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The Colonic H^+,K^+-ATPase Functions as a Na^+-dependent $K^+(NH_4^+)$-ATPase in Apical Membranes from Rat Distal Colon*

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Recent studies have suggested that the colonic H^+,K^+-ATPase ($HK\alpha_2$) can secrete either Na^+ or H^+ in exchange for K^+. If correct, this view would indicate that the transporter could function as either a Na^+ or a H^+ pump. To investigate this possibility a series of experiments was performed using apical membranes from rat colon which were enriched in colonic H^+,K^+-ATPase protein. An antibody specific for $HK\alpha_2$ was employed to determine whether $HK\alpha_2$ functions under physiological conditions as a Na^+-dependent or Na^+-independent K^+-ATPase in this same membrane fraction. K^+-ATPase activity was measured as [γ-^{32}P]ATP hydrolysis. The Na^+-dependent K^+-ATPase accounted for approximately 80% of overall K^+-ATPase activity and was characterized by insensitivity to Sch-28080 but partial sensitivity to ouabain. The Na^+-independent K^+-ATPase activity was insensitive to both Sch-28080 and ouabain. Both types of K^+-ATPase activity substituted NH_4^+ for K^+ in a similar manner. Furthermore, our results demonstrate that when incubated with native distal colon membranes, the blocking antibody inhibited dramatically Na^+-dependent K^+-ATPase activity. Therefore, these data demonstrate that $HK\alpha_2$ can function in native distal colon apical membranes as a Na^+-dependent K^+-ATPase. Elucidation of the role of the pump as a transporter of Na^+ *versus* H^+ or NH_4^+ *versus* K^+ *in vivo* will require additional studies.

Both the distal colon and renal medulla participate importantly in K^+ homeostasis (1). A unique H^+,K^+-ATPase ($HK\alpha_2$)[1] was cloned by Crowson and Shull (2) from a rat distal colon library, which was distinct at the amino acid level from both α_1-Na^+,K^+-ATPase (73% similarity) and from gastric H^+,K^+-ATPase (72% similarity). It has been demonstrated, using the *Xenopus laevis* oocyte as an expression system, that $HK\alpha_2$ can internalize Rb^+ (K^+) in exchange for H^+ when coexpressed with any known X^+,K^+-ATPase β-subunit (3–5). Furthermore, $HK\alpha_2$ was insensitive to Sch-28080, a specific inhibitor of the gastric H^+,K^+-ATPase, but partially sensitive to ouabain (IC_{50} ~ 400–600 μM) (3, 4), a specific inhibitor of the Na^+,K^+-ATPase.

$HK\alpha_2$ mRNA and protein are expressed in low abundance in the renal medulla (2, 6, 7). However, $HK\alpha_2$ mRNA and protein abundance in the kidney are dramatically augmented by chronic dietary K^+-depletion (6, 8–10). This finding indicates that $HK\alpha_2$ may play a major role in renal K^+ conservation. Furthermore, the site of this regulatory response to chronic hypokalemia has been shown to be the renal medulla (10). Based on data obtained using heterologous expression systems it has been predicted that the increase in K^+-reabsorption in the collecting tubule during chronic hypokalemia would be insensitive to Sch-28080 *in vivo*. Nevertheless, several laboratories which have evaluated HCO_3^- reabsorption during chronic hypokalemia have defined H^+,K^+-ATPase function by its sensitivity to Sch-28080. For example, Wall *et al.* (11) and Nakamura *et al.* (12) have demonstrated that the increase in bicarbonate absorption (J_{tCO_2}) observed in the medullary collecting duct during chronic hypokalemia is inhibited by Sch-28080 in low concentration (10 μM). Sensitivity to low concentrations of Sch-28080 is characteristic of the gastric H^+,K^+-ATPase (13). Nevertheless, it has been suggested that in the inner medullary collecting tubule $HK\alpha_2$ may become sensitive to low concentrations of Sch-28080 during chronic hypokalemia (12).

Cougnon *et al.* (14) demonstrated that $HK\alpha_2$ can also function to secrete Na^+ in exchange for K^+. These findings were supported by studies from Grishin and Caplan (15), which demonstrated that HEK-293 cells co-transfected with human ATP1AL1 (90% similar to $HK\alpha_2$) and the rabbit β-subunit of the gastric H^+,K^+-ATPase, grow in the presence of ouabain. This observation suggests that in transfected cells, during inhibition of the native Na^+ pump by ouabain, ATP1AL1 which is relatively insensitive to ouabain (16, 17), functions as a Na^+ pump. A possible physiological role for the rat $HK\alpha_2$ or the human ATP1AL1, as apical Na^+ pumps, is difficult to envision. Moreover, that these findings were obtained using heterologous expression systems, raises concern regarding the possibility that a similar function might not exist in native membranes.

High levels of expression of $HK\alpha_2$ have been reported in apical membranes from rat distal colon (10) where the protein has also been identified by immunolocalization (9). Taking advantage of this observation, we prepared apical membranes from rat distal colon to determine: (*a*) if under physiological conditions $HK\alpha_2$ is sensitive to low concentrations of Sch-28080, and (*b*) if $HK\alpha_2$ function, here defined as K^+-ATPase enzymatic activity, is Na^+-dependent or Na^+-independent.

Our results demonstrate that these membranes contain both Na^+-dependent and Na^+-independent ATPase activities. Using an inhibitory antibody specific for $HK\alpha_2$, we demonstrate that

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[1] The abbreviations used are: $HK\alpha_1$, α-subunit of the gastric H^+,K^+-ATPase; $HK\alpha_2$, α-subunit of the colonic H^+,K^+-ATPase; β_1, β-subunit of the Na^+,K^+-ATPase; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

this pump is responsible for Na^+-dependent activity. This finding indicates that the colonic H^+,K^+-ATPase, which has been shown to function as a proton pump, may also function as a sodium pump under certain physiological conditions. Furthermore, we also demonstrate that both Na^+-dependent and Na^+-independent activities can substitute readily NH_4^+ for K^+.

EXPERIMENTAL PROCEDURES

Preparation of Plasma Membranes (Method I)

To prepare plasma membranes (18, 19) rat distal colon (1 g of tissue) was homogenized using a Brinkmann Polytron, Model PT 10/35, followed by homogenization in a Dounce homogenizer using pestle A (4–5 strokes). The homogenization was performed in 10 ml of buffer A (10 mM Tris-HCl, pH 8.0, 1 mM EDTA-Tris, 1 mM phenylmethylsulfonyl fluoride, 3 mM benzamidine, and 1 μg/ml soybean trypsin inhibitor) containing 27% sucrose (w/v). Nuclei were removed by centrifugation at 2000 $\times$ *g* for 4 min at 4 °C, the supernatant was applied to the top of 45% (w/w) sucrose in buffer A and centrifuged at 200,000 $\times$ *g* for 45 min at 4 °C. The membranes in the interphase 27/45% sucrose were diluted in buffer A and collected by centrifugation at 25,000 $\times$ *g*, resuspended in buffer A, and stored in aliquots at −70 °C. The final protein concentration was measured using the method of Lowry *et al.* (20).

Preparation of Apical Membranes (Method II)

Apical membranes from distal colon were prepared as described by Aronson (21). Distal colon (1 g in 10 ml of buffer B: 300 mM mannitol, 1 mM Tris-HCl, pH 7.2, 1 mM phenylmethylsulfonyl fluoride, 3 mM benzamidine, and 1 μg/ml soybean trypsin inhibitor) was homogenized with a Polytron, followed by 4–5 strokes with pestle A of a Dounce homogenizer. The particulate matter was removed by centrifugation for 2 min at 200 $\times$ *g*. To the supernatant, 1 M $MgSO_4$ was added to a final concentration of 10 mM $MgSO_4$. The sample was placed on ice for 15 min and was shaken intermittently. The aggregated material was removed by centrifugation for 12 min at 2500 $\times$ *g* at 4 °C, and apical membranes from the supernatant were collected by centrifugation at 27,000 $\times$ *g* for 20 min at 4 °C. The pellet was resuspended in 5 ml of buffer B containing 10 mM $MgSO_4$, and homogenized with pestle B of a Dounce homogenizer. After removal of the aggregated material at 3100 $\times$ *g* for 12 min at 4 °C, membranes were collected by centrifugation at 27,000 $\times$ *g* for 20 min at 4 °C. The final membranes were resuspended in 10 mM Tris-HCl, pH 7.2, 1 mM EDTA-Tris, 1 mM phenylmethylsulfonyl fluoride, 3 mM benzamidine, and 1 μg/ml soybean trypsin inhibitor and stored in aliquots at −70 °C. The protein concentration was measured using the method of Lowry *et al.* (20).

Preparation of Vesicle Membranes (Method III)

Distal colon was homogenized with a Polytron in 3 volumes of iced 0.25 M sucrose, 1 mM EDTA, as described previously in our laboratory (22). The homogenate was filtered through 500-μm pore size mesh nylon and centrifuged at 8,000 $\times$ *g*. The supernatant was retained and centrifuged at 200,000 $\times$ *g*. The pellet was resuspended in 250 mM sucrose, 6 mM histidine, pH 7.0, and recentrifuged at 200,000 $\times$ *g* for 30 min at 4 °C. The new pellet was resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and stored at −70 °C. The protein concentration was measured using the method of Lowry *et al.* (20) and immunoblots were performed using a specific antibody against HKα_2 (10).

ATPase Assays

All ATPase assays were performed for 30 min at 37 °C in a final volume of 200 μl containing 30 mM Tris-HCl, pH 7.2, 1 mM EDTA-Tris, 0.1 mM EGTA-Tris, 4 mM $MgCl_2$, 3 mM ATP-Tris containing 1–10 $\times$ 10^6 cpm of [γ-^{32}P]ATP (Amersham Pharmacia Biotech, catalog number AA0068), 1 mM *N*-ethylmaleimide, 10 μg/ml oligomycin, 1 mM phenylmethylsulfonyl fluoride, 3 mM benzamidine, 1 μg/ml soybean trypsin inhibitor and, when necessary, ouabain, Sch-28080, NaCl, and/or KCl were added (see figure legends for the concentrations of ouabain, Sch-28080, NaCl, and KCl). The reaction was started by addition of rat distal colon apical membranes (5–15 μg) diluted in 10 mM Tris-HCl, pH 7.2. The reaction was stopped by addition of activated charcoal (1 ml, 50% slurry) (Fisher Scientific, catalog number C170-500) in 10 mM Na^+-phosphate, pH 7.5. The samples were vortexed, cooled in ice, centrifuged, and the supernatant (450 μl) was used to quantify the ^{32}P released during the incubation.

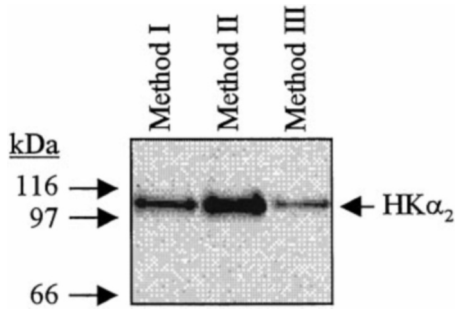

FIG. 1. **Distal colon apical membranes are enriched in HKα_2.** Membranes prepared according to Methods I, II, or III (20 μg) were resolved on a 10% SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with anti-HKα_2 (dilution 1:1000). The presence of the protein was determined using an ECL system.

K^+-ATPase Activity in the Presence or Absence of Anti-HKα_2 Antibody

Group A—Serum (15 μl) containing the anti-HKα_2 antibody was mixed with the immunizing peptide (10 μl) (1.5 mM) to inactivate the antibody (19), the antibody/synthetic peptide mixture was incubated for 1 h at 4 °C. After incubation, distal colon apical membranes (100–200 μg) were added to the mixture and incubated for 1 h at 4 °C with occasional vortexing. The membranes were diluted with 10 mM Tris-HCl, pH 7.2, to a final concentration of 0.5–1.5 μg of protein/μl and 20 μl of the diluted membranes were used in the ATPase assays.

Group B—H_2O (10 μl) was mixed with serum (15 μl) containing the anti-HKα_2 antibody. After 1 h incubation at 4 °C, rat distal colon apical membranes (100–200 μg) were added and incubated for 1 additional hour at 4 °C. The membranes were then diluted with 10 mM Tris-HCl, pH 7.2, to a final concentration of 0.5–1.5 μg of protein/μl. Finally, immunizing peptide (10 μl) was added. A graphic representation of this assay is shown in the *top panel* of Fig. 5. A volume of 20 μl of diluted membranes was used in the ATPase assays.

Other Reagents

The characterization of the antibody against HKα_2 has been described recently by our laboratory (10, 19). Immunoblots were performed as reported previously by our laboratory (10, 19). Harlan Sprague-Dawley male rats (150–200 g) were used in all experiments. Ouabain was purchased from Sigma. Sch-28080 was a gift from Dr. Kaminski at Schering-Plow Research Institute. The anti-NASE and anti-LEAVE antibodies were characterized previously (23). The amino acid alignment between different α-subunits was performed with the "Bestfit" program of the Genetics Computer Group (Madison, WI).

RESULTS

Distal Colon Apical Membranes Are Enriched in HKα_2—As displayed in Fig. 1, plasma (method I), apical (method II), and vesicle (method III) membranes (20 μg/each) were applied to a 10% SDS-PAGE, transferred to a nitrocellulose membrane and incubated with the anti-HKα_2 antibody (dilution 1:1000). As expected (10), of the three methods to prepare membranes the apical membrane method yielded membranes which were most enriched in HKα_2 protein.

K^+-ATPase Activities in Distal Colon Apical Membranes—In heterologous expression systems, the activity of rat HKα_2 has been reported to be K^+-dependent (IC_{50} < 1 mM), Sch-28080 insensitive, and only partially sensitive to ouabain (IC_{50} ~ 400–600 μM) (3, 4). To test which of the different K^+-ATPases observed in apical membranes of distal colon best fits this pharmacological profile, we measured Na^+-dependent and Na^+-independent K^+-ATPase activity in preparations of rat distal colon apical membranes in the presence of KCl (10 mM). The data displayed in Fig. 2 (*left panel*) demonstrate that the predominant K^+-ATPase in distal colon apical membranes was Na^+-dependent and was abolished by ouabain (2 mM), but was insensitive to Sch-28080 (100 μM). This same figure also demonstrates that distal colon apical membranes contain a Na^+-independent K^+-ATPase. The Na^+-independent K^+-ATPase

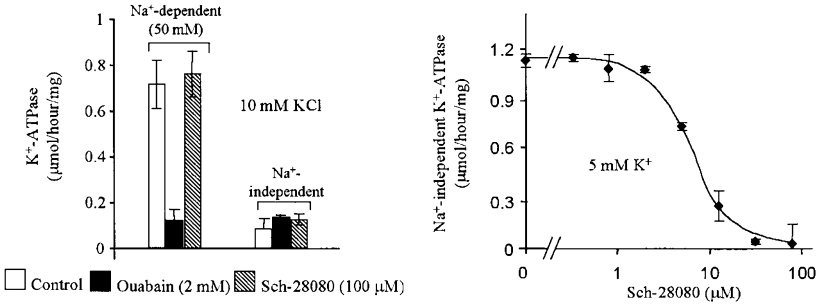

FIG. 2. *Left panel,* rat distal colon apical membranes contain both Na^+-dependent and Na^+-independent K^+-ATPases. The ATPase assay was performed as described under "Experimental Procedures." *Right panel,* rat stomach apical membrane. In contrast to the Sch-28080 insensitivity of both K^+-ATPases in distal colon, Sch-28080 has high affinity for rat gastric H^+,K^+-ATPase ($HK\alpha_1$), as expected. The assay was performed as described under "Experimental Procedures" with the only exception that all Na^+ salts were avoided. α_1, α_1-subunit of the Na^+,K^+-ATPase.

represents only 20% of the total Na^+-dependent K^+-ATPase activity (at 10 mM KCl), and is insensitive to both ouabain (2 mM) and Sch-28080 (100 μM). Treatment of the membrane preparations with CHAPS or Triton X-100 did not alter the response to Sch-28080 or ouabain for either Na^+-dependent or Na^+-independent K^+-ATPase fractions. Since Sch-28080 did not inhibit either K^+-ATPase activity in apical membranes from distal colon, we tested if the Na^+-independent K^+-ATPase ("H^+,K^+-ATPase") in apical membranes of rat stomach which are enriched in $HK\alpha_1$ is sensitive to Sch-28080. The *right panel* of Fig. 2 demonstrates that concentrations of Sch-28080 as low as 10 μM inhibit 80–90% of the Na^+-independent K^+-ATPase activity in the presence of K^+ (5 mM).

Based on the observation that many transporters of K^+, including the α_1-Na^+,K^+-ATPase, can transport either K^+ or NH_4^+ with similar affinity (24–26), we characterized the ion dependence of the K^+-ATPases present in our preparation of distal colon apical membranes. In Fig. 3 (*left panel*) apical membranes were incubated in the presence of NaCl (20 mM) and with increasing concentrations of KCl (*closed diamonds*) or NH_4Cl (*closed squares*). Na^+-dependent K^+-ATPase activity increased with increasing concentrations of K^+ and reached saturation at concentrations near 5 mM. Substitution of K^+ by NH_4 also induced an increase in Na^+-dependent NH_4^+-ATPase in a concentration-dependent manner. The V_m for K^+ and NH_4^+ are similar. However, the enzyme had a higher affinity for K^+ ($IC_{50} \sim 0.2$ mM) than for NH_4^+ ($IC_{50} \sim 2$ mM). The affinity for Na^+ was identical ($IC_{50} \sim 5$ mM) whether K^+ or NH_4^+ was used in the ATPase assay (Fig. 3, *middle panel*). Finally we determined if K^+ can be replaced by NH_4^+ in the Na^+-independent K^+-ATPase fraction. The results of a representative experiment are displayed in Fig. 3 (*right panel*). The Na^+-independent K^+-ATPase activity displayed a low affinity for K^+ (activity was not detectable until $[K^+]$ was >2.5 mM, $IC_{50} > 10$ mM, and did not reach saturation until 40 mM $[K^+]$). Similar results were obtained when K^+ was replaced by NH_4^+.

The activity of Na^+-dependent K^+-ATPase in apical membranes was inhibited by high concentrations of ouabain ($IC_{50} \sim$ 200–300 μM). Replacement of K^+ by NH_4^+ did not alter this inhibitory profile (Fig. 4). Furthermore, Sch-28080 did not inhibit K^+-ATPase activity when K^+ was replaced by NH_4^+ (data not shown).

The studies shown above (Figs. 2–4) demonstrate that apical membranes, which are enriched in $HK\alpha_2$ protein, display pharmacological properties which are virtually indistinguishable from α_1-Na^+,K^+-ATPase (27, 28). To exclude the possibility that we were studying α_1-Na^+,K^+-ATPase activity (which are contaminating the membranes preparation) rather than the activity of $HK\alpha_2$, we took advantage of two observations made by our laboratory: (*a*) our anti-$HK\alpha_2$ antibody can immunoprecipitate the $HK\alpha_2/\beta_1$-Na^+,K^+-ATPase complex in both the renal medulla and distal colon (19) and, (*b*) this same anti-$HK\alpha_2$ antibody does not cross-react or immunoprecipitate α_1-Na^+-K^+-ATPase or any other X^+,K^+-ATPase characterized thus far (19). We asked whether the anti-$HK\alpha_2$ antibody could block Na^+-dependent K^+-ATPase activity. To answer this question, the anti-$HK\alpha_2$ antibody (15 μl) was incubated in the presence (group A) or absence of immunizing peptide (500 μM) (group B) for 1 h at 4 °C (10). Incubation was followed by addition of membranes (100 μg) to each group. The mixture was then incubated for 1 h at 4 °C. Membranes were diluted to 0.5 μg/μl in the presence of 10 mM Tris-HCl, pH 7.2, and incubated at 37 °C for 30 min as described under "Experimental Procedures." In group B the immunizing peptide was added after dilution of the membranes. This approach was taken to correct for possible interference of the peptide with the ATPase assay. A schematic representation of this protocol is displayed in Fig. 5 (*top panel*). Fig. 5 (*middle panel*), demonstrates that incubation of apical membranes with the anti-$HK\alpha_2$ antibody markedly decreased Na^+-dependent K^+-ATPase activity (*closed bar*). A similar experiment was performed by substituting K^+ for NH_4^+ (Fig. 5, *lower panel*). Similar Na^+-dependent $K^+(NH_4^+)$-ATPase activity was observed in the presence of either K^+ or NH_4^+ (5 mM) under control conditions. Na^+-dependent NH_4^+-ATPase activity was blocked by the anti-$HK\alpha_2$ antibody, exactly as observed in the presence of K^+. As a control we repeated the experiment described in Fig. 5, but with plasma membranes isolated from rat renal cortex. These membranes, in contrast to distal colon membranes, are depleted of $HK\alpha_2$ but are enriched in α_1-Na^+,K^+-ATPase. The result of such an experiment is displayed in Fig. 6. This finding demonstrates that Na^+,K^+-ATPase activity in the renal cortex is not inhibited by the anti-$HK\alpha_2$ antibody. Thus, the anti-$HK\alpha_2$ antibody does not cross-react with a membrane fraction enriched in α_1-Na^+,K^+-ATPase.

To confirm further that $HK\alpha_2$ functions as a Na^+-dependent K^+-ATPase in apical membranes of distal colon origin we performed an additional experiment described in Fig. 7. In this study Na^+-dependent K^+-ATPase activity was measured in plasma membranes from renal cortex as well as apical membranes from distal colon. We reasoned that if the $HK\alpha_2$ functions in distal colon as a Na^+-dependent K^+-ATPase, more α_1-subunit from the renal cortex would be required to reach the same level of Na^+-dependent K^+-ATPase activity present in the distal colon apical membranes. Fig. 7 (*left panel*) demonstrates that both membrane fractions contain similar specific activities (expressed as nanomole of ATP hydrolyzed/hour) at any given concentration of total protein. However, the absolute level of α_1-Na^+,K^+-ATPase was much greater in renal cortex compared with distal colon (*right panel* of Fig. 7). This study was performed with two different antibodies, anti-NASE which recognizes only α_1-Na^+,K^+-ATPase and anti-LEAVE which

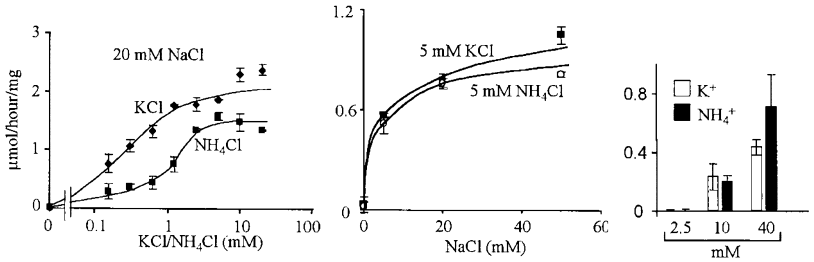

FIG. 3. *Left panel,* the Na^+-dependent K^+-ATPase has high affinity for both K^+ and NH_4^+. The assay was performed in the presence of NaCl (20 mM) with increasing concentrations of KCl or NH_4Cl. *Center panel,* $HK\alpha_2$ has similar affinity for Na^+ in the presence of either K^+ or NH_4^+. The assay was performed in the presence of KCl (5 mM) or NH_4Cl (5 mM) with increasing concentrations of NaCl. *Right panel,* the Na^+-independent K^+-ATPase fraction can employ either K^+ or NH_4^+ but at relatively low affinities. The assay was performed in the absence of Na^+.

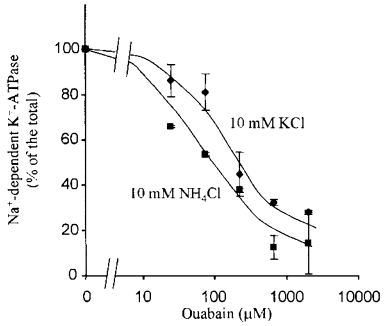

FIG. 4. **Na^+-dependent K^+-ATPase in the apical membrane has a low affinity for ouabain.** The effect of ouabain was measured in the presence of KCl (10 mM) or NH_4Cl (10 mM) plus NaCl (20 mM).

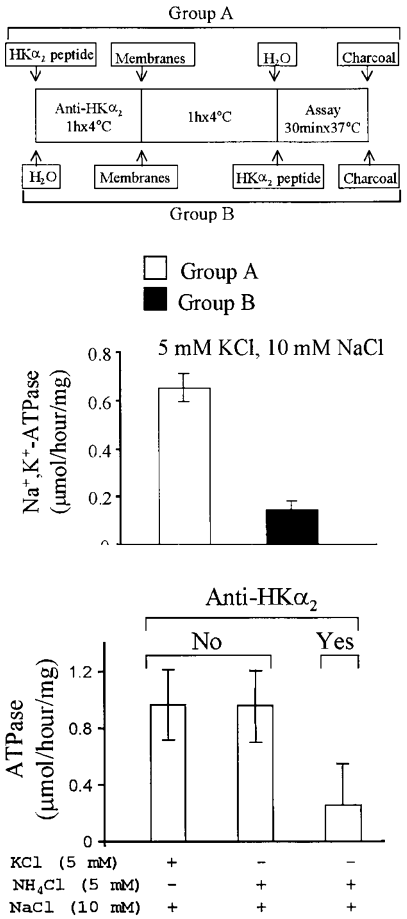

FIG. 5. *Top panel,* schematic representation of the protocol to determine if the Na^+-dependent K^+-ATPase activity in distal colon apical membranes represented $HK\alpha_2$. For differences in two groups (A and B) see "Experimental Procedures." In group A (controls) the immunizing peptide was added prior addition of the anti-$HK\alpha_2$ antibody and membranes to block recognition of $HK\alpha_2$ protein in the membrane by the antibody. Therefore, only the group B protocol allowed exposure of the native $HK\alpha_2$ to the anti-$HK\alpha_2$ antibody. *Middle panel,* the Na^+-dependent K^+-ATPase of $HK\alpha_2$ was blocked by the anti-$HK\alpha_2$ antibody. *Bottom panel,* the Na^+-dependent K^+-ATPase was blocked by the anti-$HK\alpha_2$ antibody when K^+ was replaced by NH_4^+.

should recognize α_1-, α_2-, and α_3-Na^+,K^+-ATPase (23). The observation that the immunoblots for both the distal colon and renal cortex membranes using either the anti-NASE and anti-LEAVE antibodies were indistinguishable, demonstrates that neither α_2- nor α_3-Na^+,K^+-ATPase accounts for the Na^+-independent K^+-ATPase activity in distal colon apical membranes. These findings provide additional evidence that the distal colon apical membrane fraction is enriched in $HK\alpha_2$ protein and functions as a Na^+-dependent K^+-ATPase which is distinct from α_1-Na^+,K^+-ATPase activity.

DISCUSSION

Our studies demonstrate that plasma membranes from distal colon, which are enriched severalfold in $HK\alpha_2$ protein, contain a K^+-ATPase activity which is Na^+-dependent, Sch 28080-insensitive, and partially ouabain-sensitive. To conclude that this activity represents functionally the α-subunit of the colonic H^+,K^+-ATPase, is contingent on the specificity of our anti-$HK\alpha_2$ antibody. Previous studies by our laboratory have demonstrated that the antibody used in the present study does not cross-react with any of the known X^+,K^+-ATPases (10, 19). Nevertheless, alternative explanations for this activity which were evaluated in the course of this study include all known members of the X^+,K^+-ATPase superfamily. The antibody used in these studies was raised against a synthetic peptide designed after the rat colonic H^+,K^+-ATPase the epitope of which (amino acids 686–698) is not found on any other known rat X^+,K^+-ATPases. Furthermore, there is no evidence that another H^+,K^+-ATPase or Na^+,K^+-ATPase, except $HK\alpha_2$ or α_1-Na^+,K^+-ATPase, exists in distal colon (Fig. 7). Moreover, in this, and in a previous study (10, 19), we have demonstrated that this antibody does not cross-react or immunoprecipitate other known X^+,K^+-ATPases, including the α_1-Na^+,K^+-ATPase. In addition, we now demonstrate that our anti-$HK\alpha_2$ antibody blocks Na^+-dependent K^+-ATPase activity (Fig. 5), that Na^+,K^+-ATPase activity in the renal cortex is not inhibited by the specific blocking antibody (Fig. 6), and finally that neither α_2 nor α_3-Na^+,K^+-ATPase could account for the activity in the distal colon apical membrane fraction (Fig. 7). Therefore, we conclude that $HK\alpha_2$ can function in distal colon as a Na^+-dependent K^+-ATPase.

Precedent exists for Na^+ secretion by the $HK\alpha_2$. Cougnon *et al.* (14) has demonstrated recently that $HK\alpha_2$ can secrete Na^+ in exchange for K^+ in *X. laevis* oocytes. In this study it was reported that Na^+/K^+ exchange, which was sensitive to high concentrations of ouabain, and was totally insensitive to Sch-28080, was dependent on co-expression with a β-subunit. Moreover, Kone and Higham (29) reported recently that a splice

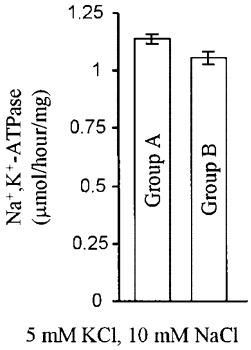

FIG. 6. **The anti-HKα_2 antibody does not block Na$^+$,K$^+$-ATPase activity in plasma membranes from renal cortex.** The assay was performed exactly as described in the legend to Fig. 5.

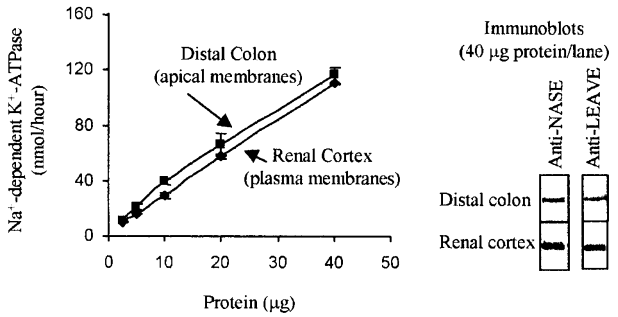

FIG. 7. **The Na$^+$-dependent K$^+$-ATPase activity in apical membranes from distal colon cannot be accounted for by α_1-Na$^+$,K$^+$-ATPase.** *Left panel,* Na$^+$-dependent K$^+$-ATPase activity was similar in both membranes at any concentration of membranes used. The assay was performed in the presence of KCl (10 mM) and in the presence or absence of NaCl (50 mM). *Right panel,* abundance of α-subunits of Na$^+$,K$^+$-ATPases in renal cortex exceeds abundance in distal colon.

variant of HKα_2 (HKα_{2b}), which is truncated by the initial 103 amino acids, supported the growth of HEK-293 cells in the presence of low concentrations of ouabain. In addition, Grishin *et al.* (17), expressed the human ATP1AL1 (90% similar to HKα_2) in HEK-293 cells, and observed that the ratio of H$^+$-secretion to K$^+$-uptake was approximately 1:10. Based on these observations he postulated that ATP1AL1 did not function solely as a H$^+$/K$^+$ exchanger. This group also reported that ATP1AL1 supported the growth of HEK-293 cells in the presence of ouabain (15). Since HEK-293 cells transfected with ATP1AL1 or HKα_{2b} grow in the presence of ouabain (15, 29), it is logical to speculate that both the rat HKα_2 and the human ATP1AL1 may function as Na$^+$ pumps. However, since these results were obtained in heterologous expression systems, questions could be raised regarding the relevance of these results to native colonic apical membranes.

It is appreciated generally that Na$^+$,K$^+$-ATPases are localized to basolateral membranes (30). In the present study, and from previous studies by our laboratory (10) it has been demonstrated that HKα_2 is enriched in apical membrane fractions from distal colon. Sangan *et al.* (8) have also demonstrated that HKα_2 protein localizes to the apical membrane of colonocytes. Moreover, Jaisser *et al.* (31) have demonstrated by *in situ* hybridization that HKα_2 mRNA localizes to surface epithelial cells of rat distal colon. Assigning a physiological role for a Na$^+$-dependent K$^+$-ATPase located in the apical membrane of colonocytes is difficult, however.

Del Castillo *et al.* (32) reported that two types of Na$^+$-independent K$^+$-ATPases are present in apical membranes from distal colon: one which is sensitive to ouabain (1 mM), and another which is insensitive to ouabain (1 mM). These K$^+$-ATPases were not present in basolateral membranes. The effect of Sch-28080 (a standard inhibitor of the gastric H$^+$,K$^+$-ATPase) was not tested. Lee *et al.* (33) reported that in preparations of "apical" membranes from distal colon, both activities (ouabain-insensitive and ouabain-sensitive K$^+$-ATPases) were inhibited by an antibody directed against the amino-terminal of HKα_2. Recently, Rajendran *et al.* (34) reported that Na$^+$-independent, K$^+$-dependent pH$_i$ recovery by rat colonocytes was ouabain-insensitive (up to 1 mM). Based on this observation, it was concluded that HKα_2 functions in colonocytes as a Na$^+$-independent, ouabain-insensitive K$^+$-ATPase.

In agreement with these findings, both Na$^+$-dependent and Na$^+$-independent K$^+$-ATPases were detected in the present study, the Na$^+$-dependent fraction, which predominated under the conditions of the assay, was relatively ouabain-sensitive. In contrast, the less abundant Na$^+$-independent fraction was ouabain-insensitive. Based on the present study it is reasonable to conclude that HKα_2 may function in native apical membranes not only as a proton pump, but as a Na$^+$ pump. Nevertheless, the physiological conditions which might serve to regulate Na$^+$/K$^+$ as opposed to H$^+$/K$^+$ exchange have not been defined. Therefore, future studies will be needed to define the relative contributions of these pumps in the distal colon as well as factors which regulate their abundance and function in physiologic and pathophysiologic conditions.

An additional finding in the present study was that the K$^+$-ATPase activity in distal colon membranes also had high affinity for NH_4^+ (Figs. 3–5). Transport of NH_4^+ by the colonic H$^+$,K$^+$-ATPase in kidney has been suggested in preliminary studies previously (35, 36). Our findings are the first to suggest that substitution of K$^+$ by NH_4^+ may occur in distal colon apical membranes. Nevertheless, the physiological role of H$^+$/NH_4^+ exchange by the colonic H$^+$,K$^+$-ATPase in either kidney or distal colon has not yet been defined clearly.

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