Na,K-ATPase activity has been identified in the apical membrane of rat distal colon, whereas ouabain-sensitive and ouabain-insensitive H,K-ATPase activities are localized solely to apical membranes. This study was designed to determine whether apical membrane Na,K-ATPase represented contamination of basolateral membranes or an alternate mode of H,K-ATPase expression. An antibody directed against the H,K-ATPase α subunit (HKα) inhibited apical Na,K-ATPase activity by 92% but did not alter basolateral membrane Na,K-ATPase activity. Two distinct H,K-ATPase isoforms exist; one of which, the ouabain-insensitive HKα, has been cloned. Because dietary sodium depletion markedly increases ouabain-insensitive active potassium absorption and HKα mRNA and protein expression, Na,K-ATPase and H,K-ATPase activities and protein expression were determined in apical membranes from control and sodium-depleted rats. Sodium depletion substantially increased ouabain-insensitive H,K-ATPase activity and HKα protein expression by 109–250% but increased ouabain-sensitive Na,K-ATPase and H,K-ATPase activities by only 30% and 42%, respectively. These studies suggest that apical membrane Na,K-ATPase activity is an alternate mode of ouabain-sensitive H,K-ATPase and does not solely represent basolateral membrane contamination.

H,K-ATPase is present in the apical membranes of the mammalian distal colon and is closely linked to active potassium absorption that most likely is the result of an apical membrane H-K exchange (1). The colonic H,K-ATPase is a member of the gene family of P-type ATPases, which include ouabain-sensitive Na,K-ATPase, gastric parietal cell H,K-ATPase that is ouabain-insensitive, and ATP1AL1 (2–6). Colonic H,K-ATPase consists of α (HKα) and β (HKβ) subunits, which have been identified and characterized, and is up-regulated by dietary sodium depletion and aldosterone (7–9).

Previous studies established that, although colonic H,K-ATPase is exclusively present in apical membranes of the rat distal colon (10), there are two distinct H,K-ATPases. One is ouabain-sensitive; the other is ouabain-insensitive (11). Subsequent investigations revealed that both ouabain-sensitive and ouabain-insensitive H,K-ATPase activities were present in apical membranes isolated from surface epithelial cells, whereas only ouabain-sensitive H,K-ATPase activity and potassium-dependent intracellular alkalization were identified in apical membranes of crypt epithelial cells (12). Because HKα mRNA and protein are selectively present only in surface (and upper 20% of crypt) epithelial cells of the rat distal colon (10, 13), it is likely that HKα only encodes the ouabain-insensitive H,K-ATPase and not the ouabain-sensitive H,K-ATPase. In contrast, HKα cRNA induces ouabain-sensitive 86Rb uptake and intracellular alkalization when expressed in Xenopus oocytes (14–16).

The studies that established the presence of H,K-ATPase in apical membranes of rat distal colon also demonstrated significant Na,K-ATPase activity in apical membranes, which was assumed to represent partial contamination of the apical membranes by basolateral membranes (11). Recently, Cougnon et al. reported that HKα cRNA could express both H-K and Na-K transport functions in Xenopus oocytes (15). As a result, it is not unlikely that our prior identification of Na,K-ATPase activity in apical membranes represented an expression of one or both H,K-ATPases and not basolateral membrane contamination. The present study was designed to explore this possibility by assessing the expression and activity of both H,K-ATPase and Na,K-ATPase and HKα and NaKα1 proteins in apical and basolateral membranes of normal and dietary sodium-depleted rats. These results have been presented in a preliminary communication (17).

EXPERIMENTAL PROCEDURES

Apical Membrane Preparation—Apical membranes were prepared from the distal colon of both normal and sodium-depleted rats (Harlan Sprague-Dawley, 200–250 g) by the method of Stieger et al. (18) as described previously (19). Sodium depletion was produced by feeding a sodium-free diet for 6 to 7 days, as described previously (20). Crude membranes were also prepared from normal rat distal colon. For crude membranes, homogenates of colonocytes were centrifuged at 30,000 × g for 30 min.

Basolateral Membrane Preparation—Basolateral membranes were prepared using the sucrose density gradient centrifugation method of Biber et al. (21), also as described previously (22). All membranes were resuspended in 20 mM Tris-HCl buffer (pH 7.4) containing 250 mM sucrose and 1 mM phenylmethanesulfonyl fluoride. Protein was assayed using the method of Lowry et al. (23).

Enzyme Assays—H,K-ATPase and Na,K-ATPase activities were determined by the method of Forbush et al. (24), as described previously (11). H,K-ATPase activity represents the difference in activity between that determined in the presence and absence of 20 mM K. Na,K-ATPase activity represents the difference in activity between that determined in the presence of both sodium and potassium and H,K-ATPase activity.

In previous studies dietary sodium depletion and aldosterone infusion via mini-pumps produced identical changes of both sodium, chloride, and potassium transport in rat distal colon (34–36). In addition, serum aldosterone levels were similar in the dietary sodium-depleted animals and in those that were infused with aldosterone via minipumps (37). Thus, in the present manuscript, aldosterone is at times used to refer to sodium-depleted animals.

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‡The abbreviations used are: HKα and HKβ, H,K-ATPase subunits α and β; AE2, anion exchange isoform 2.
ATPase activity measured in the presence of 1 mM ouabain represents ouabain-insensitive activity, whereas ouabain-sensitive activity was calculated by subtracting ouabain-insensitive activity from total activity. Preliminary studies indicated that maximal inhibition was achieved at 1 and 3 mM ouabain. Thus, the present study used 1 mM ouabain to distinguish the ouabain-sensitive and ouabain-insensitive components of H,K-ATPase. The specific activities were expressed as nanomoles of Pi liberated per milligram of protein per minute. Results presented represent mean ± s.e. of triplicate assays from at least three different membrane preparations.

### RESULTS

The results of both potassium-activated Mg-ATPase (H,K-ATPase) and Na/K-activated Mg-ATPase (Na,K-ATPase) activities in apical and basolateral membranes prepared from the distal colon of normal rat are presented in Table I. Similar to previous studies (11), H,K-ATPase activity is exclusively present in apical membranes; no H,K-ATPase activity was identified in basolateral membranes. Forty-four percent of total H,K-ATPase activity was ouabain-sensitive, while the remaining activity (56%) was ouabain-insensitive. Although Na,K-ATPase is a well-established basolateral membrane marker (26), Na,K-ATPase activity was present in apical and basolateral membranes in rat distal colon (Table I). As expected, Na,K-ATPase activity in basolateral membranes was completely ouabain-sensitive. Similar to a previous study (11), Na,K-ATPase activity was also identified in apical membranes and was also ouabain-sensitive. There are at least two possible explanations for the presence of ouabain-sensitive Na,K-ATPase activity in apical membranes: contamination of apical membranes by basolateral membranes or an alternate mode of H,K-ATPase expression. The latter would be consistent with the recent observation in Xenopus oocytes of the expression of H,K-ATPase α cRNA (HKα) as sodium-potassium dependent

### TABLE I

| Apical membrane | Basolateral membrane |
|-----------------|----------------------|
| **H,K-ATPase**  | **Na,K-ATPase**      |
| Total           | Ouabain-sensitive   | Ouabain-insensitive |
| 118.6 ± 9.2     | 52.2 ± 4.6           | 66.4 ± 6.1          |
| Na,K-ATPase     | Total                |
| 120.5 ± 19.2    | 122.3 ± 10.3         | -1.8 ± 7.5a         |

a Not detected.

b Not significantly different from zero.

H,K-ATPase activity represents ATPase activity in the presence of Mg2+, plus K+ or Mg2+ plus Na+ and K+. Total H,K-ATPase activity represents ATPase activity in the presence of Mg2+ plus K+, minus that in the presence of Mg2+ alone. Total Na,K-ATPase activity represents the difference between ATPase activity in the presence of Mg2+ plus Na+ and K+, and H,K-ATPase activity. ATPase activities were also measured in the presence of 1 mM ouabain. Activity in the presence of ouabain represents ouabain-insensitive ATPase activity. Ouabain-sensitive H,K-ATPase was determined by subtracting ouabain-insensitive activity from total activity. Mg2+-ATPase activity was 221.7 ± 13.8 and 202.6 ± 9.6 nmol of P32 liberated/mg protein · min in apical and basolateral membranes, respectively. Results represent mean ± S.E. of triplicate assays from three different membrane preparations.
ATPase activity in apical membranes of rat distal colon might represent an alternate mode of H,K-ATPase expression. There is substantial evidence for the presence of two distinct H,K-ATPases in the apical membranes of the rat distal colon; one isoform is ouabain-insensitive, is present primarily in surface cells and has been cloned and identified (5, 9, 10, 12, 13), whereas the other isoform is ouabain-sensitive, is present in both surface and crypt cells, and has not, as yet, been cloned (12). Because dietary sodium depletion and aldosterone differentially enhance the expression of these two isoforms in the rat distal colon (7, 27), the effect of dietary sodium depletion on H,K-ATPase and Na,K-ATPase activities and HKcα and NaKα1 protein expression was, therefore, determined.

Fig. 1. Effect of M1 antibody on H,K-ATPase (●) and Na,K-ATPase (○) activities in apical membranes from normal rat distal colon. H,K-ATPase and Na,K-ATPase activities were measured, as described in the legend to Table I. H,K-ATPase and Na,K-ATPase activities were measured in normal and in M1 antibody-treated apical membranes. Antibody-treated apical membranes were mixed with M1 antibody (30 μg/mL) and incubated at 37 °C for 60 min. Control membranes were mixed with PBS and were similarly incubated. ATPase activities in M1 antibody-treated membranes were also measured in the presence of 1 mM ouabain. Results represent mean ± S.E. of triplicate assays from three different membrane preparations.

Fig. 2. Effect of M1 antibody (10) on Na,K-ATPase activity in basolateral membranes from normal rat distal colon. Na,K-ATPase activity was measured, as described in the legend to Table I. Control and antibody treatment of basolateral membranes was performed as described for apical membranes in the legend to Fig. 1. Na,K-ATPase activity in M1 antibody-treated membrane was also measured in the presence of 1 mM ouabain. Results represent mean ± S.E. of triplicate assays from three different membrane preparations.

Fig. 3. Western blot analysis of apical and basolateral membranes prepared from normal and sodium-depleted rat distal colon by M1 (HKcα) antibody (10). A, blot was stained with M1 antibody (1:1000), followed by anti-rabbit IgG horseradish peroxidase conjugate as secondary antibody, as described under “Experimental Procedures.” B, relative abundance of HKcα protein in apical and basolateral membranes from normal (open bars) and sodium-depleted (cross-hatched bars) rats was quantitated using densitometry. Values represent mean ± S.E. of three experiments.
and B). In contrast, the ouabain-sensitive component of H,K-ATPase activity was enhanced by only 42%. H,K-ATPase activity was not identified in the basolateral membranes of either normal or dietary sodium-depleted rats. In contrast, HKcα protein was also found to be expressed in basolateral membranes of both normal and sodium-depleted animals (Fig. 3, A and B).

The effect of dietary sodium depletion on Na,K-ATPase activity in both apical and basolateral membranes was also examined and is presented in Fig. 7. Only ouabain-sensitive Na,K-ATPase activity was identified in basolateral membranes from either normal or dietary sodium-depleted rats (Fig. 7B). Dietary sodium depletion resulted in only a minimal (10%) increase in ouabain-sensitive Na,K-ATPase activity in basolateral membranes (Fig. 7B). Ouabain-sensitive Na,K-ATPase activity in apical membranes was increased by 30% (Fig. 7A). In contrast to HKcα protein, sodium depletion did not significantly alter the NaKα1 protein expression either in apical or in basolateral membranes (Fig. 4, A and B).

**DISCUSSION**

This study was designed to establish the nature of Na,K-ATPase activity that has been found in the apical membrane of rat distal colon (Ref. 11 and Table I). Na,K-ATPase, or the sodium pump, is a basolateral enzyme that has been frequently used as the standard basolateral membrane enzyme marker (24, 26). At the time that Na,K-ATPase activity was initially reported in the apical membrane (11), there was no acceptable apical membrane marker (18). As a consequence, the presence of Na,K-ATPase in apical membranes was considered secondary to basolateral membrane contamination (11, 17).
critical membranes of rat distal colon and in SfKc (9). Fourth, M1 antibody also inhibited potassium uptake in untransfected cells, M1 antibody identified a membrane-bound protein identified in immunohistochemical studies localized proteins to plasma membranes and cytoplasm only in SfKc (10). In contrast, M1 antibody did not inhibit NaK-ATPase activity in apical membranes and not in basolateral membranes (10), it is likely that the presence of HKc protein identified in basolateral membranes represents contamination from apical membranes. The failure to establish the presence of H,K-ATPase activity in basolateral membranes is probably due to the different techniques used to prepare apical and basolateral membranes (19, 22). This possibility is based on the recent observations that anion exchange isoform 2 (AE2)-specific protein (28) and anion exchange (Cl-HCO3) activities (29) were not detected in basolateral membranes prepared from kidney and colon, respectively, in the absence of protease inhibitors. In contrast, AE2 protein and Cl-HCO3 exchange activity were readily identified in basolateral membranes prepared in the presence of protease inhibitors. Because the method to prepare colonic basolateral membranes does not usually include protease inhibitors, we suspect that, similar to AE2 protein and Cl-HCO3 exchange activity, Na,K-ATPase was also denatured with loss of activity during the basolateral membrane preparation.

The demonstration that apical membrane Na,K-ATPase activity does not solely represent basolateral membrane contamination required studies to explain the function and origin of Na,K-ATPase in the apical membrane. Recent experiments with 86Rb uptake in Xenopus oocyes using HKc cRNA provided evidence that H,K-ATPase can be expressed both as an H-K exchange and as an Na-K exchange (15). Thus, there was a possibility that Na,K-ATPase activity in apical membrane of rat distal colon represented an alternate mode of expression of H,K-ATPase, especially as the function of H,K-ATPase in normal and sodium-depleted animals. Therefore, Na,K-ATPase activity in apical membranes represents, at least in part, contamination of apical membranes by basolateral membranes. However, these present studies with polyclonal antibody (M1) against the a subunit of H,K-ATPase provide compelling evidence that apical membrane Na,K-ATPase activity is not solely a result of basolateral membrane contamination of the apical membrane preparation (Figs. 1 and 2). First, Na,K-ATPase activity in apical membranes was virtually (92%) abolished by the M1 antibody (Fig. 1). Second, Na,K-ATPase activity in basolateral membrane was not altered by M1 antibody (Fig. 2).

HKc protein, but not H,K-ATPase activity, was identified in basolateral membranes (Fig. 3 and Table I). The presence of HKc protein in basolateral membranes was much less than that in apical membranes. Because previous immunofluorescent studies have demonstrated that HKc protein is localized only in apical membranes and not in basolateral membranes (10), it is likely that the presence of HKc protein identified in basolateral membranes represents contamination from apical membranes. The failure to establish the presence of H,K-ATPase activity in basolateral membranes is probably due to the different techniques used to prepare apical and basolateral membranes (19, 22). This possibility is based on the recent observations that anion exchange isoform 2 (AE2)-specific protein (28) and anion exchange (Cl-HCO3) activities (29) were not detected in basolateral membranes prepared from kidney and colon, respectively, in the absence of protease inhibitors. In contrast, AE2 protein and Cl-HCO3 exchange activity were readily identified in basolateral membranes prepared in the presence of protease inhibitors. Because the method to prepare colonic basolateral membranes does not usually include protease inhibitors, we suspect that, similar to AE2 protein and Cl-HCO3 exchange activity, Na,K-ATPase was also denatured with loss of activity during the basolateral membrane preparation.

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H,K-ATPase has been extensively studied during the past decade since the report of the successful cloning of its a subunit by Crowson and Shull in 1992 (5). Nonetheless, considerable controversy exists especially regarding its b subunit and its sensitivity to ouabain (8–10, 12, 14–16). Studies with HKc cRNA in Xenopus oocytes and 86Rb uptake have yielded evidence of ouabain-sensitive function (14–16). In contrast, experiments in HEK293, a mammalian cell line, and in Sf9 insect cells have demonstrated the expression of HKc cDNA as ouabain-insensitive H,K-ATPase (9, 10). Because HKc mRNA and protein are primarily expressed in surface and not in crypt epithelial cells (10, 13) and because HK function in crypt cells is solely ouabain-sensitive (12), it would be necessary to postu-

![Figure 7](http://www.jbc.org/)
late the existence of three H,K-ATPase to account for ouabain-sensitive expression of HKc cDNA.

There is, however, excellent physiological evidence for the existence of two distinct H,K-ATPases in the rat distal colon. First, ouabain-sensitive and ouabain-insensitive components of H,K-ATPases have been identified in the apical membrane of the rat distal colon (Table I and Ref. 11). Second, active potassium absorption, energized by apical membrane H,K-ATPase, has two components: a fraction that is sensitive to mucosal sodium and is ouabain-insensitive and a fraction that is insensitive to mucosal sodium and is ouabain-sensitive (27). Third, aldosterone secondary to dietary sodium depletion stimulates only one of these two components of active potassium absorption, the so-called mucosal sodium-sensitive, ouabain-insensitive sodium absorption, energized by apical membrane H,K-ATPase, and that H,K-ATPase activity was 56% ouabain-insensitive (Table I). Despite these differences, both studies concluded that apical membrane Na,K-ATPase activity represents an alternate mode of H,K-ATPase expression.

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Ouabain-sensitive H,K-ATPase Functions as Na,K-ATPase in Apical Membranes of Rat Distal Colon
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