1AL1 was

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

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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 α1-Na⁺,K⁺-ATPase/β1-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 β3-Na⁺,K⁺-ATPase could be the physiologic β-subunit for HKα₂. Therefore, uncertainty regarding which β-subunit functions as the physiologic β-subunit has endured to the present.

It is widely accepted that β₁-Na⁺,K⁺-ATPase localizes, in most cells and tissues, to the basolateral membrane, forming a complex with α₁-Na⁺,K⁺-ATPase [2]. However, if β₁-Na⁺,K⁺-ATPase functions as the physiologic β-subunit for HKα₂ 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 β₁-Na⁺,K⁺-ATPase serves as the physiologic β-subunit for HKα₂ in the distal colon. Distal colon was chosen because HKα₂ and β₁-Na⁺,K⁺-ATPase are expressed abundantly in this tissue.

METHODS

**Immunolocalization of β₁-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 (10 mmol/L Na-phosphate, 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°C 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 β₁ (cat. # 06–170; Upstate Biotechnology, Lake Placid, NY, USA) or probed with a polyclonal antibody against β₃ (cat. # 06–817; Upstate Biotechnology). The bands were visualized using an enhanced chemiluminescence (ECL) system.

Immunoblots were performed with anti-HKα₂ antibody in transiently transfected human embryonic kidney HEK-293 cells with HKα₂ plus β₁ or HKα₂ plus β₃, 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 MgCl₂, 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 MgCl₂ 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 MgCl2. The sample was centrifuged for 45 minutes at 4°C at 200,000g. The interphase between 25/45% sucrose was collected, diluted with 30 mL of homogenization buffer without MgCl2, and membranes were collected by centrifugation at 20,000g 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, HKα2 was digested overnight with XhoI plus PvuI, and cloned into the same sites of pcDNA3.1(+) Neo (cat. # V790-20; Invitrogen, Carlsbad, CA, USA). Using similar strategy β1 and β3 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 HKα2 pEGFP-2C vector

The pAGA#2 vector containing the complete cDNA for HKα2 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 HKα2 fused to fluorescent green protein at the amino-terminus (EGFP-HKα2).

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. # 10010–167; 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 NaHCO3 (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 Lipofectamine PLUS system (cat. # 10964–013; Invitrogen), and cells were grown to 80% to 90% confluence. 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% CO2. The medium was changed and 2 days later the experiments were performed. 86Rb+-uptake experiments were performed as described previously by our laboratory [15].

86Rb+-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 MgSO4, 2 mmol/L Na2HPO4, 1 mmol/L CaCl2, 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 86Rb+ (3–8 × 106 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 MgCl2, 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 86Rb+-uptake (200 µL) and protein concentration (100 µL) [11].

Fluorescence microscopy with enhanced green fluorescence protein at the amino terminus of HKα2

HEK-293 cells, growing in 3.5-cm dishes (cat. # 08–772-20; Fisher, Madison, WI, USA), were cotransfected with EGFP-HKα2 plus β1, EGFP-HKα2 plus β3, or EGFP-HKα2 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 × 1.4 numerical aperture (n.a.) objective.

Antibodies

A polyclonal antibody against rat HKα2 was raised in our laboratory [8]. Monoclonal antibody against α1-Na+,K+-ATPase, monoclonal antibody against β1-Na+,K+-ATPase, and the polyclonal antibody against β1-Na+,K+-ATPase were purchased commercially (cats. #05–369, 05–382, and 06–170, respectively; Upstate
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

HK_{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 HK_{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 images and demonstrate that antibody tissue structure was well preserved. Panel (B) displays immunolocalization using the anti-HK_{2} antibody. Panel (D) shows immunolocalization with the anti-β_{1} antibody. 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.

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 HK_{2} in the distal colon. We performed comparative experiments in HEK-293 cells cotransfected with HK_{2} plus β_{1}-Na^{+},K^{+}-ATPase or HK_{2} plus β_{3}-Na^{+},K^{+}-ATPase and measured cell surface localization and {superscript 86}Rb^{+}-uptake in transiently transfected HEK-293 cells. We reasoned that the physiologic β-subunit should support activity of HK_{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).
**86Rb**⁺-uptake by HEK-293 transiently cotransfected HKα₂/β₁ or HKα₂/β₃

Preliminary experiments (data not shown) demonstrated that **86Rb**⁺-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 (IC₅₀~0.16 µmol/L). In order to test HKα₂ functionality, HEK-293 cells in culture were cotransfected transiently with pcDNA3.1(+) plus β₁ or HKα₂/β₁. **86Rb**⁺-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 HKα₂ [12, 18, 19]. Figure 3A demonstrates that transient cotransfection of HKα₂ plus β₁-Na⁺,K⁺-ATPase produced a dramatic increase in **86Rb**⁺-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 transflecting HEK-293 with HKα₂ plus β₃-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 HKα₂ plus β₃ (second bar from the left) resulted in an increase in **86Rb**⁺-uptake compared to controls cotransfected with pcDNA plus β₃ (first bar from the left). **86Rb**⁺-uptake in cells cotransfected with HKα₂/β₃ was inhibited by ouabain in a dose-dependent manner. The results demonstrate that **86Rb**⁺-uptake was more efficient when HEK-293 cells were cotransfected with HKα₂ plus β₁ compared to HKα₂ plus β₃.

![Fig. 2. β₁ and β₃ 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 β₁ or rat β₃. The experiment was performed two times with similar results.](image)

![Fig. 3. **86Rb**⁺-uptake in human embryonic kidney HEK-293 cells transiently cotransfected with HKα₂/β₁ or HKα₂/β₃ 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 **86Rb**⁺ 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 **86Rb**⁺-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 HKα₂/β₁ (A) complex is more efficient than the HKα₂/β₃ (B) complex, as indicated by **86Rb**⁺-uptake.](image)
We also investigated the potential effect of using different plasmid preparations. In all studies cells transfected with HKα2 plus β1 revealed a higher level of 86Rb⁺ uptake than cells transfected with HKα2 plus β3.

Expression of HKα2 protein in HEK-293 cells transiently transfected with HKα2 plus β1 vs. HKα2 plus β3

We transiently transfected HEK-293 cells with pcDNA, HKα2 alone, HKα2 plus β1, or HKα2 plus β3 to test the efficiency of β1 and β3 in protecting HKα2 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-HKα2 antibody (1:1000). The results displayed in Figure 4 demonstrate that the HKα2 band was more intense when HEK-293 cells were cotransfected with HKα2 plus β1 than when the cells were cotransfected with HKα2 plus β3. These observations are compatible with the data displayed in Figure 3, and suggest that transport of 86Rb⁺ was consistently higher in the HKα2/β1 group compared to the HKα2/β3 group.

Fluorescence microscopy of HEK-293 cells cotransfected with HKα2 plus β1 or HKα2 plus β3

Intracellular localization of HKα2/β1 was performed using fluorescence microscopy (Fig. 5). HEK-293 cells were cotransfected with EGFP-HKα2 plus β1 (top) or EGFP-HKα2 plus pcDNA (bottom). In cells cotransfected with EGFP-HKα2 plus β1, the majority of the protein appeared in the plasma membrane (indicated by arrows). However, when the cells were cotransfected with EGFP-HKα2 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-HKα2 plus β3. The results of a representative experiment are displayed in Figure 5 (middle). The results demonstrate EGFP-HKα2 does not migrate efficiently to the plasma membrane when cotransfected with β3 (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 β1-Na⁺,K⁺-ATPase is expressed in apical membranes of rat distal colon, the same membrane to which HKα2 has been localized. Our results also demonstrate that β1-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 HKα2 when coexpressed with either β1-Na⁺,K⁺-ATPase or β3-Na⁺,K⁺-ATPase (the two β-subunits expressed in distal colon). The rat β1-Na⁺,K⁺-ATPase was selected because in the present study we demonstrated that β1 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 HKα2 assembles stably with β1-Na⁺,K⁺-ATPase [19] and supported 86Rb⁺-uptake. Assembly between HKα2 and β1-Na⁺,K⁺-ATPase was also observed in immunoprecipitation studies using an antibody that was specifically directed against HKα2 when membranes from renal medulla and distal colon were used [6, 7]. The rat β3-Na⁺,K⁺-ATPase was chosen because it is expressed in distal colon (Fig. 2), and there has been one report suggesting that β3-Na⁺,K⁺-ATPase could serve as the physiologic β-subunit for HKα2 [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 α₁-Na⁺,K⁺-ATPase with the B. marinus β₁-Na⁺,K⁺-ATPase, or β3-Na⁺,K⁺-ATPase or rabbit βG resulted in different K⁺-activation kinetics for the various holoenzymes. The α₁/βG 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 α₁/β1 or α₁/β3 pumps [25]. From this study it was concluded that B. marinus α₁-Na⁺,K⁺-ATPase was employing β₁- or β3-Na⁺,K⁺-ATPase as a physiologic β-subunit. Using similar criteria, through analysis of 86Rb⁺-uptake and plasma membrane localization of HKα2, the present
study provides novel evidence that β₁-Na⁺,K⁺-ATPase is more efficient than β₃-Na⁺,K⁺-ATPase in supporting HKα₂ function. Therefore, we conclude that β₁-Na⁺,K⁺-ATPase functions as the ideal β-subunit for HKα₂ in vitro. Such results are compatible with previous observations of stable assembly in a plasma membrane fraction enriched in HKα₂ derived from distal colon in vivo [6].

CONCLUSION

The present studies show that either β₁ or β₃-Na⁺,K⁺-ATPase assembles indiscriminately with HKα₂, and that each heterodimer is functional and partially sensitive to ouabain. Nevertheless, the results demonstrate that β₁-Na⁺,K⁺-ATPase localizes to the apical membrane of distal colon cells. Moreover, ⁸⁸Rb⁺-uptake is much more robust when coexpressed with β₁-Na⁺,K⁺-ATPase as compared to β₃-Na⁺,K⁺-ATPase. Furthermore, HKα₂/β₁ was translocated more efficiently to the cell surface than HKα₂/β₃. Therefore, these findings are consistent with coimmunoprecipitation experiments performed previously by our laboratory and support the view that β₁-Na⁺,K⁺-ATPase fulfills all necessary prerequisites to function as the physiologic β-subunit for assembly, translocation to the apical membrane, and function of HKα₂.

ABBREVIATIONS

X⁺,K⁺-ATPase, superfamily of proteins composed by the colonic H⁺,K⁺-ATPase, gastric H⁺,K⁺-ATPase, and the Na⁺,K⁺-ATPases; α₁, α₁-Na⁺,K⁺-ATPase; HKα₂, α-subunit of the colonic H⁺,K⁺-ATPase; β₁, β₁-subunit of the Na⁺,K⁺-ATPase; β₃, β₃-subunit of the Na⁺,K⁺-ATPase; EGFP-HKα₂/β₁, EGFP-HKα₂/β₃, EGFP-HKα₂/pcDNA.

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Fig. 5. Fluorescence microscopy of HEK-293 cells transiently cotransfected with EGFP-HKα₂/β₁ or EGFP-HKα₂/β₃. Top panel, HEK-293 cells were transiently cotransfected with EGFP-HKα₂ plus β₁ and fluorescence was measured three days later as described in Methods. The arrows demonstrate that HKα₂ localizes to the plasma membrane. The middle panel shows the same as in the top panel but the cells were transiently cotransfected with EGFP-HKα₂ plus β₃-Na⁺,K⁺-ATPase. Bottom panel, the cells were cotransfected with EGFP-HKα₂. Observe the requirement of β₁-Na⁺,K⁺-ATPase for HKα₂ translocation to the plasma membrane. The experiment was performed three times with similar results.

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